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Research Article

A novel method for delivering ramped cooling reveals rat behaviours at innocuous and noxious temperatures: A comparative study of human psychophysics and rat behaviour.

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2 Abstract

Background
Thermal sensory testing in rodents informs human pain research. There are important differences in the methodology for delivering thermal stimuli to humans and rodents. This is particularly true in cold pain research. These differences confound extrapolation and de-value nociceptive tests in rodents.

New Method
We investigated cooling-induced behaviours in rats and psychophysical thresholds in humans using ramped cooling stimulation protocols. A Peltier device mounted upon force transducers simultaneously applied a ramped cooling stimulus whilst measuring contact with rat hind paw or human finger pad. Rat withdrawals and human detection, discomfort and pain thresholds were measured.

Results
Ramped cooling of a rat hind paw revealed two distinct responses: Brief paw removal followed by paw replacement, usually with more weight borne than prior to the removal (temperature inter-quartile range: 19.1 ^{o}C to 2.8 ^{o}C). Full withdrawal was evoked at colder temperatures (inter quartile range: -11.3 ^{o}C to -11.8 ^{o}C). The profile of human cool detection threshold and cold pain threshold were remarkably similar to that of the rat withdrawals behaviours.

Comparison
Previous rat cold evoked behaviours utilise static temperature stimuli. By utilising ramped cold stimuli this novel methodology better reflects thermal testing in patients.

Conclusion
Brief paw removal in the rat is driven by non-nociceptive afferents, as is the perception of cooling in humans, in contrast to the nociceptor-driven withdrawal from colder temperatures. These findings have important implications for the interpretation of data generated in older cold pain models and consequently our understanding of cold perception and pain.

3 Keywords

pain, behaviour, rat, human, cold, nociception

4 Highlights

- Ramped cooling of a single rat hind paw elicits two distinct behaviours.
- Initial brief withdraws do not appear to be nociceptive in nature.
- Full withdrawals occur at very low temperatures and are nociceptive.
- The response profile to cooling is comparable between rats and humans.

5 Introduction

Cooling evoked behaviours in rodents are often studied with the aim of informing our incomplete understanding of thermal perception in humans. This effort is hampered by significant differences in the methodology used with the different species. The utility of results generated in rodents with respect to the above aim can, therefore, be questioned. Thus there is a need to better understand the relationships between cooling evoked behaviours in rodents and cooling evoked sensations in humans.

In human psychophysical experiments, ramped contact cooling is delivered via a thermode, usually using devices such as those made by MEDOC (http://www.medoc-web.com/medoc_en_home.aspx) or SOMEDIC (http://en.somedic.com/default.asp?pid=29). Importantly, application of ramped cooling to skin enables determination of thresholds for cold detection and pain in healthy individuals and subsequently the definition of positive and negative symptoms/sensations in patients [1, 2].

Contact thermal stimuli, often with varying ramp rates and final target temperatures, are also used in pre-clinical electrophysiological experiments in sensory primary afferent research [3-6]. This enables capture of thermal thresholds to neuronal activation and the responses to suprathreshold stimuli.

In contrast, behavioural experimentation in animals utilises significantly different cold stimuli. These include; cold plates (static temperature), evaporative cooling, or place preference tests [7-10]. The most common outputs are time-related, e.g. latency to behaviour (paw withdrawal [11] licking or flinching), behaviours per unit time [7, 12] or time spent in a particular location. It is rare that thermal behavioural thresholds per se are measured in animals, although decreasing temperature plates have been described [13], enabling differentiation between cold allodynia and cold hyperalgesia. Most thermal tests measure hyperalgesia, but milder interventions such as evaporative cooling of acetone, have also been used to elicit allodynic behaviours [8, 14].

A further important consideration is that as ramped cooling is almost never used in rodent studies there are no data regarding behaviours elicited during the transition between innocuous and noxious cooling. Given that any putative behaviours are likely to be different and that this transition must occur (albeit more rapidly) when using routine cold stimuli such as cold plates; more detailed consideration of such behaviours and the impact that they may have had on the interpretation of previous work is warranted.

Further still, most cooling evoked behaviours are elicited via cooling delivered to all four paws; the tail and also the abdomen (and testes in males). Thus, in addition to nociceptive reflexes that may be present, the observed behaviours will also be influenced by mechanisms involved in maintaining body temperature (homeostasis) [15] and may be complicated by fear/avoidance behaviours evoked by an inescapable, potentially noxious stimuli. The Hargreaves test [11, 16], by restricting heat stimulation to a single paw, removed these confounds in heat nociceptive testing.

The aim of this study was to develop methodology to address these discrepancies between rodent and human cold testing in order to better apply knowledge gained in the laboratory setting to human cold perception and pain. To achieve this, the same cooling stimulus was used to evoke behaviours in healthy rats and sensations in healthy humans. We delivered ramped cooling to a single rat hind paw and determined contact temperatures at which cooling induced behaviours

occur. This method was used in parallel to determine cool detection, cold discomfort and cold pain thresholds in healthy humans.

6 Material and Methods

6.1 Apparatus for delivering ramped thermal stimuli to glabrous skin in humans and rats

The apparatus used in all experiments was designed and built in-house, as there was no available commercial apparatus that could reproducibly and reliably stimulate both rat and human glabrous skin to enable measurement of equivalent thermal thresholds.

A Peltier unit (Supercool, Gothenburg, Sweden, (cat. no. 131-10-13); 40mm x 23mm x 3.6mm) was attached with thermally conductive adhesive (Arctic Silver 5, from Arctic Silver CA. USA) to an aluminium heat sink. The Peltier and heat sink module was mounted upon force transducers (FS series Force Transducer, Radiospares UK, cat no. 235-6210), which enabled measurement of the precise temperature at which the force transducers were unloaded. The surface temperature of the unit was recorded via a T Type thermocouple mounted on a copper plate affixed with Arctic Silver™ to the upper surface of the Peltier device. During cooling, the heat sink was flushed with 50:50 ethylene glycol:water, pre-cooled to -10°C. This enabled rapid and large reductions in temperature. During heating experiments, the heat sink was flushed with cold water. The Peltier device was driven from a control system built in house, which enabled fine control of linear heating and cooling rates.

Thermocouple and force transducer outputs were amplified and fed into a micro1401 analogue to digital converter (Cambridge Electronic Design). Data were recorded on a PC using Spike 2v6 (Cambridge Electronic Design) for subsequent off line analysis (Figure 1).

6.2 Determination of thermal thresholds in rats

All experiments involved male Wistar rats (250-350g, Harlan, UK) and were carried out with University of Bristol Ethical Review Panel approval and in accordance with the UK Animals (Scientific Procedures) Act 1986. This manuscript was prepared with reference to the ARRIVE guidelines [17]. Animals were housed in enriched environments, under 12:12 hour light:dark conditions and had ad libitum access to food and water. Prior to experimental testing, animals were habituated to both the testing apparatus and to the investigator.

Rats were placed in a Perspex enclosure similar to that used in Linton Instrumentation's Incapacitance tester (http://www.lintoninst.co.uk/). They settled into a position such that the hind paws were in contact with the Peltier device (Figure 1A, C). Occasionally it was necessary to make minor adjustments to the position of the paws. Once the animal was settled, thermal stimuli were applied to the glabrous hind paw skin, from a holding temperature of 25°C. In three rats, heating was delivered at a rate of 1°C/s, until withdrawal, upon which the plate temperature was returned to baseline. This process was repeated up to 4 times per rat. In ten rats, cooling was delivered at a rate of -1.3°C/s and continued until the animal shifted the weight off the cooled hindpaw, denoting withdrawal from the stimulus. The lowest achievable temperature was -12°C which was therefore effectively the cut-off temperature. After stimulation the Peltier device temperature was returned to baseline. A second, and occasionally a third, ramp was then delivered with an inter-stimulus interval no less than 3 minutes. Occasionally it was necessary to repeat ramps if, for example, the rat turned around in the box thus removing the hindpaw from contact surface.

Four additional rats were tested with variable cooling rates of -0.5, -1, -2 and 4°C/s. Other than the different rates, these experiments were performed as described above.
At the end of the behavioural testing the paws of the rats were inspected for signs of injury including erythema and oedema.

6.3 Relationship between surface and subcutaneous temperatures

Behavioural withdrawal to contact heating is known to depend on the subcutaneous heating rate [18]. It was therefore necessary to evaluate both the rate of subcutaneous cooling and the absolute subcutaneous temperature achieved during the rodent experiments. In order to determine the subcutaneous temperatures at which cooling behaviours occurred, one anaesthetised additional rat was used. Anaesthesia was induced and maintained with sodium pentobarbitone (induction: 60mg/kg intraperitoneal, maintenance: 20mg/kg/hr (intravenous) and the trachea was cannulated for airway maintenance. A T-Type thermocouple (made in-house) was inserted subcutaneously into the plantar skin of one hind paw. The anaesthetised rat was then positioned in the restraining box (Fig 1C) with hind paws firmly in contact with the Peltier unit, and the Peltier device was cooled at different rates. The subcutaneous paw temperatures that corresponded to the contact temperatures were determined for each ramp rate.

6.4 Determination of thermal thresholds in human volunteers

The study was given ethical approval by the Faculty of Medical and Veterinary Sciences Committee for Research Ethics, University of Bristol. Participants gave informed consent prior to testing.

Participants were excluded from the study if they suffered any neurological or other problems that could affect their ability to detect or respond to cutaneous thermal noxious stimuli.

Ten healthy participants (5 male, 5 female, 26.4 yrs + 1.2 (mean age $\pm$ SEM)) were recruited. No participant was subsequently excluded for any reason.

Participants were acclimatised to the testing facility prior to testing. The protocol was explained to them so they were aware of the procedure, and they were exposed to a familiarisation ramp prior to testing.

Participants were asked to place the pad of the right index finger on the Peltier surface, which was initially held at 30°C. The instructions were to rest / place the fingertip upon the cooling surface and not to exert any specific force (Hsin-Ni Ho and Lynette A. Jones 2008). After more than 10 seconds had elapsed, the investigator informed them that the ramp would begin within the following 10 seconds. Participants were asked to say when they detected the temperature change (detection) and when they detected the transition into an uncomfortable sensation (discomfort). Input from a foot pedal was used to capture detection and discomfort thresholds. Participants were instructed to remove their finger from the equipment when the sensation became painful; this event was recorded via the force transducer. Following withdrawal the equipment temperature was returned to 30°C. The heating rate was ~1°C/s, whereas cooling rate was -1.3°C/s. The same ramp was applied three times.

Immediately after each test, participants were asked to choose two descriptors from a predefined list that indicated the best description of the sensation(s) they experienced at withdrawal (i.e. the quality of the pain). The descriptors list was constructed with reference to previous psychophysical studies [19-21]. Four participants found it difficult to give two descriptors for each ramp. When this occurred, the single descriptor or no descriptors were recorded. One participant forgot to provide detection thresholds on 2 of 3 ramps. To keep testing number consistent between subjects, and to comply with ethics committee approval, these ramps were not repeated in this single individual and data were included as an incomplete set.

6.5 Statistical analysis

Prism 4 for Windows (Version 4, Graphpad) and SPSS (version 18; IBM, Armonk, NY) were used for statistical comparisons. Threshold and withdrawal latency at initial and full withdrawal (rats) were compared using Mann Whitney tests. Mean temperatures and latencies for evoked behaviours at different cooling rates (rats) and human psychophysical and rat behavioural data were compared using a non-parametric 1 way ANOVA (Kruskal Wallis) followed by Dunns post hoc test. Thresholds in human participants (cold detection, discomfort and noxious withdrawal) were compared using 1 way repeated measure ANOVA followed by Bonferroni's post hoc test, unless explicitly stated.

When investigating the effect of cooling rate on evoked behaviours, a linear test for trend was additionally performed as a post hoc test following 1 way ANOVA analysis of the log-log transforms of the latencies to initial and full withdrawal at the different rates.

Subcutaneous temperatures during cooling at which initial and full withdrawal occurred were interpolated offline from the data measured in un-anaesthetised rats.

Hierarchical clustering of the *latencies* of all behaviours induced during cooling at -1.3^{o}C/s was performed using the Ward method and the Squared Euclidean distance measure in SPSS. The optimum number of clusters was determined via examination of the agglomeration coefficients. SPSS was then used to assign cluster membership to the individual latencies. Frequency histograms of cluster memberships were then generated using Prism. Using SPSS, a two-cluster solution was then applied to *temperatures* of all behaviours induced by cooling at -1.3^{o}C/s. The frequency histograms of behaviours vs temperature were then generated in Prism. It should be noted that cluster analysis does not provide a statistic that can be used to determine the probability of the data set containing a certain number of clusters.

7 Results

7.1 Heating responses in rats and participants.

Heating of the plantar surface of a single rat hind paw elicited a robust withdrawal response at 47.8°C +/- 0.4°C (Mean +/-SEM) or 48.19°C (46.5°C to 49.1°C) (median (inter-quartile range)) (Figure 2A). Heating of the finger pad of the index finger of the human participants elicited detection, discomfort and withdrawal thresholds of 38.1°C +/- 1.5°C, 47.4°C +/- 1.2°C and 49.8°C +/- 0.9°C (Mean +/-SEM) respectively (Figure 2B). Whilst human detection thresholds occurred at significantly lower temperatures than rat withdrawal, no differences were found between human discomfort, human withdrawal and rat withdrawal temperatures (Figure 2C).

7.2 Cooling responses in rats

Cooling stimuli were applied following the above validation of this method of delivering an isolated thermal stimulus to a single rat hind paw. Cooling of one hind paw elicited two distinct behaviours in the rat. These behaviours were identified by observation and could also be seen in the weight bearing profile, shown as raw data in Figure 3A. Initial brief paw removals, that were not sustained, were usually, but not always, seen as the contact temperature decreased. This involved a brief removal of the paw from the plate (1-2s duration), which was then returned, often subsequently bearing more weight than before despite the continued lowering of contact temperature (Fig. 3A). As the temperature continued to decrease, a full withdrawal was evoked as the paw was removed from the plate and remained lifted until the plate was re-warmed (Fig. 3A). When paw removals were grouped into ”initial brief” if they were not sustained, and ”full” when the paw lift was sustained as determined by the trace, then initial brief and full removals occurred at contact temperatures of 14.3°C (2.8°C to 19.1°C) and -11.7°C (-11.3°C to -11.8°C) respectively (median (inter-quartile range)). Full withdrawal often occurred at temperatures approaching the lowest that was achievable (approximately -12°C, Fig. 3B). The differences in withdrawal temperatures were reflected in withdrawal latencies (initial 11.6s (8.2s-20.0s), full withdrawal 33.5s (31.3s-36.4s)). The variability in the withdrawal latency was greater than that seen in the withdrawal temperature. This probably reflects events occurring after the attainment of the lowest possible plate temperature. The latencies were also significantly different between initial and full withdrawal values (Fig. 3C).

It was evident (Figs. 3B, C) that the temperatures at which initial paw removal occurred overlapped with those temperatures eliciting full withdrawal. To determine whether: hindpaw cooling did indeed evoke two distinct behaviours in the rat; or whether the initial and full withdrawals were a continuum of the same behaviour; or whether subjective experimenter opinion was influencing interpretation, latencies and temperatures were subject to cluster analysis. Examination of the agglomeration coefficients for latencies most strongly supported a model with 2 clusters, although a 3 or 4 cluster model could not be discounted from the agglomeration coefficients alone. Examination of different cluster solutions (i.e. 2, 3 or 4 clusters) always generated a first cluster with a median (I.Q.R) latency of 11.7 (8.6 to 14.3) seconds (initial removal) and this cluster always included the same number of data points, suggesting that the two behaviours were indeed distinct. Using a two cluster model gave a median (I.Q.R) latency for the second cluster of 28 (23.4 to 31.7) seconds. Three and four cluster models all split the longer latency cluster (full withdrawal) into additional sub clusters with no effect on the first cluster. See

Figure 3E. Furthermore, the first cluster in the two cluster solution for temperature vs responses contained a similar number of responses as the first cluster generated using latencies: 38 data points for temperatures vs 36 data points for latencies. This generated a first cluster with a median (I.Q.R.) temperature of 13.6 (18.8 to 10.3) ^{o}C and a second cluster with a median (I.Q.R.) temperature of -8.1 (-2.5 to -12.3) ^{o}C.

The rate of contact heating and subsequent subcutaneous heating rate in rats affects different groups of nociceptive afferents [18]. We therefore determined the effect of different cooling rates on the evoked rat behaviours.

Increasing the rate of cooling from -0.5 to -4 ^{o}C/s reduced the latency to both initial brief removal and full withdrawal (Figs 4A & 4B), but did not affect the skin temperature at which initial or full removal occurred (Figs 4C & 4D). Interpolation of the subcutaneous temperature at which behaviours would be expected to occur, as derived from the skin and subcutaneous temperatures in the anaesthetised rat, generated subcutaneous mean initial removal temperatures of approximately 5^{o}C to 10^{o}C (Fig. 4E). These were highly variable, as seen for the skin contact temperatures (Fig. 3B). Notably interpolated subcutaneous temperatures at full withdrawal were much less variable, mean withdrawal temperatures were ~ -2^{o}C (Fig. 3F).

The subcutaneous temperature was linearly related to the surface temperature and this linear relationship was identical for rate of cooling from -0.5^{o}C/s to -2^{o}C/s. At the fastest rate of cooling, -4 ^{o}C/s, this was no longer the case, and subcutaneous temperatures were slightly higher than would be predicted from a linear relationship. See Figure 4E.

7.3 Cooling responses in participants

In human studies, participants indicated detection and discomfort to cooling and this was captured on the raw trace via a foot pedal input. Finger removal occurred when the sensation became painful (Fig. 5A). Cooling detection occurred at 24.9^{o}C (26.3^{o}C to 19.5^{o}C) (median (IQR)), discomfort at -1.2^{o}C (1.2^{o}C to -5.9^{o}C) and noxious withdrawal -6.3^{o}C (-3.5^{o}C to -10.3^{o}C) (Fig. 5B). Notably, cooling discomfort and noxious withdrawal were not evident until skin contact temperatures were less than 0^{o}C. The most frequent words chosen to describe the sensation evoked at noxious cold withdrawal were “cold”, “numbing” and “freezing” (Fig. 4C).

Due to the mismatch between human and rodent studies on cold nociception, one aim was to determine whether rat behaviours could be related to psychophysical correlates in humans. As there were potential differences in skin thickness and thermal transfer between rats and humans and we could not directly measure subcutaneous temperatures in humans, and many rat withdrawals occurred at the cut-off temperature whereas humans did not, we compared latency to withdrawal rather than contact temperatures. Comparison showed that rat full withdrawal and pain-evoked withdrawal in humans occurred at a similar latency in both species (Fig. 6A-C), indicating that this method of stimulation elicited nociception in both species at equivalent times after onset of cold ramping. In addition, initial removal latencies in rats were equivalent to cold detection latencies in humans (Fig. 5C). Probably most crucial is that the profile of responses to reducing temperature is comparable between the two species.

Interestingly, cold discomfort thresholds in humans was not clearly distinguishable from noxious cold withdrawal in humans and they were also not significantly different from full cold

withdrawals in rats. There was also no statistical difference between latencies to cold detection/initial removals in humans and rats respectively (Fig. 5C). This supports the interpretation that the initial paw removal responses in rats are associated with non-noxious rather than noxious cold. The variance of both cold measures (initial and full withdrawals) in the rat was significantly greater than in the human measures, i.e. initial rat responses to ramped contact cold stimulation were less consistent between trials than those of humans.

8 Discussion

The neurophysiology of thermal perception, particularly that of cold pain, is complex. Multiple afferent populations and ascending pathways inform perception and behavioural responses [22], and processing, particularly of noxious cold information, is also influenced by descending modulation from brainstem centres [23-25]. Further complexity is added to interpretation of findings by methodological differences between studies, including stimuli (magnitude; area; skin type) and outcomes (psychophysics, behaviour, neuronal properties, imaging). Overarching all of the above are possible differences between species.

This complex situation has led to assertions and possible over-simplifications, which, especially when taken out of context of the original work, can be contradictory to everyday experience. For example, it is commonly reported that humans have a cold pain threshold of near to 14^{o}C, a figure which has been used to relate perception to the function of individual putative transduction molecules [26]. However, it is not usually painful to handle a bottle of milk from a fridge at 4^{o}C and it is possible to handle frozen foods from a domestic freezer (-20^{o}C) for short periods of time without suffering pain, although it can be uncomfortable.

We sought to address the mismatch between experimental conclusions in rats and humans, and everyday observations on cold discomfort/pain, and to compare cold behavioural responses in humans and rats by using equivalent cold ramping stimuli in both species.

Initial experiments sought to validate this novel method of delivery of thermal stimuli. This was possible with reference to previously published heat withdrawal thresholds in rats. Heat withdrawal thresholds reported here of c. 48^{o}C are remarkably similar to the 47.6 ^{o}C +/- 2 ^{o}C generated utilising thermal stimuli from a radiant heating lamp [16], a comparable thermal stimulus. Furthermore, and as noted by Banik and Kabadi [16], these withdrawal temperatures are comparable to those generated in humans (data herein and e.g. 47.5^{o}C, [27]).

Given these positive validation experiments, we were then able to explore the unknown effects of the application of ramped cooling stimuli. In equivalent skin types, both species demonstrated that they were able to respond to the onset of cooling from ambient at equivalent latencies/temperatures, and this occurred at temperatures not perceived as painful by humans. We therefore hypothesise that the initial brief paw removal seen in rats is not a nocifensive behaviour. This hypothesis is strongly supported by the new observation, which is only possible to make using this novel apparatus, that more weight is borne by the paw after it is replaced, during these initial behaviours.

It is interesting to note that cold induced vasoconstriction during cooling of isolated paws occurs in the rat at approximately 22^{o}C [28]. It could be that the brief removal from the cooling surface represents a homeostatic response to cooling, as rodent paws and tail are critical for maintenance of body temperature in the rat [29]. In the rat, without the benefit of the additional information on increased weight borne on the cooled hind paw after these movements, the more transient behaviours evoked by non-noxious cooling could easily be misinterpreted as a full withdrawal response. It only becomes apparent that withdrawals are transient when applied temperatures are subsequently lowered to levels where complete withdrawal occurs. If a trial is terminated on initial paw removal, the cold threshold will be recorded at a higher value. Indeed, noxious cold thresholds in rats are often quoted at values much warmer than those we report (e.g. circa 4 or 5^{o}C [7, 12]), in the range in which most of the brief paw removals occurred.

The initial paw removal is clearly different from the overt full withdrawals evoked by lower contact temperatures that most obviously correlate with the noxious cold thresholds measured in humans. Such withdrawals occur at temperatures similar to those that evoke withdrawal in lightly anaesthetised animals which, by definition [30], are nociceptor driven [23, 31] . This full withdrawal behaviour and the correlate in humans is therefore more likely to be driven by cold nociceptors, and may be an appropriate measure to use in preclinical cold evoked pain research. It should also be noted that for both brief "initial" paw removal and noxious withdrawals, the responses in rats are much more variable than those elicited in humans. It is therefore clear to us that the interpretation of cold-evoked withdrawal behaviour in rodents is complicated by the more complex withdrawal behaviour than is seen with heat stimulation.

As rate of cooling increased, latency to behaviours decreased, yet the temperatures at which behaviours occurred was consistent. This finding suggests that it is the absolute temperature at the neuronal receptor (subcutaneous/dermal/epidermal etc.) that drives both initial and full withdrawals, rather than the rate of change of cutaneous temperature.

Subcutaneous temperature measurements indicate that full noxious withdrawal behaviours, in rats at least, are evoked at temperatures just below 0^{o}C, with contact/surface temperature well below 0^{o}C. It is well known that freezing is painful and may lead to tissue damage[32]. Whether the withdrawal behaviour described here reflects actual freezing pain is debatable. There was no evidence of tissue damage to the paw after testing and no sensitisation of responses was seen, even though both would be expected following tissue injury. Furthermore, it may be argued that nociceptive systems function to warn of the potential for injury and that this system would act to prevent such injury in an awake animal. It is also known that thermoregulatory mechanisms are blunted under anaesthesia [33] and it is therefore possible that the subcutaneous temperature in awake animals were not as low as in the anaesthetised rat.

While it is difficult to compare our data to previous work because of the differences in methodology, it is interesting to note that previously reported cold pain thresholds in both rats and humans occurred at warmer temperatures to those reported here. On the thenar eminence, thresholds are reported as between 10^{o}C and 15^{o}C [19-21, 34, 35]though with high variability [36], as with other skin sites, whereas our data show thresholds substantially less than zero^{o}C in both species. We suggest that these differences could be accounted for by a combination of factors, including lack of expectation/anticipation effects[37, 38] when using an escapable stimulus where termination is entirely under the participant's control; a smaller stimulus area and therefore reduced spatial summation [39], and the lower cold sensitivity of glabrous skin [20], in particular the finger tip in relation to other skin sites [36].

Which afferent types could be involved in these behaviours? INSERT ADDENDUM HERE – WILL NEED IMAGINATIVE REDUCTIONS IN WORD COUNTS.

There are obviously inherent difficulties in relating psychophysical outcomes in humans and behavioural outcomes in rats, not least that rats lack some neuroanatomical structures within the forebrain that are crucial to the human experience of pain [40]. However, withdrawal behaviours in the rat are informed by afferent populations that appear to be common across the two species [41]. Given that a major reason to undertake research in experimental animals is to inform and improve human pain management, we believe that the two species should be investigated in parallel, to highlight similarities and differences.

9 Conclusions

We have used a novel method of monitoring behavioural and psychophysical responses to cooling and noxious cold in rats and humans. By delivering ramped cooling our technique more appropriately reflects currently used clinical protocols and thus facilitates human comparative studies. This approach has revealed a cooling induced behaviour in the rat that is unlikely to be driven by nociceptive afferents. It has also revealed that full withdrawal from cold stimuli requires much colder surface, and probably subcutaneous temperatures than have previously been employed. This work has important implications for the interpretation of past and ongoing work studying physiological and pathophysiological cold pain.

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11 Conflict of interest

The authors confirm that they know of no conflicts of interest.

12 Tables, Figures and Legends

Figure legends

Figure 1. Behavioural testing apparatus.

A. Diagrammatic representation of the testing apparatus with a rat in position. Individual thermal modules consist of a Peltier device, heat sink and force transducers. The rat hind paws are located over these modules via the positioning frame. The temperature of the Peltier device and the output from the force transducer is amplified and then captured via CED's 1401 which interfaces with a PC.

B. Photograph of the apparatus illustrating the two plates independently mounted on separate thermal modules. In flow and out flow tubing for coolant for the heat sinks can be seen to the left of the photograph. Connections to amplification and recording equipment are visible to the right of the photograph.

C. Photograph of apparatus as above but including the Perspex positioning box and a rat in situ.

Figure 2. Contact ramped heating of the rat hind paw elicits withdrawal at temperatures equivalent to human discomfort and withdrawal thresholds.

A. Human. Panel shows a typical example of a digitised trace of the raw data generated from the force transducers under the heated index finger (top trace, arbitrary units), and the surface/contact temperature of the Peltier device in contact with the finger (lower trace, ^{o}C), over time (s). Detection, discomfort and withdrawals are indicated by the vertical numbered cursors and the temperature read from the lower trace as illustrated.

B. Rat. Panel shows a typical example of a digitised trace of the raw data generated from the force transducers under the heated hindpaw (top trace, arbitrary units), and the surface/contact temperature of the Peltier device in contact with the plantar hindpaw (lower trace, ^{o}C), over time (s). Withdrawal is indicated by the vertical cursor 1. Temperature is read from the lower trace.

C. Mean data from human (n=10) and rat (n=3) experiments . Human detection of heating occurs at a significantly lower temperature than rat behaviours. Human pain threshold and withdrawal threshold are not different to the rat withdrawal temperature. Kruskal-Wallis test, ** = $P<0.05$, ns = $p>0.05$).

Figure 3. Contact ramped cooling of the rat hindpaw elicits two distinct behavioural responses.

A. Panel shows a typical example of a digitised trace of the raw data generated from the force transducers under the cooled hindpaw (top trace, arbitrary units), and the surface/contact temperature of the Peltier device in contact with the plantar hindpaw (lower trace, ^{o}C), over time (s). The rate of cooling is -1.3^{o}C/s.
The vertical cursors indicate the cooling evoked behaviours. Cursors 1, 2 and 3 indicate rapid, transient removals of weight from the plate, and the contact temperature at which they occurred (22.1^{o}C, 19.2^{o}C and 10.9^{o}C). When the paw was placed back on the plate, there was often an increase in weight borne on the plate, as indicated by the upward shift of the trace most noticeable between cursors 3 and 4. The plate temperature continued to fall during this time. Vertical cursor 4 indicates a more prolonged removal of weight, full withdrawal, at -11.87^{o}C.

B. Scatter plot illustrating the temperature at which the two cooling evoked behaviours occurred. Horizontal bars show median and IQRs. The median temperature at which the initial transient withdrawal occurred was higher than that at which full withdrawal behaviours occurred ($p<0.001$, n=10 rats, up to 3 ramps per rat. Mann Whitney test). The rate of cooling is -1.3^{o}C/s.

C. Scatter plot illustrating the latencies at which the two cooling evoked behaviours occurred. Horizontal bars show median and IQRs. The median time to withdrawal (latency) was longer for full withdrawals than for initial withdrawals ($p<0.001$, n=10 rats, up to 3 ramps per rat. Mann Whitney test). The rate of cooling is -1.3^{o}C/s.

D. Frequency histogram showing the two-cluster solution provided for responses vs temperature. The open bars indicate “initial” withdrawals, associated with cluster one as detected by hierarchical clustering, and the closed bars indicate cluster 2, the full withdrawals. For full explanation of clustering please see text. The rate of cooling is -1.3^{o}C/s.

E. Frequency histogram showing the two-cluster solution provided for responses vs latencies. The open bars indicate "initial" withdrawals, associated with cluster one as detected by hierarchical clustering, and the closed bars indicate cluster 2, the full withdrawals. For full explanation of clustering please see text. The rate of cooling is -1.3^{o}C/s.

Figure 4. The effect of rate of contact ramped cooling on behavioural responses in rats.

A. As cooling rate increased, the latency to initial withdrawal decreased. (Median ± I.Q.R, n= 4 rats, 1-2 trials per rate, **p<0.01, Kruskal-Wallis + Dunn's, ***p<0.001 linear test for trend following log-log transformation).

B. As cooling rate increased, the latency to full withdrawal also decreased. (Median ± I.Q.R., n= 4 rats, 1-2 trials per rate, *p<0.05, ***p<0.001, Kruskal-Wallis + Dunn's. ***p<0.001 linear test for trend following log-log transformation).

C. Although the cooling rate increased, the plate temperatures that evoked initial withdrawal was unchanged, although this was highly variable. (Median ± I.Q.R., n=4 rats, 1-2 trials per rate, One way ANOVA with Bonferroni post hoc test).

D. The plate temperature at which full withdrawals occurred was also unchanged by the rate of plate cooling. For full withdrawals the plate temperature was highly consistent across trials. (Median ± I.Q.R., n=4 animals, 1-2 trials per rate One way ANOVA with Bonferroni post hoc test).

E. The mean (±95%CI) subcutaneous temperatures at which initial withdrawals occurred at different rates of cooling were interpolated from the contact temperatures (Fig 3C) and known values of subcutaneous measurements made in an anaesthetised rat. Unfortunately, the plate temperature was not lowered sufficiently in the subcutaneous temperature experiment to enable the extrapolation of the lower confidence interval for the -2^{o}C/s cooling rate.

F. The mean (±95%CI) subcutaneous temperatures at which full withdrawals occurred at different rates of cooling were interpolated from the contact temperatures (Fig. 3D) as above.

G. Plate temperature is plotted against sub cutaneous temperature for the 4 different cooling rates. The relationship between plate and sub-cutaneous temperature is approximately linear for cooling rates -0.5oC/s to -2oC/s.

Figure 5. Contact ramped cooling stimuli on human forefinger allow definition of cold detection, discomfort and pain thresholds and latencies.

A. Panel shows a typical example of a digitised trace of the raw data generated from the force transducers under a cooled forefinger (top trace), and the surface/contact temperature of the Peltier device in contact with the forefinger (lower trace), over time (s). Cooling rate was ~ -1.3^{o}C/s. Participants indicated cold detection and discomfort and this was recorded via a foot pedal. This is shown in the Marker channel to the top of the figure. The vertical cursors indicate readout of psychophysical events. Cursor 1 indicates cold detection at 19.1^{o}C, cursor 2 cold discomfort at -2.48^{o}C and cursor 3 cold pain at -9.1^{o}C.

B. Cold detection thresholds were significantly higher that cold discomfort and cold pain thresholds. Cold discomfort was also significantly different from cold pain (median (I.Q.R.). ***p<0.001, 1 way ANOVA + Bonferroni's test, n = 10).

C. The most common descriptors used to describe the cold pain evoked by contact cooling were "cold", "numbing" and "freezing". (n=10, 2 descriptors for each of three trials).

Figure 6. Comparison of human psychophysical and rat behavioural thresholds during contact ramped cooling.

A. Latency to psychophysical thresholds evoked by cooling in humans is shown as a frequency histogram relative to the plate temperature. The number of events (left Y axis) in each 5 second bin is shown for detection (dashed line), discomfort (dotted line) and withdrawal (solid line) (n= 10).

B. Latency to behaviours evoked by cooling in rats is shown as a frequency histogram relative to the plate temperature. The number of events in each 5 second bin is shown for initial (dashed line) and full withdrawals (solid line) (n= 10).

C. The latencies to initial brief paw removal (rats) and cold detection (human) were equivalent, although the variability of responses was much greater in the rats. The latencies for discomfort, noxious cold (humans) and full withdrawals (rats) were also not significantly different, again rat responses were more variable. The latencies for "initial response" and "pain" were significantly different in both humans and rats (p<0.0001). All other comparisons were significantly different except for those indicated (Kruskal Wallis + Dunn's test). Bars indicate medians and IQRs.

13 Bibliography

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Figure 1. Apparatus

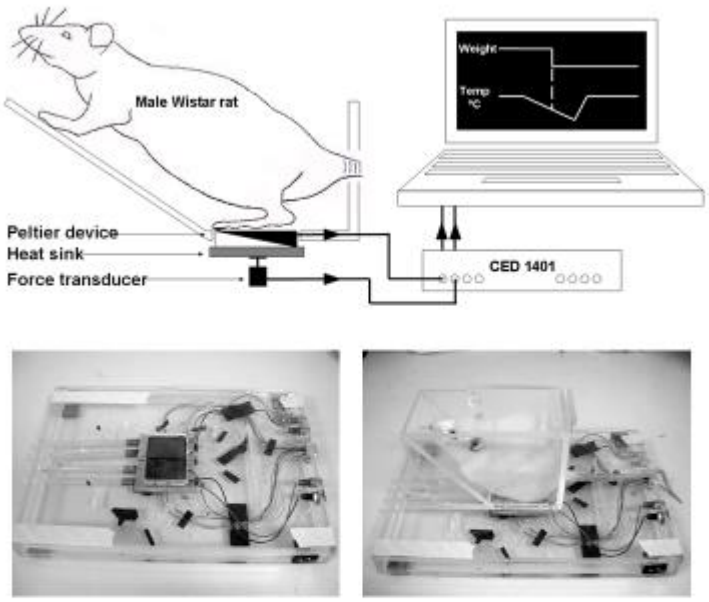

Figure 2. Validation of the thermal stimuli method.
Heat ramps applied to rats and humans.

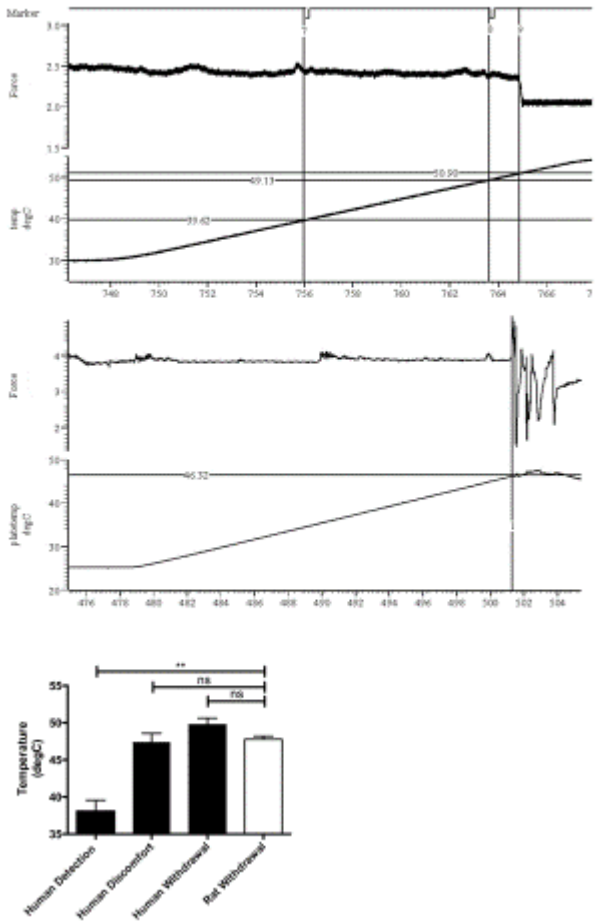

Figure 3. Rat behaviours elicited by ramped cooling of a single hind paw

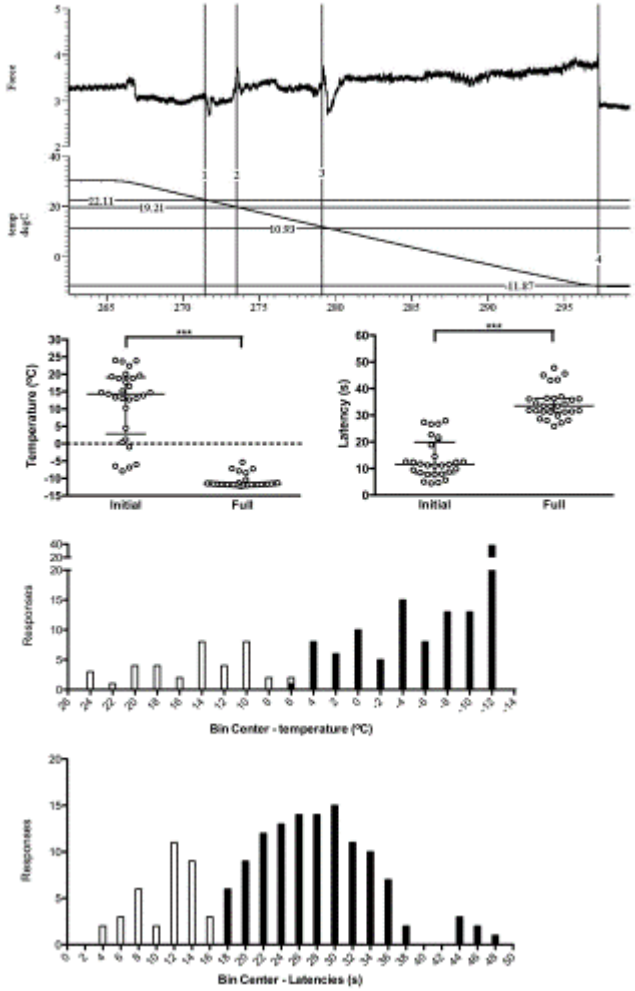

Figure 4. Rat behaviours elicited by ramped cooling of a single hind paw

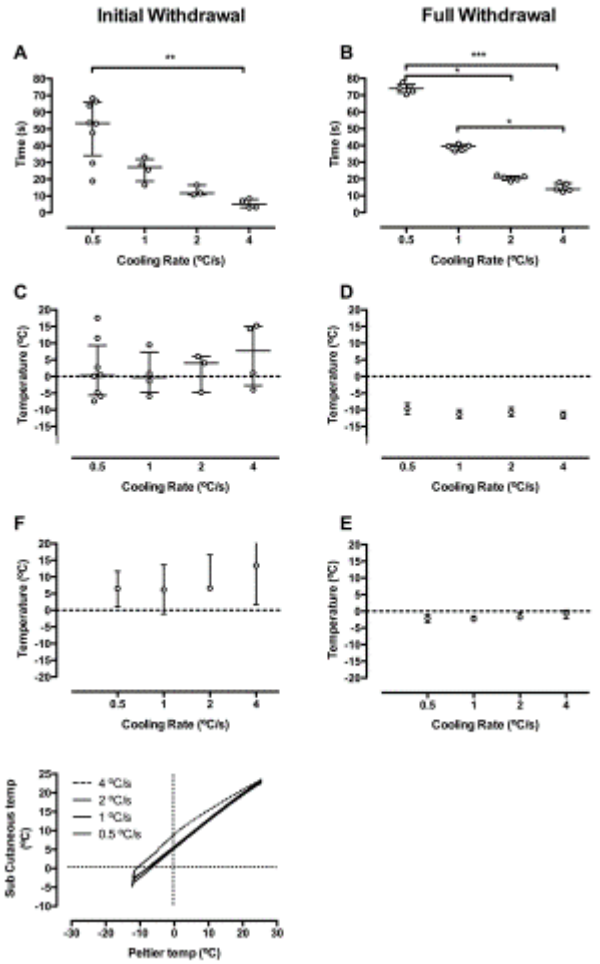

Figure 5. Human psychophysics elicited by ramped cooling of the finger pad.

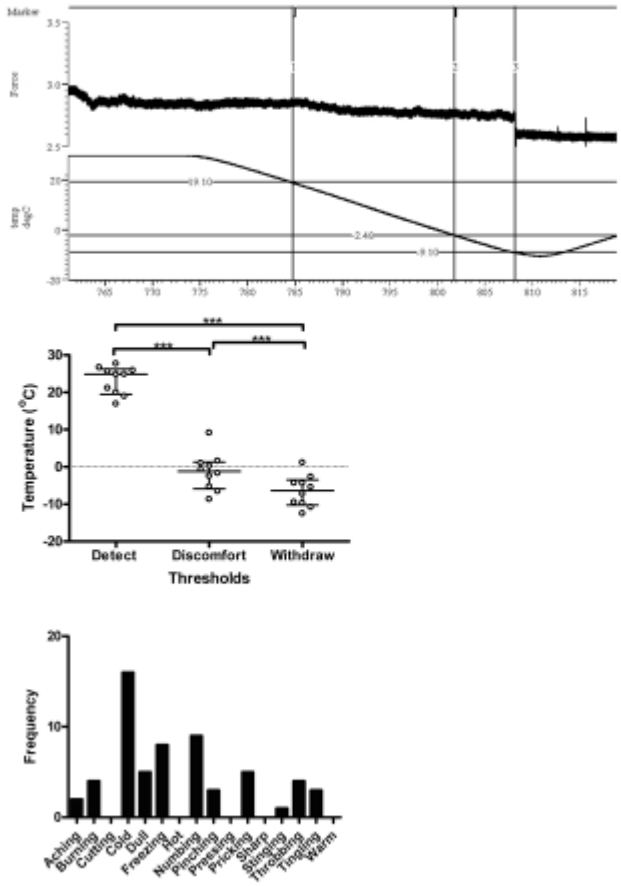

Figure 6. A comparison of rat and human responses to ramped cooling.