1 Altered Hypothalamic Inflammatory Gene Expression Correlates with Heat Stroke Severity in a
2 Conscious Rodent Model

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10 Running Title: Heat stroke alters gene expression in the brain

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23 Abstract:

24 It has been suggested that heat-induced hypothalamic damage mediates core temperature (T_c)
25 disturbances during heat stroke (HS) recovery; this is significant as hypothermia and/or fever
26 have been linked to severity and overall pathological insult. However, to date there has been a
27 lack of histological evidence in support of these claims. We hypothesized that local
28 hypothalamic cytokines and/or chemokines, known regulators of T_c , are mediating the elevation
29 in T_c during HS recovery even in the absence of histological damage. In experiment 1, the
30 hypothalamus of Fischer 344 rats was examined for 84 cytokine/chemokine genes (real-time
31 PCR) at multiple time points ($T_{c,Max}$, 1, 3, and 10 days) during mild HS recovery. In experiment
32 2, the hypothalamus of three different HS severities (MILD, moderate [MOD], and severe
33 [SEV]) in rats were examined for the same genes as experiment 1 as well as six oxidative
34 damage markers, at a single intermediate time point (1 day). Systemic cytokines were also
35 analyzed in experiment 2 across the three severities. There were significant alterations in 25
36 cytokines/chemokines expression at $T_{c,Max}$, but little or no changes in expression at longer time
37 points in experiment 1. In experiment 2 there were significant changes in gene expression in
38 SEV rats only, with MILD and MOD rats showing baseline expression at 1 day, despite an
39 absence of systemic cytokine expression in any severity. There was also no change in any
40 oxidative marker of damage at 1 day, regardless of severity. In conclusion, we show only
41 limited changes during long term recovery from HS, but demonstrate differences in
42 hypothalamic gene expression patterns that may be driving HS pathology and morbidity. These
43 findings contribute to our overall understanding of HS pathology in the CNS, as well as
44 providing avenues for future pharmacological intervention.

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46 Key words: heat stroke, hypothalamus, hyperthermia, central nervous system, cytokines

1 **Introduction**

2
3 Heat stroke (HS) is the most devastating form of heat illness, and is characterized by
4 systemic organ damage with potentially debilitating central nervous system (CNS) dysfunction
5 [1]. CNS dysfunction is characterized by cognitive and motor deficits during moderate HS cases
6 while often resulting in permanent brain damage and coma in the most severe forms of HS [2-4].
7 It has been suggested that CNS damage to the preoptic anterior hypothalamus (POAH) causes
8 temperature disturbances, such as fever and/or hypothermia in severe HS cases. While these
9 post-HS temperature responses have been linked to severity and morbidity in animal models and
10 humans there is no evidence that the POAH is damaged in even the most severe HS cases,
11 including non-survivors [2, 5-8]. For example, Malamud et al. performed autopsy on 125
12 military exertional HS cases and noted significant histological damage to several brain areas
13 whereas the POAH was spared [9]. Similarly, we have shown in rat and mouse conscious
14 passive HS models that hypothermia and fever occur in the absence of histological damage to the
15 hypothalamus or other brain regions [10, 11]. In mice, hypothermia was associated with
16 increased POAH and hippocampal gene expression of a number of cytokines (e.g., interleukin-6
17 [IL-6]) and chemokines (e.g., macrophage inflammatory protein [MIP]) suggesting this response
18 is linked to core temperature (T_c) during recovery as well as mirroring what is occurring
19 systemically [12]. However, responses in the hypothalamus beyond 24 hours of recovery remain
20 unstudied in any model; therefore it is unknown what alterations are retained through longer-
21 term recovery. In addition, what differences in hypothalamic responses are occurring between
22 different HS severities, and how these might be driving severity, remains unknown.

23 The goal of this study was to determine if molecular alterations exist in the hypothalamus
24 during 10 days of recovery from HS and examine hypothalamic inflammatory and oxidative
25 gene responses and T_c profiles across 3 HS severities. We used targeted gene analysis to examine
26 the inflammatory changes in the hypothalamus of rats at the onset of HS and at long-term
27 recovery time points (3, 5, 10 days of recovery). We then compared inflammatory and oxidative
28 hypothalamic gene responses amongst mild (MILD), moderate (MOD), and severe (SEV) HS
29 rats, which previously showed a lack of histological brain damage [6]. This research effort
30 aimed to identify neurological pathways that may play a part in determining HS severity,
31 irrespective of histological damage. Our findings showed that the majority of alterations
32 occurred early (<3 days), with a limited number of alterations still observed at 10 days post-HS.
33 However, SEV HS rats showed greater gene expression changes at 1 day of recovery compared
34 to MILD or MOD rats suggesting that hypothalamic gene expression could be influencing
35 recovery.

36 **Results**

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39 *Experiment 1.* Body weight (BW) was virtually identical between CON and MILD HS rats prior
40 to experimentation at ~270 g (Table 1). CON rats experienced ~2% dehydration, presumably
41 due to lack of food and water during experimentation, whereas MILD HS was associated with
42 ~7% dehydration (Table 1; Students t-test, $P < 0.05$). MILD HS rats required ~174 min of heat
43 exposure to reach $T_{c,Max}$ of 41.8°C and incurred an ascending and descending thermal load of
44 ~100 and ~19°C·Min, respectively (Exact values found in Table 1). These responses are similar
45 to previously reported values in MILD HS rats using our experimental protocol [10]. MILD HS
46 rats displayed sustained hyperthermia (~1°C) into 3D of recovery that is also typical of this level

1 of HS severity in the rat (Figure 2A; ANOVA, $P < 0.05$; [10]). For ease of presentation, T_c
2 responses are shown through night three only with T_c virtually identical between CON and
3 MILD HS rats beyond this time point (data not shown).

4 Plasma and brain samples were collected at $T_{c,Max}$ (most robust T_c response during
5 recovery of MILD HS rats) and compared to CON at the same time point. Figure 2B shows the
6 pattern of gene expression changes from $T_{c,Max}$ through 10D of recovery of MILD HS rats when
7 compared to CON values. The majority of gene expression changes (29%) were observed at
8 $T_{c,Max}$ with both up-regulated (33%) and down-regulated cytokine and chemokine responses
9 (67%) observed at this time point (Figure 2). Of those in the cytokine family, four were down-
10 regulated (Integrin β -2 (Itgb2), Transforming growth factor β -1 (Tgfb1), Toll-like receptor-2
11 (Tlr2), Tumor necrosis factor (Tnf) and one was up-regulated (Interleukin 1 β) at $T_{c,Max}$. Of the
12 chemokine family, 12 were downregulated (MCP-1-related chemokine [CCL2], Eotaxin-2
13 [Ccl24], Mucosae-associated epithelial chemokine [Ccl28], Macrophage inflammatory protein-1
14 [Ccl4], Monocyte chemoattractant protein-3 [Ccl7], Macrophage inflammatory protein 1 α receptor
15 [Ccr1], Macrophage inflammatory protein 1 α receptor like-1 [Ccr1l], Eosinophil eotaxin
16 receptor [Ccr3], Macrophage inflammatory protein 3 β receptor [Ccr7], Putative monocyte
17 chemotactic protein receptor [Ccr12], [Cmklr1], CKLF-like MARVEL transmembrane domain
18 containing 5 [Cmtm5]), and 7 were upregulated (Chemokine binding protein 2 [Ccbp2],
19 Monocyte chemoattractant protein 1 [Ccl2], Exoderm-2 [Ccl21], Ccl6 [Ccl6], Chemokine receptor
20 like-1 [Ccr8], CKLF-like MARVEL transmembrane domain containing 2A [Cmtm2a],
21 Fractalkine [Cxcl3]) at this time point. The largest changes were seen in the chemokines, with
22 chemokine receptor like-1 showing the greatest increase (+10.31, $P = 0.02$; Figure 2B), and
23 macrophage inflammatory protein 1 α receptor showing the largest decrease (-19.59, $P = 0.03$;
24 Figure 2C). At 10D of recovery, Macrophage inflammatory protein 3 β receptor (Ccr7) (-2.35,
25 $P < 0.05$) and plasminogen activator (Plau) (-2.42, $P < 0.05$) were the only two genes that remained
26 significantly decreased in expression compared to CON suggesting that rats may not be fully
27 recovered from MILD HS at this time point. While plasminogen activator did not reach
28 significance at either $T_{c,Max}$, 3D, or 5D, it did show a trend of decreased expression at 3D (-2.02,
29 $P = 0.08$). A summary of basic *in vivo* actions of those genes significantly altered at all time
30 points is included in table 3.

31 *Experiment 2.* For those animals in experiment 2, dehydration and heating characteristics
32 have been previously characterized [6]. In brief, while T_c recovery profiles varied for the three
33 severities in *experiment 2*, none of the heat exposure responses (thermal area, time to $T_{c,Max}$)
34 were different between the severities. This is consistent with previous findings in other animal
35 models [6, 13-15]. Figure 3 provides representative T_c profiles. SEV HS rats were characterized
36 by hypothermia, while MILD and MOD HS rats showed only brief or no period of hypothermia
37 followed by $\sim 1^\circ$ C hyperthermia, lasting through 1D (Figure 3A). Figure 3 also provides an
38 illustrative representation of the experimental design (Figure 3B). Gene expression responses of
39 MILD and MOD HS rats were not different and were combined for ease of presentation (Figure
40 3B). SEV HS rats showed ~ 2 -3-fold increased expression of six chemokines (Liver activation
41 regulated chemokine [Ccl20], Exoderm-2 [Ccl21], Chemokine (C-C motif) ligand 4 [Ccr4],
42 Monocyte chemoattractant protein receptor [Ccr7], Chemokine receptor-like 1 [Ccr8], Neutrophil-
43 activating protein 3 [Cxcr1]) and one cytokine gene (Interferon- γ [Ifng]) at 1D compared to CON
44 rats with P -values between 0.01 and 0.05. Despite these changes in inflammatory genes in the
45 hypothalamus, there were no changes in any of the oxidative damage related genes tested (data

not shown). Further, there was no change in circulating cytokines in any HS severity at 1D when compared to control animals (assessed in experiment 2 only).

Discussion

There were 3 main findings from this study: First, MILD, MOD and SEV HS were associated with chemokine and cytokine gene expression changes in the hypothalamus despite a lack of damage to this brain region at any time point. Second, the majority of gene expression changes were associated with the most robust T_c responses (hyperthermia at $T_{c,Max}$ and hypothermia in SEV HS rats), yet a very select fraction of gene alterations persisted through 10D of recovery despite normalization of T_c . Third, the only observed response during acute recovery (*experiment 2*) was associated with hypothermia in the SEV rats; despite displaying $\sim 1^\circ\text{C}$ hyperthermia through 1D, there were no gene alterations in MILD or MOD HS rats. This suggests that despite a significant body of literature showing no histological damage to the brain, and previous data in our model showing no brain damage [6], there could be molecular alterations that persist through acute recovery in the most severe animals, implicating CNS inflammation in the sequelae of HS morbidity in these severe cases.

We found that most gene alterations occurring in the hypothalamus took place at $T_{c,Max}$ (Figure 2). Interestingly, the majority of changes (70%) occurred in chemokine expression, and of these the Ccl/Ccr family represented the vast bulk of the changes (74%). Chemokines and their receptors regulate a wide range of processes, and have been shown to be critical in the inflammatory cascade [16, 17]. The Ccl family has been shown to stimulate cell migration and recruitment, act as signaling molecules in the immune system, and stimulate local inflammation [18]. While we can only speculate as to the role chemokines are playing in the hypothalamus, systemic inflammation has been shown to be a critical factor in overall HS pathology [12, 19-22], as well as in brain pathology [15, 23-27]. The Ccl family has previously been shown to be important in the inflammatory cascade in a multitude of pathologies, including HS [14, 16, 28-33]. This suggests that despite a lack of morphological damage, there could be pathologically relevant disruptions occurring in the hypothalamus after HS. It would be informative to use learning and behavioral tests, as well as brain imaging, in the future to identify causal influence(s) of increased inflammatory gene expression changes in the hypothalamus with brain function, severity and outcome.

While gene expression alterations at $T_{c,Max}$ were not unexpected based on previous work in our mouse model [12], it was surprising that most changes in *experiment 1* reflected decreases in expression regardless of time point ($T_{c,Max}$; 70% and 10D; 100%). Previous work with our mouse model showed a general increase in inflammatory gene expression at multiple time points [12]. The current findings could be caused by activation or release of chemokines/cytokines locally, causing suppression of overall gene expression (i.e. local feedback). However, we hypothesize it could also be a consequence of the hypothalamus responding to the well documented increases in circulating inflammatory proteins at this time point, such as those shown in IL-6 [13-16, 23, 34]. That is, the hypothalamus may respond to systemic factors acutely and compensate by decreasing local expression (i.e. systemic feedback). Indeed, it has been shown that there are heart, liver and gut-to-brain connections during inflammatory insults that affect homeostasis and can contribute to overall morbidity [60, 61]. Whether this is occurring in HS has yet to be shown, however there is strong evidence for a dramatic increase in

visceral inflammatory protein expression in this model, despite a lack of significantly altered systemic levels at 1D in this study [13].

It could also be that at $T_{c,Max}$ overall gene expression (not just inflammatory genes) is inhibited, as the machinery for transcription is likely suppressed by heat [22]. While this has not been measured *in vivo* with HS, it has been shown previously that *in vitro* hyperthermia inhibits DNA transcription and RNA processing, as well as other important steps in gene expression. However, two observations argue against this hypothesis. First, there were 7 genes that were significantly increased at $T_{c,Max}$ (some as much as 5- to 10-fold) indicating hyperthermia is not universally suppressing all genes. Second, two genes remained significantly suppressed at 10D of recovery despite normalization of T_c . Interestingly, hypothermia has repeatedly been associated with increased gene expression changes in the hypothalamus and other tissues [12, 32, 35-37]. More work will need to be completed to identify what mechanisms are causing the decrease in expression seen in this model at $T_{c,Max}$ and 10D.

While most of the changes occurred at $T_{c,Max}$, there were still two genes that were significantly altered at 10D, with five additional genes showing strong trends for altered responses, although these did not reach statistical significance ($P < 0.08$ & > 1.5 fold change, see Figure 1). All of these genes showed decreased expression. This could be due to several different processes. First, it could mean that there are permanent alterations in the hypothalamus transcriptional machinery, which could be causing long term dysfunction (as suggested previously). This may mean that overall hypothalamic function is permanently damaged, despite a lack of morphological abnormalities [6]. Further still, it may indicate that circulating inflammatory levels have yet to return to baseline and the hypothalamus is still responding by decreasing local expression (i.e. systemic feedback). Indeed, this is supported by a small but growing body of evidence that suggests there could be long lasting systemic organ damage that may persist months, years, or even decades after the initial insult [38]. Additional genes and overall protein expression, as well as systemic gene/protein expression at these later time points, needs to be analyzed to determine the cause of these alterations. However, our own data contradicts this, showing at 1 day there is an absence of inflammatory marker changes in plasma (experiment 2). Clearly, more work needs to be conducted to resolve these conflicting data.

It is important to note that the only rats that show an increase in gene expression at 1D (SEV animals, experiment 2) are also the only animals that developed hypothermia during recovery. This suggests that the increase in hypothalamic inflammatory gene expression may be driving hypothermia. While we did not measure responses at hypothermic depth in the SEV rats, our data at 1D and $T_{c,Max}$ in conjunction with previous mouse studies suggest that increased hypothalamic chemokine/cytokine expression is linked to the recovery T_c profile, and could be driving hypothermia in a yet undetermined manor. The key point separating our two rodent models is that hypothermia during recovery in mice is considered a regulated response and necessary for recovery [7, 34, 39], while it is a marker of severity in rats [6, 40]. However, we have previously shown in our mouse model that the greatest alterations in gene expression of the brain (and systemically) occur at hypothermia depth [12]. This may suggest that the increase in cytokines seen in rats at 1D is not a normal physiological response, but rather is a pathological condition contributing to poor overall prognosis.

In addition to measuring inflammatory gene expression, we also measured six general markers of oxidative stress in MILD, MOD and SEV animals at 1D of recovery. Contrary to previous findings from other labs [41], we found no increase in any marker of oxidative stress in any severity at this time point. While this was somewhat unexpected, especially in the SEV

animals, it does support the hypothesis that the brain is highly protected against ischemia-induced ROS damage even under severe HS conditions. It could also mean that any ROS challenges occurred at an earlier time point ($<1D$); as we did not have adequate sample to measure ROS at $T_{c,Max}$ we can only speculate to this point. Reasons for these discrepancies between our study and previous models that showed ROS damage may be that these previous models used death as an endpoint, modeled heat “shock” rather than heat stroke, and used anesthetized animals [41, 42]. The model used in the current study more accurately reflects what is occurring in human HS pathology, as the animals remain conscious and have recovery profiles that are similar to what has been documented previous in clinical case reports [8].

Experimental Procedures

Animals. Eighty-two adult male Fischer 344 rats (age 101 ± 1 days, body mass $270.9 \pm 2.1g$) were used in this study (Charles River Laboratories, Wilmington, MA). Rats were housed individually in Nalgene polycarbonate cages (Ancare, Bellmore, NY) fitted with HEPA-filter cage tops and ALPHA-dri / Cob blend bedding (PharmaServ, Framingham, MA). Environmental enrichment consisted of a rat igloo (Nalgene Nunc, Rochester, NY), a maplewood product containing a food treat to encourage foraging (W0002, BioServ, Frenchtown, NJ), and stainless steel rings for the support of postural adjustments. Rats were housed under standard laboratory conditions ($20 \pm 2^{\circ}C$, 12:12-h light:dark cycle; lights on at 0600h) in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided *ad libitum* except during heat exposure. The Institutional Animal Care and Use Committee approved all experimental procedures, which were performed in accordance with the American Physiological Society’s guiding principles for research involving animals and adhered to the *Guide for the Care and Use of Laboratory Animals*.

Radiotelemetry Measurements. Rats were intraperitoneally implanted with radiotelemetry transmitters (TL11M2-C50-PXT Physiotel® Multiplus Radiotelemetry Transmitters, Data Sciences International, St. Paul, MN) for the continuous monitoring of T_c alone (*Experiment 1*) or in combination with heart rate (HR, beats per minute [BPM]), and systolic blood pressure (SBP) as described previously [6].

Heating Protocol and Sampling Time Points. This study consisted of two phases of experimentation. For both phases, rats were acclimatized to the heating chamber (floor standing incubator; Thermo scientific; Waltham, MA) for ~ 1 day prior to experimentation to reduce potential confounding stressors (e.g., fan noises).

In *experiment 1*, conscious, unrestrained rats were exposed to an ambient temperature (T_a) of $37.3 \pm 0.2^{\circ}C$ in a floor standing incubator, in the absence of food and water, until a predetermined $T_{c,Max}$ of $41.8^{\circ}C$ was reached ($\sim 4-5$ hours; Figure 1). All rats experienced MILD HS based on the T_c response observed during recovery, which consisted of sustained hyperthermia through 2 days of recovery, as previously described [10]. Rats were sacrificed at $T_{c,Max}$ ($N=5$) for blood and whole brain harvesting or were provided food and water *ad libitum* during undisturbed recovery at T_a $20 \pm 2^{\circ}C$ until blood and whole brain were harvested at 3 days (3D, $N=7$), 5 days (5D, $N=6$), or 10 days (10D, $N=7$) of recovery (Figure 1A). Control (CON,

N=20) animals were exposed to the same experimental conditions at T_a of $20 \pm 2^\circ\text{C}$ with the timing of all procedures matched to that of a MILD HS rat.

In *experiment 2*, all rats were heated to $T_{c,\text{Max}}$ of 41.9°C (~4-5 hours; Figure 1B) with post-hoc stratification by HS severity based on maximum HR and SBP responses during heat exposure and T_c responses during recovery, as previously described [6]. Briefly, rats with MILD (N=13) HS demonstrated sustained tachycardia and normal MAP with hyperthermia ($\sim 1^\circ\text{C}$ above CON values) during recovery; MOD (N=5) HS was characterized by tachycardia and normal SBP with a biphasic rebound hyperthermia ($\sim 1^\circ\text{C}$ above CON values) during recovery; SEV (N=6) HS was characterized by the highest HR increase at $T_{c,\text{Max}}$ and hypothermia $\sim 1^\circ\text{C}$ with sustained hypotension $\sim 20\text{mmHg}$ below CON during recovery [10]. Rats were sacrificed at 1Dof recovery for blood and brain harvesting.

Blood and Brain Harvesting. Rats were deeply anesthetized under 5% isoflurane anesthesia and exsanguinated via intracardiac puncture followed by perfusion with sterile saline. Blood was placed into 1.2mL EDTA tubes and mixed. Tubes were then centrifuged for 5 minutes at 3000rcf in order to separate plasma from cellular components, and the plasma was distributed between new 1mL tubes and frozen at -80°C for further analysis.

In *experiment 1*, the whole brain was excised, frozen immediately in liquid nitrogen, and stored at -80°C for later dissection of the POAH. Briefly, each frozen brain was mounted in a cryostat and 1mm bilateral micropunches were obtained from the hypothalamus using a brain punch set (Brain Punch Set, MyNeurolab, Buffalo Grove, IL). Micropunches were transferred to a 1.5ml microcentrifuge tube on dry ice containing homogenization beads (Matrix D, MP Biomedicals, Solon, OH).

In *experiment 2*, the hypothalamus was freshly dissected upon sacrifice and the hypothalamus was frozen separately from the rest of the brain at -80°C . Previously, brain sections from those animals used in this study were assessed histologically for evidence of tissue damage by a certified veterinarian pathologist [6]. In this previous work, we found that there was no damage to any part of the brain (including hypothalamus) even in those animals with the most severe HS pathology.

Real-time PCR (rtPCR). All samples, regardless of experiment number, were analyzed using the same Profiler rtPCR arrays. All genes analyzed can be found in table 2. In short, RNA was extracted from each frozen hypothalamus using the FastPrep-24 homogenizer (MP Biomedicals, Solon, OH) and RNAqueous Micro Kit (Ambion, Grand Island, NY). RNA quantity and quality were assessed using a Nanodrop 8000 spectrophotometer (Nanodrop Products, Wilmington, DE). The 260/230 and 260/280 nm ratios were ≥ 1.8 for all RNA samples. RNA samples were reverse transcribed into cDNA (37°C for 2 h) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and the resultant cDNA was used in rtPCR experiments. Qiagen RT² Profiler PCR arrays (ARN 022ZC-12, Qiagen, Valencia, CA) were used in *experiment 1* and 2 to analyze the gene expression of cytokines and chemokines known to be important in the acute and chronic SIR (Table 1).

Further, in *experiment 2* only, the hypothalamus was also investigated for the expression of six markers of reactive oxygen species (ROS; SOD1, SOD2, SOD3, LCN2, CAT, GSR) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Changes in target gene expression were calculated as fold-change relative to CON using the $2^{-\Delta\Delta C_t}$ method. Details of gene expression analysis are described elsewhere [17].

1 *Circulating Cytokine and Chemokine Analysis (plasma).* Unfortunately, there was insufficient
2 blood plasma available to analyze circulating proteins in *experiment 1*. In *experiment 2*, a rat
3 custom multiplex kit (Bio-Rad, Hercules, CA) was used to analyze duplicate plasma samples for
4 cytokines (IFN- γ , IL-1 β , IL-6, IL-10, TNF- α) and chemokines (MCP-1, MIP-1 α , MIP-3 α) using
5 the FlowMetrix System (Luminex, Austin, TX).

6
7 *Calculations and Statistical Analysis.* Thermal strain ($^{\circ}\text{C}\cdot\text{min}$) during heating (ascending) and
8 cooling (descending) were calculated as intervals $\times 0.5$ ($^{\circ}\text{C}$ above $T_c = 40.0^{\circ}\text{C}$ at the start of the
9 interval) and Σ of the time intervals $\times 0.5$ ($^{\circ}\text{C}$ above T_c 40.0°C at the end of the interval),
10 respectively. BW was measured on a top-loading balance with an accuracy of 0.1 g. Percent
11 dehydration was calculated as $[(\text{preheat BW} - \text{postheat BW})/\text{preheat BW}] \times 100$, which is an
12 estimate that did not account for body weight loss from feces or urine. Preheat BW was obtained
13 immediately prior to heat exposure; postheat BW was obtained immediately upon removal from
14 the heating chamber at $T_{c,\text{Max}}$. Gene expression data are presented as mean fold-change. At each
15 time point, unpaired *t*-tests were conducted on normalized Ct values to compare HS to CON
16 animals only. Due to lack of responses, there was no comparison of gene expression between
17 HS animals across time points in experiment 1. Two-way ANOVA was used to discriminate
18 between the three levels of HS severity, and then unpaired *t*-tests were used to determine
19 differences between each severity group and CON, with the Bonferroni test used to help correct
20 reduce false discovery rate. Statistics were performed with Sigma Plot 12.0 (Systat Software,
21 San Jose, CA) and Qaigens publically available online analysis software (RT² Profiler PCR
22 Array Data Analysis, version 3.5). Changes in gene expression that were >2 fold with $P \leq 0.05$
23 were considered significant in experiment 1, and >2 fold with $P \leq 0.02$ in study 2. Genes with a P
24 value ≤ 0.05 were also reported for experiment 2. A heat map is included for all genes measured,
25 where intensity represents the relative magnitude of change compared to CON (higher intensity,
26 greater the magnitude of change; Figure 2). Genes highlighted in red represent decreases in
27 expression (negative fold-change), and those highlighted in green represent increases in
28 expression (positive fold-change) compared to CON.

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43
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Figure Legends

Figure 1: Experiment Outlines

A: Experiment 1: The heating protocol has been described previously [6]. Briefly, HS rats were placed in the incubator ~24hrs before the start of experimentation to provide adequate acclimatization to the new surroundings. On day 0, food and water were removed and the incubator was increased to 37°C between 0700-0730. All rats were heated to 41.8°C core temperature (T_c). Upon reaching $T_{c,Max}$ rats were removed from the heat and sacrificed at $T_{c,Max}$ or allowed to recover for 3, 5, or 10 days, at which point they were sacrificed for brain harvesting. B: Experiment 2: All protocols for experiment 2 are identical to study A, except animals were heated to 41.9°C and then sacrificed at 1D only.

Figure 2: Representative Temperature Profile through 3 Days, Heat Map and Corresponding Fold-Change Values of Genes With Significant Alterations at $T_{c,Max}$ 3, 5, & 10 Days of Recovery

A: Representative core temperature curves of control and mild heat stroke rats through 3D of recovery. Hyperthermia (solid arrow; beginning of hypothermia) persists until 3D (dashed arrow). B: Heat map showing gene expression changes in the hypothalamus of MILD HS rats at $T_{c,Max}$, 3D, 5D and 10D of recovery relative to CON values. No responses at 3D or 5D were significant, but were included in the heat map for comparison purposes. At $T_{c,Max}$ and 10D there were changes in both cytokines and chemokines, with chemokines making up the majority of significant alterations. The darker the color the greater the magnitude of change. **Green**= increased expression, **Red**= decreased expression, **White**= baseline expression (≥ -1 , ≤ 1). C: Fold change values of only those genes with significant responses (≥ 2 fold change, $P \leq 0.05$).

Figure 3 SEV HS rats were the Only Animals to Show Significant Gene Responses at 1D

A: It has been shown previously that while time to $T_{c,Max}$ does not differ between different severities, there are significant differences in T_c profiles during recovery that highly-accurately predict HS induced organ damage and overall morbidity [6]. MILD ($\blacktriangle$) animals have a significant bout of hyperthermia through 1 day of recovery, MOD ($\blacksquare$) animals have a small bout of hypothermia followed by a rebound hyperthermia, while SEV ($\bullet$) animals have significant hypothermia lasting through 1 day of recovery. Representative CON ($\blacksquare$). B: When compared to CON or MILD/MOD, SEV animals were the only animals to have any significant changes in gene expression. There were 7 genes with altered expression, representing both chemokines and cytokines. These are also the only rats to have extended bouts of hypothermia during recovery. There were no significant differences between MILD and MOD or MILD/MOD and CON. * $P \leq 0.02$ compared to CON & MILD/MOD (Bonferroni correction). $\dagger P < 0.05$ compared to CON & MILD/MOD.

Figure 1

Study A

Figure 2

B

C

	T _{max}	10D
Cebp2	2.38	
Ccl12	-15.64	
Ccl21	5.80	
Ccl24	-6.81	
Ccl28	-4.82	
Ccl3	-3.66	
Ccl4	-7.61	
Ccl6	2.03	
Ccl7	-4.06	
Ccr1	-19.59	
Ccr11	-15.78	
Ccr3	-15.81	
Ccr7	-6.96	-2.3557
Ccr8	10.31	
Ccr2	-10.67	
Cmklr1	-4.56	
Cmtm2a	3.91	
Cmtm5	-4.13	
Cxcl13	2.05	
Il1b	3.63	
Itgb2	-3.60	
Plau		-2.4209
Tgfb1	-4.91	
Thr2	-2.14	
Tnf	-10.77	

Figure 3

Rat placed in incubator for
acclimatization ~1 day
prior to heat (0700)

$T_{c,Max}$ 41.9° C
(~ 1100-1200)

1D sacrifice

End of Acclimatization,
start of heating (~0700)

B

Table 1. Characteristics of Control and Heat Stoked Rats

	Control (N=13)	Heated (N=26)
Starting Body Weight (g)	268.3 ± 3.8	270.2 ± 2.6
Dehydration at T_{c,Max} (%)	1.9 ± 0.6	7.1 ± 0.4
Time to T_{c,Max} (Min)	----	174 ± 8
Ascending Thermal Area (°·Min)	----	101.0 ± 8.8
Descending Thermal Area (°·Min)	----	18.6 ± 1.7

Data are means ± SEM. T_{c,Max}, maximum core temperature

Table 2: rtPCR Profiler Array Chemokines and Cytokines

Chemokines		Cytokines	
Gene Code	Protein Encoded Common Name(s)	Gene Code	Protein Encoded Common Name(s)
C5	Complement component 5	Fgf2	Fibroblast growth factor 2 (FGF-2)
C5ar1	Complement component 5a receptor 1 (C5a-R1)	Fpr1	Formyl peptide receptor 1 (FPR1)
Ccbp2	Chemokine binding protein 2	Gpr17	G protein-coupled receptor 17 (GPR17)
Ccl11	Eotaxin-1	Hif1a	Hypoxia-inducible factor 1, alpha subunit (HIF-1 α)
Ccl12	MCP-1-related chemokine	Ifng	Interferon gamma (IFN- γ)
Ccl17	Thymus and activation regulated chemokine (TARC)	Il10	Interleukin 10 (IL-10)
Ccl19	EBI1 ligand chemokine (ELC), macrophage inflammatory protein-3-beta (MIP-3beta)	Il16	Interleukin 16 (IL-16)
Ccl2	Monocyte chemotactic protein 1 (MCP1)	Il1b	Interleukin 1 beta (IL-1 β)
Ccl20	Liver activation regulated chemokine (LARC), Macrophage Inflammatory Protein-3 (MIP3A)	Il4	Interleukin 4 (IL-4)
Ccl21	Exodus-2, secondary lymphoid-tissue chemokine (SLC)	Il6	Interleukin 6 (IL-6)
Ccl22	Stimulated T-Cell Chemotactic Protein 1	Il8ra	Interleukin 8 receptor, alpha (IL-8R)
Ccl24	Eotaxin-2, Myeloid Progenitor Inhibitory Factor 2	Itgam	Integrin, alpha M
Ccl25	Thymus-Expressed Chemokine (TECK)	Itgb2	Integrin, beta 2 (CD18)
Ccl28	Mucosae-Associated Epithelial Chemokine (MEC)	Kdr	Kinase insert domain receptor (KDR)
Ccl3	Macrophage Inflammatory Protein-1 (MIP1A)	Mapk1	Mitogen activated protein kinase 1 (MAPK-1)
Ccl4	Macrophage Inflammatory Protein 1-Beta (MIP1 β)	Mapk14	Mitogen activated protein kinase 14 (MAPK-14)
Ccl5	Regulated on activation, normal T cell expressed and secreted (RANTES)	Pf4	Platelet factor 4 (PF4)
Ccl6	Chemokine (C-C motif) ligand 6*	Plau	Plasminogen activator, urokinase (PLAU)
Ccl7	Monocyte Chemotactic Protein 3 (MCP-3)	Ppbp	Pro-platelet basic protein (PPBP)
Ccl9	Macrophage Inflammatory Protein-1 γ (MIP-1 γ)	Prkca	Protein kinase C, alpha
Ccr1	Macrophage inflammatory protein 1 alpha receptor (MIP-1 α -R)	Slit2	Slit homolog 2 (SLIT2)
Ccr10	Chemokine (C-C motif) ligand 10*	Tgfb1	Transforming growth factor, beta 1 (TGF- β 1)
Ccr11	Macrophage inflammatory protein 1 alpha receptor-like 1 (MIP-1 α -RL1)	Tlr2	Toll-like receptor 2 (TLR2)
Ccr2	Monocyte Chemoattractant Protein 1 Receptor (MCP-1R)	Tlr4	Toll-like receptor 4 (TLR4)
Ccr3	Eosinophil Eotaxin Receptor	Tnf	Tumor necrosis factor (TNF superfamily, member 2)
Ccr4	Chemokine (C-C motif) ligand 4*	Tymp	Thymidine phosphorylase
Ccr5	HIV-1 Fusion Coreceptor	Vegfa	Vascular endothelial growth factor A (VEGF)
Ccr6	G-Protein Coupled Receptor 29	Xcll	Lymphotoxin (LTN)
Ccr7	MIP-3 Beta Receptor		
Ccr8	Chemokine Receptor-Like 1		
Ccr9	Chemokine (C-C motif) ligand 9*		
Ccr12	Putative MCP-1 Chemokine Receptor		
Cklf	Chemokine-like factor*		
Cmklrl	Chemerin Receptor 23 (ChemR23)		
Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A*		
Cmtm3	CKLF-like MARVEL transmembrane domain containing 3*		
Cmtm5	CKLF-like MARVEL transmembrane domain containing 5*		
Cx3cl1	Fractalkine		
Cx3cr1	Fractalkine Receptor		
Cxcl1	Neutrophil-Activating Protein 3 (NAP-3)		
Cxcl10	Interferon gamma-induced protein 10 (IP-10)		
Cxcl11	Interferon gamma-induced protein 9 (IP-9)		
Cxcl12	Stromal cell-derived factor 1 (SDF-1)		
Cxcl13	B lymphocyte chemoattractant (BLC), B cell-attracting chemokine 1 (BCA-1)		
Cxcl14	Breast and kidney-expressed chemokine (BRAK)		
Cxcl16	Chemokine (C-X-C motif) ligand 16*		
Cxcl2	Macrophage Inflammatory Protein 2-Alpha (MIP2 α)		
Cxcl3	Macrophage inflammatory protein-2-beta (MIP2 β)		
Cxcl5	Epithelial-derived neutrophil-activating peptide 78 (ENA-78)		
Cxcl9	Monokine induced by gamma interferon (MIG)		
Cxcr2	Interleukin 8 receptor, beta (IL-8R- β)		
Cxcr3	Interferon-Inducible Protein 10 Receptor (IP-10-R), CD183		
Cxcr4	Fusin, CD184		
Cxcr5	Burkitt lymphoma receptor 1 (BLR1), CD185		
Cxcr6	Cluster of differentiation 186 (CD186)		
Cxcr7	Chemokine Orphan Receptor 1 (CMKOR1)		

*No universally accepted common protein names

Table 3: Genes with Significant Changes and Summary of *in vivo* Actions of Encoded Proteins

Gene Code	Encoded Chemokine/Cytokine Name	Summary of Actions
<i>Chemokines</i>		
C5, c5ar1	Complement Component 5, Complement Component Receptor A	Complement immune system; chemotaxis; inflammation
Ccbp2	Chemokine binding protein 2	Immune cell recruitment; inflammation;
Ccl2, Ccl4, Ccl6, Ccl7, Ccl12, Ccl21, Ccl24, Ccl28	Chemokine (c-c motif) ligand 2, 4, 6, 7, 12, 21, 24, 28	Immune cell recruitment; inflammation; allergic reactions
Ccr1, Ccr11, Ccr2, Ccr3, Ccr6, Ccr7, Ccr8,	Chemokine (c-c motif) receptor 1, 1-like-1, 2, 3, 6, 7, 8	Chemokine receptors; recruitment of immune cells; inflammation
Cxcl16, Cxcl5	Chemokine (C-X-C motif) ligand 15, 16	Cell reruitment; inflammation
Cmklr1	Chemokine like receptor 1	Inflammation; adipogenesis signaling
Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A	Transmembrane trafficking; inflammation; development; growth
Cmtm5	CKLF-like MARVEL transmembrane domain containing 5	Transmembrane trafficking; apoptosis; inflammation
Cxcl13	Chemokine (C-X-C motif) ligand 13	Immune function; B cell chemotactic
<i>Cytokines</i>		
Il1b	Interleukin 1 beta	Pro-inflammatory; apoptotic; anti-proliferative
Itgb2	Beta 2 Integrin	Pro-inflammatory; Leukocyte recruitment
Plau	Plasminogen activator	Prevents fibrinolysis
Tgfb1	Transforming growth factor, beta 1	Pro-growth; control of immune system; apoptosis- multitude of other functions
Tlr2	Toll-like receptor 2	Immune function; antigen recognition
Tnf	Tumor Necrosis Factor -alpha	Pro-inflammatory; apoptotic; anti-proliferative; anti-growth
Ifng	Interferon gamma	Immune function; inflammation
Il8ra	Interleukin 8 receptor, alpha	Interleukin 9 receptor; chemotaxis; immune function; inflammation
PpBp	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	Mitosis; platlet activation; growth