

Sex-dependent effects of extreme temperature exposures in rats and evaluation of tDCS as a countermeasure

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This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs through the Clinical Research Intramural Initiative (CRII) Women's Health Research Award (MWHRA) under award number CDMRPL-17-0-DM170693, work unit number H1735. The views expressed in this presentation are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. The study protocol was reviewed and approved by the Wright-Patterson Air Force Base (WPAFB) Institute of Research Institutional Animal Care and Use Committee (IACUC) in compliance with all applicable Federal regulations governing the protection of animals in research and conducted in an AAALAC-accredited facility.

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REPORT DOCUMENTATION PAGE					<i>Form Approved</i> OMB No. 0704-0188							
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Service Directorate (0704-0188). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.												
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1. REPORT DATE (DD-MM-YYYY)		2. REPORT TYPE DTIC Report			3. DATES COVERED (From - To) March 2018-December 2021							
4. TITLE AND SUBTITLE Sex-dependent effects of extreme temperature exposures in rats and evaluation of tDCS as a countermeasure				5a. CONTRACT NUMBER CDMRPL-17-0-DM170693								
				5b. GRANT NUMBER								
				5c. PROGRAM ELEMENT NUMBER								
6. AUTHOR(S) Nathan M. Gargas, Victoria T. Ethridge, Molly K. Miklasevich Shawn M. McInturf, Raquel J. Moore, Candice Hatcher-Solis, Joyce G. Rohan				5d. PROJECT NUMBER								
				5e. TASK NUMBER								
				5f. WORK UNIT NUMBER H1735								
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Medical Research Unit Dayton (NAMRU-D) Environmental Health Effects Laboratories 2728 Q Street, Area B, Building 837 Wright Patterson Air Force Base, OH 45433					8. PERFORMING ORGANIZATION REPORT NUMBER							
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S) CDMRP							
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)							
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution A												
13. SUPPLEMENTARY NOTES												
14. ABSTRACT This study assessed the possibility of using transcranial direct current stimulation (tDCS) as a mitigating factor against the adverse effects of extreme temperature exposures. Repeated intermittent exposures to cold differentially affected female and male rats in short term hippocampal plasticity, water maze performance, brain levels of GABA and plasma levels of inflammatory markers. Similar effects of repeated cold exposures in female and male rats were also observed. An increase in serotonin, decrease in dopamine levels and increase in IL-4 were detected in both female and male rats. Repeated intermittent exposures to heat differentially affected female and male rats in water maze performance, brain levels of glutamate and GABA, and plasma levels of C3 and TNF- α . Similar effects of repeated heat exposures in female and male rats were also observed. An increase in motor activity, increase in serotonin, decrease in dopamine, and an increase in IFN-g, IL-10 and IL-4 were detected in both female and male rats. Female rats experienced a decreased estrogen level resulting from repeated cold or heat exposures. However, our overall data showed that the tDCS used here was not an effective countermeasure in reversing the adverse effects resulting from repeated intermittent cold exposures.												
15. SUBJECT TERMS extreme cold, intermittent cold exposure, heat exposure, tDCS, cognition, hippocampus, cytokines, rats												
16. SECURITY CLASSIFICATION OF: <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%; padding: 2px;">a. REPORT</td> <td style="width: 33%; padding: 2px;">b. ABSTRACT</td> <td style="width: 33%; padding: 2px;">c. THIS PAGE</td> </tr> <tr> <td style="text-align: center; padding: 2px;">unclassified</td> <td style="text-align: center; padding: 2px;">unclassified</td> <td style="text-align: center; padding: 2px;">unclassified</td> </tr> </table>			a. REPORT	b. ABSTRACT	c. THIS PAGE	unclassified	unclassified	unclassified	17. LIMITATION OF ABSTRACT		18. NUMBER OF PAGES	
a. REPORT	b. ABSTRACT	c. THIS PAGE										
unclassified	unclassified	unclassified										
					19a. NAME OF RESPONSIBLE PERSON							
					19b. TELEPHONE NUMBER (Include area code)							

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SUMMARY

This study assessed the possibility of using transcranial direct current stimulation (tDCS) as a mitigating factor against the adverse effects of extreme temperature exposures. Extreme temperatures were chosen as our initial stressors because they are often faced by our Service Members (SMs) and because there are well-documented differences in how men and women respond to acute and prolonged cold and heat. SMs can be deployed to places such as the western Himalayas where winter temperature ranges from -35 to -55°C and summer temperature reaching as high as 40°C in Afghanistan. Prolonged exposure to heat and/or cold can have negative impact on cognition and thus mission success. Heat related injuries have been documented in the military among service members deployed to Iraq or Afghanistan (Armed-Forces-Health-Surveillance-Branch, 2017). Cold weather injuries have also been documented in the military (Armed Forces Health Surveillance, 2020). Non-invasive brain stimulation such as tDCS can help maintain the psychological and physical fitness of military personnel during these harsh conditions which is of utmost importance in ensuring optimal performance and health of our troops as well as success of their missions.

Our first objective was to characterize the sex-dependent effects of repeated cold or heat exposures on behavioral performance and various biochemical endpoints. Our second objective was to determine whether tDCS can be used to reverse the effects that are induced by repeated exposures to a cold or hot environment. The specific aims for this study were:

- Specific Aim 1: Characterize the sex-dependent differences in tDCS effects on neuroplasticity.
- Specific Aim 2: Determine the sex-dependent differences in cognitive effects of temperature-induced stress.
- Specific Aim 3: Evaluate the mitigating effects of tDCS in cognitive decrements resulting from cold- or heat-induced stress in female rats.

We have previously published work demonstrating that tDCS can enhance synaptic plasticity of male rats. Here, we used electrophysiology and behavioral testing to show that tDCS can also enhance synaptic plasticity in female rats. We then exposed both female and male rats to either room temperature (~20-25 °C), cold (~4°C, 6 hours per day) or heat (~38 °C, 1 hour per day) for 21 consecutive days. Cognitive effects of temperature-induced stress were measured and compared using electrophysiology, behavioral testing and biochemistry assays. On a subgroup of rats we performed tDCS prior to the cold or heat exposure to determine whether tDCS was successful in mitigating the adverse cognitive effects induced by cold or heat stress.

We found that 30 min of 0.2 mA anodal tDCS enhanced LTP in female rats similar to male rats. Repeated intermittent exposures to cold differentially affected female and male rats in short term hippocampal plasticity, water maze performance, brain levels of GABA and plasma levels of inflammatory markers (C3, IFN- γ , IL-10, IL-13, TNF- α). Similar effects of repeated cold exposures in female and male rats were also observed. An increase in serotonin, decrease in dopamine levels and increase in IL-4 were detected in both female and male rats. Repeated intermittent exposures to heat differentially affected female and male rats in water maze performance, brain levels of glutamate and GABA, and plasma levels of inflammatory markers (C3, TNF- α). Similar effects of repeated heat exposures in female and male rats were also observed. An increase in motor activity, increase in serotonin, decrease in dopamine, and an increase in IFN- γ , IL-10 and IL-4 were detected in both female and male rats. Furthermore, female rats experienced a decreased estrogen level resulting from repeated cold or heat exposures.

The tDCS parameters used in this study (0.25 mA, 30 min) did not support the use of tDCS as an effective countermeasure strategy in reversing the adverse resulting from extreme temperature exposures. Overall, rats treated with tDCS experienced similar changes as control rats (SHAM) following exposures to cold or heat. However, administration of tDCS did induce small but statistically significant effects in several measured parameters that may or may not indicate partial mitigating effects. Detected effects of tDCS in this study were: (1) male rats that were exposed to room temperature (RT) when subjected to tDCS had a trending decrease in stereotypical behavior whereas male rats previously exposed to cold then tDCS had a statistically significant increase in stereotypical behavior, (2) administration of tDCS improved water maze performance in female rats but worsened water maze performance in male rats, (3) female rats exposed to cold had significant reduction in plasma estradiol level whereas female rats that were subjected to cold then tDCS had only trending reduction in plasma estradiol that were no longer statistically significant, (4) male rats that were exposed to cold or heat, when subjected to tDCS, did not experience the cold/heat-induced reduction in brain dopamine levels, (5) female rats subjected to tDCS experienced a decreased average plasma TNF- α level compared to SHAM female rats, independent of temperature exposures. Although tDCS did not reverse many of the temperature-mediated changes, our data on dopamine and estrogen showed a mitigating potential of tDCS and warrant follow-on studies using perhaps higher tDCS intensity and/or repeated consecutive tDCS sessions as a countermeasure.

BACKGROUND

Transcranial direct current stimulation

Transcranial direct current stimulation (tDCS) is a non-invasive method of brain stimulation with a promising potential as a therapeutic or countermeasure against several neurological symptoms induced by traumatic brain injury (Clayton et al., 2016) and neurological diseases such as Alzheimer's Disease, Parkinson's Disease, stroke, epilepsy, and depression (Floel, 2014, An et al., 2017, Peanlikhit et al., 2017). A majority of tDCS related studies have been performed in human subjects. There are two types of tDCS (anodal versus cathodal) differ in the direction of current flow. Anodal tDCS is where a positive stimulation is delivered inducing depolarization of neuronal membrane potential that enhances excitability of affected neurons but still is below the threshold for action potential firing (Nitsche et al., 2008). In contrast, cathodal tDCS is where a negative stimulation is delivered inducing hyperpolarization of neuronal membrane potential and decreasing the excitability of affected neurons (Nitsche et al., 2008). In this study we administered only anodal tDCS and not cathodal tDCS. The consensus in the field is that tDCS when applied at sufficient intensity and duration can alter neuroplasticity of affected neurons. Simplistically, neuroplasticity can be viewed as the ability of our brain to undergo changes. As we develop, learn and experience, the connectivity of neurons and cells within our nervous system is being altered, and we refer to this phenomena as neuroplasticity or also referred to as synaptic plasticity. Anodal tDCS is oftentimes associated with enhancement of neuroplasticity whereas cathodal tDCS has been associated with depression of neuroplasticity. Neuroplasticity can be measured in human subjects by recording evoked response following a stimulus, which is unlike the typical electroencephalography (EEG) or electromyography (EMG) which measure only spontaneous (non-evoked) activities. Evoked response recording in humans is limited in response size mainly due to the non-invasive positioning of wires typically placed on top of the scalp, and can only record the summated signal from a large population of neurons and surrounding glia. A more invasive positioning of wires or probes would elicit a stronger signal from a smaller population of neurons and glia. In an animal model, neuroplasticity can be measured more effectively mainly because we can position electrodes invasively into the animal brain, brain slices or even single neurons. Neuroplasticity in animals is often reported as long term potentiation (LTP) which corresponds to strengthening of synapse and long term depression (LTD) which corresponds to

weakening of synapse. LTP is believed to be the molecular basis for learning and memory and both LTP and LTD of synapses throughout the brain occur continuously in the developing as well as mature brain. Mechanism of LTP and LTD are believed to involve changes in expression of critical synaptic receptors at the post-synaptic dendrites mainly the glutamate receptors AMPA or NMDA. Paired pulse facilitation (PPF) is another form of neuroplasticity that can be easily assessed in animals. Whereas LTP and LTD are forms of permanent or long term neuroplasticity, PPF is a form of short term neuroplasticity. Mechanism of PPF is believed to center around the pre-synaptic terminal involving changes in the machinery and dynamics of neurotransmitter release.

While there are multitudes of studies reporting the benefits of tDCS on neurological symptoms, studies evaluating the use of tDCS to augment performance in healthy subjects are relatively sparse. A study performed at Wright Patterson Air Force base found that subjects treated with anodal tDCS (2 mA for 30 minutes, dorsolateral prefrontal cortex) significantly improved their information processing capability resulting in enhanced performance when compared to subjects that were treated with SHAM (2 mA for only 30 seconds) (Nelson et al., 2016). A separate study also reported that tDCS (2 mA, 20 min) over the same region (dorsolateral prefrontal cortex) provided some improvement in memory monitoring (Chua and Ahmed, 2016). Although tDCS has been shown to mitigate neurological impairments and enhance cognitive performance, the underlying mechanism remains unclear. We have an ongoing project to elucidate the cellular and molecular mechanism(s) by which tDCS exerts its effect in an animal model. We discovered that a 0.25 mA anodal tDCS for 30 minutes induced an enhancement in synaptic plasticity in male Sprague Dawley rats (Rohan et al., 2015, Rohan et al., 2020). However, we have only evaluated effects using exclusively male rats in those previous studies. Since there are several documented differences in the development, mechanism and modulation of neuroplasticity between the two sexes (Juraska, 1998), assessments of tDCS effects on neuroplasticity in female rats are also needed. Thus, our first aim of this study was to determine the optimal tDCS dosing that will elicit a robust enhancement of synaptic plasticity in female rats.

Extreme temperature exposure

A major environmental stressor that is often faced by military personnel is exposures to prolonged cold or heat. Troops can be deployed to places such as the western Himalayas where winter temperature ranges from -35 to -55°C in some regions (Jayaswal et al., 2001). In 2016, there were over 2,500 diagnoses of heat illness among service members (Armed-Forces-Health-Surveillance-Branch, 2017). During 2012-2016, a total of 572 diagnoses of heat injuries were documented among service members deployed to Iraq or Afghanistan. Although the heat stroke incident rates were highest among males, the incident rates of other heat illnesses were in fact highest among females in 2016 (Armed-Forces-Health-Surveillance-Branch, 2017). Therefore, the maintenance of psychological and physical fitness of military personnel during these harsh conditions is of utmost importance in ensuring optimal performance and health of our troops and success of their missions. There already exist regulations to prevent cold and heat casualties (TRADOC-Regulation-350-29, 2016) as well as limited studies assessing effects of extreme cold or heat on military performance (Jayaswal et al., 2001, Radakovic et al., 2007, Paulus et al., 2009). Excessive exposures to cold or heat can adversely impact human cognition such as accuracy, memory, attention, and/or executive function (Enander and Hygge, 1990, Taylor et al., 2015). Sensitivity and tolerance to cold and heat differ in men and women mainly due to differences in hormone, body/tissue compositions such as fat and muscle, and other physiological parameters such as blood flow and core temperature (Xiong et al., 2015). Therefore, the main objective of our proposal was to explore the possibility that tDCS can be used as a mitigating factor against neurological impairments induced by harsh environmental stressors in the female and male rats.

Stress and Cognition

Temperature-induced stress has a detrimental impact on performance and other cognitive function such as learning and memory (Enander and Hygge, 1990; Radakovic et al., 2007). According to a 1990 review by Enander and Hygge, simple reaction time increased with heat exposures and is barely affected by cold temperatures. In contrast, complex reaction time slows down with heat and greater errors were made in cold environment. Vigilance and tracking tasks were found to be optimally performed at an effective temperature of 27-32°C, where effective temperature takes into account the air temperature (dry bulb temperature), humidity and air velocity. However, learning tasks were optimized at a lower temperature of 21°C, whereas creative tasks were optimized at a higher temperature of 27°C. Memory registration progressively declined with decreasing core temperature from a level of about 36.7°C (Enander and Hygge, 1990, Radakovic et al., 2007). Heat exposure (40°C) can also impair cognitive performance in soldiers (Radakovic et al., 2007).

Using animal models, detailed characterization of the physiological responses to cold or heat stress can be performed. Cold exposures tend to decrease the rates of enzymatic reactions, diffusion and membrane transport, whereas heat exposures tend to increase those rates (Sonna et al., 2002). Interestingly cold and heat exposures can also induce similar cellular stress response such as slowing down cell cycle progression, increased denaturation and disaggregation of proteins, alterations in metabolism leading to decreased ATP, disruption in cytoskeletal elements and changes in membrane permeability resulting in increased sodium and calcium in cytosol (Sonna et al., 2002). Gene expression changes in critical protein such as heat shock factors (HSF), heat shock proteins (HSP) and cytokines have also been reported due to thermal stress (Sonna et al., 2002).

Chronic intermittent cold stress (CIC, 4°C 6 h/day, 14 days) in male Sprague-Dawley rats induced a deficit in reversal learning in the attentional set-shifting test which is a measure of cognitive flexibility in humans (Lapiz-Bluhm et al., 2009, Danet et al., 2010). Impairment in reversal learning coincided with decreased serotonin concentration and can be mitigated with the anti-depressant (selective serotonin reuptake inhibitor) citalopram or CIT (Lapiz-Bluhm et al., 2009). A separate study reported that CIC in male rats sensitized neuro-immune responses and such immune enhancement may contribute to the dysregulation of cognitive processes (Girotti et al., 2011). Neurodegeneration of CA1 cells of male Wistar rats was also observed resulting from chronic cold water exposures (Kumar et al., 2015).

Exposure of rats to 4 hours of 38°C heat resulted in increased blood brain barrier permeability with decreased blood flow in multiple regions including the hippocampus (Sharma and Dey, 1987). That same study also reported an increase in levels of brain serotonin or also known as 5-hydroxytryptamine (5-HT). A more recent study reported that continuous exposure to heat (32°C) for up to 21 days induced significant changes in neurotransmitter levels within multiple brain regions in male Wistar rats (Nakagawa et al., 2016). For example, noradrenaline level in the caudate putamen region was altered only after 3 hours of exposures. Similarly, serotonin and dopamine levels also increased within the hippocampus after 28 days of heat exposures. Intermittent exposures to 40°C over 7 or 21 days have been shown to be detrimental to central nervous system (CNS) development eliciting degeneration of dendrites and neuronal necrosis at the cerebral cortex, spinal cord as well as cerebellar layers (Ahmed, 2005). In addition, 4 days of continuous exposure to 31°C resulted in abnormal fear expression (increased freezing) as well as synaptic changes in the prefrontal cortex and hippocampus of female rats (Gruene et al., 2014). Increased blood brain permeability was also reported in rats exposed to acute heat (4 hr at 38°C) or chronic heat (34-36°C for 12 hours per day for 7 days) (Sharma and Dey, 1986). Neuro-inflammation also occurs during prolonged heat exposures resulting in increases in blood levels of IL-2 and IL-12 when rats were exposed to 8 hours of 32 °C environment for 7 days (Wang et al., 2015).

Using tDCS as countermeasure against environmental stressors

No one has yet evaluated the possibility of tDCS serving as a countermeasure against the adverse neurological effects of environmental stressors. As previously discussed, there are numerous studies employing tDCS as a therapeutic measure against several neurological symptoms from multiple diseases. One that is most relevant to this proposal is its potential benefit in combating against depression. Studies thus far have shown some promise in the ability of tDCS to counteract depression, and there are ongoing clinical studies to further characterize the scope with which tDCS can be used to mitigate depression (Shiozawa et al., 2014, Padberg et al., 2017). An effective pharmacological intervention for depression is a selective serotonin reuptake inhibitor (SSRI) and thus we hypothesize that one mechanism of tDCS-induced enhancement in neuroplasticity involves the serotonergic pathway. We know that serotonin is an essential neurotransmitter that is absolutely critical in brain development, network configuration and neuroplasticity in both the immature and mature brain (Lesch and Waider, 2012). Interestingly, healthy subjects who were chronically given citalopram (CIT), a type of SSRI, had prolonged LTP-like plasticity enhancement for over 24 hours following anodal tDCS (Kuo et al., 2016). Although increases in serotonin levels have not been reported, there is evidence that tDCS can induce increases in extracellular dopamine concentration in the rat striatum (Tanaka et al., 2013).

Previous studies also support involvement of the serotonergic and dopaminergic pathways in mediating the adverse effects of cold- or heat-induced stress (Sharma and Dey, 1986, Girotti et al., 2011, Nakagawa et al., 2016). In fact, administration of the anti-depressant CIT has been shown to ameliorate the learning impairments induced by cold exposures in rats (Lapiz-Bluhm et al., 2009, Danet et al., 2010). It is this convergence in effects elicited by tDCS as well as temperature stressors that formed the rationale for this study. Since both tDCS and extreme temperatures have the capability to modulate serotonergic and dopaminergic pathways in opposing directions, we hypothesized that tDCS can be used to at least partially reverse some of the adverse effects triggered by cold or heat stressors.

Sex-dependent differences in effects induced by tDCS and extreme temperatures

Although numerous works have documented sex-dependent differences in molecular mechanism and modulation of neuroplasticity, there are only a very limited number of studies that directly compared differences in tDCS-induced effects in male versus female human subjects or animals. We know that there are vast differences in morphology of as well as the number of cells present in several regions of the female and male brain (Juraska, 1998). More importantly, dendritic branching and patterns of network connectivity within the hippocampus of female rats differ than those male rats and it is believed that these differences may account for the differences in learning and memory between men and women (Juraska, 1998). Estrous cycle can also affect the number of dendritic spines of hippocampal neurons from female rats (Juraska, 1998). The hormone estrogen plays a critical role in synaptic plasticity and has generally been associated with having uplifting effects on mood such that it serves as an effective countermeasure against depression in human as well as animal models (Smith, 1994). Estrogen's role in hippocampus-dependent spatial learning and memory as well as sensorimotor functions has been well-established (Smith, 1994). Another study found that mice deficient in brain derived neurotrophic factor (BDNF) and serotonin transporter (SERT) have significant differences in levels of serotonin and dopamine in the hippocampus, striatum, hypothalamus and brain stem regions of the brain as well as distinct anxiety-like behaviors (Ren-Patterson et al., 2006). Thus, if the foundational mechanism of neuroplasticity is sex-dependent, it is reasonable to assume that tDCS effects on neuroplasticity are likely to be sex-dependent as well. Chaieb et al., (2008) reported some differences in which tDCS in the visual cortex of healthy human subjects altered short-term neuroplasticity (Chaieb et al., 2008). Another study demonstrated sex-dependent differences in how tDCS over the medial prefrontal cortex

influence social cognitive ability (Adenzato et al., 2017). The first aim of this proposal addressed this question, assessing the mechanism of tDCS effects in female rats and comparing them to our previous data obtained from male rats.

It has been well documented that women and men respond to an identical stressor distinctly. Although certain cellular events may be identical in both sexes such as the catecholamines (i.e., adrenaline) released from the adrenal medulla and glucocorticoids (corticosterone in rodents, cortisol in human) secreted from the adrenal cortex, how those changes in signaling pathways influence cognitive function such as learning and memory in each sex can be quite distinct (Guenzel et al., 2014). There are multiple types of learning and memory and those distinct types appeared to be susceptible to stress differentially in the two sexes. The hippocampus-dependent spatial learning and memory in women is more susceptible to disruption by stress compared to that in men whereas the striatum-dependent stimulus-response learning and memory in men is more susceptible to disruption by stress compared to that in women (Guenzel et al., 2014).

Extreme temperature exposures can induce stress by activating similar signaling pathways as other stressors (Cure, 1989, Taylor et al., 2015). Peripheral responses to extreme temperature differ between men and women, with women having lower finger skin temperature and blood perfusion rate compared to men (Bartelink et al., 1993). Sex-dependent differences in endocrine response to cold stress have also been reported. Increases in plasma level of epinephrine and serum level of cortisol in men but not in women have been reported due to cold stress (Solianik et al., 2015). Cognitive perturbation resulting from cold stress was more prominent in men compared to women (Solianik et al., 2015). In response to heat stress, men tend to lose greater body weight and plasma volume (Rocker et al., 1978).

Studies in animal models exploiting sex-dependent differences in response to cold or heat stress are somewhat more limited. A previous study using Wistar rats and synchrotron radiation microangiography revealed that arteries of female rats did not dilate as much as those of male rats in response to cold exposures, which may partially explain why women are more sensitive to cold (Sato et al., 2008, Xiong et al., 2015). Continuous heat exposure to 34°C leads to significant increases in corticosterone levels after 30 and 60 minutes (Cure, 1989). Heat shock response and heat acclimation are two mechanisms by which we adapt to chronic heat exposures. Although we are capable of compensating chronic heat exposures to some degree by physiological and molecular adaptations such as by reducing metabolic rate and modifying gene expression of critical proteins including heat shock proteins, there can still be degradation of performance and cognitive functions. Here, we characterized cognitive deficits induced by both cold and heat exposures on female and male rats and assess how they differ from male rats and evaluate the potential for tDCS as a countermeasure against those adverse cognitive effects.

The main objectives of this study were to evaluate sex-dependent cognitive effects in extreme cold and heat environments and evaluate biochemical changes in neurotransmitter and cytokine levels in female and male rats. We will investigate whether tDCS can serve as a countermeasure against the adverse effects induced by extreme temperature exposures. Our specific aims are:

Aim 1: *Characterize the sex-dependent differences in tDCS effects on neuroplasticity.* Based on our previously published data from male rats, our hypothesis was that 30 minutes of 0.25 mA tDCS would result in enhancement of long term potentiation (LTP).

Aim 2: *Determine the sex-dependent differences in cognitive effects of temperature-induced stress.* Temperature-induced stress were induced in the female and male rats by exposing them to either room temperature (20-25°C), cold (~4°C) or heat (~38°C) for 6 hours per day for 21 consecutive days. Based on

previously published work, we hypothesized that repeated intermittent exposures to cold or heat would induce measurable reductions in synaptic plasticity as assessed by electrophysiology, deficits in cognitive function as assessed by behavioral tasks, and changes in corticosterone as well as neurotransmitter levels (serotonin, dopamine) as assessed by standard biochemical assays. Degrees of thermal-induced cognitive impairment were predicted to differ slightly between the sexes.

Aim 3: *Evaluate the mitigating effects of tDCS in cognitive decrements resulting from cold- or heat-induced stress in female rats.* Our hypothesis was that rats that were given 30 minute sessions of tDCS (0.25 mA) would mitigate some of the effects of repeated cold and/or heat exposures in female and male rats. The degree of reversal by tDCS was predicted to slightly differ between the sexes.

METHODS

Animals

All animals were held and treated according to Wright Patterson Air Force Base (WPAFB) Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines. The study protocol was reviewed and approved by the WPAFB IACUC in compliance with all federal regulations governing the protection of animals and research.

Male and female Sprague-Dawley rats (*Rattus norvegicus*) were purchased from Charles River (Wilmington, MA). A total of 366 animals were used for this project. The rats were approximately 5 weeks old at receipt and were pair-housed in plastic, solid bottom cages. Upon receipt, the rats immediately entered a quarantine and acclimation period for 7-10 days. During this period, each animal was observed twice daily for mortality, morbidity, and changes in general appearance or behavior. Animals were identified by cage card and tail marking with a unique number assigned upon receipt. Rats were weighed the day of receipt and at least weekly thereafter.

Animal housing room conditions were maintained at a temperature and relative humidity in accordance with the recommendations of the National Research Council's *Guide for the Care and Use of Laboratory Animals*, with approximately 15 complete air changes per hour, and a 12 hour light/dark cycle. Animals were fed a certified rodent diet, *ad libitum*, except during stress exposures and neurobehavioral testing. Reverse osmosis purified municipal tap water was available at all times including during cold or heat exposures.

Female and male rats were assigned to the (1) control or room temperature group (RT), (2) cold group (COLD), or (3) heat group (HEAT). Rats were individually housed following surgical procedures for the tDCS/SHAM rats and at start of exposures for the remaining rats. This was needed to preserve the welfare of the animals and integrity of the study by preserving the surgical site, preventing injury from potential surgery-induced aggression, and reducing the risk of unintentional disturbance (via licking or rubbing) of surgical sites. Rats were also be housed singly once exposure was initiated. Individual housing minimized variations from social interactions that can affect study data, especially those data arising from electrophysiology, biochemical and neurobehavioral testing.

Exposures

Animals were exposed to cold (4°C), heat (38°C), or room temperature (RT, 22-24 °C) for 6 hours, 1 hour, and 6 hours, respectively. Exposures were conducted over 21 consecutive days. During exposures, all enrichment was removed. Enrichment was returned to all animals following exposures. During exposures, animals had access to food and water ad libitum. Additionally, COLD and control (RT) animals were observed every hour for clinical observations. HEAT animals were observed every 15 minutes. Following exposures, animals were returned to their home-rack. It should be noted that for Aim 3 male rats, the heat chamber malfunctioned. Therefore, the cold chamber was converted to the heat chamber. The cold animals were then exposed in the walk-in cold room that maintained the proper cold temperature of 4°C.

Environmental chambers for temperature exposures

NAMRU-D has two TSE Systems environmental chambers. Each chamber consists of a display showing temperature and humidity. In addition to the chamber display, we monitor temperature and humidity using additional sensors placed on the top and bottom shelf of each chamber. Chamber #1 was designated as the cold exposure chamber, with temperature set to 4°C. Chamber #2 was designated as the heat exposure chamber, with temperature set to 38°C. A bucket full of water was placed on each shelf of the heat chamber (chamber #2) to increase humidity. Control exposures were done at room temperature (RT, 22-24 °C). Chamber and room temperatures were monitored using MadgeTech RHTemp1000IS (Intrinsically Safe Temperature and Humidity Data Logger), ran by MadgeTech 4 Data Logger Software (Version 4.2.13.1). Chamber #1 reached 4 °C (chamber display) at approximately 89 minutes for the top shelf and at approximately 20 minutes for the bottom shelf. Animals were placed inside the cold chamber after 90 minutes so that the target temperature was reached on both shelves. Independent MadgeTech probe placed on the lower shelf read 3.6-3.7 °C, whereas probe placed on the top shelf read 4 °C ([Figure 1](#)). To compensate for this slight differential in temperature, we rotated animal placements between the bottom and top shelf. Chamber #2 reached 38 °C (chamber display) at approximately 70 minutes for the top shelf and at approximately 22 minutes for the bottom shelf. Animals were placed inside the heat chamber after 70 minutes so that the target temperature was reached on both shelves. Independent MadgeTech probe placed on the lower shelf read 39 °C, whereas probe placed on the top shelf read 38.5 °C ([Figure 2](#)). To compensate for this slight differential in temperature, we rotated animal placement between the bottom and top shelf. A bucket of water was added to each shelf to help raise the humidity within the chamber.

Figure 1. Exposure chamber #1. Graphs plotting temperature and humidity obtained from MadgeTech probes placed on the top and bottom shelves of the cold chamber (chamber #1). Animal cages were rotated from accordingly to compensate for the slight ≤ 0.4 °C difference in temperature.

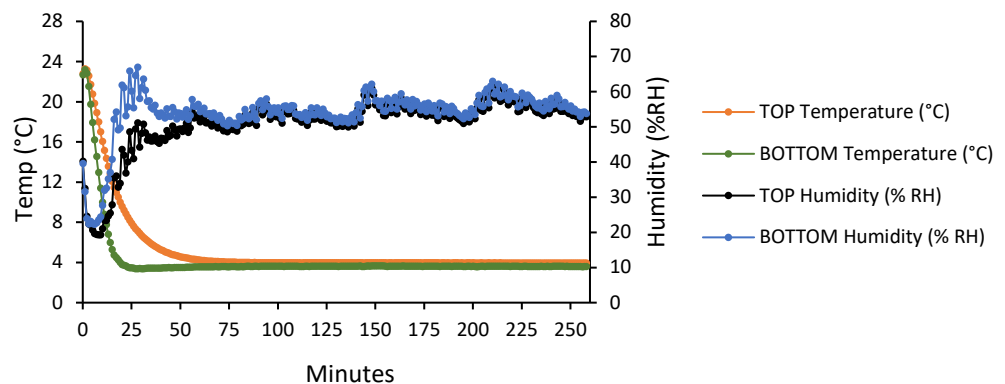
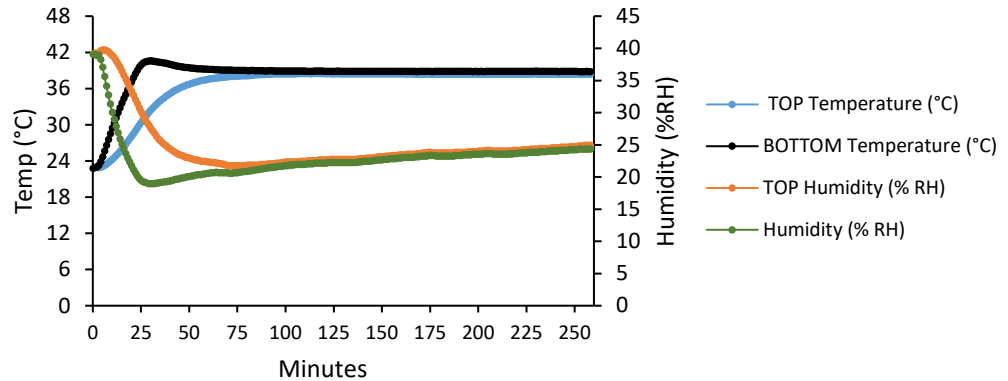


Figure 2. Exposure chamber #2.

Graphs plotting temperature and humidity obtained from MadgeTech probes placed on the top and bottom shelves of the heat chamber (chamber #2). Animal cages were rotated from accordingly to compensate for the slight $\leq 0.5^\circ\text{C}$ difference in temperature.



Behavioral Testing

Novel Object Recognition (NOR)

We used Novel Object Recognition (NOR) test in Aims 1 and 2 to evaluate memory and attention in rats based on spontaneous exploration of a novel object. Typically, a normal rat will be more interested in exploring a novel object than familiar objects. NOR test was performed in square polycarbonate boxes ($\sim 28'' \times 28'' \times 12''$ in height) containing bedding typically used in their home cages. The exterior of the boxes were painted black to minimize external cues, and testing was performed in a dimly lit room (~ 30 lux at the level of the area). Animal exploratory behavior was tracked and recorded by camera for post-testing manual or automated scoring of behavior (object contacts). Unique objects of similar materials, texture and scent were placed on the arena floor in one of four corners of the field and approximately 4'' from the field's edges. To avoid possible olfactory cues, multiple copies of the objects were used throughout the study, and the chambers were cleaned with a 70% ethanol solution between animal tests. Testing was divided into three sessions: habituation, familiarization, and a choice trial. For habituation, the animals were placed into the field containing no objects and will be allowed to explore the field for 10 min. After habituation, rats were returned to their home cage. The next day, animals were subjected to the familiarization phase. During this phase, rats were allowed to explore the same arena which contained two identical objects located at opposing corners. The familiarization phase lasted for 5 minutes. The amount of cumulative time and number of contacts exploring each object were recorded for the entire trial. Contact was defined as orientation toward the object with the nose of the rat within < 3 cm of the object. After the sample trial, rats were returned to their home cages. The next day, animals were tested in the choice trial to determine recognition memory. The choice trial consisted of a 5-min exploration of the field containing both a familiar, previously explored during the sample trial, object and a novel object. Cumulative time and number of contacts exploring both the familiar and novel object were scored. The location of the objects was counterbalanced across treatment groups but remained constant for each animal during the habituation, sample, and choice trials.

Motor Activity

Animals in Aim 2 and Aim 3 were subjected to open field and Morris water maze tests. Open field test was used to assess motor activity and was conducted one day following the last exposure day. Rats were placed in an open field to assess motor activity levels. Animals were placed into 40 cm by 40 cm open field activity chambers and allowed to move freely. Motor activities were measured using an automated photobeam data collection system (San Diego Instruments, Inc). Horizontal activity and vertical rears were scored using two separate sets of photobeams. Photobeams recorded both fine movements, reported as one broken photobeam, and ambulation, reported as two broken photobeams in sequence. Stereotypical beam breaks represented repetitive breaks of the same photon beam that are < 1 sec apart. Endpoints included ambulatory

beam breaks, stereotypical beam breaks, total distance traveled in centimeters, total time active and inactive in seconds, animal speed in centimeters per second, time spent in center versus perimeter of the open field, and number of rears on hind limbs. Each test session lasted 30 minutes and motor activity habituation was determined over six blocks of five-minute intervals.

Morris Water Maze (MWM)

Rats were also subjected to the Morris water maze (MWM) test, as previously described (Rohan et al., 2018). Briefly, the animal was placed in a cylindrical dark plastic tank 7 feet in diameter and filled with water (22-26°C). A hidden escape platform (10 cm²) was anchored to the floor of the tank but submerged 1 inch below the surface of the water. Four virtual quadrants were created inside of the tank using the SMART tracking software from San Diego Instruments. The outside of the tank contained four large distinct shapes to serve as visual cues for location of the hidden escape platform. The animal was initially placed facing the wall in one of the four external cue-based quadrants. Rats were allowed to swim until reaching the escape platform or until reaching the previously set maximum time of 60 seconds. Once the animal found the platform and was standing on it, the rat was given approximately 15 seconds to familiarize itself with its location. If an animal did not locate the platform within 60 seconds, it was led to it and also given approximately 15 seconds for familiarization. Rats were subjected to 4 training trials per day with an inter-trial interval of 15 seconds between trials, as previously described (Vorhees and Williams, 2006). Training occurred over a 5-6-day period for a total of 24 trials. The probe trial occurred approximately 24 hours after the last training run. During probe trials, the platform was removed and the animal was placed in a novel start location diagonally across from the platform quadrant. Rats were given 60 seconds to identify and reach the area where the platform was previously located. Several parameters such as latency to platform, swimming distance, time in target quadrant, and mean distance from goal were recorded automatically using a San Diego Instruments SMART tracking system and software.

Data Analysis

Behavioral data were analyzed using SigmaPlot (v13.0) and Microsoft Excel. A one-way analysis of variance (ANOVA) was used to assess statistical significance across experimental groups (RT, COLD, HEAT). For habituation data in motor activity assessments and the learning parameters across the multiple training days in the Morris water maze test, two-way ANOVA was used to evaluate statistical significance, with the experimental groups as one factor and either the 5-minute block (motor activity) or training day (Morris water maze) as the second factor.

Transcranial direct current stimulation (tDCS)

Surgery

We used a circular 2.5 mm radius electrode that is better adapted to our specialized tDCS system such that only the electrode casing is implanted onto the scalp in which it can then be connected to the tDCS electrode. Animals were anesthetized with isoflurane (Med-Vet International, Mettawa, IL) using 5% induction, followed by 2-3% isoflurane to maintain anesthetic depth. A 5 mm diameter, circular, head electrode casing (Tangible Solutions, Fairborn, OH) was attached to the skull from 0 mm to -5 mm bregma. Luting dental cement (GC Fuji I, GC America Inc., Alsip, IL) was applied to base of the head electrode casing and to the skull, followed by an acrylic dental cement (Sigma-Aldrich, St. Louis, MO) to secure the electrode. Animals were given a minimum of

7 days recovery period before tDCS treatment. Rats were randomly selected for SHAM (control) or tDCS treatment.

tDCS

On the same day, prior to stimulation, animals were acclimated to the testing room for 10 min. A conducting medium (SignaGel, Parker Laboratories, Fairfield, NJ) was placed into the head casing prior to connecting the head electrode. The reference electrode (12 mm diameter, Tangible Solutions, Fairborn, OH) was placed on the rats shaved chest with SignaGel as the conducting medium. Once the electrodes were in place, the animal was wrapped with a flexible cohesive bandage (PetFlex, Med-Vet, Mettawa, IL.) and placed into an open arena. tDCS was then applied at 0.25 mA using a constant-current stimulator (Magstim DCstimulator; Neuroconn, Ilmenau, Germany) for 30 min. The SHAM group was prepared the same way as the stimulation groups but did not receive any current and thus served as our control group. The head electrode was connected to the stimulator's negative terminal terminal to deliver anodal tDCS.

Necropsy and Sample Collection

All animals were euthanized via rapid decapitation with an approved guillotine. Animals were placed into decapicones (Braintree Scientific, Inc., Braintree, MA) immediately prior to euthanasia. Immediately, trunk blood was collected and processed to obtain plasma for biochemical assays. Blood was stored on ice until centrifugation. After centrifugation, plasma was decanted and stored at -80°C until analysis. Additionally, the brain was immediately removed and either frozen whole, halved and each half frozen, or the left half frozen and the right half used for MED-64 electrophysiology analysis. Brain samples immediately frozen were stored at -80°C until analysis.

Electrophysiology

Electrophysiology was done on a separate cohort of rats. Exposures for rats designated for electrophysiology study were staggered to accommodate the schedule of 3 rats per day: 1 RT, 1 COLD, and 1 HEAT per day.

Brain Slice Preparation

The brain was kept viable after removal by maintaining it in ice-cold artificial cerebrospinal fluid (ACSF) that was kept continuously oxygenated (95%/5% O₂/CO₂) and hippocampal slices were prepared as previously described (Rohan et al., 2015). ACSF consisted of (in mM): 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 10 D-Glucose, 1 MgSO₄, 36 NaHCO₃, and 2 CaCl₂ (pH ~7.4). To obtain hippocampal slices, the cerebellum and approximately 1 cm of frontal cortex were removed and the remaining brain sectioned at 350 µm thick using a vibratome (VT1000S Leica Microsystems) in the transverse plane, at approximately 30° angle from the horizontal axis. Brain slices were maintained in warm (37°C) oxygenated ACSF and allowed to recover for at least 60 minutes prior to recording. A new batch of ACSF was prepared each morning of experimentation and continuously oxygenated with 95% O₂ and 5% CO₂.

Brain Slice Recording

All electrophysiology data were obtained using AlphaMed's MED64 Quad II systems (Automate, Berkeley, CA). Probes containing 16 microelectrodes arranged in a 4 x 4 planar array, were initially pretreated with 0.1%

polyethylenimine (PEI) in 25-mM borate buffer for at least 24 hours prior to use. A hippocampal slice was placed onto the pre-coated microelectrode array and positioned such that the neurons from the CA1 region were recorded. A perfusion cap was used to circulate fresh oxygenated warmed ACSF into the probe to prevent the slice from drying. The flask containing the ACSF solution was kept in a 37°C water bath. Flow rates of ACSF were adjusted such that the brain slice was constantly maintained in a liquid-air interface. Typical flow rates range between 0.5 -1.5 ml/minute. In order to deliver warmed, humidified oxygen to the brain slice, a line carrying 95% O₂ and 5% CO₂ was passed through a heated water-containing flask before entering the MED64 probe. Data acquisition and stimulation protocols were performed using Mobius software (Automate, Berkeley, CA). A biphasic stimulating current of 10-120 μ A was applied to the Schaffer collateral of the hippocampus to obtain an input-output (I/O) relationship curve. Evoked field potentials mainly consisting of inward deflections were recorded from neurons within the CA1 region of the hippocampus at a sampling frequency of 20 kHz using the Quad II multichannel amplifier. Evoked responses primarily consisted of a mixtures of population spikes and field excitatory post-synaptic potentials (fEPSP). The potentials were digitized and graphically displayed using Mobius software. Paired pulse facilitation (PPF) signals were obtained by delivering sets of two consecutive stimuli of equal intensity that were 40 ms apart. Degrees of PPF were quantified by calculating the paired pulse (PP) ratio in which the response amplitude from the second stimulating pulse was divided by the response amplitude from the first pulse. Thus, a PP ratio of >1 indicates PPF whereas a PP ratio of <1 indicates paired pulse depression (PPD). Spontaneous spiking activity of neurons within the CA1 region were recorded for 10 minutes and frequency of firing was calculated using the Mobius software. A threshold value of 5 times the standard deviation was used to calculate spike frequency. For long term potentiation (LTP) experiments, the I/O function was used to determine the size of the stimulating current that resulted in half of the maximal output response. Typically, a stimulating current size of 30-50 μ A induced a half-maximal response and thus was used for LTP experiments. A baseline recording was obtained for each slice for at least 20 minute prior to LTP measurements. LTP was induced by delivering 3 trains of theta burst stimulation (TBS), consisting of 10 repeats of 4 high frequency stimulations (100 Hz) every 200 ms to the Schaffer collateral region. Evoked responses in the forms of fEPSP and population spikes were monitored at 12 second intervals for at least 20 minutes following LTP induction. Percent potentiation was calculated by computing the percent difference in population spike amplitude or fEPSP slope at 30 minutes following LTP induction by TBS from baseline. Averages of 10 data points for each were calculated to obtain baseline and LTP values.

Data Analysis

Analyses of electrophysiology data were performed using Mobius software. Microsoft Excel and SigmaPlot v.13 were used to plot analyzed data and for statistical assessments. The downward (negative, inward) field potentials (fEPSP) were used to calculate degree of evoked response. In the input-output, paired pulse and LTP analyses, amplitudes of the negative deflection of the population spikes and initial slopes of the field potentials were calculated to quantitate the size of evoked response. Amplitude and slope calculations were performed using Mobius software and its "Amplitude Minimum" and "Slope1040LinearFit" measurement options respectively. Neuronal activity was recorded from 10-15 microelectrodes within the MED64 probe chamber. For any given slice, data obtained from multiple microelectrodes covering one defined brain region (e.g. CA1) were averaged together to yield the response value for that particular slice. Statistical analyses were then performed on the data obtained from the different brain slices. Data are represented as means with the standard error of the mean (SEM) and were statistically compared using either a one-way or two-way analysis of variance (ANOVA). A calculated *p*-value of less than 0.05 is considered significantly different. All quantitation and statistical analysis as well as graphs were generated using Microsoft Excel and SigmaPlot v.13.

Biochemical Analysis

Biochemical assays were performed on plasma and brain homogenate samples. Plasma samples were diluted as needed per assay. Brain samples were homogenized in PBS with 1 mL of PBS being used per 0.5g of tissue. Samples were homogenized with a tissue tearor (Bio Spec Products, Inc., Bartlesville, OK). Following a minimum of two freeze/thaw cycles, the homogenates were centrifuged. The supernatant was decanted and used for analysis. As needed, brain homogenate samples were diluted per assay. Enzyme-linked Immunosorbent Assays (ELISA) were used to analyze plasma samples for corticosterone (Enzo Life Sciences, Inc., Farmingdale, NY and R&D Systems, Minneapolis, MN) and estradiol (female rats only) (LifeSpan Biosciences, Inc., Seattle, WA and Arbor Assays, Ann Arbor, MI). Multiple proinflammatory cytokines (IFN- γ , IL-1 β , IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF- α) were measured using a multi-spot cytokine assay system (Meso Scale Discovery, Rockville, MD). Samples were analyzed per manufacturer supplied protocols. ELISA were used to analyze brain homogenate samples for serotonin (Aviva Systems Biology, San Diego, CA), dopamine (CUSABIO, Houston, TX), glutamate (Abnova, Taipei City, Taiwan), Gamma Aminobutyric Acid (GABA) (MyBioSource, Inc., San Diego, CA), lipocalin-2 (NGAL) (MyBioSource, Inc., San Diego, CA), and complement component 3 (C3) (MyBioSource, Inc., San Diego, CA). Samples were analyzed per manufacturer supplied protocols.

RESULTS

Specific Aim 1: Effects of tDCS on female rats

Enhanced synaptic plasticity as measured by long term potentiation (LTP) was also observed in female rats following 30 min of 0.25 mA tDCS administration. All female rats underwent surgery in which a transcranial electrode is positioned onto the scalp using dental cement. Estrous cycle of all female rats were determined using vaginal lavage. Half of the animals were subjected to 0.25 mA tDCS for 30 minutes (referred to tDCS or stim rats) whereas the other half were similarly hooked up but were not given current stimulation (referred to SHAM). Animals were then euthanized 30 minutes later and brain were harvested and kept viable by constant perfusion with oxygenated artificial cerebrospinal fluid (ACSF). Horizontal hippocampal slices were prepared and synaptic plasticity was measured using a microelectrode array system. LTP was induced in the hippocampal slices using a theta burst stimulation (TBS) protocol in which 4 high frequency pulses (100 Hz) were given repetitively. Female animals that were subjected to the 0.25 mA tDCS showed a statistically significant higher percent potentiation compared to control animals ($p=0.016$, Mann-Whitney Rank sum test, $p=0.03$, t-test, $n=6$ female rats) ([Figure 3](#)). This finding in female rats is similar to our previous finding in which 30 min tDCS also induced enhancement in LTP in male rats (Rohan et al., 2015). Both stimulated and control rats showed similar preference for novel object over familiar object during the initial 90 seconds after introduction of novel object ([Figure 4](#)). Rats subjected to 30 min of 0.25 mA tDCS showed longer preference to novel object compared to control rats that were subjected to SHAM (0 mA) ($p=0.018$, t-test, $n=16$ female rats per group). For the duration of 5 minutes following novel object introduction, stimulated rats continued to show strong preference to novel object whereas control rats showed decreased preference to novel object after 90 seconds ([Figure 4](#)).

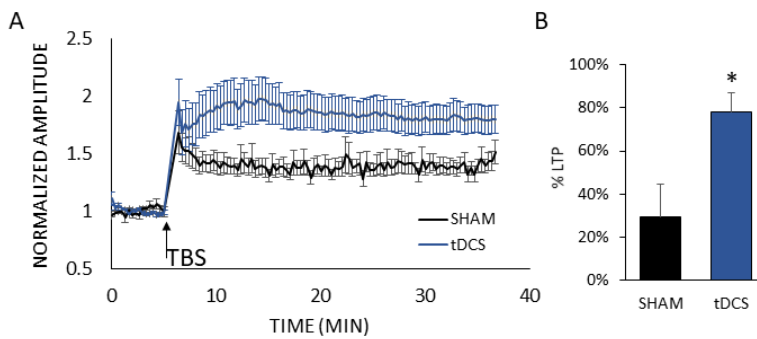


Figure 3. Long term potentiation was also enhanced in female rats by 30 minutes of 0.25 mA tDCS. (A)

Averaged normalized traces from animals subjected to 30 min of 0.25 mA tDCS (red) or SHAM (blue). LTP was induced by theta burst stimulation (TBS) as indicated by arrow. (B) Bar graph showing increased % LTP from rats subjected to tDCS (blue) compared to SHAM (black). % LTP was calculated as amplitude (averaged of 10 data points) of response 30 min following TBS minus baseline amplitude (before TBS, averaged 10 data points), divided by the baseline amplitude. * $p=0.016$, Mann-Whitney Rank sum test, $p=0.03$, t-test, $n=6$ rats.

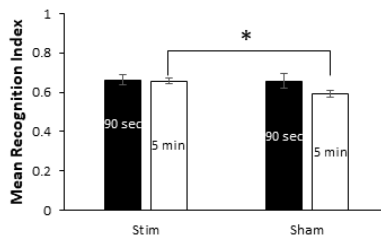


Figure 4. Novel object recognition. Summary bar graph demonstrating the effects of tDCS on NOR performance in female rats. Stimulated rats continued to show strong preference for novel object compared to control rats. * $p=0.018$, $n=16$ rats.

Specific Aim 2: Effects of repeated intermittent cold or heat on animal weights and behavioral tests

Effects on behavioral performance were evaluated using open field test to assess motor activity levels, novel object recognition (NOR) to assess recognition memory and Morris water maze (MWM) to assess spatial learning and memory. Motor activity test was performed one day following the last exposure day. NOR was performed the three days following completion of the open field test. MWM required 7 days and was initiated 8 days following the last exposure day or 4 days after completion of NOR. Rats were then euthanized the next day after the last day of MWM testing, which fell on the 15th day after the last exposure day. Plasma and brain from these rats were used in the biochemical assays and electrophysiological recordings to evaluate delayed effects or referred to in this report as effects observed two weeks following exposures.

Animal Weights

Significant reduction in body weight gain was observed for female rats exposed to heat (Figure 5). Two way ANOVA was conducted, with temperature exposures as one factor (RT, COLD, HEAT) and number of weeks as the second factor (Week 1 – Week 5). Significant difference between exposure groups was detected with $F(2, 225) = 11.769$, $p < 0.001$, $n = 16$. Post-hoc comparison analysis using Holm Sidak method yielded significant difference between HEAT vs RT and HEAT vs COLD, with $p < 0.001$. Interestingly, there was no significant difference in weight gain between COLD and RT in female rats. However, significant reduction in weight gain was observed in male rats exposed to COLD or HEAT compared to male rats exposed to RT (Figure 5). Two way ANOVA yielded significant difference among exposure groups with $F(2, 135) = 19.340$, $p < 0.001$, $n = 10$ and post hoc multiple comparison analysis revealed significant difference between COLD vs RT and HEAT vs RT with $p < 0.001$.

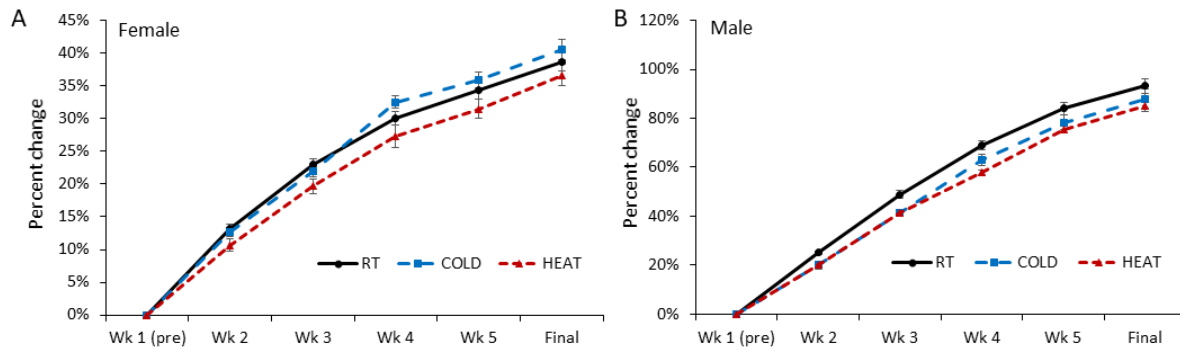


Figure 5. Effects on body weight gain. (A) Female rats exposed to heat had decreased body weight gain compared to female rats exposed to RT or cold (two way ANOVA $F(2, 225) = 11.769$, $p < 0.001$, $n = 16$ with $p < 0.001$ for HEAT vs RT and HEAT vs COLD). Week 1 data is the pre-exposure weight just prior to initiation of exposures. Week 2 and 3 are weight gains during exposures. Week 4 and 5 are weight gains after the 21-day exposures. (B) Male rats exposed to cold or heat had decreased body weight gain compared to male rats exposed to RT (two way ANOVA $F(2, 135) = 19.340$ $p < 0.001$, $n = 10$ with $p < 0.001$ for COLD vs RT and HEAT vs RT).

Motor Activity

In female rats, we saw a significant increase in motor activity of female rats exposed to heat (38°C) compared to rats exposed to cold or room temperature (Figure 6). Two way ANOVA yielded $F(2, 264) = 7.825$, $p < 0.001$ ($n=16$). Post hoc multiple comparison testing using the Holm Sidak method yielded $p = 0.019$ HEAT vs RT control, $p < 0.001$ HEAT vs COLD. Female rats exposed to heat also experienced an increase in total rears compared to female rats exposed to cold ($p = 0.014$). Similar to female rats, male rats also experienced a significant increase in motor activity of male rats exposed to heat (38°C) compared to rats exposed to cold or room temperature (Figure 6). Two way ANOVA yielded $F(2, 162) = 9.024$, $p < 0.001$, $n=10$, with $p = 0.003$ for HEAT vs RT control and $p < 0.001$ for HEAT vs COLD. Male rats exposed to heat showed a trending increase in total rears that was not statistically significant. In addition, male rats exposed to heat showed statistically significant increase in time spent in the center of the open field compared to RT control ($p = 0.038$) and decreased resting time compared to male rats exposed to cold ($p = 0.037$).

Novel Object Recognition (NOR)

There were no detectable effect in recognition index (RI) of female rats exposed to cold or heat in novel object recognition testing ($F(2, 45) = 0.687$, $p = 0.5$, one way ANOVA, $n = 16$) (Figure 7). Similarly, there were no detectable effect in RI of male rats exposed to cold or heat ($F(2, 26) = 0.744$, $p = 0.5$, one way ANOVA, $n = 10$) (Figure 7).

Morris Water Maze (MWM)

There was a significant effect in the learning phase of the MWM testing. There was a significant increase in latency to reaching the platform in female rats exposed to heat or cold compared to control female rats exposed to room temperature (RT) (Figure 8). Two way ANOVA yielded $F(2, 225) = 6.232$, $p = 0.002$, $n=16$. Post hoc multiple comparison testing yielded $p = 0.004$ for COLD vs RT and $p = 0.011$ for HEAT vs RT. There was no significant effects in the memory phase of MWM testing ($F(2, 45) = 0.956$, $p = 0.392$, $n = 16$, one way ANOVA). There was a trending but non-significant increase in percent time spent in target quadrant for female rats

exposed to cold during the memory phase ($p = 0.392$, one way ANOVA). In male rats, there was a significant increase in latency to reaching the platform in male rats exposed to heat compared to male rats exposed to cold (Figure 8). Two way ANOVA yielded $F(2,153) = 9.197$, $p < 0.001$, $n = 9$ RT, 10 COLD, HEAT. Male rats exposed to cold appeared to perform better compared to male rats exposed to room temperature, as they have decreased latency to platform compared to control rats ($p = 0.019$, two way ANOVA, post-hoc multiple comparison testing). There was a similar trending but non-significant increase in the percent spent at the target quadrant during the memory phase ($p = 0.223$, one way ANOVA) (Figure 8).

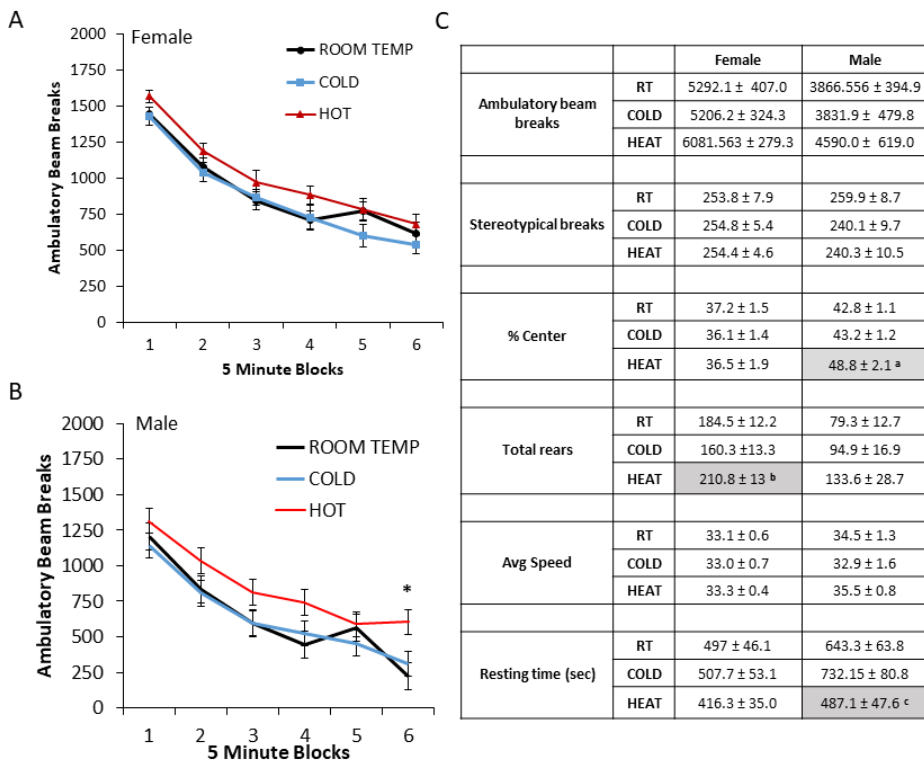


Figure 6. Motor activity data from female and male rats. (A) Motor activity habituation data from female rats showing significant increase in motor activity from rats in the HEAT group (two way ANOVA, $F(2, 264) = 7.825$, $p < 0.001$, $n=16$, Holm Sidak post hoc multiple comparison testing HEAT vs RT control $p = 0.019$, heat vs cold $p < 0.001$). (B) Motor activity habituation data from male rats showing significant increase in motor activity from rats exposed to heat (two way ANOVA, $F(2,162) = 9.024$, $p < 0.001$, $n=10$, Holm Sidak post hoc multiple comparison testing HEAT vs RT $p = 0.003$, heat vs cold $p < 0.001$). ^a $p=0.043$ heat vs RT, $p = 0.047$ HEAT vs COLD, one way ANOVA at the sixth 5-min block. (C) A summary table of additional motor activity parameters. ^a $p= 0.038$ (RT vs HEAT) and $p = 0.032$ (HEAT vs COLD), ^b $p = 0.014$ (HEAT vs COLD), ^c $p = 0.037$ (HEAT vs COLD).

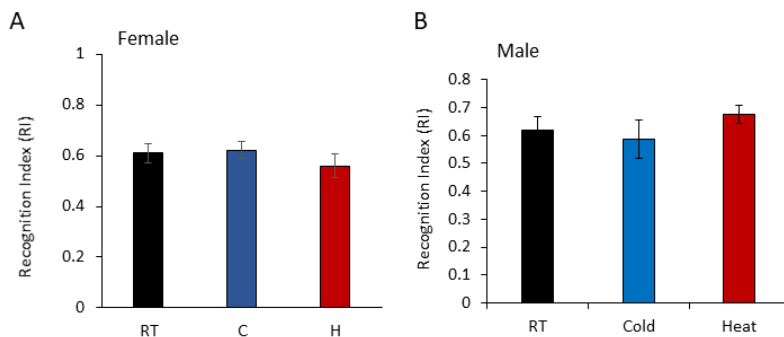


Figure 7. Novel object recognition test. (A) No significant effects of repeated cold or heat exposures on novel object recognition in female rats ($F(2,45) = 0.687$, $p = 0.509$, $n = 16$, one way ANOVA). (B) No significant effects of repeated cold or heat exposures on novel object recognition in female rats ($F(2,26) = 0.744$, $p = 0.485$, $n = 10$, one way ANOVA).

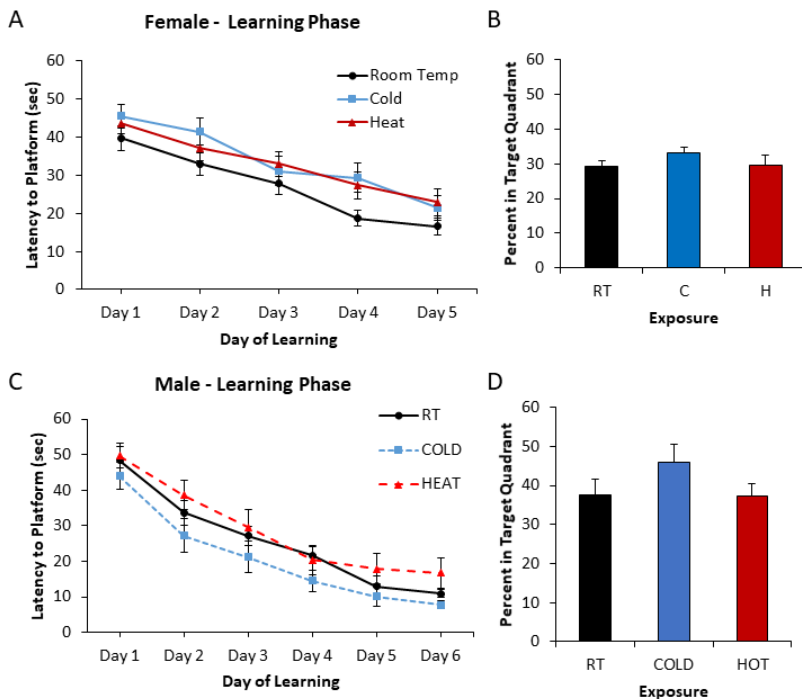


Figure 8. Morris water maze testing in female (A,B) and male (C,D) rats. (A) There were significant increase in latency to platform in female rats exposed to cold or heat during the learning phase of Morris water maze testing (two way ANOVA, $F(2,225) = 6.232$, $p = 0.002$, $n=16$, Holm Sidak post hoc multiple comparison, cold vs room temperature (RT) $p = 0.004$, heat vs RT $p = 0.011$). (B) There was no significant effect on percent spent in target quadrant in the memory phase of Morris water maze testing ($F(2,45) = 0.956$, $p = 0.392$, $n = 16$, one way ANOVA). (C) There was a significant reduction in latency to platform in male rats exposed to cold compared to animals exposed to heat or RT (two way ANOVA, $F(2,153) = 9.197$, $p < 0.001$, $n = 9$ RT, 10 COLD, HEAT). Tukey post hoc multiple comparison, cold vs RT $p = 0.019$, cold vs heat $p < 0.001$). (D) There was no significant effect on percent spent in target quadrant in the memory phase of Morris water maze testing ($F(2,26) = 1.59$ $p = 0.223$). However there was a trending increase in percent spent in target quadrant in male rats exposed to COLD.

Specific Aim 2: Effects of repeated intermittent cold or heat on hippocampal function

Hippocampal function was assessed in a separate cohort of rats using a microelectrode array system at one day post exposures. Only subtle differences were observed resulting from repeated exposures to cold or heat in female rats. There was no significant effect on input/output (I/O) function of hippocampal neurons ([Figure 9](#)). There was a slight increase in paired pulse facilitation (PPF) in female rats exposed to cold compared to rats exposed to heat or room temperature ([Figure 9](#)). Two way ANOVA yielded $F(2, 2091) = 8.246$, $p < 0.001$, $n = 16$ rats, 2-8 slices per rat. Post hoc multiple comparison testing yielded $p = 0.004$ for COLD vs RT, $p = 0.006$ for COLD vs HEAT stimulated at 40 μA , and $p = 0.012$ COLD vs HEAT stimulated at 50 μA . There was a trending but not significant reduction in the degree of long term potentiation (LTP) in female rats exposed to cold or heat compared to RT rats ([Figure 9](#)). There was also a trending but not significant reduction in the frequency of spontaneous activity of hippocampal neurons from female rats exposed to cold or heat ($p = 0.55$, one way ANOVA) ([Figure 9](#)). In male rats, there was a small increase in the I/O function from male rats exposed to heat but only when compared to male rats exposed to cold, with two way ANOVA yielding $F(2,1116) = 5.343$ $p = 0.005$ (RT: $n = 8$ rats, 32 slices, 4 slices per rat, COLD: $n = 8$ rats, 36 slices 3-6 slices per rat; HEAT: $n = 7$ rats, 28 slices 4 slices per rat) ([Figure 10](#)). Post hoc multiple comparison test using Holm-Sidak yielded significant difference only between COLD and HEAT ($p = 0.004$). No significant or trending effects in male rats exposed to cold or heat relative to RT control in I/O, PPF, LTP or spontaneous activity ([Figure 10](#)).

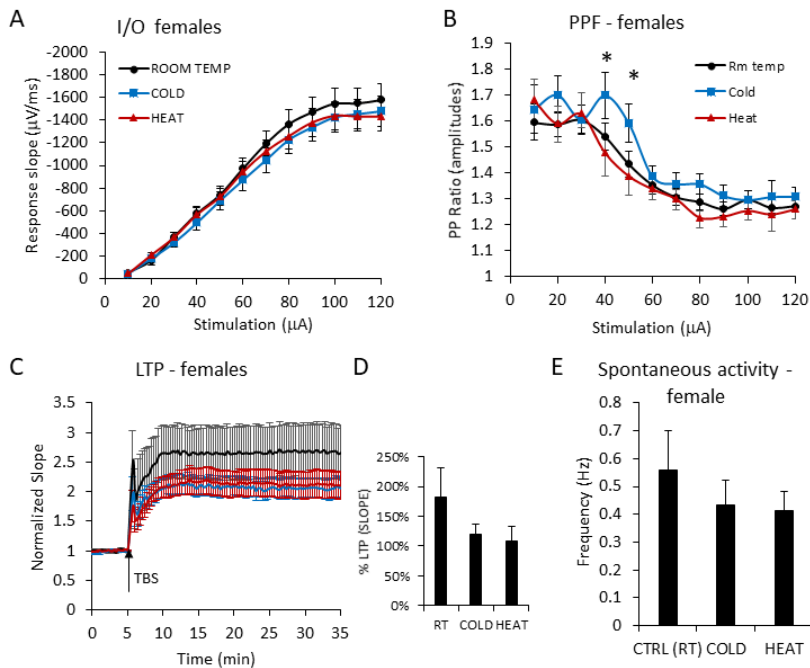


Figure 9. Electrophysiology result from female rats.

(A) There was no significant difference in the input-output (I/O) function from CA1 hippocampal neurons of female rats exposed room temperature (black, control/RT), cold (blue) or heat (red). Two way ANOVA yielded $F(2, 2146) = 2.213$, $p = 0.110$ ($n = 16$ rats, 2-8 slices per rat) (B) There was a significant increase in PPF of CA1 hippocampal neurons from female rats exposed to cold (two way ANOVA, $F(2, 2091) = 8.246$, $p < 0.001$, $p = 0.004$ COLD vs RT group, Holm Sidak post hoc multiple comparison, $*p = 0.006$ COLD vs HEAT within $40 \mu\text{A}$, $*p = 0.012$ COLD vs HEAT within $50 \mu\text{A}$, $n = 16$ rats, 2-8 slices per rat). (C) Averaged normalized slopes of field responses showing a trending reduction in LTP in female rats resulting from cold or heat exposures but reduction was not statistically significant ($F(2, 34) = 1.745$, $p = 0.187$) (RT: $n = 16$ rats 25 slices at 1-2 slices per rat, COLD: $n = 16$ rats, 26 slices, 1-3 slices/rat, HEAT: $n = 13$ rats, 27 slices at 1-3 slices/rat). LTP was induced by theta burst stimulation (TBS) in which 3 repeats of 10 bursts of 4 high

frequency pulses at 100 Hz were applied to the Schaffer collateral –CA1 region of hippocampal slices. (D) Bar graph of percent LTP at 30 minutes post theta burst stimulation (TBS). (E) There was no statistically significant difference in the frequency of spontaneous activity from hippocampal neurons in female rats ($F(2, 39) = 0.970$, $p = 0.388$) ($n = 14$ rats RT, 13 rats COLD, 15 rats HEAT).

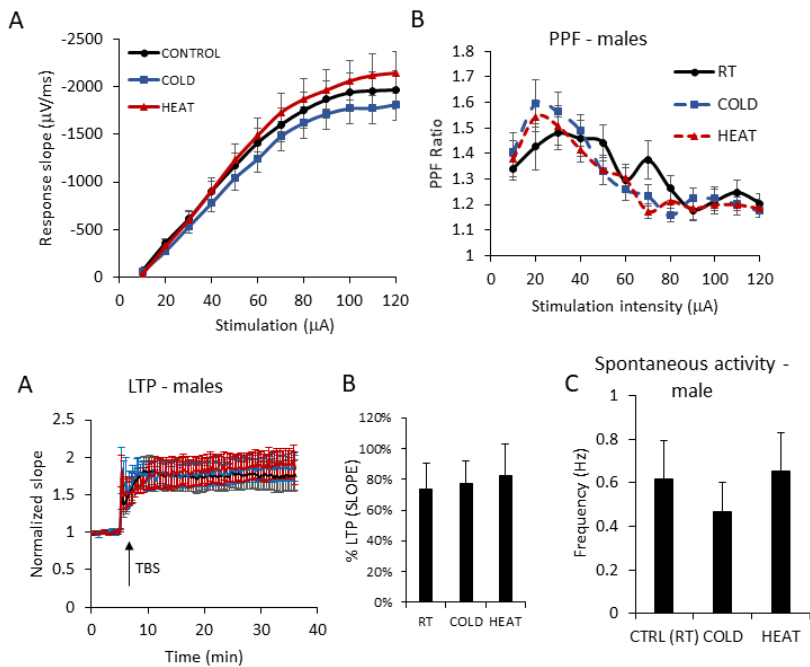


Figure 10. Electrophysiology results from male rats.

(A) There was a small reduction in the input-output (I/O) function from CA1 hippocampal neurons of male rats exposed to cold (blue) compared to male rats exposed to heat (red). Two way ANOVA yielded $F(2, 1116) = 5.343$, $p = 0.005$ (RT: $n = 8$ rats, 32 slices, 4 slices per rat, COLD: $n = 8$ rats, 36 slices 3-6 slices per rat; HEAT: $n = 7$ rats, 28 slices 4 slices per rat) across groups. Post hoc multiple comparison test using Holm-Sidak yielded significant difference between COLD and HEAT ($p = 0.004$). Comparison between COLD and RT yielded $p = 0.07$. Comparison between HEAT and RT yielded $p = 0.241$. (B) There was no significant effect of cold or heat exposures on paired pulse facilitation (PPF) of male rats ($F(2, 1116) = 0.561$, $p = 0.571$, RT: $n = 8$ rats, 32 slices, 4 slices per rat, COLD: $n = 8$ rats, 36 slices 3-6 slices per rat; HEAT: $n = 7$ rats, 28 slices 4 slices per rat). (C) Averaged normalized slopes of field responses showing no change in long term potentiation (LTP) in male rats resulting from cold or heat exposures ($F(2, 21) = 0.0717$, $p = 0.931$, $n = 8$ rats). LTP was induced by theta burst stimulation (TBS) in which 3 repeats of 10

bursts of 4 high frequency pulses at 100 Hz were applied to the Schaffer collateral –CA1 region of hippocampal slices. (D) Bar graph of percent LTP at 30 minutes post theta burst stimulation (TBS). (E) There was no statistically significant difference in the frequency of spontaneous activity from hippocampal neurons in male rats.

Specific Aim 2: Effects of repeated intermittent cold or heat on biochemical endpoints

Estradiol

Plasma estradiol levels were measured in female rats. There was a significant decrease in plasma estradiol level in female rats exposed to either cold or heat at one day post-exposures ([Figure 11](#)). One way ANOVA

yielded $F(2, 44) = 11.22$, $p < 0.001$ ($n = 16$ RT, COLD, 15 HEAT). Post hoc multiple comparison using the Holm Sidak method yielded $p < 0.001$ for HEAT vs RT, $p = 0.027$ for COLD vs RT, and $p = 0.033$ for COLD vs HEAT. However, the reduction in estradiol level was no longer detected at two weeks post-exposures.

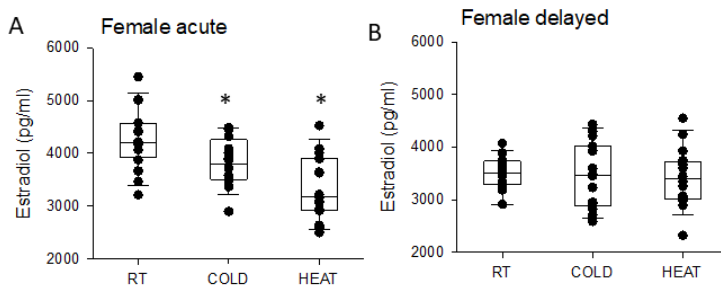


Figure 11. Estradiol levels in female rats. (A) Exposures to cold or heat significantly reduced plasma estradiol levels in female rats 1 day post exposures ($F(2, 44) = 11.22$, $p < 0.001$, one way ANOVA, $*p < 0.001$ HEAT vs RT, $*p = 0.033$ COLD vs HEAT, $*p = 0.027$ COLD vs RT, $n=16$ CONTROL, 16 COLD and 15 HEAT). (B) Reduction in plasma estradiol level was no longer observed ~2 weeks post exposures ($p = 0.906$, one way ANOVA, $n = 16$ CONTROL, 15 COLD, 16 HEAT).

Neurotransmitters

Serotonin. We detected increased brain serotonin levels from both female and male rats following exposures to cold or heat. One way ANOVA revealed significant difference between groups with $F(2, 45) = 8.881$, $p < 0.001$, $n = 16$ (Figure 12). Post hoc multiple comparison yielded significant increase in female rats exposed to heat compared to control RT rats ($p < 0.001$) and female rats exposed to cold compared to control RT rats ($p = 0.007$) at 1 day post exposures but not at two weeks post exposures (Figure 12). Similarly, one way ANOVA revealed significant increase in male rats exposed to cold or heat compared to control RT rats at one day post exposures ($F(2,21)=6.823$, $p = 0.005$, one way ANOVA, with $p = 0.005$ HEAT vs RT, $p = 0.032$, COLD vs RT, $n = 8$ per group) (Figure 12).

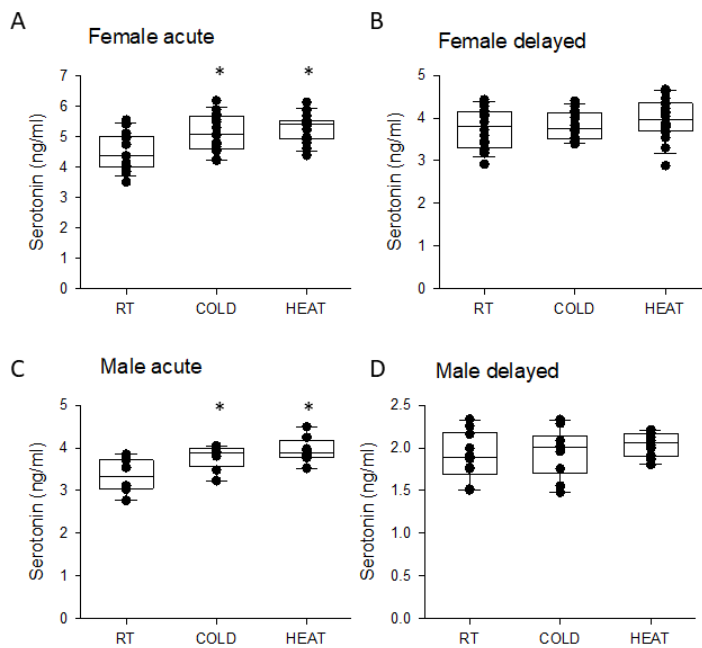


Figure 12. Serotonin levels in female and male rats. (A) Brain levels of serotonin slightly increased in female rats exposed to cold or heat 1 day post exposures ($F(2,45)=8.881$, $p<0.001$, one way ANOVA, $*P<0.001$ HEAT vs RT, $*P=0.007$, COLD vs RT, $n=16$ per group). (B) Increase in serotonin from female rats was no longer observed at two weeks post-exposures ($F(2,44)=1.028$, $p=0.366$, $n=16$ RT, 15 COLD, 16 HEAT). (C) Brain levels of serotonin also slightly increased in male rats exposed to cold or heat 1 day post exposures ($F(2,21)=6.823$, $p=0.005$, one way ANOVA, $*p = 0.005$ HEAT vs RT, $*p = 0.032$, COLD vs RT, $n=8$ per group). (D) Increase in serotonin from male rats was no longer detected at two weeks post-exposure ($F(2,27) = 0.735$, $p = 0.489$, $n = 10$ per group).

Dopamine. In contrast to serotonin levels, we detected a delayed reduction in brain dopamine levels from both female and male rats following exposures to cold or heat (Figure 13). One way ANOVA revealed significant decrease in female rats exposed to cold compared to RT rats and trending decrease in female rats exposed to heat compared to RT rats, at two weeks post-exposures ($F(2,42) = 4.669$, $p = 0.015$, one way ANOVA, with $p =$

0.013 COLD vs RT and $p = 0.091$ HEAT vs RT, $n = 10$ per group). One way ANOVA revealed significant decrease in dopamine from male rats exposed to cold or heat compared to control rats at two weeks post-exposures ($F(2,27) = 8.787$, $p = 0.001$, one way ANOVA, $p = 0.001$ HEAT vs RT, $p = 0.008$ COLD vs RT, $n = 10$ per group). No significant difference at one day post-exposures ($p = 0.5$, 0.8 for female and male rats respectively) ([Figure 13](#)).

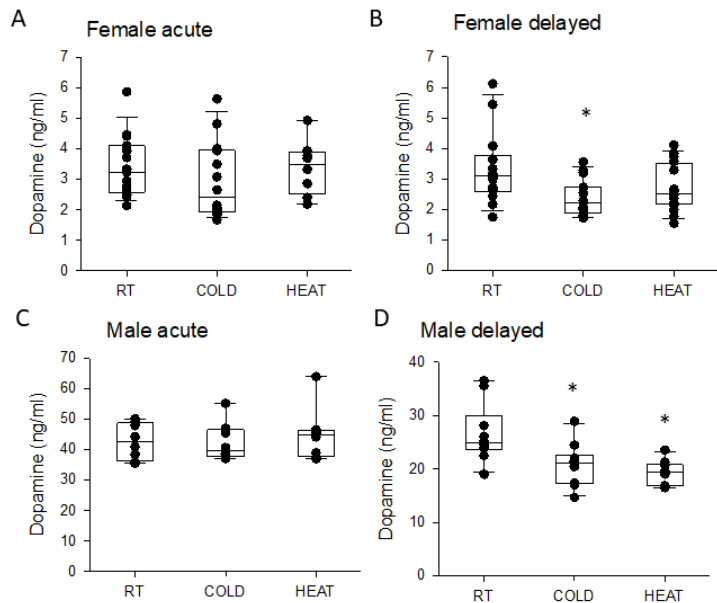


Figure 13. Dopamine levels in female and male rats. (A) Brain levels of dopamine was not significantly affected in female rats one day post-exposures ($p = 0.5$, one way ANOVA). (B) Brain levels of dopamine slightly decreased in female rats exposed to cold two weeks post-exposures ($F(2,42) = 4.669$, $p = 0.015$, one way ANOVA, $p = 0.013$ cold vs control, $p = 0.091$ HEAT vs RT, $n = 10$ per group). (C) Brain levels of dopamine was not significantly affected in male rats one day post-exposures ($p = 0.8$, one way ANOVA). (D) Brain levels of dopamine slightly decreased in male rats exposed to cold or heat two weeks post-exposures ($F(2,27) = 8.787$, $p = 0.001$, one way ANOVA, $p = 0.001$ HEAT vs RT, $p = 0.008$ COLD vs RT, $n = 10$ per group).

GABA. We saw a statistically significant change in GABA levels in the brain from female rats following exposures to cold ([Figure 14](#)). One way ANOVA revealed significant decrease in brain GABA in female rats exposed to cold compared to RT rats at one day post-exposures ($F(2, 45) = 5.388$, $p = 0.008$, one way ANOVA, $p = 0.006$, COLD vs HEAT, $p = 0.04$ COLD vs RT, $n=16$) but not at two weeks post-exposures ([Figure 14](#)). GABA levels from male rats were only measured at one day post-exposure and did not show any significant effects of repeated exposures to cold or heat ([Figure 14](#)).

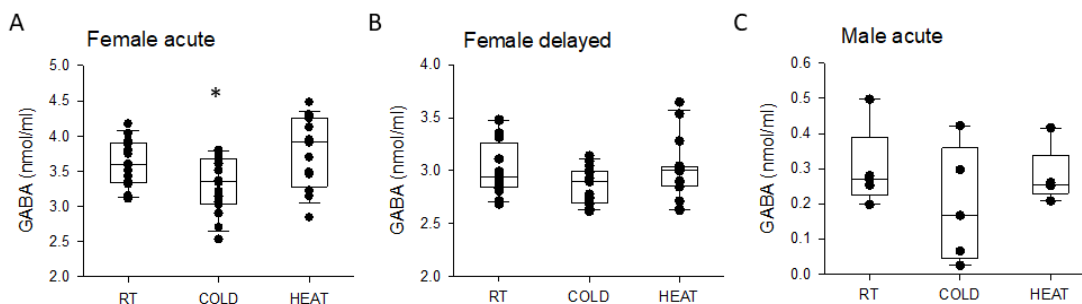


Figure 14. Brain GABA levels. (A) There was a slight decrease in GABA in female rats exposed to cold at one day post-exposures ($F(2, 45) = 5.388$, $p = 0.008$, one way ANOVA, $p = 0.006$, COLD vs HEAT, $p = 0.04$ COLD vs RT Student-Neuman-Keuls method, $n = 16$). (B) Reduction in GABA levels in female rats was no longer detected at two weeks post-exposures ($p = 0.16$). (C) There was no significant effect on male GABA level at one day post-exposures ($p = 0.973$, $n = 8$).

Glutamate. Female rats showed no significant difference in brain glutamate levels at one day post-exposures ($p = 0.1$). Interestingly, we detected a significant reduction in glutamate level from male rats exposed to heat ([Figure 15](#)). One way ANOVA revealed significant decrease in male rats exposed to heat compared to RT rats

at one day post exposures ($F(2, 21) = 4.004$, $p = 0.034$, one way ANOVA, $p = 0.032$ HEAT vs RT, $n=8$ per group) ([Figure 15](#)).

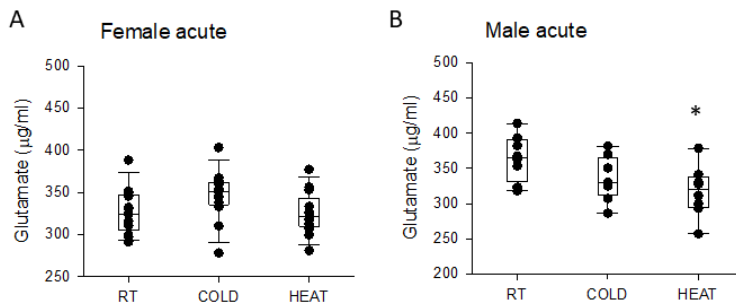


Figure 15. Glutamate levels. (A) No statistically significant effect of cold or heat on glutamate levels from female rats. (B) There was a slight reduction in glutamate in male rats exposed to heat at one day post-exposures ($F(2, 21) = 4.004$, $p = 0.034$, one way ANOVA, $p = 0.032$ HEAT vs RT, $n = 8$ per group).

Inflammatory markers

Complement 3 (C3). To assess complement activation in the brain, we measured brain levels of complement 3 (C3). At one day post-exposures, there was a significant increase in brain levels of C3 in female rats exposed to heat compared to control RT rats ([Figure 16](#)). One way ANOVA yielded $F(2, 44) = 5.970$ $p = 0.005$ ($n = 16$ RT, 15 COLD, 16 HEAT), with post hoc multiple comparison yielding $p = 0.007$ for HEAT vs COLD, $p = 0.024$ HEAT vs RT. However, at two weeks post-exposures, there was a significant decrease in C3 in female rats exposed to heat compared to RT rats ($F(2, 44) = 16.554$ $p < 0.001$, $n=16$ RT, 15 COLD, 16 HEAT, with $p < 0.001$ for HEAT vs RT and HEAT vs COLD) ([Figure 16](#)). In contrast, no significant changes were observed in plasma C3 levels from male rats at one day post-exposures or at two weeks post-exposures ([Figure 16](#)).

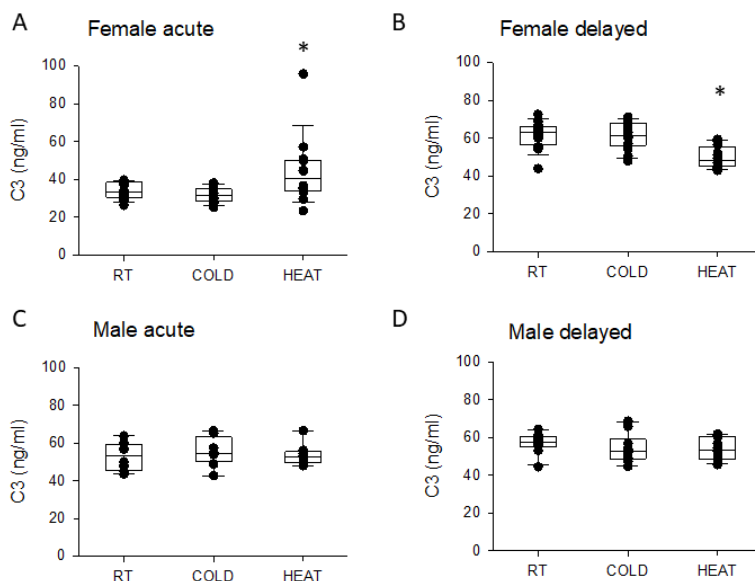


Figure 16. Complement 3. (A) There was a small increase in brain C3 levels in female rats one day following repeated exposure to heat (one way ANOVA, $F(2, 44) = 5.970$ $p = 0.00508$ ($n = 16$ RT, 15 COLD, 16 HEAT), HEAT vs COLD $p=0.007$; HEAT vs RT $p=0.024$, COLD vs RT $p=0.507$). (B) There was a small decrease in brain C3 levels in female rats two weeks following repeated exposures to heat (one way ANOVA, $F(2, 44) = 16.554$ $p < 0.001$, $n=16$ RT, 15 COLD, 16 HEAT; Holm Sidak HEAT vs RT $p<0.001$; COLD vs HEAT $p<0.001$). (C) No significant effect in brain C3 levels in male rats one day post-exposures (one way ANOVA, $F(2, 21) = 0.274$ $p=0.763$ $n = 8$). (D) No significant effect in brain C3 levels in male rats two weeks post-exposures (one way ANOVA, $F(2, 27) = 0.543$ $p = 0.587$, $n = 10$).

IFN- γ and IL-6. At one day post-exposures, there were small but statistically significant increases in IFN- γ and IL-6 in female rats exposed to heat ([Table 1](#), [Figure 17](#), [18](#)). One way ANOVA of IFN- γ data at one day post exposures resulted in $F(2, 45) = 4.974$, $p = 0.01$, $n = 16$, with post hoc multiple comparison yielding $p = 0.014$ for HEAT vs RT and $p = 0.043$ HEAT vs COLD. One way ANOVA of IL-6 data at one day post exposures resulted in $F(2, 45) = 5.065$, $p = 0.010$, $n = 16$, with post hoc multiple comparison yielding $p = 0.008$ for HEAT vs RT. At two weeks post-exposures, there were small but statistically significant increases in IFN- γ and IL-6 in female rats exposed to heat or cold, with no significant changes observed in other cytokines measured ([Table 2](#),

Figure 17, 18). One way ANOVA of IFN- γ data at two weeks post exposures resulted in $F(2, 43) = 6.375$, $p = 0.0038$, $n=15$ RT, HEAT, $n=16$ COLD, with post hoc multiple comparison yielding $p = 0.026$ for COLD vs RT and $p = 0.004$ for HEAT vs RT. One way ANOVA of IL-6 data at two weeks post exposures resulted in $F(2, 42) = 13.981$, $p < 0.001$, $n = 14$ RT, 16 COLD, 15 HEAT, with $p < 0.001$ for HEAT vs RT, $p = 0.006$ for HEAT vs COLD, and $p = 0.029$ COLD vs RT. There were no corresponding increases in IFN- γ and IL-6 at one day post-exposures for male rats exposed to heat. Similar to the acute data, there were no corresponding increases in IFN- γ and IL-6 from male rats at two weeks post-exposures.

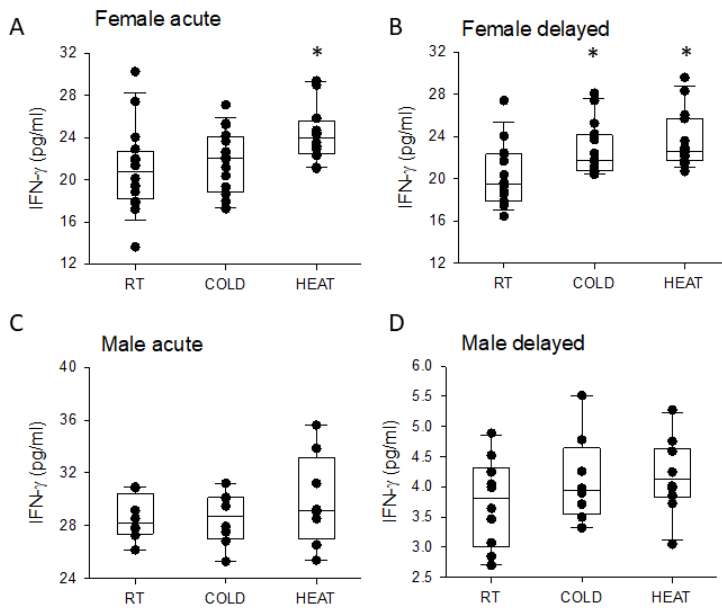


Figure 17. IFN- γ . (A) Plasma IFN- γ levels increased in female rats one day post-exposures (one way ANOVA, $F(2, 45) = 4.974$, $p = 0.01$, $n = 16$, * $p = 0.014$ HEAT vs RT, $p = 0.043$ HEAT vs COLD). (B) Plasma IFN- γ levels increased in female rats two weeks post-exposures (one way ANOVA, $F(2, 43) = 6.375$, $p = 0.0038$, $n = 15$ RT, HEAT, $n = 16$ COLD, * $p = 0.026$ COLD vs RT, * $p = 0.004$ HEAT vs RT). (C) No statistical effects on plasma IFN- γ levels in male rats one day post-exposures (one way ANOVA, $F(2, 21) = 0.782$, $p = 0.47$, $n = 8$). (D) No statistical effects on plasma IFN- γ levels in male rats two week post-exposures (one way ANOVA, $F(2, 25) = 1.26$, $p = 0.302$, $n = 10$ RT, HEAT, $n = 8$ COLD).

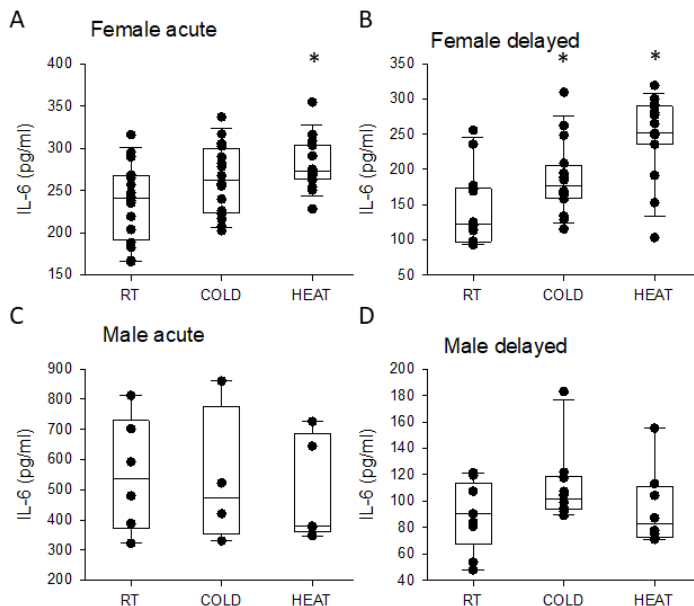


Figure 18. Plasma IL-6. (A) Plasma IL-6 level increased in female rats one day following repeated exposures to heat (one way ANOVA $F(2, 45) = 5.065$, $p = 0.010$, $n = 16$, * $p = 0.008$ HEAT vs RT). (B) Plasma IL-6 level increased in female rats two weeks post-exposures to cold or heat (one way ANOVA $F(2, 42) = 13.981$, $p < 0.001$, $n = 14$ RT, 16 COLD, 15 HEAT, * $p < 0.001$ HEAT vs RT, $p = 0.006$ HEAT vs COLD, * $p = 0.029$ COLD vs RT). (C) No significant effect was detected in plasma IL-6 from male rats one day post-exposures ($F(2, 12) = 0.109$, $p = 0.897$, $n = 6$ RT, 4 COLD, 5 HEAT). (D) No significant effect was detected in plasma IL-6 from male rats two weeks post-exposures ($F(2, 24) = 1.621$, $p = 0.219$, $n = 9$ RT, 10 COLD, 8 HEAT).

IL-10. There was a small but statistically significant increase in IL-10 from female rats exposed to heat compared to RT at one day post exposures (**Table 1, Figure 19**). One way ANOVA yielded $F(2, 44) = 3.831$, $p = 0.029$, $n = 15$ RT, 16 COLD, HEAT, with $p = 0.026$ HEAT vs RT. This increase was no longer detected at two weeks post exposures in female rats. On the other hand, IL-10 increased in male rats exposed to heat at two

weeks post exposures but not at one day post exposures ([Table 2, Figure 19](#)). One way ANOVA of male IL-10 data at two weeks post exposures yielded $F(2, 27) = 10.103$, $p < 0.001$, $n = 10$, with $p < 0.001$ HEAT vs RT and $p = 0.008$ HEAT vs COLD.

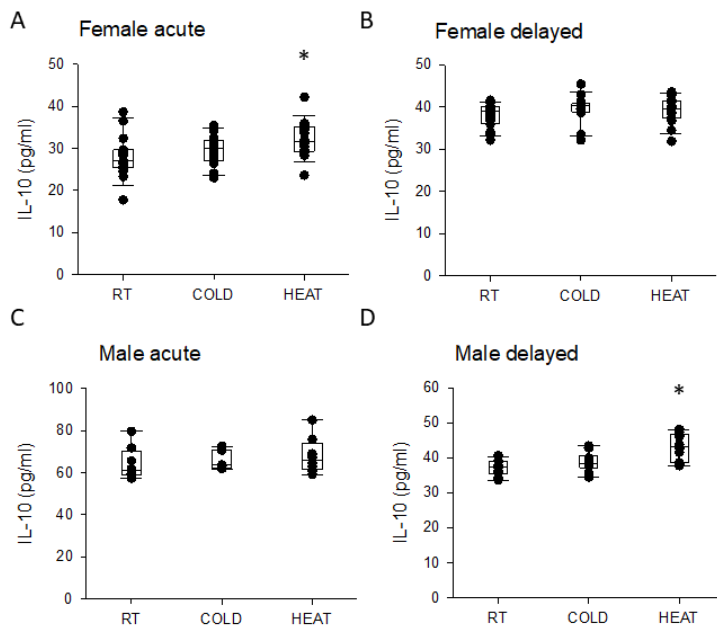


Figure 19. IL-10. (A) Plasma IL-10 level from female rats exposed to heat was increased at one day post-exposure ($F(2, 44) = 3.831$, $p = 0.029$, $n = 15$ RT, 16 COLD, HEAT, $*p = 0.026$ HEAT vs RT). (B) Plasma IL-10 level from female rats was not significantly altered two weeks post-exposures ($F(2, 45) = 1.229$, $p = 0.302$, $n = 16$). (C) Plasma IL-10 level from male rats was not significantly altered one day post-exposure ($F(2, 20) = 0.555$, $p = 0.583$, $n = 8$ RT, 7 HEAT, 8 COLD). (D) Plasma IL-10 level from male rats exposed to heat was increased two weeks post-exposures ($F(2, 27) = 10.103$, $p = 0.00053$, $n = 10$, $*p < 0.001$ HEAT vs RT, $p = 0.008$ HEAT vs COLD).

IL-4. There was a delayed increase in plasma level of IL-4 from male rats exposed to cold or heat exposure ([Table 2, Figure 20](#)). One way ANOVA of IL-4 data from male rats at two weeks post exposure yielded $F(2, 27) = 4.607$, $p = 0.019$ ($n = 10$) with post hoc multiple comparison yielding $p = 0.026$ for HEAT vs RT and $p = 0.049$ for COLD vs RT.

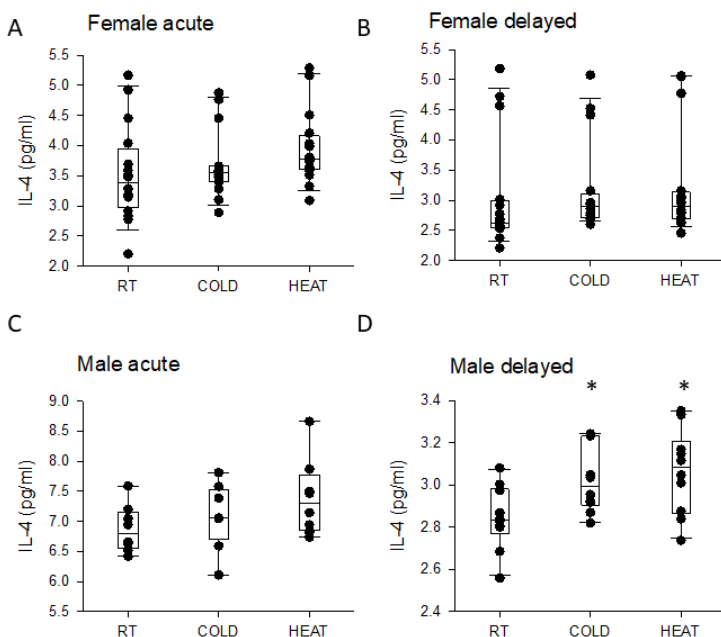


Figure 20. IL-4. (A) Plasma level of IL-4 was not significantly altered in female rats at one day post-exposures ($F(2, 44) = 1.682$, $p = 0.198$). (B) Plasma level of IL-4 was not significantly altered in female rats at two weeks post-exposures ($F(2, 45) = 0.235$, $p = 0.791$, $n = 16$). (C) Plasma level of IL-4 was not significantly altered in male rats at one day post-exposures ($F(2, 21) = 1.893$, $p = 0.175$, $n = 8$). (D) Plasma level of IL-4 was increased in male rats exposed to cold or heat at two weeks post-exposures ($F(2, 27) = 4.607$, $p = 0.019$, $n = 10$, $*p = 0.026$ HEAT vs RT, $*p = 0.049$ COLD vs RT).

IL-13. There was a delayed increase in plasma level of IL-13 from male rats exposed to cold ([Table 2](#), [Figure 21](#)). One way ANOVA of IL-13 data from male rats at two weeks post exposure yielded $F(2, 26) = 3.772$ $p = 0.036$ ($n = 9$ RT, 10 COLD, HEAT) with post hoc multiple comparison yielding $p = 0.032$ for COLD vs RT.

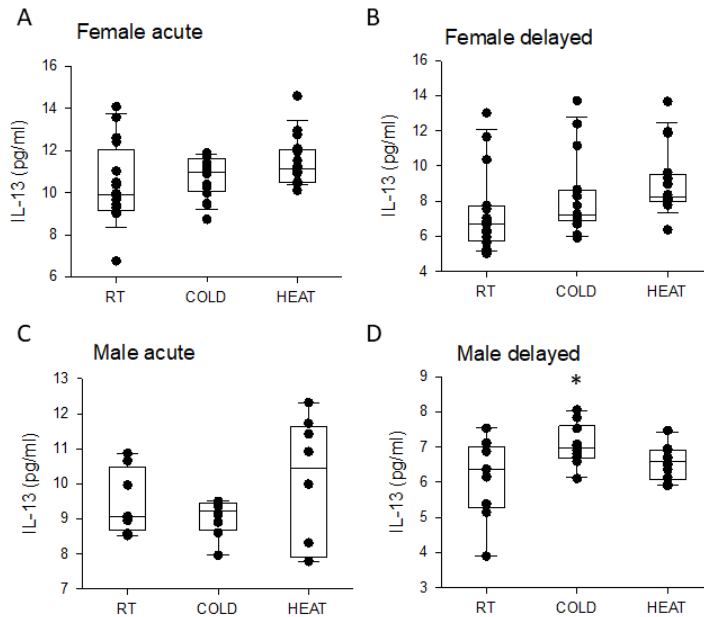


Figure 21. IL-13. (A) Plasma level of IL-13 was not significantly altered in female rats at one day post-exposures ($F(2, 45) = 2.301$, $p = 0.112$, $n = 16$). (B) Plasma level of IL-13 was not significantly altered in female rats at two weeks post-exposures ($F(2, 45) = 2.39$, $p = 0.103$, $n = 16$). (C) Plasma level of IL-13 was not significantly altered in male rats at one day post-exposures ($F(2, 21) = 1.313$, $p = 0.29$, $n = 8$). (D) Plasma level of IL-13 was increased in male rats exposed to cold at two weeks post-exposures ($F(2, 26) = 3.772$, $p = 0.036$, $n = 9$ RT, 10 COLD, HEAT, $*p = 0.032$ COLD vs RT).

TNF- α . No statistically significant effect was detected in plasma level of TNF- α from female rats following cold or heat exposures at one day or two weeks post exposures. Similarly, no statistically significant effect was detected in plasma level of TNF- α from male rats at one day or two weeks post exposures ([Figure 22](#)).

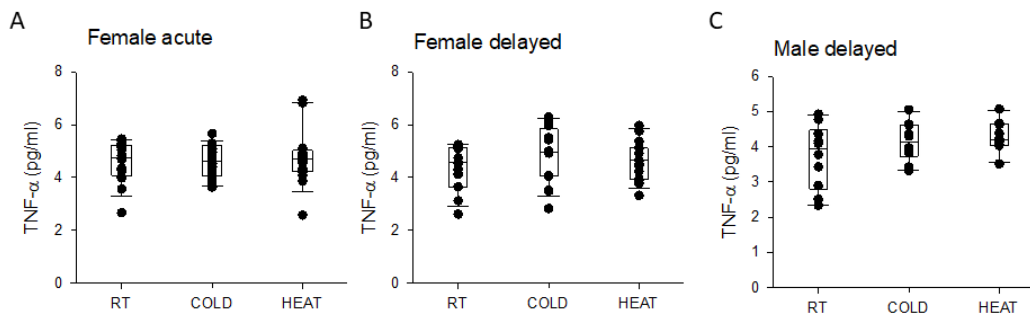


Figure 22. TNF- α . (A) Plasma level of TNF- α was not significantly altered in female rats at one day post-exposures ($F(2, 45) = 0.221$, $p = 0.802$, $n = 16$). (B) Plasma level of TNF- α was not significantly altered in female rats at two weeks post-exposures ($F(2, 43) = 0.969$, $p = 0.388$, $n = 15$ RT, 16 COLD, 15 HEAT). (C) Plasma level of TNF- α was not significantly altered in male rats at two weeks post-exposures ($F(2, 27) = 1.931$, $p = 0.164$, $n = 10$).

Table 1. Summary data from one day post-exposures. Effects of cold or heat exposures on plasma cytokine levels one day following exposures.

	DELAYED					
	Male			Female		
	Control	Cold	Heat	Control	Cold	Heat
IFN- γ (pg/ml)	3.7 + 0.2	4.1 + 0.3	4.2 + 0.2	20.2 + 0.8	22.7 + 0.6 *	23.6 + 0.7 *
IL-10 (pg/ml)	37.1 + 0.7	38.7 + 0.9	43.0 + 1.2 *	38.0 + 0.7	39.7 + 0.8	39.1 + 0.8
IL-13 (pg/ml)	6.1 + 0.4	7.1 + 0.2 *	6.6 + 0.2	7.4 + 0.6	8.2 + 0.6	9.0 + 0.5
IL-4 (pg/ml)	2.8 + 0.05	3.0 + 0.05 *	3.1 + 0.06 *	3.0 + 0.2	3.2 + 0.2	3.2 + 0.2
IL-6 (pg/ml)	88.3 + 8.6	110.3 + 8.8	94.3 + 10.3	141.9 + 14.0	186.5 + 12.7 *	247.2 + 15.0 *

Table 2. Summary data from two weeks post-exposures. Effects of cold or heat exposures on plasma cytokine levels two weeks following exposures.

	ACUTE					
	Male			Female		
	Control	Cold	Heat	Control	Cold	Heat
IFN- γ (pg/ml)	28.6 + 0.6	28.6 + 0.7	29.9 + 1.2	21.0 + 1.0	21.7 + 0.8	24.4 + 0.7 *
IL-10 (pg/ml)	64.4 + 2.7	65.5 + 1.6	68.1 + 3.1	64.4 + 2.7	65.5 + 1.6	68.1 + 3.1
IL-13 (pg/ml)	9.5 + 0.3	9.0 + 0.2	10.0 + 0.7	10.4 + 0.5	10.7 + 0.2	11.5 + 0.3
IL-4 (pg/ml)	6.9 + 0.1	7.1 + 0.2	7.4 + 0.2	3.5 + 0.2	3.7 + 0.1	4.0 + 0.1
IL-6 (pg/ml)	549.1 + 76.8	533.1 + 115.8	494.3 + 79.1	236.1 + 11.4	262.7 + 10.3	280.6 + 7.7 *

Specific Aim 3: Effects of tDCS on temperature-induced behavioral effects

All rats used to complete Specific Aim 3 underwent surgery to implant the tDCS electrode holder. Rats were then divided into RT, COLD and HEAT cohorts. Seven days after surgery, rats were exposed to RT (~22-24°C), cold (4°C, 6 hours) or heat (38°C, 1 hour) daily for 21 days. Following completion of exposures, one half of the rats from each cohort were subjected to tDCS (0.25 mA for 30 min) whereas the remaining half were connected but were not stimulated and thus served as our SHAM group. Therefore, our experimental groups are RT SHAM, RT tDCS, COLD SHAM, COLD tDCS, HEAT SHAM, HEAT tDCS. Rats were subjected to tDCS or SHAM one day following the last exposure day, then underwent motor activity testing the same day. For the next three days, rats were subjected to NOR test. One week following the last exposure day or three days after the last NOR day, tDCS/SHAM were administered for the second time. The next day, rats were subjected to 6-days of MWM testing. After completion of MWM, these rats were euthanized and plasma and brain were collected for biochemical endpoints two weeks post exposures. For female rats, we had a separate cohort in which rats were subjected to surgery, 7-day recovery period followed by 21 days of RT, cold or heat exposures. Following the last day of exposures, these female rats were administered 30 min of 0.25 mA tDCS/SHAM and were euthanized 30 min later. Plasma and brain collected from these female rats were used to obtain our biochemical endpoint and these data are referred to as our one day post exposures outcome. Experiment timeline is shown in [Figure 23](#).

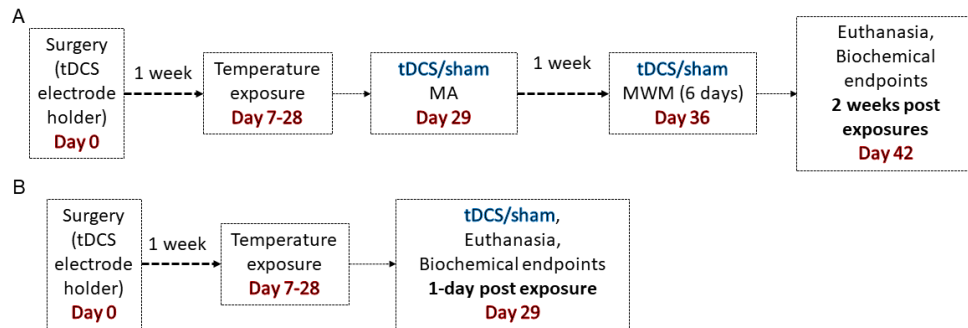


Figure 23. Experimental time line for Aim 3. All rats in this aim were subjected to surgery to implant the tDCS electrode holder onto their scalp. Following a recovery period, rats were exposed to RT, COLD or HEAT for 21 consecutive days. For female rats there were 2 separate experimental cohorts described in (A) and (B), whereas for the male rats, there were only one cohort, described in (B). (A) For the first cohort, female rats were then subjected to either tDCS (0.25 mA, 30 min) or SHAM (0 mA, 30 min) the next day followed by euthanasia for brain and blood collection. This cohort provided our one day post exposure biochemistry data. (B) For the second cohort, female rats were then subjected to either tDCS (0.25 mA, 30 min) or SHAM (0 mA, 30 min) and tested in open field chambers to assess motor activity levels. One week later, rats were subjected to a second round of tDCS or SHAM followed by 5 days of Morris water maze training. Probe test day for Morris water maze was assessed on the 6th day. The next day, which is approximately 2 week post completion of temperature exposures, all rats were euthanized. The right half of the brain was used for electrophysiology whereas the left half was used for biochemistry measurements. Plasma was also collected from trunk blood. This cohort provided our 2 weeks post exposure biochemistry data.

Animal Weights

Significant reduction in body weight gain was observed for female rats exposed to heat ([Figure 24](#)). Two way ANOVA was conducted, with temperature exposures as one factor (RT, COLD, HEAT) and number of weeks as the second factor (Week 1 – Week 5). Significant difference between exposure groups was detected with $F(2, 225) = 7.896$, $p < 0.001$, $n = 16$. Post-hoc comparison analysis using Holm Sidak method yielded significant difference between HEAT vs RT with $p < 0.001$. Similar to Aim 2 weight changes, there was no significant difference in weight gain between COLD and RT in female rats. However, significant reduction in weight gain was observed in male rats exposed to COLD or HEAT compared to male rats exposed to RT ([Figure 24](#)). Two way ANOVA yielded significant difference among exposure groups with $F(2, 181) = 41.664$, $p < 0.001$, $n = 15$ RT, 16 COLD, 10 HEAT and post hoc multiple comparison analysis revealed significant difference between COLD vs RT and HEAT vs RT with $p < 0.001$. One male rat from the RT group, one male rat from the COLD group and two male rats from the HEAT group were euthanized prior to completion of exposures due to the loss of the tDCS probe holder that was previously implanted onto the animal's scalp. Four additional male rats from the HEAT group died during the second week of heat exposure, presumably resulting from heat stroke. We also found that the temperature of one part of the chamber had arisen to 42°C. Following this finding, that chamber was no longer used. The cold chamber was then converted to the heat chamber and heat exposures took place in this chamber during the third week. We used a cold storage room that was kept at 4°C for our cold exposure on the third week.

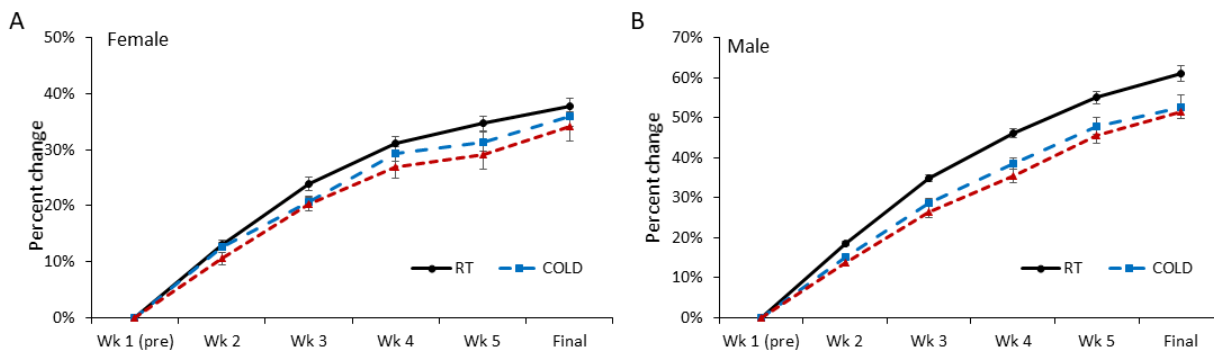


Figure 24. Body weight changes. (A) Female rats from Aim 3 exposed to heat had significant decrease in body weight gain compared to female rats exposed to RT (two way ANOVA $F(2, 225) = 7.896$ $p < 0.001$, with $p < 0.001$ RT vs HEAT, $n = 16$). Week 1 data is the pre-exposure weight just prior to initiation of exposures. Week 2 and 3 are weight gains during exposures. Week 4 and 5 are weight gains after the 21-day exposures. (B) Male rats from Aim 3 exposed to cold or heat had significant decrease in body weight gain compared to male rats exposed to RT (two way ANOVA $F(2, 181) = 41.664$ $p < 0.001$, with $p < 0.001$ for COLD vs RT and HEAT vs RT, $n = 10$).

Motor Activity

We detected distinct effects of tDCS on **motor activity levels** in female and male rats following exposures to cold. Administration of tDCS in female rats did not result in significant changes in ambulatory motor activity, in the room temperature (RT/control), COLD or HEAT groups. Data on stereotypical motor activity are shown in [Figure 25 A-C](#). Administration of tDCS in male rats in the COLD group significantly increased stereotypical activity ([Figure 25 E](#), $p = 0.03$, t-test, $n = 7$ SHAM, 8 tDCS). No significant difference was detected between the RT or HEAT group ([Figure 25 D,F](#)). Administration of tDCS did not generate statistically significant changes in other motor activity parameters (distance traveled, activity time, total rears, total ambulation).

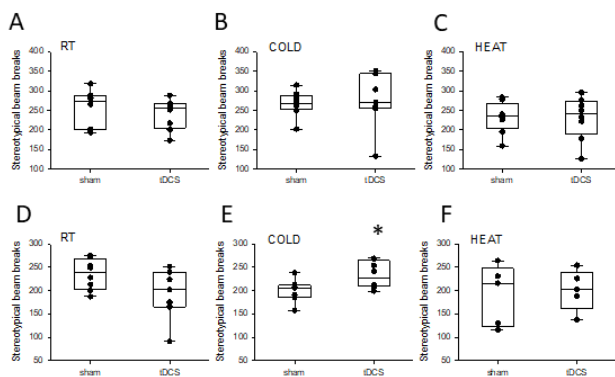


Figure 25. Effects of tDCS on stereotypical motor activity following RT (CONTROL), HEAT or COLD exposures in female (A-C) and male (D-F) rats. (A) Administration of tDCS did not induce statistically significant change in stereotypical beam breaks in control female rats (RT) ($p = 0.54$, t-test, $n = 8$). (B) Administration of tDCS did not induce statistically significant change in stereotypical beam breaks in female rats exposed to cold ($p = 0.81$, t-test, $n = 8$ SHAM, 7 tDCS). (C) Administration of tDCS did not induce statistically significant change in stereotypical beam breaks in female rats that were exposed to heat ($p = 0.96$, t-test, $n = 8$). (D) tDCS decreased stereotypical beam breaks of rats exposed to room temperature (RT), but decrease was not statistically significant ($p = 0.08$, t-test, $n = 8$ SHAM, 7 tDCS). (E) tDCS increased stereotypical beam breaks of rats exposed to COLD (* $p = 0.03$, t-test, $n = 7$ SHAM, 8 tDCS). (F) tDCS did not increase stereotypical beam breaks of rats exposed to HEAT ($p = 0.78$, t-test, $n = 5$).

Morris water maze (MWM)

We detected distinct effects of tDCS on MWM performance in female and male rats. Rats were placed in a water tank to find the hidden platform using four visible spatial cues during training days (5 days). Latency in finding the hidden platform, total distance traveled, speed were among the parameters measured to assess spatial learning. During the probe test day (6th day), the hidden platform was removed and time spent in

platform quadrant was the main parameter measured to assess memory. Administration of tDCS induced **reduced the latency** to finding the hidden platform (slight improvement) in the learning phase of the Morris water maze test in female rats that were exposed to room temperature (RT) or heat, but not cold (**Figure 26 A-C**). No significant effects of extreme temperature exposures or tDCS were observed in the memory phase (probe day). Administration of tDCS **increased the latency** to finding the platform (slight decline) in the HEAT group on day 2 and day 4 of training ($p < 0.05$, t-test) (**Figure 26 F**). Trending increase in latency was observed in CONTROL rats exposed to RT resulting from tDCS, but increase did not reach statistical significance (**Figure 26 D**). No significant effect of tDCS was seen in the COLD group (**Figure 26 E**). No significant effects of extreme temperature exposures or tDCS were observed in the memory phase.

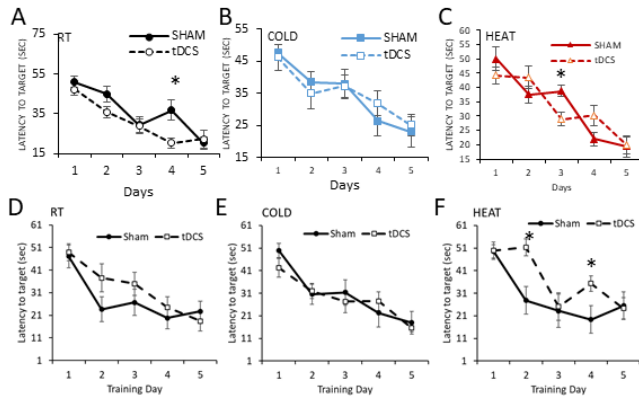


Figure 26. Effects of tDCS on water maze performance after RT, COLD and HEAT exposures on female and male rats. (A) Slight improvement in learning was observed resulting from tDCS administration in RT female rats. Administration of tDCS (0.25 mA, 30 min) on control rats exposed to room temperature (RT) resulted in improved learning performance on day 4 as they found the hidden platform (target) in less time than RT rats who were not subjected to tDCS (SHAM). $*p < 0.05$ (day 4), $n=8$. (B) No effect was observed resulting from tDCS administration in female rats exposed to cold ($n = 8$). Administration of tDCS (0.25 mA, 30 min) on rats exposed to 4°C did not result in improved learning performance as they found the hidden platform (target) in similar time as rats who were not subjected to tDCS (SHAM). (C) Slight improvement in learning was

observed resulting from tDCS administration in female rats exposed to heat. Administration of tDCS (0.25 mA, 30 min) on rats exposed to 38°C resulted in improved learning performance on day 3 as they found the hidden platform (target) in less time than rats who were not subjected to tDCS (SHAM). $*p < 0.05$ (day 3), $n = 8$. (D) tDCS induced a trending increase in latency to finding the hidden target, but increase was not statistically significant (two way ANOVA, $n=7$ SHAM, 8 tDCS). (E) tDCS had no effect on water maze performance from rats exposed to repeated intermittent COLD ($n=6$ SHAM, 7 tDCS). (F) tDCS increased the latency to finding the hidden platform in rats exposed to repeated intermittent HEAT ($*p < 0.05$, day 2 and day 4, $n = 5$ SHAM, $n = 4$ tDCS). There was no effect of tDCS on memory (probe test day).

Specific Aim 3: Effects of tDCS on temperature-induced biochemical effects

Estradiol

Acute effects on estradiol levels in female rats differed between repeated cold and heat exposures at one day post exposures. At one day post exposures, we detected a statistically significant decrease in plasma estradiol level from female rats in the COLD group compared to CONTROL (**Figure 27 A-C**). However, there was a statistically significant increase in plasma estradiol level from rats in the HEAT group compared to CONTROL (**Figure 27 A-C**). There was no difference in estradiol level from rats subjected to tDCS compared to SHAM. Therefore, at one day post exposures, tDCS administration did not reverse changes in plasma levels of estradiol in female rats induced by extreme temperature exposures. Two way ANOVA produced significant difference between groups ($F(2,42) = 36.359$ $p < 0.001$) but not between SHAM and tDCS ($F(1,42) = 0.658$ $p = 0.422$). There is no significant interaction between exposure and stimulation groups ($p = 0.564$). Post hoc multiple comparison analysis using Holm Sidak method yielded significant difference between COLD and CONTROL, HEAT and CONTROL and between COLD and HEAT ($p < 0.001$). Significant difference within SHAM between COLD vs CONTROL ($p = 0.003$), HEAT vs CONTROL ($p = 0.002$) and COLD vs HEAT ($p < 0.001$) were detected. Significant difference within tDCS between COLD vs CONTROL ($p = 0.014$), HEAT vs CONTROL ($p = 0.019$) and COLD vs HEAT ($p < 0.001$) were also detected.

Delayed reduction of estradiol was detected two weeks post exposures to cold or heat. Potential mitigating effect of tDCS on the delayed effects of temperature-induced reduction of estradiol levels was observed. At

two weeks post exposures, there was a significant reduction in estradiol level from female rats exposed to cold and heat (**Figure 27 D-F**). Although a statistical difference was not detected between tDCS and SHAM, tDCS administration reduced the effects on estradiol levels at 2 weeks post exposures such that differences in average values across exposure groups were no longer statistically significant. Two way ANOVA produced significant difference between groups ($F(2,42) = 5.369$ $p = 0.008$) but not between SHAM and tDCS ($F(1,42) = 0.0865$ $p = 0.770$). There is no significant interaction between exposure and stimulation groups ($p = 0.609$). Post hoc multiple comparison analysis using Holm Sidak method yielded significant difference between COLD and CONTROL ($p = 0.009$), and between HEAT and CONTROL ($p = 0.042$). Within the SHAM group, significant difference was detected only between COLD vs CONTROL ($p = 0.017$), but not HEAT vs CONTROL ($p = 0.101$). However, there was no significant difference within the tDCS group between COLD vs CONTROL ($p = 0.361$), or between HEAT vs CONTROL ($p = 0.316$).

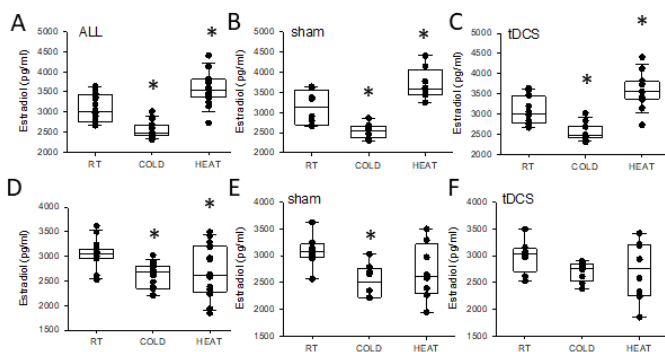


Figure 27. Differing effects of cold and heat on estradiol level in female rats at one day post exposures (A-C) and potential mitigating effect of tDCS at two weeks post exposures (D-F). (A) Acute data from female rats at 1 day post exposures, showing that exposure to COLD reduced estradiol levels but increased estradiol level in female rats exposed to HEAT ($p < 0.001$ two way ANOVA, $*p < 0.001$, $n = 16$). (B) Within the SHAM group, significant decrease and increase in estradiol level in female rats exposed to COLD ($*p = 0.003$) and HEAT ($*p = 0.002$) respectively was detected ($n = 8$). (C) Within the tDCS group significant decrease and increase in estradiol level in female rats exposed to COLD ($*p = 0.019$) and HEAT ($*p < 0.001$) respectively was still detected ($n = 8$). No significant effects of tDCS was observed at 1 day post

exposures. (D) Delayed data showing that exposure to COLD or HEAT reduced estradiol levels in female rats 2 weeks post exposures ($p = 0.008$, two way ANOVA, $n = 16$). (E) Within the SHAM group, there was significant reduction in estradiol levels from rats exposed to COLD ($*p = 0.017$). There was a trending reduction from rats in the HEAT group but was not statistically significant ($p = 0.101$) ($n = 8$). (F) Within the tDCS group, a trending reduction was still observed from female rats exposed to COLD ($p = 0.361$) or HEAT ($p = 0.316$) but reduction was no longer statistically significant ($n = 8$).

Neurotransmitters

Changes in levels of neurotransmitters in homogenized brain were detected for female and male rats following repeated exposures to cold or heat. Our data show that tDCS administration cannot reverse changes in glutamate or serotonin levels in the brain of female or male rats induced by extreme temperature exposures. However, our data indicate that tDCS induced some effect on dopamine levels in male but not female rats. Data on GABA measurements were limited and our data did not indicate significant effects of tDCS on temperature-induced changes.

Serotonin. Changes in serotonin levels were observed in female but not in male rats and with no statistical effects of tDCS (**Figure 28**). There were differential acute/immediate versus delayed effects of COLD and HEAT exposures in female rats. At one day post-exposures, there was a trending decrease in serotonin in female rats exposed to COLD (compared to CONTROL rats) but a trending increase in serotonin in rats exposed to HEAT (**Figure 28 A**). Two way ANOVA yielded significant difference between groups ($F(2,42) = 3.558$ $p = 0.037$) but not between tDCS and SHAM ($F(1,42) = 1.373$ $p = 0.248$). Post hoc multiple comparison analysis (Holm Sidak) produced significant difference only between COLD and HEAT ($p = 0.037$). Within the individual SHAM and tDCS groups, no significant difference was detected (**Figure 28 B-C**). At two weeks post-exposures, there was a statistically significant increase in serotonin level from female rats in COLD group compared to CONTROL ($P < 0.001$) but a decrease in HEAT group compared to CONTROL ($P < 0.001$), without statistical effects of tDCS (**Figure 28 D**). This is the opposite of the trending changes observed at one day post exposure (acute data).

Two way ANOVA yielded significant difference between groups ($F(2,42) = 63.762$ $p < 0.001$) but not between tDCS and SHAM ($F(1,42) = 0.270$ $p = 0.606$). There is not a significant interaction between exposure and stimulation groups ($p = 0.378$). Post hoc multiple comparison using Holm Sidak method yielded statistical significance between COLD and CONTROL ($p < 0.001$), HEAT and CONTROL ($p < 0.001$), and also between COLD and HEAT ($p < 0.001$). Significant differences between the temperature exposure cohorts were also observed within SHAM and tDCS groups ($p < 0.002$) (Figure 28 E-F). In male rats, there was no statistical effect of tDCS on serotonin levels (Figure 28 G-I).

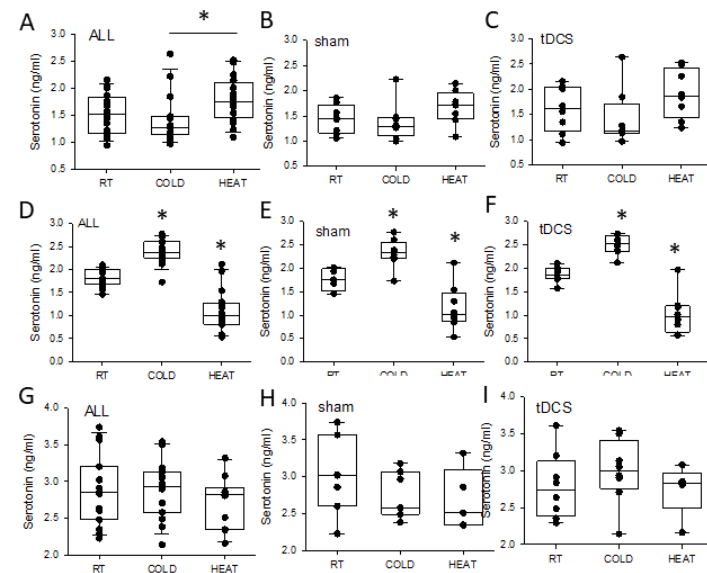


Figure 28. Delayed reduction in serotonin level in the brain from female rats resulting from COLD and HEAT exposures with no statistically significant effect of tDCS. (A-C) Data from female rats, one day post exposures. Two way ANOVA yielded significant differences between groups ($F(2, 42) = 3.558$, $p = 0.037$). (A) Acute data from combined SHAM and tDCS female rats showing trending decrease in serotonin levels in COLD group relative to CONTROL (RT) and trending increase in HEAT group relative to CONTROL ($n = 16$). There was a statistically significant difference detected between the COLD and HEAT group ($*p = 0.037$) (B,C) There were no statistically significant effects within the SHAM (B) or tDCS group (C) ($n = 8$). (D-F) Data from female rats, two weeks post exposures. Two way ANOVA yielded significant differences between groups ($F(2, 42) = 63.762$, $p < 0.001$). (D) Combined tDCS and SHAM female rats showed a statistically significant increase in serotonin in the COLD group but a significant reduction in the HEAT group ($*p < 0.05$) ($n = 16$). (E,F) Significant increase in the COLD and decrease in the HEAT group were also detected within the SHAM (E) and tDCS (F) groups, ($p < 0.05$, $n = 8$). (G-

I) Data from male rats, two weeks post exposures, showing no statistically significant effect of temperature exposures or SHAM/tDCS administrations ($F(2, 34) = 0.623$, $p = 0.542$, SHAM: $n = 7$ RT, 7 COLD, 5 HEAT, tDCS: $n = 8$ RT, 8 COLD, 5 HEAT).

Dopamine. Reduction in brain dopamine level was observed in both female and male rats in Aim 2. However, male rats subjected to tDCS after cold/heat exposures did not experience significant changes in dopamine. In female rats there was a reduction in dopamine level due to heat exposures, independent of tDCS. At one day post exposures, there was a statistically significant reduction of dopamine from female rats in the HEAT group compared to CONTROL at 1 day post exposure, without statistical effects of tDCS (Figure 29 A). Two way ANOVA resulted in significant difference between groups ($F(2,42) = 4.470$ $p = 0.017$) but not between tDCS and SHAM ($F(1,42) = 1.652$ $p = 0.206$). There is not a statistically significant interaction between exposure and stimulation groups ($p = 0.804$). Post hoc multiple comparison analysis yielded significant difference between HEAT and CONTROL ($p = 0.019$). No significant difference within SHAM and tDCS groups (Figure 29 B-C). At two weeks post-exposures, reduction in dopamine in female rats were no longer observed (Figure 29 D-F).

We detected a trending reduction in brain level of dopamine in all male rats after cold or heat exposures regardless of stimulation (Figure 29 G). Reduction in dopamine in exposed male rats was statistically significant in rats that did not undergo tDCS (SHAM) (Figure 29 H). However, male rats that were subjected to 30 min of tDCS did not have statistically significant decrease in dopamine level resulting from cold or heat exposures compared to RT. Two way ANOVA yielded statistical difference between groups ($F(2,33) = 3.318$ $p = 0.049$) but not between tDCS and SHAM ($F(2, 33) = 0.495$ $p = 0.486$). There is no significant interaction between exposure and stimulation groups ($p = 0.145$). Post hoc multiple comparison analysis using Holm Sidak method did not result in significant difference between RT and HEAT ($p = 0.051$). However, within the SHAM group, statistical difference was observed between HEAT and CONTROL ($p = 0.030$) and also between COLD and CONTROL ($p = 0.041$). Interestingly, rats that were subjected to tDCS did not show significant difference in

dopamine levels. Therefore, although there was no statistically significant difference between the overall SHAM vs tDCS groups, COLD/HEAT-mediated reduction in dopamine was no longer detected in rats subjected to tDCS

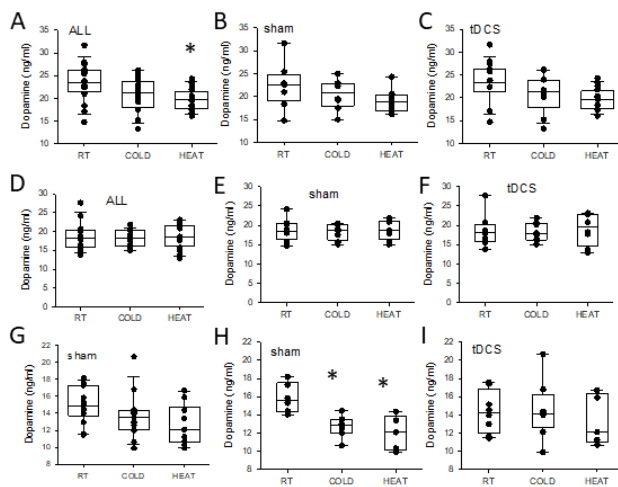


Figure 29. Reduction in brain level of dopamine from female and male rats resulting from COLD or HEAT exposures, with potential effect of tDCS in male rats. (A-C) Data from female rats, one day post exposures. Two way ANOVA yielded significant differences between groups ($F(2, 42) = 4.470, p = 0.017$). (A) Combined tDCS and SHAM female rats showed a significant decrease in dopamine levels in the HEAT group relative to CONTROL (RT) ($*p < 0.05, n = 16$). (B,C) Trending reduction in dopamine was not statistically significant within the SHAM (B) or tDCS group (C) ($n = 8$). (D-F) Data from female rats, two weeks post exposures showing no statistically significant effect in dopamine levels two week post exposures ($F(2, 42) = 0.143, p = 0.867, n = 8$). (G-I) Data from male rats, two weeks post exposures. Two way ANOVA yielded significant differences between groups ($F(2, 33) = 3.318, p = 0.049$). (G) Combined tDCS and SHAM male rat showed a trending reduction in dopamine levels in the COLD and HEAT group, without statistical significance ($p = 0.189, 0.051$) ($n = 16$). (H) Reductions in dopamine in the COLD and HEAT groups were more pronounced within the SHAM cohort ($*p < 0.05, n = 6$ RT, 7 COLD, 5 HEAT). (I) Within the tDCS cohort, there was no longer significant reduction in dopamine detected in the COLD or HEAT groups ($n = 8$ RT, 8 COLD, 5 HEAT).

GABA. There was an acute and transient reduction of GABA following exposures to cold and heat in female rats. GABA levels were below detection limit in some samples. There was a statistically significant reduction in brain GABA levels from female rats at 1 day following exposure to cold and heat, without statistical effects from tDCS ([Figure 30](#)). Two way ANOVA revealed statistically significant difference between exposure groups ($F(2, 20) = 5.601, p = 0.012$) but not between tDCS and SHAM ($F(1, 20) = 0.0181, p = 0.894$). Post hoc multiple comparison using Holm Sidak revealed statistical difference between COLD and CONTROL ($p = 0.010$) ([Figure 30 A](#)). Within the SHAM group, there were statistical differences between COLD and CONTROL ($p = 0.039$) and also between HEAT and CONTROL ($p = 0.039$) ([Figure 30 B](#)). However, within the tDCS group, there were no statistical difference between the exposure groups (limited sample per group) ([Figure 29 C](#)). There was no significant changes detected in samples obtained ~2 weeks post exposures ([Figure 29 D-F](#)). There was no statistical effect of tDCS on GABA levels from male rats. However, some of our samples were below detection limit. There was a trending increase in GABA levels from male rats exposed to COLD compared to RT and HEAT in the SHAM group ([Figure 30 E](#)).

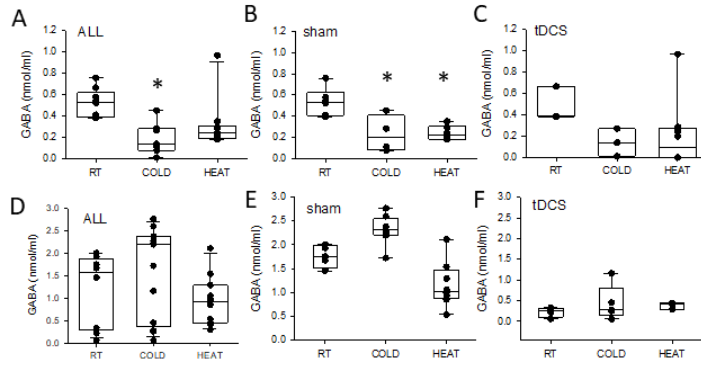


Figure 30. Transient reduction in GABA level in the brain from female rats resulting from COLD and HEAT exposures with no statistically significant effect of tDCS. (A-C) Data from female rats, one day post exposures. Two way ANOVA yielded significant difference among experimental groups ($F(2, 20) = 5.601, p = 0.012$). (A) There was a statistically significant reduction in GABA level from all rats (combined tDCS and SHAM) exposed to COLD compared to CONTROL (RT) ($*p < 0.05$) ($n = 9$ RT, 7 COLD, 10 HEAT). (B) Significant decrease in GABA relative to RT was detected in female rats exposed to COLD or HEAT within the SHAM only group ($*p < 0.05$) ($n = 6$ RT, 4 COLD, 6 HEAT). (C) Trending decrease in GABA was detected in female rats exposed to COLD or HEAT within the tDCS only group but did

not reach statistical significance, likely due to the limited samples per cohort ($n = 3$ RT, 3 COLD, 4 HEAT, many samples were measured at below the detection limit). (D-F) Data from female rats, two weeks post exposures, showing trending changes in GABA levels resulting from COLD or HEAT exposures (two way ANOVA $F(2, 20) = 2.092, p = 0.150$, SHAM: $n = 5$ RT, 4 COLD, 5 HEAT, tDCS: $n = 4$ RT, 5 COLD, 3 HEAT).

Glutamate. Exposures to heat induced a delayed decrease in glutamate level in female rats, that was unaffected by tDCS (Figure 31). No significant effects from temperature exposures or tDCS/SHAM administration were observed at one day post-exposures. However, there was a statistically significant reduction in glutamate level from female rats in HEAT group compared to CONTROL at two weeks post exposure without statistical effects of tDCS (Figure 31 D-F). Two way ANOVA yielded significant difference between exposure groups ($F(2, 41) = 6.837, p = 0.003$), but not between tDCS and SHAM ($F(2, 41) = 0.161, p = 0.691$). There was not a statistically significant interaction between exposure and stimulation groups ($p = 0.986$). Post hoc multiple comparison analysis using Holm Sidak yielded significant difference between HEAT and CONTROL ($p = 0.002$). Significant difference was also observed between HEAT and CONTROL within the SHAM group ($p = 0.05$) and within the tDCS group ($p = 0.027$). In male rats, there was no statistically significant effect on glutamate levels. Administration of tDCS did not induce significant changes in glutamate levels (Figure 31 G-I).

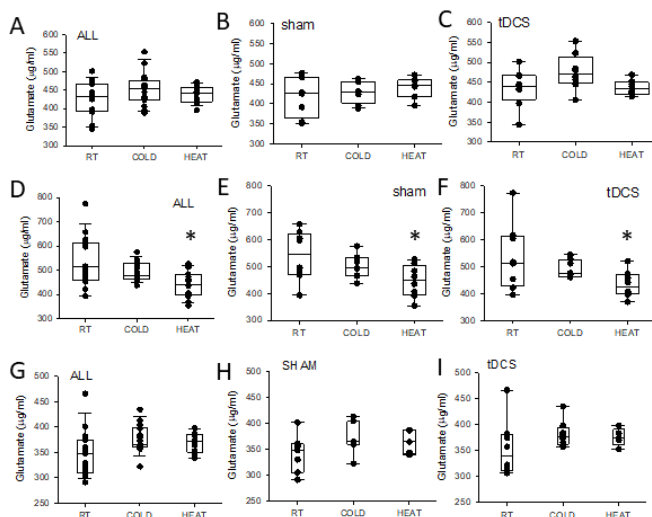


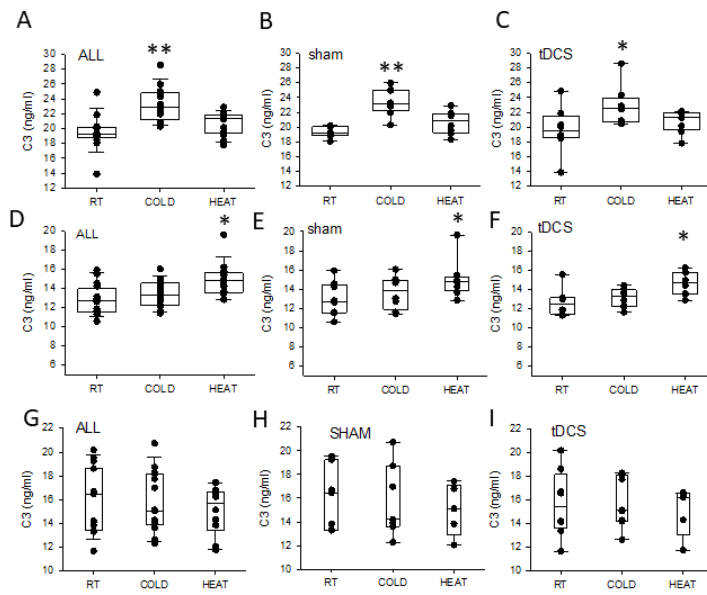
Figure 31. Delayed reduction in glutamate level in the brain from female rats resulting from HEAT exposures with no statistically significant effect of tDCS. (A-C) Data from female rats, one day post exposures, showing no statistically significant acute effect of COLD or HEAT exposures on glutamate levels with or without tDCS ($F(2, 41) = 1.721, p = 0.192$, SHAM: $n = 8$ RT, 8 COLD, 7 HEAT, tDCS: $n = 8$). (D-F) Data from female rats, two weeks post exposures. Two way ANOVA yielded significant differences between groups ($F(2, 41) = 6.837, p = 0.003$). (D) There was a statistically significant reduction in glutamate level from all rats (combined tDCS and SHAM) exposed to HEAT compared to CONTROL (RT) ($*p < 0.05$) ($n = 16$ RT, 15 COLD, 16 HEAT). (E) Significant decrease in glutamate was detected in female rats exposed to HEAT within the SHAM only group ($*p < 0.05$) ($n = 8$ RT, 7 COLD, 8 HEAT). (F) Significant decrease in glutamate was also detected in female rats exposed to HEAT within the tDCS only group ($p < 0.05$) ($n = 8$). (G-I) Data from male rats, two week post exposures, showing no statistically significant acute effect of COLD or HEAT exposures on glutamate

levels with or without tDCS. Two way ANOVA yielded $F(2, 34) = 2.571, p = 0.091$ (SHAM: $n = 7$ RT, 7 COLD, 5 HEAT, tDCS: $n = 8$ RT, 8 COLD, 5 HEAT).

Inflammatory markers

Our data showed that tDCS administration did not reverse changes in plasma levels of **inflammatory markers** induced by extreme temperature exposures in female or male rats. An exception was TNF- α level that was reduced in male rats subjected to tDCS.

Complement 3 (C3). C3 is a marker of complement activation, secreted by astrocytes and interacts with microglia. Cold or heat exposures induced an increase in C3 in female but not male rats. Administration of tDCS did not reverse changes in plasma levels of C3 in female rats induced by extreme temperature exposures. In female rats, repeated cold exposures induced an acute increase in C3 levels but HEAT exposures induced a delayed increase in C3 levels in female rats ([Figure 32](#)). We detected a statistically significant increase in brain C3 level from female rats in the COLD group compared to CONTROL at 1 day post exposure without significant effects of tDCS ([Figure 32 A-C](#)). Two way ANOVA yielded significant difference between groups with $F(2, 42) = 12.91$ $p < 0.001$, but not between tDCS and SHAM ($F(1, 42) = 0.003$ $p = 0.956$). There was not a statistically significant interaction between exposure and stimulation groups ($p = 0.822$). Post hoc multiple comparison analysis yielded significant difference between COLD and CONTROL ($p < 0.001$) and between COLD and HEAT ($p = 0.004$) but not between HEAT and CONTROL ($p = 0.108$). Within the SHAM group, there was a statistical difference between COLD and CONTROL ($p < 0.001$) and between COLD and HEAT ($p = 0.021$). Within the tDCS group, there a statistical difference between COLD and CONTROL ($p = 0.010$) and between COLD and HEAT ($p = 0.090$). Interestingly, at ~2 weeks post exposure, we detected a statistically significant increase in brain C3 level from female rats in the HEAT group compared to CONTROL, again with no significant effects of tDCS ([Figure 32 D-F](#)). Two way ANOVA yielded significant difference between groups with $F(2, 42) = 7.093$ $p = 0.002$, but not between tDCS and SHAM ($F(1, 42) = 1.003$ $p = 0.322$). There was not a statistically significant interaction between exposure and stimulation groups ($p = 0.982$). Post hoc multiple comparison analysis yielded significant difference between HEAT and CONTROL ($p = 0.002$) and between HEAT and COLD ($p = 0.023$) but not between COLD and CONTROL ($p = 0.319$). Within the SHAM group, there was a statistical difference between HEAT and CONTROL ($p = 0.043$). Within the tDCS group, there was also a statistical difference between HEAT and CONTROL ($p = 0.037$). Our data showed that temperature exposure and/or tDCS administration in male rats did not induce statistically change plasma levels of complement 3 (C3) ([Figure 32 G-I](#)). Two way ANOVA yielded no significant differences between exposure groups ($F(2, 34) = 0.324$ $p = 0.725$) or between tDCS and SHAM ($F(1, 34) = 0.036$ $p = 0.851$).



overall (J) ($n = 15$ RT, 15 COLD, 10 HEAT), within the SHAM group (H) ($n = 7$ RT, 7 COLD, 5 HEAT), or within the tDCS group (I) ($n = 8$ RT, 8 COLD, 5 HEAT).

Figure 32. Exposure to cold or heat induced an increase in C3 in female but not male rats. Administration of tDCS could not reverse C3 increases observed in female rats. (A-C) Data obtained from female rats at one day post exposures. (A) At one day post exposures, there was a statistical significant acute increase in C3 level from female rats exposed to COLD ($*p < 0.05$, $n = 16$). (B) Female rats within the SHAM group showed a statistically significant increase in C3 one day following cold exposure ($*p < 0.05$, $n = 8$). (C) Female rats within the tDCS group showed a statistically significant increase in TNF- α resulting one day after cold exposures ($*p < 0.05$, $n = 8$). (D-F) Data obtained from female rats at two week post exposures. (D) At two weeks post exposures, there was a statistically significant increase in C3 level resulting from HEAT exposures ($*p < 0.05$, $n = 16$). (E) Statistically significant increase in C3 was observed within the SHAM group ($*p < 0.05$, $n = 8$). (F) Statistically significant increase in C3 was observed within the tDCS group ($*p < 0.05$, $n = 8$). (G-I) Data from male rats at 2 weeks post exposures, showing no statistically significant change in C3 levels in male rats exposed to COLD or HEAT

IFN- γ . Our data showed that repeated exposures to COLD or HEAT induced an increase in IFN- γ level in both female and male rats, and that administration of tDCS could not reverse the temperature-induced increase in IFN- γ in female rats, but exacerbated the temperature-induced increase in IFN- γ in male rats. In female rats, administration of tDCS did not reverse the cold- and heat-induced acute increase in IFN- γ in female rats. We detected statistically significant acute increases in IFN- γ in female rats resulting from cold and heat exposures compared to control (RT), but tDCS stimulation did not induce a significant effect ([Figure 33 A-C](#)). Statistical analysis was completed using student t-test in Excel as well as two way analysis of variance (two way ANOVA) using Sigmaplot in which the two factors are the temperature exposure groups (RT/CONTROL, COLD, HEAT) and stimulation (TDCS, SHAM). Significant differences were observed between exposures groups (RT/CONTROL, COLD, HEAT) with $F(2,39) = 7.3$ $p = 0.002$. There was no statistically significant difference among the different levels of stimulation (tDCS, SHAM) ($F(1,39) = 0.80$ $p = 0.377$). The effect on the different exposure groups did not depend on the level of STIM is present. Post hoc multiple comparison testing using Holm-Sidak method yielded significant difference between COLD and CONTROL ($p = 0.015$) and between HEAT and CONTROL ($p = 0.002$). Within the SHAM group, there was no significant difference between COLD and CONTROL ($p = 0.27$) or between HEAT and CONTROL ($p = 0.095$). Within the tDCS group, there was a significant difference between the COLD and CONTROL ($p = 0.014$) and between HEAT and CONTROL ($p = 0.014$). Effect on IFN- γ level in female rats resulting from COLD/HEAT was transient and no longer observed at two weeks post exposure ([Figure 33 D-F](#)). Exposures to COLD or HEAT with or without tDCS did not induce significant effect on plasma level of IFN- γ . Two way ANOVA yielded no significant difference between groups ($F(2, 40) = 1.56$ $p = 0.223$) or between stimulations (tdcs/SHAM) ($F(1, 40) = 0.007$ $p = 0.933$). Similar to the female rat data, our data from male rats suggest that tDCS did not prevent the heat-induced delayed increase in IFN- γ . There was no statistically significant increase in the delayed plasma level of IFN- γ from male rats in the COLD group compared to RT (control) rats ([Figure 33 G,H](#)). However, we detected a statistically significant delayed increase in plasma levels of IFN- γ from male rats in the HEAT group, compared to RT and COLD rats ([Figure 33 G,H](#)). Statistically significant increase in plasma level of IFN- γ was still observed in male rats that were subjected to tDCS and heat compared to rats subjected to tDCS and either RT or cold ([Figure 33 I](#)). Two way ANOVA was performed using Sigmaplot v13 in which the two factors are the temperature exposure

groups (RT/CONTROL, COLD, HEAT) and stimulation (TDCS, SHAM). Significant differences were detected between exposure groups ($F(2,30) = 15.1$ $p < 0.001$) but were not detected between stimulation groups ($F(1,30) = 1.0$ $p = 0.326$). The effect on exposure group did not depend on stimulation (no statistically significant interaction between exposure groups and stimulation groups ($p = 0.129$)). Post hoc multiple comparison analysis using Holm Sidak method yielded significant difference between HEAT and CONTROL ($p < 0.001$) and also between HEAT and COLD ($p < 0.001$). Within the SHAM group only, significant difference was detected between HEAT and COLD ($p = 0.045$) and was nearly detected between HEAT and CONTROL ($p = 0.054$). Within the tDCS group only, significant difference was detected between HEAT and CONTROL ($p < 0.001$), HEAT and COLD ($p = 0.006$) and interestingly, also between COLD and RT ($p = 0.024$). There was no statistically significant difference between SHAM and tDCS within the HEAT or the COLD group ($p = 0.103$, 0.308).

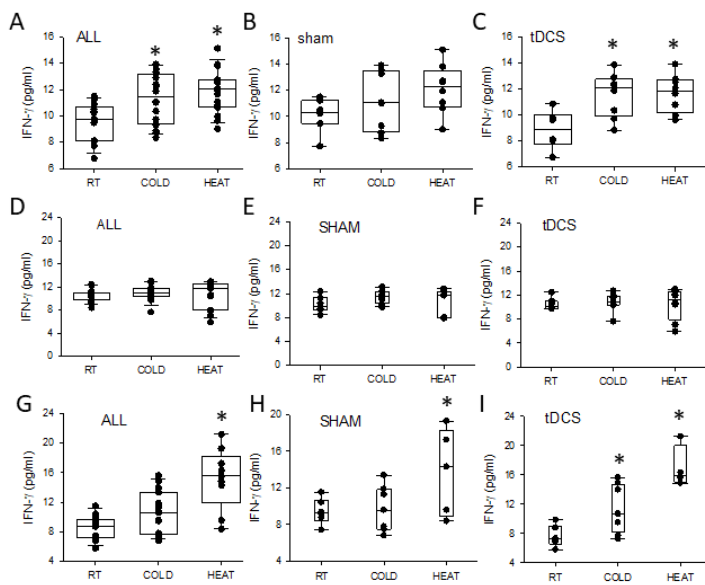


Figure 33. Administration of tDCS did not reverse the elevated IFN- γ induced by COLD or HEAT exposures in female or male rats. Samples were collected at 1 day post exposures (A-C) or two weeks post exposures (D-I). (A) At one day post-exposures, there was an overall increase in the average level of IFN- γ from all female rats (combined SHAM+tDCS groups) exposed to COLD ($*p = 0.015$) or HEAT ($*p = 0.002$) compared to CONTROL (Room Temperature or RT, $n = 13$) ($n = 13$ CONTROL, 16 COLD, 16 HEAT). (B) At one day post-exposures and within the SHAM group only, there was trending increases in IFN- γ in rats exposed to COLD ($n = 8$) and HEAT ($n = 8$) compared to CONTROL ($n = 7$) but those increases were not statistically significant. (C) At one day post-exposures, rats treated with tDCS and exposed to COLD ($n = 8$) or HEAT ($n = 8$) showed a statistically significant increase in IFN- γ relative CONTROL ($n = 6$) rats ($*p = 0.014$). (D-F) At two weeks post-exposures, increase in IFN- γ was no longer detected at 2 weeks post exposures in all female rats (D, $n = 15$ CONTROL, 15 COLD, 16 HEAT), SHAM female rats (E, $n = 7$ CONTROL, 8 COLD, 8 HEAT), or tDCS female rats (F, $n = 8$ CONTROL, 7 COLD, 8 HEAT). (G) At two weeks post-exposures, there was an increase in the average level of IFN- γ

from male rat exposed to HEAT ($*p < 0.05$) compared to CONTROL ($n = 12$ CONTROL, 15 COLD, 9 HEAT). (H) Within the SHAM group, there was a statistical difference ($*p < 0.05$) in IFN- γ level in male rats in the HEAT group but not the COLD ($n = 6$ CONTROL, 7 COLD, 5 HEAT). (I) Within the tDCS group, there were statistically significant increases in IFN- γ levels in male rats in both the COLD and HEAT group ($*p < 0.05$) ($n = 6$ CONTROL, 8 COLD, 4 HEAT).

IL-10. Repeated exposures to heat induced a significant increases in IL-10 in both female and male rats that were not affected by tDCS. A transient increase in IL-10 was also observed in female rats exposed to cold. Female rats that were repeatedly exposed to heat experienced an increased IL-10 at one day and two weeks post exposures, independent of tDCS. Increase of IL-10 due to cold exposures was observed at one day post exposures but no longer detected at two weeks post exposures. At one day post-exposures, we detected a statistically significant increase in IL-10 resulting from cold and heat exposures compared to control (RT) in female rats, but tDCS stimulation did not induce significant effect (Figure 34 A-C). Two way ANOVA revealed significant difference between exposure groups ($F(2, 31) = 16.45$ $p < 0.001$) but not between stimulation groups ($F(1, 31) = 0.354$ $p = 0.556$). Post hoc multiple comparison testing using Holm Sidak method yielded significant difference between COLD and CONTROL and between HEAT and CONTROL ($p < 0.001$). Within the SHAM group there was a significant difference between COLD and CONTROL ($p = 0.007$) and between HEAT and CONTROL ($p = 0.004$). Within the tDCS group there was a significant difference between COLD and CONTROL ($p < 0.001$) and between HEAT and CONTROL ($p = 0.007$). At two weeks post-exposures, we detected

a statistically significant delayed increase in plasma IL-10 resulting from HEAT exposure, independent of tDCS in female rats ([Figure 34 D-F](#)). Two way ANOVA revealed significant difference between exposure groups ($F(2, 38) = 4.77$ $p = 0.014$) but not between stimulation groups ($F(1,38) = 1.96$ $p = 0.170$). Post hoc multiple comparison testing using the Holm Sidak method yielded significant difference between HEAT and CONTROL ($p = 0.014$). There was no statistically significant interaction between exposure groups and tDCS ($p = 0.89$). In male rats, we detected statistically significant increases in plasma levels of IL-10 from rats in the HEAT group, compared to control rats (RT group) and also compared to rats in the COLD group ([Figure 34 G-I](#)). Statistically significant increase in plasma level of IL-10 was still observed in rats that were subjected to tDCS and heat compared to rats subjected to tDCS and either RT or cold. Two way ANOVA was performed using Sigmaplot v13 in which the two factors are the temperature exposure groups (CONTROL, COLD, HEAT) and stimulation (tDCS, SHAM). Data suggest that tDCS did not prevent the temperature induced increase in IL-10. Two way ANOVA yielded significant difference between exposure groups ($F(2, 31) = 18.149$ $p < 0.001$) but not between stimulation groups ($F(1,31) = 1.49$ $p = 0.231$). There was not a statistically significant interaction between exposure and stimulation groups ($p = 0.193$). Post hoc multiple comparison testing using Holm Sidak method yielded significant difference between HEAT and CONTROL and between HEAT and COLD ($p < 0.001$). Within the SHAM group there was a significant difference between COLD and CONTROL ($p = 0.02$) and between HEAT and COLD ($p = 0.02$). Within the tDCS group there was a significant difference between COLD and CONTROL ($p < 0.001$) and between HEAT and COLD ($p = 0.003$).

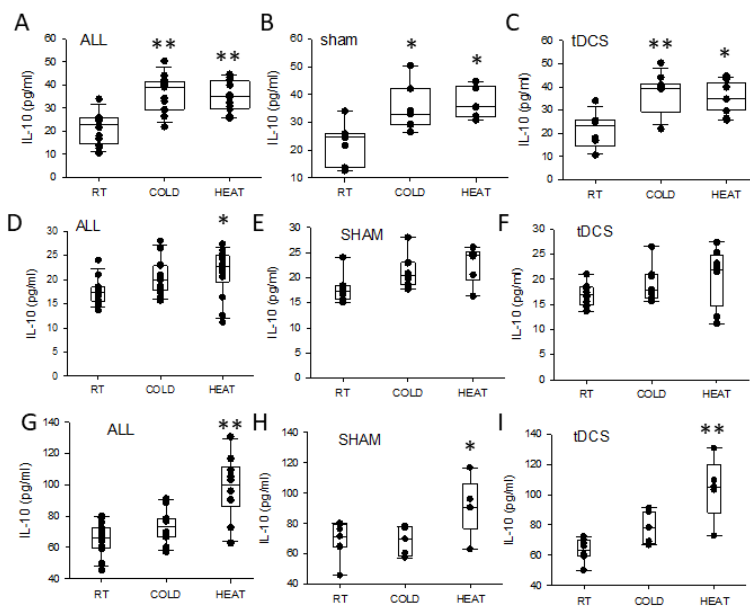


Figure 34. Administration of tDCS did not reverse the increase in IL-10 induced by COLD or HEAT exposures in female (A-F) and male (G-I) rats. (A) There was an increase in the average level of IL-10 from all female rats exposed to COLD or HEAT compared to CONTROL (RT) (** $p < 0.001$, * $p < 0.05$). (n = 12 RT, 13 COLD, 12 HEAT). (B) Within the SHAM group, significant increase was detected in COLD and HEAT female rats (* $p < 0.05$) (n = 7 RT, 7 COLD, 6 HEAT). (C) Female rats treated with tDCS and exposed to COLD or HEAT still showed statistically significant increase in IL-10 (** $p < 0.001$, * $p < 0.05$) (n = 5 RT, 6 COLD, 6 HEAT). (D) At two weeks post exposure a less pronounced increase in IL-10 was detected in the female rats exposed to cold (* $p < 0.05$) (n = 15 RT, 15 COLD, 14 HEAT). (E) Within the SHAM only group, the trending increase was not statistically significant (n = 7 RT, 8 COLD, 6 HEAT). (F) Within the tDCS only group, the trending increase was also not statistically significant (n = 8 RT, 7 COLD, 8 HEAT). (G) There was an increase in the average level of IL-10 from all male rats exposed to HEAT (** $p < 0.001$) (n = 15 RT, 12 COLD, 10 HEAT). (H) Within the SHAM group, the increase in IL-10 from male rats remained statistically significant (* $p < 0.05$) (n = 7 RT, 5 COLD, 5 HEAT). (I) Within the tDCS group, the increase in IL-10 from male rats exposed to HEAT was more pronounced (** $p < 0.001$) (n = 8 RT, 7 COLD, 5 HEAT).

IL-4. There was a significant increase detected in IL-4 from female and male rats resulting from cold or heat exposures. Administration of tDCS did not significantly affect IL-4 increase in female rats (undetermined in male rats – see below). In female rats, there was an acute increase in IL-4 due to COLD and HEAT exposures and delayed increase in IL-4 due to HEAT that were independent of tDCS. At one day post-exposures, there was a statistically significant increase in IL-4 from female rats after repeated exposures to COLD or HEAT ([Figure 35 A-C](#)). Administration of tDCS did not reverse the temperature-induced increases in IL-4 in female

rats. Two way ANOVA yielded significant difference between exposures groups (CONTROL, COLD, HEAT) with $F(2, 42) = 22.75$ $p < 0.001$, but not between stimulation groups (tDCS, SHAM) with $F(1, 42) = 0.204$ $p = 0.654$. There is not a statistically significant interaction between exposure and stimulation groups ($p = 0.914$). Post hoc multiple comparison analysis using Holm Sidak method yielded significant difference between COLD and CONTROL ($p = 0.001$) and between HEAT and CONTROL ($p < 0.001$). Within the SHAM group, there was significant difference between COLD and CONTROL ($p = 0.021$), between HEAT and CONTROL ($p < 0.001$) and also between COLD and HEAT ($p = 0.038$). At two week post-exposures, there was a slight but statistically significant increase in IL-4 in female rats exposed to HEAT ([Figure 35 D-F](#)). The acute increase in IL-4 from female rats in the COLD group was no longer detected 2 weeks post exposures. Two way ANOVA yielded significant difference between exposures groups (CONTROL, COLD, HEAT) with $F(2, 39) = 3.537$ $p = 0.039$, but not between stimulation groups (tDCS, SHAM) with $F(1, 39) = 0.0067$ $p = 0.935$. There is not a statistically significant interaction between exposure and stimulation groups ($p = 0.781$). Post hoc multiple comparison analysis using Holm Sidak method yielded significant difference between HEAT and CONTROL ($p = 0.043$). There was no statistical difference between exposure groups within the SHAM or tDCS groups.

IL-4 data from male rats were below detection limit. However, data from Aim 2 showed sig difference in plasma IL-4 levels between COLD and CONTROL and between HEAT and CONTROL (one way ANOVA $F(2, 27) = 4.61$ $p = 0.019$, post hoc comparison COLD vs CONTROL $p = 0.049$, HEAT vs CONTROL $p = 0.026$) ([Figure 35 G](#)).

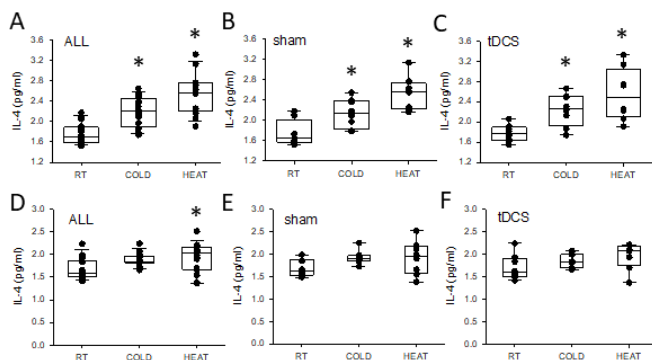


Figure 35. Administration of tDCS did not reverse the increase in IL-4 induced by COLD or HEAT exposures in female (A-F) rats at 1 day post exposures. (A) There was an increase in the average level of IL-4 from all rats exposed to COLD or HEAT compared to CONTROL (RT) at one day post exposures ($*p = 0.001$, $**p < 0.001$) ($n = 16$). (B) Within the SHAM group, there were statistically significant differences in IL-4 levels from female rats exposed to COLD or HEAT ($n = 8$). (C) Female rats treated with tDCS and exposed to COLD or HEAT still showed statistically significant increase in IL-4 relative to rats treated with tDCS and exposed to RT ($p = 0.014$, $P < 0.001$) ($n = 8$). (D) Significant increase in IL-4 was not as pronounced at 2 weeks post exposures. There was an overall slight but significant increase in IL-4 in female rats

exposed to HEAT ($*p < 0.05$, $n = 14$ RT, 15 COLD, 16 HEAT). (E) No statistical difference was observed within the SHAM only group ($n = 6$ RT, 8 COLD, 8 HEAT). (F) No statistical difference was observed within the tDCS only group ($n = 8$ RT, 7 COLD, 8 HEAT). IL-4 data from male rats subjected to tDCS or SHAM were below detection level.

IL-13. We detected some significant differences in IL-13 data from female rats across SHAM rats exposed to cold with some potential mitigating effect of tDCS ([Figure 36 A-F](#)). There was a statistically significant increase in IL-13 in female rats one day following exposures to cold in SHAM rats ($F(2, 13) = 10.598$ $p = 0.002$, $n = 5$ RT, 5 COLD, 6 HEAT, with $p = 0.049$ for COLD vs RT and $p = 0.002$ for COLD vs HEAT) ([Figure 36 B](#)). However, there was only a trending increase that did not reach statistical significance in IL-13 level following cold exposures in female rats in the tDCS group ($F(2, 16) = 0.198$ $p = 0.823$, $n = 7$ RT, 4 COLD, 8 HEAT) ([Figure 36 C](#)). Changes from cold exposures were no longer detected two weeks later. However, female rats exposed to heat induced a delayed decrease in IL-13 level two weeks following exposures relative to IL-13 from rats exposed to cold in the combined SHAM and tDCS rats and also within the SHAM group ([Figure 36 D-E](#)). Within the male SHAM group, there was a significant difference between groups ($F(2, 18) = 3.714$ $p = 0.045$, $n = 7$ RT, 8 COLD, 6 HEAT), with significant difference between COLD vs HEAT ($*p = 0.042$). This decrease in IL-13 two weeks following heat exposures was no longer statistically significant in the tDCS group ($F(2, 17) = 2.213$ $p = 0.140$, $n = 8$ RT, 7 COLD, 5 HEAT) ([Figure 36 F](#)). However, there was no statistically significant difference when we compare IL-13 levels in female rats that were subjected to tDCS and SHAM. In contrast to our Aim 2 IL-3 data,

we did not see any statistically significant effect of cold or heat exposures on IL-13 levels in male rats ([Figure 36 G-I](#)).

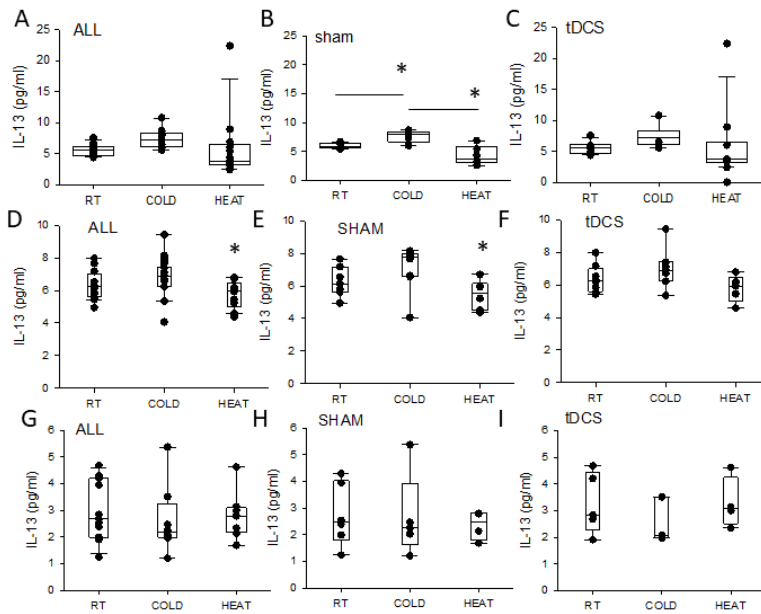


Figure 36. Potential mitigating effects of tDCS on IL-13 changes in female rats. (A-C) Female rats at one day post-exposures. (A) No statistically significant effect of temperature exposure on combined tDCS and SHAM female rats at one day post exposures. (B) Within the SHAM group, there was a significant difference between groups ($F(2, 13) = 10.598$ $p = 0.002$, $n = 5$ RT, 5 COLD, 6 HEAT). Post hoc multiple comparison indicates an increase in IL-13 level in COLD rats compared to RT rats ($*p = 0.049$) or HEAT rats ($*p = 0.002$). There was a trending decrease in IL-13 in HEAT rats compared to RT ($p = 0.072$). (C) Within the tDCS group, there was no statistically significant difference in IL-13 levels ($F(2, 16) = 0.198$ $p = 0.823$, $n = 7$ RT, 4 COLD, 8 HEAT). (D-F) Female rats at two weeks post exposures. (D) There was a statistical difference between groups in combined SHAM and tDCS female rats ($F(2, 38) = 6.4$ $p = 0.004$, $n = 15$ RT, 15 COLD, 11 HEAT), with significant difference between COLD vs HEAT ($*p = 0.003$). (E) Within the SHAM group, there was a significant difference between groups ($F(2, 18) = 3.714$ $p = 0.045$, $n = 7$ RT, 8 COLD, 6 HEAT),

with significant difference between COLD vs HEAT ($*p = 0.042$). (D) Within the tDCS group, there was no significant difference between groups ($F(2, 17) = 2.213$ $p = 0.140$, $n = 8$ RT, 7 COLD, 5 HEAT). (G-I) Male rats at two weeks post exposures. No statistical difference was observed in IL-13 levels from male rats.

TNF- α . There were distinct effects of cold and heat exposures and tDCS/SHAM administration on TNF- α levels in female and male rats ([Figure 37](#)). In female rats, there was a transient increase in TNF- α was detected in female rats one day following exposures to COLD ([Figure 37 A](#)). Two way ANOVA yielded significant difference between groups with $F(2, 39) = 4.127$ $p = 0.024$, but not between stimulation groups ($F(1, 39) = 0.370$ $p = 0.546$). Post hoc multiple comparison analysis using Holm Sidak method revealed statistical difference between the COLD and CONTROL group ($p = 0.027$). However, this difference was not detected within the SHAM or tDCS groups ([Figure 37 B-C](#)). There was also no significant difference detected between the tDCS and SHAM groups in female rats ($p = 0.546$) ([Figure 37 D](#)). At two weeks post-exposures, there was no significant effects of COLD or HEAT exposures on TNF- α levels ([Figure 37 E-G](#)). Two way ANOVA did not yield significant difference between exposure groups ($F(2,38) = 0.449$ $p = 0.641$) but showed statistical difference between the tDCS and SHAM groups ($F(1, 38) = 5.174$ $p = 0.029$) with tDCS rats showing reduced TNF- α compared to SHAM rats ([Figure 36 H](#)). In male rats, a delayed increase in TNF- α was detected in male rats two weeks following exposures to HEAT and administration of tDCS did not affect the heat-induced increase in TNF- α ([Figure 37 I-L](#)). Two way ANOVA revealed significant differences among exposure groups ($F(2, 28) = 5.421$ $p = 0.010$) but not between tDCS and SHAM ($F(1,28) = 0.475$ $p = 0.496$). Post hoc multiple comparison analysis using Holm Sidak revealed statistical difference between HEAT and CONTROL ($p = 0.017$) and between HEAT and COLD ($p = 0.017$) ([Figure 37 I](#)). Within the SHAM only group, no statistical difference was observed between groups ([Figure 37 J](#)). Within the tDCS only group, there was statistical difference between HEAT and CONTROL ($p = 0.008$) and between HEAT and COLD ($p = 0.037$) ([Figure 37 K](#)). There was no statistically significant difference in TNF- α from male rats subjected to SHAM or tDCS ([Figure 37 L](#)).

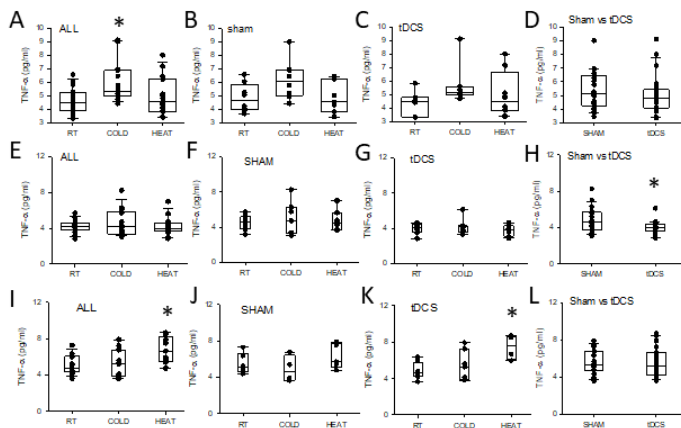


Figure 37. Administration of tDCS reduced TNF- α level independent of exposure at 2 weeks after exposures in female (A-D) but not male rats (I-L). (A) At one day post exposures, there was a statistical significant acute increase in TNF- α level from female rats exposed to COLD ($*p < 0.05$, $n = 15$). (B) Female rats within the SHAM group showed a trending increase in TNF- α one day following cold exposure but the increase did not reach statistical significance ($n = 8$ RT, 8 COLD, 7 HEAT). (C) Female rats within the tDCS group showed a trending increase in TNF- α resulting one day after cold exposures, but the increase was not statistically significant ($n = 7$ RT, 7 COLD, 8 HEAT). (D) At one week post exposure, combined female rats (RT, COLD, HEAT) subjected to 30 min of 0.25 mA tDCS showed a trending decrease in TNF- α level compared to rats subjected to SHAM, without reaching statistical significance ($n = 23$ SHAM, 22 tDCS). (E-G)

At two weeks post exposures, there were no statistically significant change in TNF- α level resulting from COLD or HEAT exposures overall (E) ($n = 15$ RT, 14 COLD, 15 HEAT), within the SHAM cohort (F) ($n = 7$), or within the tDCS cohort (G) ($n = 8$ RT, 7 COLD, 8 HEAT). (H) Combined female rats (RT, COLD, HEAT) subjected to tDCS had statistically significant reduction in TNF- α level compared to rats subjected to SHAM ($*p = 0.029$, t-test, $n = 20$ SHAM, 22 tDCS). (I-L) Data from male rats at 2 weeks post exposures. (I) There was a statistically significant increase in TNF- α in male rats exposed to HEAT ($*p < 0.05$, $n = 13$ RT, 12 COLD, 8 HEAT). (J) Significant increase in TNF- α was not detected within the SHAM group only ($n = 8$ RT, 5 COLD, 4 HEAT). (K) Significant increase in TNF- α was detected within the tDCS group after exposures to heat ($n = 8$ RT, 8 COLD, 4 HEAT). (L) Combined male rats did not show any change in TNF- α resulting from tDCS administration ($n = 17$ SHAM, 20 tDCS).

Other Cytokines. Plasma IL-1 β , IL-6, IL-5 levels were below detection limit. Since we saw significant increase in IL-6 from Aim 2, we repeated these assays but again obtained IL-6 levels that were below ELISA detection limit.

Table 3. Summary of data from rats used in Specific Aim 2 and Specific Aim 3.

	COLD		HEAT	
	FEMALE	MALE	FEMALE	MALE
MA	no change	no change	increase (Figure 6)	increase (Figure 6)
water maze latency	increase (Figure 8)	decrease (Figure 8)	increase (Figure 8)	no change
IO	no change	no change	no change	no change
PPF	increase (Figure 9)	no change	no change	no change
LTP	trending decrease (Figure 9)	no change	trending decrease (Figure 9)	no change
estradiol	decrease (Figure 11)	N/A	decrease (Figure 11)	N/A
serotonin	increase (Figure 12)	increase (Figure 12)	increase/decrease (Figure 12/Figure 28)	increase (Figure 12)
dopamine	decrease (Figure 13)	decrease (Figure 13)	decrease (Figure 13)	decrease (Figure 13)
glutamate	no change	no change	decrease (Figure 31)	decrease (Figure 15)
GABA	decrease (Figure 14)	no change	decrease (Figure 30)	no change
C3	increase (Figure 32)	no change	decrease, increase (Figure 16, 32)	no change
IFN-γ	increase (Figure 17, 33)	no change	increase (Figure 17, 33)	increase (Figure 33)
IL-6	increase (Figure 18)	no change	increase (Figure 18)	no change
IL-10	increase (Figure 34)	no change	increase (Figure 19, 34)	increase (Figure 19, 34)
IL-13	increase (Figure 36)	increase (Figure 21)	trending decrease (Figure 36)	no change
IL-4	increase (Figure 35)	increase (Figure 20)	increase (Figure 35)	increase (Figure 20)
TNF-α	increase (Figure 37)	no change	no change	increase (Figure 37)

DISCUSSION

We found in Aim 1 that tDCS induced a similar enhancement in synaptic plasticity in hippocampus from female rats, similar to male rats. Physiological responses to repeated intermittent cold or heat exposures in female and male rats were assessed in Aim 2, in which we found both sex-dependent and sex-independent effects. The objective of Aim 3 is to determine whether tDCS is an effective countermeasure against the adverse effects of temperature exposures. Thus, all Aim 3 rats were subjected to surgery to implant the casing for tDCS probe prior to exposures to RT, COLD or HEAT. Rats used in Aim 3 were then subjected to either SHAM, where they were hooked up but no current was delivered, or tDCS, where they were hooked up and 0.25mA direct current was delivered for 30 minutes. All Aim 3 rats were given 7 days to recover after surgery prior to

initiation of temperature exposures. However, since there were slight differences noted in some of the neurotransmitter and cytokine data obtained from Aim 2 and Aim 3, there may still be lingering effects from the surgery and/or the anesthesia (isoflurane) that can potentially aggravate, mask or modify the cold or heat-mediated responses from Aim 3 rats. A table summarizing our Aim 2 and Aim 3 data is shown in [Table 3](#). We will prioritize Aim 2 data when discussing the physiological responses of extreme temperature exposures and Aim 3 data when discussing the effectiveness of tDCS in mitigating cold or heat-mediated physiological changes.

Temperature Effects

Behavior and electrophysiology

Repeated heat exposures induced similar effects in motor activity levels with increased activity reported in both female and male rats ([Figure 6](#)). Repeated cold exposures did not cause significant changes in motor activity. Interestingly, repeated intermittent exposures to cold induced very distinct cognitive effects in female and male rats. Latency in finding the hidden platform significantly increased in female rats in the six training days following the 21-day cold exposures. In contrast, male rats performed better, as reported by a decrease in the latency in finding the hidden platform compared to male rats exposed to RT or heat. The impaired learning performance in female rats is corroborated by our electrophysiology data showing a trending reduction in LTP and a significant increase in PPF which typically indicate an impaired neurotransmitter release ([Figure 9](#)). Male rats did not show any trending changes in the electrophysiological parameters ([Figure 10](#)). Therefore, data indicate that female rats are more susceptible to cognitive effects resulting from repeated intermittent cold exposures. A likely explanation is that male rats had successfully acclimated during the 21-day exposure regimen to the cold, with physiological adaptations that contribute to the observed enhanced performance.

Interestingly, female rats experienced reduced weight gain when exposed to heat but did not experience weight gain changes after exposures to cold ([Figure 5, 24](#)). Male rats, on the other hand, experienced reduced weight gain when exposed to heat as well as cold. Altogether, our data suggest that weight loss does not necessarily correlate with adverse effects on cognition since male rats, which experienced significant decrease in weight gain, performed better in water maze test following exposures to cold.

Estradiol

Our data indicate that cold or hot environment can induce statistically significant changes in plasma estradiol levels from female rats ([Figure 11](#)). A previous study indicates that estradiol can regulate release of neuropeptides from large dense core vesicles in the hippocampus (May et al., 2014). Thus, the reduced estradiol level in female rats may account for the trending reduction in LTP ([Figure 9](#)) and impaired performance in the water maze test ([Figure 8](#)).

Neurotransmitter

The central nervous system maintains a stable body temperature despite a wide range of environmental temperatures. Acute physiological responses as well as adaptations to cold or hot environment are mediated through several neural pathways in different regions of the brain including the pre-optic area, and other regions within the hypothalamus and brainstem. Neurotransmitters including serotonin, dopamine, GABA and glutamate play a key role in the physiological responses and/or adaptations to cold or heat. Repeated exposures to cold or heat therefore can potentially disturb the levels of these neurotransmitters in the brain. Disruptions in any of these neurotransmitters have been correlated with a decline in cognitive function. Here, we assessed whether there are detectable changes in brain levels of serotonin, dopamine, GABA and glutamate following

repeated intermittent exposures to cold or heat in both female and male rats. Changes in brain neurotransmitter levels with the exception of GABA were mostly similar in female and male rats, with increased serotonin resulting from either cold or heat exposures, reduced dopamine from either cold or heat exposures, and decreased glutamate from the heat exposures. Decreases in brain GABA levels were only detected in female rats following exposures to cold or heat.

Serotonin. Increase in serotonin is consistent with serotonin's established role temperature regulation, described in an early study in which destruction of serotonergic neurons in the anterior hypothalamic pre-optic area led to impaired heat production during cold stress exposures and serotonin release from anterior hypothalamus was significantly enhanced when the animal was exposed to cold (Myers, 1981). A 1999 study reported that cold exposures can indeed induce serotonin activation in the brainstem and increases in 5-hydroxyindoleacetic acid (SHIAA) concentration after exposures to cold (3°C) for at least 60 min (Passerin et al., 1999). A more recent study showed that a 2-hour exposure to heat (35°C) induced an increase in extracellular levels of serotonin in the ventral tegmental area of the brain (Ishiwata et al., 2017). Here, an increase in serotonin was detected in both female and male rats following exposures to cold or heat in Aim 2 (**Figure 12**). Aim 3 female rats showed increase in serotonin following exposures to cold similar to Aim 2 female rats, but interestingly showed decreased serotonin following exposures to heat (**Figure 28**). Serotonin changes are dynamic and known to fluctuate as a result of heat exposures and the discrepancy between Aim 2 and Aim 3 rats can be attributed to the prior surgery and/or anesthesia experienced by Aim 3 rats causing a shift in the timing of the serotonergic response. Our data overall are consistent with previous reports implicating the essential role of serotonin in temperature regulation and thus can be an important therapeutic target in improving physiological performance in an extremely cold or hot environment in both men and women.

Dopamine. Similar to serotonin, dopamine has also been reported to play an important role in thermoregulation (Cox and Lee, 1980, Lee et al., 1985, Hasegawa et al., 2008). Reduction in brain dopamine has been previously reported in rats following repeated exposures to 32°C for 1-28 days in another study (Nakagawa et al., 2016). Our data also showed decreased brain dopamine levels following exposures to cold or heat in female and male rats (**Figure 13**). Our data overall indicate that dopamine is involved in cold or heat-mediated responses in male and female rats and thus potentially play a key role in the compensatory mechanism against adverse effects of repeated cold or heat exposures in both men and women.

GABA. Decrease in GABA was only observed in female rats following cold (Aim 2 and 3) or heat exposures (Aim 3) (**Figure 14, 30**). However, many samples from the male rats were below the detection limit of the GABA assay that we used, decreasing our sample size considerably. Despite the low sample size, we did detect a trending increase in GABA levels from male rats in Aim 3 following cold exposures and a trending decrease in GABA levels following heat exposures. GABAergic signaling has also been implicated in thermoregulation and thus detected differences in GABA levels may contribute to the distinct sex-dependent responses to repeated cold and heat exposures.

Glutamate. Repeated exposures to cold did not induce significant changes in brain glutamate levels in female or male rats. However, repeated exposures to heat caused a decrease in brain glutamate levels in both female and male rats (**Figure 15**). Reduced glutamate receptor expression has been previously documented in heat acclimated rats that had been exposed to 34°C for 30 days (Yacobi et al., 2014). The reduction in brain levels of glutamate observed here can potentially be a part of the physiological adaptation response resulting from the repeated intermittent heat exposures over 21 days.

Inflammatory cytokines

Previous studies indicate that cytokines play important roles in temperature regulation mediating various physiological responses resulting from infections, exercises or ambient changes in temperatures. Increase in Th2 cytokines was observed in horses 5 hours following cold weather exercises (Davis et al., 2005). Mice subjected to acute infections experienced increases in inflammatory cytokines including IL-6 and TNF- α , but cold water stress prior to infection induced a decrease in IFN- γ and TNF- α (Monroy et al., 1999). Male human subjects exposed to cold stress experienced increases in several cytokines including IL-6 and TNF- α (Rhind et al., 2001). Increases in cytokines have also been reported following heat stroke and during recovery from heat stroke (Biedenkapp and Leon, 2013, Welc et al., 2013). Overall, more cytokines were significantly changed in female rats compared to male rats following **cold** or **heat** exposures (**Table 3**). Female rats experienced increased IFN- γ , IL-6, IL-10, IL-13, IL-4 and TNF- α whereas male rats only experienced elevated levels of IL-4 and IL-13 following repeated **cold exposures**. C3, another inflammatory marker, was increased in female rats but not male rats following cold exposures. C3 has been associated with hippocampal dysfunction and Alzheimer's disease in a mouse model (Shi et al., 2015, Shi et al., 2017). **Heat exposures** induced increased IFN- γ , IL-10 and IL-4 in both female and male rats but only induced increased IL-6 in female rats and increased TNF- α in male rats. C3 levels decreased in female rats two weeks after exposures to heat in Aim 2 but C3 increased two weeks after exposures to heat in Aim 3. The differential inflammatory marker profile resulting from cold or heat exposures between female and male rats likely reflect the sex-dependent molecular mechanisms by which men and women acclimate to repeated cold or heat exposures.

tDCS effects

We previously demonstrated that tDCS enhanced LTP in hippocampus from male rats (Rohan et al., 2015). Aim 1 data verified that tDCS can also enhance LTP in hippocampus of female rats. A secondary objective of this project was to evaluate whether tDCS can be used as a countermeasure against the adverse effects of cold or heat exposures. Overall, our data did not show that tDCS can reverse the effects of cold or heat exposures. However, there were some data parameters that were affected by tDCS. Motor activity data were obtained immediately following 30 min of tDCS at 0.25 mA. The same set of rats were given a second round of tDCS one day prior to the first training day for Morris water maze. Acute biochemical assays were performed on blood and brain collected one day following tDCS administration.

Motor Activity

Male rats in Aim 3 experienced increased repetitive behaviors when they were subjected to cold and tDCS (**Figure 25**). Administration of tDCS following exposures to cold in male rats induced a statistically significant increase in the number of stereotypical beam breaks. Stereotypical beam breaks are repetitive beam breaks < 1 sec apart and represent repetitive behaviors that are typically associated with dopamine stimulation as dopamine deficient mice experienced increased stereotypical behaviors when L-DOPA was exogenously administered (Chartoff et al., 2001). Another study reported that reduced brain tissue level of dopamine correlated with increased repetitive rearing and jumps (Pogorelov et al., 2005). Therefore, it's possible that administration of tDCS following cold exposures induced a surge in brain DA level in male rats, inducing an increase in stereotypical beam breaks in male rats. Interestingly, we did not observe this effect in female rats, suggesting that perhaps there is subtle sex-dependent differences in the mechanism by which tDCS caused an increase in DA and/or differences in the sensitivity of dopamine signaling system in inducing this repetitive behaviors.

Water maze

We did observe a mild improvement in the temperature-induced spatial learning in **female rats** with a hint of mitigating effect on estradiol and TNF- α levels resulting from tDCS. Female rats in Aim 3 experienced a reduction in plasma estradiol level one day and two weeks following cold or heat exposures ([Figure 27](#)). However, the reduction at the two weeks' time point from the cold was no longer statistically significant in female rats that were subjected to tDCS, although a trending decrease was still observed. Administration of tDCS also induced a significant decrease in TNF- α level in female rats. Altogether, the mitigating effect on estradiol level and the decrease in TNF- α may account for the improved performance in female rats during water maze test. In contrast, tDCS exacerbated performance in **male rats** exposed to RT or heat ([Figure 26 A, F](#)). We are uncertain why tDCS would induce a slight learning impairment in male rats exposed to RT or heat, but one possible reason can be the additional lingering stress resulting from the prior surgery in implanting the tDCS electrode. There was a hint that tDCS had a partial mitigating effect of temperature-induced decreases in dopamine level in male rats. Male rats subjected to tDCS no longer had a statistically significant decrease in dopamine levels whereas male rats that were in the SHAM group experienced a statistically significant decrease in dopamine levels after repeated cold or heat exposures ([Figure 28 H-I](#)). Perhaps tDCS administration sufficiently modified the physiological adaptation events that partially reversed the temperature-mediated decrease in brain dopamine levels. Although rats were allowed to recover for 7 days following surgery before temperature exposures, another possible explanation for the reduced water maze performance in male rats following tDCS is a lingering stress effect of surgery that were greater than the benefits obtained from tDCS at least in the context of spatial learning.

CONCLUSION

Repeated **heat exposures** induced (1) an increase in motor activity in both male and female rats compared to rats exposed to cold or RT, (2) an increase in water maze latency in finding platform from female rats only, (3) trending reductions in LTP and spontaneous activity from female rats only, (4) reduced plasma estradiol level from female rats, (5) increased brain serotonin and decreased dopamine levels from both female and male rats, (6) decreased GABA from female rats only, (7) decreased brain glutamate levels from male rats only, (8) increased C3, IFN- γ , IL-6, IL-4, IL-10 in female rats, and (9) increased IFN- γ , IL-10, IL-4, TNF- α in male rats.

Repeated **cold exposures** induced (1) an increase in water maze latency in finding the platform from female rats and decrease in latency from male rats, (2) increase in paired pulse facilitation (PPF) from female rats only, (3) trending decrease in LTP from female rats only, (4) decrease in plasma estradiol level in female rats, (5) increased brain serotonin and decreased dopamine levels from both female and male rats, (6) decreased GABA from female rats only, (7) increased C3, IFN- γ , IL-6, IL-10, IL-13, IL-4 and TNF- α in female rats, and (8) increased IL-13 and IL-4 in male rats.

Administration of tDCS did not reverse many of the temperature-mediated changes. However, there were some exceptions. Male rats that were exposed to room temperature (RT) when subjected to tDCS had a trending decrease in stereotypical behavior compared to RT SHAM male rats, whereas male rats previously exposed to cold then tDCS had a statistically significant increase in stereotypical behavior compared to COLD SHAM rats. Administration of tDCS improved water maze performance in female rats but worsened water maze performance in male rats. Female rats exposed to cold had significant reduction in plasma estradiol level whereas female rats that were subjected to cold then tDCS had only trending reduction in plasma estradiol that were no longer statistically significant. Male rats that were exposed to cold or heat, when subjected to

tDCS, no longer experienced the statistically significant cold/heat-induced reduction in brain dopamine levels. Female rats subjected to tDCS experienced a decreased average plasma TNF- α level compared to SHAM female rats. Therefore, our data overall did not rule out the possibility of tDCS as a potential countermeasure tool, but that a stronger tDCS intensity and/or repeated tDCS sessions may produce greater mitigating effects against adverse temperature-mediated changes.

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