Hot Button: Non-invasive Approach for Studying Aggressive States

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Abstract

Aggression is an innate behavior in most vertebrate animal species, including humans. Various mental health disorders or neurological conditions can be accompanied by pathological aggression. Understanding the circuits that control normal aggression may help us in finding treatments for these conditions.

The development of psychiatric drugs that target the brain's aggression circuits requires reliable and non-invasive methods to assess the aggressive state. I will demonstrate that a fairly inexpensive thermography camera can provide a measure of the aggressive state of mice.

In my experimental trials, I use mice since researchers have identified an area of the brain in the ventromedial hypothalamus, ventrolateral subdivision (VMHvl), that contains aggression neurons that can be optogenetically stimulated. This essentially means that we have the tools to artificially stimulate hardwired primal emotions such as anger in a controlled manner.

My research provides statistical evidence that activation of VMHvl neurons leads to an increase in a mouse's head temperature. I conducted various control experiments to ensure that this temperature rise is not due to factors such as the laser energy used in optogenetic stimulation or the mouse's motion. Additionally, I repeated the experiments with a control group of mice that did not exhibit VMHvl sensitivity to laser stimulation. This suggests a scientific correlation between body temperature increase and aggression. Lastly, by comparing the temporal responses of pupil dilation and body temperature to VMHvl activation, I found that, unlike pupil dilation, which is caused by arousal, the persistent temperature response is indicative of aggression.

Introduction

The expression “getting heated,” which associates heat with anger, is not unique to English. In Spanish, we say “echar leña al fuego” (add wood to the fire), which refers to aggravating an already tense situation, in Russian, there is “Кипеть от злости” (boiling from anger), in Hebrew “חמום מוח” (heated brain), and in Hindi there is an expression “गुस्से में आग बबूला होना” (being fiery angry). Almost every culture seems to associate anger with heat, but is there a scientific link?

Aggression is an innate behavior in most vertebrate animal species, including humans. Even if we are consciously aware of our current emotional states such as anger, the mechanisms that give rise to these emotions remain largely unresolved and the subject of current research [1]. Various mental health disorders or neurological conditions can be accompanied by pathological aggression, including some forms of dementia, like Alzheimer's disease, paranoid schizophrenia and psychotic disorders. Progress in understanding these disorders requires an understanding of the brain circuits that control normal aggression [2].

Some research on humans has found a correlation between high temperatures potentially causing aggressive behavior [3]. Similarly, in an experiment in which people were asked to self-report where in the body certain emotions were felt, anger was depicted as a red hot map in the upper part of the body [4]. However, no study has produced statistical evidence of human body temperature increasing when angry.

One technique that has been instrumental in advancing our understanding of the brain and how different neural circuits contribute to emotions is optogenetic stimulation. Optogenetic stimulation allows researchers to precisely control the activity of specific neurons or cells in complex neural circuits. The method involves genetically modifying target cells to express light-sensitive proteins. When laser light of the appropriate wavelength is shone on the modified cells, it causes the neurons to activate.

This technique has been optimally applied in mice. Groundbreaking research in Professor Anderson’s lab [5] demonstrated that optogenetic stimulation of neurons in the ventromedial hypothalamus, ventrolateral subdivision (VMHvl) in mice elicits an aggression response that causes male mice to attack both females and inanimate objects, as well as males. Similarly, pharmacogenetic silencing of VMHvl reversibly inhibits inter-male aggression. It is interesting to note that male mice exhibit a preference for

aggression-seeking behavior when VMHvl is stimulated [6][7]. Other states such as fear or anxiety can also be evoked [8].

Using this type of research, we can begin modeling the brain through its aggression or fear pathways, paving the path for medical drugs to treat psychological disorders and other brain diseases.

It is important to develop non-invasive methods to correlate emotion states with other vital signs such as heart rate, breathing rate or pupil dilation. In my research, I developed a novel non-invasive method to measure body temperature, and how that correlates with aggressive states.

Previous ways to measure temperature, for example rectal thermometry [9] or subcutaneous temperature-sensitive chips [10], were highly invasive. This makes the experiment more cumbersome and expensive, and it could introduce anxiety or stress in the animals, thus impacting the data collection.

My work consists of four main parts:

1. Developing a non-invasive method to measure the mouse's temperature.
2. Conducting optogenetic stimulation experiments using this method to collect data
3. Analyzing the data to assess correlation between temperature and aggression state.
4. Understanding the impact of VMHvl activation on pupil dilation, and how it deviates from body temperature temporal response.

I demonstrate that there is statistical evidence that VMHvl neuron activation results in a mouse's head temperature increase. This temperature increase is persistent over several seconds after activation, indicating that it is attributable to an aggression state. This is in contrast with pupil dilation, which goes back to the baseline before the stimulation is over, indicating an arousal state and not anger.

Method

All test animals used in this study were laboratory mice (Mus musculus), strain C57BL/6, aged between 8 to 20 weeks. The care and experimental manipulation of the animals was carried out in accordance with the NIH guidelines and approved by the Caltech Institutional Animal Care and Use Committee.

The experimental group consisted of three mice. Researchers at Professor Anderson's Lab had expressed

channelrhodopsin-2 (ChR2) in VMHvl neurons using stereotactic co-injection of adeno-associated viral vectors fused with green fluorescent protein. Cells in this region can be selectively illuminated with a laser using an implanted fiber-optic cable [5]. The control group consisted of three mice with the green fluorescent protein but without ChR2 such that the aggression response would not be elicited with laser activation.

Results

Non-invasive apparatus to measure body temperature

My research primarily aimed to develop a non-invasive apparatus for measuring mouse body temperature, synchronized with the laser stimulation used to optogenetically elicit the anger response.

The thermographic data were collected using an inexpensive Mosaic Core Seek Thermal Camera. In order to synchronize the thermal data capture with the optogenetic neural stimulation, I used a DAQ device from Measurement Computing to receive a trigger from the laser Pulse Stimulator. Figure 1 illustrates how the lab instrumentation is connected and Figure 2 shows a picture of the set up while running a test.

Significant amount of work was initially needed to make this camera accurately capture the temperature change in mice over time. Using the Seek Thermal Software Development Kit (SDK) and Measurement Computing DAQ drivers, I created software (in C++) to capture data synchronized with the other lab equipment. I made this code openly available on github [11].

One problem we encountered early on was that this camera used a shutter to calibrate itself every few seconds, causing the camera to lose about 9 frames with each shutter action. This was problematic because it created missing frames and the recording would lose synchronization with the rest of the lab instrumentation. When we disabled the shutter, the thermal data quality was severely degraded, rendering the data too inaccurate for our needs.

For our experiment, we decided to work around these limitations by activating the shutter by software before each frame to achieve high-quality recordings while maintaining control over the video frame rate. With this approach, the frame rate was limited to 3 Hz, but this was still sufficient for our purposes, since body temperature takes several seconds to change.

Thus, I programmed two capture modes:

- Continuous mode records continuously at a 27 Hz frame rate upon receiving an external trigger signal or a keyboard press.
- Trigger mode captures a frame synchronized with each external trigger signal, allowing users to customize the frame rate for the thermal capture.

For both modes, users have several options to select the data type and filtering mode, reducing noise and enhancing data quality.

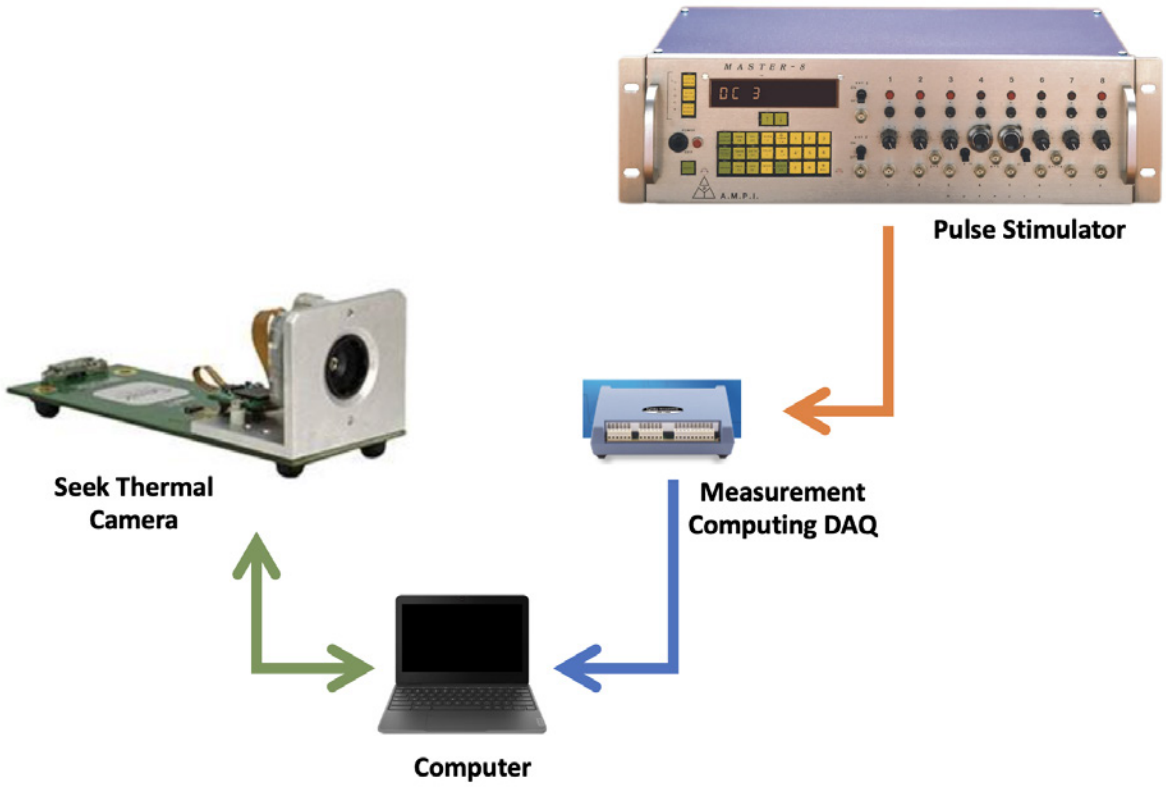


Figure 1. Diagram of experimental set-up. The computer captures a trigger signal from the pulse stimulator (orange arrow) through the DAQ device (blue arrow), and uses it to start a thermal data capture and subsequently read the thermal capture data (green arrow)

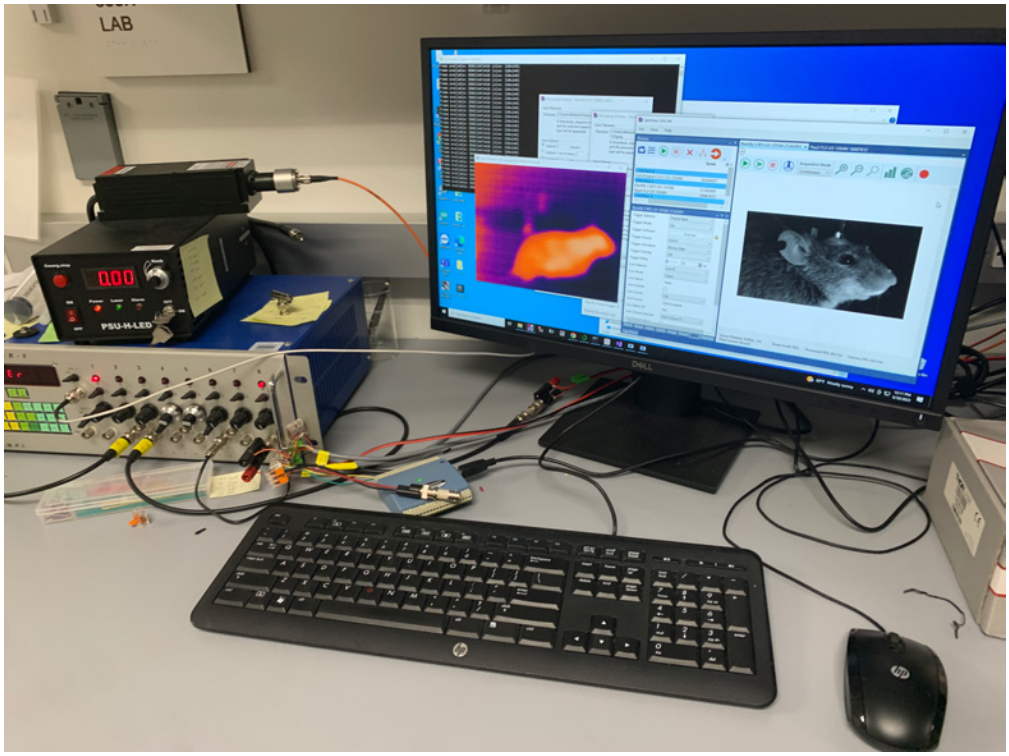


Figure 2. Picture of experimental set-up during data collection

Thermography data collection and analysis

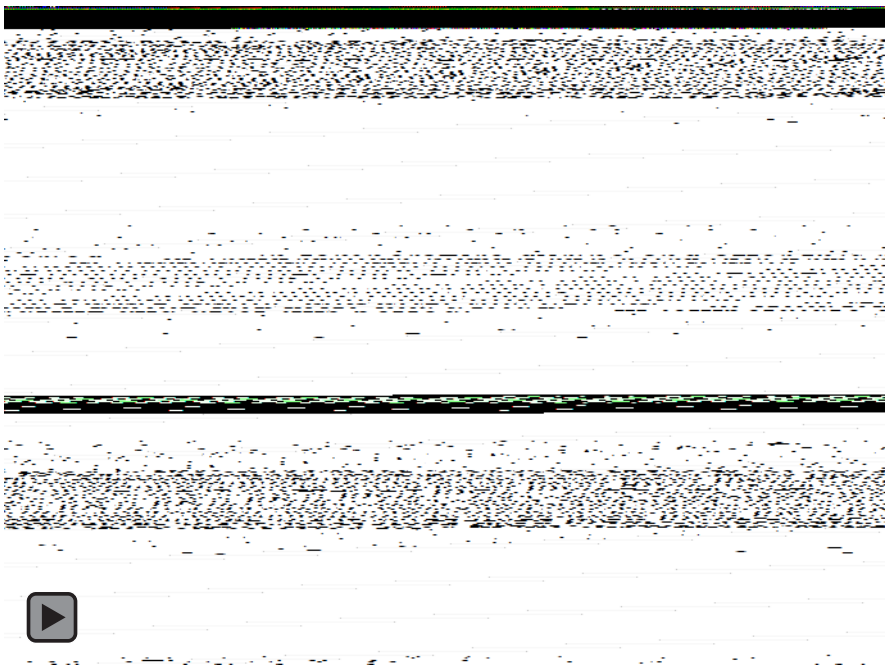
We conducted eight trials on the three mice in the experimental group at a 1Hz sampling rate for thermal recordings. We enabled VMHvl activation for a total duration of 20 seconds with 10-millisecond laser pulses at 20 Hz and 2 mW power. There were 120-second intervals between laser stimulations. I acknowledge with gratitude the tremendous help from my mentor, Dr. Amit Vinograd, in running the trials and collecting the data.

Video 1 shows the live thermal camera data from one mouse as the experiment is conducted. Note that viewing the videos requires the free Acrobat Reader; other PDF readers, such as those on Mac, will not work. There is also a black box designating the 11x11 pixel area that I used to assess the mouse's temperature. I could not use the overall temperature of the mouse because significant movement during the trials could skew the results, as different parts of the mouse have different temperatures. Instead, I took the average temperature from an 11x11 pixel area centered on the hottest point observed in the video,

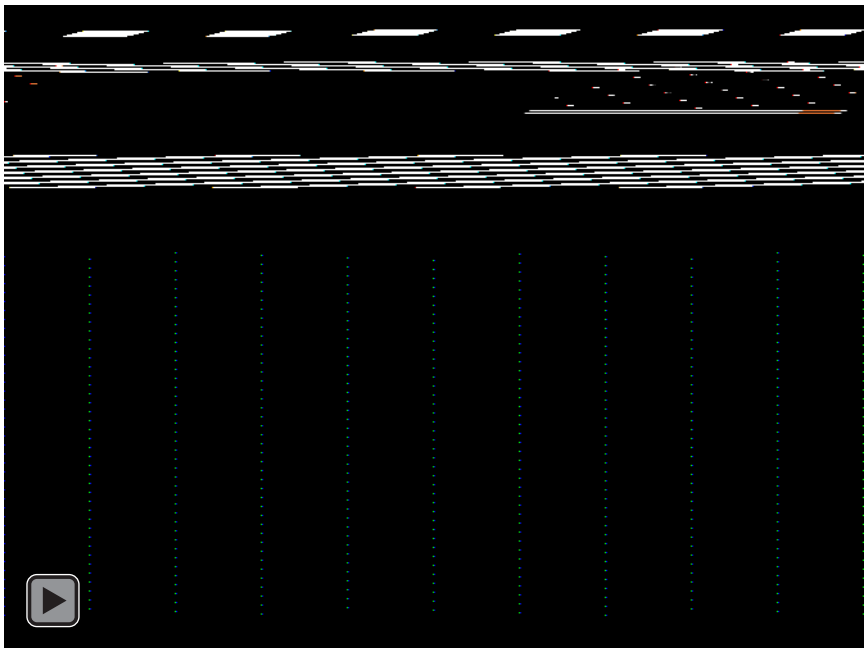
typically near the eye. Unlike the body, the head position was fairly stable during the trials, providing a more reliable temperature measurement. Furthermore, it is reasonable to assume that the head will better reflect any changes in mouse temperature, since, if anger neuron activations were to increase temperature, that change would manifest first in the head.

As an example, Video 2 shows a single trial measuring a mouse's temperature during laser activation. The area between the red lines indicates the period of laser activation, and the data clearly shows an increase in temperature during and after this period.

Figure 3 includes snapshots of the three mice used in the experiment, alongside the box selected for assessing temperature. Figure 4 displays the average temperature within the 11x11 pixel box over time (in blue) and the laser activation times (in red boxes).



Video 1. Thermal camera video of mouse. Small box corresponds to the region used to assess temperature.



Video 2. Example of increase in temperature during and after optogenetic neural stimulation.

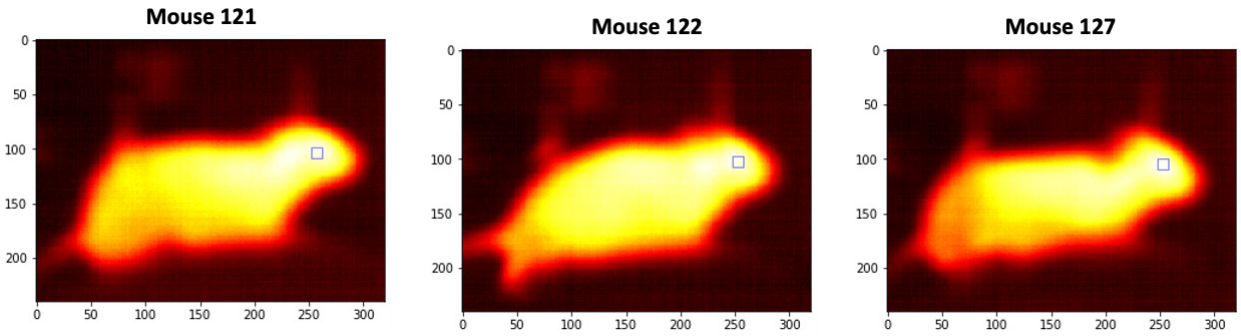


Figure 3. Thermography snapshot for each of the mice with box used to assess temperature.

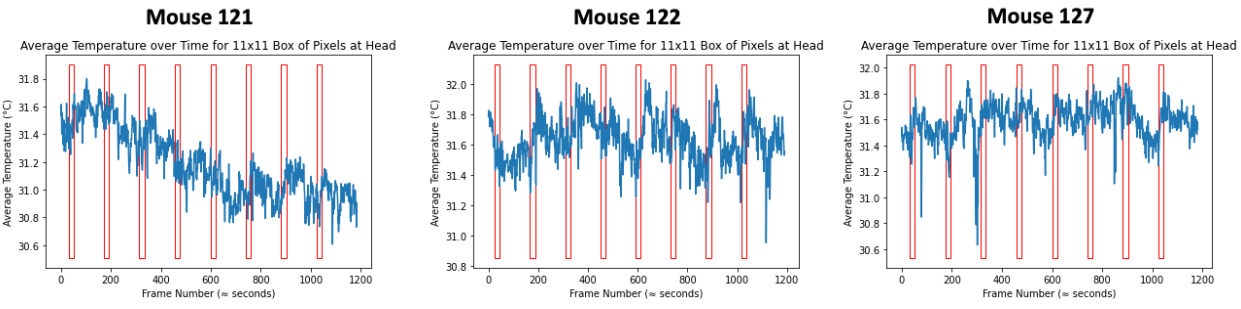


Figure 4. Mouse's temperature in blue as a function of time (average of 11x11 pixel box) for the three mice in the experimental group. The red boxes indicate the periods of laser activation.

To assess the statistical validity of my observations, I superimposed the data from all eight trials and three mice, using laser activation as the time marker.

Figure 5 displays the average of all eight trials for the three mice. It shows a clear increase in temperature during laser activation, which persists for around 20 seconds after the laser is activated, before the temperature begins to level off and decrease. The blue curve represents the average temperature in the 11x11 pixel box over time after applying a smoothing filter, with the gray area indicating the standard error of measurement (SEM). The laser activation occurs during the period indicated by the red area.

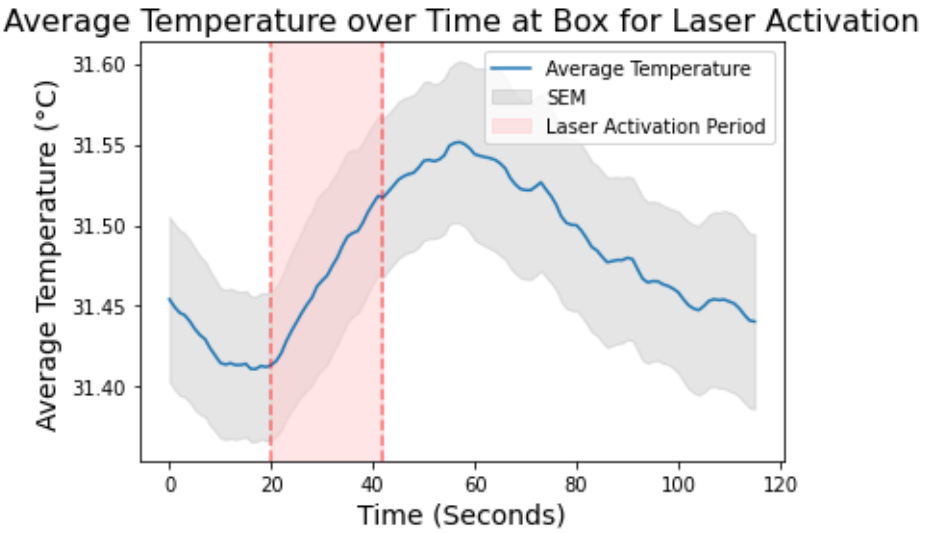


Figure 5. Average temperature in blue and standard error of measurement (SEM) in gray over time. The red area indicates the laser activation period.

Control test #1: Comparing mouse's temperature to background control temperature

To demonstrate the validity of the result, I compared the mice's temperatures to data collected over a similarly sized background box (see Figure 6). The bottom-left graph in Figure 6 includes the temperatures before and after laser activation for each trial, with the black line displaying the average of all trials. I determined the statistical significance of the results using the Wilcoxon test, which yielded a p-value of 0.002. The same test performed on the background control box resulted in a p-value of 0.7, which is not statistically significant. This validates the observation that there is a significant temperature increase in the mice that is not due to an environmental temperature increase.

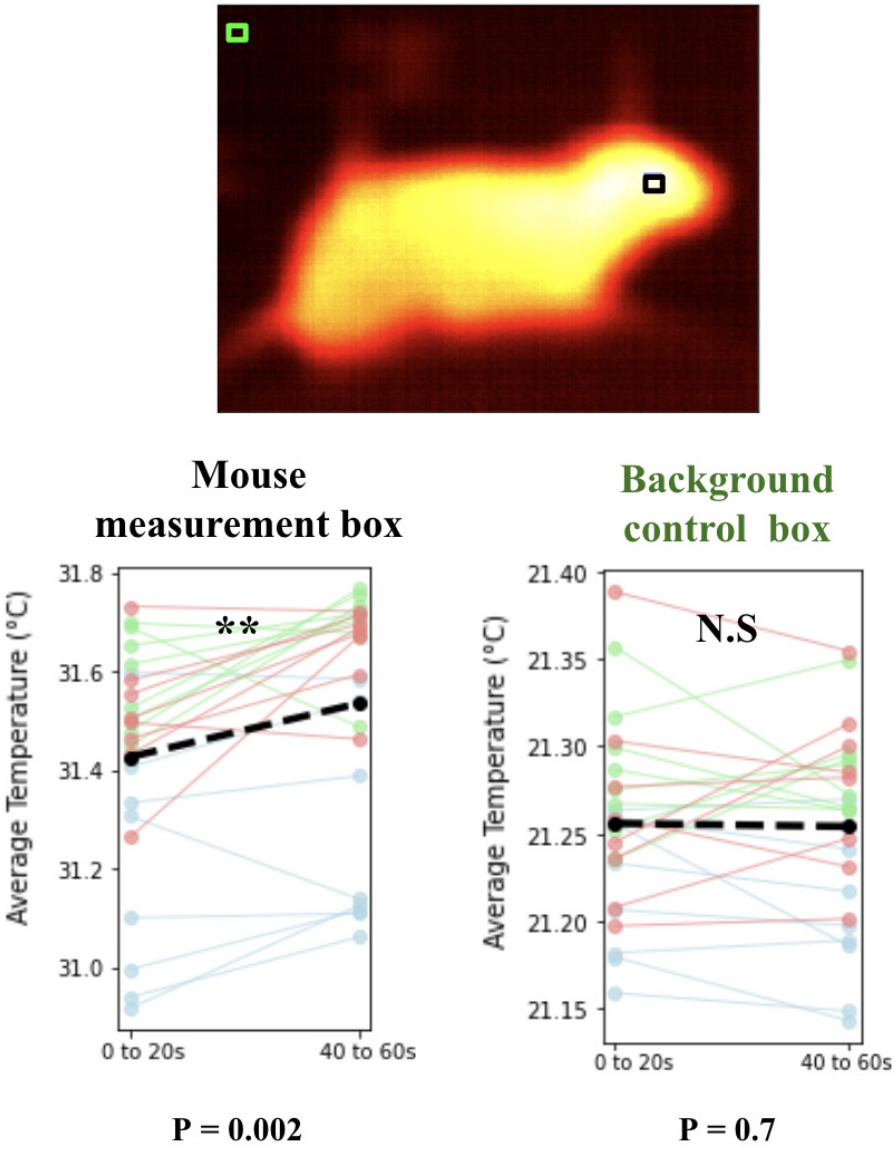


Figure 6. Control experiment using a box in the background (green in the image) compared the mouse's temperature (black box). The bottom graphs show the temperature data for each trial and all three mice (each mouse a separate color) during and after laser activation. Left is for the measurement box and right is for the control box. The dashed black line in each graph is the average. The p-value for the mouse's temperature data indicates the significance of the result, whereas the control box temperature shows no significance.

Control test #2: Increase in temperature due to laser energy

Another potential explanation for this temperature increase could be the heat energy from the laser. To assess this hypothesis, I calculated the expected temperature change in the mouse's brain by considering the energy in Joules from a 2 mW laser over 20 seconds and the average brain mass of a mouse (0.416g), using the average specific heat capacity of brain tissue (3.63 J/g°C). This calculation leads to a temperature change of 0.0265°C over 20 seconds, with a predicted slope of 0.001325°C/sec, assuming that all the energy contributed to raising the brain's temperature—a conservative assumption indeed. However, the linear regression of the measured data yielded a value nearly four times higher, as seen in Figure 7. This indicates that the observed temperature increase cannot be attributed solely to the injected laser energy.

$$\Delta T = \frac{Q}{m\,c}$$

$$= \frac{0.04\text{J}}{0.416\text{g} * 3.63\ \text{J/(g °C)}}$$

$$\Rightarrow \Delta T = 0.0265\ °\text{C}$$

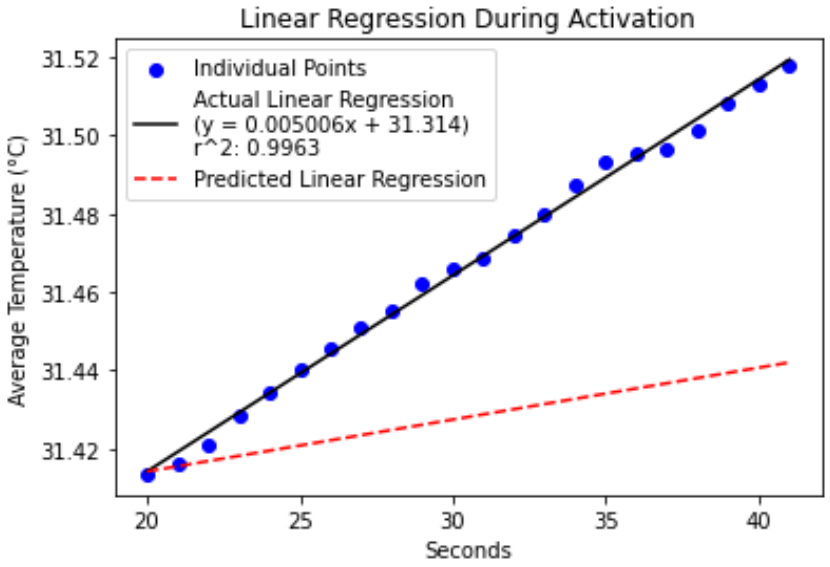


Figure 7. The estimated increase in temperature due to the laser activation energy of 0.04J (2mW over 20 seconds) is 0.0265°C. The predicted slope due to the laser energy is 0.001325°C/sec, whereas the measured slope is 0.005006°C/sec, demonstrating that this effect cannot be attributed to the laser energy.

Control test #3: Increase temperature due to mouse's body movement

Another possible explanation for the increase in body temperature could be the heat produced by the mouse's motion. To disprove this hypothesis, I utilized DeepLabCut [12], an open-source neural network-based software that estimates animal poses.

I selected a few markers to estimate the mouse's velocity (see Figure 8). Tracking the position of these pixels over time and computing the distance between frames allowed me to calculate a proxy for the mouse's velocity, by averaging all markers in units of pixels per second.

Video 3 demonstrates the validity of this algorithm: the mouse's motion, as shown, correlates with sharp velocity increases depicted on the right graph. This method yielded data like that shown in Figure 9, which displays the velocity in pixels per second as a function of time.

To assess the impact of mouse velocity on temperature, I first computed the average speed during and after laser activation and compared it to the overall speed. It is evident in Figure 10(a) that the mice generally do not run much, and their activity does not correlate with laser activation. The observed speeds range from 1 to 3.2 pixels (including the measurement error) per second. I then calculated the average temperature of the mouse during periods without laser activation. Figure 10(b) indicates that the temperature difference for mouse speeds from 0 to 1 pixel per second (orange) versus 1 to 3.2 pixels per second (red) is negligible, and much less than the temperature increases observed during laser activation.

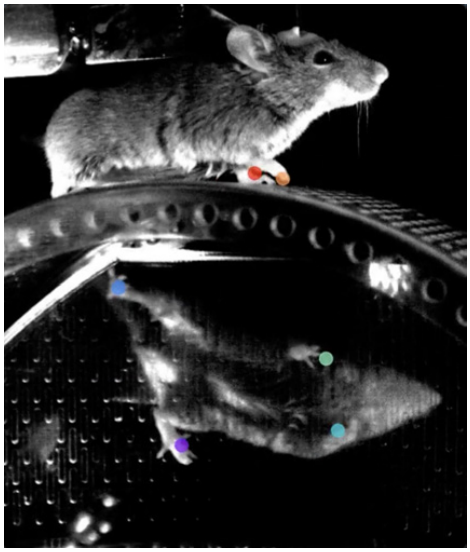
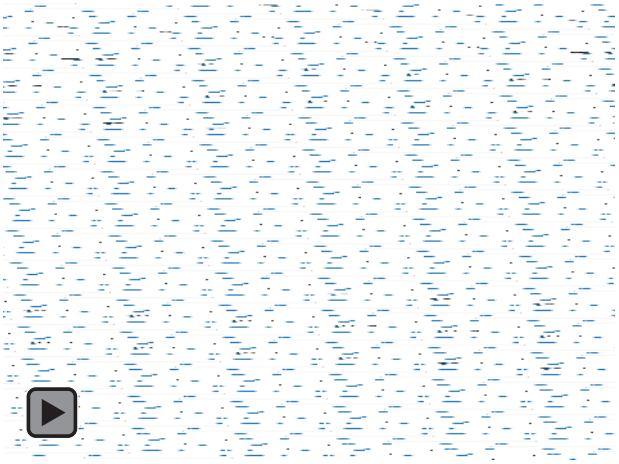
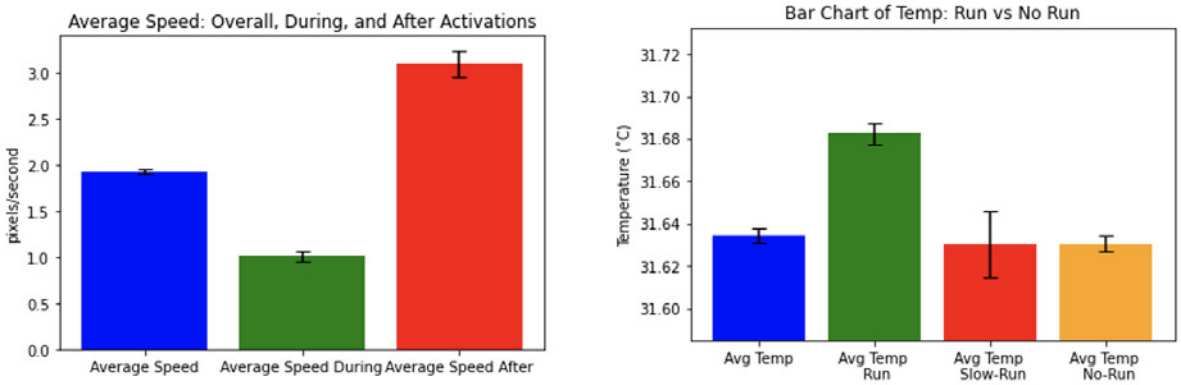


Figure 8. Markers used to estimate mouse's velocity



Video 3. Illustration of algorithm to compute mouse's velocity

Figure 9. Velocity in pixels per second as a function of time. When the mouse runs, the velocity spikes to over 15 pixels per second.



(a) **(b)**

Figure 10. (a) Overall average speed (blue), speed during laser activation (green) and speed for 20 seconds after laser activation (red) with error bars. (b) Overall average temperature (blue), average temperature for velocities larger than 3.2 pixels/second (green), average temperature for velocities between 1 pixel/second and 3.2 pixels/second (red) and temperature for velocities from 0 to 1 pixel/second (orange).

Control test #4: Impact of laser activation on control group

Ultimately, the best way to demonstrate that the increase in the mice's temperature was due to VMHvl activation was by repeating the experiment with a control mouse population without ChR2 expression, rendering the VMHvl neurons not susceptible to laser illumination. I extend my gratitude to my advisor, Dr. Vinograd, for collecting this data on the control population.

Figure 11 shows the average temperature for each mouse and all trials (top panel) and the average for all three mice (bottom) as a function of time. The temperature increase following laser activation (red area) is not statistically significant. A temperature-time plot, similar to the one for the experimental group shown in Figure 7, demonstrates temperature variability below any significant increase that could be attributed to the laser energy. This is depicted in Figure 12, with the data in blue and the regression line in black. The fluctuations of the measured temperature data as a function of time, unlike the linear trend in Figure 7, further suggests that these are random temperature variations and not significant increases that could be associated with laser activation.

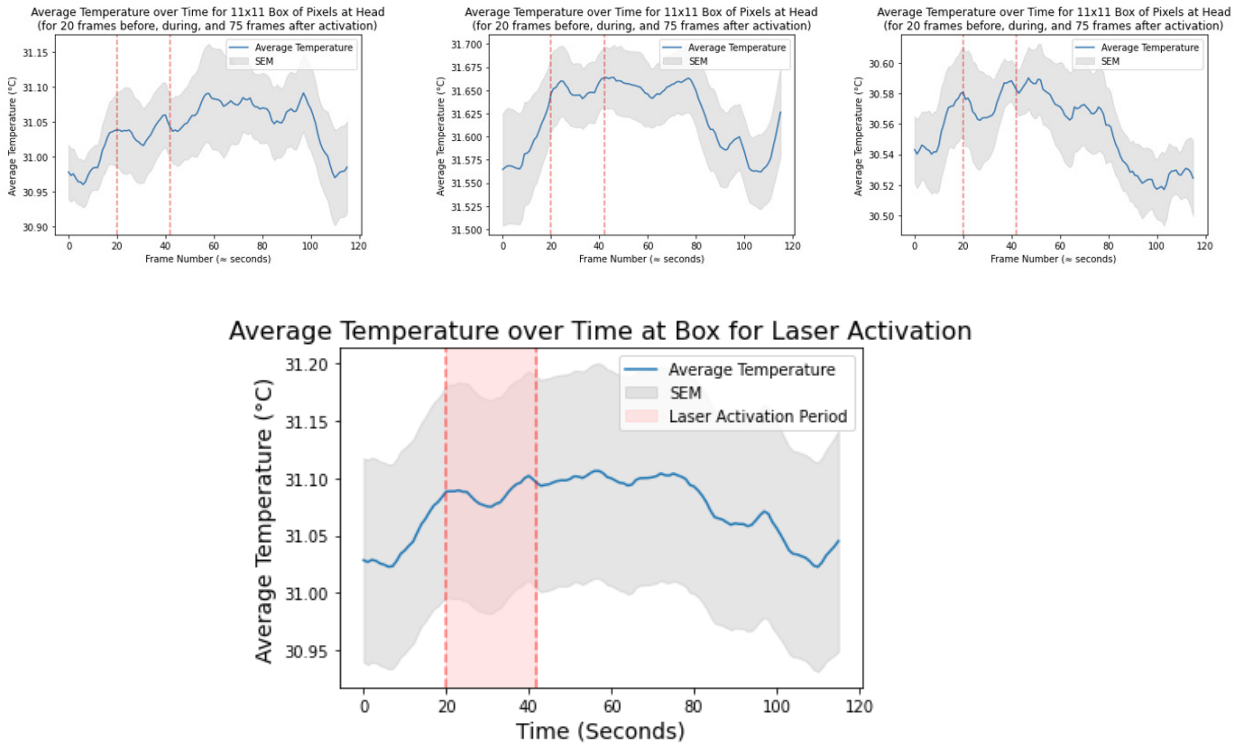


Figure 11. Average temperature as a function of time in the selected 11x11 pixel box. The top panel has the temperature for each mouse, and the bottom is the average of all three mice.

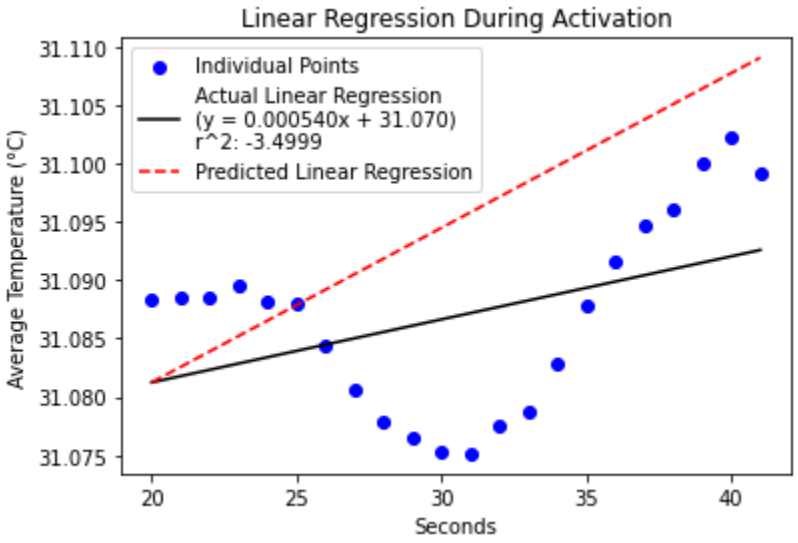


Figure 12. The estimated increase in temperature due to the laser activation energy of 0.04J (2mW over 20 seconds) is 0.0265°C. The predicted slope due to the laser energy is 0.001325°C/sec, whereas the measured slope is 0.000540°C/sec.

Correlation of VMHvl activation and pupil dilation

In order to compare the body temperature response time with that of other vital signs such as pupil dilation, I utilized a neural-network-based open-source package called Facemap [13-14] to assess pupil size during the experiment.

Figure 13 shows snapshots of the mouse's eye, with modified contrast to more clearly observe the pupil. The snapshot on the left shows the eye before activation, while the one on the right shows it 15 seconds after activation, which approximately corresponds to peak pupil size. The middle plot presents the average pupil area (in pixels squared) over time for all trials, with the laser activation time shaded in red. The three panels correspond to the three mice in the experiment. One mouse (bottom panel) exhibited pupil dilation throughout the experiment. The hypothesis is that this mouse was in an anxiety state and was therefore excluded from the conclusions.

Figure 14 displays the average pupil dilation over time for the two remaining mice. This figure clearly demonstrates a correlation between laser activation and pupil dilation.

Interestingly, the pupil dilation began to decrease before the end of the laser activation period, similar to findings reported in [15], which utilized a different neural circuit. This suggests that the response is due to an arousal state rather than anger, which is more persistent.

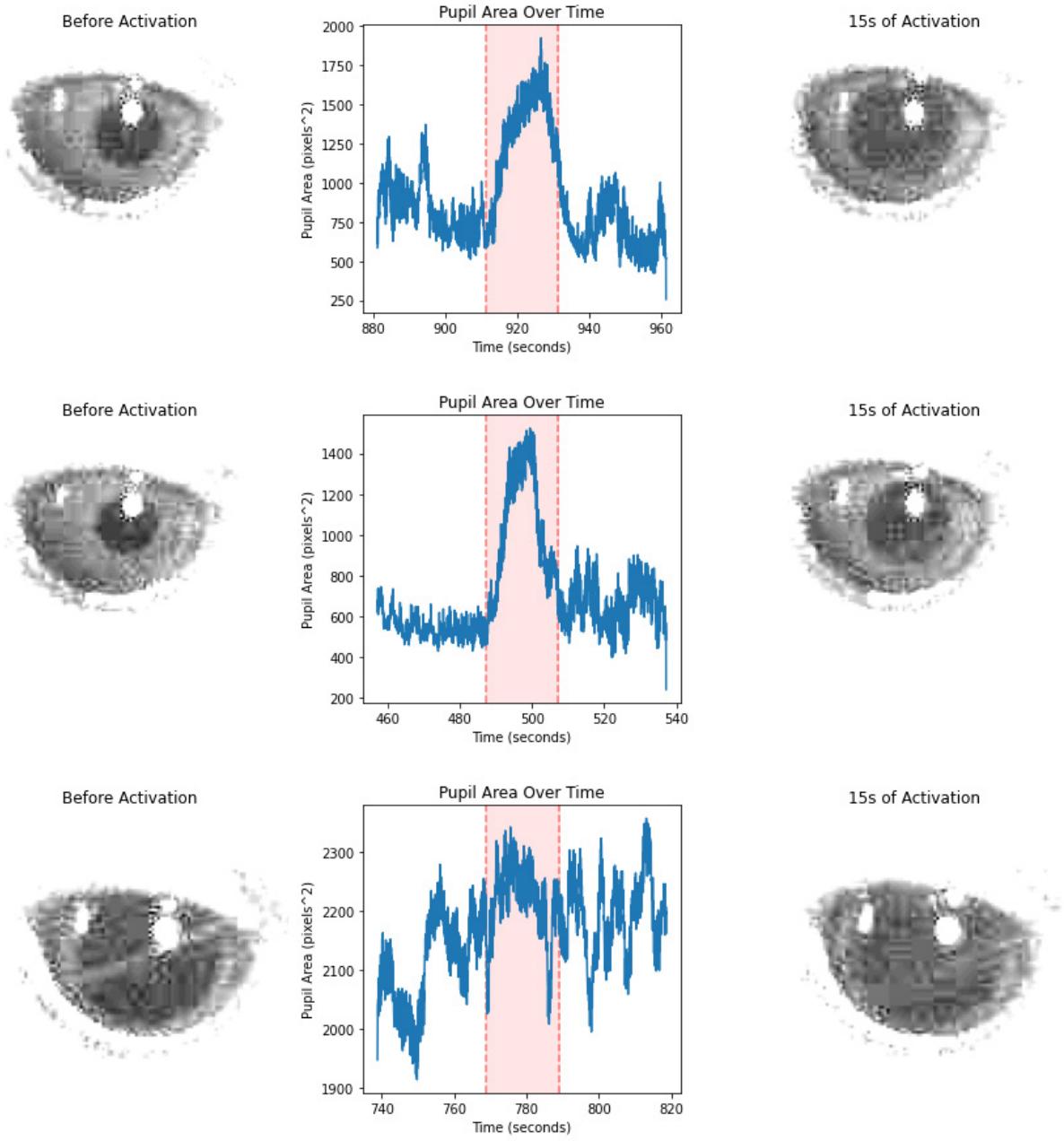


Figure 13. Average pupil area as a function of time for three mice. The first two mice clearly show pupil dilation with laser activation. The third mice (bottom panel) had a dilated pupil over the whole duration of the experiment, likely due to anxiety, so it was discarded.

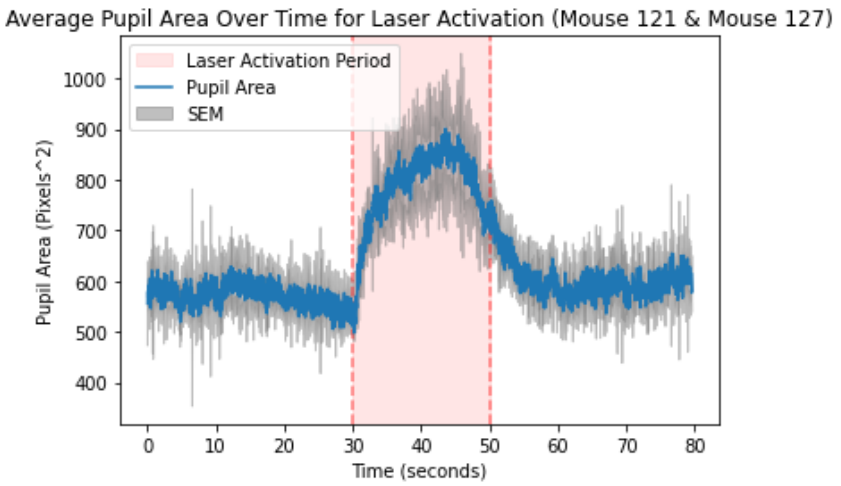


Figure 14. Average pupil area as a function of time for the two mice that were selected for this experiment. The gray area indicates the standard error measurement (SEM).

Discussion

During my research, I successfully demonstrated a novel non-invasive method using a thermography camera to capture body temperature changes in mice. Prior methods of performing these temperature measurements, such as rectal thermometers and implantable microchips, were highly invasive and could affect the mice's behavior due to increased stress or anxiety. They were also more cumbersome and required additional preparation, cost and complexity in the experiment.

Using this non-invasive method, I discovered a statistically significant increase in mice's body temperature following VMHvl activation. By scientifically linking aggressive behavior to this temperature rise, we now have a non-invasive way to quantify aggression levels in mice. This is particularly important when developing psychiatric drugs that target the brain's aggression circuits. My research indicates that body temperature may serve as a valid indicator of animal behavioral states.

Additionally, this temperature increase during activation prompts significant questions. We believe that this effect results from physiological processes triggered by anger neuron activation. Could the increase be due to neurons that cause blood vessel contraction? Might the temperature rise because anger drives increased blood flow to the head? There could be evolutionary advantages to this mechanism. An angry mouse may need to engage in strenuous activities like fighting, where enhanced blood flow could improve cognitive function and alertness, essential in stressful situations. Further research into the

mechanisms behind this temperature rise could be crucial for understanding the brain circuits involved in aggressive behavior.

Finally, evidence indicates that pupil dilation, typically associated with an arousal state, also correlates with VMHvl activation. Pupil dilation showed a different temporal pattern compared to temperature; it decreased before the activation period ended, while the temperature continued to rise for approximately 20 seconds after laser activation. This further suggests that the aggression circuit, not arousal, drives the temperature increase.

Future Work

We plan to acquire a shutterless thermography camera to enable more precise measurements at a higher frame rate. This will allow us to conduct cross-comparisons with other vital signs such as heart rate or breathing rate and determine whether temperature fluctuations can predict these signs.

Using neural networks for image processing, we also plan to correlate the temperature increase with the mouse's “anger” face. This novel result, previously unpublished, could show if a higher temperature increase correlates with a more pronounced expression of anger, further supporting the idea that body temperature can indicate aggression levels in mice.

We will then compare temperature changes against different laser strengths and durations to further explore the anger-temperature relationship and whether temperature continues to rise during extended periods of activation, or at some point it settles and/or decreases.

Finally, we plan to conduct similar investigations with other emotional states, including fear, to understand temperature variations in response to different emotional states. Professor Anderson’s laboratory has identified two fear responses—freezing and fleeing—dependent on stimulus intensity [8]. I hypothesize that freezing will lower the mouse's body temperature due to physiological changes such as decreased metabolic and muscle activity, and vasoconstriction to evade predators. Conversely, fleeing might increase the mouse's body temperature due to physiological responses like an elevated heart rate and blood flow, enhancing awareness and reaction speed. Investigating the temperature differences between aggression, freezing and fleeing could yield important insights into these emotional states. With a thermographic camera, we can measure temperature changes across these states and potentially use temperature as a general indicator of the emotional state in mice.

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