Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

James Orlando Jordano April 8, 2019

Respiration Features Reflect Stress and Inflammatory Pain Conditions in Mice

by

James Orlando Jordano

Dr. Shawn Hochman
Adviser

Biology

Dr. Shawn Hochman
Adviser

Dr. Heidi Kloefkorn

Committee Member

Dr. Ronald Calabrese

Committee Member

Dr. Sandra Garraway

Committee Member

2019

Respiration Features Reflect Stress and Inflammatory Pain Conditions in Mice

By

James Orlando Jordano

Dr. Shawn Hochman

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology

2019

Abstract

Respiration Features Reflect Stress and Inflammatory Pain Conditions in Mice
By James Orlando Jordano

Pain often occurs with physical trauma and disease. The associated pain experience can vary greatly between patients, including: the type of pain, an individual's pain sensitivity, and the subjective perception of pain. This pain experience is often studied using rodent models though quantifying pain in rodents is difficult. Accepted pain measures have failed to show consistency from multiple studies across different pain types, stunting our understanding of pain and the development of solutions for the clinic. A new method of quantifying pain would contribute a clearer picture of pain experienced across different injury types and different analgesic effects; Respiratory features are such potential measures. Respiratory rate (RR) and respiratory rate variability (RRV) are physiological variables that can be recorded in a natural environment. RRV, for instance, has previously been related to pain responses in mice with chronic inflammatory pain and spinal cord injury. To further examine RR and RRV as pain measures, it is necessary to identify other variables that may affect it—including stress. While recording mice in home cages (asleep) and in restraint tubes (awake and anesthetized), RR and RRV were studied under noxious and non-noxious conditions. A well-characterized pain model—formalin injection—was used to produce a short and robust biphasic pain response. When formalin was administered to restrained mice, respiration rate (RR) was elevated in the formalin group (n=8) relative to the baseline group (n=8). Additionally, the anesthetized mice group's (n=7) RRV was elevated after formalin injection relative to their baseline RRV—a pattern that was not present while the mice were awake and restrained. These results suggest both stress and pain affect respiratory patterns independently; RR seems to change when animals are awake and stressed, while RRV changes when animals are not awake and unstressed. Future studies of awake mice experiencing pain in non-stressful environments are necessary to illuminate the nuances of the intertwined relationship of stress and pain.

Respiration Features Reflect Stress and Inflammatory Pain Conditions in Mice

By

James Orlando Jordano

Dr. Shawn Hochman

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology

2019

Acknowledgements

My thanks to Dr. Heidi Kloefkorn, my supervisor, for her advice throughout the entire thesis process. Thank you to my principal investigator, Dr. Shawn Hochman, and my other committee members, Dr. Ron Calabrese and Dr. Sandra Garraway, who were supportive and accommodating. Finally, thank you to Alishah Lakhani, Michael Sawchuk, and William Goolsby for assisting with the experiments, administering injections, and technical support.

Table of Contents

Figures

I. INTRODUCTION

Pain often occurs with physical trauma and disease. The associated pain experience can vary greatly between patients, including: the type of pain, an individual's pain sensitivity, and the subjective perception of pain (Mogil, 2009; Nielsen, Staud, & Price, 2009). Due to this variability, and the ethical limits of human studies, pain is often researched in rodent models instead (Mogil, 2009). Even so, quantifying pain in rodent models has been difficult. Accepted pain measures—paradigms in which pain can be inferred or measured indirectly by another variable—have failed to show consistency from multiple studies across different pain types, stunting our understanding of pain and the development of solutions for the clinic (Mogil, 2009). New and consistent measures of pain would contribute a clearer picture of relative pain experiences between injury types and analgesic effects.

Physical pain can broadly be classified as nociceptive pain, inflammatory pain, and neuropathic pain. Though the full pain experience includes important psychosocial aspects, this study focuses on physical pain measures. Nociceptive pain is triggered by noxious stimuli, like heat or sharpness (Woolf 2010). As such, nociceptive pain sensitivity has been measured in rodents by tests like a heat-induced paw withdrawal test (Dirig 1997). Inflammatory pain is caused by an immune response to tissue damage or infection while neuropathic pain is induced by nervous system damage or a disease state (Woolf 2010). The physical pain experience often includes more than one type of pain which may interact to exacerbate the whole experience. Moreover, many gold standard pain assessments in the rodent field do not distinguish between results caused by nociceptive, inflammatory, or neuropathic pain. One such gold standard assessment is the Von Frey test which, by applying different filaments with known forces to the skin, can estimate a rodent's mechanical sensitivity threshold between tolerance and withdrawal and detect

pain-related behavior changes associated with all physical pain types (Chaplan 1994). Because many tests reflect effects of multiple pain types without differentiating between them, it is sometimes difficult to say with certainty what pain type is present within a model and accurately quantify it. Moreover, some pain assessments are unable to detect certain pain conditions in rodents.

Spontaneous pain is one subset of neuropathic pain that is especially difficult to quantify with current methods. Spontaneous pain results from nerve injury, and is related to the spontaneous (i.e. not evoked) firing of pain neurons (nociceptors) (Djouhri, Koutsikou, Fang, McMullan, & Lawson, 2006). There are currently several methods to infer spontaneous pain, however the most commonly accepted/widely used is the conditioned place preference test (CPP) (Tappe-Theodor & Kuner, 2014). This is an operant test in which a rodent can choose between two conditionally-paired outer chambers from a smaller neutral inner chamber (King et al., 2009). An injured animal, in theory, would spend more time in an analgesic-paired chamber while a healthy animal would explore both chambers equally. This test highlights motivation to the analgesic chamber as the indicator of spontaneous pain (Tappe-Theodor & Kuner, 2014). CPP has been validated in mice using mechanical and thermal sensitivity measures (He, Tian, Hu, Porreca, & Wang, 2012). However, CPP remains far from ideal. Currently, CPP has shown behavioral differences in mice for chronic inflammatory (complete Freund's adjuvant) injuries and spinal nerve ligation. If including rats, CPP has also shown behavioral differences for a spared nerve injury, sciatic nerve axotomy, paw incision, and osteoarthritis. The analgesic-preference behavior demonstrated, though, occurs at very specific time points, such as 24-hours after paw incision or CFA injection. These time frames do not quite match up with the previously reported periods of allodynia associated with the injuries (Tappe-Theodor & Kuner, 2014). For example, significant weight

bearing changes persisted for 21 days after a hindpaw CFA injection, indicating the allodynic period is much longer than the spontaneous pain period suggested by CPP results (Tappe-Theodor & Kuner, 2014). Next, the motivation to the analgesic chamber cannot be clearly caused by pain; the analgesic used may act on the reward circuit or other neural circuits in ways independent of the pain experience (Mucha, Van Der Kooy, O'Shaughnessy, & Bucenieks, 1982). Additionally, if a mouse is not mobile, such as after a complete spinal transection, then CPP cannot be used. Finally, since CPP is reliant on the contextual memory of the mice, if an injury or an administered drug affects their activity level or memory systems, or the association is simply lost, then the test may not accurately indicate spontaneous pain, and may inadvertently measure other complex behaviors (Tappe-Theodor & Kuner, 2014). Finally, CPP requires the use of an entirely novel environment for the mice, which can be stressful even after acclimation, and may confound the experimental results.

Instead, ideal methods to measure spontaneous pain would occur in the home cage. Some attempts have been made to observe spontaneous paw behaviors, such as the level of licking and flinching, in the home cage. Licking and flinching can be a result of other sources besides spontaneous pain, can be biased by manual scorers, and are not present in all injury types known to cause spontaneous pain. For example, licking and flinching were identified in a mouse model of cancer pain (Asai et al., 2005) but were not found in a mouse model of chronic nerve constriction injury (Tappe-Theodor & Kuner, 2014). CPP, paw licking, and flinching are useful tools but new measures are needed that are consistent across different injuries and not confounded by stress or experimenter bias.

An ideal spontaneous pain measure would 1) be recorded in a natural environment, 2) not be inhibited by the injury itself, and 3) be physiological instead of behavioral. Such a pain measure

may be respiration rate (RR) and respiration rate variability (RRV). These measures have already been shown to change with spinal cord injury (H. Kloefkorn et al., 2017) and inflammatory pain while the animal is at rest (Jordano, Kloefkorn, Goolsby, Martin, & Hochman, 2018). Respiration is also primarily under autonomic control and it can be measured non-invasively in an animal's natural home cage environment using non-contact electric field sensors (detailed below). Additionally, respiration control is largely unaffected by all but the most severe or high spinal injuries, making it a more universal measure than CPP, paw licking, or flinching.

As previously mentioned, RR and RRV have been recorded while animals are at rest in their natural home cage environment under uninjured and injured conditions (Jordano et al., 2018) . RRV was shown to correlate with increased mechanical sensitivity (Jordano et al., 2018). However, RRV was measured while the animal was at rest and has not been proven to reflect pain. To determine whether RR and RRV may reflect spontaneous pain events, a new study was designed to capture RR and RRV in awake animals.

To record RR and RRV non-invasively using non-contact electric field sensors (described later), the mice need to be still enough to capture only respiratory-related movements, and therefore restraint tubes had to be used. This also created a paradigm in which stress and pain-while-stressed could be explored. Because instances of spontaneous pain cannot yet be confidently identified nor has RR or RRV been proven to reflect any pain type, a model of inflammatory pain caused by formalin was used for the validation of this study. To clarify, we are not

proposing this study directly confirms whether RR and RRV reflect spontaneous pain, however, this study lays the ground work to answer that question in a future study.

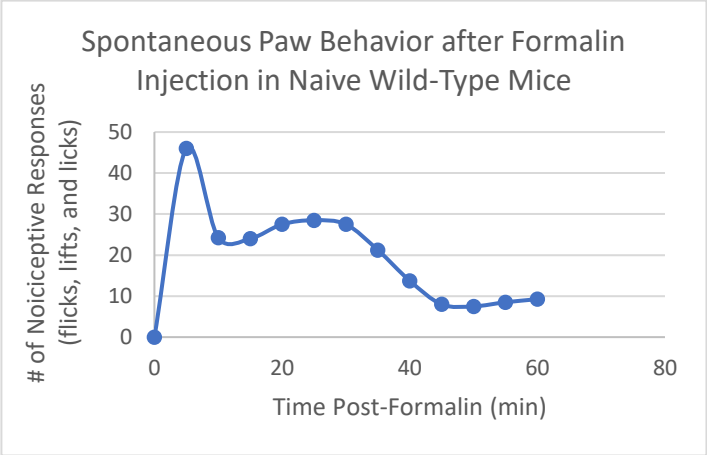

Figure 1. Biphasic Formalin Response Measured by Spontaneous Paw Behavior after a Formalin Injection. The two phases peak at about five minutes and about 30 minutes, while the entire response nearly subsides after one hour (Garraway).

Formalin causes a well-documented inflammatory pain response with two phases: one acute, stronger response five minutes after injection and one weaker response thirty minutes after injection (Figure 1) (Hunskaar and Hole 1987, Garraway)The entire response resolves after an hour (Garraway). Its well-documented nature and relatively short response allow the comparison of RR and RRV with known changes in pain experience, while not keeping the mice restrained for too long. Changes in RR and RRV are expected to reflect the biphasic pain response of formalin. Additionally, stress alone is expected to affect RR and RRV.

II. METHODS

2.1 Ethics Statement

All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

2.2 Animals

Eight C57BL/6 adult mice (four male, four female) were used to perform all experiments in this study. The age for the female mice was 140 days (over four months old). The average age for the male mice was 135±3.5 days (over four months old). The female mice all lived together, while three of the male mice lived together and one male mouse lived alone. This latter male mouse had excessive bedding in his home cage to compensate. Cages included corn cob bedding, constant food access, and water at all times. Cages were stored next to other mouse cages on a storage rack in a temperature- and humidity-controlled room in the Whitehead Biomedical Building (Emory University). The light–dark cycle was 12 h light, 12 h dark, with the lights on at 0700 h. Testing occurred in the first half of the light cycle, not starting before 0830 h.

2.3 Experimental Summary - Restraint

Animals were put into restraint tubes for two hours on each of the testing days immediately after being removed from their home cages. Baseline respiration measurements were recorded over

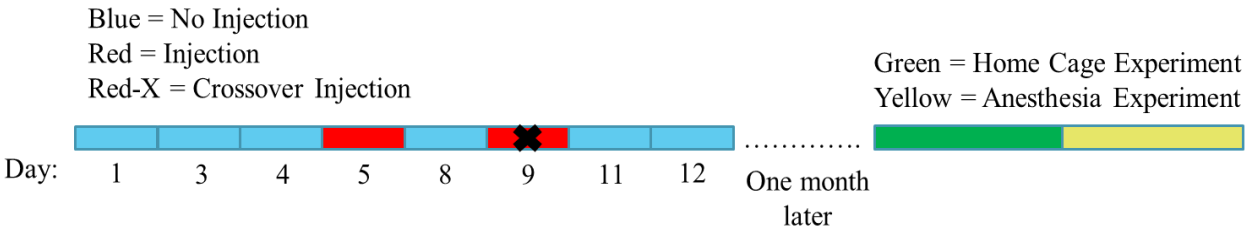

Figure 2. Restraint Tube Experimental Timeline. The restraint experiment was carried out on eight testing days over a twelve-day period. On the days highlighted in red, mice were injected with either formalin or saline in a crossover design; an animal injected with formalin on day four received a saline injection on day seven. The home cage and anesthesia experiments are also listed to put the restraint experiment in context of the other experiments.

the first three days, without any intervention (no injections). On day four, the same restraint protocol was followed but mice were additionally injected with either formalin (n=4) or saline (n=4) using a crossover experimental method immediately prior to being placed in the restraint tubes. Mice were restrained and recorded after this injection day for an additional two days without any intervention. On day seven, injections were carried out again, but the crossover groups switched; those that received saline (n=4) on day four now received formalin (n=4) on day seven. The animals were then restrained and recorded for one more day without intervention.

2.4 Restraint Tube Setup

Restraint tubes were made from 50 ml Falcon tubes by drilling air holes near the cone of the tube and a tail hole in the tube's cap.

The non-contact electric field sensors (EF sensors) were affixed to the exterior of the restraint tubes (an accelerometer was also used as described below). (Figure 3). Non-contact EF sensors measure movement-related changes in the local electric field. When the animal is still, the predominant movement has been shown to be due to respiration (Figure 4A) (Noble et al., 2017).

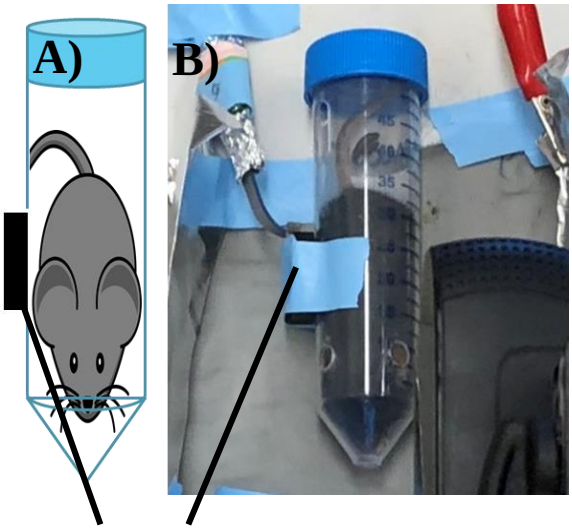

Figure 3. Restraint Tube Design. A) A diagram depicting where the non-contact electric field sensor is placed on the restraint tube. B) A photo from the experiment showing the actual restraint tube setup.

Respiration is cyclic and results in a sinusoidal voltage trace from the EF sensors (Figure 4B). To analyze RR and RRV, a respiration event is defined as a continuous cyclic trace of multiple breaths uninterrupted by non-respiratory movement such as exploration, sniffing, grooming, or chewing. Each respiration event in the voltage trace contains multiple peaks for which

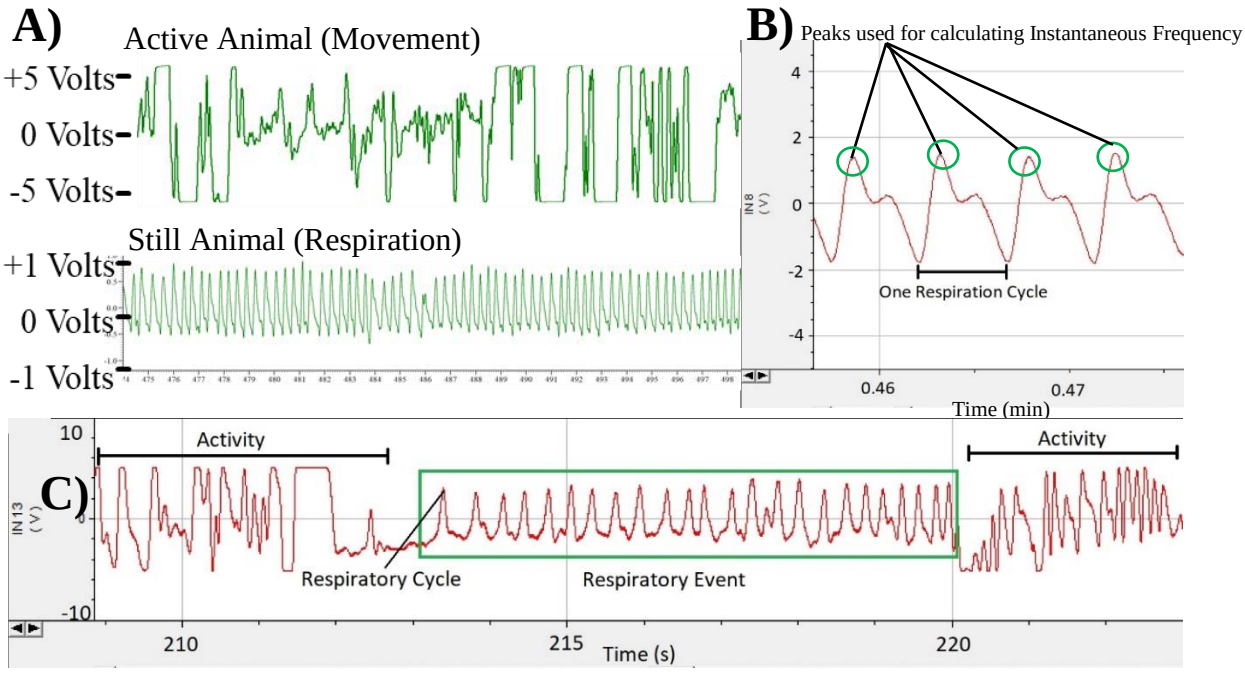

Figure 4. Non-Contact Electric Field Sensor Output. A) The top output is data from an animal active in front of the sensor. It has high amplitudes and no discernible, consistent pattern. The bottom output is from an animal that is still in front of the sensor. This pattern is respiration. B) This output is zoomed in to depict a common shape of respiration's cyclic pattern. The maximum peak of each cycle is used to calculate average respiration rate and respiration rate variability. C) This is an example of a respiration event, as depicted by a cyclic respiratory pattern flanked by two periods of activity.

instantaneous frequency can be calculated for each peak (Figure 4B). RR is calculated as the average of the instantaneous frequency of all the peaks within a respiration event. RRV is calculated as the standard deviation of the instantaneous frequency of all the peaks within a respiration event. All respiration events measured (see example in Figure 4C) were between 2.9 and 60 seconds in duration. Any event outside of this range was not recorded.

The non-contact EF sensors are connected to a filter and amplifier box then to a data acquisition box (Axon Instruments, Digidata 1440a). The data acquisition box then sends the signal to a computer where the data are recorded using the programs LabVIEW (National Instruments, Austin, TX) and Clampex (Molecular Devices, San Jose, CA) and analyzed using Clampfit

(Molecular Devices, San Jose,CA). Synced video recordings of the testing session were also recorded.

2.5 Accelerometer Setup

One tube had additional instrumentation in the form of an accelerometer inside the tube. The accelerometer has been shown to detect heart rate via subtle murine movements (Gurel, Jeong, Kloefkorn, Hochman, & Inan, 2018). The accelerometer used was an analog 3-channel microelectromechanical systems (MEMS) accelerometer (ADXL354CZ, Analog Devices, Norwood, MA). Its power supply was a 9V battery that was regulated by the LT1763 (Linear Technology, Milpitas, CA). The accelerometer was connected directly to the Digidata 1440a (Axon Instruments, San Jose, CA) for collection and followed the same set-up as EF sensors. The accelerometer's output is depicted in Figure 5. The peaks used to calculate heart rate are circled. Each peak has an instantaneous frequency; all these frequencies are averaged to find the average heart rate, just as respiration rate is calculated.

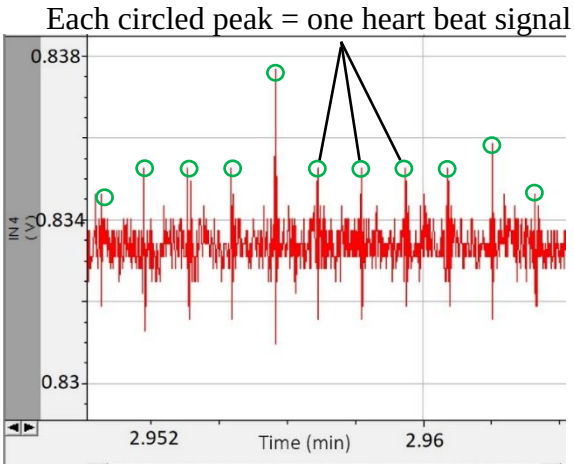

Figure 5. Accelerometer Output. The output is from a mouse positioned directly on an accelerometer while in a restraint tube. The circled peaks each have an instantaneous frequency—the average of which is the average heart rate.

2.6 Injections

On the injection days, mice were administered a subcutaneous injection on the right dorsal hind paw of either 20µL saline or 20µL formalin using a 29-gauge needle. The formalin was a 5% (v/v) solution in water. Saline was a 0.9% sodium chloride solution in water. Mice were injected

directly before being put into the restraint tubes on the appropriate testing days. One experimenter restrained the mice via neck scruffing the mice while another injected the hind paw.

2.7 Experimental Summary—Home Cage Recordings

One month after the restraint experiment, the mice were monitored in home cages using an established protocol from previous experiments in the Hochman group (Figure 6). Each cage held two mice separated into two separate chambers during testing. Each side had free water, constant food access, corn cob bedding, and a petri dish nest. Respiration was collected by using non-contact EF sensors positioned on the outside lateral wall of the cage next to the nest. They measure respiration in the same way as the restraint experiment. Respiration can be captured whenever the mouse is still enough, such as when asleep or—less frequently—when awake but not moving. This paradigm was validated by previous work looking at respiration rate variability in mice with spinal cord injury (H. Kloefkorn et al., 2017) and chronic inflammatory pain

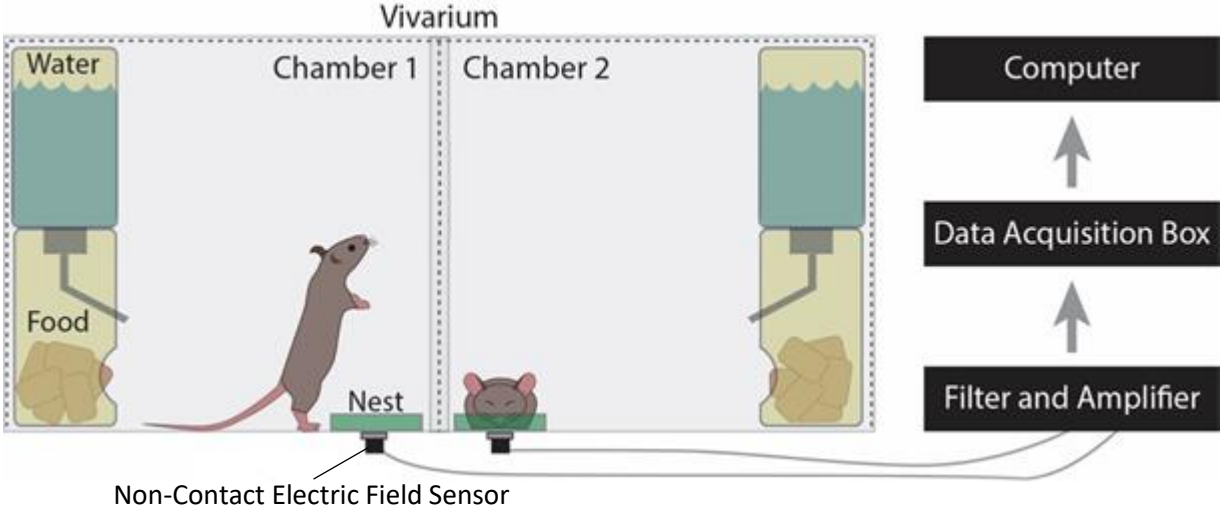

Figure 6. Model of the Instrumented Home Cage. Non-contact electric field sensors are placed on the outside wall of the home cage. In this experiment, they were placed on the lateral wall of the cages. The EF sensors can measure respiration by recording movement-related changes in the electric field. The signal is then filtered, amplified, and displayed on an attached computer.

(Jordano et al., 2018). These previous studies did not look for respiration instances where the animals were both awake and not moving, but respiration while the mice were asleep was analyzed. The current experiments' home cage recordings were compared to the baseline respiration features of a historic cohort of eight C57BL/6 adult mice (n=8). Respiration was recorded in the same way—using non-contact electric field sensors in home cages identical to those of the current experiment.

2.8 Experimental Summary—Anesthesia Endpoint

An anesthesia experiment was performed one week after the home cage measurements to attempt to create the control condition of pain-without-stress inside the restraint tubes. This "pain-without-stress" condition attempts to isolate the effects of nociceptor response from pain-induced behaviors, such as the stress response.

Testing was carried out in two cohorts (n=4 each) over 2 days. On the first day, four mice were administered gaseous isoflurane. Upon being anesthetized, each mouse was initially injected intra-peritoneally (IP) with an insulin syringe of 10% urethane by weight (1 g/kg) and then were returned to their home cages. Urethane is an anesthesia known for its minimized cardiovascular and respiratory damping effects (Hara & Harris, 2002).

After an additional ten minutes, a booster IP injection of 10% urethane (0.5 g/kg) was administered. The anesthetized mice were then put into the restraint tube for twenty minutes of baseline respiration recording. Following baseline, the mice were injected with formalin in the same foot as before (following the same protocol in the above injections section) and then returned to their respective restraint tubes for another hour of recording. Once recording was complete, mice were euthanized via a cervical dislocation.

On the second day, the remaining four mice received the same initial 10% urethane injection (1 g/kg) the same 10% urethane booster (0.5 g/kg) but also a third booster of 10% urethane (0.25 g/kg) because the animals did not appear to be responding the same as on day 1. One mouse needed to be given a fourth booster (0.25 g/kg). This mouse eventually was euthanized early—before the formalin injections—because of poor reaction to the urethane; it was excluded from the anesthesia group for analysis. The other three mice followed the same procedure once in the restraint tubes as the day one mice.

2.9 Analysis and Statistics

Four days of recording were analyzed by experimental group: day one of baseline, day three of baseline, and both injection days (n=8 for all days).

Respiration events from the first 45 minutes of each day were grouped together and used in calculating RR and RRV to capture the total formalin response. The analysis described below was performed on these data. Additionally, to see if the formalin response was biphasic, the data across the entire two hours was reviewed in ten-minute intervals. After review, there was no indication of a consistent biphasic response across animals for RR or RRV and no further analysis was performed on these data.

Unless otherwise stated, all comparisons were analyzed using a 1-way ANOVA with a Tukey's honest significant difference post-hoc test. Groups within experiments were compared; for example, in the restraint experiment, the baseline, formalin, and saline groups were compared to one another to assess differences in RR and RRV.

To attempt to recreate the condition for pain-without-stress, the respiration data from the anesthesia experiment (baseline and formalin, n=7) were compared using a student's t-test.

Heart rate data for a wake, restrained mouse (baseline and formalin) were analyzed using a two sample, equal variance t-test.

To confirm that respiration event duration did not co-vary with either RR or RRV, thus removing the need to include it in the statistical model, general regression models were performed.

All data in figures is presented as the mean value ± standard deviation.

III. RESULTS

3.1 Restraint Group Comparisons

During the first 45 minutes after formalin injection, RR was elevated in the formalin group relative to baseline ($p=0.047$) The formalin group's RR did not differ from that of the saline group ($p=0.13$). Additionally, The RR of the baseline and saline groups were also not different ($p=0.87$) (Figure 7A). There was no difference in RRV between any of the groups ($p>0.75$) (Figure 7B).

3.2 Home Cage Comparisons

When comparing respiration features of the current mice cohort and historic cohort while asleep in home cages, the respiration rate ($p=0.88$) and variability ($p=0.36$) did not differ (Figure 7)

3.3 Anesthesia Endpoint

The RR measure before and after formalin injection while anesthetized also was not different ($p=0.96$) (Figure 7A). The anesthesia formalin group RRV was elevated relative to the anesthesia baseline group ($p=0.017$). The restraint formalin group RRV did not differ from the anesthesia formalin group ($p=0.12$) (Figure 7B). The animals on the first day of the anesthesia endpoint experiment all had clearly-elevated respiration event values. These peaks occurred when the first formalin phase was expected. The three animals from the second anesthesia day did not have clearly-elevated respiration events (Figure 8).

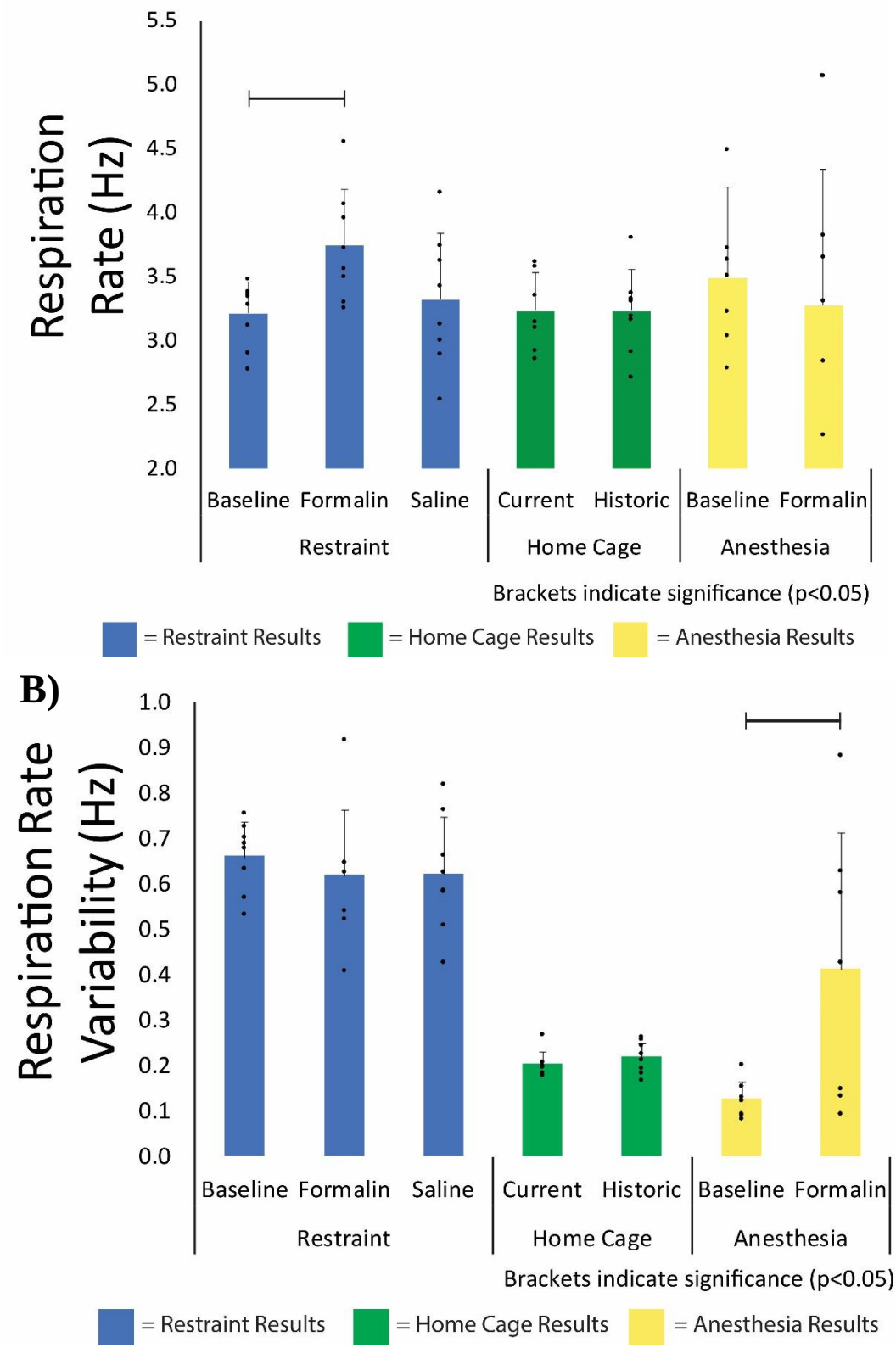

Figure 7. Respiration Rate and Variability Within-Experiment Comparisons. Mean respiration rates and variabilities are compared within their experiment (restraint, home cage, anesthesia). Values are displayed as mean ± S.D. A) The restraint formalin group's RR was elevated relative to the restraint baseline group. B) The anesthesia formalin group's RRV was elevated relative to that of the anesthesia baseline group.

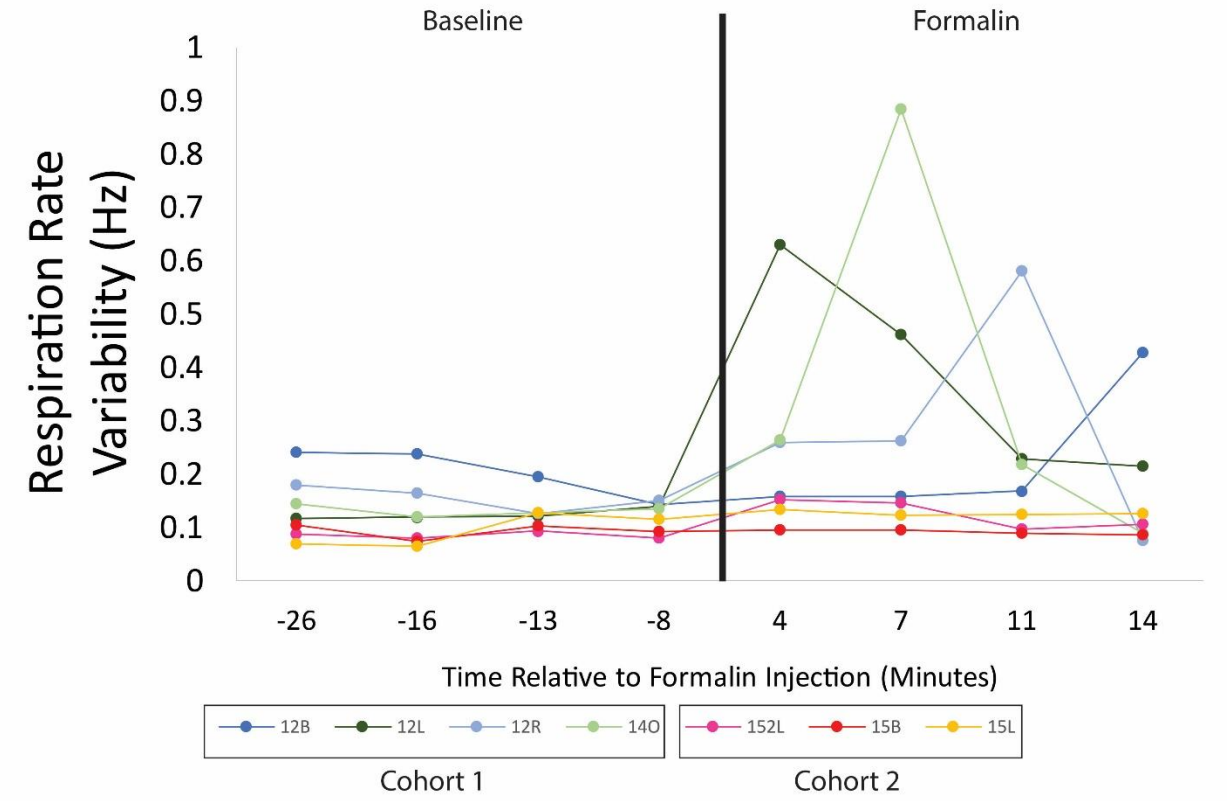

Figure 8. Maximum Respiration Event Values Under Anesthesia After Formalin Injection. Values are displayed as mean ± S.D. Each respiration event was about 3-5 minutes in duration, plotted across time relative to the formalin injection. The animals tested in the first cohort each had a respiration event much higher than their other three events, while the second cohorts' events were much more suppressed.

3.4 Restraint—Event Duration

The relationship between respiration event duration and respiration features of the restraint experiment was analyzed. Regressions between respiration event duration and RR or RRV were not significant.

3.5 Restraint—Heart Rate

Only two baseline and three formalin heart rate events were analyzed due to the difficulty in obtaining reliable positioning for heart rate detection. There was no difference between the two groups (p=0.25) (Figure 9). However, due to the small sample size and lack of power, meaningful conclusions cannot be drawn from this result. Further analysis is necessary to create a more complete picture of the effect of formalin while under restraint on heart rate.

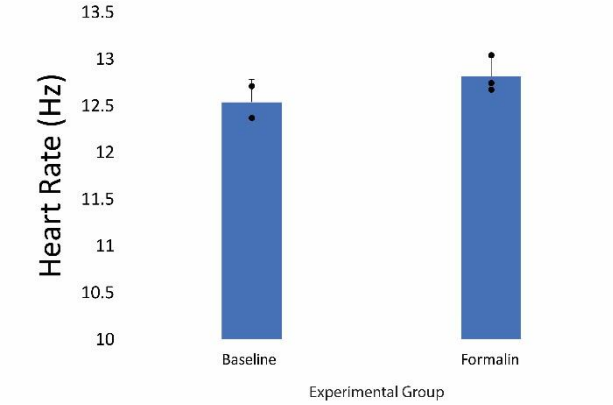

Figure 9. Heart Rate of One Animal. Values are displayed as mean ± S.D. Heart rate data from a baseline recording day and a formalin recording day were compared. There was no difference between the two experimental (p=0.25).

IV. DISCUSSION

Respiration was successfully recorded in mice under multiple environmental conditions with and without inflammatory pain. This allowed for informative comparisons to study the effect of formalin-induced inflammatory pain on respiration rate and its variability.

Under restraint, RR was significantly increased after formalin-induced pain while RRV was unchanged. The observed pain-induced increase in RR is consistent with a recent study in rats that examined RR increases associated with allodynia in spinal cord injured rats (Noble, Martin, Parvin, & Garraway, 2019). RRV being unchanged after formalin-induced pain suggests that observed changes in RRV previously associated with pain may instead be due to coexistent increases in stress (H. I. Kloefkorn, S.; Halder,M.;Goolsby,B.;Aiani,L.M.;Pedersen,N.P.;Hochman,S., 2018). However, this conclusion is premature, as observed values of RRV under restraint may be due to an already maximal effect of stress on increasing RRV. Indeed, much lower RRV values were obtained in the home-cage; the home cage RRV of the current animals was only 29% of their restraint RRV.

Additional studies are required to determine whether RRV as a variable reaches a maximal value such that the addition of pain does not produce additional response. Indeed, evidence that pain afferent signaling increases can lead to RRV increases was provided in our animals when tested under anesthesia. Anesthetized animals that received formalin responded differently. Here, after formalin injection, RR did not change, whereas RRV increased relative to anesthesia baseline values. In other words, using the same pain-test under anesthesia as a non-stress condition, the opposite effects were observed—RRV increased but RR was unchanged in response to a nociceptive stimulus.

While examination of respiration features under anesthesia does have limitations, the short formalin response time (≤60 minutes before resolution) prevented its use under a natural condition that can be removed from experience of stress. One possibility is testing during sleep, but it is unlikely that mice will fall asleep within 60 minutes of receiving an inflammatory pain injection; anesthesia was therefore the best possible pain-without-stress condition using a formalin model.

Overall, the results demonstrate that RRV changes can coincide with induction of pain but that stress also leads to increases in RRV, such that ongoing stress may mask the use of increases in RRV as an index of pain. This may indicate that RRV is a more accurate indicator of ongoing spontaneous pain than RR in non-awake, non-stressed animals—a conclusion supported by previous work (H. Kloefkorn et al., 2017). Since the relationship between RRV and pain was not apparent under restraint, the presence of stress and the cognitive recognition of pain (i.e. when mice are awake and processing pain centrally) may alter respiration rate variability's response to pain. This highlights the limitations of using respiration as a sole indicator for pain, and may be important to consider when performing other pain assessments that might include stress. As such, future experiments should study pain conditions in a non-stressful environment.

After further analysis of the anesthesia experiment, the pattern of change of RRV was also similar to the first-phase component of formalin's biphasic response (though occurring at different times for different animals). Barring unlikely gender differences (male mice happened to comprise the first cohort; females for the second), the clear maximal respiration events may have been suppressed in the second cohort because this group required an additional booster of urethane. The extra anesthesia may have too heavily sedated the mice and interfered with the natural RRV response. Despite these differences, there seems to be a relationship between the

formalin-induced pain and respiration rate variability—one that may suggest specifically when an animal is feeling pain, as opposed to just indicating the binary presence or absence of pain.

Heart rate was anticipated to change with respiration rate, but in preliminary studies heart rate did not appear to change between the baseline and formalin conditions. A larger sample size is required to determine this. The current paradigm of using accelerometry-based detection is promising, however it can be improved. Since capturing heart rate is very position-dependent in anesthetized animals, a better understanding of how to maintain the ideal position in awake animals will lead to clearer heart rate results (Gurel et al., 2018).

By providing values for RRV here under restraint stress and at rest in the home-cage, the present work may importantly inform future studies on the use of RRV as a variable indicative of magnitude of baseline activation of the stress/pain axis. In other words, RRV may be a useful biomarker of ongoing stress/pain axis activation.

Future studies will further illuminate the relationship between respiration features and activation of stress and pain. The RR and RRV findings from this study warrant further experiments in a non-stressful environment. In such an environment, respiratory patterns will be clearer and potentially indicate not only if an animal is in pain, but at what time points it experiences pain. This would be the key to transitioning to identifying spontaneous pain; respiratory events of high variability may suggest the presence of spontaneous pain.

The current experiments make progress towards this goal. By tracking respiration in mice under various conditions, respiration features were better understood—specifically, by learning that stress and the cognitive recognition of pain can confound respiration features' ability to indicate pain. These results help move the field closer to finding the ideal quantitative measure of pain.

V. SOURCES CITED

Asai, H., Ozaki, N., Shinoda, M., Nagamine, K., Tohnai, I., Ueda, M., & Sugiura, Y. (2005). Heat and mechanical hyperalgesia in mice model of cancer pain. *Pain, 117*(1-2), 19-29.

Djouhri, L., Koutsikou, S., Fang, X., McMullan, S., & Lawson, S. N. (2006). Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. *Journal of Neuroscience, 26*(4), 1281-1292.

Garraway, S. M. *Biphasic Formalin Response Measured by Spontaneous Paw Behavior after a Formalin Injection*. Physiology. Emory University School of Medicine.

Gurel, N. Z., Jeong, H. K., Kloefkorn, H., Hochman, S., & Inan, O. T. (2018). *Unobtrusive Heartbeat Detection from Mice Using Sensors Embedded in the Nest.* Paper presented at the 2018 40th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC).

Hara, K., & Harris, R. A. (2002). The anesthetic mechanism of urethane: the effects on neurotransmitter-gated ion channels. *Anesthesia & Analgesia, 94*(2), 313-318.

He, Y., Tian, X., Hu, X., Porreca, F., & Wang, Z. J. (2012). Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. *The Journal of Pain, 13*(6), 598-607.

Jordano, J. O., Kloefkorn, H. E., Goolsby, W., Martin, K., & Hochman, S. (2018). *Respiration Rate Variability Correlates with Mechanical Sensitivity in a Mouse Model of Inflammatory Pain.* Oral Presentation. Physiology. Emory University School of Medicine.

King, T., Vera-Portocarrero, L., Gutierrez, T., Vanderah, T. W., Dussor, G., Lai, J., . . . Porreca, F. (2009). Unmasking the tonic-aversive state in neuropathic pain. *Nature neuroscience, 12*(11), 1364.

Kloefkorn, H., MacDowell, C., Sawchuk, M., Goolsby, W., Halder, M., & Hochman, S. (2017). *Characterizing sleep state respiration changes after spinal cord injury using non-contact electric field sensors*. Physiology. Emory University School of Medicine.

Kloefkorn, H. I., S.; Halder,M.;Goolsby,B.;Aiani,L.M.;Pedersen,N.P.;Hochman,S. (2018). *Changes in sleep architecture, respiratory behavior, and indices of pain correlate after thoracic spinal cord contusion* Paper presented at the Society for Neuroscience.

Mogil, J. S. (2009). Animal models of pain: progress and challenges. *Nature Reviews Neuroscience, 10*(4), 283.

Mucha, R. F., Van Der Kooy, D., O'Shaughnessy, M., & Bucenieks, P. (1982). Drug reinforcement studied by the use of place conditioning in rat. *Brain research, 243*(1), 91-105.

Nielsen, C. S., Staud, R., & Price, D. D. (2009). Individual differences in pain sensitivity: measurement, causation, and consequences. *The Journal of Pain, 10*(3), 231-237.

Noble, D. J., MacDowell, C. J., McKinnon, M. L., Neblett, T. I., Goolsby, W. N., & Hochman, S. (2017). Use of electric field sensors for recording respiration, heart rate, and stereotyped motor behaviors in the rodent home cage. *Journal of neuroscience methods, 277*, 88-100.

Noble, D. J., Martin, K. K., Parvin, S., & Garraway, S. M. (2019). Spontaneous and Stimulus-Evoked Respiratory Rate Elevation Corresponds to Development of Allodynia in Spinal Cord–Injured Rats. *Journal of neurotrauma*.

Tappe-Theodor, A., & Kuner, R. (2014). Studying ongoing and spontaneous pain in rodents–challenges and opportunities. *European Journal of Neuroscience, 39*(11), 1881-1890.