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Electric Field Sensor-based Characterization of Mouse Physiology and Behavior in the Home Cage: Variability Before and After Spinal Cord Injury

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An Abstract of a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Animal models are weakened by unexplained variability in experimental outcome. For models of spinal cord injury (SCI), this variability and the lack of reproducibility in therapeutic effect is cited as a key factor inhibiting clinical translatability. The environmental influences that contribute to variability in outcome remain highly understudied. This thesis develops enabling technologies that continuously capture mouse physiology and behavior in the home-cage environment to characterize inter-animal variability before and after high thoracic spinal cord transection.

This thesis tests the hypothesis that even strictly controlled genetic and environmental conditions do not fully mitigate inter-animal variability in physiology (respiration) and behavior (motor activity). Comparisons were made between adult male C57BL/6J mice as; (i) dual-housed littermates, (ii) littermates housed in different cages, and (iii) non-littermates. Housing conditions were otherwise identical.

To avoid exaggeration of inter-animal variability due to unique animal interactions with experimenter and/or contact-based recording devices, I first developed a method for using ultra-sensitive electric field sensors to capture continuous, non-contact measurements of motor activity and respiratory rate in the home cage. Using this method, continuous 12-hour recordings of respiratory rate and motor activity were obtained daily

for seven days in six adult male mice. Resting respiratory rate was found to be highly comparable in all mice at all time periods sampled. Motor behavior was characterized by alternative bouts of activity and rest. Same-cage littermates had similar activity profiles but this profile differed considerably between cages. Non-littermates exhibited significantly different overall activity levels.

I next examined the temporal changes in respiration and activity for one-week after upper thoracic spinal cord transection. SCI led to an overt increase in inter-animal variability in both respiratory rate and motor activity. Differences in lesion completeness and extent were noted and may have contributed to the observed variability. Two characteristic features across animals were; (i) an early loss of episodic patterns of motor activity that appeared to recover after several days and (ii) respiratory rate that stabilized to a significantly lower value.

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1.0 Introduction

1.1 Overview

The overarching goal of this thesis is to better understand the role that animal life history plays in experimental outcome seen after spinal cord injury (SCI). Section 1.2 of this Introduction focuses on the important issue of reproducibility and variability in experimental outcome in animal models of SCI and the inhibitory impact of this variability on the translatability of model results to clinical applications.

A commonly held assumption is that because inbred strains of animals are genetically identical (here C57BL / 6J mice) and because vivarium housing is standardized, experimental variability in outcome is due to other factors. However, given the complex temporal interactions between environment and behavior and physiology, it seems reasonable to assume that each animal has a unique life history and therefore unique physio-behavioral phenotype. I therefore tested the hypothesis that even a strictly controlled group of animals housed in different home cages exhibit variability in behavior (motor activity) and physiology (respiratory rate). I then explored the possible association between outcome after spinal cord injury and pre-injury physio-behavioral variability.

Section 1.3 of the Introduction details specifics of my experimental design and provides background regarding my variables of interest and choice of controls.

As the current methodologies for recording these physio-behavioral parameters may themselves introduce variability, I first developed a technology to allow for continuous noninvasive and noncontact recordings of animal respiratory rate and motor activity in the home cage. I detail the limitations of other technologies and rationale for this method development in section 1.4. Therefore, a significant portion of this thesis is focused on

using movement-sensitive electric field sensors as a novel technology for the detection of physiological and behavioral variables. As such, the methods, results, and discussion sections are all divided into two parts, one dedicated to the development of this method and the other to my experimental investigation. In this way, this thesis is specifically structured to facilitate simultaneous explanation of both my experimental aims and method development.

1.2 Spinal cord injury and sources of experimental variability

There is an issue of variability of experimental results in studies employing animal models of spinal cord injuries (SCIs) (Landis et al., 2012; Nielson et al., 2014). Indeed, inter- and intra-lab variability of results has been well documented in a broad range of SCI research including investigations into neuropathic pain, functional recovery, and axonal regeneration after SCI (Mogil 2009; Callahan et al., 2008; Hulsebosch et al., 2009; Steward et al., 2008; Blesh and Tuszynki, 2009; Parker, 2005; Pinzon et al., 2008). This issue of variability has been cited as inhibiting the translatability of experimental results from SCI model systems to clinical applications (Nielson et al., 2014; Blesch and Tuszynski, 2009). Thus, understanding and accounting for factors that contribute to this variability is an important and necessary step in improving the value of future studies.

Typically, variability in outcome after SCI is attributed to the inevitable heterogeneity of animal demographics (e.g. age, sex, genetics), injury characteristics (type of SCI, location, severity), SCI-injury devices, and measurement protocols between and within labs (Steward et al., 2011; Basso et al., 2006; Parker, 2005; Nakipoglu-Yuzer et al., 2013). However, a recent review by Steward et al. suggests that other currently

unaccounted for factors may influence experimental outcome (Steward et al., 2011). The review summarized eleven studies that attempted to reproduce published results of previous SCI experiments. Each reproduction replicated, "to the best of their ability," the methodologies of the original papers; using the same animal strains, animal suppliers, animal demographics, SCI characteristics (type, location, severity), and procedures. Many even involved the original Principal Investigator to make sure methodologies were being adhered to accurately. Despite these efforts, only one original study was successfully reproduced. Six were not reproduced, four were partially reproduced, and the results of one were inconclusive. This lack of reproducibility, despite accounting for the factors to which variability is typically credited, suggests that not all potential influences are being considered and emphasizes the important need for additional research investigating and controlling for sources of this variability.

It is interesting to note that none of these studies attempted to replicate animal housing conditions. This is not uncommon. However, because of the plethora of variables present in the home cage/vivarium environment, it is unlikely that animal life histories of home cage experiences are identical. As a whole, the current research enterprise largely excludes consideration of animal housing environment (Brown et al., 2000; Burke et al., 2007). Yet animal experiences in the home cage represent the dominant portion of an animal's life history and involve a dynamic interaction of numerous variables that influence physiology and behavior (Hernandez et al., 2006; Crabbe et al., 1999). For example, the presence of simple environmental enrichment objects, such as a cage shelf or housing shelter, dramatically impacts results of common behavioral tests (Okva et al., 2013; Coombs 2014), influences animal physiology (Ravenelle et al. 2014) and affects recovery

after SCI (Fischer and Peduzzi, 2007; Berrocal et al., 2007). Even very subtle changes to home cage conditions, such as alterations to airflow or noise level, have been shown to change mouse activity and physiology (Memarzadeh et al., 2004; Reynolds et al., 2010; Turner et al., 2007). Inter-cage social dynamics and handling by researchers or vivarium personnel also impact these variables (Champagne et al., 2003; Gariepy et al., 2002; Sabine et al., 2005). In this way, combinations of variables and their temporal order imposes an additional layer of complexity that is typically not considered in variability of experimental outcome.

The focus of this thesis is to test the hypothesis that even strictly controlled genetic and environmental conditions cannot entirely mitigate inter-animal variability in physiology (respiration) and behavior (motor activity). Furthermore, I predict that physio-behavioral variability in uninjured animals is associated with variability in outcomes after SCI.

1.3 Monitoring variability in physiology and behavior: variables of interests and controls.

To assess physio-behavioral condition I continuously monitor trends in two indicators of global physiological and behavioral status – activity and respiratory rate (Cretikos et al., 2008; Grossman 1983; Foltz, 1999). Respiratory rate provides an important index of autonomic function, with increased rates associated with increased sympathetic drive (Cretikos et al., 2008;). Motor activity is a well-known indicator of arousal state and health status (Crawley et al., 1999).

I compare these parameters between adult male C57BL/6J mice as; (i) dual-housed littermates, (ii) between littermates housed in different cages, and (iii) between mice

reared by sibling dams. I chose the C57BL/6J strain because it is thoroughly inbred, thus minimizing genetic differences between individuals (Matsuo et al., 2010; Bryant et al., 2008). Furthermore, C57BL/6J dams are believed to exhibit highly stereotyped maternal behavior, thereby limiting differences in life experiences and subsequent variability in physiology and behavior between litters (Champagne et al., 2007; Van der Veen et al., 2007). To further mitigate potential environmental variability, all animals are housed in identical home cages located directly next to each other in the same vivarium room.

Animals' respiratory rate and activity are monitored for seven days, twelve hours a day. I then evaluate these parameters for an additional seven days after giving animals upper thoracic spinal cord transections. These injuries are known to disrupt both locomotor and autonomic function (Zimmer et al., 2007; Battistuzzo et al., 2012). Specifically, a complete upper thoracic transection should lead to a decline in respiratory rate and a transient decline followed by recovery of motor activity (Schilero et al., 2009; Brown and Weaver, 2012; Van Meeteren et al., 2003)

1.4 Developing methods to record physiology and behavior in the home cage.

The first part of this thesis is dedicated towards developing the use of ultrasensitive electric field sensors (EPIC sensors; Plessey Semiconductors) as a novel method for non-contact, continuous monitoring of animal physiology and behavior. This method is essential to my experimental design because in order to strictly control for animal environment and experience, measurements of activity and respiratory rate must not introduce variables that could uniquely impact individual animals. Current methods for continuous recordings of physiology, such as radiotelemetry and plethysmography do not

meet these standards. Telemetry is invasive (thus altering life history) and plethysmography requires a unique testing environment separate from animal home cages; both systems require considerable animal-experimenter interactions (Aaron & Powell 1993; Huetteman & Bogie 2009). Additionally, while many commercial systems already exist for automated rodent behavioral phenotyping such as the Phenotyper® (Noldus), LABORAS (Metris), and Intellicage (NewBehavior) (de Visser et al 2006, Krackow et al 2010, Pham et al 2009, Quinn et al 2003, Van de Weerd et al 2001), these systems are extremely cost-prohibitive (ranging in the thousands of dollars per cage) and require complex video surveillance systems in specialized cages.

The proposed use of EPIC sensors for recordings presents an alternative to the aforementioned technologies for monitoring physiology, with the additional advantage of providing concomitant measure of animal activity. Also, because recordings are continuous, this method permits investigation into the temporal dynamics of variability between animals. Particularly relevant to this study is that my proposed methodology does not require implanting or affixing any devices to the animal and can take place in a home cage that mimics social housing conditions. In this way, I attempt to limit any confounding factors that may exaggerate variability between animals.

2.0 METHODS

2.1 Experimental animals

C57BL/6J mice (20-30 g) were housed in standard cages in a vivarium with a 12:12-hour light-dark cycle and were fed ad libitum standard rodent diets. The C57BL/6J strain was chosen because it is a commonly used inbred strain with limited genetic variability

(Matsuo et al., 2010; Bryant et al., 2008). All experiments were approved by the Animal Care and Use Committees of Emory University. The experiments conformed to national standards for the care and use of experimental animals and the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

2.2 Development of Method

2.2.1 Plessey Semiconductors EPIC™ sensors

Plessey Semiconductors have manufactured a series of ultra-high impedance, dry-contact capacitive coupling electric field sensors. These high sensitivity sensors are advertised as capable of use for non-contact based detection of movements including proximity to sensor, gesture recognition, and recording electrocardiographic (ECG) activity in humans. I tested PS25251 sensors. Each Plessey PS25251 sensor is $1cm^2$ in size and has four pins: Vdd, Vss, Gnd, and output. To interface with an A/D converter and power supply, each of these pins was soldered to one of four pins on a 9-pin VGA adapter. A specialized DC power supply box was constructed to provided ± 5 volts and ground to the Vdd, Vss, and Gnd VGA pins, respectively. Epoxy was applied to each soldered joint on the sensors to provide additional strength and protection. Standard electrostatic discharge protection precautions were followed throughout the construction and handling process. A detailed description of the response characteristics of these sensors can be obtained from the manufacturer's website (http://www.plesseysemiconductors.com). The Output and Gnd pins of the VGA were soldered to a BCA adapter, which allowed them to be connected to an A/D converter (Digidata 1321A: Axon instruments, or PCI-6221 multifunction DAQ board: National Instruments). Analog signal was digitized at unity gain at sampling rate of 1 kHz.

The digitized data was continuously output to a Windows computer running pCLAMP (Molecular Devices) or LabVIEW (National Instruments) data acquisition software.

2.2.2 Comparing recorded electric field sensor responses to conventional physiological recordings

Whole body plethysmography: I first compared voltage waveform recordings obtained from EPIC sensors to plethysmography, the conventional method for recording respiration. Individual animals (n=2) were placed in a Plexiglas plethysmographic chamber (Buxco Research Systems; 12" x 12" x 9.75"; 10" diameter), and inspired ventilation was measured by continuous flow barometric plethysmography as described previously (Wilkinson et al 2010). At the same time, an EPIC sensor was affixed to the exterior wall of the chamber with tape and strategically positioned so that the animal's resting location inside the chamber was close to the sensor. Both signals were fed to a PCI-6221 multifunction DAQ board as described previously, and processed via a specialty program run in LabVIEW. To isolate respiratory rate from other high and low frequency components, recordings were bandpass filtered at 1 Hz (high pass) and 5 Hz (low pass). Recorded respiratory waveforms were compared to voltage waveforms obtained from the electric field sensor signals by calculating peak-to-peak intervals.

Comparison with electrocardiographic (ECG) recordings of heart rate in anesthetized animals: To determine whether EPIC sensors could detect heart rate, experiments were undertaken in anesthetized mice (n=3). Animals were anesthetized with ketamine and xylazine (0.3 ml/100g; IP) and placed within a 10x5cm PVC conduit, a commonly used shelter in vivarium home cages (Bennett G. Galef 2000). Sensors were positioned to cover a

small window created in the side of the conduit. For recording ECG, the animal's front right paw and back left paw were connected to disposable adhesive electrodes (3M Red Dot™). Leads were connected to a differential amplifier, then digitized (Digidata 1321A: Molecular Devices) and captured at 1 kHz using pCLAMP software (Molecular Devices). To isolate heart rate from other higher frequency components, recordings were typically low-pass filtered at 10 Hz.

2.2.3 Effect of animal location (distance and angle) on voltage waveform

Because accurate respiratory rate recordings represent an important aspect of the proposed experiments, I assessed the role of rostro-caudal and circumferential sensor placement on recorded respiratory rate. For these experiments, a sensor was placed over a hole in the side of a PVC conduit so that the front of the sensor was 1cm from the interior wall. An adult mouse (n=1) was anesthetized with ketamine and xylazine (0.3 ml/100g; IP) and placed within the PVC conduit so that its nose was 2cm away from the sensor (i.e. sensor was aimed at neck/base of head). Recordings were captured as the sensor was moved circumferentially around the animal at angles ranging from 0° to 180° with respect to vertical. To investigate sensor sensitivity to rostro-caudal positioning, the animal was initially placed so that its nose was 7cm away from the sensor, which was angled at 40° with respect to vertical. Recordings were captured as the animal was moved progressively closer to the sensor in 2cm increments. Care was taken to maintain sensor lateral proximity (1cm).

2.2.4 Modifications in home cage and optimizing EPIC sensor placement for experimental studies

Based on my characterization of EPIC sensor recording properties I designed a modified home cage environment that would allow for continuous monitoring of mouse physiology and behavior. Given that the sensors most reliably captured respiratory rate and heart rate when they were in close proximity to a resting animal, I modified a standard vivarium home cage to encourage animals to rest in a specific location near a sensor. To do this, I secured 50mm culture dishes, which prior observational studies indicated were preferred resting locations in C57BL/6J mice, to the floor of each cage. I then affixed sensors to the exterior of the cage, directly under the dishes at an angle of 0° with respect to vertical. Dishes were particularly preferred resting locations when located under a food bin and sheltered from light by opaque Plexiglas dividers. Note: I chose 50mm dishes, rather than PVC conduits for housing shelters, because the former are smaller, thereby limiting variability of animal proximity to the sensor, ensuring a more consistent signal as validated by preliminary trials. To mimic vivarium social conditions, animals were housed in littermate pairs. To allow for individual recordings from each animal an insertable piece of sheet metal was placed in the center of the cage to divide the cage and electrically isolate recordings of one side from the other. Holes in the divider permitted social touch between cage mates. Given sensor exquisite sensitivity to changes in electric field, I grounded the metal cage divider and covered the cages in electrically grounded metal baskets. To accommodate testing of six animals, a total of three of these modified home cages were constructed. A model of a complete home cage is provided in Figure 1.

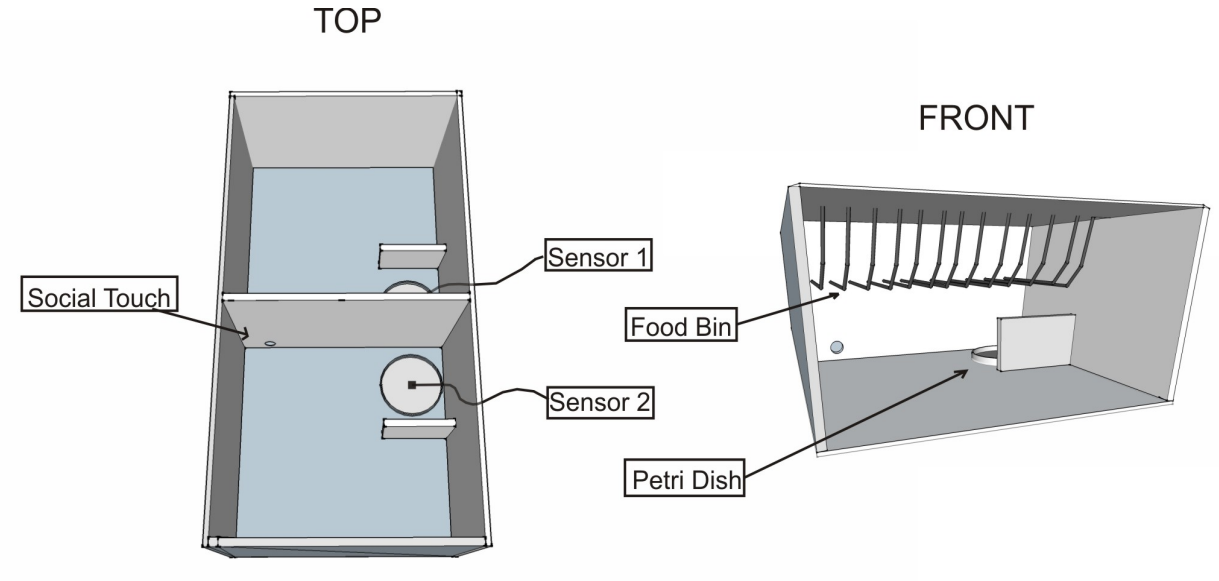

Figure 1. Model of sensor-equipped home cage. (Left) Top-view of home cage displays cage divider, petri dish, and sensor locations. Each cage permitted housing and monitoring of two animals simultaneously. **(Right)** Front view of one side of the home cage shows location of the Plexiglas divider and food bin in respect to the petri dish.

2.3 Experimental Investigation

2.3.1 Monitoring Animal Behavior and Physiology

Six adult (82-84 days) male C57BL/6J mice were used for these experiments. Four were littermates from an initial litter of five and two were littermates from an initial litter of six. The four littermates were housed in the same cage from weaning until one week prior to the experiment, at which point they were transferred to paired housing. The other two animals were housed with the rest of their original litter until one week prior to the experiment, at which point they too were transferred to paired housing. Since conditions were not available to undertake these experiments within the vivarium, I converted an area in the lab into a simulated vivarium including mimicking temperature and automated light cycle conditions. To investigate natural variability in baseline physio-behavioral condition,

animals were recorded in the sensor-equipped home cages for twelve hours, from 1:00am to 1:00pm (halfway through the dark and light phases respectively) for seven consecutive days. The specific epoch of time was chosen so as to best capture physio-behavioral differences in both light cycles. Precautions were taken to minimize exposure to light during the 1:00am transfer from vivarium to sensor-equipped home cages. In the laboratory, animals were housed in the same pairs as in vivarium and were placed in the same cage and side during each recording epoch. Additionally, to permit acclimation to the new cage environment, all animals spent 1:00am-1:00pm epochs in the sensor-equipped cages beginning three days prior to collecting data.

2.3.2 Spinal cord transection

SCIs took place after the seventh day of baseline recordings. All mice were anesthetized with isoflurane. Following a dorsal laminectomy to expose the spinal cord, a 2mm segment between thoracic spinal segments T1 and T2 was removed. Gel foam was inserted into the spinal cord to maintain the separation between rostral and caudal parts of the cord. Two animals died during surgery. For the survivors, buprenorphine (0.16mL/kg) was administered every twelve hours at 1:00am and 1:00pm for two days post-surgery per approved pain-management protocol. Manual expression of the bladder was undertaken twice daily at 7:00am and 7:00pm.

2.3.3 Post-SCI recordings

Animals were recorded in the sensor-equipped home cages according to the aforementioned twelve-hour schedule for seven days post-injury, beginning at 1:00am the

night of the SCI. At ~7:00am each day, recordings were briefly interrupted to permit bladder expression. To broadly assess severity of SCI and overall recovery, observations of animal locomotor function and bladder control were made four times a day, during bladder expression and vivarium-laboratory transfers.

2.3.4 Extent of SCI

To verify extent of injury, on day eight post-SCI all surviving animals were anesthetized with urethane (2mg/kg ip) and perfused with 1:3 volume/body weight ice-cold 0.9% NaCl, followed by equal volume/body weight of 4% paraformaldehyde. The animal that died on day two was not perfused, but instead transferred to 4% paraformaldehyde solution upon discovery of death. All spinal cords were isolated and cryoprotected in 10% sucrose one hour postfix, until injury extent was assessed histologically.

2.3.5 Analysis of Experimental Results

All automated and manual analysis of raw sensor output was accomplished using ClampFit analysis software (Molecular Devices). In addition, spectrograms of raw data were produced in MATLAB (Mathworks) using in-house code. Simultaneous video recording during the first two days of recordings confirmed that distinct waveforms in the raw traces and corresponding unique frequency components of the spectrograms were indicative of active and inactive periods. Using this information, total duration of daily activity was manually quantified from the raw data traces. Average respiratory rate was determined by manually counting number of respirations during eight, twenty-second epochs during periods of animal inactivity each day. To confirm accuracy of these manual methods,

manually quantified respiratory rate and daily activity from two animals for two days pre-injury were compared to values obtained via automated threshold detection analysis using ClampFit. In all instances, manual quantification accurately replicated automated results. All values were reported as means +/- S.D. Statistical comparisons were made using ANOVA and students' t-test.

3.0 RESULTS

3.1 Development of Method

3.1.1 Plessey EPIC sensors had characteristic properties and responded with high fidelity to external mechanical stimuli

Plessey EPIC sensors responded to changes in the surrounding electric field due to electrical and mechanical stimuli (Fig. 2). When placed between the plates of a parallel plate capacitor and exposed to induced oscillations in surrounding electric fields at behaviorally/physiologically-relevant frequencies, the sensor output accurately reflected the input command voltage across the capacitor (Fig. 2 A). Tests investigating sensor characteristics revealed that sensor output magnitude scaled linearly with command voltage (Fig. 2 A_2) and showcased sensor filtering qualities (Fig 2 A_3), confirming the sensor properties provided by the manufacturer (Plessey Semiconductors). A phase delay was apparent between command input and sensor output at lower frequencies (Fig 2 A_4). Additionally, given that the sensors are AC-coupled, strictly positive command voltage oscillations were output as voltage fluctuations about zero.

When attached to the side of an animal home cage and exposed to a mechanical stimulus (metronome), sensor output accurately reported changes in electric field

associated with the movement of a metronome lever arm (Fig. 2 B). The magnitude of this output signal was dependent on proximity of the sensor to the moving object and on the composition of the object (Fig. 2 $B_{2\text{-}4}$). I found that sensors had dramatically larger responses when the metronome was in very close proximity (1cm - 6cm) to the moving metronome arm and that the difference in response magnitude attenuated as this distance increased to greater values (Fig. 2 $B_{2\text{-}3}$). Given that these sensors reportedly monitor movement by detecting perturbations in electric field, it was important to investigate how the triboelectricity (ability to hold electric charge) of the moving material impacted sensor output. When a material with a high triboelectricity (i.e. carried increased negative charge) such as silicone was affixed to the upper arm of a metronome located 6cm from the sensor, the sensor's output was magnified compared to control. Adding a piece of polyethylene tubing (greater triboelectric effect than silicone) to the undulating metronome further increased output magnitude.

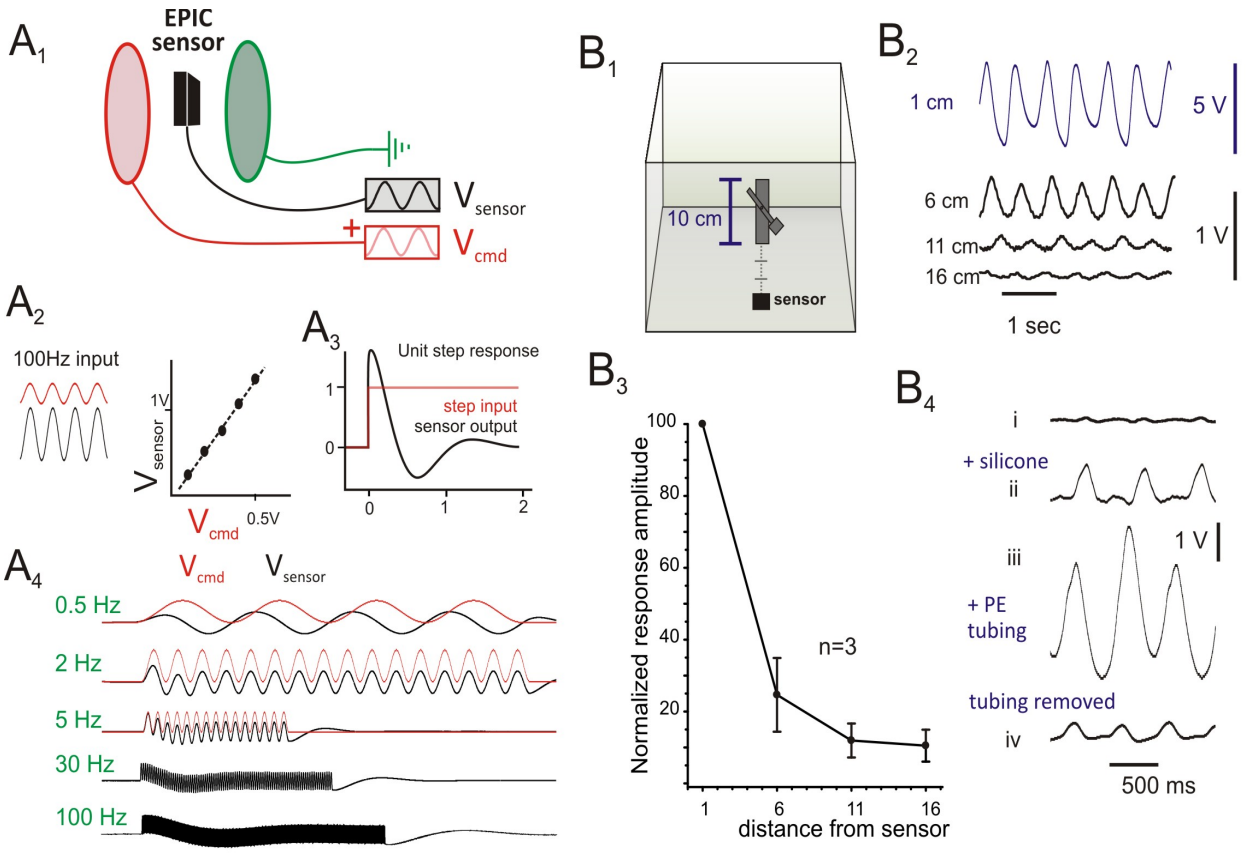

Figure 2. EPIC sensors accurately detect electrical oscillations and movement dependent disturbances in the electric field. (A1) A single sensor was placed midway between a parallel plate capacitor, and various frequency cosine voltage commands were delivered to alter the electric field. **(A2)** Sensor output frequency accurately reflected input frequency and scales linearly with the input (input-output gain differences depend on properties of capacitor and distance from sensor). **(A3)** Unit step response shows filtering qualities of sensor. **(A4)** Sensor output matched input frequencies but with phase delay at lower frequencies (e.g. 0.5 Hz). **(B)** Recordings of periodic arm movements from a mini-metronome placed in several locations within a plastic home cage. **(B1)** Schematic showing approximate metronome placed at several distances from sensor. **(B2)** The magnitude of sensor response was distance dependent as shown with placement of the metronome at 4 different locations from sensor spaced 5 cm apart as indicated. **(B3)** Normalized relative changes in voltage recorded with metronome distance from sensor. Sample is the normalized mean ± SE of conditions i-iii shown in panel **(B4)**. **(B4)** Sensor sensitivity to object movement depends on triboelectric properties of moving object. Shown are recordings of metronome pendulum arm movement 6 cm from sensor with different negatively charged materials affixed to upper arm of pendulum; **(i)** control, **(ii)** drip of silicone glue, **(iii)** 1 cm length PE tubing attached to silicone, and **(iv)** tubing removed.

3.1.2 S*ensors reliably reported cardiorespiratory variables*

I next sought to validate the ability of EPIC sensors to measure a rhythmic and continuous physiological mechanical stimulus – respiration. By comparison with the conventional methodology of whole body plethysmography, I showed that sensors are exquisitely sensitive to the respiratory rhythm in rodents. Recordings from EPIC sensors affixed to the outside of a plethysmography chamber demonstrated that sensors accurately reported respiratory rate. Using this modified plethysmography-EPIC sensor setup; raw traces were obtained from each recording method (Fig. 3B). There was a strong correspondence between plethysmographic recordings of respiratory rate and those obtained with the EPIC sensor. This was observed by plotting instantaneous frequency of

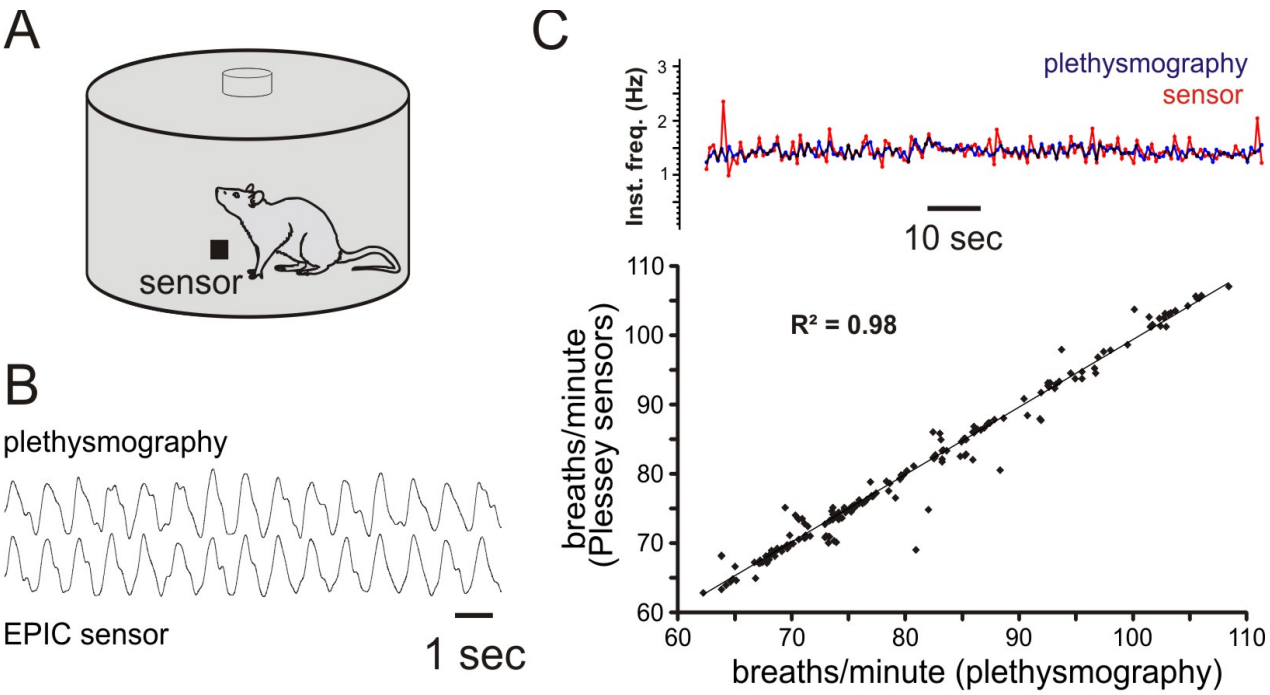

Figure 3. Plessey sensors accurately detect respiratory rate. (A) Schematic shows plethysmography chamber with Plessey sensor affixed to wall. **(B)** Representative respiratory traces from the plethysmograph **(top)** and Plessey sensor **(bottom)** in a resting animal. The signals were band-pass filtered at 1-5Hz. **(C)** Comparisons of plethysmographic and sensor-based recordings of respiratory rate. **(Top)** Threshold-based detection of interpulse intervals converted to frequency resulted in similar determinations of instantaneous respiratory rate over 105s in a resting animal. **(Bottom)** Scatterplot comparison of variability in respiratory rate measured with plethysmography (x-axis) to those recorded with an electric field sensor (y-axis) at several 10 second epochs in an individual recording. Correlation coefficient (R2) was 0.98.

the respiratory cycle (Fig. 3C). Linear regression analysis of this relationship revealed an R^2 value of 0.98, suggesting a nearly perfect alignment of sensor- and plethysmography-based calculations of respiratory rate (Fig. 3D).

Given the ease with which EPIC sensors tracked the respiratory waveform, I next sought to determine whether they could reliably detect another physiological signal indicative of global health status and valuable to physiological investigations: heart rate (Palatini, 2007; Kannel et al., 1987). By comparing raw traces captured from a conventional ECG with those simultaneously captured by a sensor affixed to the side of a standard PVC animal housing conduit, I validated the ability of Plessey sensors to monitor heart rate in anesthetized adult mice located inside the PVC (Fig. 4). It is important to note that to capture the heart rate signal, animals had to be strategically placed within the PVC conduit so that they were in close proximity to the EPIC sensor (~1cm). Additionally, to isolate heart rate from other higher frequency components, recordings were commonly low-pass filtered at 10Hz. While 10Hz is in the middle of typical heart rates for awake and behaving mice, it is well above reported values for mice anesthetized with ketamine/xylazine, thus filtering at this value was not of concern (Erhardt et al., 1984).

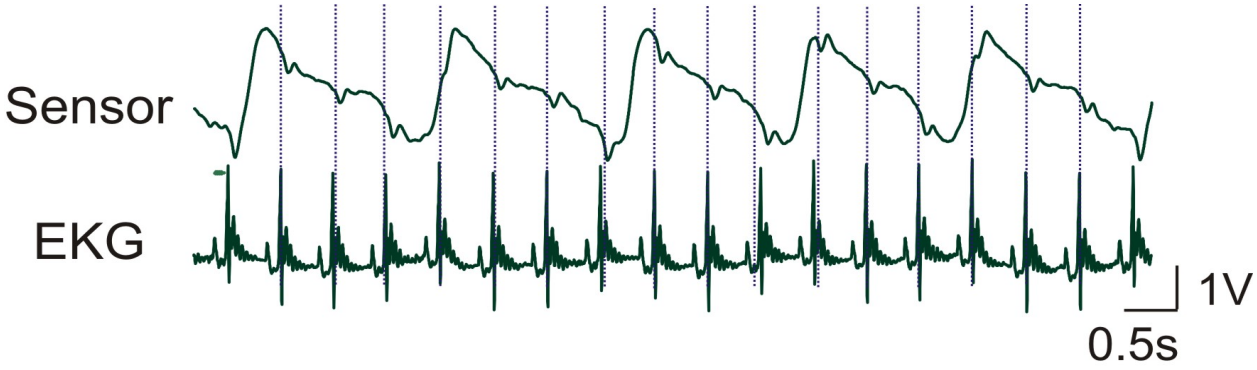

Figure 4. EPIC sensors record cardiac activity when appropriately placed. An adult mouse was anesthetized with ketamine/xylazine and placed in a standard home cage PVC conduit. An EPIC sensor was attached to the outside of the conduit. ECG leads were connected to the animal's front right and back left paws. A representative sensor and ECG recording is shown, high pass filtered at 0.1 Hz and low pass filtered at 10 Hz.

3.1.3 Animal location relative to sensors impacted respiratory waveform

Having validated the use of EPIC sensors for measuring respiratory and heart rate, I performed several experiments to better understand the impact of animal position on sensor responsiveness. In particular, I was interested in the relative rostro-caudal distance and angle of the sensor from the animal; information needed to strategically design a home cage that encouraged animals to position themselves in a location best suited for the capture of respiratory rate, heart rate, and activity. Using an anesthetized animal in PVC conduit (n=1) I recorded from the sensor while incrementally changing either sensor location or angle, as shown in schematics of my setup (Fig. 5). Voltage waveforms were dependent on both angle of the sensor and rostro-caudal animal position within the conduit (Fig. 5 $A_{1\text{-}2}$). While recording fidelity was dependent on the angle of recording, respiratory rate was captured at all tested angles and locations. Small voltage deflections from heart rate activity were most apparent when the chips were located at 0° or 40° with respect to vertical and when the base of the animal's head or top of neck was positioned next to the sensor (2-3 cm nose to sensor).

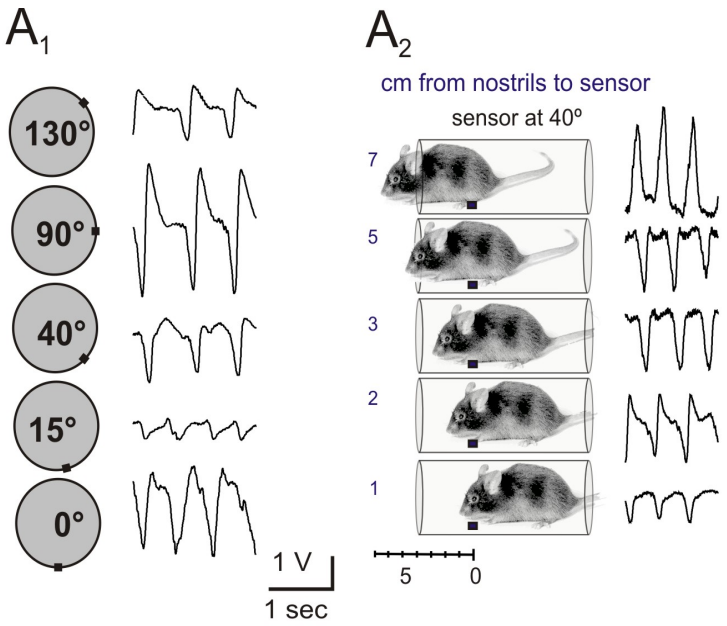

Figure 5. Effect of animal location and sensor angle on voltage waveform in mouse (A) Animals were anesthetized with ketamine/xylazine and placed in PVC conduit within the home cage. A Plessey sensor was placed over a hole in the side of the tubing so that the front of the chip was 1cm from the interior wall of the conduit. **(A1)** The animal was placed so that its nose was 2 cm away from sensor (i.e. sensor was aimed at neck/base of head). Recordings were captured as the conduit was turned, thereby situating the sensor at different angles. **(A2)** The voltage waveform changed depending on animal rostro-caudal location.

3.1.4 Continuous capture of mouse activity and respiratory rate using EPIC sensor-equipped home cages

As described in methods, I designed a modified dual-housing home cage environment that implemented EPIC sensors in a configuration that permitted continuous monitoring of mouse physiology and behavior (Fig. 1). Sensor output from these cages continuously captured epochs of animal activity and inactivity (Fig. 6A) as confirmed by simultaneous video recording (not shown). During periods of inactivity, respiratory rate could be easily observed. When at rest, corresponding spectrograms revealed high-power frequency components in discrete bands at ~2.5-4.0Hz. These bands were indicative of respiration rate and thus periods of inactivity (Fig. 6B). In comparison, high-power, wide-frequency range bands were characteristic of motor activity. Therefore, I used power-frequency spectrograms as a simple method to discriminate active from inactive periods in

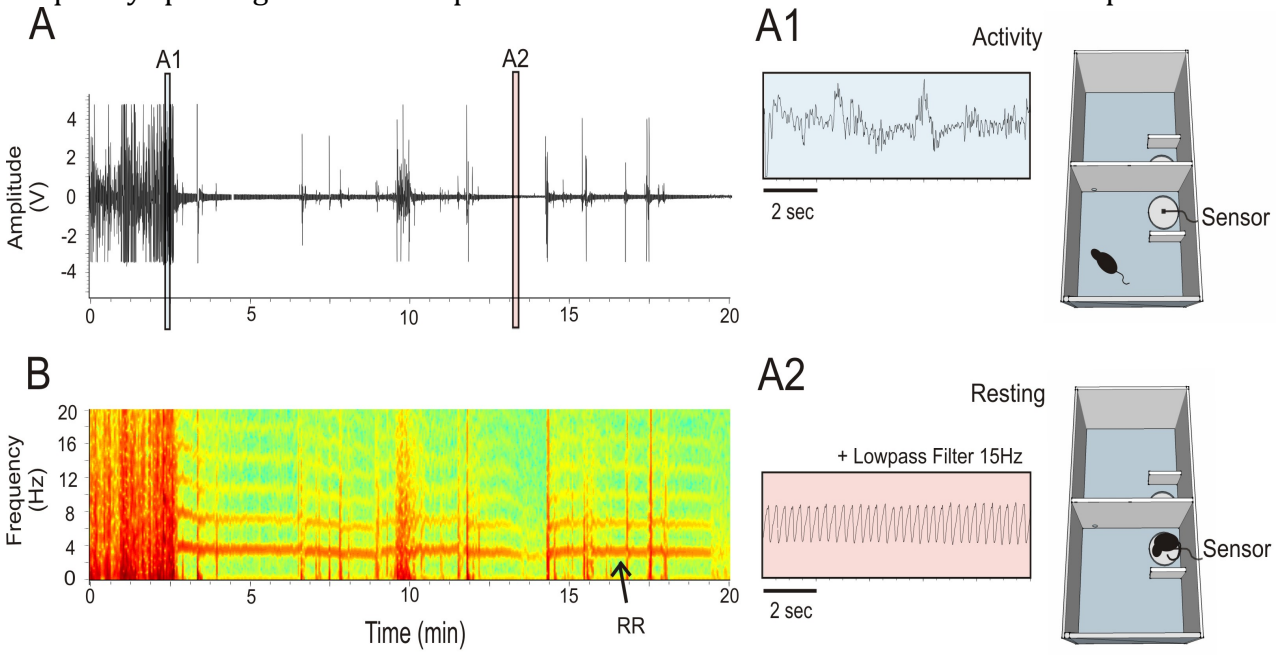

Figure 6: EPIC sensor-equipped dual-housing home cages permit the continuous capture and visualization of mouse activity and respiratory rate. (A) A 20-minute representative raw data trace from a twelve-hour continuous recording of one animal housed in a dual-housing sensor-equipped home cage. **(A1)** epochs of activity and **(A2)** inactivity can be easily differentiated. During periods of inactivity, respiratory rate is clearly present. **(B)** Corresponding spectrogram reveals unique frequency components during active, and inactive (respiratory rate) periods. The black arrow denotes frequency band associated with respiratory rate.

subsequent analysis and figures. Note that due do the sensitivity of the heart rate waveform to rostro-caudal and angular animal position, the sensor-equipped home cages were unable to reliably capture heart rate, thus it was not included in my analysis.

3.2 Experimental Investigation

3.2.1 Activity profiles appeared to differ between animal housing pairs

Spectrograms of the entire recording period for six animals on days one, three, five, and seven are shown in Figure 7. A global consideration of activity is seen and allows for the following observations: (i) All animals underwent bouts of activity separated by periods of relative inactivity. This phasic activity profile is a behavioral feature that has been documented previously (De Bono et al., 2006; Girard et al., 2001; Eikelboom 2001). (ii) The frequency and duration of these bouts appears similar between animals in the same cage but not across cages. Animals in cage one were active relatively infrequently but for fairly long durations. Conversely, animals in cage two exhibited frequent, short bursts of activity. Animals in cage three were active quite frequently and for variable durations. The close similarity in activity between cage-mates observed is not surprising given that one animal's movement in a cage likely caused vibrations and environmental stimuli that disturbed its cage-mate. This highlights the potential influence of a cage-dependent environmental variable on behavior. (iii) Mice in cage three were more active than those in cages one and two. Interestingly, these mice were littermates from a different dam than the other four animals. (iv) Animals did not exhibit higher activity during their dark cycle. It is well known that activity level is highly dependent on phases of the light cycle and circadian rhythm (De Bono et al., 2006; Loos et al., 2014). Thus, lack of differences in acitivity

between light and dark periods suggests that certain aspects of my experimental design may have disrupted circadian rhythm.

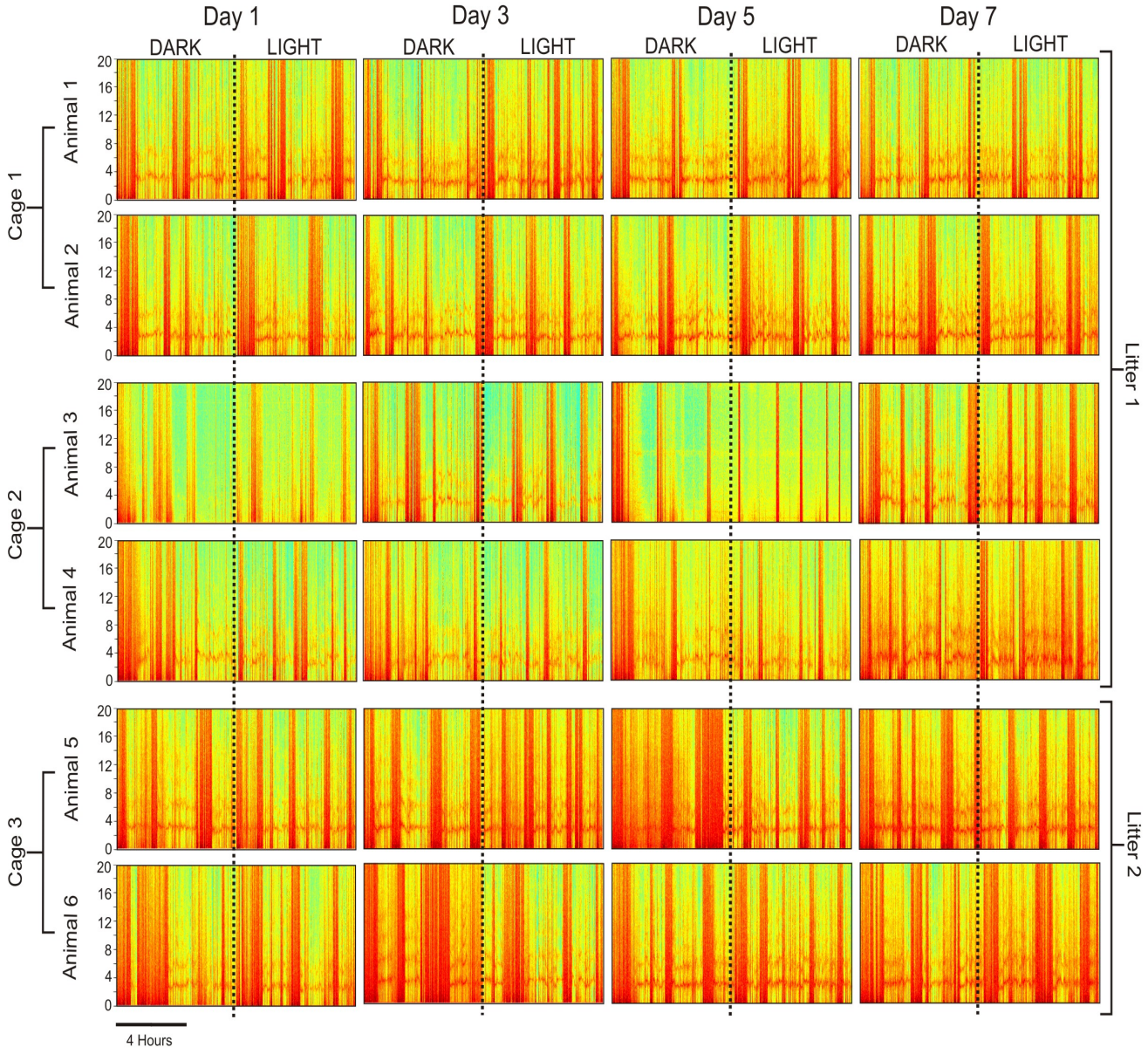

Figure 7: Spectral Analysis of six individuals during four, twelve-hour recording epochs. Spectrograms highlight differences in activity profiles between individuals, litters, and housing pairs. Note that the greatest differences appear to be between cages, and that the phasic activity of each housing pair is often synchronous. Vertical dotted lines denote the dark/light cycle of each day.

3.2.2 Respiratory Profiles

Spectral analysis also revealed variability in respiratory rate over the course of each recording session. While detection of respiration is very sensitive to animal distance from the sensor, frequency bands associated with respiratory rate (indicated on Fig.6 B) were easily detected during most resting periods. Respiratory rate generally fluctuated around 3Hz.

Given the variability in activity seen between spectrograms, I next sought to quantify and compare activity levels between animals. Figure 8 compares the proportion of time spent active for each animal over the seven-day collection period. As shown in Figure 8A, each animals' activity profile was similar throughout the seven days of sampling. Note however, that animals five and six had consistently heightened levels of activity throughout (Fig. 8 A). T-tests confirmed that these two animals spent, on average, a significantly greater proportion of their time active compared to the other four animals (Fig. 8 B).

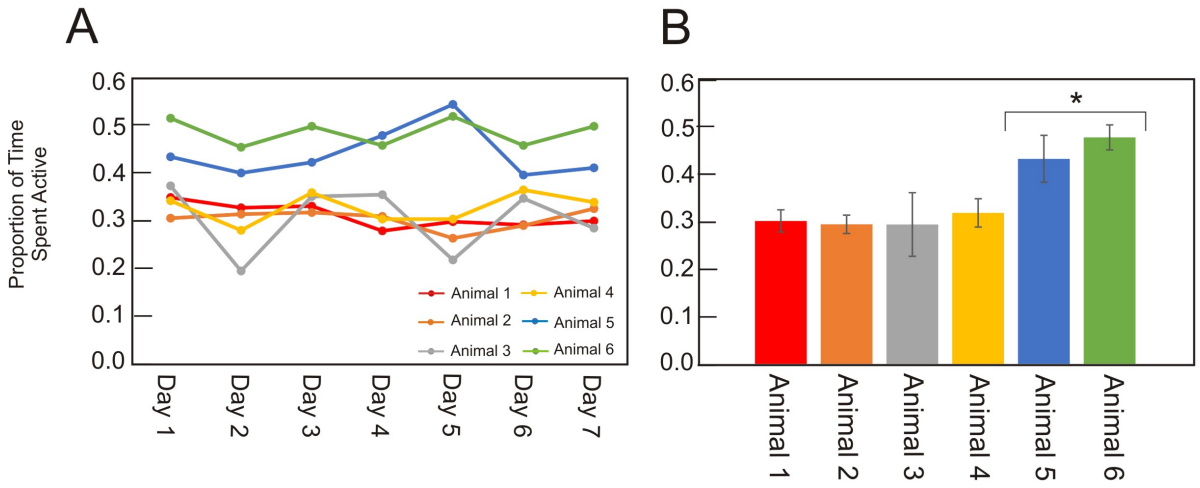

Figure 8: Comparison of the proportion of time six individuals spent active during seven, twelve-hour recording epochs. (A) A line graph depicting the daily activity of each animal. Note that animals 5 and 6 appear to have heightened levels of activity each day compared to the other four animals. **(B)** A bar graph comparing average animal activity over the seven recording epochs. T-tests confirmed that Animals 5 and 6 spent, on average, significantly greater proportions of their time active than animals 1-4 (All p values < 0.001). Error bars denote +/- SD.

3.2.3 Pre-injury respiratory rates did not differ significantly between the six subjects

Closer inspection of the spectral bands associated with respiratory rate shows that there was considerable fluctuation within a range of rates (Fig. 9 A). This is expected given that resting respiratory rates are known to vary in association with different behavioral states (Friedman et al., 2004). Nonetheless average respiratory rate was stable for each animal throughout the seven-day sample period, and mean respiratory rate was similar in all animals as confirmed by an ANOVA (Fig 9 B).

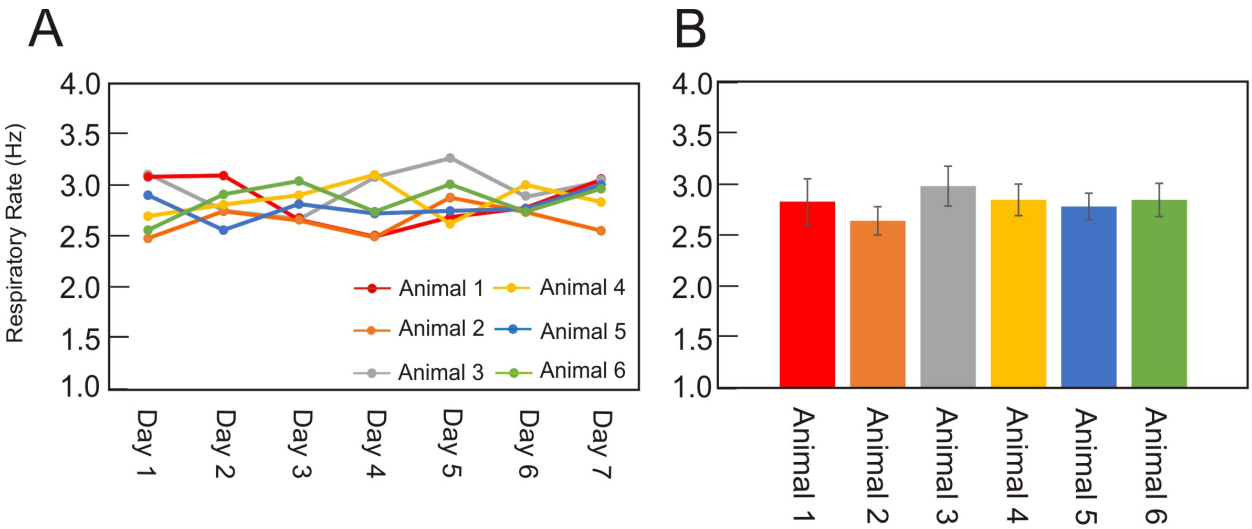

Figure 9: Comparison of average individual respiratory rates during seven recording sessions. (A) A line graph depicting the daily respiratory rates of each individual. Note that no unique trends in respiratory rates are evident. **(B)** A bar graph comparing each animal's average respiratory rate during the seven recording epochs reveals no significant variability between animals. Error bars denote +/- SD.

3.2.5 SCIs had dramatic and variable impact on activity profiles

Figure 10 compares spectrogram activity profiles in animals before and after SCI. Several observations are noteworthy. (i) There is a dramatic change in animal activity profiles in the first few days after SCI. (ii) There was an initial, complete disruption to the phasic activity patterns characteristic of uninjured animals seen in the pre-injury baseline.

Phasic patterns re-emerged by day two and appeared to stabilize by days six and seven. However, their overall signatures never returned to those seen prior to injury. (iii) Post-SCI, animals four and five appeared to have heightened levels of activity while three and six displayed lower levels of activity compared to pre-injury baseline. (iv) Animal six had the lowest activity on day one and day two after-SCI and this animal died at the end of day two. (v) After injury there was a loss of the correlated bouts of activity between housing pairs that was observed prior to injury. Quantitative comparison of activity profiles before and after SCI is shown in Figure 11. As was evident in the spectrograms, there was immense variability in individual activity levels on day one post-SCI (Fig. 11 A). Additionally, T-tests compared the mean proportion of time spent active pre-injury to two post-injury epochs, day one and two and days six and seven, shown as barplots in Figure 11B. Animal four had significantly heightened activity post-SCI at both periods while animal six had decreased activity before it died. ANOVA tests did not reveal significant differences in mean activity between animals during the two post-SCI epochs. Additionally, the average activity of the three animals that survived the duration of the experiment did not differ significantly between epochs (Fig. 11 C)

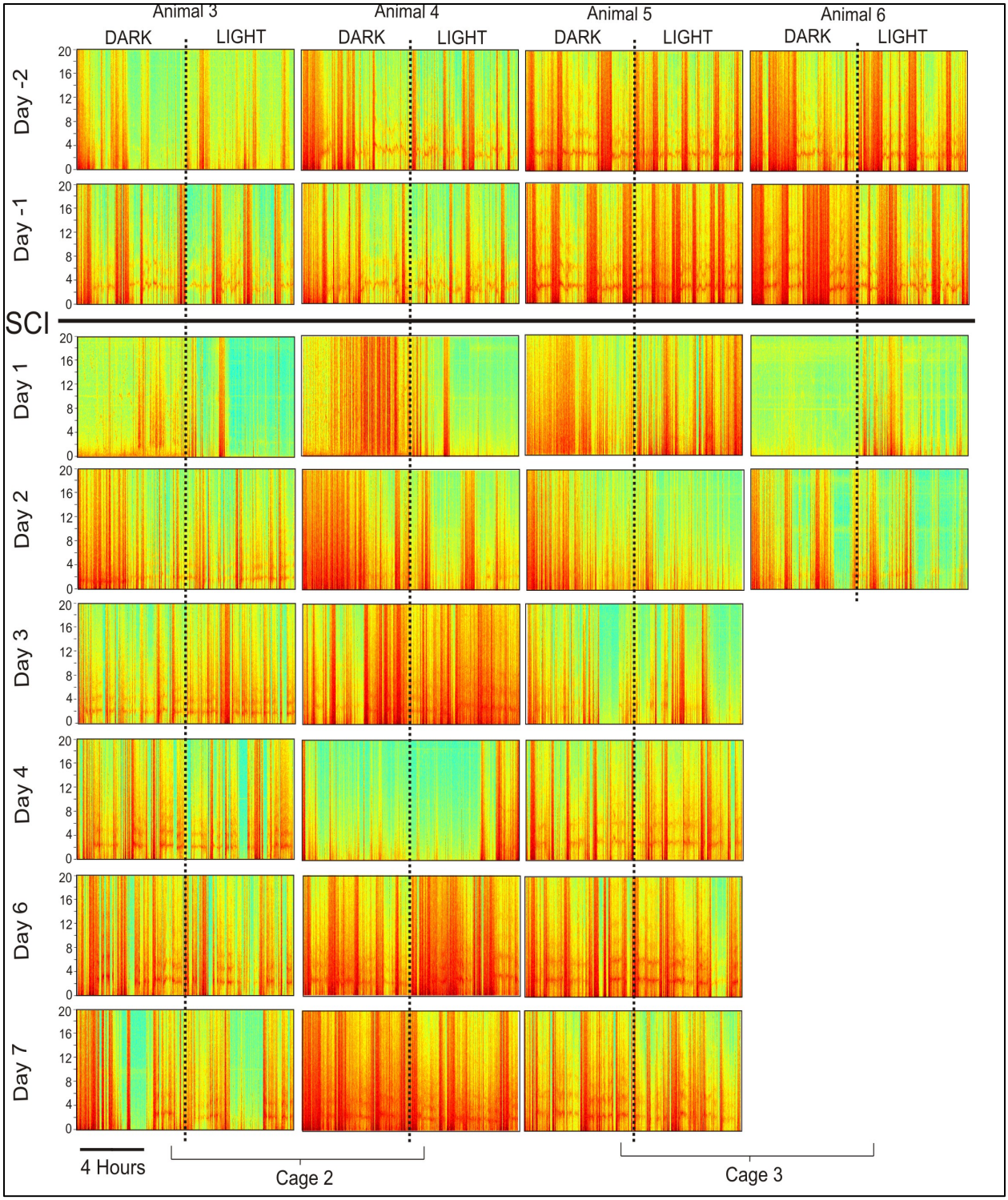

Figure 10: Spectral analysis of four individuals during twelve-hour recordings epochs, before and after SCI. Spectrograms highlight differences in activity profiles after SCI. Animals 3 and 6 exhibit reduced activity after injury while animals 4 and 5 appear to be more active compared to baseline. Note the disappearance of phasic activity patterns in the days immediately post-injury. Note: data for half of day 12 was lost, thus a spectrogram for that day is not included.

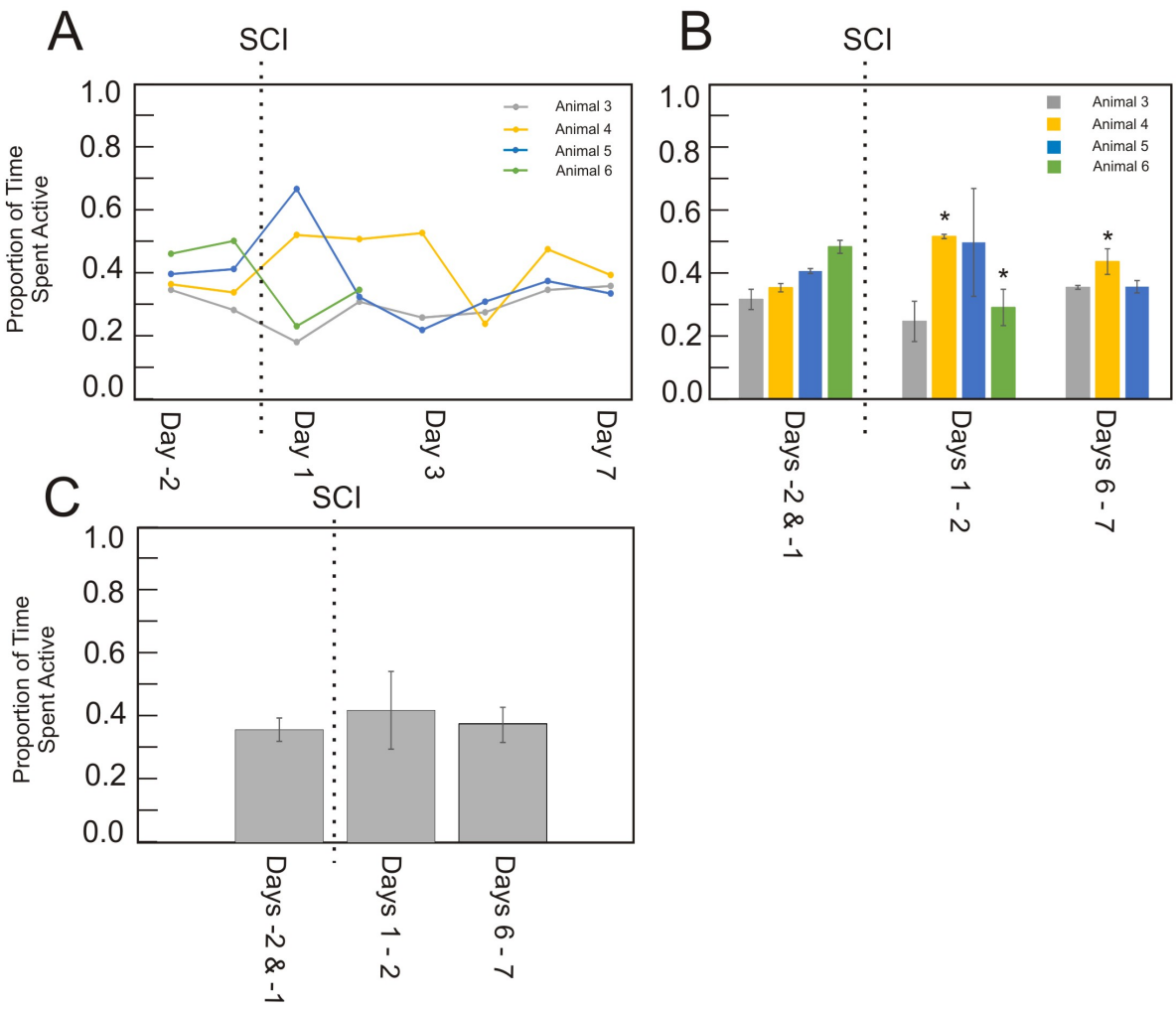

Figure 11: Comparison of the proportion of time four animals spent activity during pre- and post- SCI recording sessions. **(A)** Line graph depicting the daily activity of each animal. Note the variability between activity levels, particularly during day 1 after injury. **(B)** Bar graph comparing average animal activity at two epochs post injury to their pre-injury baseline reveals significant differences. Day 1-2: Animals 4 and 6 were significantly more and less active compared to baseline, respectively. Day 6-7: Animal 3 was significantly more active compared to baseline (p values <0.001). **(C)** Bar graph displaying average activity of the population at baseline and during two post injury epochs reveals no significant change in activity levels (excluding animal six). Error bars denote +/- SD.

3.2.6 SCIs initially triggered two distinct trends in respiratory rate followed by an overall reduction in the respiratory rate of the population

Quantitative comparison of resting respiratory rate after SCI revealed two distinct trends shown in Figure 12A. Animals three and four exhibited a significant decrease in respiratory rate in the two days following the injury. Conversely the respiratory rates of

animals five and six appeared to increase compared to baseline (Fig. 12 B). An ANOVA confirmed that overall inter-animal variability was significant (Fig. 12 C). Note that these trends were segregated by cage. By day three, the respiratory rates of the surviving animals began to converge, eventually stabilizing to similar values, all of which were significantly lower than baseline (Fig 12 B). Additionally, SCIs triggered an overall reduction in the respiratory rate of the population (Fig. 12 C).

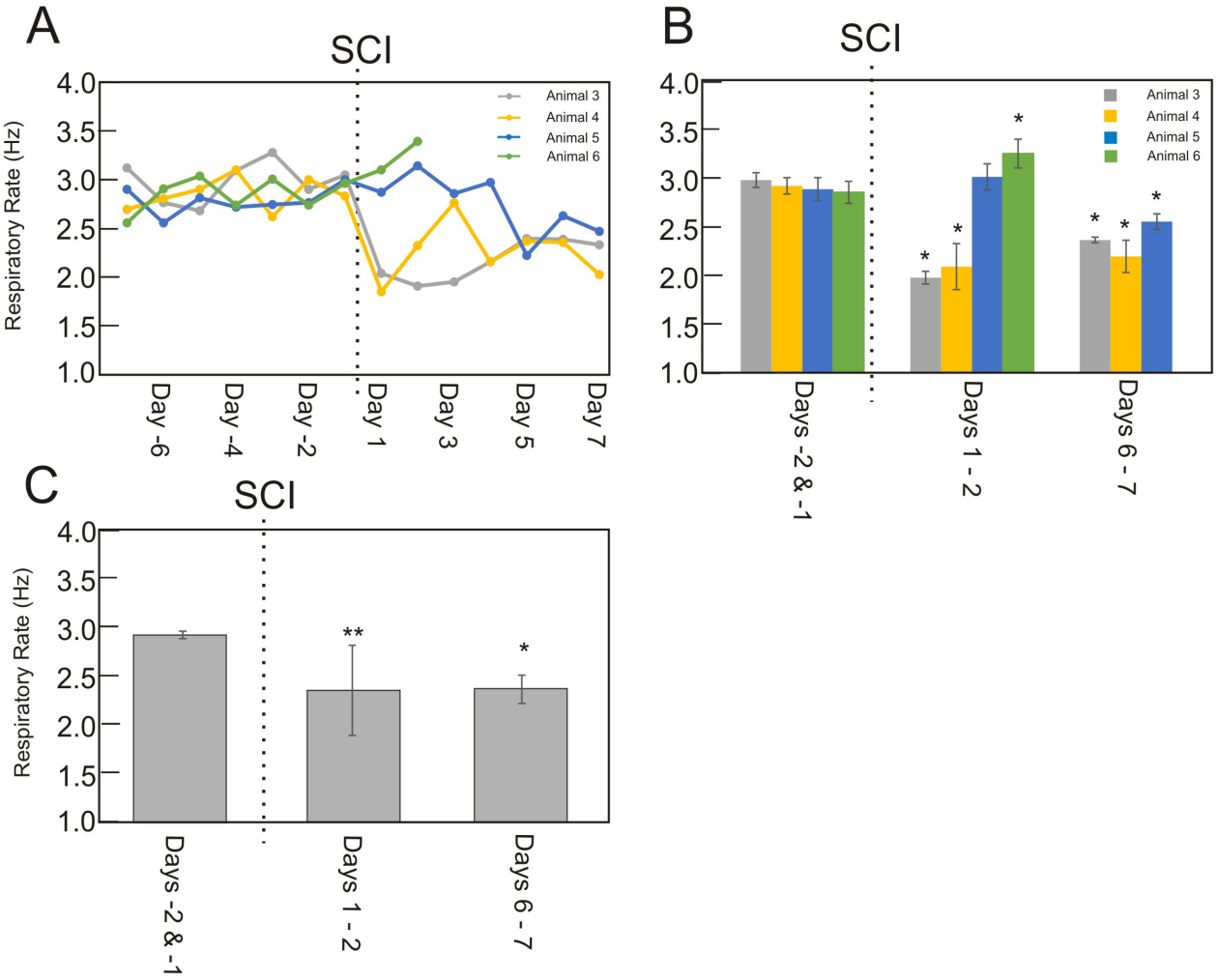

Figure 12: Comparison of average respiratory rates during pre- and post-SCI recordings epochs. (A) Line graph depicting daily average respiratory rates of each individual. Note the initial variability and subsequent stabilization of daily respiratory rates after injury. **(B)** Bar graph comparing average animal respiratory rate at two epochs post-injury to their pre-injury baseline reveals significant differences. Day 1-2: Average respiratory rates of animals 3 and 4 were significantly decreased while those of animal 6 were significantly increased compared to baseline (p-values of <0.001, 0.002, and 0.014 respectively). Day 6-7: Respiratory rates of all animals were significantly decreased compared to baseline (p values 0.005, 0.002, and 0.03 respectively). **(C)** Bar graph comparing average respiratory rates of the population (excluding animal six) at baseline and during two epochs post-injury reveals that average respiratory rate was significantly decreased by days 6-7 (p value = 0.01) ** An ANOVA confirms significant variability in average animal respiratory rates during day 1 and 2 post-SCI (p value =0.01). Error bars denote +/- SD.

3.2.4 Injury Extent and Severity

Figure 13 summarizes my histological observations on injury severity after sacrifice. All animals sustained complete transections of dorsal and ventral grey matter (Fig. 13 A). These injuries are known to severely compromise autonomic and sensory function and typically lead to autonomic dysreflexia and hyperactive reflexes (Krassioukov, 2004; Berger et al., 2014; West et al., 2012). However animal four was the only animal that sustained a complete transection of the entire spinal cord. In the others, portions of the ventral white matter remained intact, sparing of which is known to support recovery of hindlimb motor function (Schucht et al., 2002; Bachmann et al., 2013). Rostro-caudal injury location and extent was also variable as shown in Figure 13B. Observationally, there was variability in the extent and onset of hindlimb function recovery: animal three was able to undergo independent hindlimb weight bearing and locomotion by day three post SCI, though forelimb to hindlimb coordination was not assessed. Animal four showed slight hindlimb support by day seven and animal five exhibited partial hind limb support and locomotion by day five. Animal six died before any hindlimb function was recovered. Additionally, no animals recovered bladder control during the seven days post injury.

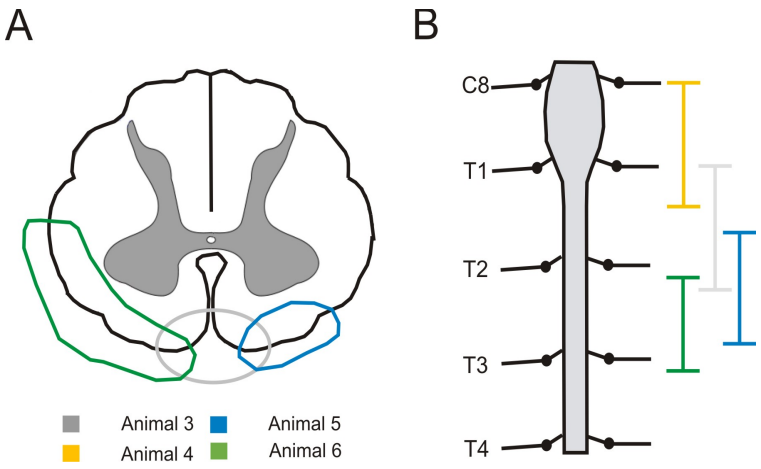

Figure 10: Extent and location of SCIs. **(A)** Cross-section diagram of a spinal cord. Outlined regions denote areas that were not fully transected. Note: Animal four had a complete transection. **(B)** Diagram of the upper thoracic spinal cord. Colored brackets denote length of individual injuries.

4.0 DISCUSSION

4.1 Summary

In this study I first sought to test Plessey Semiconductor's EPIC sensors as a method for continuous, noncontact measurement of mouse physio-behavioral status. I demonstrated the utility of EPIC sensor-equipped home cages as an inexpensive alternative to current physiological and behavioral measurement methods. I then employed this methodology to investigate the extent to which there is variability in activity and respiratory rate in dual-housed littermates in a home cage environment. I found that while activity profile differed between littermate cage-pairs, overall activity magnitude was similar among littermates from the same dam. However, both activity magnitude and temporal profile differed significantly between litters. Furthermore, I showed that pair housed animals tended to synchronize their active periods. I then explored variability in outcome after SCI and demonstrated that inter-animal variability clearly increased after injury. While changes observed evolved in an overall expected manner (recovery of activity and reduction in respiratory rate), the temporal dynamics of this change were highly variable. Changes appeared to be unrelated to cage or littermate status. Subsequent histological comparisons showed variation of lesion level. Moreover, 3 of 4 mice had some spared ventral white matter. These differences could explain the range in variability in recovery observed.

4.2 Development of Method

4.2.1 Plessey EPIC sensors permit remote monitoring of mouse physiology and behavior

First, I tested whether EPIC sensors could reliably detect respiration, heart rate, and activity of mice in appropriately equipped home cage environments. I demonstrated the fidelity of sensor output and frequency responsiveness and validated sensor sensitivity to a predictable and rhythmic mechanical stimulus. I then undertook sensor recordings in plethysmography and ECG comparison studies to validate sensor capability with the 'gold standards' of conventional physiological recording techniques. I then determined the effects of rostro-caudal and angular position on quality of sensor recordings. Using this information, coupled with knowledge of animal resting location preferences, I constructed modified sensor-equipped home cages that mimicked vivarium social housing conditions and permitted continuous capture of animal respiratory rate and activity patterns during seven, twelve-hour epochs before and after SCI.

In relation to monitoring key indices of animal physiological status, I demonstrated that EPIC sensors reliably report respiratory rate over prolonged periods of time. Based on the ease with which sensors tracked the respiratory waveform, EPIC sensors could be employed as a cheap and versatile alternative to conventional methods such a whole body plethysmography. Whole body plethysmography is a reliable but expensive technique that requires a specialized airtight chamber separate from the home cage environment (Jacky, 1978). Additionally, I compared EPIC sensor recordings to ECG and demonstrated that they accurately captured heart rate in anesthetized animals. As such they present a non-contact alternative to conventional ECG methods for monitoring heart rate in applications including surgery or during anesthetized physiological experiments. While heart rate may have been captured at times in recorded waveforms, variability in detectability associated with animal position prevented reliable capture of heart rate. Therefore further refinement

of sensor positioning and verification is required before EPIC sensors could be used as an alternative to conventional cardiac measurement methods (i.e. telemetry) in awake and behaving animals.

Many commercial systems already exist for automated rodent behavioral phenotyping (de Visser et al 2006, Krackow et al 2010, Pham et al 2009, Quinn et al 2003, Van de Weerd et al 2001). However, these systems are extremely cost-prohibitive, and commonly complex video surveillance systems. As such, EPIC sensors are a valuable new addition to the field of automatic behavioral measurements, providing an extremely low cost ($6 dollars per sensor) alternative that permits concomitant measure of animal physiology. It is important to note that in this study I have only validated EPIC sensor ability to capture animal motor activity profiles. However preliminary results in a separate collaborative study in the lab suggest that these sensors may allow for behavioral phenotyping of stereotyped behaviors including grooming, sniffing, rearing, and licking (Noble et al., unpublished).

Most importantly, particularly in the context of my research question, EPIC sensor-equipped home cages permitted non-contact monitoring of respiratory rate in the home cage. Doing so eliminated many of the confounding environmental stressors typically introduced through conventional continuous measurement methods such as telemetry (e.g. surgically implanting or affixing devices to animals) or plethysmography (e.g. handling animals and collecting measurements separate from the home cage). By eliminating these stressors, and by allowing continuous captures in multiple cages simultaneously, I have shown that sensor-equipped home cages permit insight into the temporal dynamics of animal physio-behavioral condition independent of interaction with experimentalists.

4.2.2 Significance and future applications for EPIC sensor methodology

EPIC sensors provide affordability and versatility as a novel means of measuring respiration, heart rate, and animal activity profiles. With the addition of appropriate equipment (e.g. automated data loggers), sensor-equipped home cages permit 24/7 recordings in vivarium (Hochman et al, personal observations). This will in turn allow for large scale applications. For example, incorporation of sensor technologies in all homecages within vivarium housing facilities could provide a continuous monitor of animal health and welfare. Such recordings would similarly enable high-throughput studies of physiological and behavioral concomitants of disease development and progression.

4.3 Experimental Investigation

4.3.1 Strict controls limit, but do not eliminate, inter-animal behavioral variability

I hypothesized that strict environmental and genetic controls do not fully mitigate inter-animal variability in physio-behavioral condition. Using the aforementioned sensor-equipped home cages I undertook seven days of twelve-hour continuous recordings of respiratory rate and activity in six, adult male, C57BL/6J, pair-housed animals. I found that while overall activity levels were remarkably similar between littermates, the temporal activity profile differed between littermate-pairs housed in different home cages. Furthermore, activity magnitude and profile differed significantly between mice reared by sibling dams.

Variations in maternal care are known to have long-term impacts on behavior (Francis et al., 2003; Champagne and Meaney, 2001; Champagne et al., 2003). In particular,

certain maternal behaviors are understood to elicit hyper-activity in offspring (Gammie et al., 2008; Christakis et al., 2012). However, it is widely believed that within inbred laboratory strains such as C57BL/6J, maternal care is highly consistent across litters of the same size, particularly for sibling dams (Champagne et al., 2007; Van der Veen et al., 2007). The four littermates used in this study came from a litter of five, and the other two littermates came from a litter of six. As such, the significant difference between litter activity levels observed in this study emphasize that inter-litter variability also needs to be considered and accounted for. Furthermore, the differences in activity profile observed across both littermate and non-littermate cages supports sensitivity of animal behavior to slight differences in life history.

4.3.2 Loss of circadian fluctuations in activity level.

It is well documented that mouse activity is highly circadian-dependent, with increased activity during the dark period of the light cycle (De Bono et al., 2006; Loos et al., 2014; Mistlberger et al., 1987; Solarewicz et al., 2015). Indeed, in preliminary experiments testing the sensor-equipped home cages, I observed considerable differences in activity in light versus dark cycles in two animals that were recorded during a 5:00pm to 1:00pm epoch. I anticipated that these circadian patterns would be maintained after switching to a 1:00am-1:00pm recording interval if care was taken to minimize light exposure during the vivarium to home cage transfer. However, circadian variation in activity was lost. One explanation is that the slight light exposure occurring during animal transfer disrupted the circadian structure of animal activity patterns. While it is known that exposure to light during the dark cycle disrupts mouse circadian rhythm, it is typically believed that this

exposure must be fairly long (e.g. ~30min in duration) (Shigeyoshi et al., 1997; Lewy et al., 1980). In this study, light exposure was never greater than a few minutes – at maximum five. If loss of circadian variation was due to brief light explore, light-induced changes in circadian rhythm may be more sensitive than previously thought. Alternatively, given the care taken to minimize light exposure, the observed disruption in circadian activity patterns could be due to other variables including environmental differences between the vivarium and simulated vivarium environments including presence of novel olfactory or auditory cues.

4.3.3 Strict controls mitigated physiological variability in uninjured animals

While respiratory rate fluctuated throughout the seven recording periods, it did so within a typical range and all animals exhibited similar respiratory patterns. Furthermore, my results agreed with previously published results on the average resting respiratory rates of adult C57BL/6J mice (Berndt et al., 2011). This provides evidence in support of using strict controls to minimize physiological variability.

4.3.4 Inter-animal variability increased after SCI

I suspected that inter-animal variability in outcome after an SCI could be credited in part to variability in pre-injury physio-behavioral condition. Given a range of variability in uninjured animals, I expected that there would be associated variability after SCI. Inter-animal variability clearly increased post-SCI, however it appeared largely unrelated to litter or cage-mates. As stated above, this variability seems more likely due to differences in injury location and severity.

Inter-animal variability was most evident in the temporal dynamics of activity profiles and respiratory rates after injury. To my knowledge, these observations have not been previously documented on such a short time scale after injury. However, given that extent of plasticity in both autonomic and locomotor circuits after SCI is believed to vary considerably between individuals it is not necessarily surprising to consider that the onset and time course of this plasticity is also variable (Onifer et al., 2011; Zimmer et al., 2007).

4.3.5 Potential link between pre- and post- SCI variability

Unfortunately, due to the loss of three animals, and variability in injury profile, it is difficult to draw any concrete correlations between pre- and post-SCI variability. However, it is interesting to note that prior to the death of animal six on post-SCI day two, there appeared to be a litter-associated trend in respiratory rate after injury: animals five and six both had elevated respiratory rates while animals three and four had decreased respiratory rates. Given that animals five and six had increased activity prior to injury it would be interesting to consider whether pre-injury activity level impacts respiratory response after injury. This is a reasonable possibility – it is well known that activity level has significant impacts on respiratory function (Al-Jarrah et al., 2007; Dempsey, 2012). Additionally, increased spontaneous or forced activity post-SCI impacts plasticity and recovery after SCI (Battistuzzo et al., 2012; Galea, 2012; Houle and Cote, 2013 ;Van Meeteren et al., 2003). Thus, it is possible that pre-injury activity level could impact autonomic plasticity after SCI. However, additional studies and a larger sample size would be needed to confirm this hypothesis.

4.3.6 Conclusion and significance.

To my knowledge, this thesis represents the only semi-continuous long-term monitoring of mouse activity and physiology before and after an SCI. As such, many of the results presented here are important contributions to the field that warrant future investigations. In particular, this study demonstrates just how exquisitely sensitive animal physio-behavioral condition is to possible differences in life history and experiences – even strictly controlling for environment and genetics did not entirely mitigate variability. Furthermore, activity profile was dramatically impacted by subtle changes in environment such as activity of cage-mates and brief light exposure during the dark cycle. In this way, this thesis stresses that researchers need to consider the influence of variables in the home cage as a part of an animal's life history that can alter experimental conditions and hence outcome. Inclusion of these variables would provide stricter controls that could help account for unexplained variability in outcomes observed between and within labs.

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