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Conditioning Slowed Breathing for Relaxation in the Rat

By

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Doctor of Philosophy

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Conditioning Slowed Breathing for Relaxation in the Rat

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Abstract

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By Donald J. Noble

In humans, exercises involving slowed respiratory rate (SRR) have been found to counter autonomic sympathetic bias and engage the relaxation response. This state of deep rest reduces responses to stressors, including in individuals with various degrees of autonomic dysfunction. This thesis aimed to develop an animal model of the SRR-induced relaxation response. In the rat, we used operant conditioning procedures to train SRR and examined whether conditioned reductions in respiratory rate (RR) altered the physiological and behavioral responses to stressors in a manner consistent with the relaxation response. To condition SRR, we continuously monitored RR during 20 two-hour sessions using whole-body plethysmography, with feedback provided via a customized interface in LabVIEW. Conditioned rats, but not yoked controls, were able to turn off aversive visual stimulation (intermittent bright light) by slowing their breathing. Conditioned rats decreased their average resting RR by 10.6 breaths per minute, and maintained a reduced RR during intermittent retention sessions. Subsequent testing addressed the impact of conditioned slow breathing on stress reactivity and nociceptive responses. Compared to controls, conditioned rats showed i) decreased latency to enter the center of an open field, ii) decreased RR under acute restraint, and iii) decreased mechanical sensitivity to Von Frey hairs 75 minutes after formalin injection, possibly indicating alleviated hyperalgesia. In conclusion, rats could be trained to reduce their RR, rate reductions were maintained in the absence of training, and additional behavioral changes seen following conditioning were consistent with the development of an animal model of the relaxation response. In parallel, we developed a technological approach using electric field sensors for non-contact recordings of cardiorespiratory and behavioral variables. When strategically positioned, sensors accurately recorded rat RR and heart rate, overall motor activity, and various rhythmic motor behaviors including sniffing, rearing, grooming, and chewing. We forward the view that new generation sensor technologies can be linked to subsequent respiratory conditioning studies to provide continuous home cage assessment of cardiorespiratory and behavioral changes throughout an animal's lifespan. In sum, these studies establish the use of feedback-based conditioning of RR to investigate the physiological principles of stress reduction in a well-controlled animal model.

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Figures and Tables

CHAPTER 1: Decreased respiratory rate as a physiological trigger of relaxation

Abstract

Slowing and deepening respiratory rate (RR) as a relaxation technique has shown promise in a variety of cardiorespiratory and stress-related disorders including chronic heart failure, hypertension, anxiety, and depression (Brown and Gerbarg 2005, Jerath, Edry et al. 2006). Deep breathing could serve as a voluntary portal to trigger physiological changes resulting in improved autonomic function and enhanced well-being, but to date few studies have mechanistically investigated its role in conferring these benefits. Here, I introduce the known neurophysiology of deep breathing, and outline evidence that breathing at 0.1 Hz (6 breaths per minute) is consistent with the baroreflex resonance frequency that maximizes heart rate variability (HRV) to increase ventilation-perfusion matching and promote behavioral relaxation. This frequency further entrains brainstem autonomic hubs that radiate and impose these slow-wave sleep-like oscillations to cortical circuits. Consequently, restfulness and memory consolidation are enhanced while limbic 'stress' circuitry is disengaged. Future studies on the role of deep breathing as a stand-alone therapeutic in complementary medicine are warranted. Furthermore, the integration of deep breathing into mind-body practices as a 'carrier wave' for enhancement of cognitive and behavioral therapies may further promote therapeutic outcomes. Finally, basic research in animals could provide additional insights. Although instrumental (operant) autonomic learning studies in animals have only recently regained traction, animal models using classical or operant conditioning approaches could target slow, deep breathing for its therapeutic potential. Such models that control for confounding factors such as expectancy biases, while providing additional

opportunities for experimental manipulation, would be invaluable for understanding the impact of behavioral reductions in RR on autonomic function and stress reactivity.

Introduction

Despite an abundance of mind-body practices that involve slow, deep respiration as a fundamental component, our knowledge of the 'optimal' parameters of deep breathing for therapeutic benefit is incomplete. In the broadest sense, deep breaths are those occurring more slowly than the typical rate of 12-15 breaths per minute in normal adults. Since the volume of air exchanged during each breath – or tidal volume – varies inversely with respiratory rate (**RR**) in order to satisfy relatively stable metabolic needs, these slower breaths are also necessarily deeper under resting conditions. Several yogic techniques target the specific frequency of six breaths per minute (0.1 Hz). Significantly, breathing at this unique frequency maximizes heart rate variability (**HRV**) (Eckberg, 1983) and dramatically enhances cardiorespiratory synchronization (Peng, Henry et al. 2004), the phenomenon whereby the heart rate increases during inhalation and decreases during exhalation. This phenomenon is known clinically as respiratory sinus arrhythmia

Table 1: Definitions of key cardiorespiratory terms

Heart rate variability (HRV)	The physiological phenomenon of variation in the time interval between individual heart beats. Increased HRV means that the amplitude between peak and trough heart rates is maximized.
Respiratory sinus arrhythmia (RSA)	The phenomenon whereby heart rate increases during inhalation and decreases during exhalation. Spectral analysis is an accepted measure of the HRV associated with RSA. RSA increases at slower respiratory frequencies.
Cardiorespiratory synchronization	Coupled oscillations in heart rate and respiration. RSA can provide a quantitative measure of cardiorespiratory synchronization.

(**RSA**), and is greatly exaggerated at slower respiratory frequencies as the amplitude between peak and trough heart rates is maximized. Peak-to-trough amplitude is the simplest measure of HRV; spectral analysis of heart rate variations based on ECG recordings is another commonly used quantitative method to calculate HRV and reflects the same trend.

Why study slow, deep breathing?

Breathing is fundamental to life, and the dynamics of breathing are profound in their ability to modify nervous system function for better or worse. Ventilation (ml x min/kg) consists of two independent components: tidal volume (ml/kg) – the volume of air displaced during breathing – and respiration frequency (breaths/minute). Tidal volume and respiration frequency vary inversely to provide the ventilation needed to satisfy metabolic demands for O_2 consumption. Therefore, *slower breaths are necessarily deeper under resting conditions*. There are learned and state dependent differences in tidal volume and respiratory frequency. For example, the restful state of deep sleep is characterized by high tidal volume-low respiratory frequency, whereas low tidal volume-high respiratory frequency is a characteristic feature of anxiety (Carnevali, Sgoifo et al. 2013).

Hundreds of studies have claimed that practicing mind–body interventions can effectively treat stress-related disorders like hypertension, particularly in the cases of yogic and Zen meditation (Brown and Gerbarg 2009). For example, yoga is thought to improve stress management via alterations in autonomic nervous system controls, and a yoga intervention has been reported to lower blood pressure in patients with mild

hypertension (Hagins, Rundle et al. 2014). However, a recent National Center for Complementary and Alternative Medicine (NCCAM)-funded systematic literature review of various meditation practices for health found them to be largely of poor quality, and while meta-analyses supported beneficial reductions in hypertension and stress, overall the authors concluded that "the effects of meditation practices in healthcare cannot be drawn based on the available evidence" (Ospina, Bond et al. 2007).

An American Heart Association (AHA) review more recently considered non-traditional 'alternative and complementary medicine' approaches for lowering blood pressure (Brook, Appel et al. 2013). They concluded that acupuncture, meditation, yoga, relaxation, and biofeedback-based therapies had at best modest evidence of efficacy. While one biofeedback-based approach – device-guided breathing – was a supported approach by this report, a more recent well-controlled study and subsequent review convincingly argued against the adequacy in design of prior device-guided breathing studies (Landman, Drion et al. 2013, van Hateren, Landman et al. 2014). Even though it is reasonable to assume that sustained slow respiratory rate (**SRR**) should lead to adaptive changes in autonomic balance (Bernardi, Spadacini et al. 1998, Spicuzza, Gabutti et al. 2000, Joseph, Porta et al. 2005, Pramanik, Sharma et al. 2009), human studies of SRR techniques have had difficulty ruling out extraneous variables (e.g. placebo and expectancy biases developed during training) and formulating proper control groups. Moreover, very few studies have focused on isolating SRR from attentional or emotional regulatory elements (Ospina, Bond et al. 2007).

We do know that brainstem autonomic regions are intimately integrated with cardiovascular afferent activity patterns that regulate outflow of sympathetic and

parasympathetic nervous systems as 'push-pull' components in homeostasis. Volitional deep breathing relaxation techniques (e.g. meditation) are thought to have therapeutic benefit by reducing activity in sympathetic nerve circuits associated with stress, which are the hypothalamus-pituitary-adrenal axis (**HPA axis**) and the brainstem-spinal cord sympathetic axis, while promoting activity in the parasympathetic vagal circuits. For example, rhythmic deep respiration is hypothesized to increase vagal afferent discharge to the nucleus of the solitary tract (Brown and Gerbarg 2005), leading to effects thought to be physically and emotionally calming.

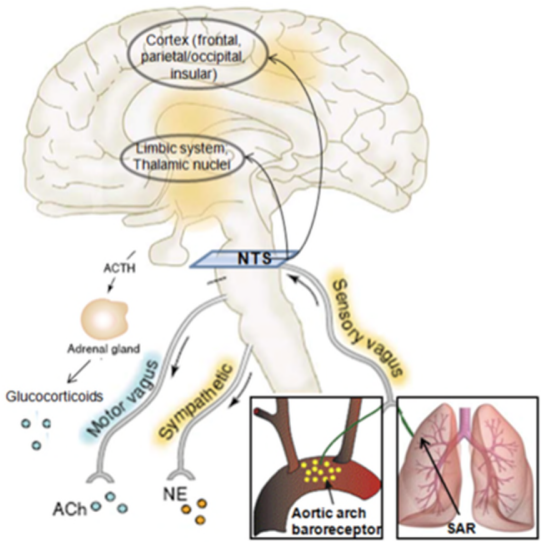
Animal experiments that test the autonomic and behavioral consequences of learning to breathe at a slower respiratory frequency could identify slower breathing as a fundamental variable of the relaxation response for subsequent complementary studies in humans. Indeed, based on the physiology of the respiratory system, control of respiration as a fundamental variable could provide a voluntary waypoint for access to autonomic nervous system modulation, with consequent impacts on cognition and behavior.

Physiology of the Respiratory System

Figure 1.1 provides a basic overview of the neurophysiological components of the respiratory system and their relationship with stress pathways. Our understanding of individual deep breaths suggests that sustained deep breathing repeatedly activates pulmonary afferents in the lungs and bronchi, and, via its effects on blood pressure, stretch-sensitive peripheral baroreceptors. These afferents project to second-order relay neurons in specific subregions of the brainstem nucleus of the solitary tract (**NTS**) via the vagus nerve (Malpas 2002, Carr and Undem 2003, Kubin, Alheid et al. 2006). Via

divergent synaptic connections to many brain regions, NTS neurons may in turn engage central parasympathetic circuitry (Petrov, Krukoff et al. 1993). One possibility is that the unique profile of sensory afferents activated during deep breathing projects along specific pathways that increase parasympathetic drive. Candidate afferents and projection systems are considered below.

Figure 1.1. Deep breathing and stress pathways – overlapping neuroanatomy.



The brainstem parasympathetic relay nucleus, the nucleus of the solitary tract (NTS), receives vagal nerve sensory afferents and extends second-order projections to limbic areas including the amygdala and hypothalamus, as well as to the thalamus and several cortical regions. NTS pathways could influence the release of acetylcholine (ACh) and norepinephrine (NE) from parasympathetic and sympathetic motor nerves, respectively, and alter plasma glucocorticoid levels by regulating pituitary release of adrenocorticotropic hormone (ACTH). (Inset) Slowly-adapting pulmonary receptor (SAR) and aortic arch baroreceptor afferents project to the NTS at the level of the medulla, and are known to innervate unique subregions. Figure modified from Raison, Capuron et al. 2006.

i) Inspiratory-activated pulmonary afferents: Normal breathing preferentially activates rapidly-adapting afferents (**RARs**). Deep inhalations also activate slowly-adapting pulmonary afferents (**SARs**) (Schelegle 2003), initiating inhibitory impulses (Jerath, Edry et al. 2006) that traverse through second-order ventrolateral NTS neuronal relays to cardioinhibitory neurons of the nucleus ambiguus (Stuesse and Fish 1984, Ezure, Tanaka et al. 2002). These in turn inhibit parasympathetic vagal outflow to the heart (Stuesse and

Fish 1984, Ezure, Tanaka et al. 2002), leading to an increase in heart rate. Conversely, inhibition of SARs following cessation of deep inhalation (Matsumoto, Saiki et al. 2006) likely results in strong rebound reciprocal changes that accentuate parasympathetic effects during exhalation, an effect that is consistent with SAR excitation evoking a tachycardia that is gradually masked by a reflex bradycardia (Schelegle and Green 2001). In summary, deep breathing may lead to a state transition in brainstem autonomic signaling due to a transition from RAR- to SAR-dependent pathways.

ii) Baroreceptors: Peripheral baroreceptors, located predominantly in the aortic arch and carotid sinus, are stretch-sensitive afferent mechanoreceptors that respond on a millisecond timescale to changes in blood pressure. Baroreceptor afferent activation typically initiates a negative feedback cycle whose final output is cardiovagal efferents from the nucleus ambiguus innervating the sinoatrial node of the heart. Like SARs, baroreceptor signals are conveyed to the NTS and project to other brainstem circuits, leading to sympathetic nervous system inhibition. As a mechanical consequence of changing intra-thoracic pressure during respiration, venous return to the heart is perturbed, altering cardiac output and thereby arterial blood pressure (Malpas 2002, Elliot 2005). Baroreceptors detect these changes, increasing afferent activity during exhalation to stimulate parasympathetic outflow to the heart through the NTS (Eckberg, Kifle et al. 1980, Ciriello 1983, Housley, Martin-Body et al. 1987, Schelegle 2003), and decreasing activity during inhalation.

iii) Nucleus of the solitary tract (NTS): The NTS is the brain's portal and relay station for all parasympathetic input. SARs project preferentially to the ventrolateral subregion of the NTS (**vlNTS**) (Berger and Dick 1987, Kubin, Alheid et al. 2006), while more medial and caudal regions of the NTS (**mNTS**) receive input from baroreceptor primary afferents (Dean and Seagard 1995) and likely house higher order neurons in pulmonary afferent pathways. NTS neurons project to central autonomic regions including monosynaptically to the amygdala and hypothalamus (Riche, De Pommery et al. 1990). In this feed-forward fashion, the strong rhythmic afferent drive initiated by deep breathing may help entrain central parasympathetic circuits (see below).

iv) Central Autonomic Pathways: The NTS projects extensively to other areas of the central autonomic network, including the central nucleus of the amygdala (**CeA**) and paraventricular nucleus of the hypothalamus (**PVN**) (Loewy 1990, Petrov, Krukoff et al. 1993), both involved in the response to stress or threat (Thayer and Lane 2000, Porges 2007). Furthermore, the NTS and nucleus ambiguus form early parts of a central autonomic network (Loewy 1990), whose CeA and PVN receive branching collaterals from the NTS (Petrov, Krukoff et al. 1993). Based on implicated roles of the CeA and PVN in response to stress or threat, models have been developed that implicate vagally-mediated HRV – intimately intertwined with the respiratory-induced activation of SARs and baroreceptors – as a marker for adaptive environmental engagement (Thayer and Lane 2000, Porges 2007). Therefore, the CeA and PVN are likely higher-order mediators of deep breathing's central autonomic effects.

Baroreceptor Resonance and Cardiorespiratory Synchronization at 0.1 Hz

All systems have at least one preferred frequency in which oscillations occur at a greater amplitude. These are the system's resonant frequencies, and independent systems with common resonant frequencies are self-reinforcing. Biologically speaking, resonance is the most energetically and thus metabolically efficient way to increase signal power. In this manner, correlated oscillations of cardiac and respiratory rhythms at one frequency (referred to as their coherence) can lead to mutually reinforcing signal amplification.

Coherence and Mayer Waves

During deep breathing, arterial blood pressure varies during the respiratory cycle as a consequence of changing intrathoracic pressures (Malpas 2002). Baroreceptor afferents respond to these changes by decreasing (inhalation) or increasing (exhalation) their activity in line with the respiratory cycle. Through direct projections to several subregions of the NTS that are known to innervate cardioinhibitory regions of the nucleus ambiguus (Ciriello 1983, Housley, Martin-Body et al. 1987), baroreceptor activation reduces blood pressure by stimulating parasympathetic outflow to the heart and inhibiting arterial sympathetic outflow. Given that cardiovagal parasympathetic output is the main determinant of HRV amplitude (Malpas 2002, Elliot and Edmonson 2006), this baroreceptor input has a strong and powerful effect on the amplitude of HRV with each cycle of the breath. At lower respiratory frequencies and for deeper breaths, changes in blood pressure will greatly accentuate changes in heart rate, leading to a wide range of observed heart rate values during respiration, and consequently a large magnitude of HRV. The arterial baroreflex exhibits positive feedback properties at a frequency of 0.1

Hz (0.4 Hz in the rat) (Bertram, Barres et al. 1998, Elliot and Edmonson 2006), resulting in self-sustained oscillations in arterial blood pressure called 'Mayer waves' (Julien 2006), and leading to high-amplitude, vagally-mediated heart rate oscillations at 0.1 Hz (Elghozi and Julien 2007). As elaborated below, voluntarily breathing at 0.1 Hz (6 breaths/minute) could entrain blood pressure and heart rate to this same resonant frequency.

Respiratory Sinus Arrhythmia (RSA)

Several distinct meditation and yoga techniques induce profound cardiorespiratory synchronization at the characteristic frequency of 0.1 Hz (Eckberg 1983, Peng, Henry et al. 2004, Cysarz and Bussing 2005). This entrainment of cardiac variability to the respiratory rhythm – so that heart rate increases during inhalation and decreases during exhalation – is referred to clinically as RSA, and is greatly exaggerated at slower respiratory frequencies, such that incremental reductions in RR result in non-linear increases in RSA; this is true in both humans and rats (Eckberg 1983, Rubini, Porta et al. 1993, Song and Lehrer 2003, Pereda, De la Cruz et al. 2005), and suggests that in both species a lower RR will lead to a corresponding increase in RSA, regardless of whether it is possible to reach the frequencies corresponding to peak RSA. Indeed, RSA appears to be a reliable physiological index of SRR's effects on autonomic function. It is decreased in individuals with depression, anxiety, and panic disorder, and enhanced in physically active postmenopausal women (Carney, Freedland et al. 1995, Davy, Miniclier et al. 1996, Beauchaine 2001). Several therapeutically relevant meditation techniques that involve slowed breathing also enhance RSA (Lehrer, Sasaki et al. 1999, Peng, Henry et

al. 2004, Cysarz and Bussing 2005), and increases are associated with improved parasympathetic function and wellness (Brown and Gerbarg 2005). In analogy with repeated exercise promoting physical fitness, voluntary and repeated lowering of RR may lead to autonomic fitness and promote well-being.

Both the frequency of Mayer waves and RR producing maximal RSA are surprisingly invariable within a given species. While the origin of Mayer waves remain incompletely understood, baroreceptor resonance patterns contribute (Julien 2006). Studies using baroreceptor denervation or alpha-adrenergic blockage (Cevese, Gulli et al. 2001) implicate peripheral baroreceptors in the corresponding 0.1 Hz rhythm in heart period. It is possible that an intrinsic central oscillator in the caudal medulla closely interacts with baroreceptor and pulmonary input in the NTS to change HRV and alter autonomic activity. This leads us to propose that breathing at six breaths per minute may maximize HRV (Eckberg 1983) by 'unlocking' baroreceptor resonance patterns to enhance inherent physiological rhythms. The end result would be increased cardiorespiratory synchronization and, via baroreceptor cardiovagal signaling pathways, autonomic balance would be shifted in the parasympathetic direction.

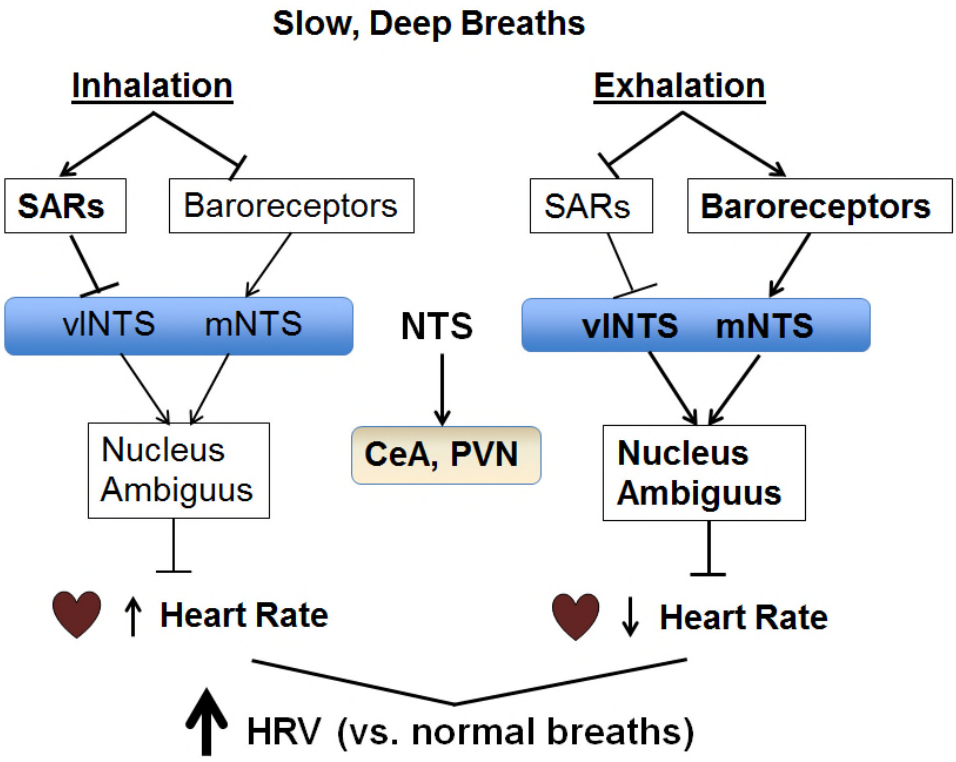


Figure 1.2. Slow, deep breaths lead to dramatically enhanced heart rate variability.

(A) Inhalations activate slowly-adapting pulmonary afferents (SARs), sending inhibitory impulses through the nucleus of the solitary trace (NTS) to inhibit parasympathetic vagal outflow to the heart. Baroreceptors decrease their activity during inhalation, inhibiting parasympathetic outflow to the heart through the NTS. **(B)** During exhalation, inhibition of SARs and activation of baroreceptors combine to accentuate parasympathetic effects. Combined, deep inhalations and exhalations lead to a wider range of heart rates and thus increased heart rate variability (HRV). Meanwhile, NTS projections to the central autonomic network, including the central nucleus of the amygdala (CeA) and paraventricular nucleus of the hypothalamus (PVN), could combine with enhanced HRV to engage adaptive systems that shift autonomic balance in the parasympathetic direction.

An Integrated View: The Physiology of Slow, Deep Breathing

Taking all of the above evidence into account, it is possible to establish a coherent model of slow, deep breathing's neurophysiology (Figure 1.2). It is known that individual slow and deep breaths engage brainstem circuitry and produce a variety of potent

downstream systemic effects. The individual components include: i) Slowly-adapting pulmonary afferents: Deep inhalations activate slowly-adapting pulmonary afferents (SARs) (Schelegle 2003), initiating inhibitory impulses (Jerath, Edry et al. 2006) that traverse through second-order NTS relays to inhibit parasympathetic vagal outflow to the heart (Stuesse and Fish 1984, Ezure, Tanaka et al. 2002). Conversely, inhibition of SARs following inhalation (Matsumoto, Saiki et al. 2006) likely results in reciprocal changes that accentuate parasympathetic effects during exhalation (Schelegle and Green 2001). ii) Baroreceptors: As a consequence of changing intrathoracic pressure and arterial blood pressure during respiration (Malpas 2002, Elliot and Edmonson 2006), baroreceptors increase their activity during exhalation, stimulating parasympathetic outflow to the heart through the NTS (Eckberg, Kifle et al. 1980, Ciriello 1983, Housley, Martin-Body et al. 1987). iii) The NTS projects extensively to other areas of the central autonomic network, including the central nucleus of the amygdala (CeA) and paraventricular nucleus of the hypothalamus (PVN) (Loewy 1990, Petrov, Krukoff et al. 1993), both involved in the response to stress or threat (Thayer and Lane 2000, Porges 2001). iv) Autonomic effects: During deep breathing, SAR and baroreceptor effects combine to enhance sympathetic activity during inhalation and parasympathetic activity during exhalation, leading to a wider range of heart rates and thus increased HRV (Elliot and Edmonson 2006), an effect that is greatest at 0.1 Hz (6 cycles/minutes) (Bertram, Barres et al. 1998, Julien 2006, Elghozi and Julien 2007). Enhanced HRV at this frequency could reflect the engagement of adaptive systems (Porges 2001) that shift autonomic balance in the parasympathetic direction.

The Impact of Slow, Deep Breathing on Systemic Function

We have discussed how slow, deep breathing can enhance RSA via increased baroreflex gain. Here we outline it's impact on optimizing ventilation-perfusion matching (Elliot and Edmonson 2006), and engaging adaptive systems (Porges 2007) to shift autonomic balance in the parasympathetic direction. Our proposal is that the signal coherence provided by cardiorespiratory synchronization at 0.1 Hz serves as a shared resonant frequency whose increased signal power leads to emergent phenomena: (i) Reduced metabolic demand in autonomic neural circuits that could also support a sense of physical relaxation. (ii) Generation of a large arterial pressure wave independent of heart function. This would facilitate tissue perfusion while reducing cardiac burden. (iii) Autonomic neural control of cortical vasculature at 0.1 Hz, generating slow cortical oscillations as seen during deep sleep. These would facilitate learning and memory. Previously, we described how deep breathing at 0.1 Hz mechanically perturbs the airways and venous return to the heart, ultimately recruiting slowly-adapting pulmonary afferents and activating arterial baroreceptors at their resonant frequency, whereby they have maximal influence on swaying heart rate in the parasympathetic direction during exhalation. Correspondingly, the oscillatory rise and fall of heart rate that varies with respiration stabilizes and further entrains to the respiratory cycle at 0.1 Hz, as its peak-to-peak amplitude is maximized (i.e. increased HRV and RSA). As described below, the resulting hike in RSA increases ventilation-perfusion matching and may entrain slow cortical rhythms akin to those observed in deep sleep, ultimately providing an ideal substratum for memory consolidation.

Ventilation-Perfusion Matching

Enhanced RSA produced by the desired cardiorespiratory synchronization at 0.1 Hz may enhance ventilation-perfusion matching (Malpas 2002, Yasuma and Hayano 2004, Thayer and Sternberg 2006). During inhalation, expansion of the chest cavity and the resulting intra-thoracic negative pressure increase would lead to expansion of blood vessel diameter, effectively increasing perfusion of freshly inhaled oxygen to vital tissues. Conversely, exhalation would correspond to the ebb in perfusion. Studies have confirmed that pulmonary gas exchange is improved with enhanced RSA, supporting an active physiological role (Yasuma and Hayano 2004). The net result would be decreased energy expenditure (eliminating unnecessary heartbeats during exhalation) and increased metabolic efficiency. The absence of alveolar dead space during inhalation and shunting during exhalation, along with downstream mechanisms, could also reduce oxidative damage and free radical formation, a finding in line with a recent study of the relaxation response that found whole-genome transcriptional changes, particularly in metabolic markers (Dusek, Otu et al. 2008).

Hypothalamic and amygdalar activations represent a neural signature of emotional engagement and 'wellness'.

Deep breathing-induced pulmonary and baroreceptor afferent activity patterns may lead to the recruitment of specific classes of NTS, amygdalar and hypothalamic neurons identifiable by their anatomical topography. Second-order neurons from the NTS project particularly densely to the central nucleus of the amygdala (CeA) and the paraventricular nucleus of the hypothalamus (PVN) (Petrov, Krukoff et al. 1993), which

may in turn engage brain circuitry involved in emotion and internal regulation (Porges 2007). In animal studies, acute social stress increases Fos expression in the CeA and PVN (Martinez, Phillips et al. 1998, Dayas, Buller et al. 2001), while depressing HRV and increasing circulating catecholamines (Sgoifo, Koolhaas et al. 1999). Given the CeA's central role in activating autonomic output pathways and centrally coordinating the fear network, as well as its particular propensity for initiating passive and reactive fear responses (Ressler 2010), stress-induced recruitment of the CeA is in line with the evolutionary idea of a polyvagal system intricately related with social adaptivity and inversely correlated with perceived threat of the environment (Thayer and Lane 2000, Porges 2007). In this case, overactivation of the CeA during acute social stress (e.g. in social anxiety disorder) would result in maladaptive behavioral responses to the threat, whereas deactivating the CeA during deep breathing would disengage sympathetic stress circuitry and increase socially adaptive behaviors (e.g. avoidance vs. freezing, or social engagement vs. disinterest). In summary, deep breathing may oppose the effects of stress-circuit activation and improve autonomic balance in response to acute social stress. A 'parasympathetic' shift in stress-related behavior would also be expected. Correspondingly, expected results would be reduced markers of sympathetic activity including galvanic skin conductance, plasma norepinephrine/epinephrine, adrenocorticotropic hormone (ACTH), salivary cortisol, and interleukin (IL-6), and increased markers of parasympathetic activity, including vasoactive intestinal peptide (VIP), RSA, and reduced heart rate and blood pressure.

Increased low frequency (LF) HRV during deep breathing reflects improved autonomic function and is predictive of meditation-derived benefits.

Traditionally, RSA has been measured as high-frequency (HF) HRV in the HRV spectrum, since a normal RR in humans (~12-15 breaths/minute) falls within this frequency domain in a Fourier-transformed HRV spectrum. However, during deep breathing at a lower rate (typically ~6 breaths/minute), RSA would shift to the lower frequency (LF) domain of the HRV spectrum, and at the most commonly cited respiratory frequency of 6 breaths/minute would coincide or overlap with the Mayer wave signature in an HRV trace. Therefore, RSA should strongly associate with LF HRV at 0.1 Hz. Indeed, several different groups have found a marked enhancement in RSA within this domain in participants engaging in relaxation practices who receive no explicit instruction to control their breathing at this frequency.

Slow Cortical Oscillations (SCOs)

There is compelling evidence of cerebral blood flow fluctuations associated with cardiovascular variability (Cencetti, Lagi et al. 1999). Furthermore, slow respiration can entrain electroencephalographic (EEG) activity (Kamei, Torui et al. 2000, Busek and Kemlink 2005). EEG recordings taken during sleep have revealed slow oscillations in membrane potential, with cortical neurons alternating between hyperpolarized and depolarized states. These slow cortical oscillations (SCOs) are known to organize other sleep rhythms, represent a fundamental cellular phenomenon (Steriade, Nunez et al. 1993), and are present in all investigated (sensory, motor, and associational) cortical areas, even surviving total lesions of thalamic perikarya projecting to the recorded

cortical neurons. They also play a pivotal role in grouping within the slow (~0.3 Hz) rhythm other sleep oscillations, such as alpha (7-14 Hz) and delta (1-4 Hz) waves. Interestingly, the deep breathing observed in meditation practices is comparable in frequency to that during deep sleep. Respiratory activity, which is fairly irregular during waking conditions, tends to stabilize during deep sleep, where it corresponds closely with heart rate, enhancing cardiorespiratory synchronization (Bond, Bohs et al. 1973). Therefore, it is possible that deep breathing-induced cardiorespiratory synchronization is entrained with SCOs. Furthermore, slow wave oscillations commonly arise from prefrontal–orbitofrontal regions (Massimini, Huber et al. 2004) that interact with the NTS. "Visceromotor" cortical areas, including specific regions of the medial prefrontal cortex (MPFC), can elicit autonomic depressor responses, an effect that is mediated in part by a projection from the MPFC to the NTS (Owens and Verberne 1996). Similarly, the orbitofrontal cortex (OFC) plays important roles in emotion and reward and is often considered to be part of the limbic system.

Of relevance to the cognitive elements of different meditation techniques is the finding that SCOs facilitate memory consolidation. While it's known that sleep contributes to the long-term consolidation of new memories, this function has recently been linked with slow (less than 1 Hz) potential oscillations, an intrinsic component of slow wave sleep that originate from the prefrontal cortical regions. Importantly, applying transcranial oscillating potentials of 0.75 Hz at the onset of slow wave sleep improves memory retention in humans (Marshall, Helgadottir et al. 2006). During the up state, synaptic activity is dramatically increased in neurons of the thalamocortical system, with firing rates approaching those observed during wakefulness (Steriade, Timofeev et al.

2001). It may be that activation of the circuitry during an up state would engage long-term plasticity mechanisms, in analogy to the hippocampal theta rhythm (Huerta and Lisman 1995). Furthermore, SCOs preferentially originate in prefrontal cortex, which is bi-directionally interconnected with the NTS. Evidence of SCOs in meditation is lacking, mostly because amplifier filters are not set to detect these slow frequencies. However, studies designed to take these frequencies into account could support coherence between cortical function and pulmonary afferent activity. Because of the presumed involvement of prefrontal-limbic circuitry in meditation techniques that emphasize emotion regulation (such as compassion meditation), the specific circuits engaged during the cognitive aspects of these techniques may be preferentially strengthened by SCOs. This line of reasoning suggests the testable hypotheses that: i) deep breathing is a core functional unit of meditation, in that it provides the physiological trigger for a large portion of meditation's therapeutic benefit; and ii) deep breathing forms an ideal substratum ('fertile soil') for the learning processes inherent in different meditation techniques, enhancing the effects of mental training. Therefore, one would expect that during a training protocol not explicitly focused on breathing control, slower and deeper breaths during the training period would predict one's ability to learn the technique and extract maximum therapeutic benefit.

Respiration in Health and Disease: Human Research

Human Studies Assessing the Benefits of Slowed Respiration

Volitional deep breathing relaxation techniques (e.g. pranayamic breathing) may be a fundamental aspect of the relaxation response observed in several forms of

meditation and yoga. Consequently, any disorder involving imbalanced autonomic activity could derive benefit from slow, deep breathing. However, the neurophysiological mechanisms through which deep breathing exerts its effects remain largely unknown. As discussed above, an emergent phenomenon at slowed RRs is cardiorespiratory synchronization. Here, slow arterial pressure waves and respiratory frequency preferentially hover at ~0.1 Hz (one breath every 10 seconds). This corresponds to the frequency of Mayer waves brought about by oscillations in autonomic cardiovascular reflex circuits. Accordingly, 0.1 Hz may be the body's physiological resonant frequency of autonomic balance where 6 breaths/minute (0.1 Hz) resonates with Mayer waves to synchronize sympathetic and vagal outflow. That these rhythms also lead to similar fluctuations in cerebral blood flow (Cencetti, Lagi et al. 1999) introduce the possibility of autonomic entrainment of the same resonant frequency to brain circuits.

It may be no coincidence, then, that two distinct meditative practices induce cardiorespiratory synchronization at a respiratory frequency of 0.1 Hz (Peng, Henry et al. 2004); even inexperienced Zen meditators lock in at 0.1 Hz (Cysarz and Bussing 2005). Intriguingly, religious and artistic practices can also facilitate 0.1 Hz cardiorespiratory synchronization, and may have evolved to promote a feeling of wellness that individuals would associate with such practices. For example, the timing of repetition of the Ave Maria in rosary prayer and of yoga mantras cycles at ~0.1 Hz and produces cardiorespiratory synchronization (Bernardi, Sleight et al. 2001). Certain forms of rhythmic poetry recitation (e.g. hexameter verse from ancient Greek literature) similarly lead to this phase-locking (Cysarz, von Bonin et al. 2004), as do specific music phrases, as in Verdi's famous arias (Bernardi, Porta et al. 2009). In sum, respiration rate and depth

(tidal volume) may insidiously control our state of well-being, as the few studies that have investigated the impact of specific respiratory frequencies have found an impressive synchronization of systemic function at the characteristic frequency of 0.1 Hz.

RSA Biofeedback and Deep Breathing as a Novel Therapeutic in Epilepsy

In the last several years, innovative biofeedback approaches have been devised based on cardiorespiratory parameters altered in meditation, such as respiratory sinus arrhythmia (RSA) (Vaschillo, Lehrer et al. 2002, Song and Lehrer 2003, Yasuma and Hayano 2004). These have proven effective in disorders as diverse as asthma, PTSD, and depression (Lehrer, Vaschillo et al. 2004, Karavidas, Lehrer et al. 2007, Zucker, Samuelson et al. 2009), but lack of a detailed mechanistic understanding has impeded progress in these areas. One popular respiratory biofeedback system that has received clinical attention in recent years is RESPeRATE (Anderson, McNeely et al. 2009), which provides auditory reinforcement when subjects breathe slower than 10 breaths/minute, with the targeted behavior varied to reflect individual baseline breathing patterns. RESPeRATE has been shown to significantly decrease resting blood pressure following several weeks of daily training sessions. Nevertheless, a previously cited study and review argued against the adequacy in design of prior device-guided breathing studies (Landman, Drion et al. 2013, van Hateren, Landman et al. 2014). A greater mechanistic understanding of deep, slow breathing would improve the design of these instruments and may suggest novel physiological parameters to target or monitor during their implementation. Based on interconnections between the autonomic nervous system, stress and inflammation (Raison, Capuron et al. 2006), these strategies would have a

range of applications in the clinic, and especially in the context of preventative medicine. For example, slow breathing exercises have been proposed as a novel therapeutic in epilepsy (Yuen and Sander 2010), where stress is the most common trigger of seizures (Sawyer and Escayg 2010) and there are measurable changes in autonomic balance (Novak, Reeves et al. 1999, Mativo, Anjum et al. 2010). Rhythmic deep breathing could be used to drive vagal afferent activity patterns that restore autonomic balance, in analogy to vagal nerve stimulation (VNS); this could in turn reduce seizure incidence and increase seizure threshold. This idea could be tested to delineate therapeutic parameters in humans or in a feedback-based animal model.

Translational studies will determine the relative impact of deep breathing as a component of different cognitive interventions.

Despite evidence of deep breathing's therapeutic benefit in disorders of autonomic imbalance (Spicuzza, Gabutti et al. 2000, Bernardi, Porta et al. 2002, Brown and Gerbarg 2005, Joseph, Porta et al. 2005, Jerath, Edry et al. 2006, Kaushik, Kaushik et al. 2006, Courtney 2009, Pramanik, Sharma et al. 2009) very few studies have focused on isolating deep breathing from attentional or emotional regulatory elements of training (Ospina, Bond et al. 2007). Ultimately, it may be possible to develop targeted interventions customized for specific disordered subpopulations and based on the relative efficacy of different cognitive techniques integrated with deep breathing. This is based on the above hypothesis that vagal afferents entrain the physiological responses of downstream areas in a manner that i) is ultimately physically and emotionally calming, reflecting induction of the relaxation response, and ii) provides an ideal substratum for

learning, as observed with slow cortical oscillations and theta frequency hippocampal long-term potentiation. We expect that engagement of the neural circuitry involved in compassion practices concurrent with deep breathing would confer a combinatorial effect, spawning a state of mental relaxation that improves cognitive and physiological outcome measures in response to a psychosocial stressor. This invokes the hypothesis that deep breathing is a core functional unit of meditation; although we predict that subsequent meditation practice will modify the basic effects of deep breathing, we also hypothesize that deep breathing by itself could provide the physiological trigger for a large portion of meditation's therapeutic benefit. Assessing breathing concurrent with meditation would make it possible to investigate the role of breathing per se, as well as its interaction with cognitive elements of the practice. Any group differences between interventions that remain after controlling for breathing could suggest an additive or synergistic effect of breathing deeply on the response to stress after meditative training.

Respiration in Health and Disease: Animal Research

Biofeedback and Instrumental Autonomic Learning

Neil E. Miller first investigated the role of instrumental learning in visceral homeostasis in the late 1960s (Dworkin 1993). In the late 60s and early 70s, his lab found impressive learning of heart rate changes in acute curarized rat preparations (Miller and DiCara 1967). Although other laboratories found similar results (Hothersall and Brener 1969, Hahn and Slaughter 1970, Slaughter, Hahn et al. 1970), several of the experiments using the curarized rat preparation were not successfully replicated (Dworkin 1973; Dworkin and Miller, 1986). Due in part to the attention garnered by unsuccessful

replication of early results, studies on instrumental autonomic learning fell out of favor for quite a few years. Based in part on subsequent studies showing instrumental learning in an array of experimental models including crustaceans, isolate insect thoracic ganglia, and the sea slug (Dworkin 1993), the idea of instrumental control of autonomic function regained traction in the early 1990s (Dworkin and Dworkin 1990, Dworkin and Dworkin 1995).

Operant (Instrumental) Conditioning of Respiration

A review by Ley concluded that "breathing behavior is amenable to the principles of Pavlovian and operant conditioning" (Ley 1999). Operant conditioning protocols have had limited success altering respiration in rats and humans (Gallego, Benammou et al. 1994, Ley 1999, Elliott and Izzo 2006), and have not been attempted to investigate SRR as a technique to induce the relaxation response. Operant procedures have been used to condition aerial respiratory behavior – decreasing the frequency and duration of the animal opening its pneumostome, or respiratory orifice – in the freshwater snail (Lukowiak, Ringseis et al. 1996), and this corresponds to plastic changes in the animal's central pattern generator for respiration (Spencer, Kazmi et al. 2002). In rats, one pilot study used electrical stimulation of the medial forebrain bundle as reinforcement to condition inspiratory duration (Gallego, Benammou et al. 1994). However, only one rat survived experimental procedures and was included in the analysis. The rat was rewarded for long inspirations (>300 ms), before authors attempted a "reversal conditioning" strategy (whereby inspirations <300 ms were rewarded) similar to our use of "up-conditioning" procedures (Chapter 2), but within the same animal. As expected, the rat

increased the frequency of long (>300 ms) inspirations during the early stages of the study, while the frequency of these events was reduced during reversal conditioning. Although the authors suggest potential improvements in experimental design for future investigation, no follow-up studies have been reported. However, the conditionability of basic reflex responses lends additional support to the idea of operantly conditioned respiration. Operant conditioning has been used to condition the monosynaptic H-reflex, the most hard-wired circuit in the central nervous system, and the effects of conditioning remain stable for months in rats and humans (Chen and Wolpaw 1995, Thompson, Chen et al. 2009, Chen, Wang et al. 2010).

Classical Conditioning of Respiration and Mechanical Ventilation Studies

Additional studies on controlled animal respiration have employed classical conditioning paradigms (Gallego and Perruchet 1991, van den Bergh, Kempynck et al. 1995, Nsegbe, Vardon et al. 1997), or externally-induced ("forced") respiration paradigms using anesthetized and mechanically ventilated preparations, intubated rats, or hypoxic/hypercapnic animals. In one classical conditioning study, Nsegbe et al. (1997) paired hypercapnia (8.5% CO_2) as an unconditioned stimulus with a one-minute tone, and then tested the respiratory response to the tone alone (Nsegbe, Vardon et al. 1997). Compared to a control group receiving a noncontingent presentation of the stimuli, conditioned rat breath duration increased and mean ventilation decreased. The authors concluded that this respiratory inhibition may have been due to the tone associating with the aversive effects of CO_2. However, in another study that paired odors with hypoxia (8% O_2) as the unconditioned stimulus, presenting the odor alone dramatically increased

ventilation over the group receiving noncontingent presentation of an odor and hypoxia, supporting feedforward or anticipatory responses to the hypoxic stimulus (Nsegbe, Vardon et al. 1998). In Appendix 1, we describe pilot studies from our lab in which we mechanically ventilated anesthetized rats at rates and depths corresponding to normal or slow, deep breathing in humans, to investigate the central autonomic circuitry recruited. However, anesthetized studies do not possess the ecological validity of an awake and behaving model since no known anesthetic leaves cardiorespiratory parameters completely unaltered. Classical conditioning approaches, on the other hand, tend to confound a range of respiratory responses depending on the unconditioned stimulus used, and remove the volitional component of slow, deep breathing techniques in humans.

A well-controlled animal model will help guide future clinical research.

After many research reports, there is still enormous variability and ambiguity on the merits of alternative approaches that include RR slowing as either implicit (e.g., yoga, meditation) or explicit (device guided breathing) to the therapeutic procedure. The development of a novel animal model provides for exquisite control of genetic and environmental variables, and represents the first step toward mechanistic studies aimed at developing a unifying theory to account for the acute and adaptive effects of slow, deep breathing on central autonomic function and behavior.

Specific Aims of this Dissertation

i) Development of an animal model of the relaxation response

The first objective of this dissertation was to establish a novel animal model of the relaxation response for present and future inquiry into the psychophysiological concomitants of wellness. Relaxation techniques that incorporate slowed RR may be effective in controlling autonomic dysfunction, but inherent difficulties in experimental design, including identification of controls, has greatly hampered the strength of published claims. As a major use of animal models is to provide greater experimental control, this dissertation aimed to develop an operant conditioning approach to slow RR in order to directly assess the role of maintained reduction in RR on physiological and behavioral indices of stress. Based on the proposed therapeutic impact of SRR, we decided to examine several outcomes that would address whether we had successfully developed an animal model congruent with the relaxation response.

ii) Construction of enabling technologies for next generation studies on the "quantified self"

While we were developing the first operant conditioning approach to train rats to slow their RR, we also pioneered the use of low-cost, ultra-sensitive electric field sensors for simultaneous non-contact reporting on autonomic, motor, and affective behavioral states. The low cost of the sensors enables chamber multiplication for parallel animal experimentation, thus offering high-throughput analyses. Simultaneously, they can be embedded within an animal's home cage environment for continuous measurement via a low-cost data logger. Sensor measures can be collectively synthesized to quantify phenotype changes in the animal's physio-behavioral profile subsequent to SRR

conditioning. Feedback-based modification of these variables promises to provide innovative solutions in prevention, detection, and correction of various disease states.

Layout of This Dissertation

The detailed layout of this dissertation is described below. Briefly, Chapters 2 and 3 document our efforts to train awake and behaving animals to slow their breathing via operant conditioning procedures, and to measure several behavioral and physiological outcomes indicative of relaxation. Chapter 4 describes the development of a novel and affordable non-contact methodology for monitoring animal physiology and behavior in real time. Chapter 5 summarizes my dissertation research and proposes future directions. Appendix 1 outlines early studies in anesthetized, mechanically ventilated animals to identify the neural circuitry engaged by deep breathing.

In *Chapter 2*, I outline successful attempts to condition reduced RR in an animal model. I describe the experimental procedures used and address several confounding factors that could explain the behavioral reduction in RR.

In *Chapter 3*, I present evidence that decreased RR was retained in the absence of conditioning, and test the hypothesis that trained reductions in RR induce behavioral and physiological changes consistent with the relaxation response.

In *Chapter 4*, I describe parallel work that was undertaken using electric field sensors to collect RR and other physio-behavioral variables in an animal's home cage to allow for measurements in the vivarium independent of experimenter interactions.

In *Chapter 5*, I summarize the presented work and propose future directions. In brief, I conclude that concurrent physiological recording via electric field sensors will

ultimately establish the impact of SRR on cardiovascular parameters and capture changes between and after training sessions to reveal whether sustained SRR evokes lasting autonomic changes that lead to reductions in disease and stress-related behaviors.

In *Appendix 1*, I detail a pilot study investigating the activation of brainstem and limbic circuitry downstream from deep breathing-recruited respiratory afferents.

CHAPTER 2: Aversive bright light can be used to condition reduced respiratory rate

Abstract

Relaxation-promoting techniques incorporating slowed respiratory rate (SRR) lower physiological indices of stress, entrain heart rate to the respiratory cycle, and have been associated with numerous therapeutic benefits. Furthermore, respiratory behavior has been successfully trained using conditioning procedures in the past (Ley 1999), but never to investigate the role of SRR in reducing behavioral and physiological responses to stress. Thus, I proposed to develop an operantly conditioned animal model of SRR. I conceptualized using operant conditioning with visual stimuli, such that awake rats would be rewarded for achieving a threshold respiratory rate (RR) corresponding to high tidal volume (deep) breaths, while yoked controls would receive response-independent reinforcement. To achieve this, I used dual chamber whole-body plethysmography and light-based feedback as negative reinforcement. Automated detection of RR was performed using the Buneman Frequency Estimator in LabVIEW, and a set threshold RR was chosen as the feedback trigger. Training sessions were run for two hours, approximately five days per week, for 20 sessions. I found that SRR conditioned rats reduced their RR over 20 sessions, with conditioning effects beginning within 4 sessions and peaking at 15 sessions. In n=11 pairs of animals, the mean decrease in RR was 10.6 breaths/minute in conditioned rats, compared to 3.6 breaths/minute in yoked controls, and conditioned rats breathed at slower RRs over the course of training. SRR conditioned rats, but not yoked controls, also increased the regularity of their breathing over training (reflected in decreased standard deviation of resting RR), but did not spend a greater percentage of each session at rest. A separate subgroup of n=4 rats that received reinforcement for fast breaths (‘FRR conditioned’ rats) successfully learned the task as

well, suggesting that stimulus controllability in SRR conditioned rats did not account for their reduced RR. Interestingly, SRR conditioned and yoked control rat average resting RRs within individual sessions were correlated ($R^2 = 0.26$), suggesting an impact of shared experience. In conclusion, i) I successfully trained rats to slow their breathing using operant conditioning procedures, ii) success in conditioning was mediated in part by increased regularity of respiration, iii) conditioned rats and yoked controls spent similar amounts of time at rest, and iv) differential stimulus controllability was not responsible for group differences in resting RR.

Introduction

Despite evidence of SRR's therapeutic benefit in disorders of autonomic imbalance (Spicuzza, Gabutti et al. 2000, Bernardi, Porta et al. 2002, Brown and Gerbarg 2005, Joseph, Porta et al. 2005, Jerath, Edry et al. 2006, Kaushik, Kaushik et al. 2006, Courtney 2009, Pramanik, Sharma et al. 2009), human studies of SRR techniques have had difficulty ruling out extraneous variables (e.g. expectancy biases developed during training) as contributors to these changes. Moreover, it has proven difficult to formulate proper control groups, and very few studies have focused on isolating SRR from attentional or emotional regulatory elements (Ospina, Bond et al. 2007). Sustained SRR could be sufficient to evoke both acute and adaptive changes in the central nervous system reflecting shifted autonomic balance (Bernardi, Spadacini et al. 1998, Spicuzza, Gabutti et al. 2000, Harinath, Malhotra et al. 2004, Pal, Velkumary et al. 2004), but new approaches are needed to isolate the impact of SRR from confounding variables.

Behavioral conditioning of respiration

We set out to resolve this issue in an animal model using behavioral conditioning procedures. Previous investigators have applied the principles of operant and classical conditioning to respiration with varying degrees of success (see Chapter 1). In brief, although classical conditioning approaches have been effective and have provided valuable information, they i) remove the volitional component involved in human SRR techniques, and more importantly ii) produce a range of respiratory responses that are not limited to changes in RR (Gallego and Perruchet 1991, van den Bergh, Kempynck et al. 1995, Nsegbe, Vardon et al. 1997), and that strongly depend on the unconditioned stimulus used to initially provoke the response. As mentioned, operant conditioning protocols have had limited success altering respiration in rats and humans (Gallego, Benammou et al. 1994, Ley 1999, Elliott and Izzo 2006), and have been used to condition respiratory behavior in the freshwater snail (Lukowiak, Ringseis et al. 1996). The only known attempt at (indirectly) reducing RR is one pilot study that conditioned inspiratory duration using electrical stimulation of the medial forebrain bundle as reinforcement (Gallego, Benammou et al. 1994) – and this study only included one rat. Avoiding brain stimulation, we sought to take a non-invasive approach to study autonomic dysfunction.

Selection of an appropriate reinforcement stimulus

Our first consideration in conditioning rat respiratory behavior was the selection of an appropriate reinforcement stimulus. We considered the merit of a large number of diverse stimuli, including but not limited to: footshock, medial forebrain bundle (MFB) stimulation, sensory stimuli including odorants (e.g. household peanut butter), sugar

water or treats (only provided after criterion is reached, e.g. through a trap door), auditory stimuli (e.g. white noise or some frequency of sound as an affective stimulus that is turned on/off when the required number of deep breaths is reached, such as aversive or appetitive ultrasonic vocalizations), pharmacological stimuli (e.g. drugs such as opioids that alter respiration), and visual stimuli (e.g. bright light or operant sensation seeking). The manner in which the stimulus would be applied could vary according to a number of considerations. For an auditory stimulus, some frequency of sound could serve as a negative stimulus that is turned off when a predetermined number of deep breaths is reached. For odorants, a fan could be triggered to waft odor into the chamber, or else the odor would be prevented from reaching the chamber by ongoing vacuum activity.

Shock and intracranial self-stimulation were ruled out due to their incredibly potent short- and long-term effects on animal physiology and behavior (Van Dijken, Van der Heyden et al. 1992, Burgess, Davis et al. 1993, Louvart, Maccari et al. 2005). We ruled out odorants and treats due to the inherent difficulty in matching their discrete administration to a continuous behavior (respiration), whereas white noise and ultrasonic vocalizations were ruled out as first-pass attempts due to possible habituation effects, and relative novelty as experimental reinforcement in the latter case. While bright light could result in a long-term impairment of visual function, previous studies have shown that it maintains its aversive salience for at least 60 hours in albino rats (Barker, Sanabria et al. 2010). For these reasons, and additional ones documented below, we chose bright light as our first pass negative reinforcement stimulus.

Bright light is aversive to rats, and has previously been used as a primary operant (i.e. rats will perform a task to avoid it) (Flynn and Jerome 1952, Kaplan 1952, Barker,

Sanabria et al. 2010). Work in the 1940s demonstrated that rats will press a lever to turn off a light, and that increased light intensity leads to a greater rate of responding (Keller 1941). However, the impact of light as a negative reinforcer depends on the particular paradigm used, and the schedule of reinforcement (Kaplan 1952, Kaplan 1956, Kaplan, Jackson et al. 1965). Light may lose its aversive salience at lower intensities, although this conclusion is debated (Lockard 1963, Lockard 1964, Campbell and Messing 1969). Interestingly, in certain cases light may even act as a positive reinforcer (Roberts, Marx et al. 1958, Barry and Symmes 1963, Goodrick 1970). More recently, Olsen and Winder developed the operant sensation seeking (OSS) paradigm (Olsen and Winder 2009), whereby mice acquired operant responding for the presentation of dynamic visual stimuli. Therefore, light's use as a reinforcer can vary depending on the specific parameters used.

It may be that providing intermittent bright light (strobe) maximizes the efficacy of bright light as an aversive stimulus to alter animal behavior. Strobe light is a common component of chronic mild stress paradigms (Forbes, Stewart et al. 1996, Bortolato, Mangieri et al. 2007) and is conventionally regarded as a mild stressor to the rodent; however, it can also provoke changes in the electrical activity of the brain that mimic those of a pre-seizure state, with repeated exposure sensitizing this response (Uhlrich, Manning et al. 2005). Despite concern about seizure susceptibility, we expected that the sensitizing effects of strobe could result in maintained aversive salience over many sessions of conditioning, thereby overcoming potential habituation effects. We did not observe any behavioral indicators of seizures in our animals, and noted similar activity levels throughout the duration of training (see Results).

Therefore, following extensive reading of the literature and several early experiments using bright light, we decided to attempt a conditioning approach focused on one specific respiratory parameter (RR) and a relatively novel, mildly aversive reinforcement stimulus – strobe light. We expected that continuous RR-based control of the light over many hours would gradually train rats to slow their breathing.

Materials and Methods

Experimental Animals

Adult male Sprague-Dawley rats (~90 days old at the start of experimental procedures) were housed in standard cages in a vivarium on a reverse 12:12-h light-dark cycle and were fed *ad libitum* standard rodent diets. All experiments were approved by the Animal Care and Use Committee 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."

Developing an Experimental Paradigm for Light-Based RR Conditioning

Whole-Body Plethysmography Setup for Recording Respiration

Using whole-body plethysmography (WBP), it is possible to monitor RR in freely behaving animals (Bartlett and Tenney 1970). During WBP, inhaled air warms to the animal's body temperature and is humidified, an effect that is reversed during exhalation. When an animal is inside an airtight WBP chamber, these changes in air temperature, moisture, and correspondingly volume, induce concomitant changes in chamber pressure

(Drorbaugh and Fenn 1955), according to Boyle's law. A pressure transducer can measure these changes and convert them to an accessible voltage readout in LabVIEW.

We continuously monitored RR using WBP. Respiratory measurements during a given session were collected similarly to those described previously (Wilkinson, Huey et al. 2010). Individual rats were placed in Plexiglas plethysmography chambers (PLY3215, Buxco Research Systems), and respiration was measured using continuous flow barometric plethysmography (Jacky 1980). A flowmeter (Cole-Parmer) was set to provide compressed air at a constant rate of 3 liters/minute, while PE-20 tubing filling the top 7 cm of the inflow line provided high input impedance. Air exited the chamber via a vacuum valve (SS-4MG, Nupro) connected to a vacuum pump. We adjusted the vacuum valve (while keeping the inflow set to 3 liters/min) in small increments as necessary to maintain a chamber pressure near atmospheric level, as monitored via water manometer. Pressures remained relatively stable and adjustments were rarely necessary once a given two-hour conditioning session had commenced. Pressure changes were measured using a differential pressure transducer referenced to atmosphere (DP45, Validyne); output from a connected carrier demodulator (CD15, Validyne) was sent to a digital data acquisition system (PCI-6221 multifunction DAQ board, National Instruments). Analog signal was digitized at unity gain and a sample rate of 1 to 10 kHz. The digitized data was continuously output to a Windows computer running LabVIEW (National Instruments). Prior to each experiment, calibration air pulses ranging from 0.5-2 ml were injected into the chamber using a syringe, to confirm signal fidelity.

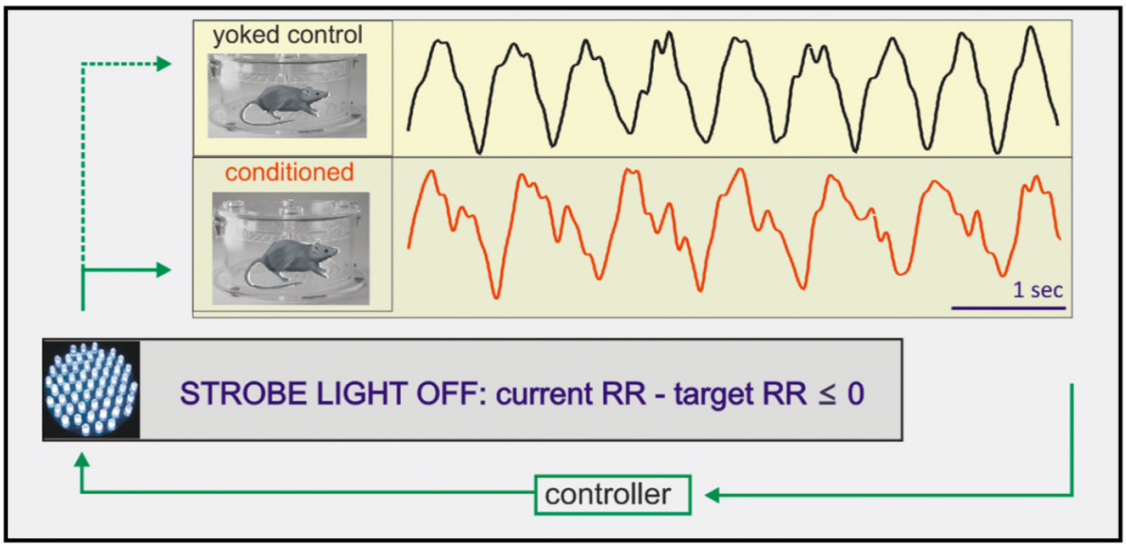


Figure 2.1. Use of light as negative reinforcement for respiratory rate feedback.

Schematic depicts feedback procedures and representative respiratory traces from control (*top*) and experimental (*bottom*) animals over identical five-second time intervals. Respiratory rate (RR) was continuously recorded via whole-body plethysmography. For conditioning, real-time RR was compared to a predetermined target RR by a controller coupled to a transducer to control light intensity, such that the strobe light was turned off whenever current RR ≤ target RR. Animals thereby experienced real-time variation in light intensity corresponding to their RR, representing a closed-loop feedback control system for conditioning of SRR. In the example traces shown, the average RRs were 70.5 (conditioned) and 91.0 (yoked control) breaths/minute.

For lighting, a specialty built array of LED lights was used for visual reinforcement (Figure 2.1). The array consisted of two floodlights projecting from above and below the plethysmography chamber, in order to prevent animals from burrowing to escape the stimulus (Barker, Sanabria et al. 2010). When the strobe light was on, light intensity at the chamber floor was 8073 lux, measured with a digital light meter (Extech Instruments). A red LED bulb provided constant background lighting largely outside of the rat visual spectrum (Burn 2008). Because LEDs do not produce heat in the form of infrared radiation, there was negligible increase in the surrounding temperature. Due to

our desire to follow a noninvasive protocol, we did not measure animal body temperatures during experimentation. Ambient chamber temperature was measured at several time points and remained relatively constant.

LabVIEW Feedback Program and Behavioral Conditioning Paradigm

Recorded data was processed by a customized software interface in LabVIEW (Bill Goolsby) to monitor respiration and provide visual feedback concurrent with plethysmographic recordings. There were a number of controllable parameters, as shown in Figure 2.2. Knowing the physiological range of respiratory frequencies, we preset the interface to sample within this range (low-pass: 5 Hz, high-pass: 1 Hz), and for a user-defined number of breaths to be captured in a sample block. For conditioning, measured RR was compared to a preset target value in the 'test' SRR conditioned population and rates below this value turned the light off. The Buneman Frequency Estimator subroutine was called to find the dominant peak, with the output being RR in breaths/minute. This value was fed into a feedback controller, which evaluated it against a user-defined target RR and activated the array of LEDs to provide animals with time-varying light intensity cues throughout a given trial. The Buneman estimate is better than the traditional fast Fourier transform (FFT) because it will interpolate the peak in the frequency spectrum, even if it is between discrete points on the spectrum graph.

Real-time RR was compared to a desired target rate by a controller that was coupled to a transducer to control light intensity, according to the equation: Light Intensity = Feedback gain x (Current RR - Target RR). For the experiments presented here, we chose infinite gain in order to provide all-or-none light intensity. Animals

thereby experienced real-time variation in light intensity (on or off) corresponding to their RR, representing a closed-loop feedback control system for conditioning of RR. Adjustable variables included target RR, gain (change in light intensity per unit increase in RR), and maximal light intensity.

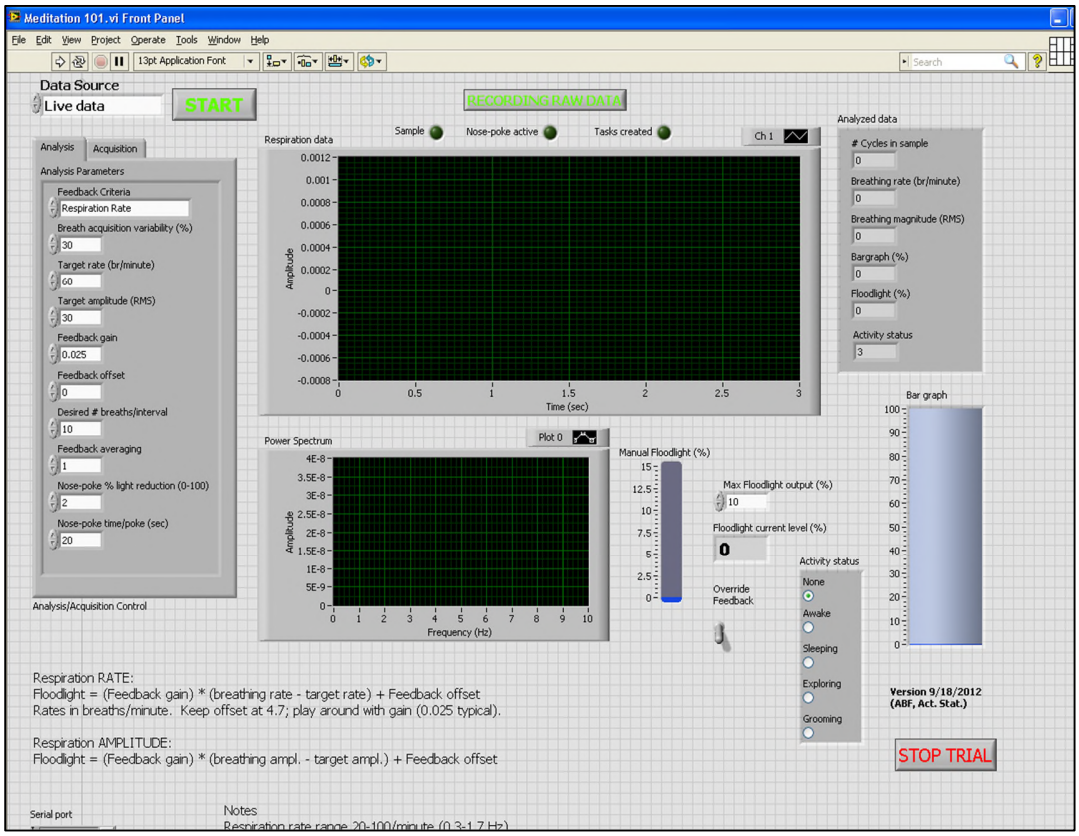


Figure 2.2. The LabVIEW feedback program interface.

Our customized LabVIEW program allows a number of parameters to be set by the user. These include target rate, feedback gain, feedback offset, maximum floodlight output, and the desired number of breaths per interval. Throughout the session, RRs are monitored by using the Buneman Frequency Estimator to determine the dominant respiratory rate over a predetermined interval or number of breath cycles. Upon session termination, raw voltage traces are automatically saved for subsequent analysis with pCLAMP Analysis Software.

Once parameters were set for a given session, the program was started and allowed to run for the allotted time. Throughout the session, animal activity levels and RRs were monitored and compared to voltage readouts in the interface by an observer remotely following the animals in an adjacent room. Upon termination, trial results were automatically saved as .abf and .txt files (for analysis with pCLAMP Analysis Software or for import into Microsoft Excel, respectively). The corresponding video file for the trial was also saved. Simple calculations in Excel produced a value of '% of samples meeting criterion' for each session, for each rat, to assess the efficacy of strobe-based negative reinforcement.

Measurement of Resting RR and Determination of % Resting vs. Movement

Dependent variables measured were average RR (breaths per minute) and the percentage of samples meeting criterion (the percentage of captured epochs, each 10 breaths long, where current RR $\leq$ target RR). Although % of samples meeting criterion provided a direct measure to assess the success of our training paradigm, it did not address the key question of whether operant procedures resulted in a trained reduction in resting RR over time. This question is important since slowed baseline respiration appears to be a trigger for a number of therapeutic benefits associated with the relaxation response, as outlined in the Introduction and Chapter 1. Therefore, we also measured resting RR.

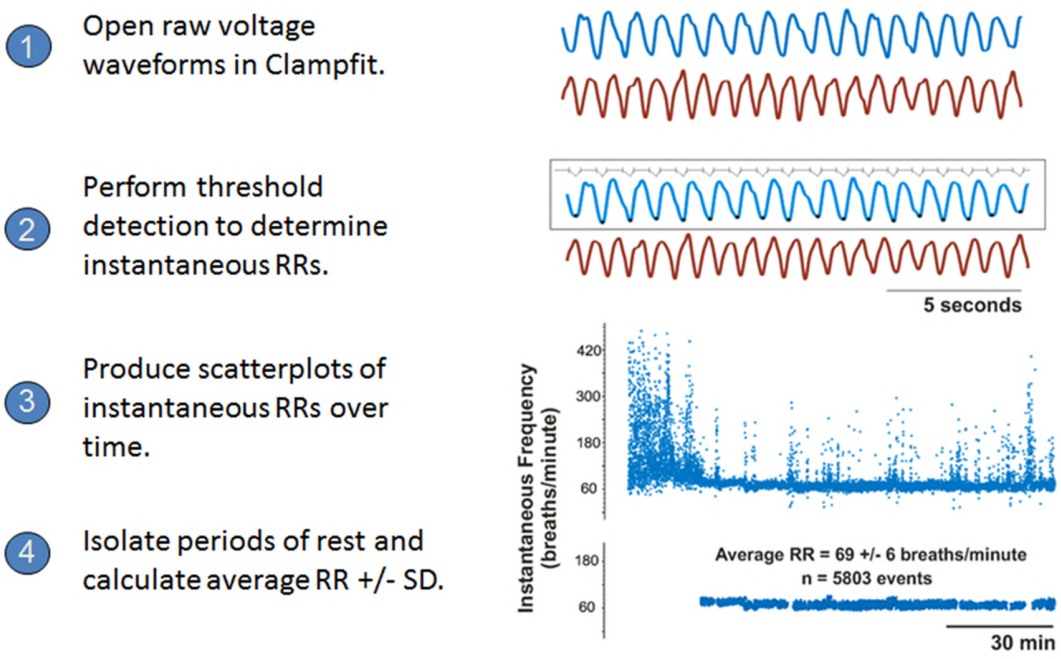


Figure 2.3. Calculation of average resting RR and % of trial at rest.

To determine an animal's average resting RR and % of time at rest for each session, we detected individual breaths and removed movement-related values to isolate periods of rest. After opening raw voltage waveforms in Clampfit (1), we applied a 5 Hz low-pass Gaussian filter to the full voltage traces. We then performed threshold detection to determine instantaneous RRs (2), which can be displayed over time in scatterplot form (3). Removal of the first 20 minutes of events for each trial (acclimation period) as well as movement events (high-variability periods of instantaneous RR in the scatterplot) (4) left only periods of resting RR. Clampfit then reported the average resting RR +/- standard deviation values for these remaining events. % resting was subsequently calculated as (1/average resting RR in Hz) x (number of events) / (total session time in seconds) x 100.

Raw plethysmographic recordings output from LabVIEW provided adequate information to score an animal's average resting RR (Figure 2.3). I applied a 5 Hz low-pass filter to these raw recordings and then performed threshold-based analysis to obtain instantaneous frequencies over individual two-hour trials, excluding the first 20 minutes of each trial as acclimation. Threshold-based analysis was performed in Clampfit

(pCLAMP Analysis Software). Briefly, for each session, for each rat, a low voltage threshold was set, such that a complete respiratory cycle (inhalation and exhalation) triggered the detection of a breath at the peak or trough of the cycle. The program automatically calculated the instantaneous frequency of these events. Scatterplots of instantaneous frequency over time were then produced to isolate periods of resting RR. Periods of rest were confirmed via video recordings to correspond to easily identified epochs of low variability in the instantaneous frequency scatterplots. The other epochs of instantaneous frequency in the scatterplots were not indicative of resting RR and were assumed to be movement based on video recordings. Resting periods were isolated from the scatterplots, and average RR (± standard deviation) was calculated. Therefore, values represented mean resting RRs over the course of ~100 minute periods, with animals divided into SRR conditioned rats and yoked controls. For the determination of % time at rest, I multiplied the inverse of average instantaneous frequency (as determined above) by the number of samples to calculate the total time at rest, divided this value by the total session time, and multiplied by 100 to get the value '% resting'.

Experimental Timeline

Baseline plethysmographic recordings to establish the timeline of RR stabilization

It was first important to record baseline measurements to establish the timeline of RR stabilization. This was because, if slow breathing alleviates stress, it is important that adaptation of RR to stress or novelty of experimental context not be a confounding factor in conditioning studies. Our paradigm consisted of running four rats (separate from those used in conditioning studies) in plethysmography chambers with only a red background

light, for two-hour periods, over the course of many days. During these sessions, RR was recorded for each individual animal.

Assignment of experimental groups – SRR conditioned rats vs. yoked controls

Prior to acclimation of experimental animals – so as not to bias group assignments – paired rats were randomly assigned as "SRR conditioned" or "yoked control," and were then placed in dual chambers (counterbalanced for side) to begin acclimation. SRR conditioned rats were so-named because during later conditioning they were able to turn off the strobe light when current RR $\leq$ target RR, while yoked control rats passively received the same stimulus (Figure 2.1). During acclimation, nose poke, and RR conditioning, an opaque barrier prevented animals from seeing each other, while the vacuum used for air outflow provided constant background noise to prevent auditory communication (although ultrasonic vocalizations could not conclusively be ruled out) during experimental procedures.

Acclimation to plethysmographic chambers and nose poke sessions for trainability

Based on results from our baseline plethysmographic recordings (described above and presented under Results), we acclimated all animals to be used for conditioning studies to the plethysmography setup for 12 hours over 3-6 sessions prior to commencement of experimental procedures. During at least the last 8 hours of this time (when animals typically began to spend substantial amounts of time at rest), RR was recorded in the plethysmography chambers. The last two hours of acclimation (once RR had stabilized, according to our results below) were used for analysis as our "Baseline"

period. 1-2 days following the final acclimation session, paired animals were once again transferred to plethysmography chambers, and conditioned rats were trained in a simple nose poke task to establish animal trainability. This task lasted for 30 minutes, and was repeated over three days. During these sessions, the strobe light turned off whenever the animal poked its nose into a small aperture with an infrared beam designed to detect each poke. All conditioned rats that performed ≥ 20 nose pokes per session by the 3rd 30-minute session, and their yoked controls, were included in the study. These sessions also had the effect of acclimating animals to all experimental conditions (including differential stimulus controllability) except for RR-based conditioning.

Exclusion Criteria

In order to prevent baseline data biases and screen for rats able to learn an operant task, we preset exclusion criteria for respiratory conditioning. Experimental rats were excluded if baseline differences in RR were greater than 20 breaths/minute following acclimation (n=2 pairs met this criterion), or if animals performed less than 20 nose pokes by the third and final nose poke session (n=2 pairs met this criterion). Typically, baseline differences were observed when animals of different ages (3-6d) were housed together by animal facility staff, and therefore this was avoided as much as possible. All other animals presented in the following results had baseline respiratory differences ≤ 20 breaths/minute and achieved ≥ 20 nose pokes by the final session, and therefore underwent a full 20 sessions of RR conditioning.

SRR Conditioning Sessions

Feedback-based conditioning of reduced RR was accomplished as described above, over the course of 20 sessions, approximately 5 days per week. During the study's earlier stages, animals occasionally received two training sessions per day under the premise that this would hasten learning. Due to i) concerns about overexposing animals to strobe light and its impact on learning, behavior, and circadian rhythms, and ii) our desire to mimic chronic mild stress paradigms by increasing the unpredictability of our reinforcement schedule, we occasionally performed less than 5 sessions per week later on in our study. Therefore, conditioning procedures were conducted on a pseudo-random schedule to maximize strobe's aversiveness as a stressor; while chronic mild stress paradigms often vary the modality of experimental stressors, we instead varied the schedule of administration. Data from all animals was pooled for experimental analysis because there were no quantifiable differences in SRR conditioning performance between animals conditioned more or less regularly.

Controllability and Learned Helplessness

Since the degree of control an animal has over its environment influences the autonomic response to negative (Maier 1986) or positive (Burgess, Davis et al. 1993) reinforcement, we established an additional control population to exclude the possibility that reinforcer controllability was an essential variable contributing to the reduction of RR in SRR conditioned rats. A separate subgroup of n=4 'FRR conditioned' rats were assigned to receive "up-conditioning" for fast (instead of slow) breaths. Procedures were identical to those for SRR training (described above), except that high (vs. low) RRs were

reinforced, so that the aversive strobe light turned off when current RR ≥ target RR. Any remaining 'parasympathetic' shift in outcome measures could suggest that stimulus controllability, rather than slow breathing, was responsible for improvements associated with training in SRR conditioned rats. The target RR for up-conditioning was determined such that rats received approximately the same amount of light as during SRR conditioning. Following identical acclimation and nose poke procedures as for SRR conditioning, paired FRR conditioned rats and yoked controls underwent 20 sessions of up-conditioning to control for the learned helplessness effects inherent in our yoked reinforcement paradigm.

Statistical Analysis

Data are presented as mean ± SEM, unless otherwise noted. Average resting RR and % under threshold values were analyzed with repeated measures (RM) ANOVA with session as a within-subjects factor, and group (SRR or FRR conditioned vs. yoked) as a between-subjects factor when 2-way RM ANOVAs were used. *Post-hoc* tests (Holm-Sidak method) corrected for multiple comparisons were performed in the case of significant results. When Normality or Equal Variance tests failed, the Friedman RM ANOVA on Ranks (one-way ANOVA alternative) or linear regression analysis (2-way ANOVA alternative) were performed. Statistics were performed with SigmaPlot 13 with significance set at $p<0.05$ and two-tailed tests.

Results

Baseline RR stabilizes over six two-hour sessions.

There was a gradual decrease and stabilization of RR. It took between 6-8 two-hour sessions to reach a stable baseline RR (Figure 2.4), much longer than typical experimental acclimation periods (see Discussion). Average RR decreased linearly over the first six sessions ($R^2 = 0.93$, $p<0.05$), with a slope of RR reduction of 8.84 breaths per two-hour session and a final average of 86.6 breaths/minute by the eighth two-hour session, at which point RR had plateaued. The average value of RR for the first two sessions was 125.0 and for the last two was 89.4 breaths/minute. The reduction was significant ($P < 0.001$; Student's t-test). Subsequent studies confirmed that the period of acclimation required to reach steady state RR (i.e. 12 hours) was also observed over an equivalent number of hours when they were condensed into three or four (instead of six) sessions. This work established the required acclimation period to achieve a stable RR for subsequent conditioning studies described below. Because RR reached a stable baseline by 12 hours, all subsequent animals were acclimated for 12 hours prior to conditioning. Importantly, these results guided the experimental design for conditioning procedures, with average values of RR (and their variability) during acclimation hinting at what would be a realistic target RR for feedback procedures.

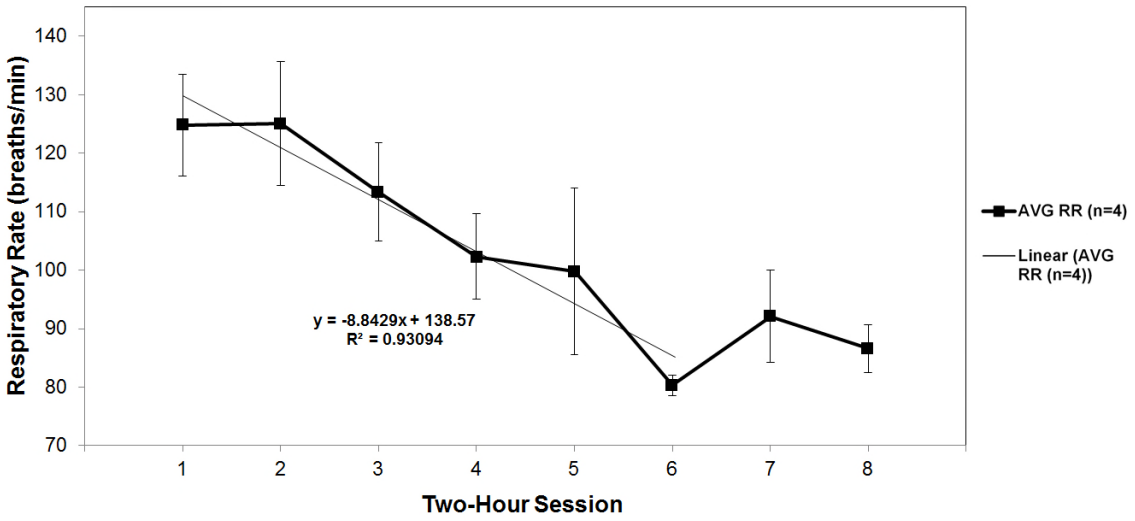


Figure 2.4. Baseline acclimation takes approximately twelve hours.

Average ± SEM values for RR are shown for n=4 rats over eight two-hour sessions, with an overlaid line of best fit for the first six sessions. RR reached an apparent plateau of 86.6 breaths/minute by the 8th session. This was a dramatic reduction from RRs during the earlier sessions: some examples were 124.8 breaths/minute (1st session), 125.1 breaths/minute (2nd session), and 102.3 breaths/minute (4th session). Calculating the line of best fit over the first six sessions demonstrated a clear linear trend in RR reduction during this time, of 8.84 breaths per two-hour acclimation session. This plateaued starting at Session 6, such that incorporating additional points into the trend line weakened the linear correlation ($R^2 = 0.93$ through Session 6 vs. $R^2 = 0.91$ through Session 8).

Conditioned rats spend progressively more time breathing below the conditioned RR threshold of 80 breaths/minute.

As described in Methods, strobe light was used as negative reinforcement for conditioning reduced RR. The preset criterion to turn off the strobe was RR ≤ 80 breaths/minute. Figure 2.5A shows the percentage of samples where RR met this criterion value in SRR conditioned and yoked control animals over the training sessions. SRR conditioned rats displayed a sharp initial increase in the incidence of breaths below the target RR of 80 breaths/minute followed by a more gradual increase over training

sessions. This increase was significant ($p<0.001$, one-way RM ANOVA). The greatest inter-session % increase occurred between baseline and Session 1 (57.3% increase), while the magnitude of the dependent variable peaked during Session 15 (60.9% of samples under threshold). For yoked controls, the greatest % change also occurred between baseline and Session 1, but was much smaller (13.9% increase), as was the highest magnitude reached (37.7% of samples under threshold during Session 15). Nonetheless, conditioned rats had a greater incidence of RR events below the preset light-off value than yoked rats ($P = 0.015$, 2-way RM ANOVA: significant main effect of Group). This effect was especially conspicuous during Session 14, when conditioned animals spent 58.6% of the session, on average, below the target RR vs. 32.2% for yoked controls. Multiple comparisons analysis using the Holm-Sidak method revealed a significantly higher % of samples below threshold in SRR conditioned vs. yoked rats during Sessions 4, 6, 8-10, 12, 14-17, 19, and 20.

Interestingly, there was a progressive increase in incidence of lower RR events in yoked rats over sessions ($P = 0.003$, one-way RM ANOVA), which was broadly comparable in shape to that in SRR conditioned rats. While our dependent variable – % of samples under threshold – directly related to dark periods in the SRR conditioned population by definition, there was also a correlation between these dark periods and the incidence that yoked rats spent under SRR threshold ($R^2 = 0.23$, $p<0.05$; Figure 2.6A). This observation suggests that yoked rats had increased RRs during sessions with greater strobe light exposure, possibly reflecting a respiratory response to the stressor. Alternatively, other shared experiences between paired yoked and conditioned rats (e.g.

handling, animal facility care) could have contributed to parallel changes in respiratory behavior (see Chapter 3 Discussion).

Conditioned rats slow their breathing over the course of training using respiration-based feedback to reduce RR.

Resting RR values during the baseline period (i.e. the final two hours of acclimation) were statistically indistinguishable from those established in Figure 2.4 ($P = 0.224$), suggesting that all animals were acclimated prior to commencing conditioning procedures. SRR conditioned animals decreased their resting RR over 20 conditioning sessions ($p<0.001$, one-way RM ANOVA; Figure 2.5B). Although the dependent variable – average resting RR – was determined independently of % of samples under SRR-conditioned threshold (Figure 2.3), resting RR nicely mirrored the trend for % of samples under threshold. Effects were greatest during Session 15, when average resting RR was lowest in SRR conditioned rats (75.9 breaths/minute). The decrease in resting RR in SRR conditioned rats was also substantial in magnitude, reaching a peak of 16.1 breaths/minute during Session 15 (from 92.0 breaths/minute at baseline, a 17.5% decrease). While yoked rats also showed a decrease in RR over sessions ($p<0.001$, one-way RM ANOVA), SRR conditioned rats underwent significantly greater reductions in RR. This was seen as an overall difference ($P = 0.043$, 2-way RM ANOVA: significant main effect of Group) and was most apparent during the later sessions (Session 10 and on). Specifically, during Sessions 10, 12, 14-16, and 20, SRR conditioned rat resting RR values were ≥ 6 breaths/minute lower than yoked control values. Indeed, follow-up testing using the Holm-Sidak method for pairwise multiple comparisons procedures

revealed a significant difference between SRR conditioned and yoked animals in resting RR during Sessions 10, 14-16, and 20 ($p<0.05$). Note that our data did not pass a normality test (Shapiro-Wilk). However, the requirement of normality for a two-factor ANOVA is debatable, and linear regression analysis (a non-parametric substitute for two-factor ANOVAs) confirmed all significant results reported here. Individual RRs for SRR conditioned and yoked rats for each two-hour session were also correlated ($R^2 = 0.26$, $p<0.05$), suggesting, as above, that yoked rats may have either found sessions with less light to be less stressful, or that shared experiences (e.g. handling, animal facility care) on each day of testing led to parallel changes in respiratory behavior (see Chapter 3 Discussion). Furthermore, note that the results for % of samples under threshold and resting RR both suggest that the efficacy of conditioning began to wane slightly during the final few sessions. This is perhaps indicative of overexposure to the sensitizing strobe stimulus or, conversely, to diminished aversive salience of the strobe. Possibilities are addressed in the Discussion.

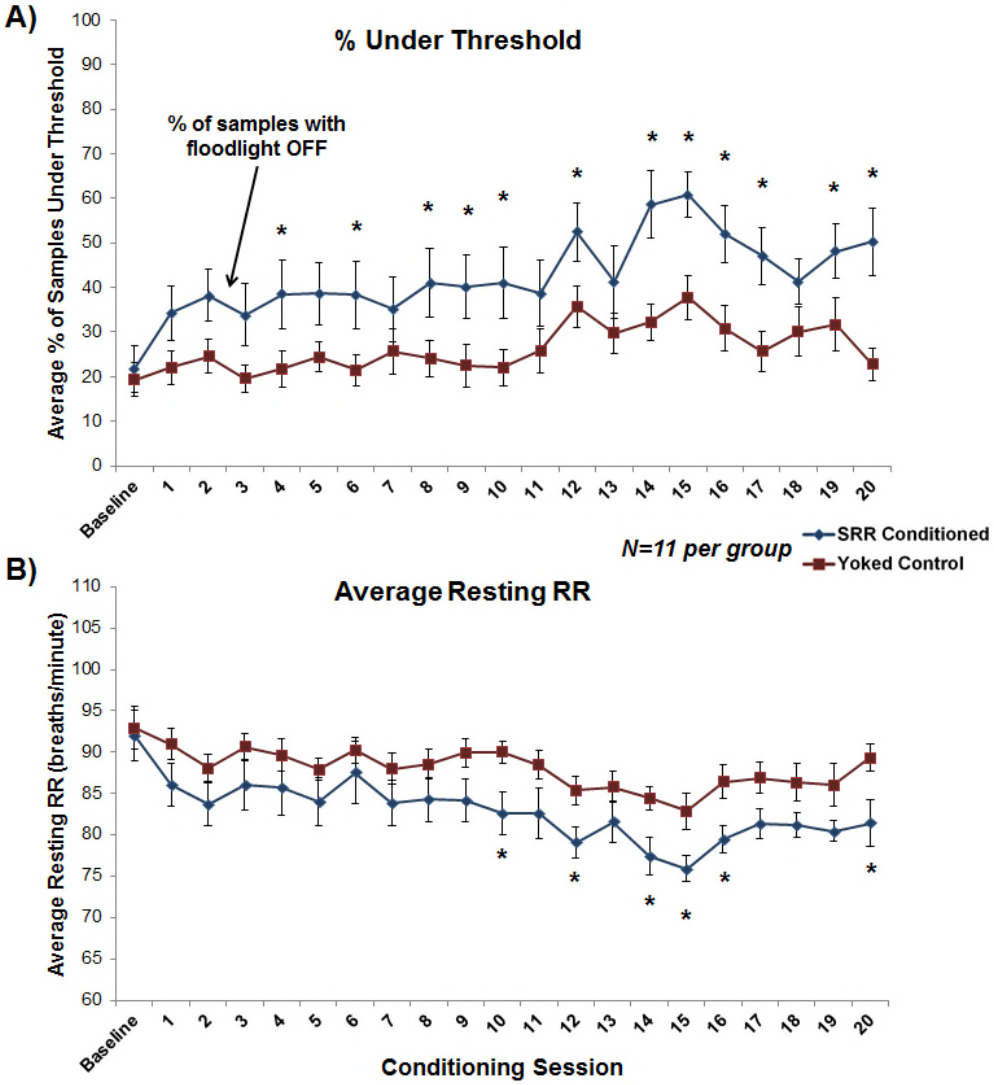


Figure 2.5. SRR conditioned rats breathe more slowly and spend more time breathing below the conditioned RR threshold of 80 breaths/minute.

Group differences in respiration emerged over the course of 20 sessions. **(A)** The percentage of samples where RR met criterion (≤ 80 breaths/minute). SRR conditioned rats displayed an increasing incidence of breaths below the target RR (*1-factor RM ANOVA*), and a greater % of samples under threshold than yoked controls (*2-factor RM ANOVA: significant main effect of Group*) over training. **(B)** SRR conditioned rats decreased their average resting RR by 10.6 breaths/minute (*1-factor RM ANOVA*) and their RR was lower than that of yoked controls (*2-factor RM ANOVA: significant main effect of Group)* over training. Note in (A) that % of samples under threshold for SRR conditioned rats is by definition % of samples in darkness, whereas for yoked rats the % of samples under SRR threshold did not have a direct relationship with the light being on or off. * *$p<0.05$ SRR conditioned vs. yoked, post-hoc Holm-Sidak comparisons*

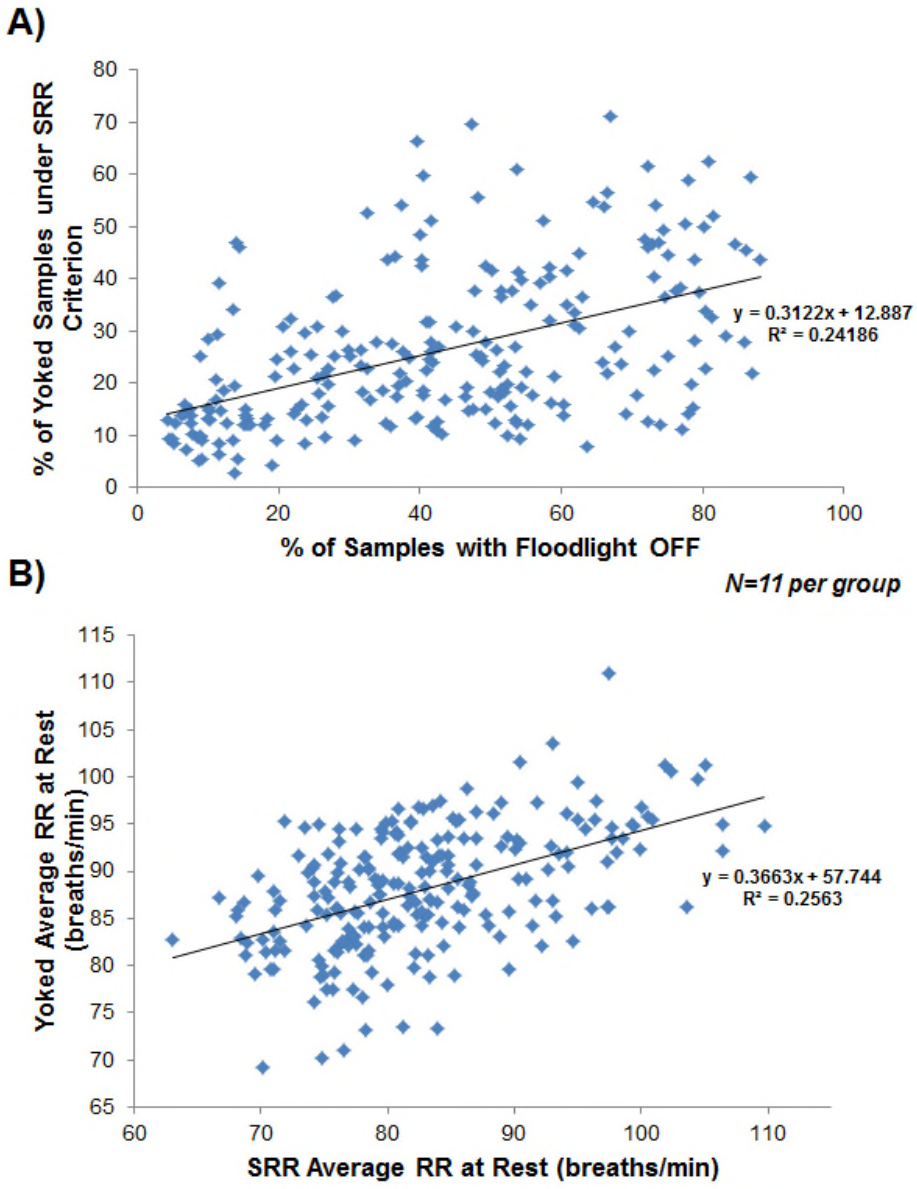


Figure 2.6. SRR conditioned and yoked rat respiratory measures are correlated.

(A) Correlation analysis shows that yoked control % under threshold values correlated with the % of samples with floodlight turned off during conditioning ($p<0.05$). This correlation also represents that between % of yoked samples under SRR threshold and % of SRR samples under threshold, since the latter directly determined the % of samples with the floodlight turned off. **(B)** SRR conditioned and yoked rat RRs at rest were also correlated. Plotting average resting RRs (determined as shown in Figure 2.3) and performing correlation analysis reveals that SRR conditioned and yoked control rats tended to breathe at similar rates during a given session ($p<0.05$).

The distribution of instantaneous RRs shifts over the course of 20 sessions and conditioned rats breathe more regularly.

SRR conditioned and yoked rat RR distributions shifted over the course of 20 training sessions (Figure 2.7). This was controlled by two different and potentially related changes that occurred during conditioning: i) decreased RR in SRR conditioned rats, as shown in Figure 2.5B, and ii) decreased variability of RR in conditioned rats. Conditioned rats, but not yoked controls, breathed more regularly at rest over training; quantitatively, they had a reduced respiratory 'Regularity Index' – that is, mean variability (measured with standard deviation values) in RR – over the course of 20 sessions ($P = 0.027$, Friedman RM ANOVA on Ranks). Although a two-factor repeated measures ANOVA did not reveal statistically significant difference in the Regularity Index between groups ($P = 0.152$), there was a trend toward reduced values – indicating greater regularity of RR – for SRR conditioned vs. yoked rats that was especially apparent during middle to later sessions, with the standard deviation decreasing from 12.4 breaths/minute to 8.4 breaths/minute by Session 15 (Figure 2.8). SRR conditioned rats showed significantly increased regularity of breathing vs. controls during Sessions 14, 15, and 20 ($p<0.05$, *post-hoc Holm-Sidak comparisons*).

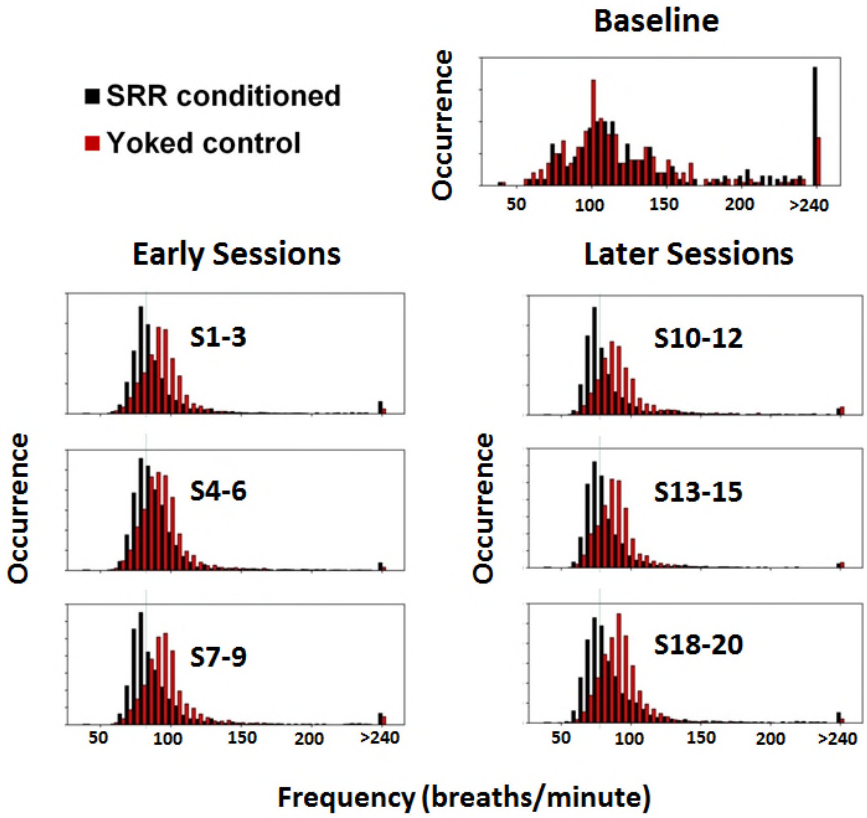


Figure 2.7. SRR conditioned and yoked rat RR distributions differ over the course of training.

Frequency histograms show the gradual divergence of RRs in a representative pair of SRR conditioned (*black*) and yoked control (*red*) animals. Decreased rates (on the x-axis) and variabilities (represented here by the relative width or narrowness of the distribution) are easily visualized in conditioned rats.

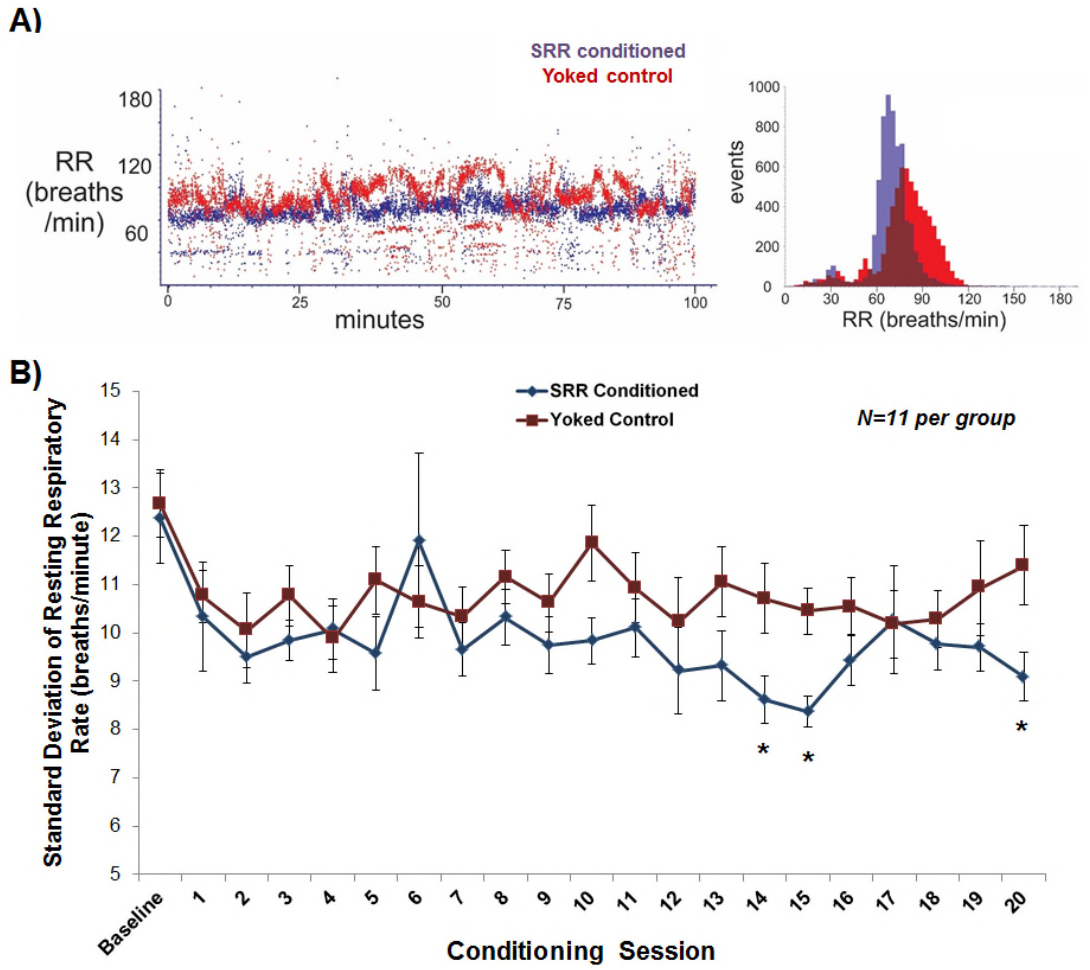


Figure 2.8. Respiration becomes more regular over 20 sessions in SRR conditioned rats.

(A) Scatterplots (produced as shown in Figure 2.3) and frequency histograms show the distribution of instantaneous RRs over a representative two-hour session. Note greater variability in RR in the yoked animal. **(B)** Mean variability (measured with standard deviation values) of resting RR was used as a 'Regularity Index' of breathing. This index was reduced at Sessions 14, 15, and 20 in SRR conditioned vs. yoked rats, indicating more regular respiration. **p<0.05 SRR conditioned vs. yoked, post-hoc Holm-Sidak comparisons*

Changes in the % of time at rest do not account for the learned reduction in RR.

By performing the calculation described in the legend of Figure 2.3, we obtained values for % of time resting for each rat over the course of baseline and 20 training

sessions. There were no differences between SRR conditioned and yoked rats in % at rest during any of these sessions ($p>0.05$, 2-way RM ANOVA; Figure 2.9), despite low variability in these values within each group, and yoked rats frequently spent similar or greater amounts of time at rest, irrespective of early vs. late sessions. For instance, this occurred during Sessions 12 and 18. The result for Session 12 is particularly striking, since post-test comparisons following a two-way RM ANOVA revealed that SRR conditioned rats breathed at a reduced rate and had a greater % of samples under threshold during Session 12, when yoked rats actually spent more time at rest. This suggests that SRR rats did not learn to spend more time at rest – when RR would more likely be under training criterion (Pappenheimer 1977) – in order to turn off the aversive strobe light.

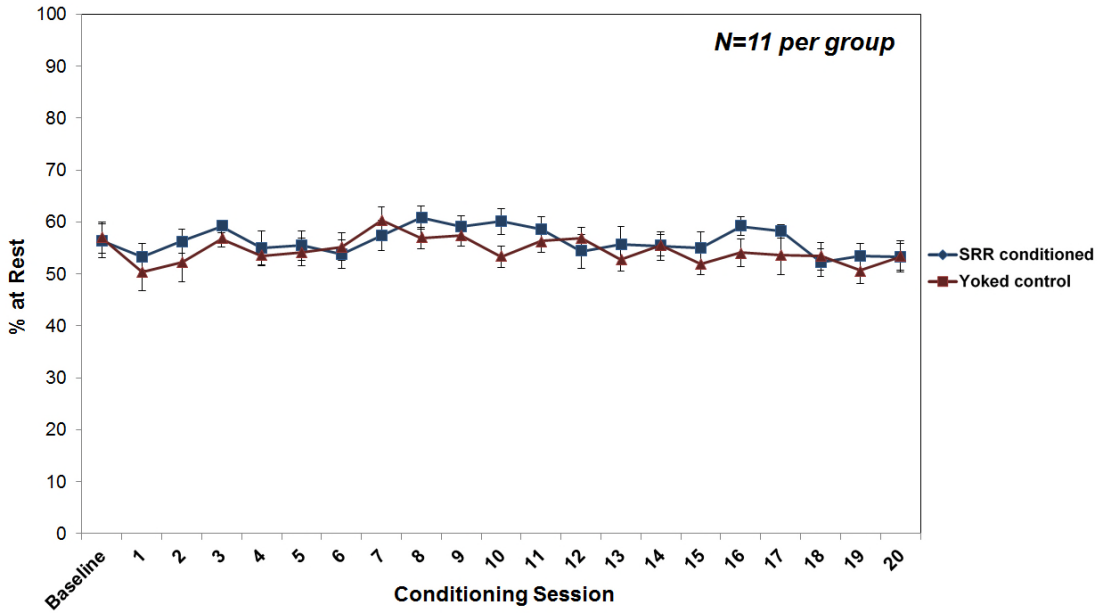


Figure 2.9. SRR conditioned and yoked rats spend similar amounts of time resting.

Average percentages of each two-hour session spent resting (% resting) for SRR conditioned rats and yoked controls are shown for baseline and Sessions 1-20. Animals typically spent between 50-60% of each trial at rest, with no differences between groups over the course of training. This suggests that factors other than differential activity levels were responsible for reducing resting RR in SRR conditioned rats.

Conditioning RR increase controls for controllability and learned helplessness effects.

In a subset of rats, conditioning procedures were modified such that aversive bright light turned off when current RR ≥ target RR, in order to control for controllability and learned helplessness effects. This was called fast respiratory rate (FRR) conditioning. FRR conditioned rats successfully learned the modified training criterion over 20 sessions (Figure 2.10), spending an increased percentage of each session over the desired target by Session 20 (87.4 ± 7.5% in FRR conditioned rats vs. 51.3 ± 7.3% in yoked controls). A 2-way RM ANOVA revealed a significant main effect of group ($P = 0.045$). *Post-hoc* multiple comparisons tests (Holm-Sidak method) revealed significant group

differences in the average % of samples meeting criterion during Sessions 12-17 and 19-20, with a maximum difference of 46.3% samples over threshold (FRR minus yoked) occurring during Session 19. FRR conditioned animals also showed a trend toward increased resting RR over 20 conditioning sessions ($P = 0.065$, one-way RM ANOVA; Figure 2.10), with RR increasing from 89.5 ± 2.8 to 91.4 ± 1.7 breaths/minute from baseline to Session 20. Although this was only a trend, the absence of any RR reduction from acclimation through Session 20 is impressive, and in fact habituation effects may have precluded an absolute increase in RR (see Discussion). In contrast, yoked controls showed a slight trend toward *decreased* resting RR over 20 conditioning sessions ($P = 0.158$, one-way RM ANOVA), with RR decreasing from 86.2 ± 4.3 to 79.7 ± 4.1 breaths/minute from baseline to Session 20). Comparison between FRR conditioned and yoked rat RRs revealed a statistically significant interaction between Group and Session ($P = 0.022$, 2-way RM ANOVA). As for SRR conditioning, this was most apparent during the later sessions (Session 12 and on), although there were also impressive group differences during Session 5. Pairwise multiple comparisons tests (Holm-Sidak method) revealed a significant difference between FRR conditioned and yoked animals in resting RR during Sessions 5, 19, and 20 ($p<0.05$), with the maximal 13.7 breaths/minute difference occurring during Session 19. As for SRR conditioning, the FRR conditioned rat resting RR "learning curve" nicely resembled that for % of samples meeting threshold (*not shown*), with early group differences diminishing during middle sessions and then reemerging around Sessions 12-20. Not shown is that respiration also showed a trend toward becoming more variable (larger Regularity Index, i.e. increased standard deviation of RR within a given session) in FRR conditioned vs. yoked rats over the

course of training (P = 0.127, 2-way RM ANOVA), with *post-hoc* analyses revealing significantly more variable breathing in FRR conditioned rats during Sessions 5, 8, 16, and 18 ($p<0.05$, Holm-Sidak method).

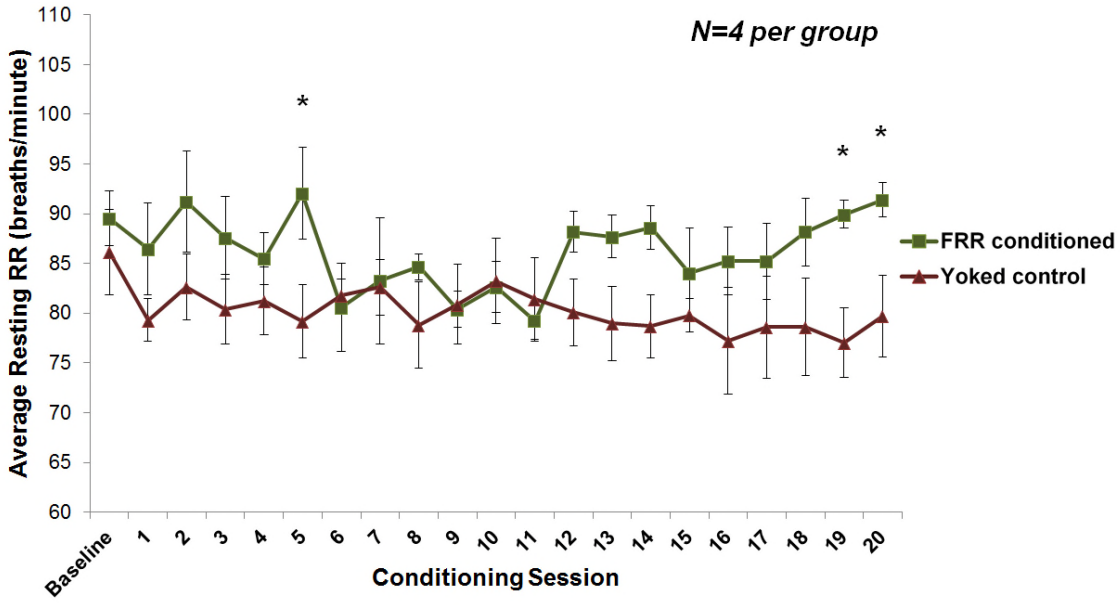


Figure 2.10. FRR conditioning increases the occurrence of fast breaths.

FRR conditioned vs. yoked control rat resting RR increased over 20 Sessions (*2-factor RM ANOVA: significant Group x Session interaction).* FRR conditioned rats breathed faster at rest during Sessions 5, 19, and 20, opposing proposed effects of habituation and controllability on reducing resting RR. **p<0.05 FRR conditioned vs. yoked, post-hoc Holm-Sidak comparisons*

Discussion

We were successful in using operant procedures to reduce respiratory frequency in adult rats, building on an early case report (Gallego, Benammou et al. 1994) that used brain stimulation reinforcement. We employed a chronic mild stressor, aversive intermittent bright light, as negative reinforcement in an automated feedback paradigm

triggered by real-time ventilatory measurements. Although not all SRR conditioned animals appeared to learn the task, a clear reduction in group average RRs was observed by the later sessions, with maximal reduction observed around Sessions 14-16. Interpretive difficulties remain that should be considered in light of our subsequent investigation of relaxation-related outcomes measures (Chapter 3), and in the case of attempted replication of the present work.

Limited Absolute Magnitude of RR Reduction

Our more impressive result for the dependent variable % of samples meeting criterion than for RR decrease (Figure 2.5) suggests that animals may have additionally learned strobe light control via some factor other than their rate of respiration. This discrepancy could be explained by their increased consistency of respiration. Indeed, we found an impressive disparity between SRR conditioned rats and yoked controls when measuring the standard deviation of resting RR values over 20 sessions (Figure 2.8); it appears that RR became more regular over 20 sessions, an effect that was especially conspicuous during Sessions 14, 15, and 20.

It is also important to emphasize that % of samples under criterion (Figure 2.5A) may be more important to consider than average resting RR (Figure 2.5B) in determining the success of conditioning, due to the fact that average RR during baseline (92.0 breaths/minute) was only marginally above the criterion for RR training (80 breaths/minute). In other words, the requirement for reinforcement may not have been sufficiently challenging, and animals may have found spending time just under 80 breaths/minute to be a less taxing strategy than progressively decreasing their rate beyond

the demands of the task. However, early attempts to condition animals using a more stringent criterion, over a smaller number of sessions, were not successful. An advantage of setting the criterion at 80 breaths/minute was the increased frequency of the strobe light turning off under this condition, thereby providing more opportunities for SRR conditioned rats to learn the response criterion. We also considered applying a "moving" target RR, akin to operant conditioning of the H-reflex (Chen and Wolpaw 1995). We attempted this strategy for a small number of animals without success, potentially due to the uncertainty invoked by changing the response criterion prior to full manifestation of a conditioned response. As ours was the first study of its kind, it was essential to establish guidelines and change variables only sparingly. Future studies attempting to replicate or extend our work should consider choosing from a range of starting target RRs, or changing criterion once conditioning has been fully established, e.g. sometime after Session 10 in our study.

Related to this consideration, SRR conditioned rats could have learned to turn off the light by spending more time sleeping or at quiet rest, when breathing is most regular and RR is more likely to be hovering just under criterion (Pappenheimer 1977). Alternatively, increased movement in yoked controls could have led to higher RRs during epochs of rest due to behavioral arousal; we often observed heightened animal RRs at rest following periods of movement in the plethysmography chamber. Therefore, one explanation for the disparity between % under threshold and average resting RR is the time animals spent at rest vs. in motion over 20 sessions. We scored the amount of time moving (including periods of exploration, as well as "active unrest" – shifting, grooming, sniffing, or any other behaviors during which stable resting respiration was not apparent)

versus resting for each session, validated via video recordings in a subsample of animals, and found that SRR conditioned rats and yoked controls spent comparable amounts of time at rest throughout training (Figure 2.9).

Finally, given the gradual shift in RRs observed during normal rat development, and the limited amount of variability in RR observed in our SRR conditioned rats (Figure 2.5B), there may be age-dependent physiological constraints limiting the lower and upper RRs an adult rat can sustain based on metabolic demands or other factors. It would therefore be informative to train younger, developing rats in our respiratory conditioning task.

Diminished Efficacy of Conditioning during Sessions 17-20

Our results for % of samples under threshold and resting RR both suggest that the efficacy of conditioning began to wane slightly during the final few sessions (Sessions 17-20). This is perhaps indicative of i) overexposure to the sensitizing strobe stimulus, ii) conversely, diminished aversive salience of the strobe, and/or iii) long-term disruption of circadian rhythms. Indeed, the observation that RR was maximally reduced during Sessions 14-16 suggests that some factor limited the retention of learning over the last 3-4 sessions. i) We suggest that strobe stimulation may have had a long-term sensitizing effect on the rats, possibly resulting in increased cortical excitability and behavioral arousal by completion of Session 20. This could also explain the negative results we found for several of the open field variables and nociception assays, and the corticosterone response to restraint (Chapter 3). This hypothesis is based in part on evidence of increased temporal lobe excitability in rats following exposure to repeated

intermittent bright light (Uhlrich, Manning et al. 2005). ii) Another possibility is that repeated strobe exposure resulted in retinal deterioration and a corresponding loss of visual function, and therefore diminished the aversive salience of the negatively reinforcing strobe stimulus. As we did not measure animal EEG and did not directly measure visual function or retinal atrophy, we cannot rule out either possibility as contributing to our waning effect magnitude following Sessions 14-16. iii) It is also possible that animals experienced a long-term disruption of circadian rhythms as a result of light exposure during the dark half of their reverse light-dark cycle. It has been shown that a single exposure to bright light during the dark cycle can drastically alter melatonin levels and sleep cycles (Lewy, Wehr et al. 1980). Although we did not measure melatonin levels or home cage activity in our study, the latter would be an intriguing future application for our remote sensor technologies (Chapter 4).

Optimization of Operant Conditioning Paradigm

Modifications of our conditioning protocol may increase its effectiveness, including altering reinforcement schedules (e.g. providing reinforcement based on dominant respiratory frequency for every 5 vs. 10 breaths, reinforcing slow breaths that are also "coherent" – i.e. conditioned rats only turn the floodlight off when the Buneman frequency-converted dominant respiratory peak is above a certain amplitude – or changing from fixed to variable ratio reinforcement). Furthermore, conditioning down to 0.4 Hz, or 24 breaths/minute (the Mayer wave frequency in rats, as discussed in Chapter 1), may not be achievable. Nevertheless, based on the inverse relationship between RR and cardiovascular synchronization (Rubini, Porta et al. 1993, Pereda, De la Cruz et al.

2005), incremental reductions in RR should still lead to increased RSA and improved autonomic function. If light reinforcement does not consistently lead to SRR, we may need to switch to an alternative reinforcing stimulus or to a positive reinforcement paradigm. One possible alternative reinforcer would be ultrasonic vocalizations (Knutson, Burgdorf et al. 2002, Hegoburu, Shionoya et al. 2011). Based on our goal of continuously reinforcing RR in freely behaving animals, discrete, rapidly habituating, or invasive reinforcement stimuli such as food reward or intracranial self-stimulation (Gallego, Benammou et al. 1994) were avoided in our study. However, the use of brain stimulation as in the only previous pilot study to operantly condition respiration in rats (Gallego, Benammou et al. 1994), or novel optogenetic approaches targeting dopaminergic reward pathways (Adamantidis, Tsai et al. 2011, Rossi, Sukharnikova et al. 2013) could be tremendously effective methods of reinforcement.

Inability to Measure Tidal Volume and Metabolic Changes

One important consideration relates to a pivotal assumption we are making considering slow breathing. Because we are only reinforcing one respiratory parameter – reduced rate – we are not rewarding or assessing deep breathing per se, but rather making the assumption that slower breaths are also deeper, i.e. that minute ventilation remains constant. This is normally the case due to relatively stable animal metabolic demands, if activity levels are similar between animals (as was the case in our study), but it is important to acknowledge that our paradigm cannot rule out metabolic changes between groups as contributing to our observed results.

A central reason for these metabolic concerns was our inability to reliably measure tidal volume (V_T) during conditioning. While the varying fidelity of day-to-day recordings precluded an absolute readout of V_T, within a given session voltage amplitudes were also relatively consistent and didn't reveal relative differences in V_T. Measuring only one of the two components leaves open the possibility that minute ventilation (= RR x V_T), and concomitant metabolic activity, were also affected by conditioning procedures. Chamber temperature (and humidity) were checked at several time points during a random sample of conditioning sessions, and remained stable throughout the two-hour period. It is possible that animal body temperature changed due to altered metabolic function or a number of factors related to differential ventilatory efficiency. Indeed, it is proposed that slowing respiration leads to enhanced ventilation-perfusion matching and possibly enhanced metabolic efficiency (Yasuma and Hayano 2004, Anderson, McNeely et al. 2009).

Additionally, there were no procedures in place to monitor possible metabolic changes in conditioned animals. While the plethysmography setup prevented us from monitoring end-tidal CO_2, as we did during experiments in anesthetized animals (Appendix 1), future studies could use pulse oximetry to measure O_2 saturation, or monitor base excess or carbonate levels in the blood to indicate kidney compensation to changes in in CO_2. Putting a mask on the rat and using capnography to record end-tidal CO_2 could be done after sessions and/or during baseline. In summary, future studies will need to successfully capture tidal volume in addition to RR, and find a way to quantify metabolic changes occurring during operant conditioning.

Baseline Acclimation and RR as an Index of Stress

The timeline of RR stabilization during early baseline recordings (Figure 2.4) was much longer than most experiments that include an acclimation period. Behavioral studies commonly use short periods of acclimation (on the order of 20-30 minutes to one hour) once animals have been accustomed to experimenter handling. In as much as RR stabilization is a reflection of return to baseline state, our observations suggests that many studies using rodent models begin experimental procedures on still non-acclimated animals. The gradual RR response to novelty suggests that lack of acclimation to experimental conditions could be a confounding variable in the interpretation of experimental results. Given that RR indexes stress levels to at least some extent (Suess, Alexander et al. 1980), and stress can dramatically impact physiology and behavior, more attention should be paid to study conditions prior to commencement of experimental procedures. Here, we used a long (12 hour) acclimation period to achieve a stable RR for subsequent conditioning studies, supporting statistical comparisons between the last two hours of acclimation (our baseline) and individual two-hour conditioning sessions. Furthermore, FRR conditioned rats actually underwent a slight increase in RR from acclimation to Session 20, supporting the idea that they began conditioning studies at a true baseline. Finally, it should be pointed out that our 12-hour acclimation period was essential for animals acclimating to an airtight whole-body plethysmography chamber with connections for the constant inflow and outflow of air, the latter of which was sent to a vacuum that provided constant, potentially aversive, background noise throughout each session. Therefore, the timeline of acclimation to this particular environment,

gauging by RR, exploration, or other stress-related metrics, may be different from that to other testing environments.

Up-Conditioned FRR Rats Support Learning Effects in SRR Conditioned Rats

Another potential confound we addressed was the impact of stimulus controllability on differences in RR and behavioral outcomes. Anticipation and learned helplessness may engage the sympathetic nervous system and HPA axis (Maier, 1984), while, inversely, control over one's environment is known to be therapeutic (Rodin and Langer 1977). Therefore, because yoked rats lacked control over experimental reinforcement, one might have expected their RRs to gradually increase relative to those of SRR conditioned rats simply due to the impact of learned helplessness on sympathetic arousal. If this were the case, the progressive decrease in SRR conditioned rat RR might be explained not by operant learning, but by differences in stimulus controllability between paired animals. Reinforcing fast vs. slow breaths in an "up-conditioned" FRR control group was used to rule out learned helplessness effects as the key factor, since the proposed impact of controllability on RR would counteract an RR increase in FRR conditioned rats; in fact, these rats may have even been expected to have a *lower* RR than yoked controls by Session 20, based on the controllability hypothesis. However, there was a trend toward increased RR in FRR conditioned rats from baseline to Session 20, and they breathed more quickly than their yoked controls throughout conditioning. The absence of any RR reduction from baseline through Session 20 is impressive, and habituation effects (instead of or in addition to controllability) may have precluded an absolute increase in RR. Together, these results suggest that differential stimulus

controllability did not account for RR reduction in SRR conditioned rats.

Acknowledgements

I would like to thank Shawn Hochman and Michael Sawchuk for helping to devise experiments, providing resources, and troubleshooting early difficulties, and Bill Goolsby for developing our LabVIEW feedback program.

CHAPTER 3: Operantly conditioned slow breathing in the rat modifies baseline respiration and induces behavioral changes consistent with the relaxation response

Abstract

In Chapter 2 we demonstrated successful operant conditioning of SRR in adult rats. Here, we set out to investigate whether conditioned SRR results in long-term respiratory changes and reduces reactivity to experimental stressors and noxious stimuli. After conditioning was established, a subset of the conditioned rats underwent approximately weekly two-hour retention sessions in the absence of reinforcement, to monitor the persistence of reduced RR 24 to 48 hours after the previous training session. Following Session 20 of conditioning, we sought to establish the impact of conditioned slow breathing on the response to stressors: i) anxiety-like behavior in an open field test, and ii) RR response during 10 minutes of acute restraint. We used electric field sensors (Chapter 4) to non-invasively monitor RR during two individual 10-minute restraint sessions, and assayed for plasma corticosterone levels following a third, 30-minute session. The 10-minute restraint tests were interspersed with a series of nociception assays (the Von Frey, Tail Flick, Hargreaves, and formalin assays) two days to one month following conditioning, to evaluate the impact of training on the response to noxious stimulation. Animals conditioned to slow their breathing maintained a reduction of 8.6 breaths/minute relative to baseline, with a reduction of 3.7 breaths/minute not reaching significance in yoked controls. SRR conditioned rats also entered the center of an open field 39.2 seconds earlier than yoked controls, on average, following training, and tended to travel a smaller total distance. Furthermore, trained rats showed a trend toward reduced respiratory response to restraint stress that became significant on the second day of testing (116.6 vs. 147.2 breaths/minute in yoked controls). Although SRR conditioned and yoked rats had mostly similar reflex responses to thermal and

mechanical nociceptive stimuli, SRR conditioned rats showed a trend toward increased sensitivity to the Hargreaves Test on the first day of testing and the Tail Flick Test on the second day of testing. Upon receiving an injection of formalin to induce central hyperalgesia, SRR conditioned rats responded to Von Frey hair presentation on the injected paw similarly to before the injection, whereas yoked controls showed the expected increase in sensitivity, leading to group differences. Taken together, these results suggest that conditioned RR reduction i) is maintained in the absence of reinforcement, ii) may have an anxiolytic-like impact on animal behavior and physiology in response to experimental stressors, and iii) alters reflex responsiveness following formalin-induced hyperalgesia.

Introduction

Are we truly modeling the relaxation response?

An emerging view is that mind-body techniques involving slow, deep breathing lead to an array of benefits in humans. Deep breathing exercises such as those in pranayama ("manipulation of the breath movement") yoga have been found to improve stress-related physiological functions including autonomic imbalance, cardiopulmonary and neuroendocrine function, and mood (Brown and Gerbarg 2005, Jerath, Edry et al. 2006, Kaushik, Kaushik et al. 2006, Courtney 2009, Pramanik, Sharma et al. 2009). Many of these effects are substantial; deep breathing increases cardiac variability by a factor of two and cuts self-reported depression in half (Janakiramaiah N 1998, Lehrer, Sasaki et al. 1999). This suggests that the sympathetic–parasympathetic balance is modifiable, with deep breathing reducing stress and decreasing sympathetic tone (Jerath,

Edry et al. 2006). Herbert Benson called this global state change the 'relaxation response', defined as "a physical state of deep rest that changes the physical and emotional responses to stress" (Benson, Rosner et al. 1974). Although our conditioned rats spent similar amounts of time at rest (Figure 2.9), reduced RR in conditioned rats may behaviorally simulate the "state of deep rest" signified in the definition. It is commonly understood that RR is heightened during stress and reduced during slow wave sleep (Pappenheimer 1977, Suess, Alexander et al. 1980). Furthermore, given previously described evidence of slow breathing's therapeutic benefits, as well as the suggestion that high RR predicts a number of negative cardiopulmonary outcomes (Fieselmann, Hendryx et al. 1993, Hodgetts, Kenward et al. 2002), RR appears to be an important index of stress and its deleterious impact on behavior. We speculate that SRR conditioned rats are more "restful" and will correspondingly show reduced physiological and behavioral responsiveness to experimental stressors – i.e. reactivity to novelty stress (open field), restraint, and nociceptive stimuli will be reduced in these animals. Although resting RR only serves as a proxy of rest, to the extent that our conditioned rats have reduced stress reactivity we can say that our animal model is *consistent* with the relaxation response.

Long-term effects of slow, deep breathing on behavior and physiology

Based on the impressive plasticity of the respiratory system and its amenability to learning (Ley 1999), I expected that SRR and any associated benefits would be maintained following training, in the absence of continued reinforcement. It has been shown that remedial breathing in humans (Han, Stegen et al. 1996) and chemically-altered ventilation in rodents (Powell, Milsom et al. 1998) result in long-lasting

respiratory changes. I therefore expected that any benefits associated with – and presumably triggered by – SRR would last into the post-training period.

I proposed the additional hypothesis that conditioned SRR would reduce the behavioral and physiological responses to acute stress. Given the numerous benefits of SRR, I expected that it would improve behavioral performance on the open field test of anxiety, i.e. SRR conditioned rats would show reduced anxiety-like behavior, including a greater duration spent in and number of visits to the center, and decreased latency to enter the center. I also expected conditioned rats to have a reduced respiratory response to acute restraint for the same reason. To address whether trained slow breathing improves pain tolerance, we devised a series of nociception assays that clearly distinguished basic nociceptive reflexes from altered responses following chemically induced hyperalgesia. I originally proposed that SRR conditioning would decrease the sensitivity to all of these stimuli, in line with its impact on the responsiveness to experimental stressors. However, differential results on these several tests could provide important distinctions regarding the impact of SRR on acute vs. tonic nociception. Finally, I assayed blood samples for corticosterone (CORT) levels following a 30-minute restraint test, to test the expectation that conditioned rats would have a reduced neuroendocrine response to physical stress.

In summary, the objective of these experiments was to determine the short- and long-term effects of SRR on stress-related behavior, nociception, and plasma measures of stress. Following initial pilot studies for operant conditioning of SRR in n=3 pairs of rats, we undertook outcome measures on the remaining n=8 pairs presented in this chapter.

Materials and Methods

Experimental Animals

Adult male Sprague-Dawley rats (~90 days old at the start of experimental procedures) were housed in standard cages in a vivarium on a reverse 12:12-h light-dark cycle and were fed *ad libitum* standard rodent diets. All experiments were approved by the Animal Care and Use Committee 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."

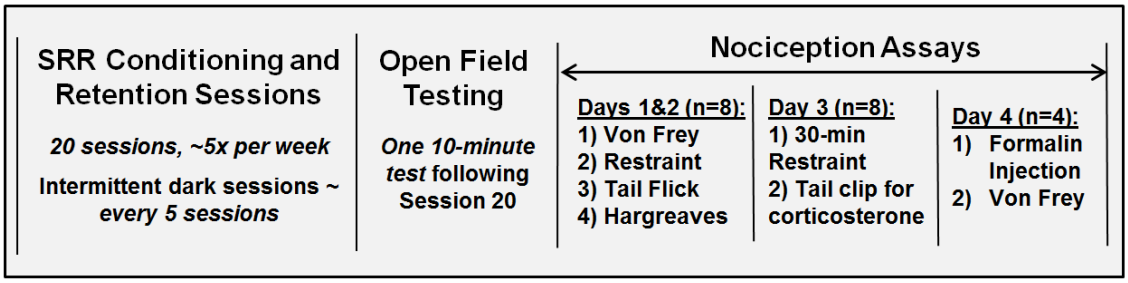


Figure 3.1. Timeline for experimental procedures.

Animals underwent SRR retention testing with no reinforcement and only background red light ("dark sessions"), intermittent with conditioning. Open field testing occurred following Session 20 of conditioning, while nociception assays took place several days to a month later. Not shown here are acclimation and nose poke sessions (described in Chapter 2) and SRR 'reminder' sessions prior to nociception assays, which confirmed the maintenance of reduced RR during this time.

SRR Retention Sessions

Based on evidence of respiratory plasticity outlined in the Introduction, I hypothesized that RR would be lower than initial baseline (the last two hours of acclimation) following training. Intermittent with training sessions, a subgroup of rats

(n=8) underwent approximately weekly two-hour respiratory recording sessions in the absence of visual reinforcement ('SRR Retention Sessions'). Each of these sessions occurred 24 to 48 hours after the previous SRR conditioning session. During retention sessions, we analyzed resting RR to address whether reduced RR was maintained following successful acquisition of respiratory learning (i.e. after Session 4, when a difference in % of samples under threshold became apparent, as revealed in Figure 2.5A). These sessions therefore monitored the persistence of SRR into the post-training period to determine whether a stable baseline reduced RR was reached; if a persistently lower RR was reached, this could in turn mediate any differences observed during open field testing or subsequent outcome measures.

Open Field Test of Anxiety

Although rats do not experience anxiety and depression in the same manner that humans do, there are methods for the assessment of both anxiety-like and depressive-like behavior in the rat. These tests are established assessments of affective-like behavior. In the Open Field Test, perhaps the most common of these, rats are tested for their exploratory and anxiety-like behavior in a large novel environment (Broadhurst 1957, Ennaceur, Michalikova et al. 2006). Following Session 20 of SRR conditioning, rats were transferred to a large open field apparatus and allowed to freely explore the environment for 10 minutes while being recored over video. Rats placed in the center of the field quickly ran out to the periphery; analysis began once the experimenter was off screen and the animal was out of the center. I worked closely with several collaborators to determine the effects of conditioned SRR on the Open Field Test. With the help of Christina

Nemeth (Gretchen Neigh's lab) and Catherine Barrett (Donald Rainnie's lab), CleverSys TopScan software was used to analyze the corresponding video files. The software allows display of the paths taken by the rats, and it measures the total distance traveled, the number of entries into the center of the arena (center 50% area of the surface), the duration of the trial spent in the center of the arena, latency to enter the center of the arena, velocity, and several additional variables, over the course of the 10-minute session. The four variables most typically reported are center field bouts (entries and exits) and duration, latency to enter the center, and distance traveled. For open field testing and Days 1-4 of nociception assays (described below), a given animal in each pair was always run first; however, order was counterbalanced such that half of the animals run first were SRR conditioned rats and half were yoked controls. The animal not being tested was placed in an adjacent room for the duration of testing.

Nociception Assays and Acute Restraint Stress

Nociception assays are often used to evaluate the response to noxious stimuli, i.e. those that stimulate peripheral nociceptors and may cause the sensation of pain (Carter and Shieh 2010). There are many nociception tests that provide complementary information and therefore are often run together. Our collaborators Sandra Garraway and Karmarcha Martin performed a series of nociception assays (the Von Frey, Tail Flick, Hargreaves, and formalin assays) following conditioning to evaluate the impact of training on withdrawal responses to noxious stimulation. Rats also underwent 10-minute acute restraint sessions in between the Von Frey and Tail Flick Tests, simultaneous with

respiratory recordings via remote electric field sensors, to determine their respiratory response to restraint.

At variable time points following 20 sessions of conditioning (two days – one month), rats were transferred to another room in the Physiology Department and acclimated in small acrylic chambers for 30 minutes. Subsequently, a series of nociception assays were performed over the course of four days. In brief, during the first two days of testing, thermal nociception in open or restraining chambers was assayed using the Hargreaves and Tail Flick Tests, respectively, while mechanical nociception was assayed in open chambers using the Von Frey Test. On the fourth day (following 30 minutes of restraint to assess CORT levels on Day 3), the formalin assay (the most common chemical assay of nociception) was used to induce central hyperalgesia over the course of 90 minutes; animal responsiveness to mechanical stimulation with Von Frey hairs was tested during the final 15 minutes. The procedures, in detail, were:

Days 1 and 2: Following the 30-minute acclimation period, individual animals were sequentially tested using the mechano-nociceptive Von Frey Test, during which Von Frey hairs (small pieces of nylon rod) were used to test each rat's sensitivity to mechanical stimulation. For Von Frey testing, rats were moved to an elevated mesh platform, and the Von Frey hairs were inserted through the mesh to poke the animal's hindpaw. Typically, rats withdraw their paw upon receiving a threshold level of measured force, at the stimulus intensity (in grams) that we report here as our dependent variable.

After completing Von Frey testing in both animals, they were sequentially (counterbalanced for group) moved to a small restraining cylindrical tube for a 10-minute acute restraint test. During this test, remote electric field sensors (Chapter 4) affixed to

the side of the tube recorded animal respiration for analysis of RR response to restraint. Immediately following restraint, rats remained in the same tube for another 10 minutes for the Tail Flick Test, which involved shining a beam of light aimed at a rodent's tail to induce a withdrawal response. For this test, latency to withdraw (in seconds) was recorded as our dependent variable.

Immediately following 20 minutes in the cylindrical chamber (pre-Tail Flick restraint test and Tail Flick Test) for both animals, rats were moved to acrylic chambers identical to those used for Von Frey testing and our second thermonociceptive assay, the Hargreaves Test, was performed. This test also used a beam of light to induce withdrawal, but in this case the light was directed at the hindpaw rather than at the tail. Therefore, we again recorded latency to withdraw (in seconds) as our dependent variable, here pertaining to the hindpaw. Note that in contrast to the Tail Flick Test, rats were in an open chamber (i.e. not restrained) during the Hargreaves Test.

On *Day 3*, a 30-minute acclimation period was followed by individual 30-minute restraint sessions with subsequent tail clip for CORT (see below).

On *Day 4* of testing, animals were again acclimated for 30 minutes, and then immediately underwent the formalin assay. Sandra Garraway or one of her lab members injected a 50 µl solution of 5% formalin into the dorsal surface of the animal's right hindpaw, inducing stereotypical behaviors such as licking and biting the affected paw. Importantly, the formalin assay produces a response in two discrete phases, with the early phase reflecting activation of peripheral nociceptors, and the later one reflecting activation of central inflammatory pathways (Carter and Shieh 2010). This separation

into acute and tonic responses allows us to differentiate the impact of SRR conditioning on nociceptive reflexes vs. centrally mediated hyperalgesia.

Blood Collection and Corticosterone Assays

Based on the proposed role of SRR in relaxation and in attenuating the response to environmental stressors, we expected that SRR conditioned rats would exhibit reduced plasma CORT levels in response to an acute restraint stressor administered after operant training. Following the operant training regimen of 20 two-hour sessions and a priming exposure to restraint stress, we expected that SRR conditioned rat plasma would contain reduced CORT levels (vs. yoked controls), indicative of reduced sympathetic activation and shifted autonomic balance. Plasma was assayed for CORT levels following a 30-minute restraint test on Day 3. The same cylindrical tube used for acute restraint during nociception assays was used for the 30-minute restraint session, and the test was administered in an identical manner; only the duration of the restraint was different, to allow timing for sufficient CORT elevation. Blood sampling for CORT was accomplished using the tail clip method. I collected blood at a single time point following the 30-minute restraint stressor, in order to prevent potentially confounding repeated exposure to the tail clip stress. Testing for CORT prior to or intermittent with earlier conditioning procedures was ruled out in order to prevent animals from developing anticipatory responses to the tail clip procedure or a fear response that they could associate with respiratory conditioning. For blood collection, animals were restrained in a narrow cylindrical tube and all blood was collected within 3 minutes to avoid elevated CORT from the collection procedure. To perform tail clip, a straight razor was

used to clip the tip of the tail (~2 mm up). Blood was collected by gently stroking the tail from base to tip. Once collected, samples were processed for blood plasma. To obtain plasma, blood was collected in EDTA-coated tubes, which were inverted several times to coat all of the blood with EDTA, and then immediately put on ice. At some point in time within the next 4 hours, the EDTA-blood was spun at 1300 rcf for 10 minutes at 4˚C. Supernatant was pulled (kept cold at all times), flash frozen, and stored at -80˚C. Plasma CORT levels were then assessed by enzyme-linked immunosorbent assay (ELISA). Gretchen Neigh's lab in the Department of Physiology performed these assays for us. Plasma samples were diluted 1:40 and assayed with a commercially available kit (Enzo Life Sciences ADI-900-097) in duplicate (sensitivity: 27.0 pg/ml; intra-assay coefficient of variation: 3.0%).

Statistical Analysis

Data are presented as mean ± SEM, unless otherwise noted. Despite the inherent advantages of a yoked design (control over all extraneous variables other than stimulus controllability), independent (vs. paired) t-tests were used to compare groups since paired tests required unvalidated assumptions about the amount of variability between experimental subjects (paired rats) at baseline. For open field analysis, variables (including bouts in center, duration in center, and total distance traveled) were scored across the 10-minute period, and planned Student's t-tests were performed between groups (SRR conditioned rats vs. yoked controls). Open field latencies and CORT levels were analyzed using a non-parametric Mann-Whitney Rank Sum test, as data failed to reach normality. Average resting RR and % under conditioning threshold during retention

sessions did not depend on when these sessions were run ($p>0.05$), and so these values were averaged and compared to baseline within a given group using paired t-tests; SRR conditioned vs. yoked rat retention session comparisons were made using Student's t-tests. Nociception responses and acute restraint RRs were analyzed with planned Student's t-tests when comparing SRR conditioned rats and yoked controls, and paired t-tests when comparing between days within a given group. A one-way ANOVA (with a follow up Holm-Sidak multiple comparisons test) was used to compare CORT response across cohorts to assess the impact of shared experience. Statistics were performed with SigmaPlot 13 with significance set at $p<0.05$ and two-tailed tests.

Results

Retention of Learned Slow Respiration between Training Sessions

As described in Methods, 3-4 non-conditioning two-hour 'SRR retention testing' sessions were undertaken on pseudo-random days interspersed between SRR conditioning sessions. These occurred in the dark in the absence of visual reinforcement. Frequency histograms for RR (Figure 3.2A) and a bar graph (Figure 3.2B) confirm that SRR conditioned rats retained reductions in RR during these sessions. Values averaged across SRR retention testing sessions were 87.7 ± 2.5 breaths/minute for SRR conditioned rats and 91.4 ± 2.0 breaths/minute for yoked controls. During SRR retention sessions, SRR conditioned rats significantly reduced their average resting RR (compared to baseline values) by 8.6 breaths/minute ($P = 0.002$, paired t-test). Yoked controls showed a reduction of 3.7 breaths/minute, which was not significant ($P = 0.299$, paired t-test). Interestingly, standard deviation of resting RR did not differ between groups during

retention sessions ($p>0.05$, Student's t-test), suggesting that increased regularity of breathing did not contribute to group differences as it did during conditioning sessions (see Figure 2.8). The observed magnitude of RR reduction was less than during conditioning, as the subset of n=8 animals undergoing SRR retention testing procedures reduced their resting RR by 15.9 breaths/minute during SRR conditioning, but only 8.6 breaths/minute during the SRR retention testing sessions (yoked values for these n=8 pairs were 6.5 and 3.7 breaths/minute for RR reduction during training and SRR retention sessions, respectively). This suggests that there may have been some extinction of the conditioned response, but it was incomplete (see Discussion). The pseudo-randomness of the two-hour dark sessions, interspersed with normal conditioning procedures, may have induced an anticipatory lowering of RR in SRR conditioned rats. Our findings indicate a learned, context-specific retention of reduced RR, but to a lesser extent than during the actual conditioning sessions.

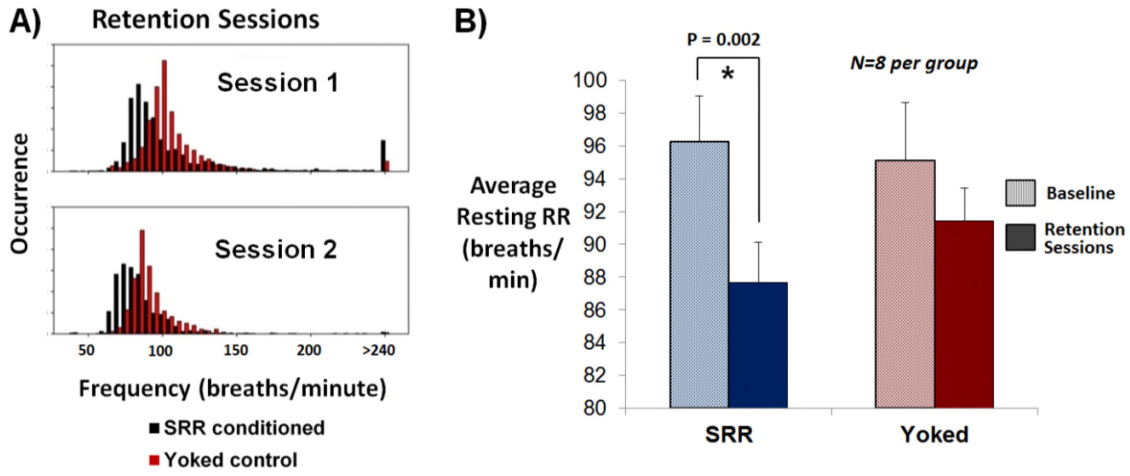


Figure 3.2. Reduced RR is retained between sessions.

SRR conditioned rats maintained slowed resting respiration in the absence of conditioning. (**A)** Individual frequency histograms for one pair of SRR conditioned (*black*) and yoked control (*red*) rats during two representative retention sessions interspersed with normal conditioning, and approximately a week apart from one another (following Sessions 6 and 12, respectively). **(B)** Trained reductions in RR were maintained across SRR conditioned rats during these SRR retention sessions (**p<0.05, paired t-test)*.

Open Field Test of Anxiety

Ten-minute Open Field Tests were used to assess whether a conditioned decrease in RR would lead to a reduction in anxiety-like behavior immediately following the 20th SRR conditioning session (Figure 3.3). SRR conditioned rats took less time to enter the center of the open field (Figure 3.3A): a Mann-Whitney Rank Sum Test (due to failed normality) revealed a significantly reduced latency to enter the center of the field in SRR conditioned rats vs. yoked controls (31.1 ± 6.3 vs. 70.2 ± 16.0 seconds, respectively; P = 0.021), with SRR conditioned rats entering the center 39.2 seconds earlier, on average. As shown in Figure 3.3B, there was also a trend toward a smaller distance traveled by SRR conditioned rats (SRR: 8079 ± 363 mm, Yoked: 9199 ± 407 mm; P = 0.059,

Student's t-test). Although we found no statistical difference in the number of center field bouts or the duration of time spent in the center between groups, the trend toward greater total distance traveled in yoked rats could have obscured differences in these variables (see Discussion).

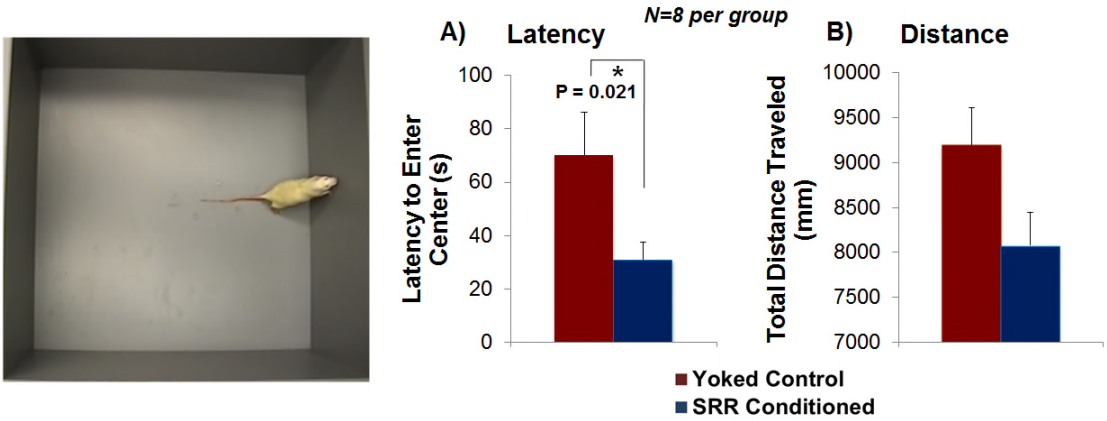


Figure 3.3. Conditioning reduces anxiety-like behavior in an open field.

Following 20 sessions of conditioning, rats were transferred to an open field and anxiety-like behavior was scored during 10-minute sessions. **(A)** SRR conditioned rats showed a reduced latency to enter the center of the field. **(B)** SRR conditioned rats also showed a trend toward reduced total distance traveled.

RR Response to Acute Restraint

In order to characterize the respiratory response to restraint stress, rats were placed in small cylinders and RR was monitored with electric field sensors (Chapter 4) during the 10-minute "pre-Tail Flick" period of nociceptive testing (described above). All eight rats that underwent outcome testing provided sufficient RR recordings during restraint. The mean RR response to restraint was significantly lower in SRR conditioned vs. yoked rats on the second of two consecutive days of testing (116.6 ± 8.2 vs. 147.2 ±

7.5 breaths/minute, respectively; P = 0.016, Student's t-test). This is shown in Figure 3.4. Day 1 values were 142.2 ± 9.4 and 155.7 ± 6.7 breaths/minute for SRR conditioned rats and yoked controls, respectively. Additionally, SRR conditioned rats decreased their RR by 25.6 breaths/minute from Day 1 to Day 2 (P < 0.001, paired t-test), while the decrease of 8.6 breaths/minute in yoked controls did not reached significance (P = 0.084, paired t-test).

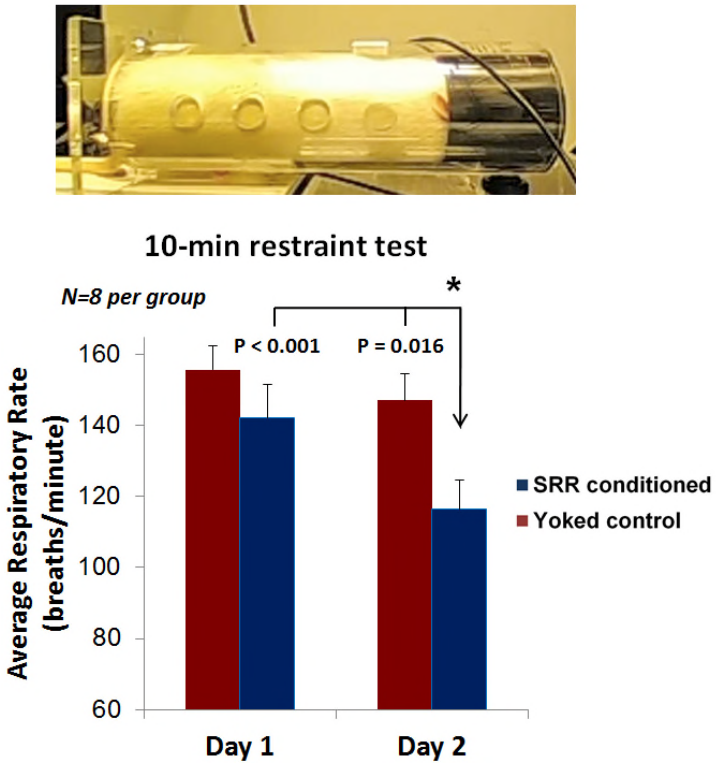


Figure 3.4. Conditioning reduces the respiratory response to acute restraint.

During the period preceding the Tail Flick Test on Days 1 and 2, rats underwent 10-minute acute restraint sessions (top panel) simultaneous with respiratory recordings. SRR conditioned rats (*blue*) had a lower RR than yoked rats *(red*) on the second day, and decreased their RRs from Day 1 to Day 2 (bottom panel); this reduction was more than twice as large as the non-significant reduction in yoked controls.

Nociception Assays

Following a variable number of 'reminder' conditioning sessions to establish maintenance of reduced RR, rats (n=8 per group) underwent a battery of nociceptive assays. Nociceptive assays performed in an open chamber included the Von Frey and Hargreaves Tests for mechanical and thermal withdrawal reflexes in the hindpaw, respectively, while the Tail Flick Test of thermal reflex withdrawal was undertaken with rats restrained in a conical Plexiglas cylinder. A subset of animals (n=4 per group) also underwent injection of formalin into the right hindpaw followed by Von Frey testing, to probe their sensitivity in an inflammatory pain model (chemical nociception). Analysis of nociception assay results was performed according to an existing testing algorithm (Chaplan, Bach et al. 1994). There were no differences in withdrawal sensitivity to mechanical or thermal nociceptive stimuli at baseline, as shown in Figure 3.5A and the left panel of Figure 3.5B, despite SRR conditioned rats showing a trend toward reduced latency to paw withdrawal on the first day of Hargreaves testing (SRR: 10.4 ± 0.8 seconds, Yoked: 13.2 ± 1.1 seconds; P = 0.063, Student's t-test; Figure 3.5A, left panel) and tail withdrawal on the second day of Tail Flick testing (SRR: 5.0 ± 0.8 seconds, Yoked: 6.8 ± 0.5 seconds; P = 0.079, Student's t-test; Figure 3.5A, right panel). However, SRR conditioned rats displayed decreased mechanical sensitivity of the formalin-injected paw during a Von Frey assay performed 75 minutes following formalin injection, requiring a greater amount of force to initiate responding (SRR: 10.2 ± 2.0 grams, Yoked: 2.5 ± 0.8 grams; P = 0.011, Student's t-test; Figure 3.5B, right panel). We interpret the mostly negative results on nociceptive assays, and the significant result following

formalin injection, to support a selective role for SRR in alleviating centrally mediated hyperalgesia (see Discussion).

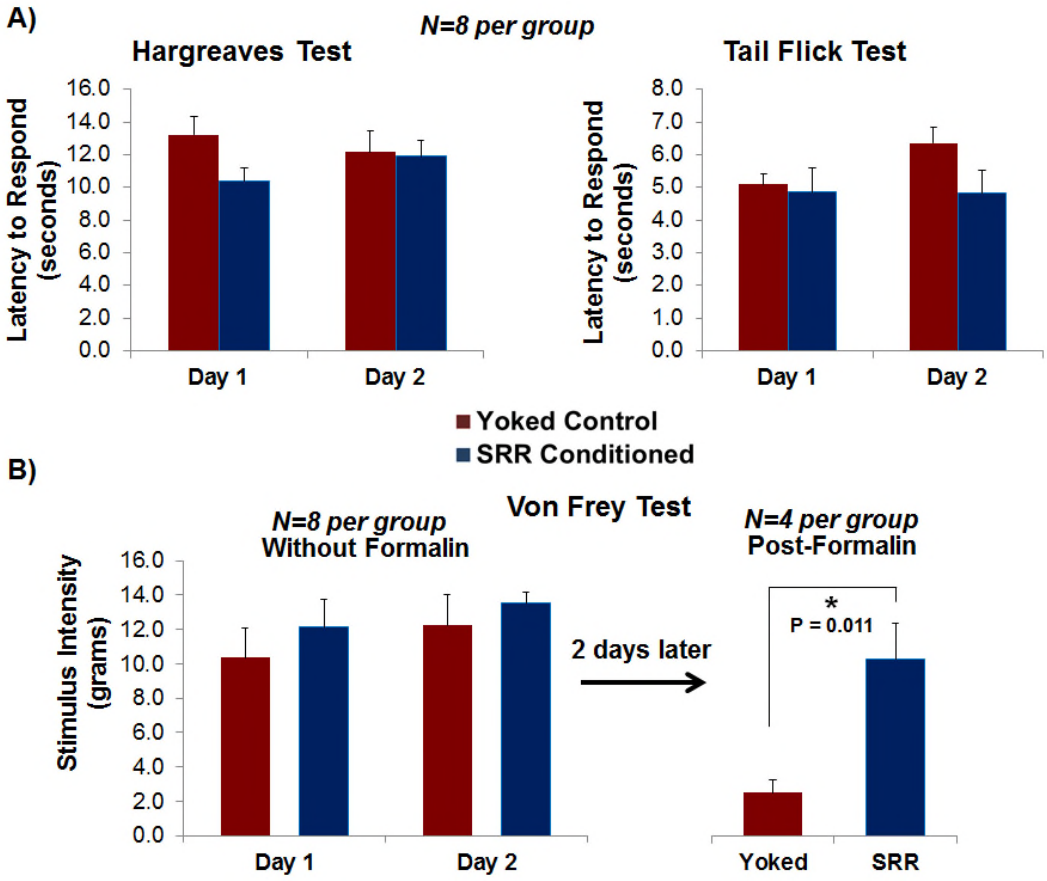


Figure 3.5. Respiratory training may impact nociceptive responses and alleviate hyperalgesia.

Shown are results for tests of thermal nociception in an open chamber (Hargreaves) or under restraint (Tail Flick), and mechanical nociception in an open chamber (Von Frey) with or without prior formalin injection into the rat's paw. **(A)** SRR conditioned rats showed a trend toward increased responsiveness to the thermal Hargreaves Test on Day 1 and Tail Flick Test on Day 2. **(B)** Although results from Days 1 and 2 on the mechanical Von Frey Test did not clearly differentiate the two groups of rats, mechanical nociceptive responses to formalin injection were reduced in SRR conditioned vs. yoked rats two days later (*$p<0.05$, Student's t-test)*. While yoked controls displayed the expected enhanced sensitivity of the injected paw, SRR conditioned rats had a threshold of responsiveness to Von Frey hairs that approached Days 1-2 levels.

Corticosterone Response to 30-Minute Restraint Test

We were not able to assess whether respiratory conditioning significantly impacted CORT levels in response to 30 minutes of restraint stress in the sample population (n=8). This is because the range of CORT values varied 9-fold over the population (from 98.4 to 886.0 ng/ml), strongly suggesting that other unknown variables contributed significantly to measured values. The consequent large values of standard deviation (Yoked: 371±98 ng/ml, SRR: 317±54 ng/ml; $P>0.05$, Mann-Whitney Rank Sum Test; Figure 3.6A) led to a reported power of 0.07 - well below the desired power of 0.80, indicating that the statistical test was greatly underpowered. Possible reasons include increased variability of CORT with animal age (~120 days at the time of experimental testing) or the large intervals of time that elapsed between running animal pairs (other possibilities are addressed in the Discussion). These results fail to support an attenuated neuroendocrine response to stress in SRR conditioned rats. However, significant differences in CORT levels were seen between animals run in different cohorts ($P = 0.002$, one-way ANOVA; Figure 3.6B). In one cohort, three of four rats had CORT values greater than 600 ng/ml, drastically higher than all animals tested in the other cohorts (the average across all animals was 224.7 ng/ml). Clearly, common environmental experiences in a given cohort had an enormous influence on CORT outcome (see Discussion).

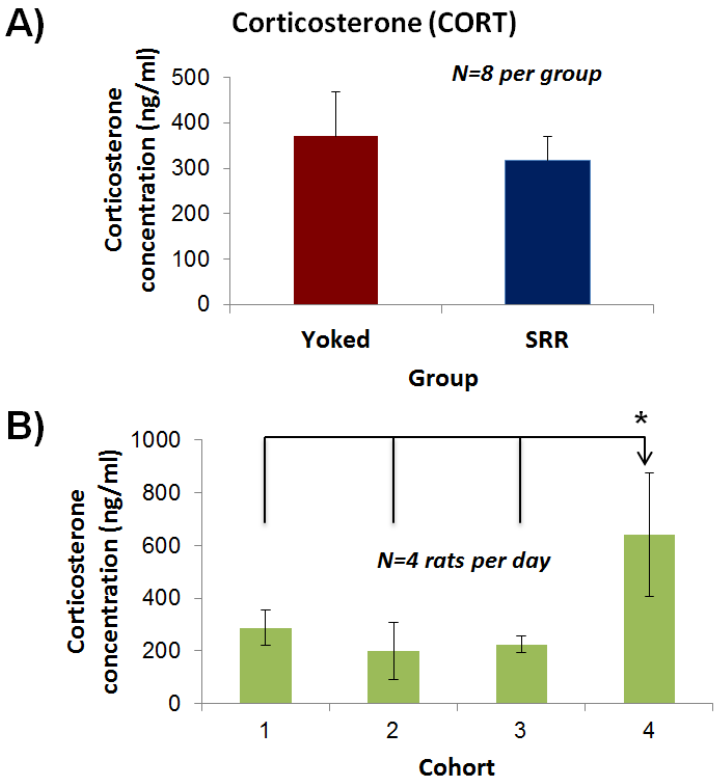


Figure 3.6. Corticosterone levels are similar in SRR conditioned rats and yoked controls.

(A) Although there was a trend toward reduced corticosterone (CORT) expression in SRR conditioned rats vs. yoked controls, high variability between animals may have obscured group differences. **(B)** Interestingly, CORT response depended on the day animals were tested. Animals tested for CORT in the final cohort exhibited higher CORT levels than the previous three cohorts (*$p<0.05$, 1-factor ANOVA with post-hoc Holm-Sidak comparisons*), hinting at the importance of shared experience. **p<0.05 vs. Cohort 4*

Correlations between Strength of Conditioning and Outcome Measures

We attempted to relate strength of performance in SRR conditioning with our behavioral outcomes. Peak success in SRR conditioning (% of samples under threshold during Sessions 14-16) correlated with open field latencies ($R^2 = 0.32$, $p<0.05$), and weaker correlations were observed for latency when plotted against absolute resting RR

or the SRR "learning index," which was calculated as RR (baseline) - RR (min). Despite correlations for open field latencies, preliminary analysis did not indicate a relationship between strength of conditioning and other outcome measures. It is possible that both learning itself (possibly a metric for controllability or a reverse metric for learned helplessness) and the absolute magnitude of RR during Session 20 (closest in time to outcome measures) impacted our behavioral results.

FRR Conditioning Outcome Measures

Outcome measures (retention sessions, open field testing, acute restraint, CORT response to stress, and nociception assays) were also run for FRR conditioned rats (Chapter 2), but only n=4 pairs were run for each test. Because of the small sample sizes and large variability in performance, statistical analyses for FRR conditioning (conditioned vs. yoked) were generally underpowered, and did not detect any significant group differences. In the Open Field Test, only SRR conditioned animals showed reduced latency to enter the center of the field; FRR conditioned rats showed a similar latency to enter the center as their yoked controls. Nociception assays suffered from large amounts of variability between animals and required larger sample sizes to assess significance. While FRR conditioning results from Chapter 2 suggest that differential stimulus controllability did not account for RR reduction in SRR conditioned rats, we do not have sufficient preliminary evidence against a role for controllability in contributing to significant results on SRR conditioning outcome measures (i.e. retention of learned SRR, open field latencies, post-formalin nociception, and RR response to acute restraint). This is clearly a weakness of the present study that must be addressed to determine the extent

to which our findings are attributable to learned respiratory changes versus controllability itself. While it is important to further investigate the impact of controllability in additional FRR conditioned rats or those undergoing a simpler operant task such as nose poke (prior to or concurrent with the future directions discussed in Chapter 5), identifying a substantial 'controllability effect' would not invalidate present results but instead would suggest novel avenues for research that could extend the applications of our model.

Discussion

Retention sessions suggest long-term maintenance of learned slow breathing.

Lingering differences in RR from baseline to retention sessions conducted at several time points between conditioning sessions supports the context-specific retention of learned reductions in RR, since yoked controls did not show the same effect. The diminished absolute magnitude of these changes compared to those observed in the presence of reinforcement suggests that there may have been some extinction of the conditioned response, but that it was incomplete even a full 24 to 48 hours after the previous conditioning session, when retention sessions were performed. Although expectation of respiratory-based feedback could have initially controlled this effect, it seems likely that animals would have quickly appreciated the complete absence of visual stimulation, especially since a small cue light on the bottom of each plethysmography chamber was left on during conditioning sessions but turned off during these retention sessions. Still, it is possible that the pseudo-randomness of the two-hour dark sessions, interspersed with normal conditioning procedures, may have induced an anticipatory lowering of RR in SRR conditioned rats. Whatever the mediating factors, the finding of a

context-specific retention of learned reductions in RR, but to a lesser extent than during the actual conditioning sessions, is in line with our initial hypothesis.

Interpretation of reduced open field latencies and distances traveled

As previous literature suggests a latency to enter the center of an open field of 40-80 second for controls animals (Nautiyal, Ribeiro et al. 2008), the value of 31.1 ± 6.3 seconds in SRR conditioned rats is surprising, especially in light of yoked control performance (70.2 ± 17.8 seconds). This occurred despite other parameters having similar values to previous reports, e.g. center field bouts: 10.5 ± 1.7 (SRR) vs. 12.0 ± 4.8 (yoked) in our animals compared to 10.2 ± 1.9 (experimental) vs. 12.7 ± 1.3 (control) in a previous study (Smith, Li et al. 2007). Therefore, we interpret this difference as indicative of decreased anxiety-like behavior in response to novelty stress in SRR conditioned rats.

Perhaps the most intriguing of our findings is the trend toward decreased distance traveled in the open field in SRR conditioned rats, especially since no activity differences were observed between animals during conditioning (Figure 2.9). However, motor activity during conditioning was defined as the absence of rest, and therefore included grooming behavior, exploratory sniffing, and vertical rearing, whereas distance traveled exclusively captured horizontal locomotion, possibly accounting for this discrepancy between measures. It may be that our conditioned animals were generally more restful following conditioning, a finding that agrees with the subjective interpretation of a blind experimenter who worked with the animals during nociceptive assays and consistently noted that SRR conditioned rats were more restful than yoked controls during and

between tests. To determine whether this finding holds across different contexts, it will be important for future studies to implement a variety of stressors, as well as to run general tests of locomotion, such as the rotarod test, which is commonly used to monitor deficits in animal models of injury (Hamm, Pike et al. 1994).

Although we found no statistical difference in the number of center field bouts or the duration of time spent in the center between groups, the trend toward greater total distance traveled in yoked rats could have obscured differences in these variables. While dividing the number of center field bouts or duration in the center by total distance did not result in significant group differences, it is possible that there is a non-linear relationship between distance traveled and tendency to explore the center of the field, for instance if rats strategically tend to explore areas of the field only once before moving on to unexplored areas (more likely to be in center, since the periphery is explored first). An alternative possibility, supported by preliminary power analyses, is that several of the variables measured during open field testing may require larger sample sizes to assess significance.

Finally, although we did not perform additional stress tests with a similar novelty component due to the already complex battery of physiological and nociceptive tests we scheduled following open field testing, future studies would benefit from additional tests of anxiety, such as the elevated plus maze, to validate our conclusions. An additional possibility would be administration of a psychosocial stressor, such as resident-intruder stress, which has been shown to potently activate neurons in the CeA and PVN (Martinez, Phillips et al. 1998), and could be repeated over the course of several sessions with intermittent blood collection for ACTH or CORT (Heinrichs and Koob 2006).

Social anxiety could be examined using either an acute or chronic design (Bhatnagar, Vining et al. 2006).

The impact of conditioned SRR on nociception and central hyperalgesia

Although nociceptive responses to thermal and mechanical stimuli were unchanged, responses to a formalin inflammatory pain model revealed significant group differences, with SRR conditioned rats having an increased threshold of responsiveness to Von Frey hairs. We interpret this significant result following formalin injection to support a role for SRR in alleviating centrally mediated hyperalgesia. Because nociception involves the activation of nociceptors upon administration of some mechanical, thermal, or chemical stimulus, and transduction of potentially harmful stimuli into adaptive reflex responses, it makes sense that long-term respiratory training would have negligible impact on these basic nociceptive responses. Hyperalgesia with formalin may be one nociceptive condition where it is adaptive to regulate pain from descending pathways, whereas during nociceptive reflexes (such as those occurring during the Tail Flick Test), this is not advantageous. One candidate brain region that could be contributing to this effect is the anterior cingulate cortex, a limbic area implicated in the perceived unpleasantness of pain when its perceived intensity remains unchanged (Rainville, Duncan et al. 1997). Interestingly, this selective responsiveness for the unpleasantness of noxious stimulation has parallels in human meditation studies (described below) and could account for our finding that basic nociceptive reflexes were unaltered in SRR conditioned rats despite diminished mechanical hyperalgesia. Descending pathways could be recruited to reduce hyperalgesia-related neural activity

patterns, heal the responsible injury, and control pain, perhaps inhibiting responsiveness to subsequent "painful" stimuli.

Interpretation of unidirectional changes in response to tail flick and acute restraint

Several interpretations could explain the result (from Day 2 of nociceptive testing) that conditioned animals showed a trend toward increased sensitivity to thermal stimulation to the tail, despite an attenuated physiological response to the preceding restraint stress. i) It may be adaptive to remove the tail from the Tail Flick testing apparatus upon detection of noxious heat, and/or ii) yoked animals may lack responsiveness due to increased anticipatory anxiety following the first day of testing. One might have expected decreased sensitivity and an increased threshold of responsiveness to the Tail Flick Test in SRR conditioned rats, which would have potentially been indicative of enhanced coping with anticipatory anxiety (since the animals were already familiar with the Tail Flick Test on Day 2, having undergone the same testing protocol one day before). However, our observation of a trend toward enhanced sensitivity in SRR conditioned rats may actually reflect adaptive nociceptive responses that prevent continuous exposure to noxious heat stimulation. Since greater levels of restraint may lead to greater tail flick latencies due to heightened stress levels (Bannon and Malmberg 2007), differential stress responses between SRR conditioned and yoked rats may have been the main contributor to borderline group differences. Indeed, tail flick latencies went up slightly from Day 1 to Day 2 in both groups, with the magnitude of the increase being larger in yoked rats (5.7 to 6.8 seconds vs. 4.6 to 5.0 seconds in SRR conditioned rats). Furthermore, this increase was significant in yoked rats

(P = 0.008, Wilcoxon Signed Rank Test due to failed Normality Test) but not SRR conditioned rats (P = 0.685, paired t-test). This could suggest that anticipatory anxiety caused a stress response in yoked rats that delayed their responsiveness on the second day of Tail Flick testing.

CORT and conditioning results highlight the importance of shared experience.

In Chapter 2, we suggested that correlations between the % of samples with the floodlight off in a given trial and the % of samples yoked rats spent under SRR threshold (Figure 2.6A), and between SRR conditioned and yoked rat average RRs (Figure 2.6B), indicated that yoked rats may have found sessions with less light to be less stressful, or that shared experiences (e.g. handling, animal facility care) on each day of testing led to parallel changes in respiratory behavior. The parallel yoked animal RR decrease could be explained by the progressive reduction in strobe light exposure during later conditioning sessions, in combination with strobe's nature as a chronic mild stressor. SRR conditioned rats' increased success in reaching criterion resulted in a gradual decrease in the percentage of each trial with the strobe light on, which correlated with yoked animal RR and % under threshold. Although we'd expect both animals to be more active and to experience an arousal-related increase in RR during periods of darkness (van den Buuse 1994) the impact of long-term strobe exposure on general activity levels and RR has not been previously reported. In summary, the parallel trends in SRR conditioned and yoked rat respiratory parameters may have been due to reduced exposure to light or some other shared experience.

Similarly, since outcome measures in different animal cohorts were run several

months apart due to the timeline of conditioning procedures, the large variability observed in CORT levels (as well as other outcome measures) could be explained in part by common environmental experiences that differed between animals run on different days. CORT levels were similar to those previously reported following a 30-minute restraint test (Bourke and Neigh, 2011). Although there was a trend toward reduced CORT expression in SRR conditioned rats, there was high variability between animals and inadequate power to detect group differences. Some of this variability seemed to originate from the day animals were tested (Figure 3.6B). This result points to a larger issue that needs to be addressed: because all of our outcome measures depend at least to some extent on human interaction with the rats (either on the part of the experimenter or animal facility staff), and we only have a readout of each animal's physiology and behavior during a small portion of the day, we ultimately possess an incomplete picture of baseline state – which can dramatically impact testing performance. Our study design would benefit from a greater understanding of how shared experience impacts our animals on a day-to-day basis. In the next chapter we discuss our lab's development and refinement of a non-contact remote monitoring system for this purpose.

Absence of heart rate and RSA readouts during conditioning

Due to our desire to study freely behaving rats and avoid invasive telemetry procedures, combined with our ongoing refinement of electric field sensors for monitoring heart rate (Chapter 4), we did not measure cardiac activity during the present study. Next generation operant conditioning studies will not only remotely monitor respiratory and heart rate using remote sensors, but will also provide targeted feedback of

RR and RSA (Chapter 5).

Although we expect SRR conditioning to dramatically increase RSA, it is also possible that rat autonomic function is already optimally tuned for peak RSA, such that SRR will not enhance it. While this is unlikely given evidence of Mayer waves at low frequencies of arterial blood pressure in these animals and of enhanced cardiorespiratory synchronization at slower respiratory frequencies (Rubini, Porta et al. 1993, Pereda, De la Cruz et al. 2005), we could precondition rats in an environment that is likely to promote stress. Many options are available (Heinrichs and Koob 2006). For example, exposing rats to resident-intruder stress pre-training would be expected to prime a baseline stress response.

Inflammation and autonomic outcome measures

Although we chose to measure a battery of basic stress-related outcomes, it is possible that additional or alternative markers may provide a better metric of the therapeutic impact of SRR. These could include adrenocorticotropic hormone (ACTH), or markers of sympathoadrenomedullary stress axis function (e.g. norepinephrine or epinephrine) or immune activity. For instance, stress is associated with activation of inflammatory pathways implicated in the pathophysiology of depression (Raison, Capuron et al. 2006). In the case that autonomic changes are not as predicted, or as a future direction, we could measure stress-induced inflammation as a marker of autonomic imbalance. This is an especially important consideration due to our finding the SRR conditioned rats have an increased threshold of responsiveness to nociception during the chemical formalin assay (Figure 3.5B), which recruits central inflammatory pathways

during its later stages, when animals were tested with Von Frey hairs. Furthermore, our collaborators recently found that frequently practicing meditation reduced the plasma interleukin-6 (a marker of inflammatory activity) response to psychosocial stress, independent of HPA axis activation (Pace, Negi et al. 2009). Although respiration was not monitored during practice, differences in RR may represent an underlying confounding variable.

To what extent does our model accurately depict the relaxation response?

While a central concern is the extent to which physiological and behavioral changes in our model are congruent with the relaxation response in humans, it is important to note that we have also set out to *establish an operational definition of relaxation and the relaxation response in the rodent*, since our study is the first to operantly condition SRR and observe its effects. Our results from Chapters 2 and 3 cast light on the physio-behavioral changes that may in combination define our model. We conceptualize "relaxation" as involving slowed respiration (Figure 2.5B) and possibly increased time at rest (e.g. in the open field, Figure 3.3), and the relaxation response as involving reduced physiological and behavioral stress responses, including reduced latency to enter the center of an open field (Figure 3.3), decreased RR response to restraint (Figure 3.4), and potentially decreased sensitivity to painful mechanical stimulation following formalin injection (Figure 3.5).

As pertains to this last point, evidence from human studies informs the conception of our model. Decreased anxiety and stress reactivity are well documented in meditation, yoga, and with paced breathing (McCaul, Solomon et al. 1979, Sakakibara and Hayano

1996, Brown and Gerbarg 2005, Pace, Negi et al. 2009). While there is also evidence that the intensity of pain (pain response thresholds) may be unaltered, its perceived unpleasantness is reduced in meditation (Lutz, McFarlin et al. 2013); indeed, it may be adaptive to avoid painful stimuli. Furthermore, the finding from the same study that meditation reduces the anticipatory representation of aversive events supports our results in the Tail Flick Test, where latencies increased significantly from Day 1 to Day 2 in yoked rats – possibly indicative of anticipatory anxiety inducing a passive and maladaptive "freezing" response to the nociceptive stimulation – but not in SRR conditioned rats.

Interestingly, our negative findings for CORT also have precedent in human meditation studies, which have found the cortisol response to acute stress to be similar between meditators and control subjects (Pace, Negi et al. 2009), or even greater in meditators despite decreased self-reported psychological stress reactivity (Creswell, Pacilio et al. 2014). It is also possible, however, that our CORT responses were artificially low because animals had previously been exposed to the restraint apparatus on the two previous days.

Taken together, our findings presented in Chapters 2 and 3 are consistent with the human literature and our understanding of the relaxation response as a state of rest that alters stress responsiveness (Benson, Rosner et al. 1974). Running additional tests of anxiety, locomotion, and pain responsiveness will help narrow our operational definition of relaxation and enable next generation studies to precisely address a variety of mechanistic questions (see Chapter 5).

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CHAPTER 4: Plessey EPIC sensors permit remote monitoring of respiration, heart rate, and stereotyped behavior in the rodent

A modified version of this chapter will be submitted for publication as:

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Abstract

Numerous environmental and genetic factors can contribute significantly to behavioral and cardiorespiratory variability observed in experimental tests. Technologies that allow for continuous home cage recordings of an animal's physio-behavioral ethogram should enhance our understanding of inter-animal variability while providing a platform to more uniquely evaluate experimental results. Current systems for quantification of behavioral variables require specialty equipment and software, while measures of respiratory and heart rate are often provided by surgically implanted or chronically affixed devices. We assessed whether inexpensive electric field sensors (EPIC sensors, Plessey Semiconductors) embedded in a rodent's home cage could accurately record respiration, heart rate, and motor behaviors. EPIC sensors were first shown to accurately encode imposed sinusoidal changes in electric field tested at frequencies ranging from 0.5-100 Hz. Metronome arm movements were easily detected, but response magnitude was highly distance dependent. In anesthetized rats and mice, a nearby embedding site allowed for accurate mechanical detection of both respiratory and heart rate. Comparable success was seen in naturally behaving animals at rest or sleep when embedded sensors were nearby. Video-verified motor behaviors (sniffing, grooming, chewing, and rearing) were detectable and uniquely separable by their characteristic voltage fluctuations. Larger movement-related events had comparably larger voltage dynamics that allowed for a broad approximation of overall motor activity. Spectrograms accurately detailed an animal's physio-behavioral ethogram, while filtering and thresholding software allowed for detection and quantification of identifiable movement-related events. We conclude that EPIC electric field sensors provide a means for non-contact detection of

cardiorespiratory and motor events in a home cage and should allow for inexpensive high-throughput capture of animal life history, including detection of unexpected changes and responses to experimental interventions.

Introduction

Non-contact continuous physiological monitoring

Respiratory and heart rate are two of the most reliable markers of general health status, with both measures heightened during stress or in a number of neuropsychiatric conditions (Grossman 1983, Kannel, Kannel et al. 1987, Fieselmann, Hendryx et al. 1993, Gillman, Kannel et al. 1993, Jouven, Zureik et al. 2001, Fox, Borer et al. 2007, Palatini 2007, Cretikos, Bellomo et al. 2008, Hegoburu, Shionoya et al. 2011). To this end, there is great demand for increasingly versatile and economical methods of monitoring cardiorespiratory parameters. The last few decades have seen leaps and bounds in the development of novel technologies for monitoring rodent physiology (Hegoburu, Shionoya et al. 2011, Zehendner, Luhmann et al. 2013). While whole-body plethysmography (Jacky 1978, Aaron and Powell 1993, Wilkinson, Huey et al. 2010) and arterial transducer-coupled radiotelemetry (Huetteman and Bogie 2009, Kuwahara 2011) remain the "gold standards" for monitoring respiratory and cardiovascular variables, respectively, these methodologies are expensive and have additional limitations. Plethysmography requires animal placement in chamber environments that are separate from home cages, and cardiorespiratory monitoring requires use of expensive and surgically invasive approaches. These limitations restrain experimental design by increasing cost and decreasing throughput.

Non-contact continuous behavioral monitoring

Certain rodent motor behaviors occur at characteristic frequencies. Sniffing and whisking are commonly synchronized rhythmic motor activities occurring at 5-11 Hz that enable rodents to localize and track objects in their environment and are an expression of reward anticipation (Welker 1964, Deschenes, Moore et al. 2012). Grooming is a self-directed response to novelty and includes licking, scratching, and face-washing (Bolles 1960, Jolles 1979). 'Protracted grooming' begins with 5-9 forepaw licking strokes at 6-7 Hz, followed by a short series of 2-4 Hz strokes and ending with licking the ventrolateral body surface (Fentress 1988). Licking during drinking ranges from 4-7.5 Hz (Weijnen 1998).

Tracking motor behaviors in the home cage environment may provide important predictive and diagnostic information. For example, analysis of grooming behavior may be able to discriminate between highly stereotyped chain movements and different levels of anxiety in the rat (Fentress 1988, Kalueff and Tuohimaa 2005). The 'sickness behaviors' characterizing ill animals administered infectious agents frequently involve decreased grooming (Hart, 1988; Aubert, 1999; Weary et al., 2009); healthy rodents spend a large portion of their waking time grooming, which aids in thermoregulation (Gaskill et al., 2013). In recent years there has been an increased focus on generalizing the behavioral changes that occur in a variety of disease states (Weary, Huzzey et al. 2009). For instance, mice with cancer (developing SL2 lymphoma) spend less time rearing as the disease progresses (van Loo, Everse et al. 1997), reflecting common changes in activity and exploratory behaviors. Changes in grooming (e.g. reduction) are

also particularly common in animal models of disease (van Loo, Everse et al. 1997, Paumier, Sukoff Rizzo et al. 2013).

While novel technologies abound for detecting animal behavior and its alteration in models of stress and disease (Spruijt and DeVisser 2006, Richardson 2015), these technologies are expensive and typically require additional equipment or concomitant video monitoring, making their use impractical for large-scale studies or in animal welfare applications where low cost and high throughput are desirable. Partly for this reason, and despite what is known about changes in behavior that accompany disease, behavioral phenotyping of rodent models of disease is relatively rare (Richardson 2015). Most disease studies with behavioral phenotyping make use of existing technologies such as Phenotyper® (Noldus), LABORAS (Metris), or the Intellicage (NewBehavior) (Van de Weerd, Bulthuis et al. 2001, Quinn, Stean et al. 2003, de Visser, van den Bos et al. 2006, Pham, Cabrera et al. 2009, Krackow, Vannoni et al. 2010). More recent studies have made use of high-throughput systems such as the Behavioral Spectrometer and automated behavior recognition (ABV) (van Dam, van der Harst et al. 2013, Brodkin, Frank et al. 2014), but these require concomitant video recording and may be tedious or costly for long-term animal monitoring applications on a large scale.

EPIC electric field sensors

Proposed here is the use of inexpensive EPIC electric field sensors as a multipurpose alternative to the aforementioned technologies. We sought to answer the question: Do EPIC sensors accurately record respiration, heart rate, and stereotyped rodent behavior or general activity levels in controlled environments, and in a

conventional home cage environment? To answer this question, we took the following approach: we i) compared sensor recordings to the conventional methodology for monitoring respiration and cardiac activity, in awake and anesthetized animals; ii) determined distance and location dependence of sensor recordings by plotting the response to simple mechanical motion and to rhythmic physiological activity in ketamine/xylazine anesthetized animals; and iii) extended sensor application to a home cage environment to monitor stereotyped animal behaviors and activity levels, as well as the physiological response to experimental stressors.

Here we demonstrate that EPIC electric field sensors can be embedded into the home cage environment to provide for non-contact ultrasensitive detection of movement-related events including respiratory and heart rate as well as various motor behaviors. Further studies are warranted to better understand the strengths and limitations of this technology and to develop analytics for automated detection, quantification, and sorting of observed cardiorespiratory and motor events.

Materials and Methods

Experimental animals

Male Sprague-Dawley rats (300–550 g) and C57/B6 mice (20-30 g) were housed in standard cages in a vivarium with a 12:12-h light-dark cycle and were fed *ad libitum* standard rodent diets. All experiments were approved by the Animal Care and Use Committee 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."

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 proximity to sensor, gesture recognition, and electrocardiographic (ECG) activity in humans. We tested Electric Potential Integrated Circuit (EPIC) PS25251 sensors. Each Plessey PS25251 sensor was 1 cm^2 in size and had 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 DB rectangular connector (DB-9). A DC power supply box provided ± 2.5 or ± 5 volts and ground to the Vdd, Vss, and Gnd DB-9 pins, respectively. The output and Gnd pins of the DB-9 were soldered to a BNC 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 and a sample rate of 1 to 10 kHz. The digitized data was continuously output to a Windows computer running pCLAMP data acquisition and analysis software (Molecular Devices) or LabVIEW (National Instruments). A dab of Epoxy was applied to each soldered joint on the sensors to provide additional strength and protection. Standard electrostatic discharge precautions were followed throughout the construction and handling process, which was an essential step.

A detailed description of the response characteristics of the sensors can be obtained from the manufacturer's website (http://www.plesseysemiconductors.com). Relevant to the current application are its lower and upper -3dB points of 0.2 Hz and 10

kHz, respectively. Unless otherwise stated, sensors were embedded in PVC tubing or taped externally to the chamber, with their wires run along the length of the cage and connected to their power supply box. This box also adapted connections to BNC outputs for subsequent signal digitization and data collection as described above.

Characterizing EPIC sensor frequency response properties

Plessey EPIC sensors respond to changes in the surrounding electric field due to movement. A description of the frequency response properties of EPIC sensors is provided by the manufacturer (Plessey Semiconductors). Given an understanding of these properties (e.g. filters) as according to sensor specifications, we sought to determine the response to strategically chosen external stimuli and to understand the relationship between signal amplitude and distance. Given that the sensors are AC-coupled at a -3dB low of 0.2 Hz, we applied a series of voltage waveforms across the frequency range thought relevant to the cardiorespiratory and motor behaviors to be assessed. Specifically, we investigated how sensors picked up changes in the surrounding electric field induced by periodic variation in a nearby capacitor or the movement of a metronome lever arm.

Comparing recorded electric field sensor responses to conventional recordings

Whole-body plethysmographic recording of respiration

For comparison of EPIC sensors with conventional methodology for recording respiration, individual rats (n=2) were placed in a Plexiglas chamber (PLY3215, Buxco Research Systems), and respiration was measured using whole-body plethysmography, as described previously (Chapter 2, Wilkinson, Huey et al. 2010). An

EPIC sensor was affixed to the exterior wall of the chamber with tape and strategically repositioned following the acclimation period so that the animal's resting location inside the chamber was closely adjacent to sensor placement. Both signals were fed to a PCI-6221 multifunction DAQ board as described previously, and processed via a customized program in LabVIEW. To isolate RR from other high and low frequency components, recordings were bandpass filtered at 1 Hz (high-pass) and 5 Hz (low-pass). LabVIEW data were converted to .abf files and subsequently imported into pCLAMP software for analysis. Recorded respiratory waveforms from plethysmography were compared to those obtained from the electric field sensors by calculating peak-to-peak intervals and exporting values obtained to Microsoft Excel to plot their correspondence as scatterplots. To show the accuracy of EPIC sensors over a wider range of respiratory rates, we used a frequency subroutine in LabVIEW to find dominant peak frequencies (in breaths/minute) for consecutive 10-breath intervals, which were typically 5-10 seconds in length per interval. We then produced a scatterplot of paired plethysmographic and sensor values and performed correlation analysis to determine the relationship and strength of correlation between methods.

Electrocardiographic (ECG) recording of heart rate in anesthetized animals

For assessment of sensor accuracy in detecting heart rate, experiments were undertaken in anesthetized rats (n=4; 3 adults and one 30-day old rat) and mice (n=3 adults). Animals were anesthetized (ketamine/xylazine; 0.3 ml/100g, IP) and placed within cylinders of PVC tubing, which is commonly used as a shelter in vivarium home cages (Bennett G. Galef 2000). For rats, the PVC tubing was 20 cm long and 9 cm in

diameter. For mice, it was 12 cm long and 4 cm in diameter. To test whether continuous recordings of heart rate could be accomplished in the home cage, sensors were placed over a bored hole in the side of the tubing. For recording ECG, the animal's front right paw and back left paw were connected to disposable adhesive electrodes (3M Red Dot^{TM}) with signal amplified using a custom built amplifier, digitized (Digidata 1321A, Molecular Devices), and captured at 1 kHz using pCLAMP software. To isolate heart rate from other higher frequency components, recordings were commonly low-pass filtered at 10 Hz.

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

For these experiments, sensor placement was also over a bored hole in the side of PVC tubing so that the front of the sensor was 1 cm from the interior wall of the tube. Adult animals (one rat and one mouse) were anesthetized with ketamine/xylazine as above for ECG experiments. The animals were placed so that their noses were 2 cm away from the sensor (i.e. sensor was aimed at the neck/base of the head). Recordings were then captured as anesthetized animals were moved rostrocaudally in 1 cm increments, with the sensor angled upwards at 40 degrees with respect to the vertical. Relative rostrocaudal position was measured from the tip of nose to middle of the sensor. Recordings were also captured as the tube was turned, situating the sensor at different angles while the animal remained in same position. Several circumferential angles were tested ranging from 0º to 180º from the horizontal plane.

Detection of stereotyped behavior in naturally behaving rats and mice

To determine the ability of EPIC sensors to accurately measure stereotyped behaviors in a home cage setting, sensors were affixed by tape to the front right exterior wall of the long side of home cages (46 x 30 x 30 cm), and we attempted to detect several behaviors in adult rats (n=2) and mice (n=2). EPIC sensors were used to measure stereotyped animal behaviors including vertical rearing, sniffing (largely a respiratory behavior), grooming, and chewing over the course several hours, with recordings compared to video in order to determine their accuracy in predicting these behaviors.

In several experiments, cages were shielded with wire mesh to ensure that detected events arose from the home cage. For the experiments presented here in adult rats, two sensors were recorded simultaneously. Both were positioned 7 cm from the right side of the chamber, at heights of 4 cm and 8 cm from the bottom. A video camera (Logitech) was positioned to simultaneously record animal activity. Recorded behaviors were tagged in real-time by an experimenter, with the most common behaviors observed being vertical rearing (upward exploratory activity, typically with paws on the side of the chamber), sniffing (high frequency respiration with corresponding head movements), grooming (self-directed licking or scratching), chewing (typically gnawing at food held in between the paws), and resting (lack of movement). Scoring categories represent some of the core behaviors from more nuanced behavioral ethograms (Van de Weerd, Bulthuis et al. 2001). Raw recordings were cross-validated with video analysis to confirm the precise timing of observed behaviors. Recorded data was then analyzed for its predictive value using a combination of manual scoring of video and sensor recordings combined with spectrographic analysis, to determine the correspondence between the observed behaviors

and recorded signal.

Sensor application to detecting respiratory and activity changes in response to experimental stressors

To validate sensor utility in tracking predictable changes in activity and respiration upon administration of experimental stimuli, recordings were undertaken in two naturally behaving adult rats in home cages. In the first, EPIC sensors affixed to the side of the cage were placed to capture broad movement events while the animal freely explored the cage unperturbed or following administration of a diet gel. In the second case, the home cage was equipped with PVC tubing and embedded sensors that were affixed to the bottom of cage walls, to allow sensors and wiring to be placed through adjacent windows cut into the cage. The rat was allowed to freely explore the cage and, once acclimated and in the PVC tubing, resting RR was monitored before two serial experimental perturbations ("stressors") were applied. Following administration of each stressor, changes in the animal's RR were monitored via sensor recordings and cross-validated with real-time experimenter scoring. Experimental manipulations included either a cage flick administered by the experimenter or an air puff delivered via a pressurized canister to elicit a startle response.

Analysis of experimental results

Subsequent signal analysis was accomplished in Clampfit (part of pCLAMP data acquisition and analysis software, Molecular Devices) or MATLAB (Mathworks). Using Clampfit, raw signal was analyzed and filtered, power spectra were produced and

analyzed, and threshold-based detection of movement events was performed. Spectrograms of the raw data were obtained in MATLAB using a customized code developed in our lab, and were produced from temporally averaged data (2 second windows with no window overlap, unless otherwise stated).

Results

EPIC sensors have characteristic properties and respond with high fidelity to external mechanical stimuli.

An EPIC sensor was placed between a parallel plate capacitor and subjected to a changing electric field (Figure 4.1A_1). As shown, voltage amplitude of sensor output was linearly related to magnitude of capacitor voltage (Figure 4.1A_2) with known filtering properties (Figure 4.1A_3). EPIC sensors accurately followed sinusoidal voltage commands at frequencies tested (.05 to 100 Hz) but with a phase delay at lower frequencies and a distorted waveform due to the filter design of the sensor integrated circuit (Figure 4.1A_4). Next, a Wittner 903012 Taktell Super-Mini Metronome was placed within a home cage at different locations to determine location and distance dependence of the sensor voltage waveform (Figure 4.1B_1). Lever arm movement ranged between 1.5 and 3 Hz with corresponding upper and lower arm swing magnitudes being 7 and 3 cm or 9 and 4.5 cm, respectively. Movement-related changes in the electric field accurately reflected the periodic metronome movement. Waveform appearance was observed to vary, presumably due to differences in pendulum trajectory coordinates associated with slight changes in the metronome face (Figure 4.1B_2). A sensor was positioned at one of four distances from the metronome and response amplitude was

quantified (Figure 4.1B$_{1-3}$). Normalized amplitude was plotted to reveal a 5-fold decrease when the sensor was moved from 1 cm to 6 cm away from the metronome, and thereafter amplitude appeared to taper off asymptotically. Finally, we showed that the magnitude of sensor recordings also depended on the static charge of the moving material (Figure 4.1B$_4$). Silicone and PE tubing have high capacity for static negative charge accumulation. Both materials were shown to increase the amplitude of the recorded response considerably, and appeared to transfer static charge to the metronome arm, since voltage amplitude remained larger than control values after their removal. Thus, changes in electrostatic field may significantly alter the magnitude of response and must be considered in recordings where response amplitude consistency is important.

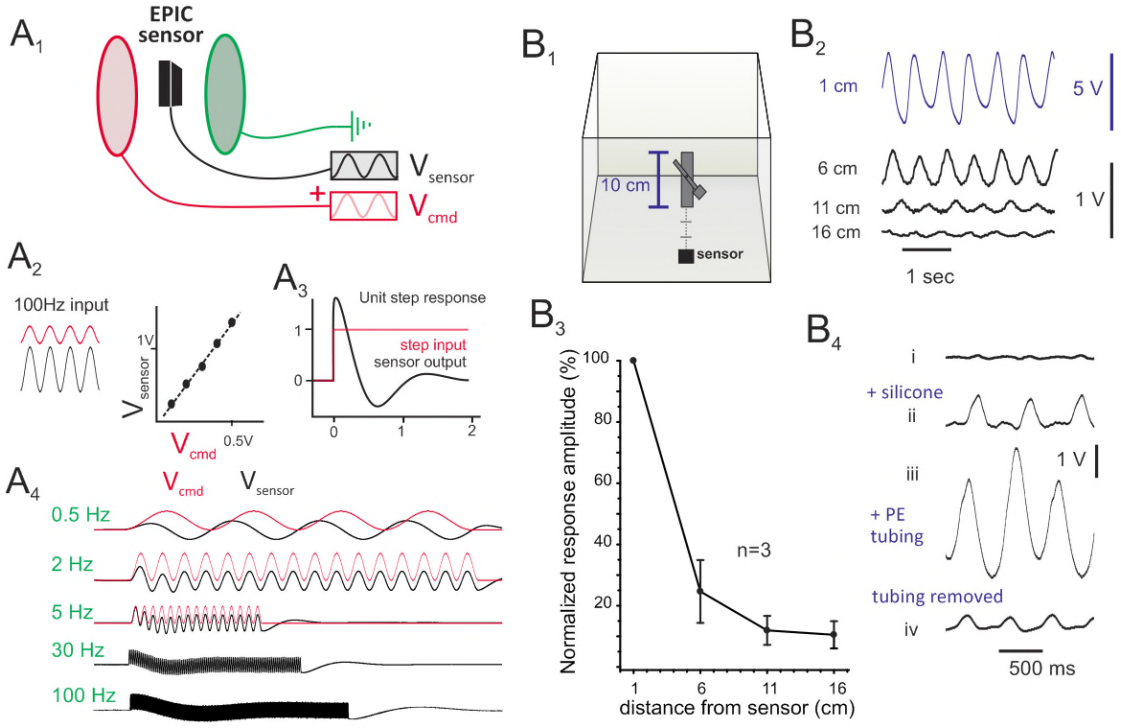


Figure 4.1. EPIC sensors accurately detect electrical oscillations and movement-dependent disturbances in the electric field.

(A$_1$) A single sensor was placed midway between a parallel plate capacitor, and various frequency cosine voltage commands were delivered to alter the electric field. **(A$_2$)** Sensor output frequency accurately reflected input frequency and scaled linearly with the input (input-output gain differences depended on properties of the capacitor and distance from the sensor). **(A$_3$)** Unit step response to show filtering qualities of the sensor. **(A$_4$)** Sensor output matched input frequencies but with phase delay at lower frequencies (e.g. 0.5 Hz). Note also distortion of the waveform due to the 0.2 Hz high-pass filter design of the sensor. **(B)** Recordings of periodic arm movements from a mini-metronome placed at several locations within a plastic rat cage. **(B$_1$)** Schematic showing approximate metronome placement at several distances from the sensor. **(B$_2$)** The magnitude of sensor response was distance dependent, as shown with placement of the metronome at 4 different locations from the sensor spaced 5 cm apart as indicated. **(B$_3$)** Normalized relative changes in voltage recorded with metronome distance from the sensor. Shown is the normalized mean ± SEM of conditions i-iii shown in panel **(B$_4$)**. **(B$_4$)** Sensor sensitivity to object movement depended on the triboelectric properties of the moving object. Shown are recordings of metronome pendulum arm movement 6 cm from the sensor with different negatively charged materials affixed to the upper arm of the pendulum: (i) control, (ii) drop of silicone glue, (iii) 1 cm length of PE tubing attached to silicone, or (iv) tubing removed.

Sensors reliably measure respiratory rate in mice and rats.

With the above caveats in mind, we next sought to validate the ability of EPIC sensors to monitor a rhythmic and continuous physiological mechanical stimulus, respiration. By comparison with the conventional methodology of whole-body plethysmography, we showed that sensors can accurately detect the respiratory rhythm in rodents. Using a modified plethysmography-EPIC sensor setup (schematized in Figure 4.2A), raw traces were simultaneously collected, showing the strong correspondence between plethysmographic recordings of RR and those obtained with the EPIC sensor (Figure 4.2B). This was clearly observed when plotting both instantaneous frequency of the respiratory cycle (Figure 4.2C, *top*), and dominant RRs over longer (~5-10 second) periods of time (Figure 4.2C, *bottom*) (n=2 rats for each). Correlation analysis of this relationship (Figure 4.2C, *bottom*) revealed an R^2 value of 0.98, suggesting a nearly perfect agreement between sensor- and plethysmography-based calculations of RR. The sensors faithfully reported a wide range of RRs, from approximately 60-120 breaths/minute. It is worth noting that, since we waited until the rat was resting and relatively close to the sensor, proximity may be an important consideration for accurate RR determination (see Figures 4.1 and 4.4).

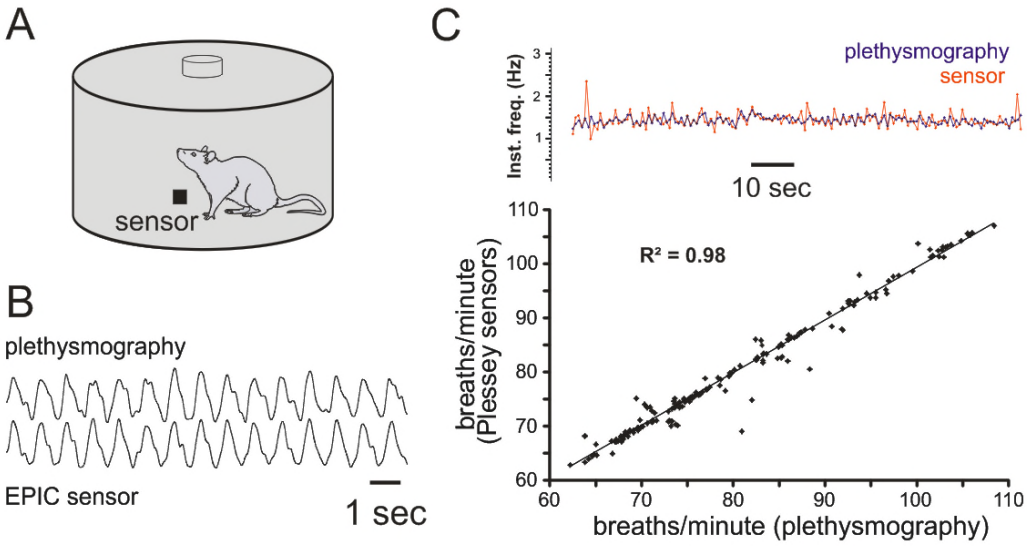


Figure 4.2. EPIC sensors accurately detect respiratory rate.

(A) Schematic shows plethysmography chamber with Plessey sensor affixed to the wall. **(B)** Representative respiratory traces from the plethysmograph (*top*) and Plessey sensor (*bottom*) in a resting rat. The signals were low-pass filtered at 4 Hz. **(C)** Comparisons of plethysmographic and sensor-based recordings of RR. *(Top)* Threshold-based detection of interpulse intervals converted to frequency resulted in similar determinations of instantaneous RR over 105s in a resting rat. *(Bottom)* Scatterplot comparison of dominant RRs measured with plethysmography (x-axis) to those reported from an electric field sensor (y-axis), with points representing 10-breath epochs in an individual recording. The R^2 value was 0.98.

Sensors reliably measure heart rate in mice and rats.

We next sought to determine whether EPIC sensors could reliably detect heart rate by comparison with ECG. Using a modified home cage setup with PVC tubing, we obtained raw traces from EPIC sensors and electrodes for ECG in anesthetized animals. In separate experiments, mice (n=3 adults) and rats (n=4, three adults and one 30-day old rat) were positioned inside the PVC tubing in close proximity to EPIC sensors. ECG and raw sensor recordings are shown for mouse and rat (Figure 4.3A_1 and A_2, respectively). Smaller events superimposed on respiratory waveforms were shown to match the period

of ventricular contraction on the ECG. Spectrograms further confirmed that these higher frequency components were identical to those of the ECG (Figure 4.3B). To isolate heart rate from other higher frequency components, recordings were commonly low-pass filtered at 10 Hz. Subsequent signal analysis was accomplished in Clampfit and MATLAB, as described previously. Spectrograms produced over longer time periods (Figure 4.3B) revealed respiratory and heart rate frequency bands in all of the animals at characteristic frequencies. However, spectrograms for both ECG and sensor recordings often contained strong bands at higher-frequency values that represented harmonics of the actual heart rate, potentially due to the unique shape of the cardiac waveform. Therefore, it was necessary to analyze raw recordings, possibly in combination with power spectra, for a precise determination.

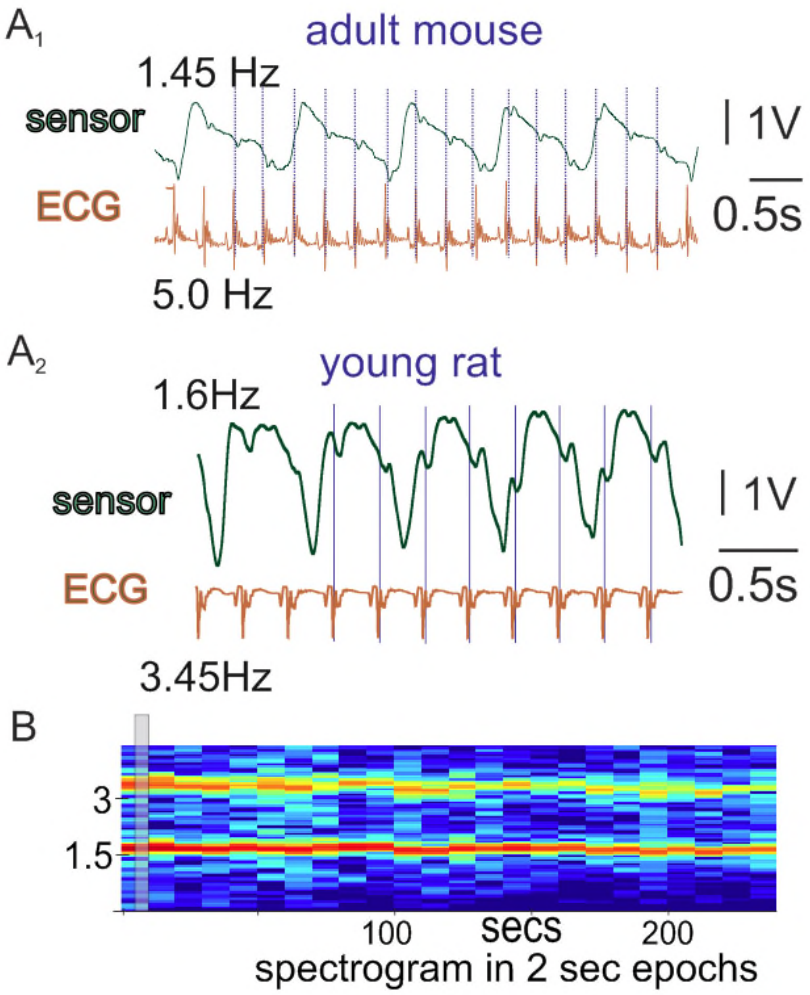


Figure 4.3. EPIC sensors record cardiac activity when appropriately placed.

EPIC sensors were attached to the outside of standard home cage PVC tubing, where rodents typically spend their resting time. A small hole drilled into the tubing in front of the sensor increased signal strength. **(A_1)** An adult mouse was anesthetized with ketamine/xylazine and strategically placed in the PVC tubing, with ECG leads connected to the front right and back left paws. A representative sensor recording is shown (*green*), high-pass filtered at 0.1 Hz and low-pass filtered at 10 Hz, for comparison with ECG (*orange*). **(A_2)** Representative sensor recording for a young rat, using the same setup as for mouse. **(B)** Spectrogram produced from the raw recordings sampled in **(A_2)** shows a predictable frequency band above 3 Hz for heart rate (y-axis) that decreases in frequency over time (x-axis).

Animal location relative to sensors impacts respiratory and cardiac waveform.

Having validated the use of EPIC sensors for measuring respiration and heart rate, we performed several experiments to better understand the impact of animal position on sensor responsiveness. In particular, we were interested in the effects of relative rostrocaudal distance and the angle of the sensor relative to the animal. Using anesthetized animals in PVC tubing (n=1 mouse and n=1 rat), we recorded at various sensor positions while incrementally changing sensor location or angle, as shown in schematics of our setup (Figure 4.4). While voltage waveforms were dependent on rostrocaudal animal position within the tubing, RR was captured at all tested locations (Figure 4.4A_2, 4.4B_1 and 4.4B_2). In the examples shown, when the animal's flank or stomach was positioned near the sensor, respiratory activity saturated the signal.

Regarding heart rate, the strongest recordings (largest signal amplitude of cardiac protrusions) were obtained when the sensor was angled upwards at 40 degrees with respect to the vertical (Figure 4.4A_1). In the rat, optimal rostrocaudal location was at the animal's shoulder region (8-12 cm nose-to-sensor). In the mouse, it was when the base of the animal's head or top of the neck was positioned next to the sensor (2-3 cm nose-to-sensor). These results demonstrate the importance of sensor placement for accurately monitoring cardiac activity.

Therefore, while RR was easily captured regardless of animal position, voltage deflections due to cardiac activity were extremely sensitive to location.

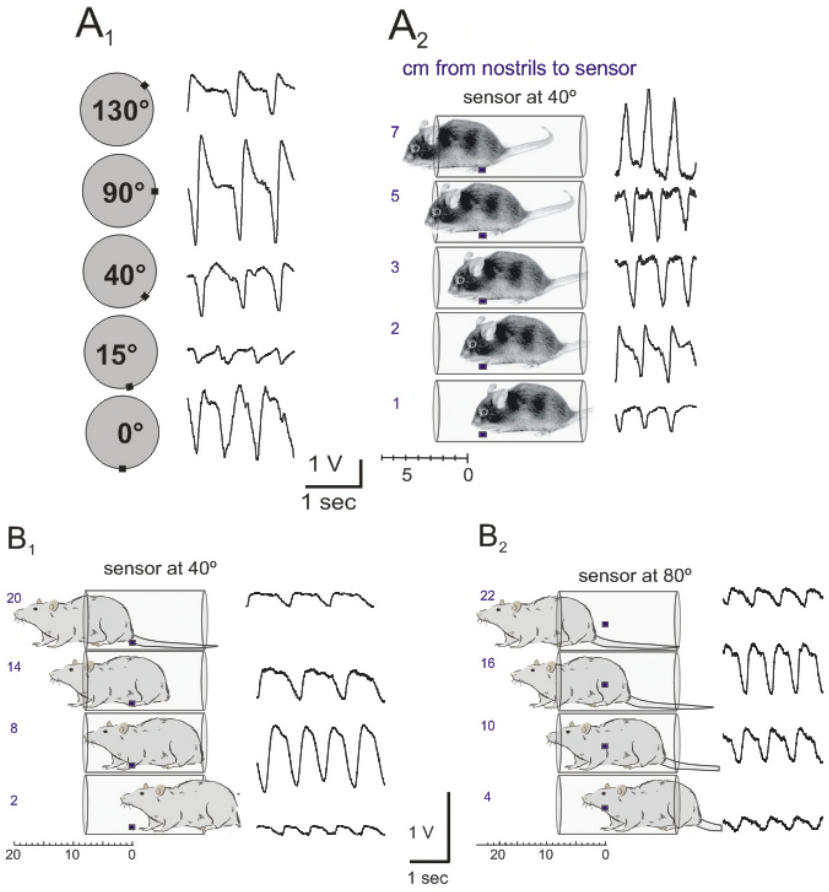


Figure 4.4. Effect of animal location and sensor angle on voltage waveform in both mice and rats.

Animals were anesthetized with ketamine/xylazine and placed in PVC tubing within the home cage. An EPIC sensor was placed over a hole in the side of the tubing so that the front of the chip was 1 cm from the interior wall of the tube. **(A)** Recorded waveforms in a mouse when the sensor was placed at various angles **(A_1)** or at 40º **(A_2)** upwards with respect to the bottom vertical. **(A_1)** The animal was placed so that its nose was 2 cm away from the sensor (i.e. sensor was aimed at the neck/base of the head). Recordings were captured as the tube was turned, thereby situating the sensor at different angles (animal remained in the same position). The voltage waveform changed depending on the angle of the sensor. RR was captured at all tested angles, while small voltage deflections from cardiac activity were most apparent when the sensor was located at 40º. **(B)** Recorded waveforms in a rat when the sensor was placed at 40º **(B_1)** or 80º **(B_2)** upwards with respect to the bottom vertical. Recordings were captured as the rat was moved progressively closer to the sensor with distance in cm measured from the tip of nose to the middle of the sensor. RR was captured at all tested locations, while small voltage deflections reflecting cardiac activity (as shown in Figure 4.3) were extremely sensitive to location.

Sensors reliably measure stereotyped rodent behaviors in a home cage setting.

In a modified setup with EPIC sensors affixed to the sides of a home cage, we were easily able to detect respiration in adult rats (n=2) and mice (n=2). EPIC sensors were also shown to accurately measure stereotyped rodent behaviors including vertical rearing, sniffing (largely a respiratory behavior), grooming, and chewing (Figure 4.5). Representative example traces from an individual rat of these four different stereotypical behaviors were obtained (Figure 4.5A, *bottom*) over the course of a three-hour period, and indicate their relative predictability. Spectrograms produced over shorter (Figure 4.5A, *middle*) and longer (Figure 4.5B) recording periods delineated epochs of stereotyped rodent behavior, as well as general activity levels. While EPIC sensor recordings clearly distinguished between sniffing and rearing, grooming and chewing were harder to isolate but appeared to be distinguishable based on unique spectrogram frequency band signatures and power spectra (see below).

We found that frequency, which was analyzed through a combination of observer scoring and power spectral analysis for individual samples, was consistently a more reliable predictor of animal behavior than peak amplitude, reported from the analysis software, which was dependent on animal distance from the sensors. The most common behavior picked up by the sensors during periods of free exploration was sniffing, with an average frequency (from all analyzable recordings) of 6.5 ± 0.9 Hz. Exploratory sniffing was isolatable by the presence of ~6 Hz oscillations concurrent with high-amplitude voltage deflections characteristic of rearing or movement.

Although grooming and chewing were sometimes predictable when the rat was positioned in certain orientations relative to the sensors, they were not generally distinguishable based on the raw signal. However, the similar motor pattern of rostral grooming and chewing (animal assumes vertical orientation, with weight on rear and hindpaws, and repeatedly brings forepaws to mouth) often resulted in a characteristic power spectral signature when the raw voltage signal was converted to the frequency domain, which tended to consist of several frequency peaks presumably corresponding to the different subcomponents of the behavior, typically in the range of 2-10 Hz. We were not able to identify a clear signature for chewing that occurred on the cage floor (where diet gel was placed), as opposed to chewing that occurred while the rat was in the rostral grooming orientation. However, comparison of spectrograms for cage floor chewing and rostral grooming revealed a range of low-frequency, high-intensity activity for grooming that was absent during chewing (Figure 4.5A, *middle*), suggesting that the behaviors could potentially be distinguished by appropriately processing the raw recordings.

Rears were quantified in an awake rat in the home cage, using video combined with the raw concatenated files. Rearing events on the side of the cage produced high-amplitude voltage peaks, and were easily recognized in raw recordings of animal home cage activity. We quantified the number of rearing events over the course of a one-hour recording period, recording 73 clear rearing events of variable duration. Rearing was considered in progress as long as animal forepaws made contact with the side of the cage. We found that under our particular experimental conditions, an upper threshold value of 0.3V for sensor recordings faithfully reported 67 (91.78%) of all rearing events. Of the 68 periods of crossing encountered at this threshold, only one event (1.47%) was a false

positive. For more refined analysis of movement over time, graphical analysis demonstrated an increased density of high-amplitude events (scatterplots) and high power across frequencies (spectrograms) during broad movements in general and rearing in particular. Therefore, plotting the density of high-amplitude events or high-power frequency bands could serve as an approximation of home cage activity levels in freely behaving animals (also see below).

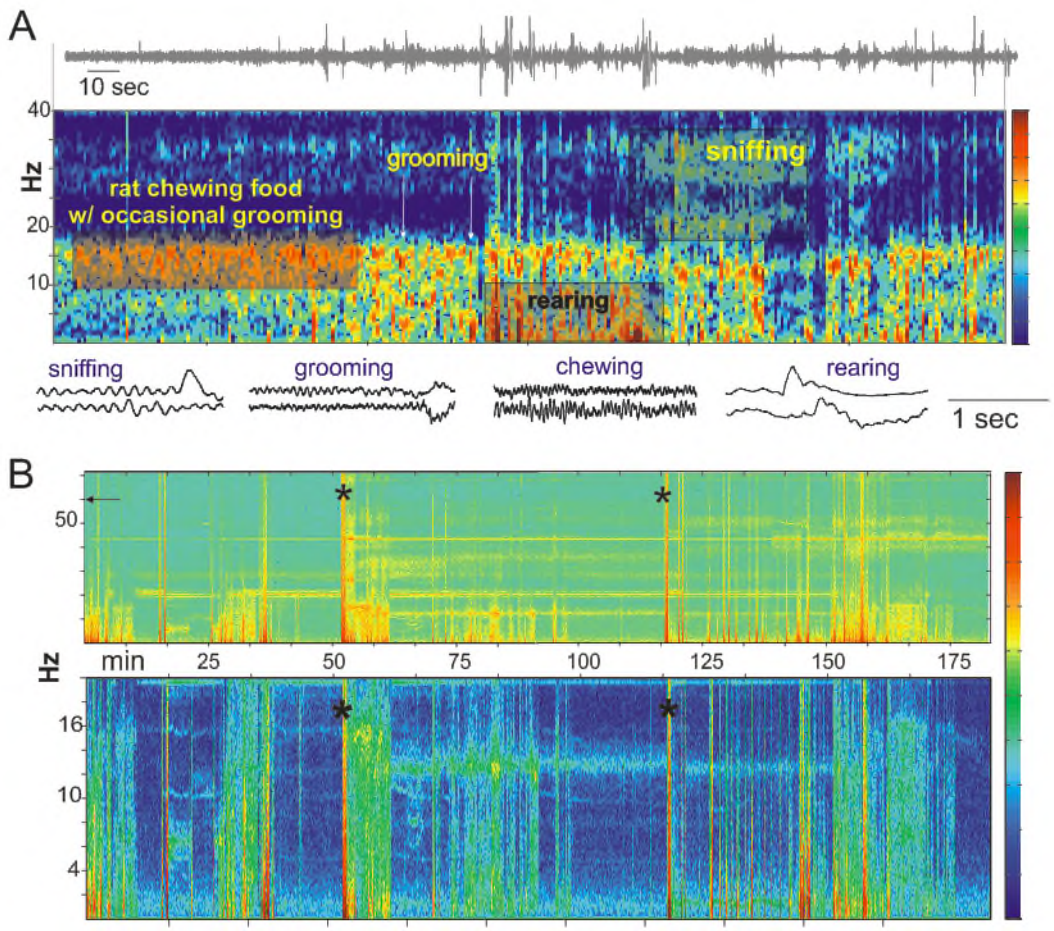
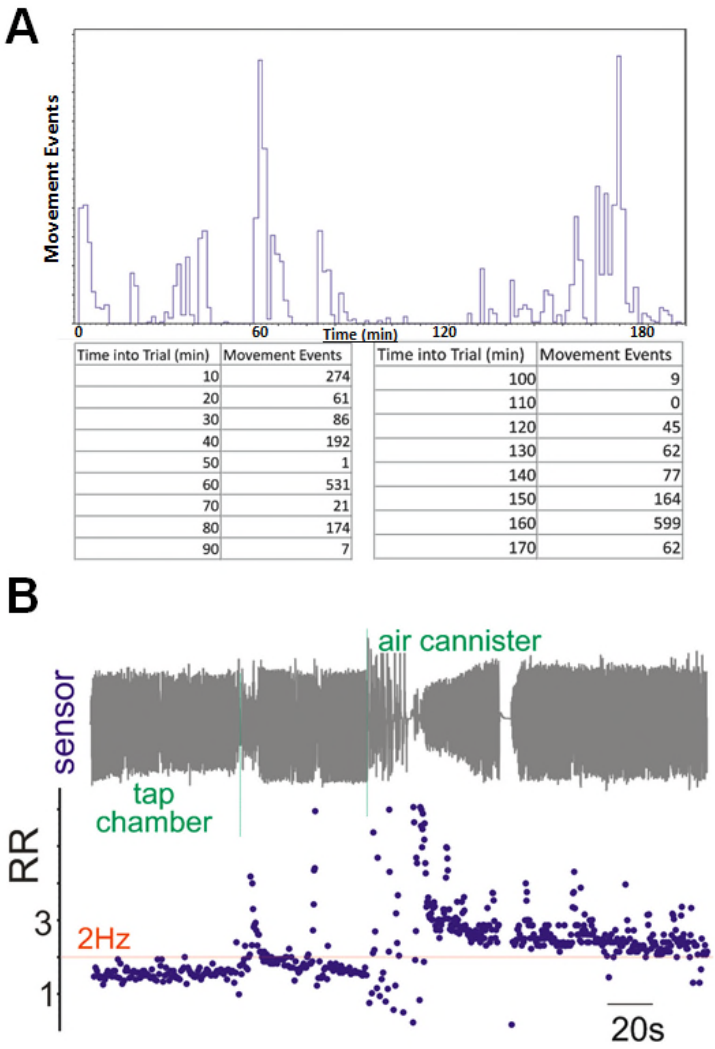


Figure 4.5. EPIC sensors record stereotyped behaviors in awake rodents in their home cages.

(A) *Top:* Raw 10-minute voltage recording of rat behavior in the home cage from a sensor affixed to the wall exterior. *Middle:* Spectrogram of the recording (2 sec windows), tagged for different behaviors. Video recordings associated observed motor behaviors with distinct spectral heat map signatures. *Bottom:* Epochs of raw voltage recordings of different behaviors from above. **(B)** Three-hour recording highlighting the multitude of unique spectra observed. *Top:* 0-70 Hz (note: home cage electrical shielding abrogates 60 Hz interference). *Bottom:* 0-20 Hz. Asterisks identify experimenter manipulation of the cage and its subsequent effect on spectral signature. Heat maps were intensity adjusted to optimize contrast.

Sensors reveal changes in respiration and activity following experimental perturbation.

To investigate sensor feasibility in an experimentally relevant paradigm, several experimental perturbations were applied to induce changes in physiological variables and activity during ongoing sensor recordings (n=2 rats). For the first of these, an adult rat was placed inside a home cage and allowed to explore freely for several hours. One hour into this period, a carton of diet gel was placed in the cage. Animal exploration in the area of the gel increased, as reflected in a greater density of high-amplitude events in the raw recording, captured via threshold-based detection (Figure 4.6A). For stressor administrations, an awake adult rat was placed in a home cage with EPIC sensors affixed to the outside of PVC tubing, and was allowed to acclimate prior to administration of a mechanical cage flick stimulus. Following the cage flick and during a subsequent period of rest, an air canister in close proximity to the rat was used to provoke a startle response. The air puff startle resulted in a dramatic increase in RR and increased activity after 500 seconds in the recording, when the animal began to explore. These phenomena were reflected clearly in raw sensor recordings and scatterplots (Figure 4.6B). Therefore, in distinct scenarios, sensors can capture elevated activity (diet gel stimulus) or RR (cage flick and air puff startle stimuli) immediately following experimental perturbations; in the latter case this response scaled with stimulus intensity, since the more stressful air puff stimulus resulted in a greater elevation in RR.



Time into Trial (min)	Movement Events
10	274
20	61
30	86
40	192
50	1
60	531
70	21
80	174
90	7

Time into Trial (min)	Movement Events
100	9
110	0
120	45
130	62
140	77
150	164
160	599
170	62

Figure 4.6. EPIC sensor-based recordings of changes in activity and RR in awake and behaving rats.

(A) A freely behaving rat was recorded for 3 hours in the home cage and threshold-based detection was used to isolate movement events. We set a high voltage threshold such that the animal traveling around the cage or other broad changes in movement triggered detection of an event. The rat moved most frequently during acclimation, around 60 minutes in, when a carton of diet gel was placed inside the cage, and near the end of the recording. **(B)** A rat was placed in its home cage with a sensor affixed to the side of the PVC tubing shelter. The grey trace shows raw sensor output (*top*), while inter-breath instantaneous RRs were auto-detected with threshold setting software and are shown in scatterplot form (*bottom*). The front of the cage was tapped at the time indicated by the first vertical green line, while a compressed air canister delivered an aversive stimulus at the time indicated by the second vertical green line.

Discussion

In summary, our lab sought to test Plessey Semiconductors EPIC sensors as a low-cost alternative method to monitor physiological and behavioral variables non-invasively. We hypothesized that these sensors, which measure changes in the surrounding electric field, could reliably detect respiration, heart rate, and behavior from rodents in the confines of a small experimental chamber, or in an appropriately equipped home cage environment. Above, we demonstrated the fidelity of sensor output and frequency responsiveness and validated sensor responses to a predictable and rhythmic mechanical stimulus. We then undertook sensor recordings in plethysmography and ECG experiments to validate sensor capabilities with conventional recording techniques, and further determined the rostrocaudal and angular positional requirements for high-fidelity sensor recordings. Finally, we obtained recordings in modified animal home cages with or without PVC tubing, mimicking housing conditions in animal research facilities, and showed that sensors detect stereotyped rodent behaviors, as well as general activity levels and stress-induced changes in activity and respiration.

In relation to monitoring key physiological indices ("vitals") of health and disease, we demonstrated that EPIC sensors reliably monitor respiration and accurately report RR, instantaneously and over prolonged periods of time. We validated sensors by comparing their accuracy with the "gold standard" conventional method of whole-body plethysmography, a reliable but expensive technique that requires an airtight chamber. Based on the ease with which sensors tracked the respiratory waveform, we would expect them to accurately detect a behavior such as freezing, which is characterized by lack of motion and presence of stereotyped respiration (Hegoburu, Shionoya et al. 2011). EPIC

sensors could therefore present a cheap alternative to plethysmography for measuring respiration during fear conditioning. Additional applications are up to the imagination of the experimenter (Sousa, Almeida et al. 2006). While more sensitive to proper positioning, sensors also tracked another key physiological index of health, heart rate. For assessment of sensor accuracy in detecting heart rate (when embedded into PVC tubing home cage shelters), we compared sensor-based detection of heart rate to ECG. EPIC sensors recorded cardiac activity when appropriately positioned relative to either a rat or mouse. In our recordings, placement of the sensors through a hole in standard home cage PVC tubing was sufficient to acquire a clear signature for heart rate in anesthetized rats and mice. This required relatively precise placement, and the signal was clearest when the sensor was located 2-3 cm from mice and 8-12 cm from rats. Because of difficulties of interpretation posed by multiple strong harmonics in spectrograms of these recording periods, heart rate was best analyzed from the raw sensor recordings. We conclude that sensors provide a cheap, non-contact means of measuring heart rate in mice and rats at rest in standard housing PVC tubing, although appropriate positioning is paramount. Sensors could represent a low-cost, non-invasive alternative to telemetry for measuring heart rate.

EPIC sensors, which are commercially available for movement detection in humans, also accurately reported stereotyped behaviors and differential activity levels in rodents. Adult rats placed in a home cage and allowed to explore freely for three hours displayed a range of conspicuous and repetitive behaviors including vertical rearing, sniffing, grooming, and chewing. EPIC sensors provided characteristic signatures for sniffing and rearing, which are typically evoked in response to novelty or stress, and

could therefore be useful for biobehavioral research and animal monitoring applications. While sniffing was characterized by a specific frequency pattern, vertical rearing led to large-amplitude voltage swings, which were accentuated when the behavior occurred in close proximity to the sensors. Against background noise and other reported behaviors, periods of rearing were easily detected using threshold-based detection of high-amplitude events in Clampfit, and were characterized by high-power activity across frequency bands in a corresponding spectrogram. Weaker signatures for grooming and chewing were observable upon processing the raw recordings. Given that rearing and grooming behavior can be indicative of an animal's stress state (Kalueff and Tuohimaa 2005), having affordable and effective ways to monitor them on a long-term basis could be invaluable for stress-related research or as a basic index of animal wellness. In conclusion, sensors may provide a low-cost means of monitoring stereotyped animal behaviors over time, and have practical uses for tracking changes in activity levels in a home cage environment following various manipulations.

However, the extent to which EPIC sensor signatures for stereotyped behaviors generalize across days, animals, strains, and different sensor placements remains to be established; it may be the case that concomitant video recordings are necessary to identify the characteristics of signature waveforms in a particular experimental context prior to commencing long-term home cage recordings. It also remains to be determined whether the sensors can pick up more subtle behavioral changes such as animal shivering or motor activity during sleep. Future experiments applying EPIC sensors to study a wider range of experimentally relevant behaviors, across different species and strains of animals, will ultimately resolve the full scope of sensor capabilities and whether their

applicability generalizes from one context to another. We propose that any behavior with a clear motor signature will be isolable upon appropriate processing of the sensor signal, since sensors are exquisitely sensitive to movement-based changes in the electric field.

Finally, as the combined measurement of autonomic and motor parameters may complexly define behavioral state, EPIC sensor recordings could permit characterization of animals predisposed to develop disease, or identification of the stress state of an animal. In our final set of experiments, we used EPIC sensors to detect changes in activity and respiration following the administration of experimental perturbations. Sensors accurately reported an increased frequency of movement events and heightened RR following these stimuli, with the RR response graded by stressor intensity. This is of relevance to animal models of disease, where behavioral and physiological changes may accompany disease onset as well as its alleviation with various therapeutics.

Despite advances in home cage monitoring of rodent behavior and physiology, automated behavioral technologies have still not been used to characterize the behavior of rodents with many different systemic diseases (Richardson 2015). Indeed, many studies using rodent disease models do not even consider key behavioral and physiological variables. This is often due to the high cost or cumbersomeness of available methods. EPIC sensors provide affordability and adaptability as a novel means of measuring respiration, heart rate, and stereotyped rodent behaviors including rearing, sniffing, grooming, and chewing. The sensors are able to effectively isolate bouts of activity (typically including rearing, sniffing, and general exploration) from relative restfulness (resting respiration). This technique is cheaper than conventional methodology, would permit near-continuous home cage recordings in the vivarium (e.g. using data loggers),

and may also confer ability to monitor movement and predict animal location. We expect that refinement of the sensors for home cage studies will ultimately allow for real-time feedback-based control of physiological parameters as a preventative therapeutic approach to promote autonomic balance and facilitate wellness. Potential applications range from large-scale animal housing facility implementation of sensor technologies to monitor animal health and welfare, to high-throughput studies of the physiological and behavioral concomitants of disease development and progression. As elaborated upon in Chapter 5, remote non-contact sensors could eventually permit home or office monitoring of health status in humans as a basis for conscious or subconscious feedback-based strategies. There are tremendous clinical and technological implications for 'smart' feedback-based technologies in wellness enhancement.

Acknowledgements

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CHAPTER 5: General Conclusions and Future Directions

Summary

Here, we used operant conditioning procedures combined with whole-body plethysmographic recordings of respiration to lower respiratory rate (RR) in adult rats. Our study employed a dual-chamber, yoked design, such that only slow respiratory rate (SRR) conditioned rats were able to control an aversive strobe light by slowing their breathing. SRR conditioned rats successfully learned the task, with average RR decreasing by more than 10 breaths per minute over the course of training. Conditioned rats also breathed more regularly at rest, and spent a similar amount of time at rest compared to their yoked controls. A small control group of rats that learned to breathe faster instead of slower (FRR conditioned rats) controlled for learned helplessness effects in our experimental design.

SRR conditioned rats maintained slower breathing during retention sessions intermittent with conditioning. Following conditioning, they took less time than yoked controls to enter the center of an open field, and had a diminished respiratory response to stress-inducing restraint. Although SRR conditioned rats had unaltered reflex responses to thermal and mechanical nociception, they did not respond with increased sensitivity to mechanical stimuli following chemical-induced hyperalgesia, as yoked rats did. Overall, our findings were consistent with the development of an animal model of the relaxation response focused on slowed respiration as the triggering stimulus.

Our inability to monitor heart rate (and consequently respiratory sinus arrhythmia) noninvasively led to our lab's recent development of low-cost remote sensing technologies (Plessey Semiconductors) to report on key physiological and behavioral indices of an animal's state. We found that these electric field sensors could accurately

measure rodent respiratory and heart rate, as well as general activity levels and stereotyped behaviors, in specific experimental setups or home cage environments. Applying sensors to monitor these metrics on a 24/7 basis, while providing targeted cardiorespiratory feedback, will ultimately establish a next-generation model of the "quantified self" for basic studies aimed at predicting and preventing autonomic dysfunction.

Our animal model will also lend itself to mechanistic studies investigating the neuroanatomy and neurobiology of slow, deep breathing. This could be studied independent of or in combination with studies on the neurocircuitry of associative learning. For instance, it would be informative to study the cellular and molecular basis of neuroplastic changes resulting from the pairing of respiratory afferent activations with brain systems mediating the responses to rewarding or fearful stimuli. Some of the many possibilities are described below.

The Neural Basis of Conditioned SRR: Review and Proposal

Although the current state of knowledge in the field requires that any detailed statements on the neurobiological basis of conditioned slow breathing remain speculative, we propose the involvement of at least four functionally integrated pathways or systems, namely those implicated in: i) slow, deep breathing (e.g. pulmonary afferents, the NTS, CeA, and PVN), ii) response to intermittent bright light (e.g. visual cortex and amygdala), iii) avoidance learning (e.g. basolateral amygdala), and iv) reward pathways (e.g. nucleus accumbens).

As detailed in Chapter 1, we know that slow, deep breaths activate pulmonary afferents and baroreceptors at different points in the respiratory cycle to increase HRV (Figure 1.2). Afferent synapses with the NTS are glutamatergic (Reis, Granata et al. 1981, Carr and Undem 2003). In the rat, GABAergic neurons in the vlNTS receive input from SARs (Kubin, Alheid et al. 2006), thereafter sending inhibitory impulses to other NTS neurons that relay messages to subcortical and cortical components of the central autonomic network (Chapter 1).

The CeA and PVN in particular receive dense catecholaminergic inputs from the NTS, which may lead to synchronized responses for the maintenance of homeostasis. Furthermore, there may be specific roles for norepinephrine and galanin. Norepinephrine is the major neurotransmitter involved in these NTS pathways, based on retrograde labelling in the A2 catecholaminergic cell group (Petrov, Krukoff et al. 1993). Norepinephrine likely exerts its effects by acting on α1 receptors in the CeA to alter behavioral and neuroendocrine responses, an effect that occurs following experimental stressors (Cecchi, Khoshbouei et al. 2002). On the other hand, amplifying the stress-induced norepinephrine response leads to a galanin-mediated anxiolytic effect in the CeA (Khoshbouei, Cecchi et al. 2002). Similarly, in the PVN, norepinephrine microinjection induces corticotropin-releasing factor (CRF) gene expression in rats (Itoi, Suda et al. 1994). It's possible that Gq-coupled α1-adrenergic receptors also mediate this action.

Therefore, our proposal for the neural mechanisms of slow, deep breathing's behavioral effects is that: i) Activation of SARs inhibits norepinephrine release from catecholaminergic NTS neurons via GABAergic vlNTS relay neurons; ii) Diminished activation of α1-adrenergic receptors prevents recruitment of PVN CRF-synthesizing

neurons and CeA neuropeptidergic neurons; iii) Inhibitory drive extends to spinal sympathetic neurons to inhibit systemic release of norepinephrine and epinephrine; and iv) Effects oppose the impact of stressors on downstream markers (e.g. immune activity) and behavioral outcomes. To aid in these effects, galanin release from noradrenergic NTS neurons could reduce behavioral stress responses.

Furthermore, specific cell types in the PVN are involved in CRF synthesis and regulate the hypothalamic component of the HPA axis (Sawchenko, Li et al. 2000), as outlined in Appendix 1. While not enough is known to predict the specific subtypes of CeA neurons recruited by slow breaths, the NTS sends a direct projection to the medial portion of the CeA (Schwaber, Kapp et al. 1982, Sawchenko 1983), and cells in this subdivision are the only ones that project to the startle circuit (Rosen, Hitchcock et al. 1991). Several neuropeptides could be recruited to modulate the CeA's downstream effects: galanin and substance P are known to be located in the medial subdivision, while CRF and somatostatin have been located in both the medial and lateral subdivisions (Gray and Magnuson 1987). While the specific CeA cell types recruited by SARs are unknown, slow, deep breaths may counter CeA-induced autonomic effects.

There could also be involvement of the periaqueductal gray (PAG), since the CeA extensively innervates the PAG along its rostrocaudal axis (Rizvi, Ennis et al. 1991). The PAG projects to several brainstem nuclei that could exert downstream effects to aid in the recovery of homeostasis following stress. In particular, the raphe magnus releases serotonin, while the locus coeruleus releases norepinephrine; serotonergic and noradrenergic projections to the spinal cord could in turn produce analgesia.

The neurocircuitry recruited by intermittent bright light could overlap with that recruited during respiration to initially mediate stress responses that are gradually converted to conditioned responses via established neural mechanisms of associative learning. While basic visual input is integrated in V1, repeated exposure to strobe light can lead to a sensitized visual response in rats (Uhlrich, Manning et al. 2005) that may prolong the aversive impact of the stimulus and result in a sustained conditioned response when the light is paired with an initially neutral stimulus. Strobe light and respiratory afferent activations would presumably combine to recruit brain regions implicated in associative learning, thereby continually reinforcing the conditioned response.

While slow, deep breathing could be therapeutic in itself, associative learning could also discourage fast or normal breaths, since these would be associated with the stressful effects of repeated visual stimulation. In other words, slow, deep breaths could alter the fear responses provoked by strobe light or acute stress by virtue of being intrinsically rewarding or therapeutic (some potential mechanisms are described in Chapter 1), or alternatively, "punishment" of normal or fast breaths could occur via NMDA-receptor dependent associative learning such that breathing slowly achieves the avoidance of negative consequences. These effects could occur alone or in combination.

This leads us to propose the amygdala-mediated association of aversive strobe light with "non-slow" (fast or normal) breaths. While the basolateral nucleus of the amygdala (BLA) is involved in instrumental avoidance learning, the CeA has been implicated in more passive or Pavlovian responses (e.g. freezing) (Ressler 2010). Active avoidance of strobe light could be adaptive, as it would lead to decreased exposure to

stressful stimulation and potentially to improved physiological and psychological function (the latter being mediated through cortical areas).

Classical dopamine "reward" systems could also play a role. This would likely entail reward processing in the nucleus accumbens upon removal of aversive strobe stimulation, and could associate with CeA-mediated disinhibition of the BLA. In this scenario, removal of strobe light would release dopamine from the ventral tegmental area (VTA) to act on D1 and D2 dopamine receptors in the medial core of the nucleus accumbens. These effects could combine with slow, deep breaths releasing glutamate from the BLA to act on AMPA/kainate and NMDA receptors in the same location. The combined integration of mesoaccumbens dopamine reward pathways and glutamatergic projections from the BLA would cause NMDA-dependent plasticity in medium spiny neurons of the nucleus accumbens, which in turn could lead to an array of changes potentially ranging from dendritic spine alterations to neurogenesis, and ultimately resulting in learning of the conditioned response.

Taking the above scenarios together, we propose a model to explain the neurobiological basis of operantly conditioned slow, deep breathing (Figure 5.1) that involves several integrated components: i) Strobe light impinges on autonomic circuitry via the BLA; aversive effects are then mediated via the CeA. ii) Associative learning in the BLA pairs respiratory input with the response to strobe light by an NMDA-dependent mechanism, resulting in the operant aversion to normal or fast breathing. iii) Removal of aversive strobe light is intrinsically rewarding and associates with the neurocircuitry of slow, deep breathing.

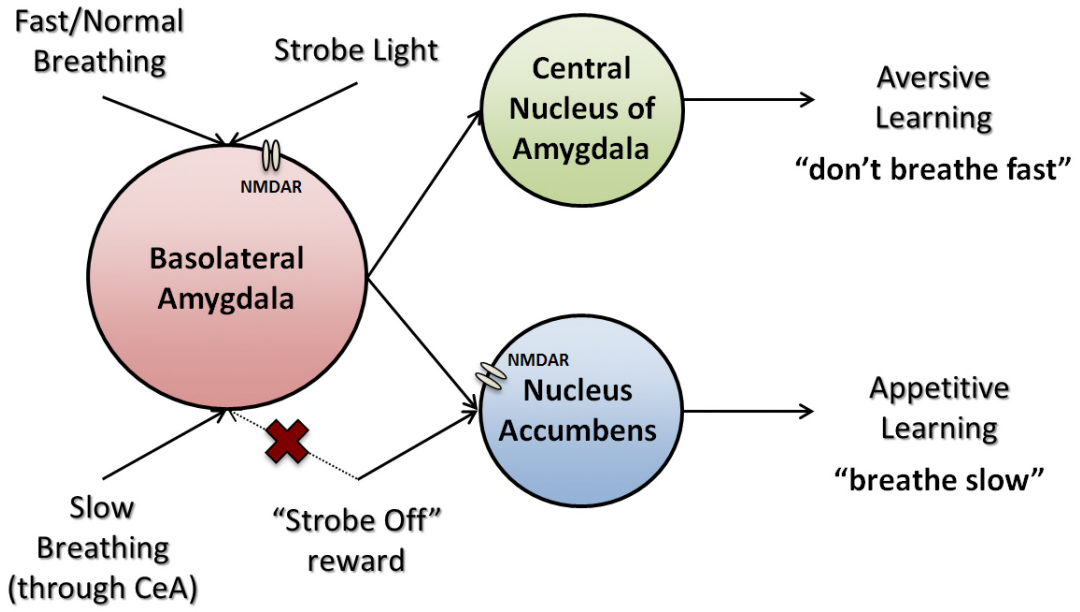

Figure 5.1. Proposed neurobiological mechanisms of SRR conditioning.

During fast breaths, strobe light effects and respiratory circuitry interact to activate BLA and CeA stress responses. During slow breaths, the absence of strobe light prevents associative stress responses in the BLA. Glutamate from the BLA then integrates with the nucleus accumbens dopamine response to associate with "strobe off" reward.

Based on this proposal, we envision several promising future directions, involving cellular, molecular, or genetic approaches, to get at neurophysiological mechanisms. One idea is that protective galanin (a peptide co-released at high frequencies with norepinephrine) is released upon activation of SARs during inhalation or upon rebound inhibition during exhalation, leading to anxiolytic effects. As a future direction, it would be informative to perform microdialysis for galanin and norepinephrine following conditioning sessions and during stress, at several different time points. Pharmacological approaches could also be employed (e.g. galaninergic and noradrenergic agonists and antagonists) to determine their effect on behavior and a variety of outcome measures.

Another idea is that aversive learning could be sufficient to evoke a conditioned response, without any involvement of conventional dopaminergic reward pathways. To address whether respiratory conditioning still occurs in the absence of dopamine reward systems, antagonists or conditional knockout against dopamine receptors in the nucleus accumbens could be attempted to establish their importance in mediating conditioned slow breathing, i.e. to establish necessity. Complementarily, novel approaches involving optogenetics or DREADDS (Rogan and Roth 2011) to activate ion channels or G-protein coupled dopamine receptors (D1/D2) with slow, deep respiration, in the absence of other stimuli, could be undertaken to establish sufficiency and optimize behavioral conditioning paradigms. α1-adrenergic receptors in the CeA or PVN could represent an additional promising target for experimental manipulation. All of these approaches would ideally be combined with high-resolution physio-behavioral monitoring to establish their impact on appetitive or aversive learning and relaxation-related outcomes.

Ultimately, optogenetic conditioning approaches that activate dopamine reward pathways could increase the efficiency of respiratory training (Adamantidis, Tsai et al. 2011, Rossi, Sukharnikova et al. 2013). Combined with EPIC sensor-based recording and feedback of behavioral and cardiorespiratory variables (Chapter 4), these approaches could dramatically improve study design and suggest novel mechanisms for respiratory conditioning and slow breathing-induced relaxation.

Chapter 2 Future Directions

Feedback-based control of respiratory rate

We propose to develop closed-loop control of physiological parameters as a preventative therapeutic approach to normalize autonomic function. In recent years, slow-breathing-based feedback devices for lowering blood pressure have shown therapeutic potential in clinical studies (Grossman, Grossman et al. 2001, Rosenthal, Alter et al. 2001, Schein, Gavish et al. 2001, Viskoper, Shapira et al. 2003, Meles, Giannattasio et al. 2004, Anderson, McNeely et al. 2009, Anderson, McNeely et al. 2010, Oneda, Ortega et al. 2010, Sharma, Frishman et al. 2011), and radiotelemetric studies in rats suggest that relaxation practices can modulate cardiovascular activity in a manner compatible with stress reduction (Baldwin, Wagers et al. 2008). Thus, operantly conditioning SRR in rats should reproduce autonomic and cardiovascular changes that occur during and following SRR in humans (Lehrer, Sasaki et al. 1999, Peng, Henry et al. 2004, Anderson, McNeely et al. 2010). One of the most promising applications of our sensors is the measurement of respiratory sinus arrhythmia (RSA), an index of cardiorespiratory synchronization. Several distinct relaxation techniques that involve slowed breathing enhance RSA (Eckberg 1983, Rubini, Porta et al. 1993, Lehrer, Sasaki et al. 1999, Song and Lehrer 2003, Peng, Henry et al. 2004, Brown and Gerbarg 2005, Cysarz and Bussing 2005, Pereda, De la Cruz et al. 2005, Jerath, Edry et al. 2006), and RSA appears to be a reliable physiological indicator of cardiovascular health and balanced autonomic function (Brown and Gerbarg 2005). With EPIC sensors, we will investigate whether the performance of slow and deep breaths corresponds to enhanced RSA. In the long run, we may be able to complement respiratory conditioning with closed-loop feedback of RSA. Since substantial reductions in blood pressure have been found following SRR in patients with high baseline blood pressure (Mori, Yamamoto et al. 2005), we also expect the

therapeutic impact of SRR to be large in hypertensive animals. In short, we will derive RSA from sensor recordings as a reliable index of autonomic balance, and our long-term objective is to continuously monitor cardiorespiratory variables as a means to developing a feedback-based model with relevance to variety of conditions characterized by autonomic dysfunction. Below I describe these directions in more detail.

The next generation of operant conditioning studies

I. Over the long-term, we aim to develop a versatile model of the "quantified self" based on our ability to continuously and non-invasively monitor RR and heart rate. Recordings could be combined with 24/7 feedback-based conditioning of RSA, one of the most reliable indices of wellness, and a variety of experiment manipulations, such as speakers for delivering ultrasonic vocalizations that provoke aversive or appetitive behavior in rats (Knutson, Burgdorf et al. 2002). Combined with measuring indicators of health, such an approach would allow us to model the transition from stress or disease to wellness.

II. Along with cerebral blood flow fluctuations associated with cardiovascular variability (Cencetti, Lagi et al. 1999), there is evidence that slow respiration can entrain electroencephalographic (EEG) activity (Kamei, Torui et al. 2000, Busek and Kemlink 2005). It may be possible to refine our electric field sensors to record EEG in restrained animals or to implement telemetric procedures for this purpose.

III. SRR conditioned rats in our study slow and deepen their breathing while other respiratory parameters remain unaltered. However, it is possible that SRR techniques with a focus on prolonged exhalation confer selective benefits over others

(Cappo and Holmes 1984). A straightforward manipulation based on this idea is to shape the operantly conditioned response so that this parameter is selectively reinforced. Combining selective training with autonomic outcome measures, we could more precisely delineate the respiratory criteria to condition for maximal therapeutic effect.

IV. By taking these steps in normal adult rats and in animals models of disease (e.g. hypertension, anxiety, or depression) (Frank, Salchner et al. 2006), research will inspire new pharmacological and technological strategies for alleviating autonomic imbalance.

Chapter 3 Future Directions

Transitioning from normal animals to models of disease

As respiratory measures provide a clear quantitative appraisal of stress-related behavioral state (Hegoburu, Shionoya et al. 2011) and slow, deep breathing is a therapeutic stress reduction technique (Grossman, Grossman et al. 2001, Rosenthal, Alter et al. 2001, Schein, Gavish et al. 2001, Viskoper, Shapira et al. 2003, Meles, Giannattasio et al. 2004, Anderson, McNeely et al. 2009, Anderson, McNeely et al. 2010, Oneda, Ortega et al. 2010, Sharma, Frishman et al. 2011), we expect that real-time closed-loop control of RR could prove especially beneficial in disorders of the autonomic nervous system, which are often characterized by sympathetic overactivity. For example, we have previous proposed to test closed-loop feedback control using light to reduce RR and assess its ability to prevent or minimize the effects of stress in an animal model of hypertension. Several different animal models could be integrated with SRR conditioning paradigms and EPIC sensor remote monitoring applications to reveal the impact of

feedback-based conditioning on outcomes in disorders of the autonomic nervous system. Possibilities are discussed below.

Home cage sensor-based identification of behavioral state changes associated with chronic social stress and hypertension

Hypertension in America affects a shocking ~1/3 of adults, with another 1/3 having prehypertension that in sum leads to a profound disease burden (CDC 2011, Roger, Go et al. 2012). Prehypertension is an example of a continuously monitored variable that could serve as a predictor for therapeutic closed-loop control systems. There is a well-known link between stress and hypertension, which is characterized by sympathetic overactivity (Guzzetti, Piccaluga et al. 1988) and parasympathetic underactivity (Langewitz, Ruddel et al. 1994). However, the physiological basis of this association between stress and hypertension has not been investigated at the level of detail that a well-controlled animal model provides. There are several possibilities for an animal model of hypertension (Sarikonda, Watson et al. 2009), including the popular spontaneously hypertensive rat (SHR) model of essential or primary hypertension (Okamoto and Aoki 1963), or the borderline hypertensive rat (BHR), which accounts for the impact of environmental factors in the genesis of hypertension (Sanders and Lawler 1992). Alternatively, rats could be exposed to prenatal dexamethasone (Neigh, Owens et al. 2010), a synthetic glucocorticoid that predisposes stressed rats to develop hypertension (O'Regan, Kenyon et al. 2008), to evoke sympathetic autonomic dysfunction. Cardiorespiratory variables would be monitored continuously while tracking for the development of hypertension. Changes in physical activity would address the

behavioral impact of experimental hypertension, while home cage recordings of autonomic and behavioral variables collected continuously from weaning would be analyzed and related to an animal's vulnerability to the development of elevated behavioral distress and hypertension following exposure to a chronic social stressor. We could then study whether operantly conditioned reductions in RR reverse adverse changes associated with hypertension. In summary, we are in a position to provide a detailed readout of stress burden from weaning onwards and test for unknown events that are predictive for subsequent resilience or disease vulnerability.

Can continuous recordings of autonomic and behavioral variables predict and prevent neurogenic disease and dysfunction?

The neurohumoral paraventricular nucleus of the hypothalamus contains the presympathetic neuronal populations innervating the spinal cord/brainstem as well as the neuroendocrine neurons comprising the hypothalamic-pituitary-adrenal (HPA) axis. Collectively, these pathways provide for integrative homeostatic feedback, including in response to stress. While historically thought of as acting in parallel but independently, new observations of dendritic crosstalk between hypothalamic presympathetic and neuroendocrine neurons support a binding of the systems as one orchestrated unit (Son, Filosa et al. 2013). Thus, measures of fundamental autonomic variables that report on sympathetic neural activity (heart rate, RR, and blood pressure) may proxy for HPA axis tone. Our proposed studies will relate fundamental measures of autonomic function to HPA status. We assert that continuous recordings of autonomic and behavioral activity will enable predictions of disease onset in many conditions. For example, stress is the

most common trigger of seizures in epilepsy (Sawyer and Escayg 2010) suggesting that measurable autonomic changes precede epileptogenesis. Strikingly, with temporal lobe complex partial seizures autonomic changes can precede electroencephalographic epileptiform discharges. These transitional autonomic changes take place over several minutes (Novak, Reeves et al. 1999), and are measurable as reduced HRV (Mativo, Anjum et al. 2010). Sudden unexplained death in epilepsy may also arise from predictable autonomic dysfunction (Mukherjee, Tripathi et al. 2009). Thus, approaches that can detect autonomic imbalance and promote feedback that restores balance, could significantly reduce disease burden in epilepsy. Most epileptics believe seizures are stress-related, and are willing to try stress reduction techniques for seizure control (Haut, Vouyiouklis et al. 2003).

Individualized preclinical control of the "quantified self"

Animal models of autonomic dysfunction offer unsurpassed resolution and versatility for conducting controlled studies that invoke disease as a means to study its evolution and identify therapeutic or preventative feedback strategies. The present idea is to develop and apply affordable new technologies for studies on prediction and prevention of disease. Normal, hypertensive, or epileptic animals could undergo behavioral conditioning in a well-controlled setting to establish the relationship between lowered RR, cardiorespiratory synchronization, and long-term stress reduction. We have obtained preliminary data demonstrating that ongoing recording of these variables can be used to identify autonomic nervous system dysfunction, and now aim to guide real-time

feedback of external stimuli for inducing physiological changes that normalize autonomic function.

Chapter 4 Future Directions

Embedding sensors and actuators in rat home cages for continuous non-contact recording of physiological variables

We aimed to establish an integrated method for remote non-contact physiological monitoring that allows affordable real-time detection and control of cardiovascular and behavioral indices of wellness. Using EPIC sensors, physical activity from behaving animals is easily measurable when sensors are attached to the chamber exterior. Moreover, strategic placement permits reliable detection of respiration and heart rate, as well as quantification of cardiorespiratory synchronization, an important derived measure of autonomic balance. That individual non-contact sensors reliably monitor motor and cardiovascular activity provides an opportunity for continuous high-throughput monitoring in rat home cages. This could represent a landmark development in the fields of behavioral and cardiorespiratory neuroscience.

Indeed, biosensor technologies have arguably reached a ‘tipping point’ in resolution and affordability. Opportunities now exist to implement a paradigm shift in health management towards individualized physiological monitoring – to predict, prevent, and better manage disease. Current strategies focus on wearable technologies and active interactions with personal devices (e.g. cell phones and smartwatches). An alternative approach involves embedded monitoring and feedback (via sensing, computing, and actuation) within a home environment to allow for experimentally

rigorous studies on disease prevention *in situ*. These studies are an essential first step in an ambitious broader agenda – leveraging affordable miniaturized sensor technologies that report on a variety of variables associated with well-being. Although home-based research is already underway including at our own universities (e.g. the Aware Home Research Initiative at Georgia Tech), significant opportunities exist to expand this research agenda to facilitate improvements in the fidelity and diversity of health-based assessment and modulation (via feedback) techniques. We propose to develop an animal model prototype – in a *home cage* – of such an environment and to test its efficacy in addressing hypertension or other disorders characterized by autonomic dysfunction.

Specifically, we propose to embed EPIC sensors within the home environment for continuous measurement via data logging of an animal's autonomic, motor, and affective behavioral states. These measures will be collectively synthesized to quantifiably phenotype the animal's physio-behavioral profile. Analytics derived from such composite signal analyses may predict subsequent disease onset. Accordingly, data will be mined for diagnostic behavioral state changes that associate with consequent expression of chronic stress and/or hypertension. Next generation studies using real-time closed-loop feedback can then be employed as 'smart' technologies in predictive and preventative health approaches.

Next generation home cage construction and sensor development

We will optimize recording fidelity via construction of home cages with two pairs of electric field sensors. One pair will be aimed at optimizing detection of cardiorespiratory parameters in resting animals, and will require cage inclusion of smaller

cave-like shelters with embedded sensors for needed proximity. Accordingly, rat home cages will be equipped with a manipulable barrier that separates rats but is transparent and contains large holes to allow for visual, olfactory, and tactile interaction between animals. The other pair will be aimed at identifying specific motor behaviors and broad indices of physical activity. Physiological variables will be recorded in rats continuously throughout the study period. We will use the sensors to record RR, heart rate, and physical activity. To compare activity differences between animals, movement-related voltage signatures will first be determined using simultaneous video capture. As seen in Chapter 4, the magnitude of movement onset is well above that of the much smaller but readily detected respiration events and thus movements are easily discriminated. The total duration associated with these large movement-related voltage fluctuations will be determined and defined as 'level of activity'. Exploratory analyses will be performed to detail group differences in stereotyped rodent behaviors.

Cage embedded multisensor/actuator systems for optimized rodent monitoring and real-time feedback

At minimal cost we will pair a data logger device to a pair of EPIC sensors strategically affixed to chamber walls to detect overall activity level, animal location, characteristic movement behaviors, as well as respiratory and heart rate. The small size of the data logger (~ 6 x 2 x 2 cm) allows it to be attached and detached from the rodent cage as necessary. Our feedback monitoring system will possess several properties: (a) Adapts to currently used DAR home cages. (b) Allows for cage washing. (c) Has detachable electronic components. (d) Contains PVC tubing with a barrier in the cage

center to allow for individual monitoring and testing. All sensors associated with monitoring and correlating heart rate, RR, and blood pressure at rest should be placed in the PVC tubing. PVC tubing will be red filtered but sufficiently transparent to allow visualization of the animal's placement in the tubing. It will also be important to record video of animals in their home cages using various designs before deciding on sensor placement, and to monitor ultrasonic vocalizations (USVs) as an additional index of stress. Microprocessor-based data collection and feedback nodes (DCFN) mounted on each cage will have outputs to drive any of a variety of effectors, such as LEDs, beepers, and vibrating motors; LED light-based feedback will initially be applied.

Physiological and behavioral sensing and analysis in 'smart' home cages

The 'smart' home cages will include EPIC sensors to provide physiological and behavioral assessments including (i) heart rate and RR, (ii) general activity levels, and (iii) various motor behaviors. These assessments will be derived from the sensors using signal-processing techniques. For example, RSA will be derived from heart rate recordings using a fast Fourier transform to convert raw heart rate data into a frequency spectrum that displays how heart rate changes over time (its variability). We will then use power spectral analysis to relate HRV to respiratory frequency; the amplitude of this frequency band provides a quantitative readout of cardiorespiratory synchronization (Peng, Henry et al. 2004). The home cages will also be augmented with these additional sensing modalities:

(i) Blood pressure: Floor contact-based recording of blood pressure via arterial pressure pulse wave velocity (PWV) (EPIC sensors or sensitive force transducers). PWV is estimated from pulse transit time (PTT) – the relative timing between proximal and distal cardiac pressure waveforms. PTT shows a tight relationship with blood pressure, and has consequently been pursued by many researchers over the past century. We will measure the arterial pulse waveform timings at the front and back feet of the animal using optical transducers, similar to a reflectance photoplethysmogram measurement (Mendelson and Ochs 1988), then calculate the time delay between the troughs of the waveforms (PTT) to estimate PWV. Previous studies have measured PWV from anesthetized rats by recording the pressure waves at the aortic arch and abdominal aorta, successfully capturing changes in arterial stiffness due to pharmacological intervention (Fitch, Vergona et al. 2001). Our non-invasive approach will allow frequent measurements of PWV while the rat remains in its home environment, enabling quantitative assessment of closed-loop therapies designed to reduce blood pressure.

(ii) Ultrasonic vocalizations: Combined recordings of ultrasonic vocalizations and respiration accurately report magnitude of stimulus novelty and arousal level. Another lab recently created an experimental chamber that allowed for simultaneous measurement of respiration, ultrasonic vocalizations (USVs), and behavior (Hegoburu, Shionoya et al. 2011). Testing in a plethysmographic chamber allowed for RR measurement. The chamber was equipped with a microphone to record USVs, and four separate video cameras for capturing behavior. A floor plate enabled foot shock and a dorsal tube allowed for delivery of odorants. The authors related properties of USVs and respiration

observed in different behaviors to contextual fear conditioning and showed that respiratory measures provided a fundamental and clear quantitative appraisal of the physiological and behavioral changes seen. Co-measurement of respiration and USVs demonstrated that the presence of USVs during freezing induced a lowering in respiratory frequency due to a profound lengthening of expiration during call emission (22 kHz), and concluded this to be a good index of anxiety levels and aversive memory (Hegoburu, Shionoya et al. 2011). Previous studies reported that deep exhalations associated with USVs during immobility corresponded to a parallel increase in blood pressure and heart rate (Frysztak and Neafsey 1991, Antoniadis and McDonald 1999, Walker and Carrive 2003). Thus, it will be very important to measure USVs. In order to achieve this, we will implement a further function of the DCFN to detect USVs.

(iii) Ground-contact forces: The home cage will be outfitted with force sensors on the cage floor and in the sleeping tubes. The resulting contact forces will be used for monitoring changes in the speed or regularity of locomotion affected by psychological state, and activity during rest in the tube. The frequency domain components (extracted from the power spectral density, PSD, of the ground force signals) would change based on the rhythmicity or irregularity of the movements. Rhythmic movements would lead to focused energy in specific bins of the PSD plot, while irregular movements would spread the frequency domain energy across a wider spectrum, with potentially a greater number of peaks. In all cases we will need to compare obtained results to standard recording technologies in the field for accuracy.

Stimulation, actuation, and closed-loop autonomic control

Systems to stimulate, encourage, and perturb behaviors (stimulation systems) will be included in the home cages. (1) An LED array capable of spanning the rat visual range from near-UV to green (and any combination) will be installed as a method of introducing novel visual stimuli or to produce bright light for negative reinforcement. (2) Speakers will be attached to the cages, allowing music, sound effects, rat vocalizations, or loud noises to be played either independently, or in combination with, the light from the LED array. (3) A capacitive touch sensor will allow motor behavior to control the light color emitted from the LED array or sound effects. (4) A small vibrator will be attached to the touch sensor, allowing for haptic explorations. (5) A Peltier device will allow changes in the temperature of the rats' environment. (6) Motorized food pellet dispensers will allow for food rewards to be given in response to desired activities. All or a subset of these systems will be included in each cage.

These systems will be used to encourage the rat to achieve specific autonomic control objectives through feedback. Once the rat is habituated to the cage and a suitable calibration period for the autonomic measurements is complete, an exploratory algorithm will test the effects of different stimuli on the animal. First, adaptive control will be used to move the RR towards a set point using light, sound, and temperature separately, then in combination, modeling the system response mathematically during the process. Next, novelty rewards will be tested through the use of the capacitive touch system, connecting it to dim light, music, and vibratory stimuli in turn. The autonomic and behavioral effects of each touch-to-stimulus connection will be recorded, with an analysis of the decay constant of the rats' interest level – and the underlying autonomic effects of that interest

level. Both the choice of which stimuli to connect to the touch sensor, and the frequency of switching, will be optimized based on this exploratory phase. Throughout the remainder of the study, this closed-loop "quantification" will continue, improving both the accuracy of the model and the effectiveness of the environment-to-autonomic control.

Developing an Animal Model of Psychophysiological Coherence

The term 'psychophysiological coherence' has been coined to explain the emergence of global order resulting from the entrainment of physiological systems (McCraty 2009). Different meditation and yoga techniques appear to evoke similar large-scale changes resulting in coherence between multiple systems and pathways. Many of these techniques involve slowed, deep breathing. While 'positive' emotions such as compassion generate a similar state of coherence, it is possible that this effect is at least partly based on the ability of positive emotions to slow respiration. In turn, the profound cardiorespiratory synchronization that occurs during these diverse practices may serve a physiological function via enhanced ventilation-perfusion matching or vascular release of nitric oxide. As discussed in Chapter 1, breathing slowly at 6 breaths per minute (0.1 Hz) unlocks baroreceptor resonance effects that may amplify rhythmic neural activity throughout central systems, ranging from spinal autonomic circuits to higher cortical networks. Although evidence of the role of psychophysiological coherence in the benefits of meditation and yoga is limited, i) basic scientific studies that monitor physiological parameters concurrent with practice, with or without manipulations such as paced breathing or heart rate variability biofeedback, and ii) animal models that permit

characterization of the underlying neurocircuitry, will provide an avenue for future research to explore the impact of psychophysiological coherence on holistic wellness.

APPENDIX 1: Isolating a role for deep breathing in reducing activation of stress-related limbic circuits

Abstract

Meditation and yoga techniques involving deep breathing (DB) have shown promise for treating a variety of cardiorespiratory and stress-related disorders, but to date no studies have mechanistically investigated the role of DB in conferring these benefits. The brainstem nucleus of the solitary tract (NTS) is the major relay nucleus for all vagal afferent input including stretch-sensitive pulmonary afferents. The NTS projects densely to the central nucleus of the amygdala (CeA) and the paraventricular nucleus of the hypothalamus (PVN), both implicated in the response to threatening or stressful stimuli. As Fos immunolabeling can be used to assay stress-evoked neuronal activation, we hypothesized that, via NTS projection pathways, DB would suppress Fos expression in these circuits. In anesthetized, mechanically ventilated rats, we compared the number of Fos-positive neurons following a two-hour period of DB vs. normal breathing. Preliminary results revealed reduced Fos expression following DB in the CeA (14.3 vs. 24.1 Fos+ cells per section) but not the PVN (70.7 vs. 62.6 Fos+ cells per section), and a nonsignificant slight preferential activation of the ventrolateral NTS (vlNTS) in DB and the medial NTS (mNTS) in control animals, gauging by Fos-positive cell counts in DB vs. control rats (vlNTS: 23 vs. 21 Fos+ cells/animal; mNTS: 60 vs. 74 Fos+ cells/animal). Future studies should be aimed at verifying these preliminary findings and identifying mechanisms that link DB to stress circuit deactivation.

Introduction

Despite known projections to central autonomic pathways, the neurophysiological

mechanisms through which SRR could support autonomic balance and wellness remain unknown. Complex and highly variable factors associated with human studies complicate interpretations and greatly reduce statistical power. For example, meditation and yoga techniques involving SRR are typically integrative blends of multiple subcomponents (Woolfolk 1975). Several reviews have recognized the need for new methodologies that allow systematic isolation of functional components (Woolfolk 1975, Jerath, Edry et al. 2006, Courtney 2009), but this has remained an unresolved challenge in human studies (Ospina, Bond et al. 2007).

We know that deep breaths activate slowly adapting pulmonary receptor (**SAR**) and arterial baroreceptor afferents (Brown and Gerbarg 2005, Jerath, Edry et al. 2006), which project to unique subregions of the nucleus of the solitary tract (**NTS**) in the brainstem. NTS neurons extend particularly dense second-order projections to the central nucleus of the amygdala (**CeA**) and paraventricular nucleus of the hypothalamus (**PVN**) (Petrov, Krukoff et al. 1993), both implicated in the response to acute stress or threat. NTS pathways recruited during deep breathing could disengage the neural circuitry of acute stress to shift autonomic imbalance in the parasympathetic direction (see Chapters 1 and 5 for additional detail on proposed mechanisms).

Researchers could investigate the implicated subsystems in greater depth using a variety of electrophysiological, immunohistological, and pharmacological approaches. As a pilot study to determine the feasibility of long-term mechanistic inquiry in freely behaving conditioned rats (Chapters 2 and 3), I performed c-Fos (**Fos**) immunolabeling procedures in a small sample of rats following brief animal handling and mechanical ventilation, in order to establish whether recruitment of central autonomic neurocircuitry

is attenuated following different depths and frequencies of breathing. Fos is the protein product of an immediate early gene whose expression is induced following activation of neurons, particularly in response to sensory stimulation or stress. I planned to use methods similar to previous investigators (Osharina, Bagaev et al. 2006, Spirovski, Li et al. 2012) to probe regional Fos expression in the NTS, CeA, and PVN. Fos expression has been used to map central nervous system recruitment following a single exposure to resident-intruder stress, revealing increased activation in each of these regions (Martinez, Phillips et al. 1998). Therefore, in anesthetized, mechanically ventilated rats, we sought to answer the question: does sustained slow, deep breathing reduce activity in the neural circuitry involved in the response to acute stress? To achieve this aim, we compared the number of Fos-positive neurons following a two-hour period of DB vs. normal breathing. We hypothesized that, via NTS projection pathways, deep breathing suppresses Fos expression in the CeA and PVN.

In summary, our neurophysiological understanding of individual deep breaths suggests that sustained DB preferentially activates SARs and arterial baroreceptors (Brown and Gerbarg 2005, Jerath, Edry et al. 2006) which we hypothesize leads to recruitment of specific subpopulations of neurons in the NTS, amygdala and hypothalamus, identifiable by their anatomical topography.

Materials and Methods

Experimental Animals

Adult male Sprague-Dawley rats (~90 days old at the start of experimental procedures) were housed in standard cages in a vivarium on a 12:12-h light-dark cycle

and were fed *ad libitum* standard rodent diets. All experiments were approved by the Animal Care and Use Committee 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." Rats were weighed prior to the commencement of experimental procedures in order to determine anesthetic doses.

Surgical Procedures

Animals were first prepared by acclimation to the environment for 12 hours. Surgical procedures then commenced. Surgical procedures were similar to those used in a previous publication (McGuire, Zhang et al. 2005). Rats were placed in a small closed chamber and anesthetized with isoflurane. Once anesthetized, anesthesia was maintained via a facemask that delivered gas mixtures (2.5–3.0% isoflurane; mixed with O_2). Femoral catheters were inserted into isoflurane-anesthetized rats for fluid administration and blood pressure readouts. The anesthetic was then slowly converted to urethane (1.6 g/kg in distilled water, IV) over the course of 20 minutes, so that the animal was stably anesthetized without isoflurane, breathing room air. We then cannulated animal tracheas and connected the rats to mechanical ventilators (Harvard Apparatus). We tested animals throughout surgical procedures for adequate anesthesia by suppression of the corneal reflex and lack of behavioral or blood pressure response to toe pinch (before and after neuromuscular blockage, respectively). After tracheotomy, pancuronium bromide (2.5 mg/kg followed by additional doses as needed) was given to obtain neuromuscular blockade. Thereafter, supplementary doses of urethane (0.16 g/kg) were given as needed

to provide stable anesthesia for the experiment. Throughout experimental procedures, we maintained rectal temperature near 37.5°C with a heating pad, and infused sodium bicarbonate and lactated Ringer's solution to maintain fluid and acid–base balance starting approximately one hour after stable anesthesia was achieved. End-tidal CO_2 partial pressure ($P_{ET,CO2}$) and blood pressure were monitored throughout mechanical ventilation procedures using a flow-through capnograph (Novametrix Medical Systems) and blood pressure transducer, respectively, and were kept relatively constant (around 40 mmHg for $P_{ET,CO2}$ and 80-100 mmHg for blood pressure). At the end of the experiments, rats were killed with an overdose of urethane (3.2 g/kg, IV).

Mechanical Ventilation Protocol

For mechanical ventilation, anesthetized rats mechanically ventilated on room air were administered one of two respiratory patterns. Normal and deep/slow tidal volumes and respiratory rates were determined for adult humans and rats, and administered for mechanical ventilation procedures (Figure A1.1). To obtain rat respiratory parameters, we calculated the percentage change in tidal volume during deep breathing in humans, and used the same percentage increase in rats, keeping overall ventilation constant. Animals were then ventilated for two hours at normal or deep tidal volumes; respiratory frequency was also experimentally altered (reduced) for DB rats in order to keep overall ventilation constant. For perfusion and tissue preparation, we overdosed rats on urethane and subsequently perfusion-fixed the tissue with paraformaldehyde in PBS (pH 7.4). Tissue was post-fixed overnight and was cryoprotected in sucrose solution. We then cut brains into coronal sections 40 μm thick using a vibratome.

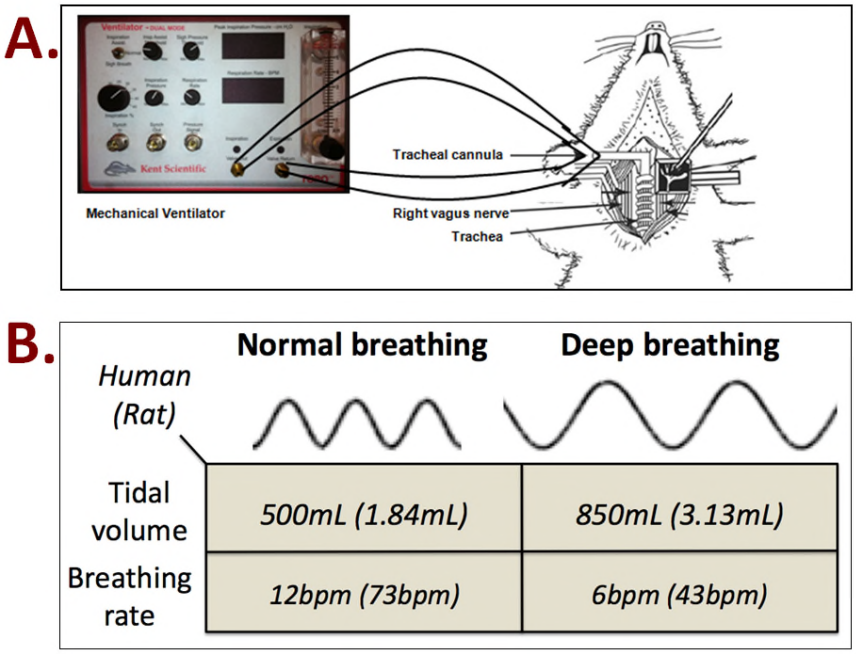

Figure A1.1. Mechanical ventilation procedures.

(A) Anesthetized, mechanically ventilated rats were administered one of two respiratory patterns. **(B)** Determination of respiratory rate and volume for mechanical ventilation procedures. Normal and deep/slow tidal volumes and respiratory rates (bpm = breaths per minute) are shown for adult humans (and rats). To obtain rat respiratory parameters, we calculated the % change in tidal volume during deep breathing in humans, and used the same value in rats, keeping overall ventilation constant.

Immunohistochemistry and Fos Quantification

For Fos immunohistochemistry, we added primary antibody (rabbit anti-Fos) to free-floating brain sections and incubated for 48 hours, then washed with TPBS. We then added a secondary antibody (Cy3-conjugated donkey anti-rabbit) for Fos detection, and incubated overnight. Sections were then mounted on glass slides and coverslipped. For imaging and quantification, we visualized sections with an epifluorescence microscope. We first captured the images and imported them to NIH Image J. We then quantified Fos-

positive (Fos+) cells bilaterally in every 5th rostrocaudal section, with regions of interest delineated (the NTS, CeA and PVN).

Pilot Fos Studies

cFos is an immediate early gene whose expression is induced following activation of neurons, particularly in response to sensory stimulation or stress (Dayas, Buller et al. 2001). Since Fos is commonly used as an activity-dependent marker of stress-induced neuronal activation, we used immunolabeling procedures to measure Fos expression in the NTS, CeA, and PVN, following acute handling and either deep or normal respiration. Recruitment of central parasympathetic circuitry was assessed by examining Fos expression in NTS termination regions including the amygdala and hypothalamus. All brains were examined 2 hours following the onset of mechanical ventilation (when Fos is maximally expressed). Brain sections were mounted on glass slides and examined using an epifluorescence microscope. Regional expression was examined throughout the NTS, CeA, and PVN.

Statistical Analysis

Data are presented as mean ± SEM, unless otherwise noted. Differences in regional expression of Fos in the CeA, PVN, medial NTS (mNTS), and ventrolateral NTS (vlNTS) were determined using two-sample independent t-tests comparing deep vs. normally breathing mechanically ventilated rats, with n=3 animals per group in each case. Statistics were performed with SigmaPlot 13 with significance set at $p<0.05$ and two-tailed tests.

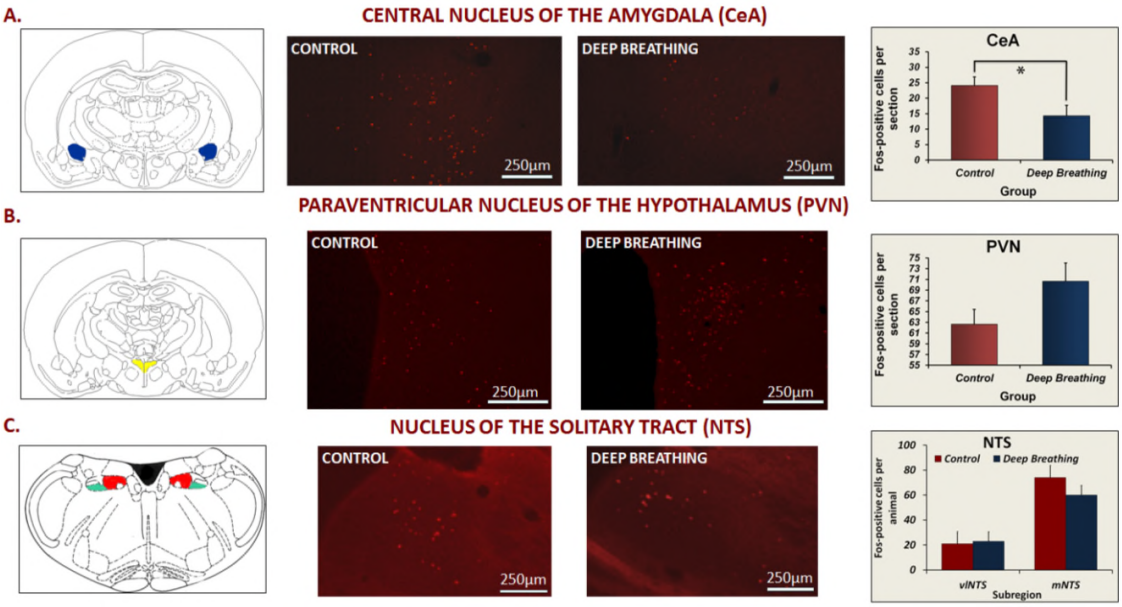

Figure A1.2. Fos immunolabeling in regions of interest.

(A) CeA. **(B)** PVN. **(C)** NTS. *Left.* Schematic plates adapted from a rat brain atlas (Paxinos and Watson 1986) display the neuraxial locations of regions of interest (Bregma: -2.12 [A], -1.80 [B], -12.72 [C]). *Middle.* Epifluorescence photomicrographs show Fos immunolabeling in representative sections. *Right.* Graphical display of average Fos+ neuronal numbers in deep breathing vs. control animals (CeA: 14.3 vs. 24.1 Fos+ cells/section; PVN: 70.7 vs. 62.6 Fos+ cells/section; vlNTS: 23 vs. 21 Fos+ cells/animal; mNTS: 60 vs. 74 Fos+ cells/animal). * *$p<0.05$ between groups using two-sample independent t-test; n=3 rats per group*

Results

Deep breathing decreases activity in the CeA, but leaves PVN and NTS activity unchanged.

Preliminary results (Figure A1.2) in three rats support reduced activity in the CeA in DB vs. control animals, as well as a slightly preferential activation of the vlNTS vs. mNTS during DB. There was a significant reduction in Fos+ cells/section in the CeA in DB vs. control rats ($p<0.05$, n=3, Student's t-test), with the average Fos+ neuronal numbers being 14.3 (DB) vs. 24.1 (control) Fos+ cells/section. There was also a trend

toward reduced Fos+ cells/animal in the mNTS in DB vs. control rats, with the average Fos+ neuronal numbers being 60 (DB) vs 74 (control) Fos+ cells/animal. For other regions, the average Fos+ neuronal numbers were 70.7 vs. 62.6 Fos+ cells/section (in the PVN) and 23 vs. 21 Fos+ cells/animal (in the vlNTS). We propose possible reasons for the lack of differences in Fos+ cell numbers in the PVN in the Discussion.

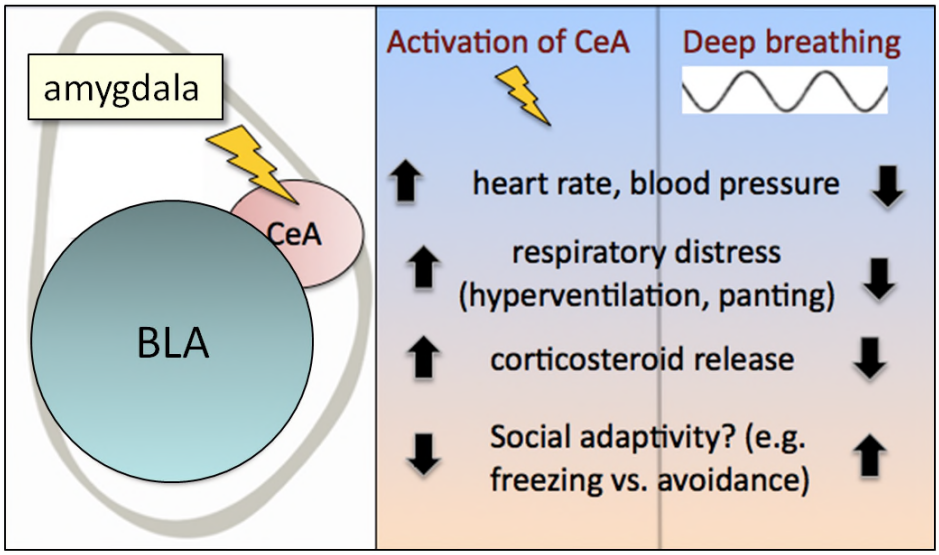

Figure A1.3. Deep breathing opposes the effects of CeA activation.

Reduced activation of the CeA by deep breathing is consistent with reduced sympathetic stress circuit activation. The CeA has known roles in cortisol release and the reaction to fear, and activates autonomic pathways implicated in a variety of emotional responses. Figure based on Ressler 2010.

Discussion

Fos as a marker of neuronal activation

There are several potential issues with using Fos as a proxy for neuronal activity. Although Fos staining generally provides a reliable readout of neuronal activation, it does

not identify neurons undergoing synaptic or transcriptional inhibition, and does not provide any indication that specific genes have been transcriptionally activated (Kovacs 1998). Furthermore, it is possible that different populations of neurons have distinct thresholds or time courses of Fos induction, such that Fos is not expressed in all types of neurons equally. Furthermore, Fos studies allow researchers to get at activation but not chemical phenotype (e.g. whether excitatory or inhibitory neurons have been activated). A final limitation is that using Fos+ neuronal number as a dependent variable does not address relative expression levels in individual neurons.

Although these disadvantages should be taken into account, comparison of experimental and control animals rules out several potential difficulties, as does an understanding of the neurobiology of the investigated circuitry and in particular the neurochemical composition of functional subpopulations. It is also experimentally important to prime Fos activation with an appropriate stressor. While we used acute handling for this purpose, another laboratory investigated Fos staining in the PVN and CeA following repeated resident-intruder stress (Martinez, Phillips et al. 1998). Therefore, an appropriately designed study should satisfactorily overcome the above limitations to address stress-related neuronal activations.

Observed results for Fos+ neuronal numbers in the NTS, CeA, and PVN following DB

Positive results in the CeA are in line with the evolutionary idea of a polyvagal system intricately related with social adaptivity and inversely associated with perceived threat of the environment (Porges 2007). The CeA has an implicated role in passive and reactive fear responses, regulates cortisol release from the PVN, and activates autonomic

output pathways implicated in emotional responses including fear (Ressler 2010). Therefore, reduced activation of the CeA by deep breathing is consistent with reduced sympathetic stress circuit activation (Figure A1.3).

Lack of expected reductions in PVN activity may be due to the heterogeneity of functional subpopulations (magnocellular vs. parvocellular). A unique population of magnocellular neurons in the PVN projects to the posterior pituitary to control oxytocin and vasopressin release, while parvocellular neurons release CRF into the portal vein circulation and project to the brainstem and spinal cord to regulate autonomic preganglionic neurons implicated in the stress response. Electrophysiological or immunological techniques could help delineate changes in these subregions (e.g. double-labeling procedures to determine whether activity in CRF-synthesizing neurons is reduced by DB).

No global changes in NTS activity were observed between groups, and similar means for Fos+ cell numbers were found between the ventrolateral and medial subregions. It is possible that electrical patterns are altered without gross changes in the number of activated neurons in this region. Our lab is pursuing electrophysiological recordings in brainstem NTS neurons to reveal changes in cellular excitability. Further studies should be aimed at verifying preliminary findings and identifying mechanisms linking deep breathing to subregion-specific NTS recruitment patterns and stress circuit deactivation. Investigating the effects of DB on activity in these subregions using electrophysiological or immunological techniques could reveal changes not detected in the present study.

Relating the acute neurophysiological effects of DB to markers of autonomic imbalance is an essential next step in building a detailed mechanistic understanding of DB's effects on well-being. Once it is understood how DB exerts its effects, pharmacological or technological manipulation of the relevant circuitry could result in novel therapeutic and preventative strategies for stress-related disorders.

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