Opioids, Obesity, and the Prefrontal Cortex: Relevance for the Behavioral Control of Breathing

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Zachary Taylor Glovak

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Dedication

This dissertation is dedicated to my best friend Sam whose love, support, competitive spirit, laughter, and wit continue to enrich my life every day. Sitting behind Sam on the first day of statistics class during our first semester of graduate school will forever be the best decision I have ever made. The rest, as they say, is history. While this dissertation represents a significant accomplishment, I am certain it pales in comparison to what the two of us will continue to achieve together personally and professionally.

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Abstract

This dissertation was motivated by the societal context of the threat to public health posed by the opioid and obesity epidemics. Both opioids and obesity have deleterious effects on breathing. Breathing is a key component of an organism's behavioral state and its survival. Significantly depressed breathing can lead to a hypoxic brain state resulting in decreased cognition and ultimately death. Opioids are effective for the management of acute pain, but they depress breathing and behavioral arousal and can cause opioid use disorder. Obesity is often comorbid with chronic pain and respiratory disorders such as sleep disordered breathing. Leptin is a hormone produced by adipose tissue that stimulates breathing by acting on leptin receptors in the central nervous system. Leptin has been implicated as a modulator of the physiological intersections between opioids and obesity, but the underlying mechanisms are not known.

The prefrontal cortex facilitates executive function and integrates autonomic output with behavioral states. The prefrontal cortex is particularly sensitive to hypoxic episodes caused by opioids or sleep disordered breathing secondary to obesity. Furthermore, opioid use disorder and obesity are both diseases that can be attributed to loss of prefrontal cortex regulation of behavioral reward pathways. Thus, the prefrontal cortex is one brain region of interest in the study of the behavioral control of breathing as well as opioid and obesity-induced changes in breathing.

The studies in this dissertation describe the respiratory effects of opioids and obesity. After a brief introduction, Chapters 2, 3, and 4 show that obese mice with dysfunctional leptin receptors exhibit reduced thermal nociception and more

pronounced buprenorphine-induced disruptions in breathing. Chapter 5 demonstrates for the first time in B6 mice that fentanyl and acetylcholine in the prefrontal cortex modulate breathing. These results encourage future studies characterizing the complete mechanism by which the prefrontal cortex regulates the behavioral control of breathing. Taken together, the findings from this dissertation highlight leptin and the prefrontal cortex as potential targets for therapeutic interventions for opioid and obesity-induced changes in breathing.

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Chapter 1 Introduction

Respiration is tightly controlled via a distributed and interconnected network of mechanical, neurochemical, and behavioral mechanisms. The foundational work describing these networks began at the start of the twentieth century when carbon dioxide was identified as the major stimulus for breathing (Haldane & Priestley, 1905). Over a century of pioneering work followed that further elucidated the neural mechanisms underlying the respiratory control networks with the goal of improving the treatment of respiratory disorders (Del Negro et al., 2018; Remmers, 2005).

Today, a persisting challenge for the field of respiratory control is opioid-induced respiratory depression. Opioid use concomitant side effects have become a major public health emergency in the United States (Rudd et al., 2016). Despite a plethora of data existing on the respiratory effects of opioids on humans and animal models, a comprehensive and unifying mechanism by which opioids depress breathing has yet to be established (Ramirez et al., 2021).

Another persisting challenge for the field of respiratory control is the rapidly increasing prevalence of individuals with obesity (Allen et al., 2016; Ward et al., 2016). Obesity is a disease that negatively impacts many aspects of physiology, including respiratory function (Conway & Rene, 2004). The current opioid and obesity crises in the United States reflect an incomplete understanding of how opioids affect human physiology, and how the effects of opioids vary with obesity. Both crises are also worsened by the ongoing COVID-19 pandemic (Gao et al., 2020; Stefan et al., 2021; Volkow, 2020). Additional data regarding the effects of opioids and obesity on

respiratory control could elucidate the underlying mechanisms and inform clinical care (Stokes et al., 2019; Volkow & Collins, 2017).

This dissertation was motivated by the context of these continuing societal challenges. The present chapter begins by introducing the unifying themes of opioids, obesity, leptin, and breathing that are present throughout this dissertation. Second, this chapter briefly overviews the major areas known to contribute to the control of breathing and provides a novel review of the research describing the respiratory effects of opioids and leptin in these areas. Third, this chapter concludes with an outline of the hypotheses, main findings, and thematic relationships of the studies included in the subsequent chapters of this dissertation.

Opioids and Breathing

Figure 1.1 displays the major unifying themes of this dissertation and illustrates the interactions among them and the primary focus of this dissertation: breathing. One unifying theme of the experiments described in this dissertation is opioids (Fig. 1.1A). Opioids are effective for relieving pain (Dahan et al., 2006; Pattinson, 2008), but in some individuals opioids lead to opioid use disorder (Dunlap & Cifu, 2016; Strang et al., 2020) and potentially lethal respiratory depression (Angel et al., 2018; Dahan et al., 2010; Glovak et al., 2022). Opioids exert physiological effects primarily by hyperpolarizing neurons (Lalley, 2008) via activation of endogenous mu, delta, and kappa G-protein-coupled opioid receptors (Dahan, 2006; Laycock & Bantel, 2019). Endogenous opioid receptors are distributed throughout the central and peripheral respiratory networks that control breathing which provides a multitude of sites for

exogenous opioids to disrupt these networks (Ramirez et al., 2021). Current understanding of opioid behavioral pharmacology, including how opioids affect human respiratory physiology, remains incomplete (Nolan et al., 2018; Ramirez et al., 2021; Scholl et al., 2019; Volkow & Collins, 2017). Thus, the experiments in this dissertation were designed to quantify the respiratory effects of opioids as a function of obesity or leptin status.

Obesity and Breathing

Another unifying theme of the studies included in this dissertation is obesity (Fig. 1.1B). The sequelae caused by obesity are exacerbated by opioids (Stokes et al., 2019). Figure 1.2 illustrates the cyclical pattern by which opioid use may be perpetuated in individuals with obesity. For example, humans with obesity report more pain (Allen et al., 2016; Okifuji & Hare, 2015) and are more likely to experience opioid-induced respiratory depression secondary to opioid pain treatment (Dowell et al., 2016; Lee et al., 2015). Obesity is comorbid with other respiratory disorders such as sleep disordered breathing (Gilat et al., 2014), and disrupted sleep resulting from obesity (Singh et al., 2005) has been associated with delayed wound healing (McLain et al., 2018) and increased pain sensitivity (Okifuji & Hare, 2015). Furthermore, the psychosocial impact of obesity and chronic pain (Vincent et al., 2011; Zdziarski et al., 2015) may precipitate opioid use disorder (Stokes et al., 2019).

Obesity and Leptin

Obesity is characterized by increasing levels of adipose tissue resulting in a proinflammatory syndrome (Cullen & Ferguson, 2012). Adipose tissue acts as an endocrine organ that regulates energy storage and metabolism through the secretion of over 600 hormones (Kunath & Klöting, 2016). Of these hormones, leptin is of particular interest given its stimulatory effect on breathing (Bassi et al., 2016; Do et al., 2020; Freire et al., 2020). Leptin (Fig. 1.1C) regulates satiety (Alhadeff et al., 2014) and alters metabolism primarily by activating leptin receptors in the hypothalamus (Paz-Filho et al., 2012). Leptin receptors are also located centrally in the cortex (Udagawa et al., 2006), and brainstem respiratory nuclei (Gauda et al., 2020), as well as peripherally in the lungs (Jutant et al., 2021) and carotid body (Yuan et al., 2018). There are six isoforms of the leptin receptor. The long form, Ob-Rb, has the most physiological influence of the six, while the shorter Ob-Ra form is the most commonly expressed (Gorska et al., 2010). Activation of Ob-Rb receptors binds Janus kinase tyrosine kinases (JAK) and the signal transducer and activator of transcription (STAT) factor which increases transcription of proopiomelanocortin and suppresses cytokine signaling and agouti-related peptide (Gauda et al., 2020).

Opioids, Obesity, Leptin, and Breathing

Emerging evidence suggests that the interactions between obesity and opioids may be modulated, in part, by leptin (O'Donnell et al., 2000; Okifuji & Hare, 2015). Leptin has been shown to alter nociceptive processing (Li et al., 2013; Maeda et al., 2009), but exactly how leptin alters nociceptive processing is not well understood. Over

time, individuals with obesity become leptin resistant (Balland & Cowley, 2015; Caro et al., 1996; Enriori et al., 2006) which attenuates the respiratory stimulant effect of leptin (O'Donnell et al., 2000; Polotsky et al., 2004; Tankersley et al., 1998). Leptin resistance also exacerbates the respiratory disorders commonly experienced by individuals with obesity (Gilat et al., 2014), including sleep disordered breathing (Pho et al., 2016; Yao et al., 2016) and opioid-induced respiratory depression (Lee et al., 2015). The relationships among leptin, obesity, and opioids motivated the subsequent review of the respiratory effects of leptin and opioids on the respiratory control network. This review highlights what is currently known and not known about the respiratory effects of opioids and leptin acting at specific nuclei within the respiratory control network.

The Respiratory Control Network

The respiratory control network is distributed and integrated throughout multiple organ systems (Feldman & Ellenberger, 1988). This review focuses on the major divisions of respiratory neurons localized to the pontomedullary brainstem and on peripheral chemoreceptors. Directly relevant to experiments reported in this dissertation is recent evidence that breathing is modulated by the prefrontal cortex (Fig. 1.1D). Novel findings from the present research show that breathing varies with leptin status and that fentanyl and an acetylcholinesterase inhibitor delivered to the prefrontal cortex alters breathing. The prefrontal cortex is a brain region of special interest given its role of integrating autonomic function with behavioral states (Fuster, 2015). In accordance with the One Health initiative (Gibbs, 2014), the subsequent review considers data reported from the humane study of a multitude of female and male

animals including mice, rats, goats, cats, and humans with the goal of improving interdisciplinary, comparative medicine for all species.

Pontine Respiratory Group

The pontine respiratory group, also known as the pneumotaxic center, consists of the Kölliker-Fuse nucleus and the parabrachial nuclei (Dutschmann & Dick, 2012; Lydic & Orem, 1979). In mammals, the pontine respiratory group primarily integrates information from other brain areas and modifies respiratory rhythm (Dhingra et al., 2017; Feldman, 1986; Jiang et al., 2004) and chemosensory reflexes (Damasceno et al., 2014). Consistent with these roles, the pontine respiratory group contains inspiratory and expiratory neurons (Alheid et al., 2004), and receives afferent input from the vagus nerve, hypothalamus, nucleus of the solitary tract, and cerebral cortex (Alheid et al., 2004). The bulk of the efferent projections from the pontine respiratory group originate in the Kölliker-Fuse nucleus and project to the respiratory rhythm generating neurons in the medulla (Alheid & McCrimmon, 2008).

Neurons within the pontine respiratory group also are activated by leptin to modulate food intake (Alhadeff et al., 2014; Benarroch, 2016). To my knowledge, no studies exist examining the respiratory effects of direct leptin administration into the pontine respiratory group (Bassi et al., 2016). However, several studies have examined the role of leptin signaling in the pontine reticular formation, which is an area that modulates nociceptive responses in mice (Wang et al., 2009), rats (Watson et al., 2008), and cats (Kshatri et al., 1998; Tanase et al., 2002). Delivery of the acetylcholinesterase inhibitor neostigmine to the pontine reticular formation is known to

decrease breathing in B6 mice (Lydic et al., 2002). The cholinergic-induced changes in breathing were attenuated in obese mice with genetically endowed leptin deficiency compared to B6 mice (Douglas et al., 2005).

Opioids have a well-documented depressive effect on neurons within the pontine respiratory group primarily via activation of mu opioid receptors (Saunders & Levitt, 2020). In addition to slowing the respiratory rhythm (Hurlé et al., 1985; Prkic et al., 2012; Varga et al., 2020), activation of mu opioid receptors hyperpolarizes Kölliker-Fuse neurons which may affect airway patency (Levitt et al., 2015). The respiratory depression elicited by opioids is likely due to the disruption of neuronal signaling in multiple respiratory centers (Pattinson, 2008).

Medulla Oblongata

The major stimulus for breathing comes from pH changes in blood caused by metabolically produced carbon dioxide. Increases in carbon dioxide (hypercapnia) is detected on the ventral surface of the medulla (Kawai et al., 1996) and in the retrotrapezoid nucleus (Guyenet et al., 2008). This sensory input is transmitted to neural networks in the medulla which generate the respiratory rhythm (Onimaru et al., 1987; Richter et al., 1975; Smith et al., 1991). The output of the medulla is modulated by respiratory nuclei within the pontine respiratory group (Bonham, 1995). The respiratory neurons within the medulla can be divided into two regions (Bonham, 1995; Speck & Feldman, 1982): the dorsal respiratory group and ventral respiratory group.

Dorsal Respiratory Group

The dorsal respiratory group was identified by Merrill (Merrill, 1970) and colleagues and is located within the ventrolateral nucleus of the solitary tract. The dorsal respiratory group receives notable projections from the pontine respiratory group, as well as the vagus and glossopharyngeal cranial nerves which enable the dorsal respiratory group to integrate sensory information from the lungs and carotid body (de Castro et al., 1994; Ezure, 1990). The nucleus of the solitary tract synthesizes the respiratory rhythm output from the ventral respiratory group (Hilaire et al., 1990; Paton et al., 1994) and peripheral sensory input to provide the primary inspiratory motor drive to breathe by activating neurons in the spinal cord to stimulate the respiratory muscles (Ezure, 1990).

Leptin stimulates breathing (Bassi et al., 2016; Gauda et al., 2020), and there is a high concentration of leptin receptors in the rodent nucleus of the solitary tract (Elias et al., 2000; Hosoi et al., 2002; Mercer et al., 1998; Shioda et al., 1998). Leptin functions as a neuropeptide modulator of breathing and metabolism as evidenced by the localization of the Ob-Rb leptin receptor to proopiomelanocortin, proglucagon, and cholecystokinin neurons in the nucleus of the solitary tract (Garfield et al., 2012). Microinjection of leptin into the nucleus of the solitary tract increases inspiratory behavior (Inyushkin et al., 2009; Inyushkina et al., 2010) and alters chemosensory responses (Ciriello & Moreau, 2012) via activation of the Ob-Rb receptor (Yu et al., 2021). Interestingly, leptin activation of nucleus of the solitary tract neurons does not increase breathing during sleep (Amorim et al., 2021), indicating a state-dependent

phenomenon. These findings are consistent with leptin's role as a regulator of energy homeostasis (Bassi et al., 2016; Gauda et al., 2020) because respiratory behavior must adapt to accommodate changes in metabolic demands (Bassi et al., 2012). However, more studies are needed to further elucidate leptin's effects on neurons in the dorsal respiratory group in relation to the rest of the respiratory control network.

In contrast, the data regarding opioidergic effects on respiration that are mediated by the nucleus of the solitary tract remains limited (Pattinson, 2008; Ramirez et al., 2021). Neurons within the nucleus of the solitary tract respond differentially to different opioid receptor agonists, with mu and kappa opioid receptors hyperpolarizing various sub-groups of neurons within the nucleus of the solitary tract and delta opioid agonists having no effect (Poole et al., 2007; Rutherfurd & Gundlach, 1993; Xia & Haddad, 1991). Activation of mu opioid receptors in the nucleus of the solitary tract decreases inspiratory drive (Iniushkin, 1997) and chemosensory reflex (Zhuang et al., 2017), but the exact mechanisms are not known.

Ventral Respiratory Group

The ventral respiratory group contains the nucleus ambiguus and nucleus retroambiguus, as well as the pre-Bötzinger and Bötzinger complexes (Alheid & McCrimmon, 2008; Cinelli et al., 2020). This area contains both inspiratory and expiratory neurons and produces the base respiratory rhythm via the pre-Bötzinger and Bötzinger complexes (Feldman et al., 2003). This rhythm is then modified by input from surrounding areas, such as the pontine respiratory group, retrotrapezoid nucleus,

nucleus of the solitary tract, and parafacial respiratory group to produce coordinated respiratory function (Onimaru & Homma, 2003). Although this area has been extensively studied, the exact mechanisms by which the central respiratory pattern generated by the ventral respiratory group is modulated by surrounding areas to produce breathing behavior remain to be identified (Feldman, 1986; Feldman & Ellenberger, 1988; Feldman et al., 2003).

The long form of the leptin receptor is widely distributed throughout the medulla (Barnes et al., 2010). Within the ventral respiratory group, the retrotrapezoid nucleus has been identified as a chemoreceptive site that responds to activation of leptin receptors. Repeated injections of leptin into the retrotrapezoid nucleus over three days increased ventilation and carbon dioxide sensitivity in leptin deficient ob/ob mice (Bassi et al., 2014). However, acute leptin administration to neurons within the retrotrapezoid nucleus of rat pups *in vitro* did not alter breathing (Bassi et al., 2014). The contrast between these two findings could be attributed to species differences or the timescale required for leptin to alter breathing. Future experiments are needed to determine whether the different results reflect developmental effects or result from species differences (Bassi et al., 2016; Gauda et al., 2020).

The effects of opioids on respiratory networks within the ventral respiratory group vary between *in vitro* and *in vivo* studies. *In vitro* studies show that activation of mu opioid receptors in the pre-Bötzinger complex slows respiratory output by decreasing excitatory neurotransmission (Mellen et al., 2003; Wei & Ramirez, 2019). However, activation of mu opioid receptors *in vivo* has yielded different results. Delivery of mu

opioid receptor agonists into the pre-Bötzinger complex slows respiratory activity of rats (Montandon et al., 2011) but not goats (Krause et al., 2009; Langer et al., 2017) or mice (Varga et al., 2020). These findings suggest that opioid binding in the pre-Bötzinger complex is not sufficient to produce opioid-induced respiratory depression *in vivo*. This interpretation aligns with evidence that opioid-induced respiratory depression emerges from opioid actions across multiple respiratory control networks, rather than from one single site of action (Varga et al., 2020).

Peripheral Control

There are several peripheral regions that contribute to the control of breathing, most notably the lungs and carotid body (Gautier & Bonora, 1979; Knowlton & Larrabee, 1946). The carotid body is a chemosensory area at the bifurcation of the carotid arteries that increases activity in response to hypoxia and to a lesser extent hypercapnia (Bonham, 1995). This feedback is provided mainly to the nucleus of the solitary tract via the glossopharyngeal nerve where the information can be further integrated into the respiratory network (Biscoe, 1971; Ciriello & Caverson, 2014). It has been estimated that the carotid bodies provide about 10% of the drive to breath (Javaheri & Kazemi, 1987).

Besides carrying out the physical act of air turnover, the lungs also provide mechanosensory feedback to the respiratory network (Pelosi et al., 1998). This is achieved via stretch receptors in the lungs and upper airways (Sant'Ambrogio & Mortola, 1977). Together, the lungs and carotid body provide critical information to the

brainstem respiratory networks regarding the position of the peripheral respiratory components and their overall function by monitoring carbon dioxide levels.

The long form of the leptin receptor has been localized in the carotid body (Caballero-Eraso et al., 2019) and the lungs (Tsuchiya et al., 1999). Data are available showing that leptin stimulates breathing by facilitating chemoreception via the carotid body to nucleus of the solitary tract pathway during spontaneous breathing conditions (Ciriello & Caverson, 2014; Porzionato et al., 2011; Ribeiro et al., 2019; Ribeiro et al., 2018; Yuan et al., 2018). However, leptin's role in respiratory control during hypercapnic or hypoxic conditions remains unclear (Gauda et al., 2020). Research has shown that leptin is involved in lung development, surfactant function, immune response, upper airway control, and gas exchange (Carron et al., 2018; Jutant et al., 2021; Pho et al., 2016; Torday et al., 2010). Evidence for leptin's involvement in the peripheral processes of respiration is abundant, however, the mechanisms remain to be fully elucidated (Bassi et al., 2016; Gauda et al., 2020; Jutant et al., 2021; Sato et al., 2011).

Opioids depress respiratory responses to hypoxia and hypercapnia (Pattinson, 2008). Studies indicate that opioids may depress the chemosensory reflex via opioid receptors in the carotid body (Baby et al., 2018; Kirby & McQueen, 1986; Weil et al., 1975), but the precise mechanisms remain unclear (Pattinson, 2008; Ramirez et al., 2021). Opioid receptors have also been localized throughout the respiratory tract, including the lungs (Cabot et al., 1994; Zebraski et al., 2000), where they have been shown to cause impaired immune function and alter bronchoconstriction upon activation

(Kuo et al., 1992; Yamanaka & Sadikot, 2013). Further research is needed to better identify and understand the pathways and the mechanisms by which peripheral opioids alter the central respiratory network (Ramirez et al., 2021).

Prefrontal Cortex and the Behavioral Control of Breathing

There is increasing evidence that the cortex has the capacity to alter the respiratory output generated by the pontomedullary brainstem. Recent research in humans (Pattinson & Wise, 2016), rats (Hassan et al., 2013), and mice (Howell et al., 2017) has identified the prefrontal cortex as one cortical region that may modulate breathing. The prefrontal cortex facilitates many complex behaviors (Fuster, 2015). The inclusion of the prefrontal cortex in the respiratory control network is consistent with the view that breathing is one component trait of the overall behavioral state (Del Negro et al., 2018; Fink, 1961). For example, the prefrontal cortex modulates many behavioral functions known to impact breathing, such as emotion regulation (Adhikari et al., 2015), arousal (Muzur et al., 2002; Van Dort et al., 2009), and executive function (Alizadehgoradel et al., 2020; Pattinson et al., 2009). The prefrontal cortex and behavior can also be altered by breathing (Herrero et al., 2018; Yuan et al., 2013). Although not yet fully characterized, these functional data suggest a bidirectional relationship between the prefrontal cortex and respiratory output (Lydic & Baghdoyan, 2021).

Anatomical evidence also supports the interpretation that the prefrontal cortex participates in the top-down and bottom-up modulation of breathing (Yackle et al., 2017). Studies of multiple animal models show pathways from the prefrontal cortex to

brainstem respiratory nuclei. In B6 mice, there are projections from subregions of the medial prefrontal cortex such as the infralimbic, prelimbic, and cingulate cortices to the amygdala (Adhikari et al., 2015) and the nucleus of the solitary tract (Howell et al., 2017). There are also connections from the lateral prefrontal cortex (frontal association cortex; FrA) of B6 mice to the nucleus pontis oralis (Van Dort et al., 2009). Studies of cat confirm reciprocal connections between the nucleus pontis oralis and the respiratory nuclei in the pontine, dorsal, and ventral respiratory groups (Lee et al., 1995). In rats, similar efferent and afferent projections exist between the infralimbic, prelimbic, and cingulate cortices and the pontine, dorsal, and ventral respiratory groups (Gabbott et al., 2005; Hoover & Vertes, 2007; Sesack et al., 1989; Terreberry & Neafsey, 1987). Finally, volitional breathing associated with human speech is facilitated by prefrontal cortex to brainstem pathways (Holstege & Subramanian, 2016). Despite this abundance of functional and anatomical data, the exact mechanisms underlying the prefrontal cortex control of breathing are not yet known (Lydic & Baghdoyan, 2021).

Leptin receptors are widely expressed throughout brain, including the prefrontal cortex (Ates et al., 2014). However, there are no studies that specifically investigate the direct interaction between leptin receptors in the cortex and breathing (Bassi et al., 2016; Gauda et al., 2020). Opioid receptors are also prevalent throughout the prefrontal cortex (Giacchino & Henriksen, 1998; Mansour et al., 1995; Peciña et al., 2019). Opioids are known to reduce electroencephalographic measures of cortical arousal in mice (O'Brien et al., 2021) and rats (Montandon & Horner, 2019). Functional magnetic resonance imaging of human cortex and brainstem also show reduced cortical activity

following opioid administration (Pattinson et al., 2009). Reduced cortical arousal has been associated with decreased breathing (DeMarco et al., 2004; Montandon & Horner, 2019; Pal et al., 2018). This is consistent with the construct of the "wakefulness stimulus for breathing" whereby the cortical activity associated with the state of wakefulness stimulates the drive to breathe (Fink, 1961). Decreased cortical arousal in the prefrontal cortex caused by opioids may contribute to opioid-induced respiratory depression, but additional studies are needed to confirm this hypothesis (Lydic & Baghdoyan, 2021; Ramirez et al., 2021; Sun et al., 2022). Chapter 5 of this dissertation describes the first measures of the respiratory effects of opioids microinjected into the mouse prefrontal cortex. Given the documented effects of leptin (Bassi et al., 2016; Gauda et al., 2020) and opioids (Ramirez et al., 2021) on the respiratory control network, continued research on their cortical effects is needed in order to enhance our understanding of the behavioral control of breathing (Lydic & Baghdoyan, 2021).

Summary

Considerable research exists linking leptin and opioids to the peripheral and central control of breathing. Depending on the brain site of action, leptin and opioids can have opposite effects on breathing. Leptin has been shown to have a stimulatory effect on respiration while opioids are notorious for eliciting dose-dependent respiratory depression. More research is required to understand the interactions between leptin and opioids. A more complete understanding of opioid action over the entire respiratory network could uncover pathways to help diminish opioid-induced respiratory depression while retaining beneficial antinociception (Bateman et al., 2021). Similarly, a greater

knowledge of leptin's role in the control of respiration could help treat respiratory disorders associated with obesity or opioid-induced respiratory depression (Freire et al., 2020). Additional research regarding the networks controlling respiration holds great promise for improving the treatment of respiratory disorders (Del Negro et al., 2018; Remmers, 2005).

To that end, the growing prevalence of obesity (Gao et al., 2020; Stefan et al., 2021; Wang et al., 2020) and the ongoing opioid crisis (Rudd et al., 2016) encouraged the studies in this dissertation examining the interactions among opioids, obesity, leptin, and breathing with the potential for translational relevance. The following summary outlines the **hypotheses** of the studies in this dissertation and how they relate to this goal.

Chapter 2: Leptin Status Alters Buprenorphine-induced Antinociception in Obese Mice with Dysfunctional Leptin Receptors

My dissertation research began with the goal of understanding the relationship between nociception, leptin, obesity, and buprenorphine. Buprenorphine is a partial mu opioid receptor agonist and a kappa opioid receptor antagonist that is used to treat opioid use disorder (Schuckit, 2016). In contrast with other full mu agonists such as morphine and fentanyl, buprenorphine is reported to have a ceiling effect for respiratory depression but not for analgesia (Dahan et al., 2006). This unique pharmacology makes buprenorphine ideal for treating the chronic pain (Lazaridou et al., 2020) commonly comorbid in individuals with obesity, however, most studies of buprenorphine

have been conducted on normal weight, male subjects. The studies described in Chapter 2 used four lines of female and male mice, all congenic with B6 mice, to test the **hypothesis** that buprenorphine-induced antinociception varies as a function of body weight, sex, or leptin status. The results showed that disrupted leptin signaling decreased baseline acute thermal nociception and attenuated buprenorphine-induced antinociception (Glovak et al., 2017).

Chapter 3: Buprenorphine Depresses Respiratory Variability in Obese Mice with Altered Leptin Signaling

My next goal was to examine the relationship between buprenorphine, obesity, leptin, and breathing. Using the same congenic lines of mice and antinociceptive dose of buprenorphine identified in Chapter 2, the study comprising Chapter 3 tested the **hypothesis** that systemic administration of buprenorphine differentially alters breathing in wild-type B6 mice compared to the three congenic lines of mice with leptin dysfunction. The results show that an antinociceptive dose of buprenorphine did not alter respiratory rate, tidal volume, or minute ventilation. However, buprenorphine significantly decreased breathing variability in all congenic lines of mice (Angel et al., 2018). The magnitude of the decrease in breathing variability was greater in mice with disrupted leptin signaling. Breathing variability is vital to maintain metabolic homeostasis and normal gas exchange (Bien et al., 2004; Van Diest et al., 2006).

Chapter 4: Buprenorphine Differentially Alters Breathing Among Four Congenic Mouse Lines as a Function of Dose, Sex, and Leptin Status

Having shown that buprenorphine depresses breathing variability in wild-type mice and obese mice with disrupted leptin signaling, my next goal was to investigate if the effects of buprenorphine on breathing varied as a function of dose. This study tested the **hypothesis** that the respiratory effects of buprenorphine varied as a function of dose, sex, body weight, and leptin status. The results showed that buprenorphine caused dose-dependent decreases in respiratory rate while increasing tidal volume, minute ventilation, and duty cycle. Buprenorphine also significantly decreased breathing variability in all mice. Overall, buprenorphine caused greater breathing changes in male mice and in mice with disrupted leptin signaling. The findings of this study further characterized the relationship between buprenorphine, breathing, sex, and leptin status (Glovak et al., 2022). These studies support and extend the findings from Chapters 2 and 3 by characterizing the respiratory effects of buprenorphine as a function of increasing doses. Doing so provided vital normative data on the interaction between obesity, leptin, and buprenorphine using one of the most widely studied animal models.

Chapter 5: Fentanyl and Neostigmine Delivered to Mouse Prefrontal Cortex Differentially Alter Breathing

The findings from Chapters 2, 3, and 4 stimulated my interest in identifying potential brain regions that contribute to opioid-induced changes in breathing. The

studies of prefrontal cortex described in Chapter 5 culminate my graduate research. I chose to study the prefrontal cortex because it contains opioid receptors (Giacchino & Henriksen, 1998; Mansour et al., 1995; Peciña et al., 2019) and has been shown to modulate drug seeking behavior in humans (Ceceli et al., 2022), breathing in rats (Hassan et al., 2013), and behavioral arousal in B6 mice (Van Dort et al., 2009). My studies were encouraged by preliminary data showing that opioids delivered to the prefrontal cortex of B6 mice altered breathing (Glovak et al., 2021; Glovak et al., 2019; Sun et al., 2022). The study described in Chapter 5 tested the **hypothesis** that compared to saline control, breathing is significantly altered by microinjection of fentanyl and the acetylcholinesterase inhibitor neostigmine into the prefrontal cortex of freely behaving B6 mice. The results show that fentanyl caused site-specific changes in breathing. Fentanyl microinjected into the lateral prefrontal cortex decreased breathing variability, whereas fentanyl microinjected into the medial prefrontal cortex increased tidal volume and respiratory duty cycle. Neostigmine microinjected into the medial prefrontal cortex significantly increased respiratory rate, tidal volume, and minute ventilation. This study showed, for the first time in B6 mice, that breathing is altered by activation of opioid and cholinergic receptors in the prefrontal cortex.

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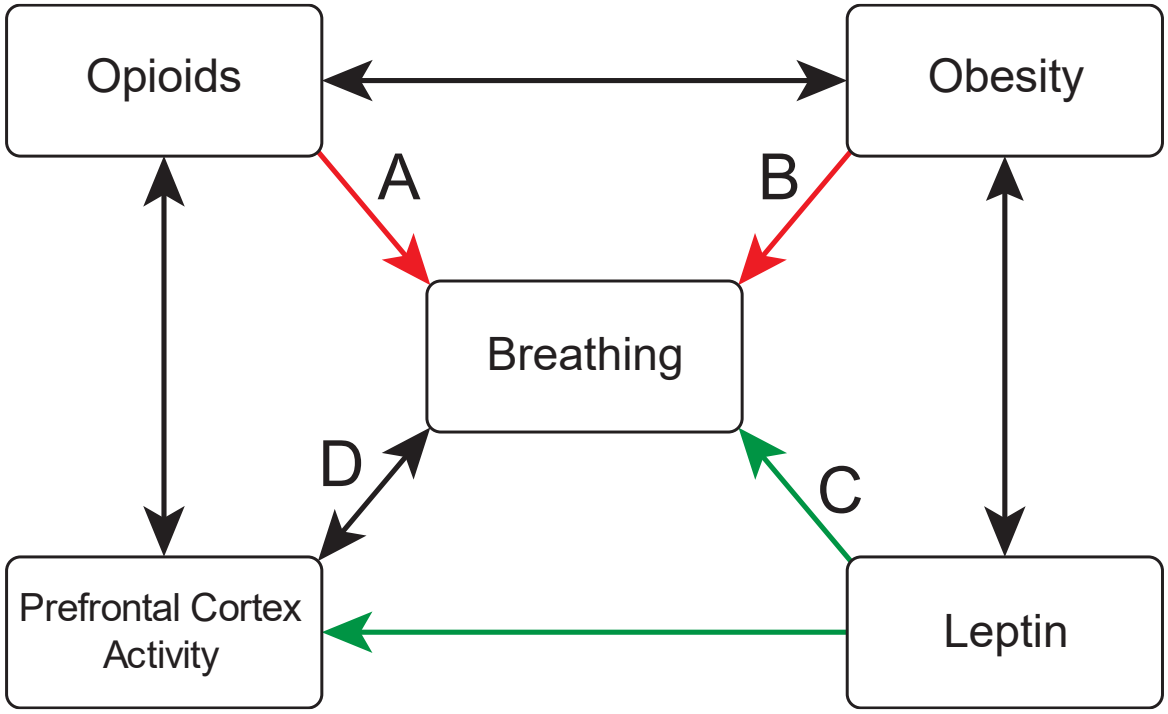
Appendix

Figure 1.1 Schematic diagram of the themes of this dissertation.

Opioids are known to depress breathing (A) and cause opioid use disorder, both of which are major public health concerns. Another major public health concern is the increasing prevalence of individuals with obesity. Obesity is characterized by increased levels of adipose tissue, reporting of pain, and respiratory disorders (B). Adipose tissue secretes leptin which regulates energy homeostasis, modulates pain, promotes neural plasticity in the prefrontal cortex, and stimulates breathing (C). Individuals with obesity become leptin resistant, which further exacerbates the respiratory effects of obesity and disrupts the normal function of leptin. The prefrontal cortex is one brain region of interest because it has been implicated in the process of opioid addiction, and the behavioral control of breathing (D). Black arrows indicate bidirectional relationships. Red arrows indicate decreases in breathing. Green arrows show that leptin promotes breathing and activity in the prefrontal cortex.


Opioids
Obesity
A
B
Breathing
D
C
Prefrontal Cortex Activity
Leptin

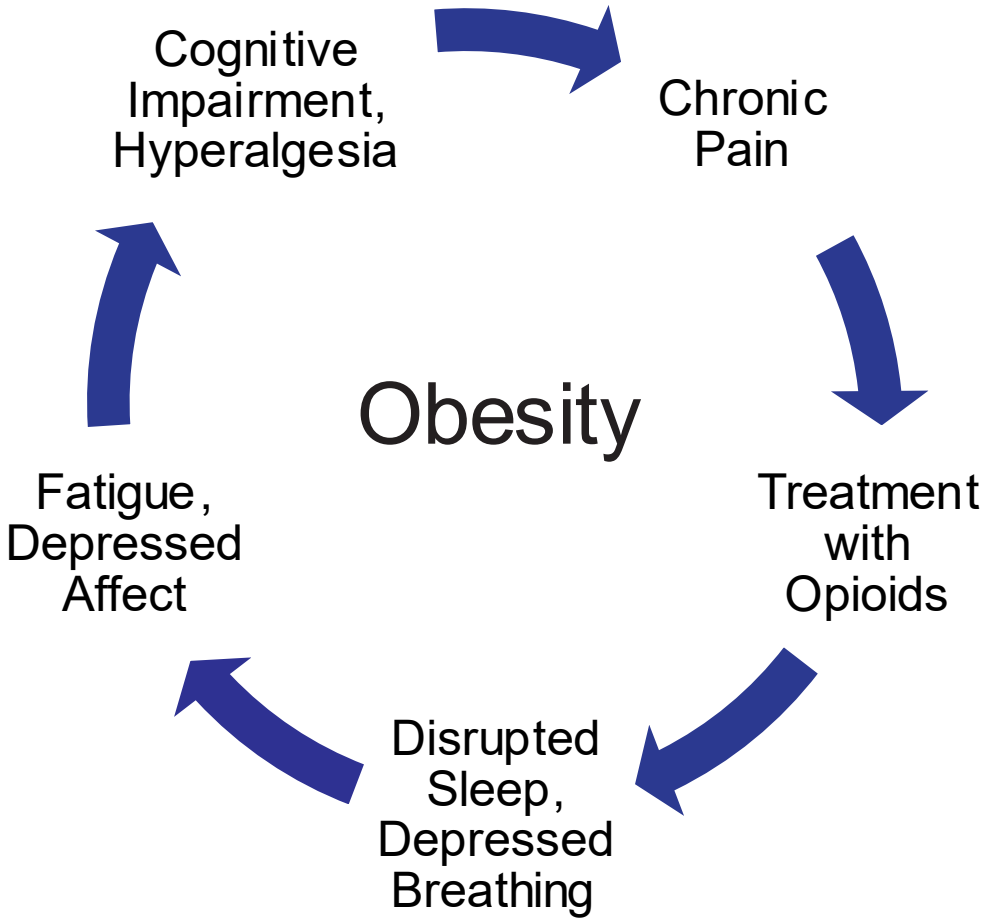


Figure 1.2 Opioid treatments may be perpetuated in individuals with obesity.

Obesity is comorbid with increased chronic pain that may require treatment with opioids. Opioids disrupt sleep, and obesity and opioids depress breathing. Over time, disrupted sleep and breathing increases fatigue and can cause depression. Depression and fatigue contribute to cognitive impairment and hyperalgesia which leads to increased levels of pain and may perpetuate opioid treatments.

Chapter 2 Leptin Status Alters Buprenorphine-induced Antinociception in Obese Mice with Dysfunctional Leptin Receptors

This chapter is adapted for dissertation formatting from the following publication:

Glovak ZT, Mihalko S, Baghdoyan HA, Lydic, R. Leptin status alters buprenorphine induced antinociception in obese mice with dysfunctional leptin receptors. Neurosci Lett 660: 29-33, 2017. https://doi.org/10.1016/j.neulet.2017.09.012.

I collected, analyzed, and interpreted the data presented in the following chapter, as well as contributed to the writing of the original manuscript from which the following chapter is adapted.

Abstract

Buprenorphine is an opiate used for pain management and to treat opiate addiction. The cytokine leptin can modulate nociception, but the extent to which buprenorphine-induced antinociception varies as a function of leptin signaling has not been characterized. Four congenic mouse lines with phenotypes that include differences in body weight and leptin status were used to test the hypothesis that the antinociceptive effects of buprenorphine vary as function of sex and leptin signaling. Each mouse line was comprised of males (n = 12) and females (n = 12) for a total of 96 animals. Groups included C57BL/6J (B6) mice (wild type), B6 mice with diet-induced obesity (DIO), obese B6.Cg-Lep^{ob}/J (ob/ob) mice lacking leptin, and obese B6.BKS(D)-Lepr^{db}/J (db/db) mice with dysfunctional leptin receptors. The dependent measure was tail flick latency (TFL) in seconds for mouse-initiated tail removal from a warm water bath. Independent variables were intraperitoneal administration of saline (control) or buprenorphine (0.3

mg/kg). Within every mouse line, buprenorphine significantly increased TFL relative to saline. Compared to the other mouse lines, db/db mice with dysfunctional leptin receptors had a significantly longer TFL after saline and after buprenorphine. TFL did not vary significantly by body weight or sex. The results provide novel support for the interpretation that acute thermal nociception is associated with altered leptin signaling.

Introduction

Buprenorphine is a mu opiate receptor agonist and kappa opiate receptor antagonist used for pain management (Campbell & Lovell, 2012) and for treating opiate use disorder (Dowell et al., 2016; Dunlap & Cifu, 2016). Most data regarding buprenorphine have been derived from studies of normal weight males. In contrast, morphine is known to cause sex-specific differences in control of human breathing (Dahan et al., 1998). Closed claims analyses reveal that being female and obese are the greatest risk factors for opiate-related adverse events (Lee et al., 2015). Obesity is co-morbid with increased reports of pain in humans (Allen et al., 2016; Okifuji & Hare, 2015) and with altered nociception in mice (Sullivan et al., 2007; Wang et al., 2009; Watson et al., 2014).

Obesity is a proinflammatory disorder with the potential to modulate nociception via multiple tissue systems (Cullen & Ferguson, 2012). Adipocytes secrete leptin, a proinflammatory cytokine that also functions to signal satiety (Balland & Cowley, 2015). Nociceptive processing is altered in obese mice (Rossi et al., 2016; Rossi et al., 2013; Tramullas et al., 2016) and previously we have shown that leptin replacement can restore thermal nociception in obese, leptin-deficient mice (Wang et al., 2009; Watson

et al., 2014). We are aware of no comparable data from mice determining whether the antinociceptive effects of buprenorphine are altered by obesity and/or sex. The current study used four congenic lines of mice with normal or dysfunctional leptinergic transmission. These four lines of mice made it possible to test the hypothesis that the antinociceptive effects of buprenorphine vary as a function of sex and leptin signaling. Preliminary results have been presented (Mihalko et al., 2016).

Materials and Methods

Animals

All procedures using animals were approved by the University of Tennessee Institutional Animal Care and Use Committee and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (The National Academies Press, 8th Ed., Washington, D.C., 2011). Adult male and female mice age 6–8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The congenic mouse lines included: C57BL/6J (B6) mice (wild type), B6 mice fed a 60% fat diet (D12492, Research Diets, Inc., New Brunswick, NJ, USA) to create diet-induced obesity (DIO), obese B6Cg-Lep^{ob}/J (ob/ob) mice that do not produce leptin, and obese B6.BKS(D)-Lepr^{db}/J (db/db) mice that produce leptin and have dysfunctional leptin receptors. None of these mice are transgenic animals. The ob/ob and db/db mice were discovered as spontaneous mutations in the B6 line. Quality control protocols at The Jackson Laboratory regularly confirm genetics and leptin status of mouse lines (Morgenweck, 2017) via End Point Genotyping to insure the presence of the ob/ob and the db/db

mutations. Additionally, genome scans confirm that these congenic lines are on a B6 background.

Each mouse line included 12 males and 12 females for a total sample size of 96. Mice were housed in a temperature-controlled environment and provided with ad libitum access to food and water. B6, ob/ob, and db/db mice were fed Teklad 22/5 rodent chow containing 5.5% fat.

Nociceptive testing and drug administration

The dependent measure was time in seconds for mouse-initiated tail removal from a water bath maintained between 48° and 50 °C. These measures of tail flick latency (TFL) provide an established index of acute thermal nociception (Cowan et al., 1977; Raffa & Ding, 2007). For one week prior to quantifying TFL, mice were conditioned for 2 min to being placed in an acrylic tube with their tails extending outside the tube. During data collection, the distal third of the tail was lowered into the water bath. A timer was started when the tail was immersed in the water. The timer was stopped when the mouse flicked its tail out of the water. Each of three TFL measures was separated by a 10-s interval of no stimulation. A cutoff time of 15 s was implemented to prevent tissue damage, and this 15-s interval served as the maximum possible TFL value. Injection volume was 0.3 mL. The 0.3 mg/kg dose of buprenorphine reliably produces antinociception in mice (Lutfy et al., 2003; Roughan & Flecknell, 2002) and buprenorphine can have an antinociceptive effect on mouse TFL for as long as 270 min after administration (Gades et al., 2000). Based on these

previous findings, TFL measures were collected 120 min after intraperitoneal (i.p.) administration of saline (vehicle control) or buprenorphine (0.3 mg/kg). Studies of acute thermal nociception often compare pre-injection (baseline) and post-injection (treatment) measures in order to normalize the data as percent maximum possible effect. As cautioned previously (Allen & Yaksh, 2004), normalization using percent maximum possible effect is misleading for nociceptive studies involving subjects that are known to differ by strain or line. Therefore, it was not appropriate in the present study to normalize the TFL measures.

Data analysis

Statistical analyses were performed using Prism 7.0b. To circumvent the potential confound of inflated degrees of freedom, all statistical analyses were performed using TFL values expressed as a mean for each mouse. The data from each of the four lines of mice were confirmed via D'Agostino and Pearson tests to satisfy the assumption of a normal distribution. The hypothesis that the antinociceptive effects of buprenorphine varied as a function of sex and leptin signaling was evaluated using two-way analysis of variance (ANOVA) followed by post hoc multiple comparisons tests. The magnitudes of the treatment effects within each mouse line were quantified with Cohen's *d* statistic. For each of the four mouse lines and for each drug condition, regression coefficients (r^2) quantified the amount of variance in TFL that was accounted for by body weight.

Results

Comparisons of nociception after saline or buprenorphine administration revealed no significant differences in TFL between males and females (Table 2.1). Subsequent analyses thus combined TFL measures from male and female mice within each congenic line (Fig. 2.1). The increase in TFL caused by buprenorphine for each mouse line was B6 = 55.8%, DIO = 47.0%, ob/ob = 43.8 %, and db/db = 23%. Two-way ANOVA showed statistically significant differences in TFL as a function of mouse line ($F = 32.09$; $df = 3,184$; $P < 0.0001$) and buprenorphine ($F = 102.3$; $df = 1,184$; $P < 0.0001$). Post-hoc analyses indicated that db/db mice had significantly ($P < 0.0001$) longer TFLs than B6, DIO, and ob/ob mice after administration of saline or buprenorphine.

Cohen's *d* statistic provided an additional metric for phenotyping the magnitude of the treatment effect. All Cohen's *d* values were large, indicating non-overlap in the TFL distributions after buprenorphine relative to saline: DIO ($d = 2.5$), B6 ($d = 2.0$), ob/ob ($d = 1.6$), and db/db ($d = 0.88$). Expressed as percent of non-overlap, the Cohen's *d* values revealed non-overlapping distributions ranging from >98% percent non-overlap for B6 and DIO mice to 73% for ob/ob, and 52% for db/db mice. Thus, the significant differences in TFL as a function of mouse line (Fig. 2.1) were not an artifact of inflated degrees of freedom associated with large sample sizes.

ANOVA confirmed that body weight varied significantly ($F = 142$; $df = 3,92$; $P < 0.0001$) as a function of congenic line. Body weights (mean ± standard deviation in g) at the time of TFL testing were B6 female (19.8 ± 1.2), B6 male (27.4 ± 1.9); DIO female (30.7 ± 2.9), DIO male (43.5 ± 5.4); ob/ob female (48.4 ± 2.9), ob/ob male (46.4 ± 2.3);

db/db female (49.4 ± 2.0), db/db male (48.0 ± 1.9). Post hoc analyses showed that body weights of DIO, ob/ob, and db/db mice were significantly ($P < 0.0001$) greater than body weight of control (B6) mice. Our assessments of higher-level phenotypes such as body weight, hyperphagia, elevated blood glucose levels, and coat condition were consistent with genotypes comprising the four congenic mouse lines used in the present study https://www.jax.org/jax-mice-and-services/find-and-order-jax-mice/why-jax-mice/genetic-quality-control-program.

Post hoc tests found that body weight of females was significantly ($P < 0.0001$) less than males for B6 and DIO mice, but not for ob/ob and db/db mice. Regression analyses were used to evaluate the percent of variance in TFL that was attributed to body weight. Body weight did not account for a significant amount of variance in TFL in any mouse line after administration of saline or buprenorphine (Fig. 2.2).

Discussion

The interacting epidemics of opiate abuse (Califf et al., 2016) and obesity (Swinburn et al., 2011; W.H.O., 2018) accentuate the need to better understand the association between opiates, obesity, and nociception. Given that leptin is produced by adipocytes, differentiating the extent to which nociception is modulated by leptin per se versus obesity is not trivial (Watson et al., 2014). In an effort to evaluate the contributions of obesity and/or leptin to buprenorphine-induced antinociception, the present study used mice that share the obesity phenotype but differ in leptin signaling. The discussion focuses on the novel finding that obese mice with dysfunctional leptin receptors had the longest latency for antinociception under control conditions and a

diminished antinociceptive response to buprenorphine. Differences in nociception identified in the present study also are relevant for mouse models of prevalent human disorders such as diabetes (O'Brien et al., 2014) and alterations in morphine pharmacokinetics associated with obesity (Dalesio et al., 2016).

Sex-dependent differences in TFL were not observed in any of the four mouse lines (Table 2.1). The absence of a sexually dimorphic effect of buprenorphine on TFL can be contrasted with results from 12 strains of rats in which opiates had a more potent antinociceptive effect in males than females (Terner et al., 2003). In addition to species differences, comparison of the present and previous (Terner et al., 2003) studies reveals significant differences in methodology. In the rat study TFL was assessed 15 min after opiate administration, whereas in the present study TFL was measured 2 h after buprenorphine administration. We reasoned that testing 2 h after injection would provide a more conservative test of our hypothesis and be consistent with previous data indicating long-duration antinociceptive effects of buprenorphine administered to mice (Gades et al., 2000).

DIO, ob/ob, and db/db mice share the obese phenotype, but only mice with dysfunctional leptin receptors (db/db) had a diminished antinociceptive response to buprenorphine (Fig. 2.1). In normal weight B6 mice that produce leptin, buprenorphine increased TFL by about 56%. In obese db/db mice, buprenorphine increased TFL only by 23%. The mechanisms causing the diminished antinociceptive effects of buprenorphine in db/db mice are unknown. Many studies have used db/db mice as a model of type-2 diabetes (Sullivan et al., 2007). Previous data, however, have shown

that when db/db mice are fed a normal diet, they become euglycemic and do not develop peripheral neuropathy (Sullivan et al., 2007). In the present study only DIO mice received a high fat diet.

Effect size statistics revealed that the well-documented antinociceptive actions of buprenorphine were greatest in lean B6 and obese DIO mice that produce leptin. Although the mechanisms are unknown, these results are consistent with recent evidence that obesity decreases the metabolism of morphine and that leptin normalizes morphine metabolism (Dalesio et al., 2016). The extent to which buprenorphine metabolism is or is not altered in the congenic lines used in the present study is not known.

As noted previously (Watson et al., 2014) the relationship between leptin and obesity adds to the complexity of determining the degree to which leptin status and/or obesity alter nociception. The Fig. 2.1 findings prompted analyses quantifying the percent of variance in TFL that could be attributed to body weight (Fig. 2.2). For no treatment condition and for no congenic mouse line did body weight account for a significant amount of variance in TFL (Fig. 2.2). Considered together, the results shown in Fig. 2.1, Fig. 2.2 support the novel conclusion that differences in leptin signaling among the four lines of mice had a greater impact on buprenorphine modulation of TFL than did body weight.

TFL was altered to a greater extent by leptin status (Fig. 2.1, ob/ob vs db/db) than body weight (Fig. 2.2) and this is noteworthy relative to mechanistic data. Leptin inhibits the excitability of neurons in the peribrachial pons (Beck et al., 2013). Neurons

in the adjacent and overlapping parabrachial pons contribute to ascending and descending nociceptive signaling (Gauriau & Bernard, 2002). Coupled with evidence that inflammatory cytokines promote hyperalgesia (Watkins et al., 1995), these foregoing studies of pontine neurons raise the question of whether the hypoalgesia displayed by db/db mice (Fig. 2.1, Fig. 2.2) is related to dysfunctional leptin signaling in peri- and parabrachial neurons. This possibility is open to future investigation and the present results encourage such studies.

In summary, the present use of four congenic mouse lines permits novel inferences regarding the relative influence of sex, leptin status, and body weight on the antinociceptive effects of buprenorphine. The results support the conclusion that leptin signaling among the four lines of mice had a greater impact on buprenorphine modulation of TFL than did body weight. The results should not be misinterpreted, however, as inferring that only leptin modulates TFL. Adipose tissue functions as an endocrine organ secreting more than 600 different adipokines (Kunath & Klöting, 2016). Thus, leptin signaling is likely only one of many adipocyte-derived molecules that influence nociception. Administering leptin protein to leptin-deficient ob/ob mice (Campfield et al., 1995) or neuron-specific LEPR-B transgenes to db/db mice (de Luca et al., 2005) has long been known to reverse the obesity phenotype and associated dysfunctions. Serum leptin levels are routinely measured during studies designed to test the hypothesis that administering exogenous leptin to leptin-deficient mice modulates breathing (O'Donnell C et al., 1999; Tankersley et al., 1998) nociception (Wang et al., 2009; Watson et al., 2014) or opiate pharmacokinetics (Dalesio et al.,

2016). Measuring leptin in the present study was not appropriate, and to do so would have comprised duplicative research for two reasons. First, the experiments were not designed to evaluate whether exogenous leptin administration could rescue nociception. Second, there was good agreement between our assessment of higher-level phenotypes, such as body weight, food consumption, and blood glucose levels, and The Jackson Laboratory's allelic assays confirming the identity of wild type and mutant mice. The present success in differentiating the effects of leptin status versus body weight on thermal nociception is an essential first step encouraging future studies of nociception in relation to functional differences in white, beige, and brown adipose tissue (Wang & Seale, 2016).

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Appendix

Table 2.1 TFL did not differ significantly between males and females.

Values (s) are based on 12 male and 12 female mice per congenic mouse line. B6 = C57BL/6J (wild-type mice); DIO = B6 mice with Diet-Induced Obesity (mice are obese); ob/ob = B6.Cg-Lep^{ob}/J (mice are obese and leptin deficient); db/db = B6.BKS(D)-Lepr^{db}/J (mice are obese and lack normal leptin receptors).

Treatment	Sex	B6	DIO	ob/ob	db/db
Saline	Males	3.11 ± 0.67	2.94 ± 0.59	3.27 ± 0.51	5.04 ± 0.58
	Females	3.40 ± 0.85	3.23 ± 0.39	2.99 ± 0.62	4.85 ± 1.16
Buprenorphine	Males	5.09 ± 1.33	4.75 ± 0.90	4.48 ± 0.71	5.83 ± 1.73
	Females	5.05 ± 0.72	4.32 ± 0.44	4.52 ± 1.22	6.33 ± 1.97

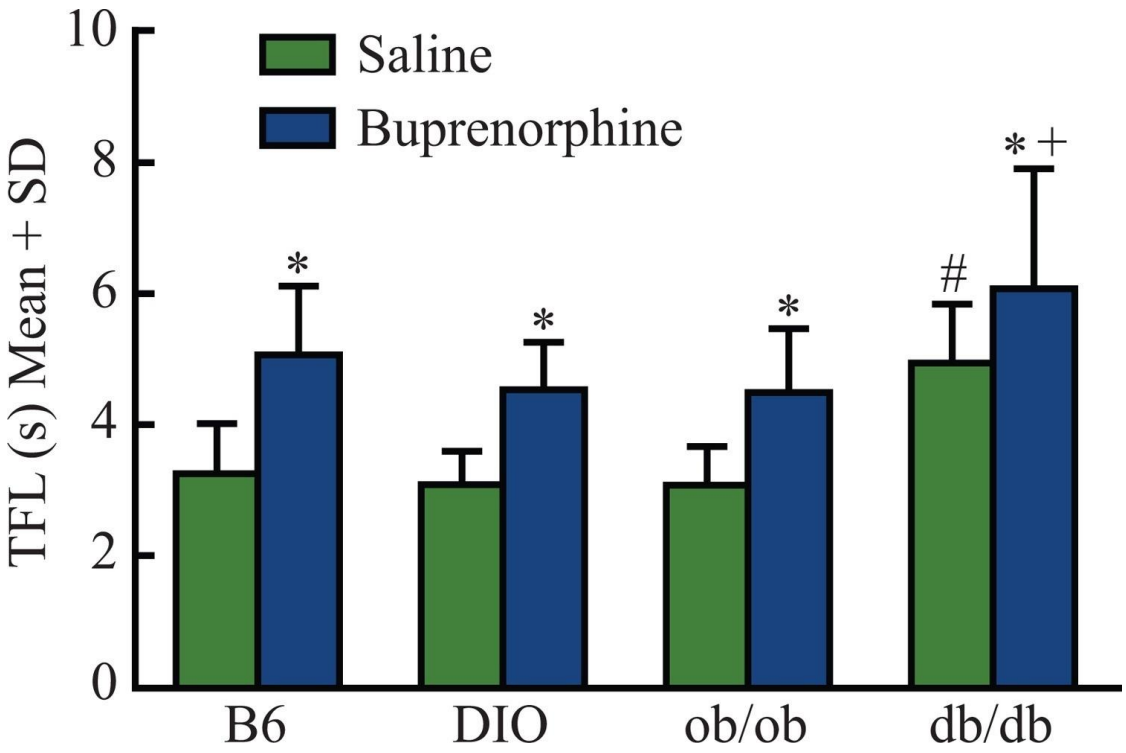
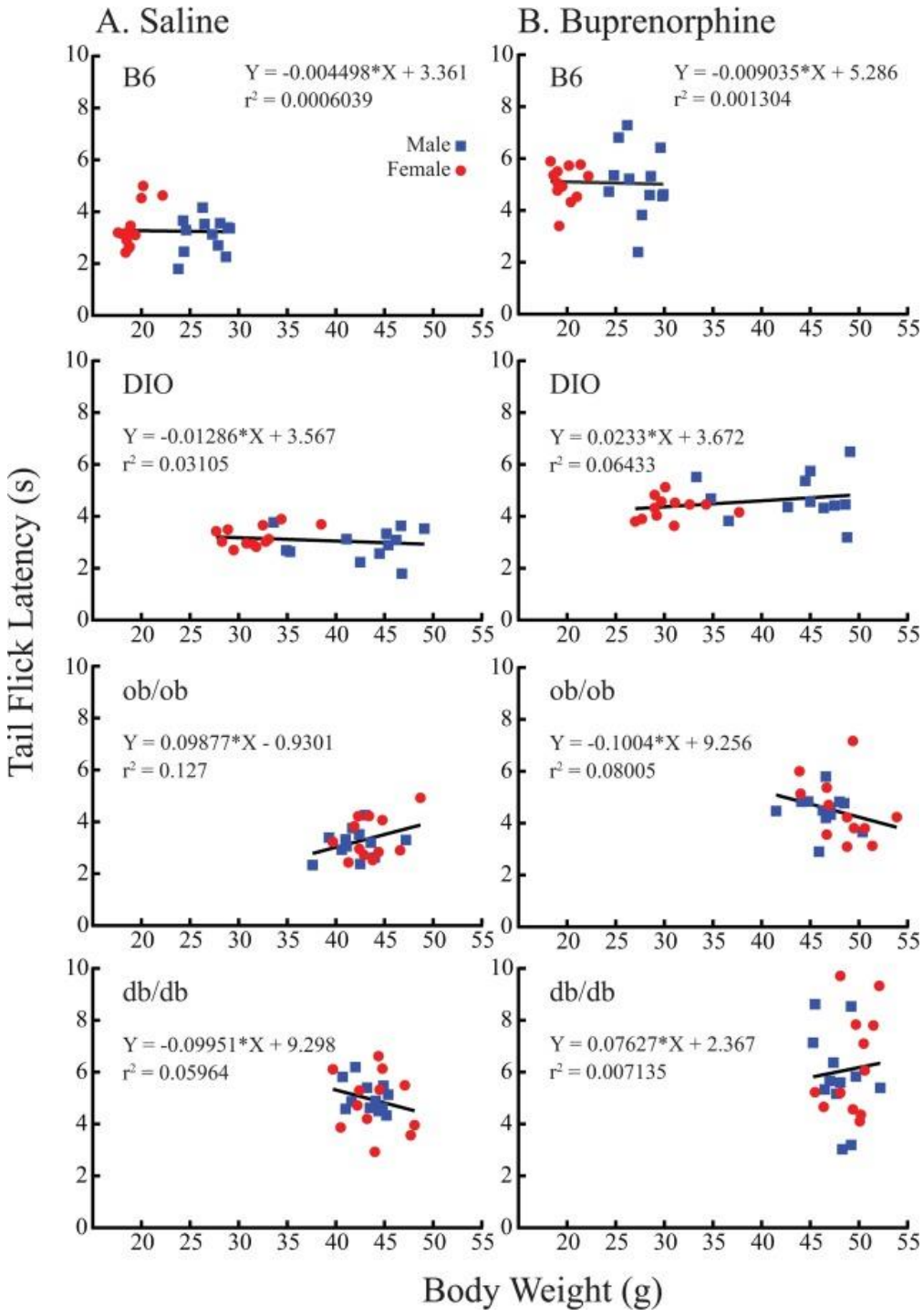


Figure 2.1 Buprenorphine significantly increased TFL.

Mean and standard deviation (SD) tail flick latency (TFL) in seconds among four congenic lines of mice. In all four lines of mice buprenorphine significantly (*) increased TFL. Mice (db/db) with dysfunctional leptin receptors displayed a significantly longer TFL than the other three congenic lines after saline (#) and after buprenorphine (+) administration.

Figure 2.2 Body weight did not account for a significant amount of variance in TFL.

Tail flick latency in seconds plotted as a function of body weight for each congenic line of mice after administering saline (A) or buprenorphine (B). Each frame provides the solution to $y = mx + b$ and the amount of variance in TFL accounted for by body weight (r^2). The slopes of each of the eight functions did not differ from zero.


A. Saline
B. Buprenorphine
B6
Y = -0.004498*X + 3.361
r² = 0.0006039
Male
Female
B6
Y = -0.009035*X + 5.286
r² = 0.001304
DIO
Y = -0.01286*X + 3.567
r² = 0.03105
DIO
Y = 0.0233*X + 3.672
r² = 0.06433
ob/ob
Y = 0.09877*X - 0.9301
r² = 0.127
ob/ob
Y = -0.1004*X + 9.256
r² = 0.08005
db/db
Y = -0.09951*X + 9.298
r² = 0.05964
db/db
Y = 0.07627*X + 2.367
r² = 0.007135
Tail Flick Latency (s)
Body Weight (g)

Chapter 3 Buprenorphine Depresses Respiratory Variability in Obese Mice with Altered Leptin Signaling

This chapter is adapted for dissertation formatting from the following publication:

Angel C, **Glovak ZT**, Alami WH, Mihalko S, Price J, Jiang Y, Baghdoyan HA, Lydic R. Buprenorphine depresses respiratory variability in obese mice with altered leptin signaling. Anesthesiology 128: 984-991, 2018. https://doi.org/10.1097/ALN.0000000000002073.

I collected, analyzed, and interpreted the data presented in the following chapter, as well as contributed to the writing of the original manuscript from which the following chapter is adapted.

Abstract

Opiate-induced respiratory depression is sexually dimorphic and associated with increased risk among the obese. The mechanisms underlying these associations are unknown. The present study evaluated the two-tailed hypothesis that sex, leptin status, and obesity modulate buprenorphine-induced changes in breathing. Mice (n = 40 male and 40 female) comprising four congenic lines that differ in leptin signaling and body weight were injected with saline and buprenorphine (0.3 mg/kg). Whole-body plethysmography was used to quantify the effects on minute ventilation. The data were evaluated using three-way analysis of variance, regression, and Poincaré analyses. Relative to B6 mice with normal leptin, buprenorphine decreased minute ventilation in mice with diet-induced obesity (37.2%; $P < 0.0001$), ob/ob mice that lack leptin (62.6%; $P < 0.0001$), and db/db mice with dysfunctional leptin receptors (65.9%; $P < 0.0001$).

Poincaré analyses showed that buprenorphine caused a significant ($P < 0.0001$) collapse in minute ventilation variability that was greatest in mice with leptin dysfunction. There was no significant effect of sex or body weight on minute ventilation. The results support the interpretation that leptin status but not body weight or sex contributed to the buprenorphine-induced decrease in minute ventilation. Poincaré plots illustrate that the buprenorphine-induced decrease in minute ventilation variability was greatest in mice with impaired leptin signaling. This is relevant because normal respiratory variability is essential for martialing a compensatory response to ventilatory challenges imposed by disease, obesity, and surgical stress.

Introduction

This study was motivated by two imperatives. First, the ongoing epidemics of opiate abuse (Volkow & Collins, 2017) and obesity (Swinburn et al., 2011) reinforce our interest in opiate research with potential for translational relevance (Cronin et al., 1995; Gauthier et al., 2011; Glovak et al., 2017; Hambrecht et al., 2007; Lydic R, 2007; Lydic et al., 2017; Nelson et al., 2009; Osman et al., 2005; Wang et al., 2009; Watson et al., 2014). Second, opiate-induced alterations in the ventilatory response to hypercapnia are sexually dimorphic (Dahan et al., 1998), consistent with evidence that respiratory control is altered by sex steroid hormones (Behan et al., 2003). Additional data show that opiate-induced respiratory depression is associated with increased risk among subjects who are female (Sarton et al., 1999) and/or obese (Domi & Laho, 2012). There is consensus that gaps in knowledge regarding mechanisms by which opiates alter breathing “warrant further study of the sex-dependent and sex-independent genetic

factors” and that “inbred strains of mice and rats may serve as the best model” (Dahan et al., 1998). We concur, and the present study quantified the effects of buprenorphine on minute ventilation variability in obese and normal weight male and female mice. The obese mice used for this study were characterized by either disrupted leptin signaling or diet-induced obesity. This design made it possible to use a three-way, mixed effects analysis of variance model to evaluate the two-tailed hypothesis that buprenorphine causes breathing to vary significantly due to sex, leptin status, and obesity. Buprenorphine was a logical choice because it is used for pain management in addicted patients (Bryson, 2014) and because it is one of three medications approved for treating opiate use disorder (Schuckit, 2016; Volkow & Collins, 2017).

Materials and Methods

Animals

Experiments and procedures were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (Knoxville, Tennessee). All phases of the experiments adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 8th edition, Washington, D.C., 2011). Mice had *ad libitum* access to food and water and were housed under 24-h illumination to promote free-running rhythms and offset potential circadian confounds (Watson et al., 2014). Adult mice (n = 80) were purchased from The Jackson Laboratory (USA). These congenic mice (10 males and 10 females of each line) included normal weight C57BL/6J (B6) wild-type mice that produce leptin; B6 mice with diet-induced obesity (DIO) that may lead to leptin resistance; obese B6.Cg-Lep^{ob}/J mice (ob/ob) that do not

make leptin; and B6.BKS(D)-Lepr^{db}/J (db/db) mice that are obese, produce leptin, and lack functional leptin receptors. None of these mice are transgenic animals, and the ob/ob and db/db mice were derived from spontaneous mutations in the B6 line. These congenic lines are maintained at The Jackson Laboratory by a multigenerational breeding program that backcrosses a mutation of interest into different mice, thereby transferring the mutations of interest. The B6, ob/ob, and db/db mice were fed normal Teklad 8640 rodent chow comprised of 5% fat. The DIO mice were fed a 60% fat diet for 20 weeks before and during the testing period. Both rodent chows were purchased from Research Diets, Inc. (USA).

Congenic mice in relation to experimental design

The four congenic lines of mice (B6, DIO, ob/ob, and db/db) are ideally suited to calls for studies “of the sex-dependent and sex-independent genetic factors” (Dahan et al., 1998) contributing to opiate-induced changes in breathing. Efforts to understand the sexually dimorphic response to opiates (Sarton et al., 2000; Sarton et al., 1999) are scientifically sound (Klein et al., 2015) and consistent with the 2016 policy mandating that National Institutes of Health–funded studies include sex as a biologic variable (Shansky & Woolley, 2016).

Among the four mouse lines, the obesity and leptin phenotypes were regarded as sex-independent factors that may contribute to buprenorphine-induced alterations in breathing. This possibility is suggested by the fact that leptin, produced by adipocytes, covaries with obesity and modulates breathing (Malli et al., 2010; O'Donnell C et al.,

1999; Tankersley et al., 1996; Yao et al., 2016) and nociception (Glovak et al., 2017; Wang et al., 2009; Watson et al., 2014). Table 3.1 summarizes similarities and differences between the obesity and leptin phenotypes in the four congenic mouse lines studied. Use of three-way analysis of variance made it possible to evaluate the effects of buprenorphine on breathing as a function of sex, leptin status, and obesity.

Buprenorphine administration and measures of breathing

Mice were injected (intraperitoneally) with saline or buprenorphine (0.3 mg/kg) (Sigma–Aldrich, USA). Buprenorphine was dissolved in saline to yield the desired concentration while holding injection volume (0.3 ml) constant. The 0.3-mg/kg dose of buprenorphine has been shown to be antinociceptive in B6 mice (Lutfy et al., 2003) and in males and females of the four congenic mouse lines used in this study (Glovak et al., 2017). Before starting data collection, the mice were habituated to being placed in whole body plethysmography chambers (Data Sciences International, USA), where they were free to move and groom. After 1 week of habituation, the mice were randomly assigned to receive either saline or buprenorphine injection and were placed in the plethysmography chambers where breathing was automatically recorded for 1 h. Testing occurred at the same time of day, and all mice had 1 week for recovery between injections. Breathing signals from mice in the plethysmography chambers were automatically digitized by the software, circumventing the need to blind experimenters to injection condition (saline vs. buprenorphine). Pioneering studies of rodent breathing across genetic lines (Strohl et al., 1997) demonstrated that measures

must be scaled to body mass, differences in CO2 production, hypoxic and hypercapnic ventilatory responses, dead space, fat distribution, and many other factors. The present measures of the effects of buprenorphine on minute ventilation across four congenic lines of mice were therefore expressed as ml/min/g body weight, consistent with our previous studies on the respiratory effects of isoflurane (Icaza et al., 2009) and sedative/hypnotics (Filbey et al., 2014).

Statistical analyses

Sample sizes (n = 80 mice consisting of 10 males and 10 females representing each of the four congenic mouse lines) were based on previous experience (Filbey et al., 2014; Glovak et al., 2017; Wang et al., 2009; Watson et al., 2014). The data were analyzed with Statistical Analysis System v9.4TS1M3 (SAS Institute, Inc., USA), using a three-way mixed-effect, repeated-measures ANOVA model with two between-subject factors (sex and mouse line) and one within subject factor of injection condition (saline vs. buprenorphine). Mouse nested within sex by mouse line was included as a random effect. After a log transformation was applied to minute ventilation values, the data satisfied all assumptions of the ANOVA model including normal distribution of residuals, similar variances, and no major outliers. No other transformations were required. Bonferroni adjustment was used to correct the P values for all post hoc comparisons.

Poincaré as exploratory data analysis

Poincaré analysis is well established and permits novel diagnostic and prognostic insights (Khandoker et al., 2013). The heuristic value of Poincaré analysis also makes it clinically relevant for diverse disorders ranging from locomotion (Xia et al., 2016) to sleep (Brignol et al., 2013). Poincaré analysis can even help identify phenotypes of metabolic syndrome (Kubickova et al., 2016). Relative to anesthesiology, Poincaré analysis has been used as a weaning predictor for some postoperative patients (Bien et al., 2004), for estimating levels of sedation analgesia (Bolaños et al., 2016), and to characterize anesthesia-induced changes in electroencephalogram (Hayashi et al., 2015).

By plotting a dependent measure relative to the previous dependent measure, Poincaré analysis makes it possible to quantify and visualize variability within a data set (Khandoker et al., 2013). The resulting dispersion of points conveys novel information about variability in the data (Fig. 3.1). For the present study, Poincaré analysis was used to characterize how buprenorphine alters the variability of minute ventilation. Whole-body plethysmography data were averaged every 5 min for 1 h. Poincaré analysis was used to quantify respiratory variability among male and female B6, DIO, ob/ob, and db/db mice in response to injection of saline and buprenorphine. Points in the Poincaré plots of minute ventilation represent time intervals of an inspiratory and expiratory cycle. In the present study, Poincaré short-term variability (SD1) quantified the variation in breathing during the 5-min measurement intervals. Long-term variability (SD2) quantified the overall variability in breathing throughout the 1-h measurement

period. SD1 describes more rapid changes, and SD2 describes long-term changes. Short-term variability (SD1) also is described by the SD of successive differences in a breath-to-breath cycle, and long-term variability (SD2) also can be described by the SD in breath-to-breath intervals (Brennan et al., 2001). Both analytic approaches were applied to the present data set.

Results

Figure 3.2 shows that DIO, ob/ob, and db/db mice had decreased minute ventilation under the control (saline) condition. Compared to the B6 (wild-type) mice, buprenorphine decreased average minute ventilation by 37.2% in DIO mice ($t = 7.68$, $df = 72$, $P < 0.0001$), 62.6% in ob/ob mice ($t = 16.61$, $df = 72$, $P < 0.0001$), and 65.9% in db/db mice ($t = 18.21$, $df = 72$, $P < 0.0001$). There was no significant sex main effect on minute ventilation. Regression analyses revealed that in no case did body weight alone account for a significant decrease in minute ventilation.

Buprenorphine decreased variability in minute ventilation across all congenic mouse lines

Figure 3.3 shows Poincaré plots illustrating features of the buprenorphine-induced decrease in minute ventilation variability. The collapse in short-term variability (SD1), visualized in Figure 3.3 as the distribution of points perpendicular to $x = y$, is not apparent in Figure 3.2. The Poincaré plots show that compared to saline, buprenorphine caused a collapse in minute ventilation variability across all four lines of mice. Three-way ANOVA indicated that short-term variability (SD1) was significantly

decreased ($F = 45.03$; $df = 3,72$; $P < 0.0001$) as a function of mouse line and injection condition (saline or buprenorphine) ($F = 148.98$; $df = 1,72$; $P < 0.0001$). The only significant interaction was between mouse line and injection condition ($F = 5.30$; $df = 3,72$; $P = 0.0023$). Post hoc comparisons showed that buprenorphine caused a greater compression of short-term variability of minute ventilation in the DIO, ob/ob, and db/db mice than in the B6 mice. Figure 3.4 shows that when short-term variability was expressed as the SD of successive differences in breath-to-breath intervals (SD of successive differences), mice with disrupted leptin signaling had lower minute ventilation variability than B6 (wild-type) mice.

Long-term variability (SD2) of minute ventilation (Fig. 3.3, dispersion of points along the line $x = y$) differed significantly by mouse line ($F = 52.66$; $df = 3,72$; $P < 0.0001$), sex ($F = 9.05$; $df = 1,72$; $P = 0.0036$), and injection condition ($F = 184.42$; $df = 1,72$; $P < 0.0001$). There was a significant ($F = 4.02$; $df = 3,72$; $P = 0.0105$) sex-by-mouse-line interaction for long-term changes in variability of minute ventilation. Post hoc analyses showed that long-term variability in minute ventilation of normal weight B6 mice differed from that of DIO, ob/ob, and db/db mice. Applying three-way ANOVA to long-term variability data calculated as the SD of breath-to-breath intervals revealed similar results (Fig. 3.5). Considered together, the results of analyses illustrated by Figures 3.4 and 3.5 support the conclusion that buprenorphine caused the greatest decrease in minute ventilation variability in mice with impaired leptin signaling.

Discussion

Three major findings emerged from this study. First, minute ventilation was significantly altered by leptin status in the absence of buprenorphine. Second, minute ventilation did not vary significantly due to sex. Third, Poincaré plots illustrate that buprenorphine causes a collapse in minute ventilation variability. The findings are discussed below relative to the relationship between leptin and obesity, making the point that studies of congenic mice can provide unique insights into this relationship. Finally, we describe the previously unrecognized effect of buprenorphine to decrease respiratory variability. Limitations of the present study also are addressed.

Leptin, obesity, and murine models of disease

The perspective that obesity is a disease satisfies multiple criteria, the most stringent of which is the increased risk that obesity conveys for premature death (Conway & Rene, 2004; Fryhofer SA, 2017). Analysis of closed claims cases identified obesity as a risk factor for opiate-induced respiratory depression resulting in severe brain damage or death (Lee et al., 2015). Leptin covaries with obesity, making it difficult to quantify the exact degree to which breathing varies as a function of obesity, leptin, or both. The present study confronted this complexity in two ways. First, the experiments were specifically designed to compare the respiratory effects of buprenorphine in normal weight B6 mice possessing normal leptin to the respiratory effects of buprenorphine in three congenic lines of obese mice, each with different obesity/leptin profiles. The four mouse lines made it possible to relate buprenorphine-induced

changes in breathing (Figs. 3.2–3.5) to obesity and leptin status (Table 3.1). B6 mice provided an ideal control group because they are among the lines of healthy mice that the Mouse Phenome Committee recommend receive the highest priority for phenotyping (Bogue & Grubb, 2004). B6 mice with diet-induced obesity are genetically identical to normal weight B6 mice. DIO mice are homologous to the vast majority of obese humans in that the mice possess both leptin and leptin receptors that, over time, may become leptin resistant. Regression analyses provided a second approach for evaluating the degree to which buprenorphine-induced changes in breathing were altered by obesity or leptin. Those analyses showed that for none of the four mouse lines did body weight account for a significant change in minute ventilation. This finding is consistent with evidence that body weight alone does not significantly alter the antinociceptive effects of buprenorphine (Glovak et al., 2017).

Human obesity due to congenital, monogenic leptin deficiency is rare (Farooqi et al., 2002) and leptin-deficient ob/ob mice do not emulate most cases of human obesity. The ob/ob mice, however, provide a valuable animal model with the potential to clarify the multiple functions of leptin. The db/db mice produce leptin but fail to express the long form of the leptin receptor (Banks et al., 2000; Bjorbaek et al., 1997). Studies by others have used DIO, ob/ob, and db/db mice to gain insights into metabolic syndrome (Sinasac et al., 2016) and type 2 diabetes (O'Brien et al., 2014; Sullivan et al., 2007). To our knowledge, the present data provide the first evidence that leptin signaling alters the effects of buprenorphine on minute ventilation by reducing minute ventilation variability.

Poincaré analysis of minute ventilation variability

Comparing Figure 3.3 with Figures 3.2, 3.4, and 3.5 illustrates the ability of Poincaré plots to provide a nuanced visualization of respiratory variability. In the Materials and Methods section, the description of Poincaré as exploratory data analysis refers to an "attitude, a state of flexibility, a willingness to look for those things that we believe are not there, as well as those we believe to be there" (Tukey, 1977). The present application of Poincaré analysis demonstrates that buprenorphine significantly reduces minute ventilation variability. Figure 3.3 also makes clear that after saline administration, the mice with functional leptin systems (B6 and DIO (Ottaway et al., 2015)) had significantly greater variability in minute ventilation than the mice with dysfunctional leptin transmission (ob/ob and db/db). Consequently, the decrease in minute ventilation variability caused by buprenorphine was greatest for B6 and DIO mice that express both leptin and functional leptin receptors. Statistical analyses showed that leptin status played a central role via its ordinal interaction with sex and injection of buprenorphine. This finding supports the interpretation that leptin dysfunction (ob/ob and db/db), rather than obesity in the presence of leptin (DIO), conferred the greatest risk for decreased minute ventilation variability (Fig. 3.3). The present results are consistent with evidence that leptin status rather than obesity causes an impaired ventilatory response to hypercapnia in ob/ob mice (Bassi et al., 2016).

Limitations

Potential limitations of the present study include the murine model, unknown mechanisms of leptin signaling, and the mathematics underlying the present discovery. The limitations of mice as models of human obesity (Nilsson et al., 2012) and obesity-related diseases derive from evolutionary differences in gene expression between mouse and human (Perlman, 2016). While acknowledging these limitations, the National Institutes of Health consider the mouse as "the premier mammalian model system for genetic research" (https://www.genome.gov/10005834/background-on-mouse-as-a-model-organism/; accessed July 23, 2017). The genetics of human obesity reveal good homology with genotypes known to promote obesity in mice (Farooqi & O'Rahilly, 2006). In addition, the mechanisms by which leptin, a protein produced by adipocytes, influences breathing remain unknown (Bassi et al., 2016; Bassi et al., 2012; Malli et al., 2010; Pho et al., 2016; Yao et al., 2016). It is clear, however, that leptin function is not limited to energy homeostasis (Friedman & Mantzoros, 2015). Leptin modulates neuronal excitability (Sutton et al., 2016) and long-standing evidence shows that leptin stimulates breathing (O'Donnell C et al., 1999; Tankersley et al., 1996). The present results should not be misinterpreted as indicating that leptin is the only adipocyte-derived molecule modulating the buprenorphine-induced collapse in minute ventilation variability. The potential involvement of additional cell signaling proteins is suggested by the fact that adipose tissue functions as an endocrine organ and secretes hundreds of different adipokines (Kunath & Klöting, 2016). Furthermore, inferring that only leptin from adipocytes modulates the effects of buprenorphine may be too

simplistic in view of the discovery that leptin also is produced by parathyroid glands (Hoang et al., 2017). A third potential limitation is interpreting and communicating time-domain data. The present study included Poincaré methods because the analyses (Fig. 3.3) “correspond so conveniently to time domain summary statistics” (Brennan et al., 2001). The strengths and limitations of Poincaré analysis and its numeric descriptors (Figs. 3.4 and 3.5) have been reviewed in detail (Karmakar et al., 2009; Khandoker et al., 2013).

Conclusions

The results show that buprenorphine causes a collapse in minute ventilation variability, with the greatest collapse occurring in mice that lack normal leptin signaling. The results are consistent with evidence that leptin signaling alters the antinociceptive effects of buprenorphine (Glovak et al., 2017). Respiratory variability is clinically relevant because such variability is an essential aspect of homeostasis (e.g., increasing tidal volume or respiratory rate) in response to challenges imposed by disease, obesity, surgical stress, and opiates. In cases where buprenorphine does cause respiratory depression, pharmacokinetic data make the cautionary point that buprenorphine-induced respiratory depression “outlasts the effects of naloxone-induced reversals from bolus injections” (Dahan et al., 2010). Thus, the present findings encourage future studies to characterize the time course of the buprenorphine-induced decrease in respiratory variability. The novelty of the present findings is further illustrated by the fact that all previous clinical studies of the respiratory effects of buprenorphine have involved normal weight individuals. Maintaining ventilation within a normal range is key in the

perioperative setting. However, that goal is complicated by the increasing number of obese patients and by patients who chronically use opiates or who are prescribed buprenorphine to manage their opiate use disorder (Volkow & Collins, 2017). Poincaré analysis of heart rate variability has a long record of diagnostic and prognostic success. The present results encourage future human studies to determine whether Poincaré plots have diagnostic or prognostic value relevant to respiratory depression caused by opiates.

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Appendix

Table 3.1 Congenic mouse lines differ in body weight and leptin status.

			Body Weight in g (means ± SD)	
Congenic Line	Abbreviation	Description	Males	Females
C57BL/6J	B6	• Normal body weight • Produce leptin • Have leptin receptors • Wild type	24.6 ± 1.2	18.6 ± 1.2
C57BL/6J with diet-induced obesity	DIO	• Obese • Produce leptin • Have leptin receptors • Wild type fed a 60% fat diet	46.9 ± 5.4	41.4 ± 5.2
B6.Cg-Lep^{ob}/J	ob/ob	• Obese • Do not produce leptin • Have leptin receptors • Spontaneous mutation on a B6 background	46.1 ± 1.9	44.5 ± 4.10
B6.BKS(D)-Lepr^{db}/J	db/db	• Obese • Produce leptin • Have dysfunctional leptin receptors • Spontaneous mutation on a B6 background	44.7 ± 2.1	40.9 ± 5.0

Values are based on 10 male and 10 female mice per group.

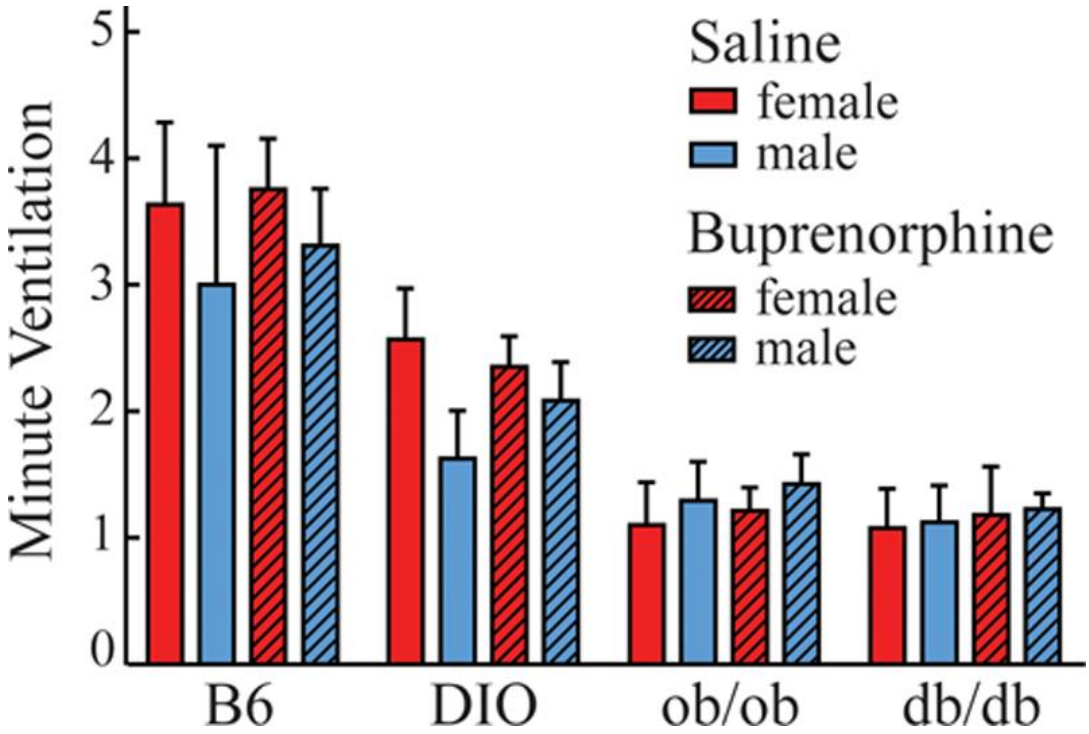
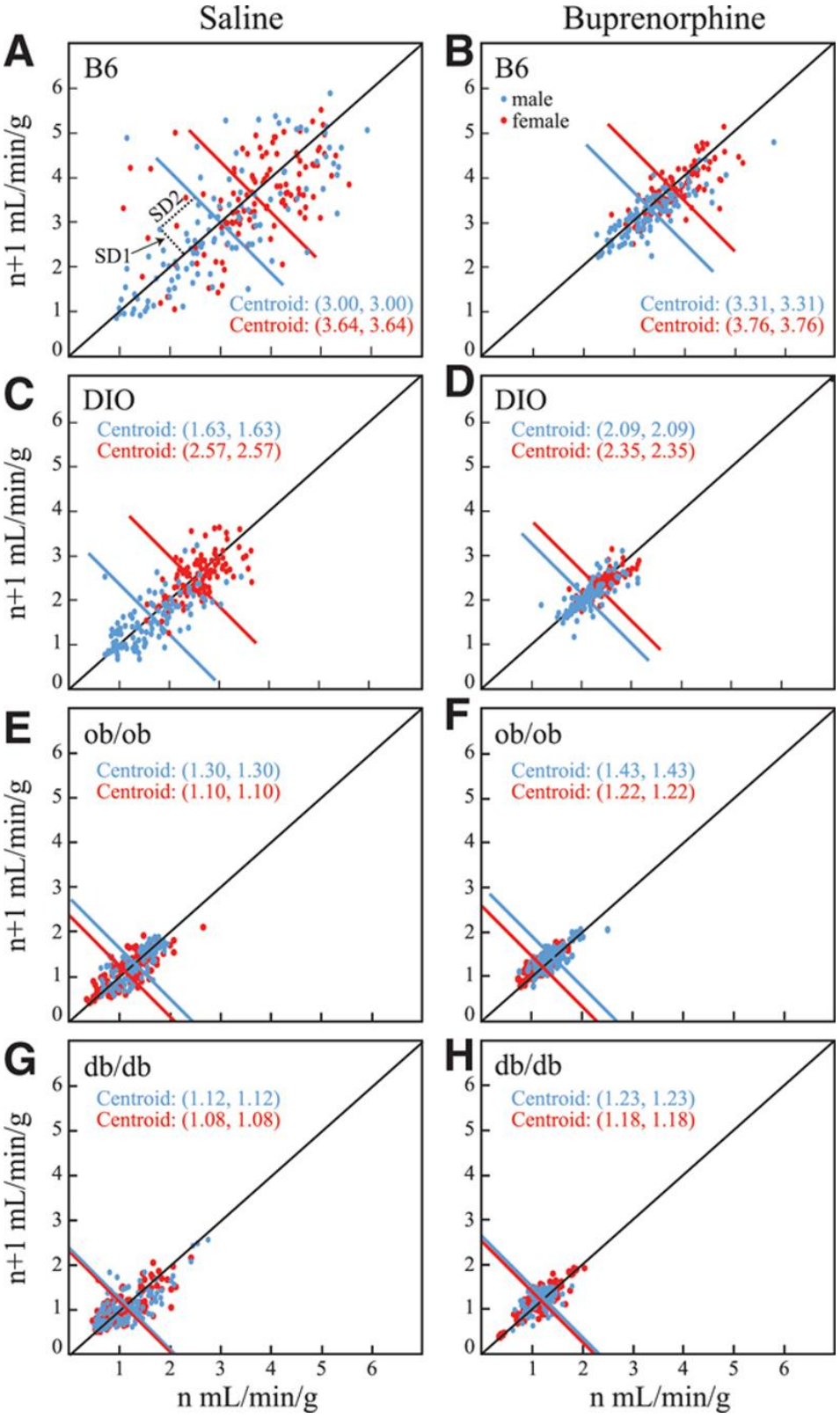


Figure 3.1 Minute ventilation varied significantly among congenic mice.

Mice with leptin dysfunction (ob/ob and db/db) had the lowest minute ventilation (means ± SD ml/min/g body weight). B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

Figure 3.2 Poincaré plots of buprenorphine-induced decrease in minute ventilation variability.

Each frame plots data from 10 male (blue dots) and 10 female (red dots) mice for 1 h after injection of saline (A, C, E, G) or an antinociceptive dose of buprenorphine (0.3 mg/kg; B, D, F, H). Also shown in black for each frame is the line of identity (x = y) and coordinates (x, y) of the average position of all data points (centroid) for males (blue type) and females (red type). The red and blue lines placed perpendicular to the line of identity mark the centroid for males and females, respectively. The dotted lines in A indicate how short-term variability (SD1) and long-term variability (SD2) are calculated for a single data point. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.


Saline
Buprenorphine
A
B6
SD2
SD1
Centroid: (3.00, 3.00)
Centroid: (3.64, 3.64)
B
B6
male
female
Centroid: (3.31, 3.31)
Centroid: (3.76, 3.76)
C
DIO
Centroid: (1.63, 1.63)
Centroid: (2.57, 2.57)
D
DIO
Centroid: (2.09, 2.09)
Centroid: (2.35, 2.35)
E
ob/ob
Centroid: (1.30, 1.30)
Centroid: (1.10, 1.10)
F
ob/ob
Centroid: (1.43, 1.43)
Centroid: (1.22, 1.22)
G
db/db
Centroid: (1.12, 1.12)
Centroid: (1.08, 1.08)
H
db/db
Centroid: (1.23, 1.23)
Centroid: (1.18, 1.18)
n+1 mL/min/g
n mL/min/g

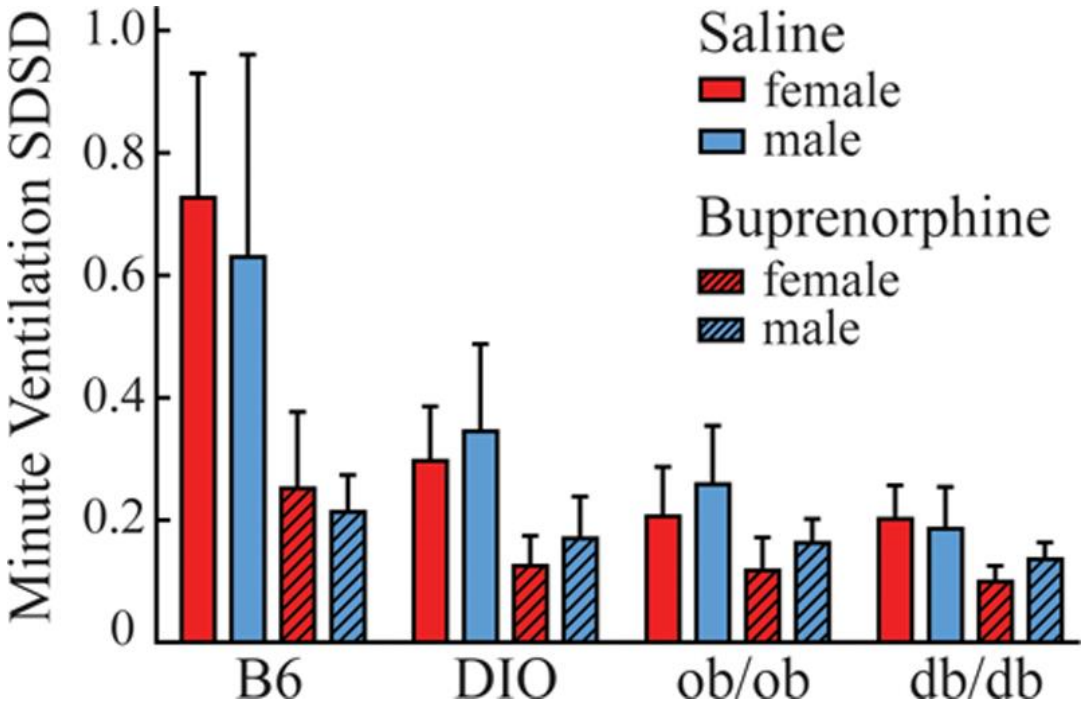


Figure 3.3 SD of successive differences in breath-to-breath intervals (SDSD) indicates short-term variability.

Histograms plot the means ± SD of SDSD for mouse line by sex and injection condition (saline vs. buprenorphine) as does SD1 in Figure 3.3. In all mouse lines, buprenorphine caused a collapse in breath-to-breath variability of minute ventilation. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

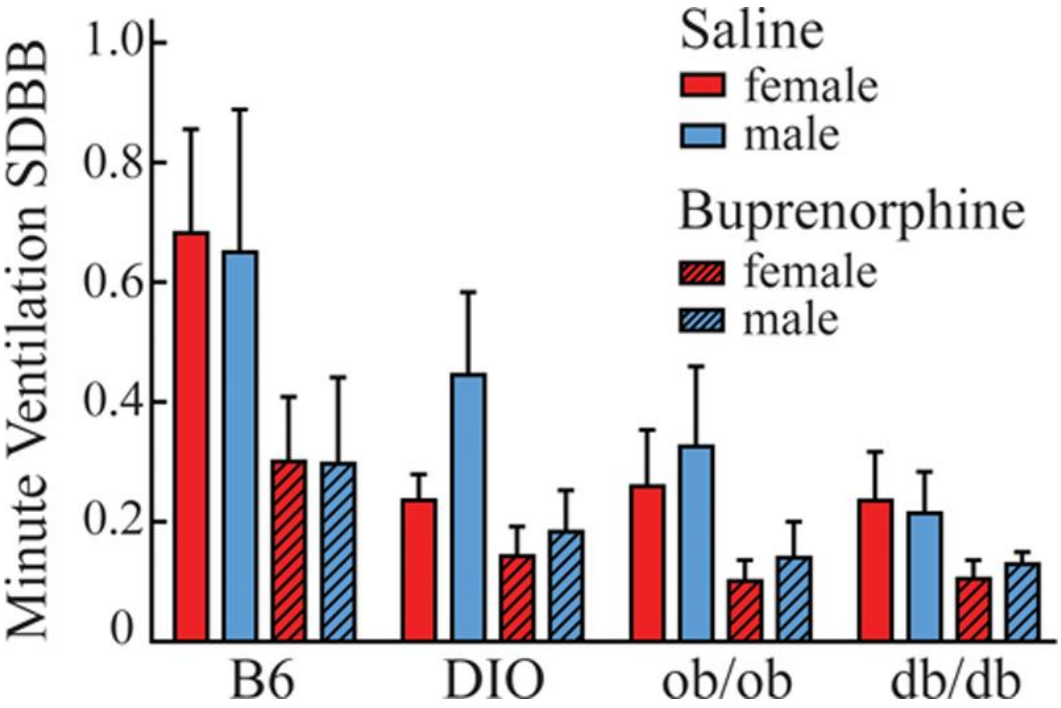


Figure 3.4 Buprenorphine decreased overall (long-term) variability of minute ventilation.

Changes in variability of the SD of breath-to-breath intervals (SDBB) are plotted as means ± SD. These histograms correspond to the variability along the line x = y in fig. 3. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

Chapter 4 Buprenorphine differentially alters breathing among four congenic mouse lines as a function of dose, sex, and leptin status

This chapter is adapted for dissertation formatting from the following publication:

Glovak, Z. T., Angel, C., O'Brien, C. B., Baghdoyan, H. A., & Lydic, R. (2022). Buprenorphine differentially alters breathing among four congenic mouse lines as a function of dose, sex, and leptin status. Respir Physiol & Neurobiol, 103834, online ahead of print: PMID: 34954128. https://doi.org/10.1016/j.resp.2021.103834.

I collected, analyzed, and interpreted the data presented in the following chapter, as well as wrote the original manuscript from which the following chapter is adapted.

Abstract

The opioid buprenorphine alters breathing and the cytokine leptin stimulates breathing. Obesity increases the risk for respiratory disorders and can lead to leptin resistance. This study tested the hypothesis that buprenorphine causes dose-dependent changes in breathing that vary as a function of obesity, leptin status, and sex. Breathing measures were acquired from four congenic mouse lines: female and male wild type C57BL/6J (B6) mice, obese db/db and ob/ob mice with leptin dysfunction, and male B6 mice with diet-induced obesity. Mice were injected intraperitoneally with saline (control) and five doses of buprenorphine (0.1, 0.3, 1.0, 3.0, 10 mg/kg). Buprenorphine caused dose-dependent decreases in respiratory frequency while increasing tidal volume, minute ventilation, and respiratory duty cycle. The effects of buprenorphine varied significantly with leptin status and sex. Buprenorphine decreased minute ventilation

variability in all mice. The present findings highlight leptin status as an important modulator of respiration and encourage future studies aiming to elucidate the mechanisms through which leptin status alters breathing.

Introduction

Obese individuals have chronically increased levels of circulating leptin and become leptin resistant (Balland & Cowley, 2015). A growing body of data show that leptin stimulates breathing (Do et al., 2020; Freire et al., 2020; Pho et al., 2021; Wei et al., 2020). Stimulation of breathing by leptin is diminished in obese individuals who have become leptin resistant (O'Donnell et al., 2000). Individuals with obesity are at an increased risk for developing breathing disorders (Gilat et al., 2014), but exactly how altered leptin signaling contributes to respiratory dysfunction remains poorly understood.

In humans (Lee et al., 2015) and mice (Angel et al., 2018), obesity is associated with a greater risk for opioid-induced respiratory depression (OIRD). Buprenorphine, a partial mu opioid receptor agonist and kappa opioid receptor antagonist (Cowan et al., 1977; Lutfy et al., 2003), is one of three medications utilized to treat opioid use disorder (Campbell & Lovell, 2012; Mattick et al., 2014). Most rodent studies of buprenorphine have been conducted using normal weight, male subjects. In contrast, the present study utilized female and male mice from four congenic lines that differ in body weight and leptin status. We have previously shown that systemic administration of an antinociceptive dose of buprenorphine (Glovak et al., 2017) caused minute ventilation variability to decrease as a function of leptin status in obese mice (Angel et al., 2018). The interacting global epidemics of obesity (Stefan et al., 2021) and opioid use disorder

(Nolan et al., 2018; Volkow & Collins, 2017) encouraged the present study evaluating the hypothesis that buprenorphine causes dose-dependent changes in breathing that vary as a function of body weight, sex, and leptin status.

Materials and Methods

Animals

All procedures involving mice were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 8th edition, Washington, D.C., 2011). This study adhered to the ARRIVE guidelines (Percie du Sert et al., 2020). Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The congenic mouse lines used included: 1) C57BL/6J (B6; Stock #000664; age: 6 wk) mice (wild type control); 2) B6 mice that were fed a 60% fat diet (D12492, Research Diets, Inc., New Brunswick, NJ) to create a model of diet-induced obesity (DIO; Stock #380050; age: 20 wk); 3) obese B6.BKS(D)-Lepr^{db}/J (db/db; Stock #000697; age: 6 wk) mice that produce leptin but have dysfunctional leptin receptors, and 4) obese B6Cg-Lep^{ob}/J (ob/ob; Stock #000632; age: 6 wk) mice that do not produce leptin but do possess functional leptin receptors (Table 4.1). The db/db and ob/ob mice are not transgenic animals. Obese ob/ob mice originated from a spontaneous mutation of the leptin gene in the B6 mouse (Ingalls et al., 1950). Obese db/db mice were derived from a spontaneous mutation of the leptin receptor gene (Hummel et al., 1966). Ten female and 10 male B6, db/db, and ob/ob mice were used, as well as 12 male DIO mice for a total of n = 72. Female DIO mice do not reliably gain weight on a high fat diet and were

not used in this study. The B6, db/db, and ob/ob mice were fed Teklad 8640 (Envigo, Madison, WI) rodent chow and all mice had ad libitum access to food and water. Mice were group housed (<5 mice per cage) with same sex and congenic line littermates in a temperature (20-24±C) and humidity (25%-75%) regulated facility under constant light. Enrichment was provided via paper cones, nestlets, and igloos. These were alternated during the weekly change of the Harlan Soft Cob Enrichment Bedding (Envigo). Upon arrival from The Jackson Laboratory, the mice were given one week to acclimate to the animal facility before conditioning began.

Congenic mice in relation to experiment design

The increasing prevalence of human obesity has led to the projection that 78% of adults in the U.S. will be obese by 2030 (Wang et al., 2020). Retrospective clinical studies identifying obesity as a risk factor for OIRD highlight the need for genetic animal models that enhance understanding of OIRD (Gupta et al., 2018). Mice have a homologous gene for every human gene (O'Brien et al., 1999), and have been described as one of the most important animal models for biomedical research (Justice & Dhillon, 2016). This unique homology provides a potential opportunity to advance scientific understanding of human disease, including obesity and OIRD. Although respiratory depression elicited by opioid administration to mice is well established (Fechtner et al., 2015; Haouzi et al., 2021; Ramirez et al., 2021), no previous studies have examined the potential for buprenorphine to depress breathing in a dose-dependent manner in the B6 mouse. Thus, this study utilized four congenic lines of

mice to unmask interactions among sex, body weight, and leptin status as potential contributors to buprenorphine-induced alterations in breathing.

Whole-body plethysmography for quantification of breathing

Previous studies of breathing in B6 mice (Friedman et al., 2004) report average ± SD respiratory frequency and tidal volume to be 177.6 ± 16.2 breaths/min and 18.3 ± 2.0 µL/breath, respectively. Consequently, minute ventilation can most accurately be measured using whole-body plethysmography (WBP). In accordance with Boyle's Law, WBP utilizes a sealed chamber with a fixed volume of room air and a steady leak of a known quantity to calculate volumetric changes based on the air pressure within the chamber. These volumetric changes correspond to inspiratory and expiratory lung volume from a freely moving mouse (Criee et al., 2011). Consistent with our previous studies of opioid effects on breathing (Angel et al., 2018), the Data Sciences International (DSI, St. Paul, MN) WBP system was calibrated before each experiment, and all flow and volume data were corrected for room temperature and humidity. WBP measurements were conducted in room air.

Mice were conditioned for one week to being handled and placed in a WBP chamber. Conditioning duration was increased by 15 min per day until mice were conditioned for a maximum of 60 min per day in the chamber. Daily handling simulated intraperitoneal (IP) drug delivery. Mice were returned to their home cage following conditioning procedures.

Opioid administration

Experiments began at approximately 9:00 am. On the day of an experiment, mice were habituated to the chambers for 15 min. Then, mice were removed from the chamber and received an IP injection (0.3 mL) of saline (vehicle control) or buprenorphine. Mice were then returned to the WBP chambers and breathing was recorded for 1 h. An experimenter observed the mice during the entirety of the 1-h recordings to ensure mice stayed awake. Additionally, the DSI data acquisition software prunes the data in accordance with various rejection indices, which include potential artifacts introduced by movement, sniffing, grooming, etc.

These studies used a crossover design in which mice underwent control and treatment conditions to reduce the number of animals needed for the study (Piantadosi, 2017). Each mouse received one IP injection of saline and 5 increasing doses of buprenorphine: 0.1, 0.3, 1.0, 3.0, 10 mg/kg. Poincaré analyses of the 0.3 mg/kg buprenorphine dose have been reported (Angel et al., 2018). Mice were given one week to recover between injections. To minimize the likelihood of tolerance, buprenorphine was administered in order of lowest to highest dose.

Poincaré analysis of minute ventilation

Poincaré analysis is a visual and quantitative tool for assessing variability within a time series dataset (Angel et al., 2018; Khandoker et al., 2013). This tool generates a “return plot” by plotting each value as a function of its chronologically preceding value (Khandoker et al., 2013). The present minute ventilation measures were averaged

every 60 s over the course of the entire 1-h recording. The results of these analyses were expressed numerically as short-term, or minute-to-minute, variability (SD1), and long-term breathing variability over the course of the 1-h recording (SD2). Poincaré analysis was utilized to evaluate minute ventilation variability in response to increasing doses of buprenorphine. Poincaré analysis was performed using custom MATLAB (R2020b, MathWorks, Natick, MA) scripts.

Statistical analysis of drug effects

Measures of respiratory frequency (breaths/min; fR), tidal volume (mL/breath/g body weight; VT), minute ventilation (mL/min/g body weight; $\dot{V}E$), and duty cycle (ratio of inspiratory time to total inspiratory plus expiratory cycle time; Ti/Ttot) were digitized and expressed numerically for statistical analyses. Tidal volume and minute ventilation were normalized for body weight. Although opioids can cause dose-dependent increases in apneas (Fechtner et al., 2015; Yamauchi et al., 2008), opioid-induced apneas were not observed and were not quantified in the present study. The lack of apneas may be due to the ceiling effect of buprenorphine on respiratory depression (Dahan, 2006). Measures of breathing were averaged in 60-s intervals over the course of the 1-h recording. No blinding was necessary because WBP measures of breathing are not subject to measurement bias originating from the experimenter. Data were analyzed using two or three-way, mixed model repeated measures ANOVAs to quantify the relationship between sex and mouse line with respect to buprenorphine dose. A Geisser-Greenhouse correction was applied when data violated the sphericity

assumption. A log transformation was applied to the data to satisfy distribution assumptions of the model. No data were excluded by the investigators. Data that were missing at random (< 2% of total dataset) were replaced with mean centered values from the same group (mouse line, sex, and dose). Post hoc comparisons were made via Dunnett's or Bonferroni tests. All p values were adjusted for multiple comparisons and cutoff for significance was set at $p < 0.05$. All analyses were conducted using Prism 9.0.0 (GraphPad; San Diego, CA) or Statistical Product and Service Solutions (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY).

The present respiratory measures were not adjusted for estrous cycle stage. The estrous cycle in the B6 mouse lasts approximately 4-5 days (Byers et al., 2012), and the cycles of group housed females become more irregular (https://www.jax.org/news-and-insights/jax-blog/2014/september/six-steps-for-setting-up-timed-pregnant-mice). Taken together, it is likely that the cycles of females within and between cages were randomly distributed, thus spreading any potential variance due to estrous cycle across the entire sample. Furthermore, there is no consensus on whether estrous cycle has a significant effect on breathing in the B6 mouse (Gargaglioni et al., 2019).

Results

The effects of sex, mouse line, and buprenorphine dose on measures of respiratory frequency, tidal volume, minute ventilation, and respiratory duty cycle were analyzed using a three-way repeated measures mixed ANOVA (Table 4.2). For each of the four dependent measures of breathing, ANOVA revealed a significant main effect of

buprenorphine dose and mouse line, as well as a significant sex main effect for respiratory frequency and duty cycle. There were no significant three-way interactions of mouse line by sex by buprenorphine dose for any of the breathing measures. Thus, statistically significant two-way interactions were evaluated using repeated measures two-way ANOVAs. All two-way ANOVA results are reported in Supplemental Tables 4.3, 4.4, and 4.5, and are illustrated by Figs. 4.1, 4.3, and 4.5, respectively.

Buprenorphine caused dose-dependent changes in breathing

Figure 4.1 plots mean ± SD frequency, tidal volume, minute ventilation, and duty cycle for female and male mice in response to administration of saline and five doses of buprenorphine. Table 4.3 summarizes the repeated measures two-way ANOVA results for the Fig. 4.1 data. In males, the mouse line by dose interaction was significant for all four dependent measures of breathing. In females, the mouse line by dose interaction was also significant, except for breathing frequency. Dunnett's post hoc test evaluated buprenorphine-induced changes in breathing compared to saline within each congenic mouse line. Measures of respiratory frequency in female B6, db/db, and ob/ob mice were not significantly altered by any dose of buprenorphine (Fig. 4.1A). For males, the 1.0, 3.0, and 10 mg/kg doses of buprenorphine significantly decreased frequency in DIO mice relative to saline (Fig. 4.1B). Buprenorphine (3.0 and 10 mg/kg) significantly decreased frequency for male ob/ob mice (Fig. 4.1B). Buprenorphine significantly increased tidal volume compared to saline for all mice (Fig. 4.1C, D). Relative to saline (0 mg/kg), buprenorphine significantly increased minute ventilation for female db/db

(0.3, 1.0, and 3.0 mg/kg) and ob/ob (0.3 mg/kg) mice (Fig. 4.1E), as well as male DIO (0.1 and 0.3 mg/kg) and db/db (1.0, 3.0, and 10 mg/kg) mice (Fig. 4.1F). All doses of buprenorphine significantly increased respiratory duty cycle for db/db and ob/ob females (Fig. 4.1G) and db/db males (Fig. 4.1H). Only the 0.1 and 0.3 mg/kg doses significantly increased duty cycle for the ob/ob males (Fig. 4.1H). The 10.0 mg/kg dose of buprenorphine significantly decreased duty cycle for DIO males (Fig. 4.1H).

Buprenorphine decreased minute ventilation variability

Figure 4.2 displays two Poincaré plots of minute ventilation recorded from B6 females (Fig. 4.2A) and B6 males (Fig. 4.2B) in response to saline (0 mg/kg) and the lowest (0.1 mg/kg) and highest (10 mg/kg) doses of buprenorphine. Minute-to-minute (short-term, SD1) variability in minute ventilation is visualized by the spread of the points perpendicular to the line of identity (defined as x = y). Long-term variability (SD2) is visualized by the parallel spread of the points along the line of identity. The semi-minor and semi-major axes of the ellipses represent group means for SD1 and SD2, respectively, for the three treatment conditions. Smaller ellipses signify a decrease in minute ventilation variability relative to control. Averages of minute ventilation for each treatment are visualized by the center of the ellipse position on the line of identity, with lower averages being represented by ellipses that are closer to the bottom left of the graph. Comparing the smaller size and the leftward and downward shifts of the green and red ellipses to the black ellipse illustrates the significant decrease in minute ventilation variability caused by buprenorphine relative to saline.

Figure 4.3 illustrates the buprenorphine-induced decrease in SD1 and SD2 metrics of minute ventilation variability. Two-way ANOVA showed no sex differences in minute ventilation variability. Therefore, data from females and males were combined and analyzed by a subsequent two-way ANOVA (Table 4.4), which demonstrated a significant dose main effect, mouse line main effect, and mouse line by dose interaction. Results from Dunnett's post hoc tests revealed that increasing doses of buprenorphine significantly decreased minute-to-minute variability (Fig. 4.3A, SD1) and long-term variability (Fig. 4.3B, SD2).

Effects of body weight vs. leptin status on breathing

Figure 4.4 plots the regression of minute ventilation versus mouse body weight for the four lines of congenic mice comparing saline (Fig. 4.4A) to the 10 mg/kg dose of buprenorphine (Fig. 4.4B). Figure 4A illustrates that after saline administration, body weight did not account for a significant amount of minute ventilation variance in any group of mice. In contrast, after buprenorphine administration body weight accounted for a significant amount of variance in minute ventilation in the ob/ob and db/db mice, but not in the DIO or B6 mice (Fig. 4.4B).

Sex differences in respiratory response to buprenorphine

The Figure 4.5 radar plots illustrate sex-dependent differences in breathing identified by repeated measures two-way ANOVAs (Supplemental Table 4.5). Bonferroni post hoc tests for B6 mice (Fig. 4.5A) and ob/ob mice (Fig. 4.5B) indicate

significant changes in breathing caused by buprenorphine. Each radius displays increasing buprenorphine doses beginning at 0 mg/kg (saline) and proceeding clockwise around the plot. Asterisks indicate a significant difference between females and males at specific buprenorphine doses. Male B6 mice had significantly lower frequency than female mice after the 3.0 and 10 mg/kg dose (Fig. 4.5A, left). Male B6 mice had significantly lower minute ventilation than female mice after 10 mg/kg buprenorphine (Fig. 4.5A, right). Only ob/ob mice exhibited sex-dependent differences in tidal volume response to buprenorphine. Male tidal volume was significantly higher than female tidal volume after the 3.0 and 10 mg/kg doses of buprenorphine (Fig. 4.5B, left). Male ob/ob mice had a significantly lower duty cycle after the 3.0 and 10 mg/kg doses of buprenorphine compared to females (Fig. 4.5B, right).

Discussion

This study reports four main findings. First, buprenorphine caused dose-dependent and differential changes in frequency, tidal volume, minute ventilation, and duty cycle among four congenic mouse lines. Second, buprenorphine significantly decreased minute ventilation variability in a dose-dependent manner in all mice. Third, leptin status, but not body weight, accounted for a significant amount of variance in minute ventilation after buprenorphine administration. Fourth, dose-dependent changes in breathing caused by buprenorphine varied significantly between female and male mice.

Buprenorphine differentially altered breathing

Buprenorphine had the greatest effect on tidal volume, as most doses caused a significant increase in tidal volume in all mouse lines when compared to saline (Fig. 4.1). The corresponding partial eta squared values indicate a large effect size for buprenorphine dose and mouse line (Table 4.2). Buprenorphine decreased respiratory frequency in the obese DIO and ob/ob mice but did not alter frequency or minute ventilation in the normal weight B6 mice. Thus, the increase in tidal volume likely reflects a compensatory response to the decreased frequency caused by buprenorphine. These changes in tidal volume are of interest relative to human data showing that increases in tidal volume are predictive of OIRD (Barbour et al., 2004). The B6 mice maintained normal minute ventilation and duty cycle after buprenorphine administration despite having an increased tidal volume. Conversely, in the db/db and ob/ob mice, buprenorphine increased minute ventilation above what was observed after saline administration. This result stands in contrast to the well-established respiratory depression caused by full mu opioid receptor agonists. Although buprenorphine is a partial mu receptor agonist and a kappa opioid receptor antagonist, the mechanism underlying this seemingly paradoxical effect remains unclear. Impaired leptin signaling in the retrotrapezoid nucleus combined with mu-opioid receptor binding by buprenorphine could have resulted in significantly blunted chemoreception and caused the observed hyperventilation (Bassi et al., 2014; Pattinson, 2008). Another possible explanation is that dysfunctional leptin receptors in the lungs (Malli et al., 2010) affected gas exchange by altering surfactant function (Carron et al., 2018; Torday et al., 2010).

Buprenorphine depressed breathing variability

Poincaré analysis has been used to examine respiratory variability and quantify OIRD (Ermer et al., 2020). Our previous study showed that the 0.3 mg/kg dose of buprenorphine significantly decreased minute ventilation variability, and that altered leptin status was the largest risk factor for buprenorphine-induced decrease in minute ventilation variability (Angel et al., 2018). The present study shows that higher doses of buprenorphine also depress minute ventilation variability in normal weight mice and mice with altered leptin status (Fig. 4.3). The finding that buprenorphine significantly decreased minute ventilation SD1 and SD2 indicates that the drug had a significant effect on the respiratory rhythm (SD1), and that this effect lasted for at least 1 h (SD2). This is physiologically relevant for two reasons. First, respiratory variability is necessary to maintain homeostasis in response to physical, emotional, or opioid-induced stressors (Angel et al., 2018; Bien et al., 2004; Van Diest et al., 2006). Second, buprenorphine is clinically relevant due to its ceiling effect for respiratory depression but not for analgesia (Dahan et al., 2006), as well as its FDA approved use as a treatment for opioid use disorder (Campbell & Lovell, 2012; Mattick et al., 2014). Despite its potential clinical relevance, little is known about how the respiratory effects of buprenorphine differ as a function of leptin status versus obesity. This study begins to address that gap in knowledge by measuring the dose-dependent effects of buprenorphine and by quantifying the interactions among buprenorphine, obesity, leptin status, and breathing.

Leptin modulates buprenorphine-induced changes in minute ventilation

Leptin is produced by adipose tissue (Morris & Rui, 2009) and is circulated to the brain where it functions to regulate energy homeostasis (Do et al., 2020) and stimulate breathing (Inyushkin et al., 2009; Wei et al., 2020). Given the interconnected processes regulating breathing, obesity, and leptin, the present study utilized DIO mice as an appropriate control for the obese, leptin deficient ob/ob and db/db mice that have dysfunctional leptin receptors. Our previous work demonstrated that for a buprenorphine dose of 0.3 mg/kg, body weight did not account for a significant amount of variance in breathing (Angel et al., 2018) or nociception (Glovak et al., 2017) among the four congenic lines of mice. The present study showed that, following 10 mg/kg buprenorphine, body weight accounted for a significant amount of variance in minute ventilation in db/db and ob/ob mice, but not in B6 or DIO mice. This result is of interest given that the db/db, ob/ob, and DIO mice exhibit the obesity phenotype, but differ in leptin status. This result supports the interpretation that leptin, and not body weight alone, modulates buprenorphine-induced changes in breathing (Angel et al., 2018; Polotsky et al., 2004). Future studies may confirm or refute this interpretation by quantifying circulating leptin levels in DIO mice.

Sex differences in breathing responses to buprenorphine

Sex differences in breathing responses to opioids are well-established in humans (Dahan et al., 1998; Dahan et al., 2005; Lee et al., 2015). The direction and magnitude of the differences as a function of sex vary across the literature (Dahan et al., 1998;

Kest et al., 2000). Data on sex-related variations in respiratory responses to buprenorphine in mice are scarce (Angel et al., 2018; Bartok & Craft, 1997), and, to our knowledge, no previous study has quantified sex differences in breathing responses in B6 mice as a function of buprenorphine dose. Data from the present study indicate that buprenorphine had a greater effect on respiration in males than in females (Fig. 4.5). Normal weight B6 males exhibited significantly lower minute ventilation than females after the highest dose of buprenorphine (Fig. 4.5A) indicating baseline differences in respiratory response to the drug. These results conflict with similar studies of Swiss mice that report greater buprenorphine respiratory potency in females than in males (Alhaddad et al., 2013). We speculate this may reflect differences between B6 and Swiss mice. The current opioid crisis, the use of buprenorphine as a treatment for opioid use disorder, and the present results encourage additional studies into sex differences in breathing responses to opioids (Unger et al., 2010).

Limitations and Conclusions

One limitation of this study is the gap in our crossover design resulting from the omission of female mice fed a high fat diet. Female B6 mice with diet-induced obesity do not express features of metabolic syndrome at the same rate as males, due to physiological traits that have been partly characterized (Pettersson et al., 2012). Obesity is a proinflammatory disorder characterized by an excessive accumulation of adipose tissue (Cullen & Ferguson, 2012). Adipose tissue functions as an endocrine organ by secreting up to 600 different adipokines (Kunath & Klöting, 2016). One of these adipokines is leptin, which alters metabolism by acting on leptin receptors in the

hypothalamus (Paz-Filho et al., 2012). Although the current results provide novel insights into the interactions among buprenorphine, leptin status, body weight, and sex, these findings do not directly address underlying mechanisms. How leptin affects the control of breathing is an active area of research (Angel et al., 2018; Bassi et al., 2016; Do et al., 2020; Freire et al., 2020; Wei et al., 2020), and we are unaware of any studies that directly measure the effect of buprenorphine on leptin receptor function.

Proper ventilation is vital for maintaining critically important functions such as cognition (Pattinson et al., 2009), metabolism (Do et al., 2020), and sleep (Douglas et al., 2005). B6 mice exhibit sleep-dependent respiratory depression (Friedman et al., 2004) and, as noted in the section entitled "*Opioid administration"*, care was taken to ensure that measures of breathing were obtained during wakefulness. Antinociceptive doses of fentanyl, morphine, and buprenorphine all disrupt sleep, and buprenorphine (0.3 mg/kg) significantly alters the temporal organization of wakefulness, NREM, and REM sleep of B6 mice (O'Brien et al., 2021).

Even with the foregoing limitations, this study is the first, to our knowledge, to quantify the effects of five buprenorphine doses on breathing in obese and leptin disrupted mouse lines. Breathing varied significantly in response to buprenorphine, with the greatest disruption occurring in the db/db and ob/ob mice that have genetically endowed leptin dysfunction (Figs. 4.1 and 4.4). Buprenorphine caused dose-dependent decreases in minute ventilation variability in all lines of mice (Figs. 4.2 and 4.3). Future studies are needed to characterize the mechanism by which leptin signaling affects buprenorphine-induced changes in breathing and how exogenous leptin attenuates

OIRD (Freire et al., 2020). Analysis of circulating leptin levels in conjunction with buprenorphine-induced changes in breathing would also provide important mechanistic insight into the role of leptin as a potential modulator of OIRD. As a crucial first step, the present study provides normative data from congenic lines of male and female B6 mice with and without normal leptin status. Finally, genetic leptin dysfunction is rare in humans (Paz-Filho et al., 2010). This fact encouraged the use of mice with diet-induced obesity as a homologous model for some aspects of human obesity. Comparison of the genetically leptin deficient mice and leptin receptor deficient mice with the DIO mice supports the conclusion that leptin modulates buprenorphine-induced changes in breathing.

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Appendix

Table 4.1 Congenic mouse lines differ in body weight and leptin status.

Adapted from (Angel et al., 2018).cc

			Body Weight in g (Mean ± SD)	
Congenic Line	Abbreviation	Description	Females	Males
C57BL/6J	B6	• Lean wild type • Produce leptin • Have functional leptin receptors	18.6 ± 1.2 N = 10	24.6 ± 1.2 N = 10
C57BL/6J with diet-induced obesity	DIO	• Obese • Produce leptin • Have functional leptin receptors	N/A	46.8 ± 4.8 N = 12
B6.BKS(D)-Lepr^{db}/J	db/db	• Obese • Produce leptin • Have dysfunctional leptin receptors	40.9 ± 5.0 N = 10	44.7 ± 2.1 N = 10
B6Cg-Lep^{ob}/J	ob/ob	• Obese • Do not produce leptin • Have functional leptin receptors	44.5 ± 4.1 N = 10	46.1 ± 1.9 N = 10

Table 4.2 Three-way ANOVA Results.

Results of three-way mixed model ANOVA with two between subject factors of mouse line and sex and one within subject factor of dose. *p < 0.05.

Source	F (DFn, DFd)	p value	Sum of squares	Partial eta squared
Respiratory Frequency				
Dose	F (3.3, 179.5) = 9.691	< 0.0005*	0.109	0.152
Sex	F (1, 54) = 7.022	= 0.011*	0.045	0.115
Mouse line	F (2, 54) = 65.547	< 0.0005*	0.843	0.708
Dose*Sex	F (3.3, 179.5) = 3.762	= 0.009*	0.042	0.065
Dose*Mouse line	F (6.6, 179.5) = 2.832	= 0.009*	0.064	0.095
Sex*Mouse line	F (2, 54) = 1.917	= 0.157	0.025	0.066
Dose*Sex*Mouse line	F (6.6, 179.5) = 0.574	= 0.768	0.013	0.021
Tidal Volume				
Dose	F (3.9, 212.4) = 143.1	< 0.0005*	1.213	0.726
Sex	F (1, 54) = 3.809	= 0.056	0.050	0.066
Mouse line	F (2, 54) = 273.662	< 0.0005*	7.145	0.910
Dose*Sex	F (3.9, 212.4) = 1.680	= 0.157	0.014	0.030
Dose*Mouse line	F (7.9, 212.4) = 18.231	< 0.0005*	0.309	0.403
Sex*Mouse line	F (2, 54) = 4.363	= 0.018*	0.114	0.139
Dose*Sex*Mouse line	F (7.9, 212.4) = 1.239	= 0.278	0.021	0.044
Minute Ventilation				
Dose	F (3.36, 181.5)=20.449	< 0.0005*	0.496	0.275
Sex	F (1, 54) = 0.076	= 0.784	0.001	0.001
Mouse line	F (2, 54) = 434.808	< 0.0005*	13.120	0.942
Dose*Sex	F (3.4, 181.5) = 0.762	= 0.530	0.018	0.014
Dose*Mouse line	F (6.7, 181.5) = 6.159	< 0.0005*	0.299	0.186
Sex*Mouse line	F (2, 54) = 7.903	= 0.001*	0.238	0.226
Dose*Sex*Mouse line	F (6.7, 181.5) = 0.761	= 0.616	0.037	0.027
Duty Cycle				
Dose	F (4.0, 216.7) = 26.351	< 0.0005*	0.82	0.328
Sex	F (1, 54) = 14.402	< 0.0005*	0.042	0.211
Mouse line	F (2, 54) = 56.506	< 0.0005*	0.327	0.677
Dose*Sex	F (4.0, 216.7) = 4.226	= 0.003*	0.013	0.073
Dose*Mouse line	F (8.0, 216.7) = 6.788	< 0.0005*	0.042	0.201
Sex*Mouse line	F (2, 54) = 0.936	= 0.399	0.005	0.034
Dose*Sex*Mouse line	F (8.0, 216.7) = 0.975	= 0.457	0.006	0.035

Table 4.3 ANOVA Results shown in Figure 4.1.

Results from repeated measures two-way ANOVAs for results shown in Fig. 4.1. *p < 0.05.

Source	Sum of squares	Mean square	F (DFn, DFd)	p value
A. Females: Respiratory Frequency				
Mouse line*Dose	0.02997	0.002997	F (10, 135) = 1.076	= 0.3848
Mouse line	0.5778	0.2889	F (2, 27) = 33.28	< 0.0001*
Dose	0.01688	0.003376	F (3.37, 90.95) =	= 0.3107
Subject	0.2344	0.008681	1.212	< 0.0001*
Residual	0.3759	0.002784	F (27, 135) = 3.118	
B. Males: Respiratory Frequency				
Mouse line*Dose	0.1156	0.007708	F (15, 190) = 5.082	< 0.0001*
Mouse line	0.3322	0.1107	F (3, 38) = 22.15	< 0.0001*
Dose	0.2683	0.05365	F (3.04, 115.5) =	< 0.0001*
Subject	0.1900	0.005000	35.37	< 0.0001*
Residual	0.2882	0.001517	F (38, 190) = 3.296	
C. Females: Tidal Volume				
Mouse line*Dose	0.1420	0.01420	F (10, 135) = 9.358	< 0.0001*
Mouse line	4.454	2.227	F (2, 27) = 109.7	< 0.0001*
Dose	0.4937	0.09874	F (3.90, 105.3) =	< 0.0001*
Subject	0.5479	0.02029	65.08	< 0.0001*
Residual	0.2048	0.001517	F (27, 135) = 13.37	
D. Males: Tidal Volume				
Mouse line*Dose	0.2166	0.01444	F (15, 190) = 9.029	< 0.0001*
Mouse line	2.872	0.9573	F (3, 38) = 132.2	< 0.0001*
Dose	0.9523	0.1905	F (3.78, 143.7) =	< 0.0001*
Subject	0.2752	0.007242	119.1	< 0.0001*
Residual	0.3039	0.001600	F (38, 190) = 4.527	
E. Females: Minute Ventilation				
Mouse line*Dose	0.1866	0.01866	F (10, 135) = 3.696	= 0.0002*
Mouse line	8.371	4.186	F (2, 27) = 211.9	< 0.0001*
Dose	0.2889	0.05778	F (3.48, 93.88) =	< 0.0001*
Subject	0.5332	0.01975	11.44	< 0.0001*
Residual	0.6816	0.005049	F (27, 135) = 3.912	

Table 4.3 continued

Source	Sum of squares	Mean square	F (DFn, DFd)	p value
F. Males: Minute Ventilation				
Mouse line*Dose	0.3105	0.02070	F (15, 190) = 5.415	< 0.0001*
Mouse line	4.988	1.663	F (3, 38) = 114.0	< 0.0001*
Dose	0.1909	0.03819	F (2.81, 106.6) =	< 0.0001*
Subject	0.5544	0.01459	9.990	< 0.0001*
Residual	0.7263	0.003823	F (38, 190) = 3.817	
G. Females: Duty Cycle				
Mouse line*Dose	0.02344	0.002344	F (10, 135) = 3.380	= 0.0006*
Mouse line	0.1468	0.07338	F (2, 27) = 19.38	< 0.0001*
Dose	0.07754	0.01551	F (3.86, 104.1) =	< 0.0001*
Subject	0.1022	0.003787	22.36	< 0.0001*
Residual	0.09361	0.0006934	F (27, 135) = 5.461	
H. Males: Duty Cycle				
Mouse line*Dose	0.03988	0.002659	F (15, 190) = 5.469	< 0.0001*
Mouse line	0.2253	0.07511	F (3, 38) = 38.08	< 0.0001*
Dose	0.009944	0.001989	F (3.53, 134.1) =	= 0.0053*
Subject	0.07496	0.001973	4.091	< 0.0001*
Residual	0.09236	0.0004861	F (38, 190) = 4.058	

Table 4.4 ANOVA Results shown in Figure 4.3.

Results from repeated measures two-way ANOVAs for results shown in Fig. 4.3. *p < 0.05.

Source	Sum of squares	Mean square	F (DFn, DFd)	p value
A. Females and Males Combined: Minute Ventilation SD1				
Mouse line*Dose	1.837	0.1225	F (15, 340) = 16.19	< 0.0001*
Mouse line	6.463	2.154	F (3, 68) = 116.0	< 0.0001*
Dose	4.738	0.948	F (2.46, 167.5) =	< 0.0001*
Subject	1.263	0.019	125.2	< 0.0001*
Residual	2.572	0.008	F (68, 340) = 2.454	
B. Females and Males Combined: Minute Ventilation SD2				
Mouse line*Dose	1.330	0.0886	F (15, 340) = 5.258	< 0.0001*
Mouse line	10.88	3.625	F (3, 68) = 124.7	< 0.0001*
Dose	7.910	1.582	F (3.54, 240.9) =	< 0.0001*
Subject	1.977	0.029	93.85	= 0.0009*
Residual	5.732	0.017	F (68, 340) = 1.725	

Table 4.5 ANOVA Results shown in Figure 4.5.

Results from repeated measures two-way ANOVAs for results shown in Fig. 4.5. *p < 0.05.

Source	Sum of squares	Mean square	F (DFn, DFd)	p value
A. B6: Respiratory Rate				
Dose*Sex	0.008581	0.001716	F (5, 90) = 0.7957	= 0.5556
Dose	0.02995	0.005991	F (2.834, 51.02)	= 0.0534
Sex	0.06293	0.06293	=2.778	= 0.0037*
Subject	0.1019	0.005660	F (1, 18) = 11.12	= 0.0014*
Residual	0.1941	0.002157	F (18, 90) = 2.624	
B. db/db: Respiratory Rate				
Dose*Sex	0.01414	0.002828	F (5, 90) = 1.146	= 0.3423
Dose	0.02044	0.004087	F (2.918, 52.53) =	= 0.1889
Sex	0.003493	0.003493	1.656	= 0.5615
Subject	0.1796	0.009979	F (1, 18) = 0.3500	< 0.0001*
Residual	0.2222	0.002469	F (18, 90) = 4.042	
C. ob/ob: Respiratory Rate				
Dose*Sex	0.03258	0.006516	F (5, 90) = 3.055	= 0.0136*
Dose	0.1226	0.02451	F (2.470, 44.45) =	< 0.0001*
Sex	0.003369	0.003369	11.49	= 0.3492
Subject	0.06564	0.003647	F (1, 18) = 0.9238	= 0.0518
Residual	0.1920	0.002133	F (18, 90) = 1.710	
D. B6: Tidal Volume				
Dose*Sex	0.01181	0.002362	F (5, 90) = 1.419	= 0.2249
Dose	0.09399	0.01880	F (2.830, 50.94) =	< 0.0001*
Sex	0.01538	0.01538	11.30	= 0.0789
Subject	0.07979	0.004433	F (1, 18) = 3.470	= 0.0012*
Residual	0.1498	0.001664	F (18, 90) = 2.664	
E. db/db: Tidal Volume				
Dose*Sex	0.02033	0.004065	F (5, 90) = 2.403	= 0.0429*
Dose	0.6288	0.1258	F (3.648, 65.67) =	< 0.0001*
Sex	0.02562	0.02562	74.34	= 0.3293
Subject	0.4587	0.02549	F (1, 18) = 1.005	= 0.0001*
Residual	0.1522	0.001692	F (18, 90) = 15.07	
F. ob/ob: Tidal Volume				
Dose*Sex	0.003112	0.0006225	F (5, 90) = 0.3596	= 0.8748
Dose	0.7995	0.1599	F (3.315, 59.67) =	<0.0001*
Sex	0.1226	0.1226	92.38	= 0.0019*
Subject	0.1664	0.009243	F (1, 18) = 13.27	<0.0001*
Residual	0.1558	0.001731	F (18, 90) = 5.340	

Table 4.5 continued

Source	Sum of squares	Mean square	F (DFn, DFd)	p value
G. B6: Minute Ventilation				
Dose*Sex	0.01340	0.002680	F (5, 90) = 0.5105	= 0.7676
Dose	0.04017	0.008034	F (2.685, 48.33) =	= 0.2216
Sex	0.1610	0.1610	1.530	= 0.0029*
Subject	0.2454	0.01363	F (1, 18) = 11.81	= 0.0016*
Residual	0.4725	0.005250	F (18, 90) = 2.597	
H. db/db: Minute Ventilation				
Dose*Sex	0.01488	0.002976	F (5, 90) = 0.5105	= 0.7676
Dose	0.4432	0.08865	F (2.685, 48.33) =	= 0.2216
Sex	0.005124	0.005124	1.530	= 0.0029*
Subject	0.3536	0.01964	F (1, 18) = 11.81	= 0.0016*
Residual	0.4545	0.005050	F (18, 90) = 2.597	
I. ob/ob: Minute Ventilation				
Dose*Sex	0.02712	0.005424	F (5, 90) = 1.275	= 0.2815
Dose	0.3113	0.06226	F (2.532, 45.57) =	< 0.0001*
Sex	0.07347	0.07347	14.64	= 0.0235*
Subject	0.2157	0.01199	F (1, 18) = 6.130	= 0.0007*
Residual	0.3828	0.004253	F (18, 90) = 2.818	
J. B6: Duty Cycle				
Dose*Sex	0.002202	0.0004404	F (5, 90) = 1.164	= 0.3333
Dose	0.006492	0.001298	F (2.661, 47.89) =	= 0.0287*
Sex	0.01709	0.01709	3.431	= 0.0127*
Subject	0.04018	0.002232	F (1, 18) = 7.654	< 0.0001*
Residual	0.03406	0.0003784	F (18, 90) = 5.899	
K. db/db: Duty Cycle				
Dose*Sex	0.005486	0.001097	F (5, 90) = 1.429	= 0.2215
Dose	0.07578	0.01516	F (3.135, 56.44) =	< 0.0001*
Sex	0.003666	0.003666	19.74	= 0.3361
Subject	0.06757	0.003754	F (1, 18) = 0.9766	< 0.0001*
Residual	0.06911	0.0007679	F (18, 90) = 4.888	
L. ob/ob: Duty Cycle				
Dose*Sex	0.01158	0.002315	F (5, 90) = 3.191	= 0.0107*
Dose	0.04228	0.008457	F (3.548, 63.86) =	< 0.0001*
Sex	0.02629	0.02629	11.66	= 0.0058*
Subject	0.04835	0.002686	F (1, 18) = 9.787	< 0.0001*
Residual	0.06529	0.0007255	F (18, 90) = 3.703	

Figure 4.1 Buprenorphine caused dose-dependent changes in breathing that varied by mouse line.

Data were analyzed using repeated measures two-way ANOVAs with post hoc Dunnett's multiple comparisons tests to assess breathing changes relative to saline (0 mg/kg) within each sex and mouse line (n = 20 for B6, db/db, and ob/ob, and n = 12 for DIO mice). Buprenorphine had no significant effect on respiratory frequency (fR) in female mice (A). Higher doses of buprenorphine decreased respiratory frequency in DIO and ob/ob mice (B). Buprenorphine caused significantly increased tidal volume (VT) relative to saline for female and male mice across all mouse lines (C, D). Female db/db mice showed significantly elevated minute ventilation ($\dot{V}E$) caused by higher buprenorphine doses (E). Lower doses of buprenorphine significantly increased minute ventilation in male DIO mice, and higher doses increased minute ventilation for male db/db mice (F). Obese female ob/ob and db/db mice displayed significantly higher duty cycle (Ti/Ttot) after buprenorphine (G). All doses of buprenorphine significantly increased duty cycle in male db/db mice, whereas only the lower doses of buprenorphine increased duty cycle in ob/ob males (H). Asterisks indicate significant buprenorphine-induced changes compared to saline within each mouse line ($p < 0.05$ adjusted for multiple comparisons).

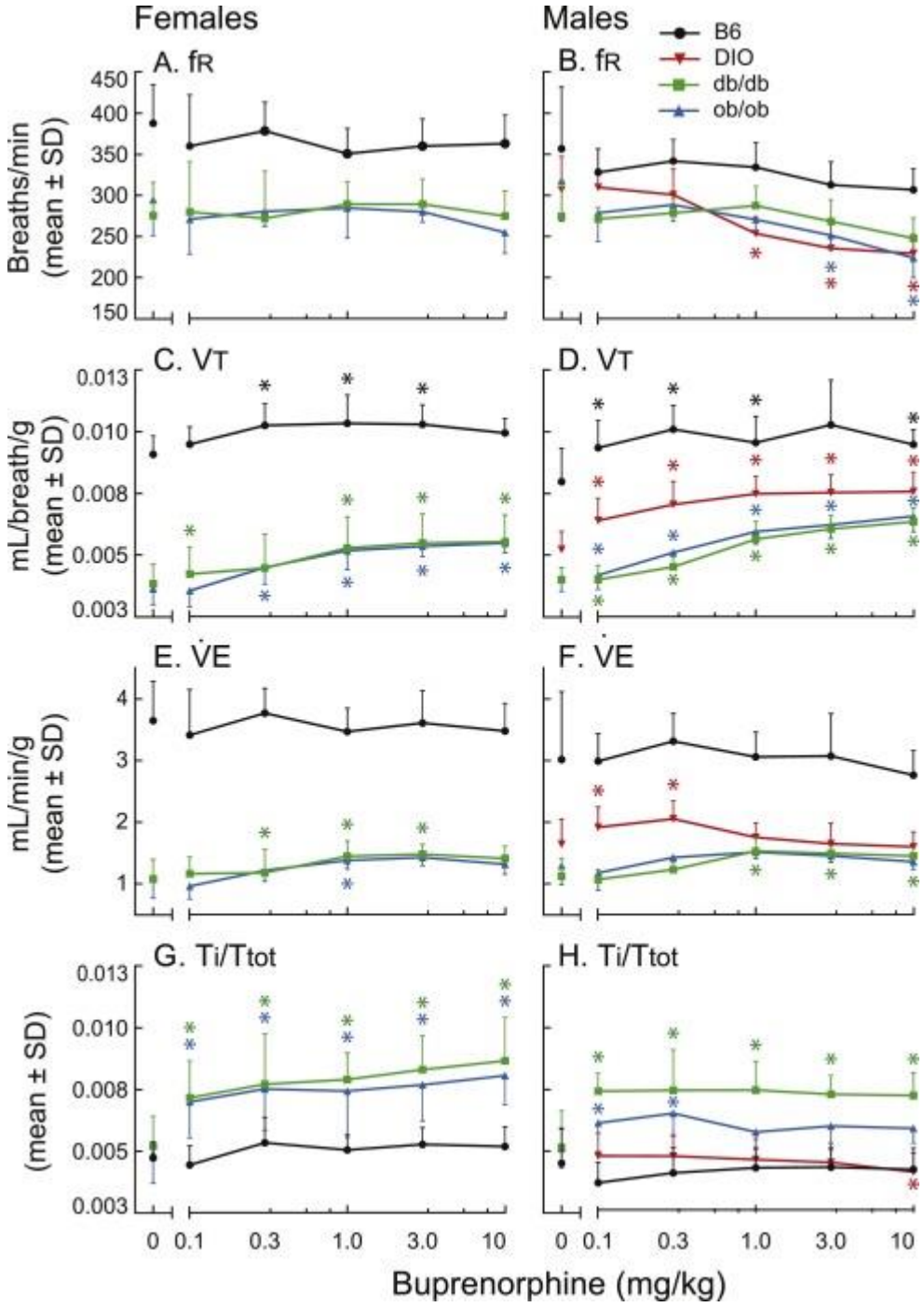

Females
Males
B6
DIO
db/db
ob/ob
A. fR
B. fR
C. VT
D. VT
E. V̇E
F. V̇E
G. Ti/Ttot
H. Ti/Ttot
Breaths/min (mean ± SD)
mL/breath/g (mean ± SD)
mL/min/g (mean ± SD)
(mean ± SD)
450
400
350
300
250
200
150
0.013
0.010
0.008
0.005
0.003
4
3
2
1
0
0.1
0.3
1.0
3.0
10
Buprenorphine (mg/kg)

Figure 4.2 Buprenorphine decreased minute ventilation variability.

Poincaré plots show minute ventilation measured during the 1-h recording following injection of saline (black dots and ellipse), 0.1 mg/kg buprenorphine (green dots and ellipse), and 10 mg/kg buprenorphine (red dots and ellipse) plotted for n = 10 B6 females (A) and n = 10 B6 males (B). The ellipses are plotted relative to the black line of identity (x = y). Short-term (minute-to-minute) variability, referred to as SD1, is visualized by the perpendicular spread of the points away from the line of identity. Long-term variability (during the 1-h recording) is referred to as SD2 and is visualized by the spread of points along the line of identity. The semi-minor axis of the ellipses corresponds to mean SD1 for that treatment condition, while the semi-major axis corresponds to mean SD2. Poincaré plots provide multiple perspectives on the collapse in minute ventilation variability caused by two doses of buprenorphine relative to saline.

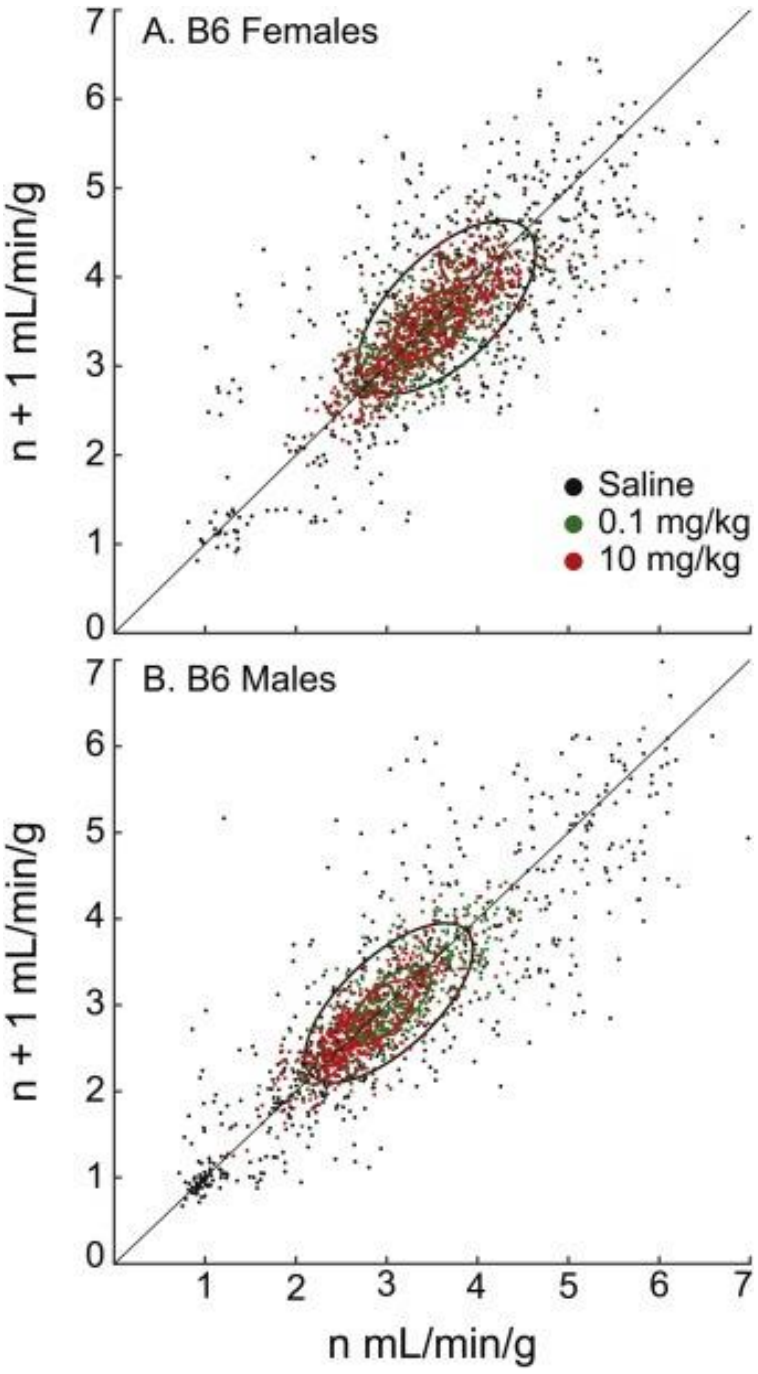

A. B6 Females
B. B6 Males
Saline
0.1 mg/kg
10 mg/kg
n + 1 mL/min/g
n mL/min/g
0
1
2
3
4
5
6
7

Figure 4.3 Buprenorphine caused dose-dependent decreases in minute ventilation variability.

Minute ventilation variability was quantified by SD1 (short-term variability, A) and SD2 (long-term variability, B). Each box plot represents the median and interquartile range of n = 20 mice (10 males and 10 females) for B6, db/db, and ob/ob congenic mouse lines and n = 12 male DIO mice. The bottom and top of each box represent the first and third quartiles, respectively. The horizontal bar within the box marks the median (second quartile). Box plot whiskers illustrate the 5th and 95th percentiles of the data. Data were analyzed via repeated measures two-way ANOVAs and Dunnett's post hoc tests. Asterisks indicate significant buprenorphine-induced changes compared to saline within each mouse line ($p < 0.05$ adjusted for multiple comparisons).

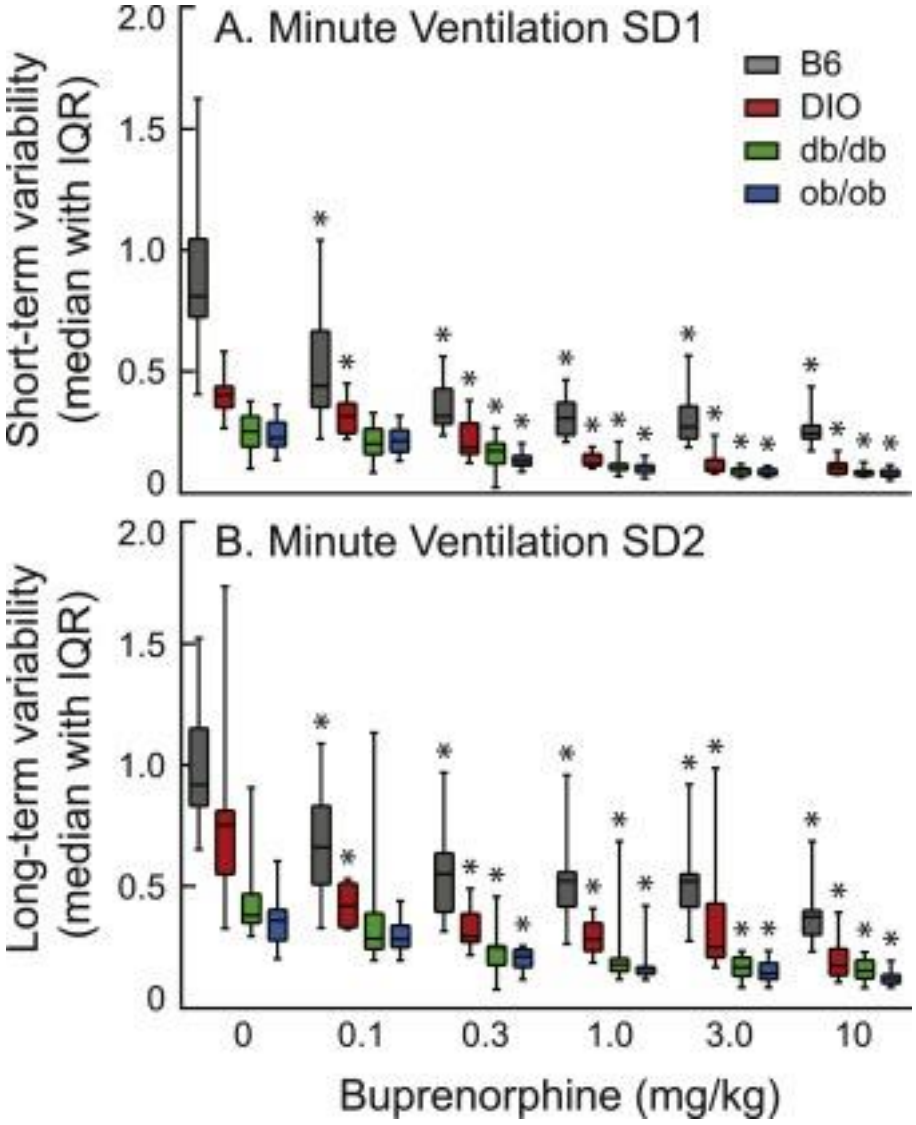

A. Minute Ventilation SD1
B6
DIO
db/db
ob/ob
Short-term variability
(median with IQR)
2.0
1.5
1.0
0.5
0
B. Minute Ventilation SD2
Long-term variability
(median with IQR)
2.0
1.5
1.0
0.5
0
0
0.1
0.3
1.0
3.0
10
Buprenorphine (mg/kg)

Figure 4.4 Minute ventilation plotted as a function of mouse body weight.

Mean minute ventilation plotted as a function of mouse body weight for 10 female and 10 male B6 ($n = 20$), db/db ($n = 20$), ob/ob ($n = 20$), and for DIO ($n = 12$, males only) mice after injections of saline (A) or 10 mg/kg buprenorphine (B). Linear regression equations for the line of best fit and goodness of fit (r^2) quantify the amount of variance in minute ventilation accounted for by body weight. Slopes of the regression lines did not differ from zero for any group of mice after saline administration. Following buprenorphine administration, body weight accounted for a significant amount of the variance in minute ventilation for the db/db and ob/ob mice ($p < 0.05$).

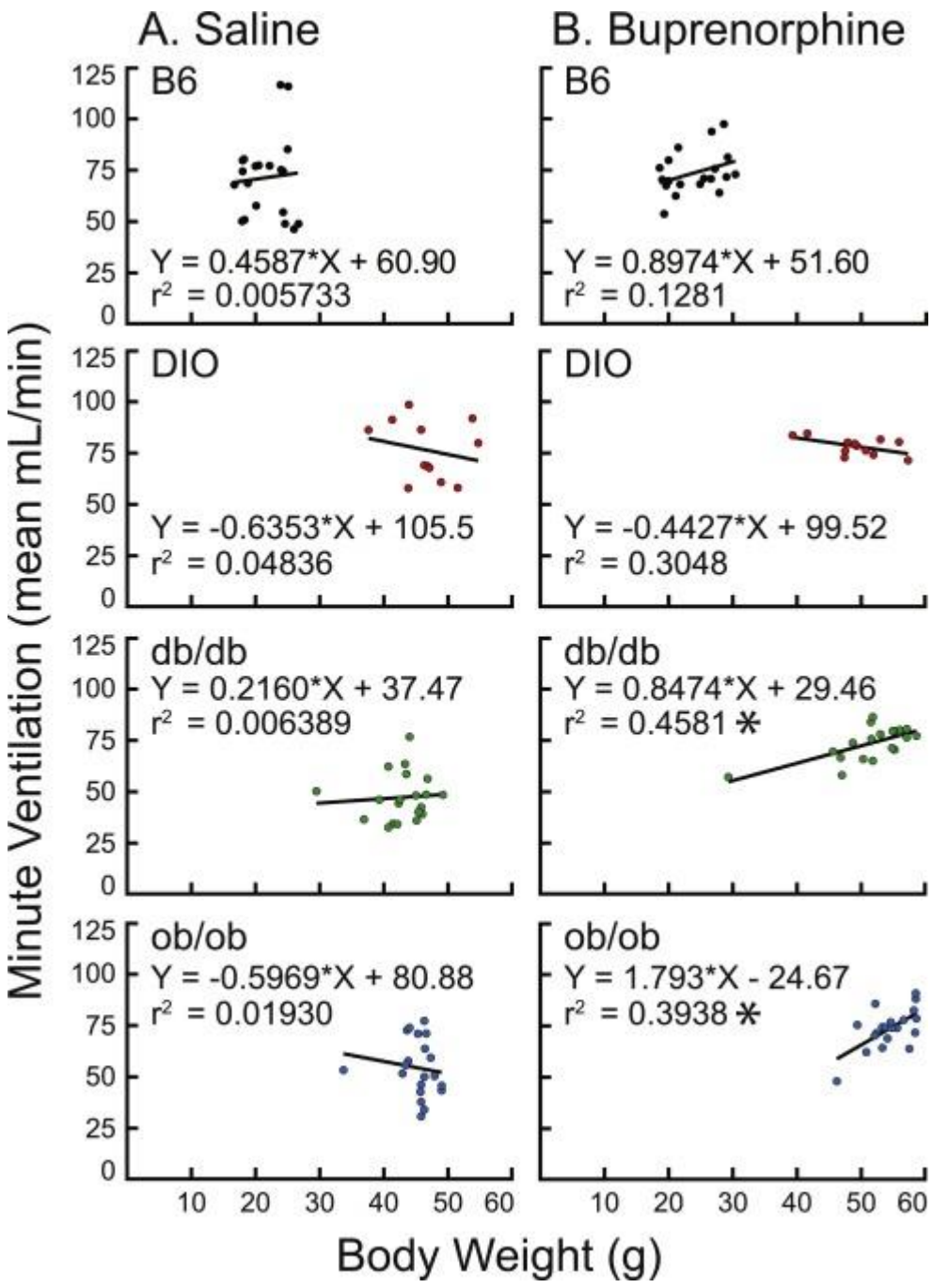
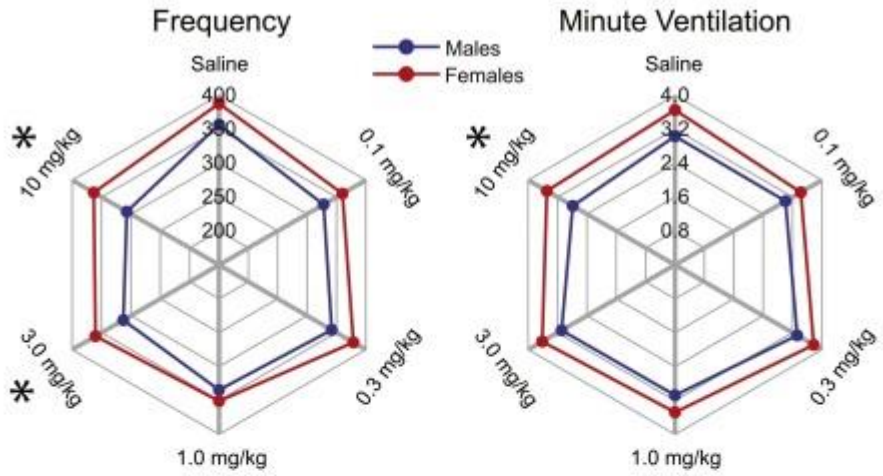

A. Saline
B. Buprenorphine
B6
Y = 0.4587*X + 60.90
r² = 0.005733
B6
Y = 0.8974*X + 51.60
r² = 0.1281
DIO
Y = -0.6353*X + 105.5
r² = 0.04836
DIO
Y = -0.4427*X + 99.52
r² = 0.3048
db/db
Y = 0.2160*X + 37.47
r² = 0.006389
db/db
Y = 0.8474*X + 29.46
r² = 0.4581 *
ob/ob
Y = -0.5969*X + 80.88
r² = 0.01930
ob/ob
Y = 1.793*X - 24.67
r² = 0.3938 *
Minute Ventilation (mean mL/min)
Body Weight (g)
0
25
50
75
100
125
10
20
30
40
50
60

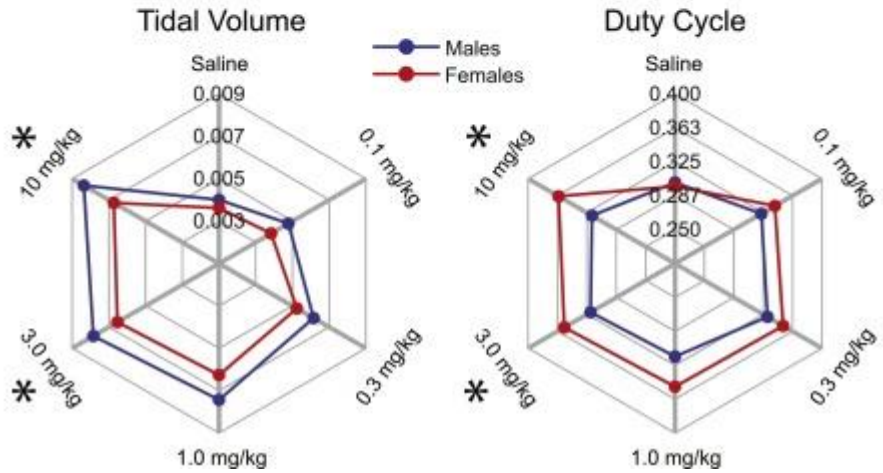
Figure 4.5 Buprenorphine caused sex-dependent changes in breathing.

Radar plots summarize sex-dependent differences in breathing for B6 (A) and ob/ob (B) mice. Data were analyzed via repeated measures two-way ANOVAs with a post hoc Bonferroni multiple comparisons test to assess differences between females (n = 10) and males (n = 10) at each dose. Buprenorphine significantly decreased respiratory frequency (A left, 3 and 10 mg/kg), and minute ventilation (A right, 10 mg/kg) in males relative to females. The lower two radar plots show that buprenorphine (3.0 and 10.0 mg/kg) significantly increased tidal volume in male ob/ob mice (B, left) and significantly increased duty cycle in female ob/ob mice (B, right). Asterisks indicate significant differences between females and males within buprenorphine dose ($p < 0.05$ adjusted for multiple comparisons).

A. B6


Frequency
Males
Females
Saline
400
350
300
250
200
0.1 mg/kg
0.3 mg/kg
1.0 mg/kg
3.0 mg/kg
10 mg/kg
*
*
Minute Ventilation
Saline
4.0
3.2
2.4
1.6
0.8
0.1 mg/kg
0.3 mg/kg
1.0 mg/kg
3.0 mg/kg
10 mg/kg
*

B. ob/ob


Tidal Volume
Males
Females
Saline
0.009
0.007
0.005
0.003
0.1 mg/kg
0.3 mg/kg
1.0 mg/kg
3.0 mg/kg
10 mg/kg
*
*
Duty Cycle
Saline
0.400
0.363
0.325
0.287
0.250
0.1 mg/kg
0.3 mg/kg
1.0 mg/kg
3.0 mg/kg
10 mg/kg
*
*

Chapter 5 Fentanyl and Neostigmine Delivered to Mouse Prefrontal Cortex Differentially Alter Breathing

This chapter is adapted for dissertation formatting from the following publication:

Glovak ZT, Baghdoyan HA, and Lydic R. Fentanyl and neostigmine delivered to the prefrontal cortex of C57BL/6J mice differentially alter breathing. (Under review March 9th, 2022).

I collected, analyzed, and interpreted the data presented in the following chapter, as well as wrote the original manuscript from which the following chapter is adapted.

Abstract

Opioids impair many functions modulated by the prefrontal cortex (PFC), including wakefulness, cognition, and breathing. In contrast, increased cholinergic activity in the PFC increases wakefulness. This study tested the hypothesis that microinjecting the opioid fentanyl and the acetylcholinesterase inhibitor neostigmine into the PFC of awake C57BL/6J (B6) mice alters breathing. The lateral (n = 7 mice) and medial (n = 19 mice) PFC of male B6 mice were unilaterally microinjected with saline (control) and fentanyl. The medial PFC received additional microinjections of neostigmine. The results show that fentanyl caused site-specific changes in breathing. Fentanyl delivered to the lateral PFC significantly decreased minute ventilation variability, whereas fentanyl delivered to the medial PFC significantly increased tidal volume and inspiratory duty cycle. Neostigmine microinjected into the medial PFC significantly increased breathing. Considered together, the present results provide the novel mechanistic insight that breathing was disrupted by fentanyl and enhanced by neostigmine when administered to the PFC of B6 mice.

Introduction

The prefrontal cortex (PFC) is the most evolutionarily recent development of the mammalian brain, and humans possess the largest PFC relative to total brain volume (Carlen, 2017; Fuster, 2015). The PFC modulates neurobehavioral functions that are negatively impacted by opioids, including behavioral and cortical arousal in mice (O'Brien et al., 2021; Van Dort et al., 2009) and rats (Montandon & Horner, 2019; Pal et al., 2018), autonomic physiology (Hassan et al., 2013; Lydic & Baghdoyan, 2021), and impulse control (Alizadehgoradel et al., 2020; Ceceli et al., 2022). In mice, systemically delivered opioids diminish acute nociception (Glovak et al., 2017), decrease EEG power during wakefulness (O'Brien et al., 2021), disrupt sleep (Lydic et al., 2022), and cause respiratory depression (Angel et al., 2018; Glovak et al., 2022). The high density of mu opioid receptors in the PFC (Giacchino & Henriksen, 1998; Mansour et al., 1995) suggests the potential for the PFC to contribute to opioid-induced changes in breathing. A role for the PFC in respiratory control is also consistent with neuroanatomical evidence of efferent and afferent projections between the PFC and brainstem respiratory nuclei (Gabbott et al., 2005; Hoover & Vertes, 2007; Howell et al., 2017). Although the pontomedullary brainstem mechanisms by which opioids depress breathing are well studied, relatively little is known about the cortical mechanisms through which opioids alter breathing (Ramirez et al., 2021).

Cholinergic neurotransmission in the PFC increases cortical EEG activation (Douglas et al., 2002) and behavioral markers of consciousness (Pal et al., 2018), both of which are decreased by opioids (Montandon & Horner, 2019; Osman et al., 2005).

Anatomical connections (Terreberry & Neafsey, 1987) and functional data (DeMarco et al., 2004; Van Dort et al., 2009) indicate there are descending projections from the PFC to cholinoceptive regions of the pontine reticular formation. These pontine areas are reciprocally connected with multiple brainstem nuclei that control breathing (Lee et al., 1995). Taken together, these findings suggest that cholinergic neurotransmission in the PFC could be one mechanism by which the PFC modulates breathing. To our knowledge, no previous studies have quantified breathing in response to direct injection of opioids or cholinergic enhancers into mouse PFC. To further elucidate the role of the PFC in modulating breathing, this study evaluated the hypothesis that breathing is altered by microinjecting the mu opioid receptor agonist fentanyl and the acetylcholinesterase inhibitor neostigmine into the PFC of freely behaving C57BL/6J (B6) mice.

Materials and Methods

Animals

All procedures involving mice were approved by the University of Tennessee Institutional Animal Care and Use Committee. This study followed the Guide for the Care and Use of Laboratory Animals (Council, 2011) and adhered to the ARRIVE guidelines (Percie du Sert et al., 2020). Efforts were made to minimize the number of animals used and their suffering. Adult, male, C57BL/6J (B6; Stock # 000664; n = 26) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mean ± SD age and weight of mice at time of surgery to implant microinjection guide tubes were 18.1 ± 9.4 weeks and 30.0 ± 5.3 g respectively. Mice were group housed in N10 mouse cages

(Ancare Corporation, Bellmore, NY). The animal facility was temperature (20-24°C) and humidity (25-75%) controlled with the lights operating on a 12:12 h light-dark cycle. Mice were given ad libitum access to food (Teklad 8640 rodent chow, Envigo, Madison, WI) and water. Harlan Soft Cob Enrichment Bedding (Envigo) and enrichment objects (paper cones, nestlets, igloos) were changed weekly. Following guide tube implantation, mice were housed individually to prevent removal of headcaps by cage mates.

Surgical procedures for microinjection guide cannula implantation

Mice were anesthetized with 2-3% isoflurane (Henry Schein, Melville, NY) delivered in 100% oxygen at a flow rate of 1 L/min. Loss of consciousness was confirmed via paw withdrawal test, the head was shaved, and ketoprofen (5 mg/kg) was delivered subcutaneously. Mice were then transferred to a stereotaxic frame (model 962; David Kopf Instruments, Tujunga, CA) and scrubbed for surgery. Isoflurane concentration was maintained at 1.3% via a mouse adaptor (model 921) and anesthesia mask (model 907) and monitored via spectrophotometry (CardioCap/5; Datex-Ohmeda Inc., Madison, WI). The mice rested on a warm water heating pad (Gaymar T/Pump TP-400; Gaymar Industries Inc., Orchard Park, NY) during surgery to maintain core body temperature (36-37°C).

After the mouse was positioned in the stereotaxic frame, the scalp was opened to expose the skull. A 26-gauge stainless steel guide cannula (C315; Plastics One, Roanoke, VA), with obturator, was implanted above the lateral (n = 7) or the medial (n =

19) PFC. Aim sites were chosen due to their homology with human PFC (Carlen, 2017) and based on previous data showing evoked autonomic responses from chemical manipulation of these regions (Hassan et al., 2013; Ramirez et al., 2021). Stereotaxic coordinates for the lateral PFC aim site were 3.0 mm anterior to bregma, 1.5 mm lateral (right) to the midline, and 1.0 mm below the surface of the skull. Coordinates for the medial PFC aim site were 1.9 mm anterior to bregma, 0.3 mm lateral (right) to the midline, and 3.3 mm below the surface of the skull (Paxinos & Franklin, 2007). The guide cannula was secured to the skull using dental acrylic (Jet Acrylic Self Curing Resin and Liquid; Lang Dental Manufacturing Co. Inc., Wheeling, IL) and four stainless steel anchor screws (Antrin Miniature Specialties, Fallbrook, CA). A unique radio frequency identification chip (ID-100B 1.4, MICROCHIPID, Lake Zurich, IL) was implanted subcutaneously and the incision was closed with sutures (Prolene 6-0; Henry Schein). Mice recovered in a heated cage and were returned to their home cages once they ambulated normally.

Behavioral conditioning

All conditioning procedures began one week after implantation and continued for two weeks before mice received a microinjection. Mice underwent daily handling to simulate drug delivery into the PFC. First, the obturator was removed from and reinserted into the guide tube. Mice then were placed inside a whole-body plethysmography (WBP) chamber (Data Sciences International, St. Paul, MN) for 15 min. The duration of the WBP conditioning was increased by 15 min each day up to the

maximum duration of 60 min. Mice were placed back in their home cages after WBP conditioning.

Whole-body plethysmography for quantification of breathing

Consistent with our previous work (Angel et al., 2018; Glovak et al., 2022), this study utilized WBP to quantify drug-induced changes in breathing. WBP measured inspiratory and expiratory lung volume in the freely behaving mice as a function of volumetric changes in air pressure within the sealed chamber (Criee et al., 2011). The WBP system was calibrated before each experiment began. All WBP measurements were corrected for air temperature and humidity, and acquisition software pruned the measurements for potential artifacts introduced by movement (sniffing, grooming, etc.). Figure 5.1 shows three representative beathing traces.

Intracranial drug administration and experimental design

All experiments began approximately 2 h after light onset. Mice acclimated to the WBP chamber for 30 min prior to receiving a PFC microinjection. During the microinjection, the obturator was removed and a 26-gauge microinjector (Plastics One) was inserted into the guide cannula. The microinjector was connected via PE50 tubing (Plastics One) to a 1-µL syringe (86200; Hamilton Co., Reno, NV) held in a manual microdrive. Microinjection duration was 60 s, and the microinjector remained in the guide cannula for an additional 30 s. The microinjector then was removed and the obturator was reinserted before returning the mouse to the WBP chamber to record

breathing for 15 min. The 15-min recording duration was chosen to quantify respiratory effects before the drug concentration was diluted due to drug diffusion away from the PFC (Baghdoyan et al., 1987). An experimenter was present during the 15-min recording period to ensure mice remained awake.

All mice (n = 26) received at least one unilateral microinjection (50 nL) of saline (vehicle control) and fentanyl (1 nmol; 530 ng; F3886; Sigma-Aldrich, St. Louis, MO). In separate experiments the PFC was microinjected with the acetylcholinesterase inhibitor neostigmine bromide (N2001, Sigma Aldrich) to increase endogenous acetylcholine. Four of the 19 mice with guide tubes aimed for the medial PFC received a concentration of neostigmine (0.5 nmol; 150 ng) that caused convulsions. Those four mice were euthanized. The remaining 15 mice received microinjections of neostigmine in lower concentrations (0.005 nmol; 1.51 ng and 0.016 nmol; 4.84 ng) that did not cause convulsions. The concentration of fentanyl was based on the minimum antinociceptive concentration delivered to the periaqueductal gray of rats (Bobeck et al., 2012). Concentrations of neostigmine were derived from previous studies in mice (Lydic et al., 2002) and pilot data (Glovak et al., 2021). This study used a repeated-measures, within-subjects design. The saline and drug injections were randomized to minimize any potential confounds due to injection order. Microinjections in the same mouse were separated by a recovery period of at least one week.

Following completion of the experimental protocol, mice were euthanized via isoflurane overdose followed by rapid decapitation. The brains were freeze-sectioned at 35-µm intervals, stained with Perls DAB to reveal iron, and counterstained with Thionine

to reveal cell bodies. Digital images of the tissue sections were obtained, and HuronViewer 1.31 (Huron Digital Pathology, St. Jacobs, Ontario) was utilized to localize the injection sites by comparing each section with a mouse brain atlas (Paxinos and Franklin, 2007). Histology was analyzed by two individuals, one of whom was blinded to treatment condition and aim site.

Statistical analysis of drug effects

No blinding was necessary for the analyses of the breathing measures because instrumentation and algorithms underlying WBP measures are not subject to experimenter bias. Respiratory rate (breaths/min; fR), tidal volume (mL/breath/g body weight; VT), minute ventilation (mL/min/g body weight; $\dot{V}E$), and inspiratory duty cycle (ratio of inspiratory time to total inspiratory plus expiratory cycle time; Ti/Ttot) were averaged in 60-s bins over the course of the 15-min recording. Data from mice that received repeated injections of the same drug and concentration were averaged to avoid inflated degrees of freedom. Poincaré analysis quantified minute ventilation variability in response to drug delivery to the PFC (Angel et al., 2018; Ermer et al., 2020; Glovak et al., 2022). The Poincaré results were expressed as minute-to-minute variability (SD1), and long-term breathing variability over the course of the 15-min recording (SD2). Poincaré analysis was performed using MATLAB custom scripts (R2020b; MathWorks, Natick, MA). To quantify breathing changes as a function of drug delivery to PFC, respiratory measures were analyzed via Wilcoxon matched-pairs test, two-tailed repeated measures t-test, and one-way repeated measures ANOVA. No data

were missing or excluded by the investigators. All data were log transformed, and a Geisser-Greenhouse correction was used so that data met the assumptions of the ANOVA model. Post hoc comparisons were made via Dunnett's post hoc test and p values were adjusted for multiple comparisons. Cutoff for significance was set at $p < 0.05$. All analyses were conducted using Prism 9.0.0 (GraphPad, San Diego, CA). Preliminary results have been published (Glovak et al., 2021; Glovak et al., 2019).

Results

Microinjection of fentanyl into lateral PFC decreased minute ventilation variability

The Poincaré plots in Fig. 5.2A show minute ventilation variability after PFC microinjection of saline (left) and fentanyl (right) into the lateral PFC. Dots in the Poincaré plots represent the nth min of minute ventilation (abscissa) versus nth + 1 min (ordinate). Each dot on these return plots is from one of the 7 mice with guide tubes aimed at the lateral PFC. Dots summarize minute ventilation variability during the 15 min of recording after PFC microinjection. The decrease in minute-to-minute variability ($\dot{V}E$ SD1) of minute ventilation is visualized by the dispersion of dots perpendicular to the reference line of identity (x = y). Long-term variability ($\dot{V}E$ SD2) is illustrated by the dispersion of dots along the line of identity. The group mean variability for $\dot{V}E$ SD1 and $\dot{V}E$ SD2 for each treatment condition is reflected by the semi-minor and semi-major axes of the ellipses.

Figure 5.2B plots the median and IQR for $\dot{V}E$ SD1 and $\dot{V}E$ SD2 after saline (gray bars) and fentanyl (red bars) microinjection. As visualized in Fig. 5.2A, Wilcoxon matched-pairs test revealed that fentanyl microinjected into the lateral PFC significantly

(Z = -2.1974, n = 7, p = 0.03) decreased minute-to-minute variability ($\dot{V}E$ SD1) and did not alter long-term variability ($\dot{V}E$ SD2).

Figure 5.2C summarizes the locations of the PFC microinjection sites as confirmed by histological analysis. Mean ± SD of the microinjection locations was 2.4 ± 0.2 mm anterior to bregma, 1.0 ± 0.2 mm lateral to the midline, and 2.3 ± 0.2 mm below the surface of the skull. Both right hemispheres illustrate 2.46 mm anterior to bregma (Paxinos & Franklin, 2003). Each circle on brain sections represents the injection site in one mouse and circles are color coded based on the percent change from saline in minute-to-minute ($\dot{V}E$ SD1) and long-term ($\dot{V}E$ SD2) variability after fentanyl microinjection. Linear regression of microinjection site coordinates revealed minute-to-minute and long-term breathing variability did not vary significantly in the anteroposterior, mediolateral, or dorsoventral dimensions within the lateral PFC. Taken together, functional (Fig. 5.2A, B) and anatomical (Fig. 5.2C) data demonstrate that fentanyl delivered to the lateral PFC decreased minute-to-minute variability of minute ventilation.

Microinjection of fentanyl into medial PFC altered breathing

All data in Fig. 5.3 were collected from mice (n = 19) with guide tubes aimed for the medial PFC. Mean ± SD of the microinjection site coordinates was 1.9 ± 0.4 mm anterior to bregma, 0.3 ± 0.2 mm lateral to the midline, and 2.5 ± 0.4 mm below the surface of the skull. The Fig. 5.3A hemispheres schematize coronal sections at 1.94 mm anterior to bregma (Paxinos & Franklin, 2003). As with Fig. 5.2C, each circle

represents the injection site in one mouse and circles are color coded to reflect percent change in each breathing variable compared to saline. Within the medial PFC, linear regression analysis of the microinjection coordinates confirmed that the effects of fentanyl on frequency, tidal volume, and minute ventilation did not vary significantly in any dimension. Only duty cycle varied significantly in the dorsoventral dimension following fentanyl microinjection ($Y = -0.044*X + 0.40$, $r2 = 0.35$, $p = 0.008$).

Figure 5.3B shows, from left to right, median respiratory rate (fR), tidal volume (VT), minute ventilation ($\dot{V}E$), and inspiratory duty cycle (Ti/Ttot) with interquartile range in response to medial PFC administration of saline and fentanyl. The whiskers denote the 5th and 95th percentiles. Data were analyzed via a repeated measures two-tailed t-test. Relative to saline, fentanyl caused a significant increase in tidal volume ($t = 4.41$, $df = 18$, $p = 0.0003$) and duty cycle ($t = 3.56$, $df = 18$, $p = 0.0023$) (Fig. 3B). Respiratory rate and minute ventilation were not significantly altered by fentanyl microinjection into the medial PFC (Fig. 5.3B). Minute ventilation variability was not significantly changed by fentanyl microinjection into the medial PFC (data not shown). Each breathing variable is plotted beneath its corresponding hemisphere to facilitate the combined interpretation of the anatomical (Fig. 5.3A) and functional (Fig. 5.3B) data

Microinjection of neostigmine into medial PFC increased breathing

Figure 4 summarizes the respiratory effects of medial PFC neostigmine administration in 15 of the 19 mice described in Fig. 5.3. Mean ± SD of the microinjection locations was 1.8 ± 0.4 mm anterior to bregma, 0.3 ± 0.1 mm lateral to

midline, and 2.4 ± 0.5 mm below the surface of the skull. Figure 5.4A hemispheres schematize the PFC at 1.94 mm anterior to bregma (Paxinos & Franklin, 2003). Each right hemisphere displays neostigmine (0.016 nmol) microinjection sites corresponding to a different breathing variable. Similar to Figs. 5.2C and 5.3A, each circle represents a microinjection site in one mouse. Circles are color coded to show percent change in breathing caused by neostigmine relative to saline. Linear regression indicated that frequency, tidal volume, minute ventilation, and duty cycle following neostigmine microinjection into the medial PFC did not vary significantly along any coordinate dimension.

Figure 5.4B plots respiratory rate (fR), tidal volume (VT), minute ventilation ($\dot{V}E$), and duty cycle (Ti/Ttot) in response to microinjection of saline and two concentrations of neostigmine into the medial PFC. Repeated measures one-way ANOVA showed that neostigmine caused a significant increase in frequency ($F_{(1.60, 22.4)} = 10.53$, $p = 0.001$), tidal volume ($F_{(1.57, 22.0)} = 6.0$, $p = 0.012$), and minute ventilation ($F_{(1.52, 21.2)} = 9.08$, $p = 0.003$). Compared to saline, Dunnett's post hoc test revealed that 0.005 and 0.016 nmol of neostigmine significantly ($p < 0.05$) increased respiratory rate and minute ventilation, but only the 0.016 nmol concentration significantly increased tidal volume (Fig. 5.4B). Duty cycle was not affected by neostigmine (Fig. 5.4B). Poincaré analysis revealed no significant changes in breathing variability as a function of neostigmine microinjection (data not shown).

Discussion

The discovery that administering ng amounts of fentanyl into the mouse PFC caused significant changes in breathing is of interest given that there are no respiratory neurons in the PFC. Changes in breathing were observed when fentanyl was delivered into lateral (Fig. 5.2) and medial (Fig. 5.3) regions of the PFC. The final section considers the finding that cholinergic stimulation of the medial PFC significantly increased breathing (Fig. 5.4).

Fentanyl delivered to the PFC altered breathing in a site-specific manner

The ability of an organism to vary respiratory rate and volume in response to environmental challenges is an essential neurobehavioral trait. Previous studies showed that opioids administered systemically to mice caused a significant decrease in respiratory variability (Angel et al., 2018; Fechtner et al., 2015; Glovak et al., 2022; Lydic & Baghdoyan, 2021). These opioid-induced decreases in respiratory variability encouraged the first set of experiments designed to determine whether breathing variability would be altered by fentanyl delivery into the lateral PFC. The dependent measure was minute ventilation (mL/min/g body weight), and breathing variability was quantified using Poincaré analyses. Compared to vehicle control, microinjection of fentanyl into the lateral PFC decreased minute-to-minute variability of minute ventilation (Fig. 5.2). Interestingly, the decrease in minute ventilation variability caused by the administration of the mu opioid receptor agonist fentanyl into the lateral PFC (Fig. 5.2B) is consistent with the decrease in minute ventilation variability caused by systemic

administration of the mu agonist and kappa antagonist buprenorphine to B6 mice (See Glovak et al., 2022, Fig. 3A). The fentanyl-induced decrease in breathing variability is also consistent with the decrease in breathing variability following microdialysis delivery of fentanyl to the PFC of awake mice (Fig. 7 of Ramirez et al., 2021).

Fentanyl delivered into the medial PFC caused a significant increase in tidal volume (Fig. 5.3B). Previous studies show that systemic administration of buprenorphine to intact, behaving B6 mice also increased tidal volume (Glovak et al., 2022, Fig. 1D). One measure of inspiratory drive is the ratio of inspiratory time to total respiratory cycle time (Ti/Ttot). Systemic administration of buprenorphine caused a decrease in Ti/Ttot in B6 mice (Glovak et al., 2022, Fig. 3H). In contrast, medial PFC fentanyl delivery caused an increase in Ti/Ttot (Fig. 3B), indicating increased respiratory effort.

Fentanyl decreased breathing variability when microinjected into the lateral PFC (Fig. 5.2) and increased tidal volume and duty cycle when microinjected into the medial PFC (Fig. 5.3). These site-specific results align with the altered respiratory activity following selective stimulation of pyramidal neurons in the medial PFC of Rett syndrome mice (Howell et al., 2017). There are well-documented efferent connections from the PFC to other brain areas that modulate breathing such as the nucleus of the solitary tract (Gabbott et al., 2005; Howell et al., 2017), the dorsomedial medulla (Terreberry & Neafsey, 1987), and the amygdala (Adhikari et al., 2015). These connections are vital for PFC integration of behavioral state, volitional control, and cardiopulmonary homeostasis (Fuster, 2015).

The neuronal substrates by which PFC fentanyl altered breathing in B6 mice (Figs. 5.2 and 5.3) are not known. Systemically administered opioids depress breathing via activation of mu opioid receptors that are widely distributed throughout the pontomedullary brainstem (Varga et al., 2020). The presence of mu-opioid receptors in the PFC (Light et al., 2017; Mansour et al., 1995) illustrates a potential receptor mechanism through which opioids may alter PFC neuronal activity. Opioids reduce cortical arousal in mice (O'Brien et al., 2021), rats (Montandon & Horner, 2019), and humans (Pattinson & Wise, 2016). Decreased cortical arousal has been associated with decreased respiratory output (DeMarco et al., 2004; Pal et al., 2018). Thus, the present data (Figs. 5.2 and 5.3) support the interpretation that the fentanyl-induced changes in breathing were caused, in part, by decreased neuronal activity in the PFC. Future studies simultaneously quantifying cortical EEG and breathing following opioid delivery to the PFC are needed to confirm this hypothesis.

Neostigmine delivered to medial PFC stimulated breathing

Having discovered that breathing is altered by PFC administration of fentanyl (Figs. 5.2 and 5.3), additional experiments involved a gain of function study by testing the hypothesis that microinjection of neostigmine into the PFC stimulates breathing. This hypothesis is based on two lines of evidence. First, are data showing that increasing cholinergic activity in the PFC causes EEG activation of the PFC EEG (Douglas et al., 2002; Van Dort et al., 2009), behavioral arousal (Pal et al., 2018; Parkar et al., 2020), and stimulates breathing (DeMarco et al., 2004). Second are decades of

evidence showing that beathing is depressed by the loss of wakefulness (Lydic & Baghdoyan, 2021) and that systemically administered opioids decrease acetylcholine release in the PFC (Osman et al., 2005). The Fig. 5.4 finding that PFC administration of neostigmine to the medial PFC caused an increase in breathing is consistent with the construct of a "wakefulness stimulus for breathing" whereby the state of wakefulness is associated with an enhanced drive to breathe (Lydic & Baghdoyan, 2021). The Fig. 5.4 data further implicate cholinergic neurotransmission in the PFC as one mechanism contributing to the wakefulness stimulus for breathing.

Limitations, strengths, and conclusions

The present study was not designed to fully characterize the mechanisms underlying the changes in breathing caused by microinjection of fentanyl and neostigmine into the PFC. The PFC of rodents contains mostly excitatory pyramidal neurons that communicate with distant cortical and sub-cortical regions (Xu et al., 2019). As noted above, many studies have identified projections from the PFC of rat to multiple nuclei within the respiratory control network, but more research is needed to characterize these pathways in mice (Carlen, 2017). The present results also demonstrate the feasibility of using mice to determine which respiratory effects are mediated by which subtypes of opioid and cholinergic receptors in the PFC. The sexually dimorphic respiratory effects of systemically administered opioids vary by species (Dahan et al., 1998; Gargaglioni et al., 2019). The present results, limited to

male B6 mice, encourage future studies of respiratory modulation by the PFC in female mice.

How the PFC integrates neurobehavioral functions into respiratory output is an active area of research (Lydic & Baghdoyan, 2021). The present data show for the first time in B6 mice that fentanyl significantly decreased breathing variability when delivered to the lateral PFC, and increased tidal volume and respiratory duty cycle when delivered to the medial PFC. Additionally, neostigmine microinjected into the medial PFC significantly increased breathing. The results support the interpretation that mu opioid receptors and cholinergic receptors in the PFC of B6 mice modulate breathing. This interpretation is consistent with the well-established fact of gain modulation by cortical neurons (Ferguson & Cardin, 2020). GABAergic (Zuperku & McCrimmon, 2002) and orexinergic (Kuwaki, 2008) transmission provide gain modulation of respiratory premotor neurons. The present results support the working hypothesis that opioidergic and cholinergic transmission in the PFC provide gain modulation of pontomedullary respiratory neurons that generate breathing.

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Appendix

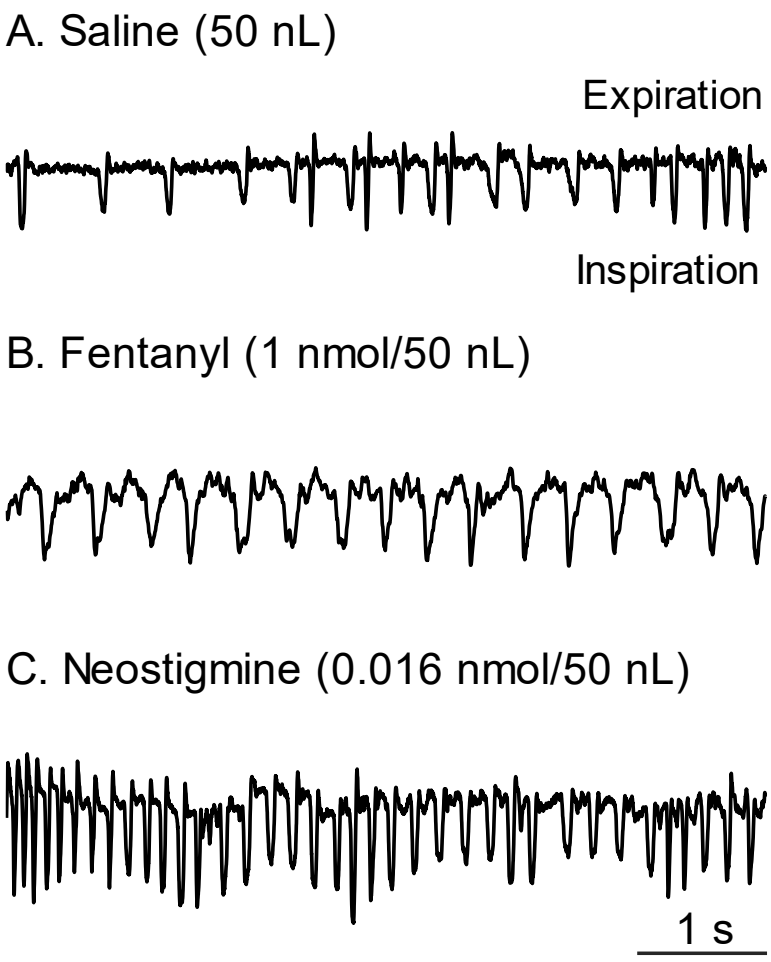


Figure 5.1 Representative breathing traces recorded using whole-body plethysmography.

These traces were recorded from the same mouse seven minutes after microinjection of saline (A), fentanyl (B), and neostigmine (C). Raw breathing traces illustrate drug-induced changes in breathing compared to saline.

Figure 5.2 Microinjection of fentanyl into the lateral PFC decreased breathing variability.

Microinjection of fentanyl into the lateral PFC decreased breathing variability. Dots in the Poincaré plots illustrate minute ventilation variability after microinjection of saline (left) and fentanyl (right) into the lateral PFC (A). Minute-to-minute ($\dot{V}E$ SD1) variability and long-term variability (across the 15-min recording, $\dot{V}E$ SD2) are visualized by dot distributions relative to the semi-minor and semi-major axes (green) of the ellipses. Group data are plotted as median with interquartile range (B). The whiskers correspond to the 5th and 95th percentiles. Compared to saline, fentanyl significantly (*$p < 0.05$) decreased $\dot{V}E$ SD1 but did not alter $\dot{V}E$ SD2. Histologically confirmed locations of microinjection sites are plotted as circles on drawings of coronal sections at 2.46 mm anterior to Bregma (modified from Paxinos and Franklin, 2003). Each circle represents the injection site in one mouse, and circles in the same place on each right hemisphere are from the same mouse. Circles are color coded to illustrate the percent change in $\dot{V}E$ SD1 or $\dot{V}E$ SD2 caused by fentanyl compared to saline (C). Anatomical abbreviations: AI, agranular insular cortex; LO, lateral orbital cortex; MO, medial orbital cortex; M2, secondary motor cortex; PrL, prelimbic cortex; VO, ventral orbital cortex.

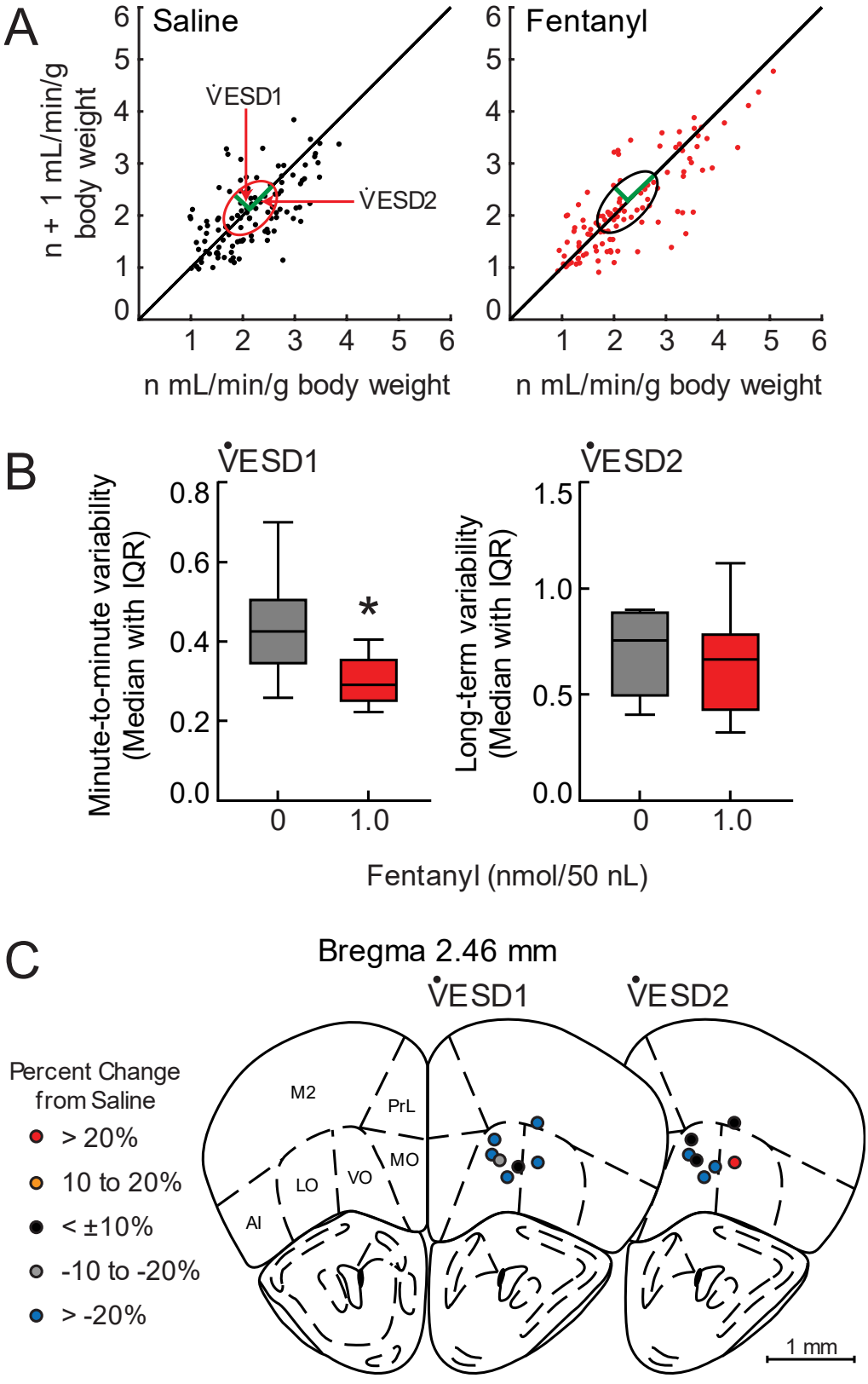

A
Saline
Fentanyl
V̇ESD1
V̇ESD2
n + 1 mL/min/g body weight
n mL/min/g body weight
B
V̇ESD1
V̇ESD2
Minute-to-minute variability (Median with IQR)
Long-term variability (Median with IQR)
*
Fentanyl (nmol/50 nL)
C
Bregma 2.46 mm
Percent Change from Saline
> 20%
10 to 20%
< ±10%
-10 to -20%
> -20%
M2
PrL
MO
VO
LO
AI
1 mm

Figure 5.3 Fentanyl delivery into the medial PFC altered breathing.

Fentanyl delivery into the medial PFC altered breathing. All coronal sections are from 1.94 mm anterior to Bregma (modified from Paxinos and Franklin, 2003). Each circle illustrates the injection site in one mouse and is color coded to show the percent change in breathing relative to saline control (A). Circles in the same place on each hemisphere represent different breathing measures from the same mouse (n = 19). Each right hemisphere maps fentanyl-induced changes in a different breathing measure: respiratory rate (fR), tidal volume (VT), minute ventilation ($\dot{V}E$), or inspiratory duty cycle (TI/Ttot). The median with interquartile range of the breathing measures is plotted below the anatomical data. The whiskers denote the 5th and 95th percentiles. Compared to saline, fentanyl significantly increased VT, and TI/Ttot. Asterisks indicate significant ($p < 0.05$) fentanyl-induced changes in breathing compared to saline (B). Anatomical abbreviations: Cg1, cingulate cortex area 1; DP, dorsal peduncular cortex; IL, infralimbic cortex; M1, primary motor cortex, M2, secondary motor cortex; PrL, prelimbic cortex.

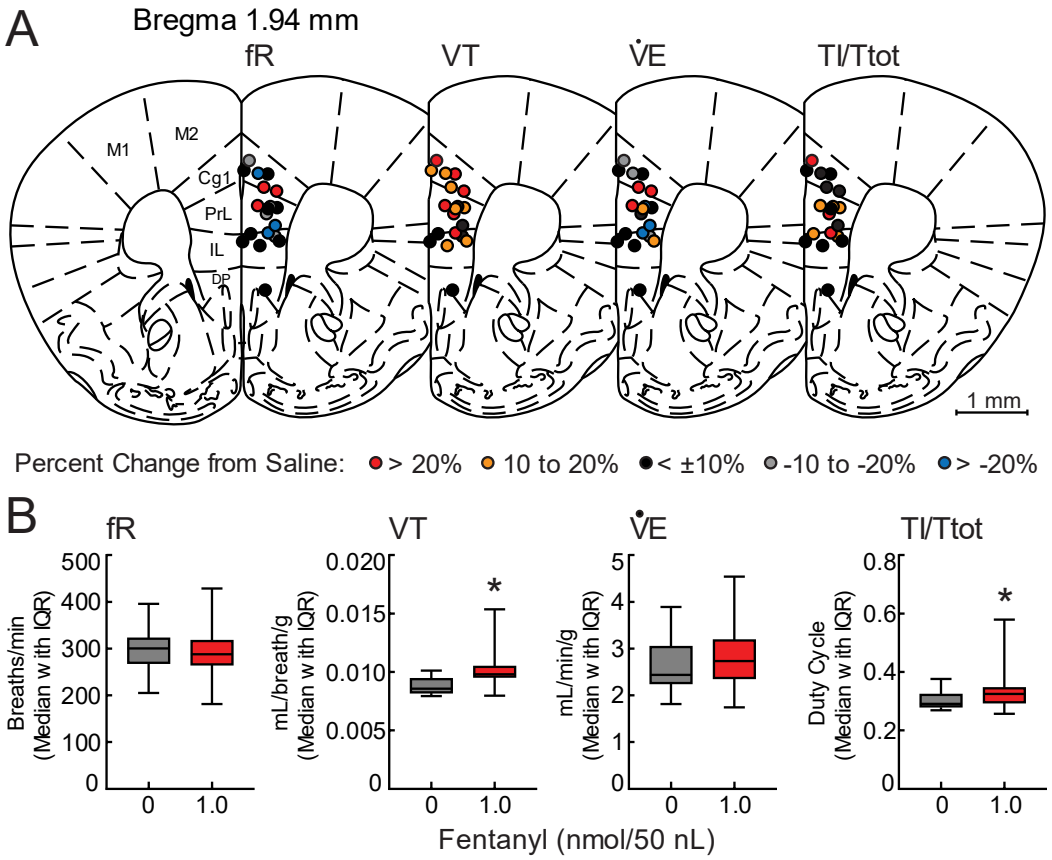
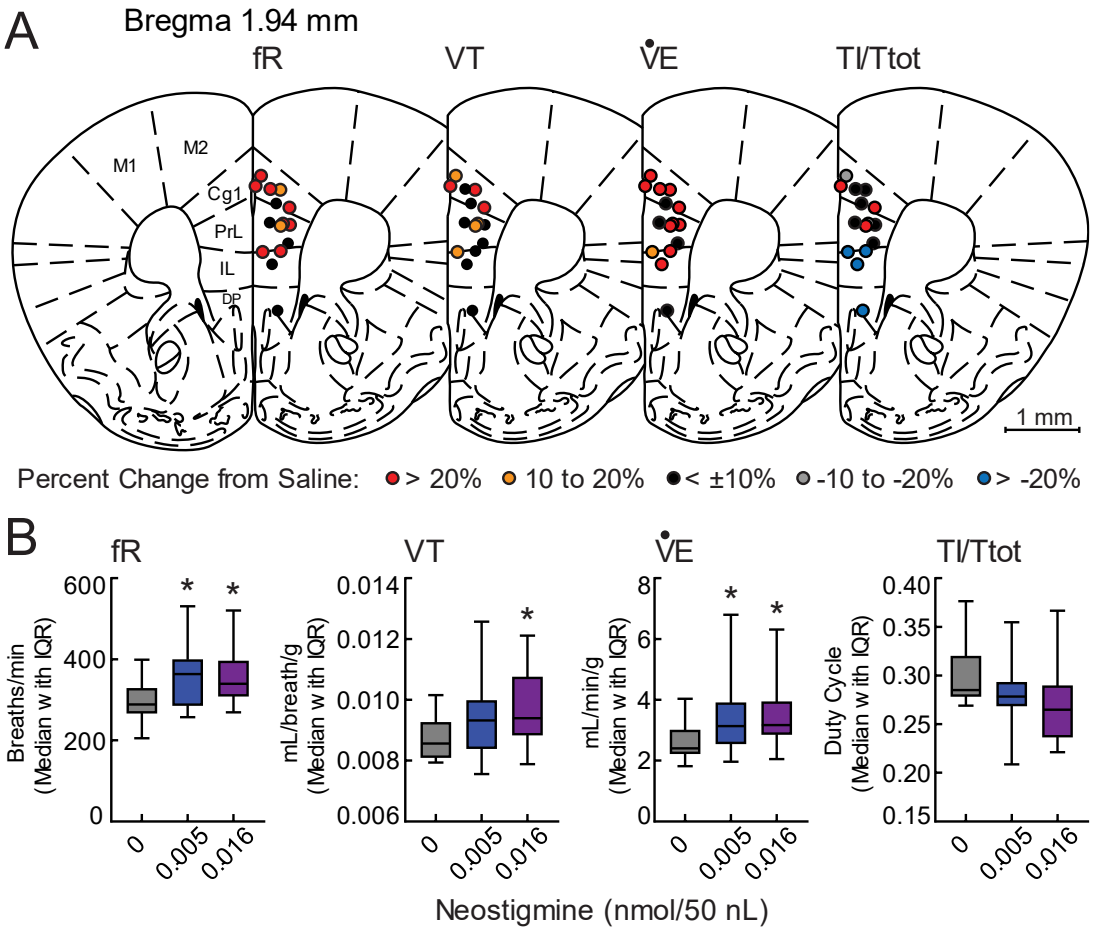

A
Bregma 1.94 mm
fR
VT
V̇E
TI/Ttot
M1
M2
Cg1
PrL
IL
DP
1 mm
Percent Change from Saline:
> 20%
10 to 20%
< ±10%
-10 to -20%
> -20%
B
fR
VT
V̇E
TI/Ttot
Breaths/min (Median with IQR)
mL/breath/g (Median with IQR)
mL/min/g (Median with IQR)
Duty Cycle (Median with IQR)
Fentanyl (nmol/50 nL)

Figure 5.4 Neostigmine increased breathing when microinjected into the medial PFC.

Neostigmine increased breathing when microinjected into the medial PFC. The drawings of coronal sections are from 1.94 mm anterior to Bregma (modified from Paxinos and Franklin, 2003). Each circle shows percent change in breathing caused by neostigmine compared to saline for one mouse (n =15), and circles in identical locations on each hemisphere represent the same mouse (A). Each right hemisphere maps neostigmine-induced changes in a single breathing measure: respiratory rate (fR), tidal volume (VT), minute ventilation ($\dot{V}E$), or inspiratory duty cycle (TI/Ttot). Functional data are plotted as median with interquartile range. The whiskers correspond to the 5th and 95th percentiles. Neostigmine significantly increased fR, VT, and $\dot{V}E$ compared to saline. Asterisks indicate significant ($p < 0.05$) changes in breathing caused by neostigmine compared to saline (B). Anatomical abbreviations: Cg1, cingulate cortex area 1; DP, dorsal peduncular cortex; IL, infralimbic cortex; M1, primary motor cortex, M2, secondary motor cortex; PrL, prelimbic cortex.


A
Bregma 1.94 mm
fR
VT
VE
TI/Ttot
M1
M2
Cg1
PrL
IL
DP
1 mm
Percent Change from Saline: > 20% 10 to 20% < ±10% -10 to -20% > -20%
B
fR
VT
VE
TI/Ttot
Breaths/min (Median with IQR)
mL/breath/g (Median with IQR)
mL/min/g (Median with IQR)
Duty Cycle (Median with IQR)
0
0.005
0.016
Neostigmine (nmol/50 nL)

Chapter 6 Conclusions

This chapter summarizes the studies described in Chapters 2, 3, 4, and 5 and synthesizes their contributions to the field and potential translational relevance. Next, limitations for the studies in this dissertation are discussed followed by suggested future directions. Finally, the results are integrated into an overarching and unifying conclusion.

Summary of Findings and Potential Translational Relevance

Chapter 2: Leptin Status Alters Buprenorphine-induced Antinociception in Obese Mice with Dysfunctional Leptin Receptors

The data presented in this chapter support the hypothesis that buprenorphine-induced antinociception varies as a function of leptin status. There were no sex differences in acute thermal nociception after saline or buprenorphine. Body weight was also ruled out as a significant predictor of thermal nociception (Fig. 2.2). The DIO, ob/ob, and db/db mice all share the obesity phenotype, but only the ob/ob and db/db mice have disrupted leptin signaling. Of note, the db/db mice exhibited significantly blunted acute thermal nociception after saline and received significantly less antinociception from buprenorphine compared to the other three congenic lines of mice (Fig. 2.1). The db/db mice produce leptin but have dysfunctional Ob-Rb leptin receptors. Thus, the decreased thermal sensitivity displayed by the db/db mice suggests that the Ob-Rb receptor modulates thermal nociception and buprenorphine-induced antinociception.

The findings from this study support and extend evidence from the literature that the interactions between opioids and obesity are modulated, in part, by leptin (Hu et al.,

2018). Leptin dysfunction has been associated with upregulated opioid receptors (Khawaja et al., 1989) and endogenous opioids (Margules et al., 1978). Individuals with obesity experience leptin dysfunction secondary to prolonged hyperleptinemia (Caprio et al., 1996; Kirchgessner et al., 1997; Lee et al., 2001). Over time, hyperleptinemia leads to leptin resistance which is a reduced cellular and physiological sensitivity to the effects of endogenous leptin (Balland & Cowley, 2015). Leptin resistance disrupts the functions modulated by leptin such as nociceptive processing (Caro et al., 1996; Enriori et al., 2006; Morris & Rui, 2009; Wang et al., 2009). This may be one interpretation as to why individuals with obesity report more pain (Hu et al., 2018; Younger et al., 2016). This is relevant given that the higher incidence of pain in obese populations has been identified as a potential contributor to the ongoing opioid crisis (Stokes et al., 2019). Taken together, the findings from Chapter 2 and other studies highlight the leptin signaling pathway as a potential therapeutic target for the treatment of neuropathic pain, or as an adjunct target to opioid pain therapies (Freire et al., 2020; Rodgers et al., 2014).

Chapter 3 and 4: Buprenorphine Depresses Respiratory Variability and Differentially Alters Breathing as a Function of Leptin Status and Dose

The findings from Chapters 3 and 4 will be discussed together because both provide evidence supporting the hypothesis that the respiratory effects of buprenorphine vary as a function of leptin status. The opioid buprenorphine was chosen due to its clinical relevance via its ceiling effect for respiratory depression but not for analgesia

(Dahan et al., 2006) and because it is used to treat opioid use disorder (Schuckit, 2016). Chapter 3 builds on the findings from Chapter 2 by showing that systemic administration of an antinociceptive dose of buprenorphine depressed breathing variability more in obese mice with dysfunctional leptin signaling (Fig. 3.2). Furthermore, Chapter 4 extends this result by showing that higher doses of buprenorphine caused dose-dependent disruptions in breathing (Fig. 4.1) and decreased breathing variability (Fig. 4.2 and 4.3). Chapter 4 also demonstrates that the respiratory effects of buprenorphine were modulated by leptin status and not body weight (Fig. 4.4). Overall, higher doses of buprenorphine disrupted breathing to a greater degree with the greatest disruption occurring in mice with dysfunctional leptin signaling.

The findings from Chapters 3 and 4 further implicate leptin as a potential modulator of the interactions between obesity and opioids. As described in Chapter 1, leptin has been shown to stimulate breathing via activation of Ob-Rb receptors located in multiple sites within the respiratory control network. The respiratory stimulant effect of endogenous leptin may be impaired in individuals with obesity due to persistent hyperleptinemia and leptin resistance (Bassi et al., 2016). This hypothesis is supported by the fact that individuals with obesity have diminished respiratory function and thus are diagnosed with more respiratory disorders (Jubber, 2004; O'Donnell et al., 2000).

Multiple studies using animal models have demonstrated that exogenous leptin administration reverts the respiratory dysfunction caused by obesity and disrupted leptin signaling (Gauda et al., 2020). One barrier to extending this treatment to human populations is the saturable leptin transport mechanism across the blood brain barrier

(Van Heek et al., 1997). Intranasal administration bypasses the blood brain barrier which may circumvent this problem. In fact, intranasal administration has been shown to attenuate sleep-disordered breathing and opioid-induced respiratory depression during sleep without reducing analgesia in mice with diet-induced obesity (Berger et al., 2019; Freire et al., 2020). Translation of these preclinical outcomes to humans holds great promise for the treatment of respiratory disorders in obese populations, as well as an adjunct therapy to reduce opioid-induced respiratory depression associated with opioid pain treatment.

Chapter 5: Fentanyl and Neostigmine Delivered to Mouse Prefrontal Cortex Differentially Alter Breathing

The prefrontal cortex was chosen due to mounting evidence that this brain region has the capacity to alter breathing (Hassan et al., 2013; Howell et al., 2017; Pattinson & Wise, 2016). The experiments in Chapter 5 were designed to evaluate the role of the prefrontal cortex as a modulator of breathing and opioid-induced changes in breathing. The technique of microinjection allowed for the targeted administration of drugs to specific regions of the prefrontal cortex of freely behaving mice. The results show that fentanyl caused site-specific changes in breathing. Relative to saline, fentanyl delivery into the lateral prefrontal cortex decreased breathing variability (Fig. 5.2). Fentanyl administration into the medial prefrontal cortex increased tidal volume and inspiratory duty cycle (Fig. 5.3). Administering fentanyl into the prefrontal cortex (Figs. 5.2 and 5.3) has the same effects on breathing as systemic administration of buprenorphine (Figs

4.1 and 4.2). Although fentanyl and buprenorphine have different mechanisms of action, both drugs are mu opioid receptor agonists (Dahan, 2006; Laycock & Bantel, 2019). The finding that similar changes in breathing were observed after central administration of opioids into the prefrontal cortex and systemic opioid administration supports the interpretation that the prefrontal cortex may be one brain region that contributes to opioid-induced changes in breathing.

Opioids are known to cause sedation (Montandon & Horner, 2019; Pattinson et al., 2009). Conversely, cholinergic activity in the prefrontal cortex is positively correlated with arousal and increased breathing (DeMarco et al., 2004; Pal et al., 2018; Parkar et al., 2020). Microinjection of the acetylcholinesterase inhibitor neostigmine into the medial prefrontal cortex significantly increased breathing (Fig. 5.4). This result aligns with the literature and highlights cholinergic transmission in the prefrontal cortex as a potential therapeutic target for offsetting opioid-induced respiratory depression. Preliminary efforts have been successful in attenuating opioid-induced respiratory depression without reducing analgesia by increasing cholinergic neurotransmission in the cortex (Kimura et al., 2013; Sun et al., 2022; Tsujita et al., 2007). As described in Chapter 1, many pathways have been documented that show that the prefrontal cortex is integrated within the established respiratory network. These anatomical data combined with the findings from Chapter 5 support the interpretation that the prefrontal cortex modulates the behavioral control of breathing.

Furthermore, the results from Chapter 5 provide additional insight into the interactions among opioids, obesity, leptin, and breathing that are discussed in previous

chapters. The prefrontal cortex is one brain region that underlies the pathogenesis of opioid use disorder (Baldo, 2016) and obesity (Lowe et al., 2019). Both opioid use disorder (Alizadehgoradel et al., 2020; Ceceli et al., 2022) and obesity (Schmidt et al., 2018; Wakabayashi et al., 2015) are appetitive disorders arising from the loss of impulse control that can be attributed to prefrontal cortex dysfunction. This prefrontal cortex dysfunction is compounded by the depressed breathing (Ramirez et al., 2021) and obtunded sleep (Lydic & Baghdoyan, 2021) caused by opioids and obesity (Spruyt & Gozal, 2012) which further disrupts normative prefrontal cortex function and leads to cognitive impairment. Leptin has been shown to reduce the respiratory depression secondary to obesity and opioids (Berger et al., 2019; Freire et al., 2020). Although not specifically addressed by studies in this dissertation, leptin may also be a potential treatment to correct the decreased neural plasticity underlying prefrontal cortex dysfunction and depression (Ge et al., 2018). The aforementioned comorbidities of respiratory depression, sleep disruption, and cognitive impairment frequently form a reciprocal and cyclic pattern that perpetuates the ongoing opioid and obesity public health crises (Stefan et al., 2021; Stokes et al., 2019; Volkow, 2020). More data are needed to describe these interactions and isolate novel targets for therapeutic interventions.

Limitations and Future Directions

Limitations and future directions for individual chapters are included within each chapter. This section will focus on overall limitations for this dissertation that have not already been discussed as well as potential future directions.

Pruning of Whole-Body Plethysmography Data

Chapters 3, 4, and 5 used whole-body plethysmography to quantify drug-induced changes in breathing in freely behaving mice. Unrestrained whole-body plethysmography is a technique that derives tidal volume from air pressure changes within a sealed chamber in accordance with Boyle's Law. The changes in air pressure cause a flexible screen to bow in or out. The flexion of the screen is transduced via an electrical potential applied to the screen. These electrical potentials correlate to inspiratory and expiratory lung volume from the mouse. This generates a raw breathing recording (Fig. 5.1) that can be analyzed to calculate various aspects of breathing behavior such as respiratory frequency, tidal volume, minute ventilation, inspiratory time, and expiratory time.

Due to the high variability in mouse breathing behavior, whole-body plethysmography data are often pruned to reduce artifacts potentially originating from behaviors such as sniffing, grooming, movement, etc. These pruning criteria, however, can vary between different investigators. This decreases the comparability of the data collected with different equipment or pruning criteria. Thus, one limitation of the studies in this dissertation is the lack of standardization in the field of unrestrained whole-body plethysmography. While all whole-body plethysmography data in this dissertation were collected by the same person and equipment, other whole-body plethysmography data in the literature may vary slightly because equipment and pruning criteria can vary. Some investigators in the field utilize custom built unrestrained whole-body plethysmography setups with custom pruning software to collect breathing data.

Companies that provide whole-body plethysmography solutions often make their pruning criteria available (https://support.datasci.com/hc/en-us/articles/115003784588-Understanding-the-FinePointe-Rinx-Parameter) and there are established rejection indices that are commonly used as well (Drorbaugh & Fenn, 1955). One way to move the field ahead would be to report any pruning criteria along with the whole-body plethysmography data. This would aid in the synthesis of the findings using different equipment and improve the rigor and reproducibility of the results.

Behavioral Breathing Differences Between Mouse Strains

The experiments described in this dissertation utilized C57BL/6J (B6) mice to study the interactions among obesity, opioids, leptin, and breathing. This animal model was chosen due the genetic homology between mice and humans (O'Brien et al., 1999) and the high prevalence of preclinical research utilizing mice (Justice & Dhillon, 2016). However, there are well-documented differences in ventilatory behavior between among mouse strains (Fechtner et al., 2015; Friedman et al., 2004; Yamauchi et al., 2008). These differences limit the comparison of these data with other breathing studies of different strains of mice. The exact mechanisms of the variations in ventilatory behavior are not known, although it is likely due to genetic differences between the strains (Han et al., 2002; Tankersley, 2002). Future studies describing how these genetic differences alter breathing could increase our knowledge of the neurobehavioral control of breathing in mice and other animal models (Gibbs, 2014).

Systems Biology Approach to Physiology and Behavior

The studies in this dissertation were not designed to completely characterize the mechanism by which the prefrontal cortex or leptin participates in opioid-induced changes in breathing. Leptin regulates many aspects of physiology including breathing, nociception, metabolism, neural plasticity, and immune function (Fuster, 2015; Gauda et al., 2020; Maurya et al., 2018; Okifuji & Hare, 2015). This versatility makes leptin a vital component of many physiological systems and grants it unique explanatory power in the field of pathophysiology. Similarly, the prefrontal cortex facilitates many neurobehavioral functions and is directly and indirectly involved in many aspects of physiology (Fuster, 2015). This chapter frequently highlights leptinergic pathways as both a potential explanation and therapeutic target for the respiratory effects of obesity and opioids described in this dissertation. However, it should be noted that this is likely one of many possible interpretations. The exact mechanisms by which leptin alters breathing are largely unknown. The same logic applies to the results of Chapter 5. Chapter 5 shows that the prefrontal cortex is one brain region contributing to opioid-induced changes in breathing, but it is likely not the only brain region. More research is needed to fully characterize the mechanisms by which the prefrontal cortex and other brain regions modulate breathing and behavior. Therein lies a limitation of this dissertation and, perhaps, the discipline of reductionistic physiology (Lydic & Baghdoyan, 2021).

Reductionist approaches have been vital for understanding individual nodes of the respiratory control network over the past century (Remmers, 2005). However,

synthesis across these nodes is necessary to push the field forward (Del Negro et al., 2018; Remmers, 2005). Reductionist approaches are not well equipped to adequately explain the biological complexity proffered by multiple complex nodes simultaneously interacting within the entire respiratory control network. To that end, a systems biology approach (Hood et al., 2008) to the neurobehavioral control of breathing may be necessary to understand the emergent properties of the respiratory control network (Lydic & Baghdoyan, 2021). A complementary and interdisciplinary combination of reductionistic and systems biology methodologies is a promising future direction for the study of the behavioral control of breathing.

Conclusions

The interacting and exacerbating epidemics of obesity and opioid abuse in the United States indicate a poor understanding at the intersection of these fields. One similarity between both epidemics is the impaired ventilatory function often experienced by individuals with obesity or those receiving treatment with opioids. Diminished breathing can lead to decreased cognitive function and ultimately death which constitutes a public health burden. Breathing is a complex behavior that emerges from the dynamic interactions among the plurality of respiratory control networks located throughout the peripheral and central nervous system. This dissertation integrates the respiratory effects of obesity and opioids with what is currently known about the behavioral control of breathing. The studies described in Chapters 2, 3, and 4 reflect a novel effort to elucidate the effects of obesity and opioids across an array of physiological conditions. The data reported in Chapter 5 are the first to describe the

respiratory effects of opioids microinjected directly to the prefrontal cortex of mice. The prefrontal cortex modulates a large and diverse collection of neurobehavioral traits, including breathing and impulse control. Opioids and obesity disrupt these traits, which makes the prefrontal cortex an area of interest for studying opioid and obesity-induced changes in neurobehavioral control. This dissertation identifies the prefrontal cortex as one potential brain region that holds promise as a therapeutic target for respiratory disorders caused by obesity and opioid use. This line of inquiry may grant insight into the complexity of the respiratory control network while also contributing to the discovery of new treatments for respiratory disorders.

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Vita

Born in Lapeer, Michigan, Zack Glovak grew up in Mooresville, Indiana before attending high school in Seymour, Tennessee. After graduating valedictorian from Seymour High School, Zack attended the nearby University of Tennessee, Knoxville to pursue an undergraduate degree in Neuroscience. As a senior undergraduate student, Zack discovered his passion for research under the tutelage of Drs. Helen Baghdoyan and Ralph Lydic. After obtaining his Bachelors' degree in Neuroscience, Zack worked as a laboratory manager for Dr. Baghdoyan and Dr. Lydic to hone his research skills and develop his graduate school application. He then began pursuing his Doctor of Philosophy degree in Experimental Psychology under the continued mentorship of Dr. Lydic and Dr. Baghdoyan. Zack's research focused on the physiological effects of opioids and obesity and characterized the role of the prefrontal cortex in opioid-induced respiratory depression. After graduating, Zack accepted a postdoctoral fellowship at the Seattle Children's Hospital Center for Integrative Brain Research.