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The Hypercapnic Ventilatory Response and Behavior in Ca2+-Activated K+ (BK) Channel Knock Out Mice And T-Cell Death-Associated Gene 8 (TDAG8) Receptor Knock Out Mice

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THE HYPERCAPNIC VENTILATORY RESPONSE AND BEHAVIOR IN CA^{2+}-ACTIVATED K^+(BK) CHANNEL KNOCK OUT MICE AND T-CELL DEATH-ASSOCIATED GENE 8 (TDAG8) RECEPTOR KNOCK OUT MICE

A thesis in partial fulfillment of the requirements for the degree of Master of Science

By

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ABSTRACT

Ratliff-Rang, Christine Annette. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2017. The hypercapnic ventilatory response and behavior in Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channel knock out mice and T-cell death-associated gene 8 (TDAG8) receptor knock out mice.

Some acid sensing areas in the brain control the expression of breathing and anxiety/fear including the locus coeruleus (LC) (Redmond & Huang, 1979) and the nucleus tractus solitarius (NTS). It has been found that knocking out T-cell death-associated gene 8 (TDAG8), a chemosensor, attenuates CO\textsubscript{2} induced fear phenotypes in mice. However their hypercapnic ventilatory response (HCVR) has not yet been looked at. Also, BK channels are large-conductance, calcium-activated potassium channels that are activated by increases in concentration of intracellular calcium ions. It has been found that BK KO rats have an increase in their HCVR (Patrone et al., 2014) however their CO\textsubscript{2} induced anxiety/fear has not been looked at yet. In this thesis, we found that the BK channel is involved in the HCVR in mice and that the CO\textsubscript{2} induced anxiety/fear pathway and the HCVR pathway are separate pathways in the BK and the TDAG8 mice.
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For my loving husband, my amazing mom, encouraging dad and BOB.
INTRODUCTION
Central nervous system neurons that respond to CO$_2$/H$^+$ are termed central chemoreceptors. Carbon dioxide (CO$_2$) provides a vital chemical stimulus to breathing, through the activity of CO$_2$/H$^+$ sensors located in the brainstem and in the carotid bodies (Mouradian et al., 2012). Acid sensing in the brain is also involved in the expression of many disorders such as generalized anxiety, panic disorder, sleep apnea, stress, mental retardation, autism, schizophrenia, learning and attention deficits (Lousberg et al., 1988; Stein et al., 1995; Verbraecken et al., 1995; Ryan & Bradley, 2005; Wang et al., 2007; Valentino & Van Bockstaele, 2008). Additionally, there are many acid-sensing areas that are involved with both the control of breathing and the expression of anxiety, including the locus coeruleus (LC) (Redmond & Huang, 1979) and the nucleus tractus solitarius (NTS) (Ghosal et al., 2014).

T-cell death-associated gene 8 (TDAG8), a pH-sensing G-protein coupled receptor (GPCR) on T-cells and microglia, has been identified as a chemosensor. It contributes to the behavioral and physiological effects of CO$_2$ inhalation by detecting and translating hypercarbia to fear (Vollmer et al., 2016). In this model, CO$_2$-evoked behavioral (freezing) and physiological (cardiovascular) responses are triggered via activation of pro-inflammatory responses in microglia localized to the subfornical organ (SFO) (Vollmer et al., 2016). TDAG8 knock out (KO) mice have been found to have a lower anxiety phenotype, however their hypercapnic ventilatory response (HCVR) has not yet been looked at. Thus, in this thesis I will be investigating the HCVR and activity level in TDAG8 KO versus control mice, to examine if the respiratory and anxiety phenotypes involve the same neural and signaling pathways.
Additionally, large-conductance, calcium-activated potassium channels (BK channels) are activated (opened) by increases in concentration of intracellular calcium ions (Ca\(^{2+}\)) (Miller et al., 2000; Putnam et al., 2004; Putnam, 2010). Since activation of these channels results in decreased neuronal firing rate in response to elevated CO\(_2/H^+\), they are thought of as a “brake” in the neuronal chemosensitive response. When inhibited, they cause LC neurons to increase their firing rate in response to elevations in CO\(_2\) (Imber & Putnam, 2012; Imber et al., 2014).

In the present study, I investigated the effects of BK channel deletion on the HCVR. I also examined the role of the BK channel on anxiety in hypercapnic and in normoxic conditions, and the activity level of the mouse, to determine if the respiratory and anxiety phenotypes involve similar sensing pathways in this model as well.
Control of Ventilation

Findings in central chemoreception support a complex, cohesive system for the detection of CO₂/H⁺ and the stimulation of breathing (Coates et al., 1993; Huang et al., 1997; Putnam et al., 2004; Nattie & Li, 2009). The major stimulus for an increase in breathing is an elevation of CO₂/H⁺ (hypercapnic acidosis, HA). CO₂ increases breathing in order to blow off CO₂, which results in decreased H⁺ (increased pH). Furthermore, respiratory chemosensitivity is the ability of the brain to detect changes in CO₂/H⁺ and alter physiological systems to regulate H⁺ within tightly controlled values (Feldman et al., 2003). For ventilation to be increased, HA must be detected by specialized sensory elements (Putnam et al., 2004). These sensory elements are cells that are sensitive to CO₂/H⁺ (chemosensitive cells) and have been identified both peripherally (glomus cells within the carotid body) (Gonzalez, 1992; Peers, 1995) and centrally (neurons localized within various regions of the brain stem) (Coates et al., 1993; Nattie et al., 1998; Nattie et al., 1999; Feldman et al., 2003).

Chemosensitive neurons have been reported in many regions of the brain, including the LC (Pineda & Aghajanian, 1997; Filosa & Putnam, 2003), the medullary raphé (Richerson, 1995; Wang et al., 1998), the nucleus tractus solitarius (NTS) (Dean et al., 1989; Conrad et al., 2009; Nichols et al., 2009) and the retrotapezoid nucleus (RTN) (Mulkey et al., 2004; Ritucci et al., 2005) (Fig 1). Furthermore, the NTS is suggested to be an important site of ventilatory and cardiovascular control (Barraco et al., 1990; Mifflin, 1992) since it obtains inputs from peripheral chemoreceptors and lung stretch receptors (Donoghue et al., 1984, Bonham & McRimmon, 1990, Mifflin, 1992; Koshiya & Guyenet, 1996).
Figure 1: The rat brainstem (sagittal view) displaying the locations of multiple areas involved in central chemosensitive respiratory control, including the locus coeruleus (LC), nucleus tractus solitarius (NTS), caudal ventral respiratory group (cVRG), rostral ventral respiratory group (rVRG), retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), VII facial nucleus (VII), motor nucleus cranial nerve 7 (7n), nucleus ambiguous (Amb), Bötzinger complex (BC), caudal ventrolateral medulla (CVLM), dorsal vagal motor nucleus (DVMN), Kölliker-Fuse nucleus (K-F), lateral reticular nucleus (LRt), motor trigeminal nucleus (Mo5) parabrachial nucleus (PB), pre-Bötzinger complex (preBötC), rostral ventrolateral medulla (RVLM), medullary raphé (MdR). This figure is adapted from Spyer and Gourine. Philos Trans R Soc Lond B Biol Sci. 2009 Sep 12; 364(1529): p. 2603.
FIGURE 1:

Central chemosensitive respiratory control areas in rat brain stem (sagittal view).
There are three things that define a chemosensitive area. First, acidification of just that area (e.g. the LC, or NTS) results in increased ventilation. To demonstrate this, in previous research, acetazolamide was injected focally into various brain stem areas in anesthetized animals, and the ventilatory response was measured (Coates et al., 1995; Nattie et al., 2001). Locally injecting acetazolamide into various brain stem regions to produce focal acidosis is actually used as a probe for chemosensitive areas (Coates, 1991; Coates, 1993; Nattie, 1998; Nattie, 1999). The second characteristic defining a chemosensitive area is, when that area is lesioned (i.e. killing most neurons within a given area), a decreased HCVR will result (Curran et al., 2000). For instance, lesion experiments in anesthetized animals have indicated that the rostral ventral medulla (RVM) is important in the maintenance of normal breathing and the normal ventilatory response to systemic CO₂ (Nattie et al., 1988; Nattie and Li, 1990; Nattie et al., 1991, 1992; Nattie and Li, 1994). Also the ventilatory response to CO₂ is reduced when the LC is substantially lesioned (Li and Nattie, 2006; Biancardi et al., 2008). Lastly, the third feature that defines a chemosensitive area is that area must contain neurons whose firing rates are affected by exposure to hypercapnic solutions (Putnam et al., 2004). Most neurons that respond, respond with an increase in their firing rate. It also needs to be shown that the neuron is intrinsically sensitive to CO₂/H⁺, and not merely responding to altered synaptic input from other neurons that are chemosensitive. The neuron responds to an acid challenge in the lack of all potential input from both electrical and chemical synapses. Intrinsically CO₂-sensitive neurons have been located in numerous regions including the NTS (Dean et al., 1990), the medullary raphé (Richerson et al., 1995), the LC (Oyamada et al., 1998), the RTN (Mulkey et al., 2004; Guynete et al., 2008; Takakura
et al., 2014) the nucleus ambiguus (Rigatto et al., 1994), and the ventrolateral medulla (VLM) (Wellner-Kienitz et al., 1998).

CO₂ is a by-product of cellular respiration and is dissolved in the blood, then taken up by red blood cells and gets converted to carbonic acid (H₂CO₃), a reaction catalyzed by carbonic anhydrase (CA) (Fig 2). Most of H₂CO₃ then dissociates to bicarbonate (HCO₃⁻) and hydrogen ions (H⁺). Thus, the level of CO₂ affects the levels of H⁺ (i.e., pH) and HCO₃⁻. Numerous potential signals (e.g., changes in extracellular pH (pHo), intracellular pH (pHi), CO₂ and HCO₃⁻) in response to hypercapnia exist and there are also numerous ion channel targets (e.g., K⁺, Ca²⁺, cation channels) whose activity could be affected by these various signals (Putnam et al., 2004). The activities of some of these channels are altered by changes in either pHi or pHo. For instance decreases in pHi and/or pHo can inhibit many kinds of K⁺ channels (Putnam et al., 2004; Li & Putnam, 2013) or inhibit Ca²⁺-activated non-selective (CAN) cation currents (Richerson, 2004; Wu et al., 2010). Moreover, Ca²⁺ channels, at least in LC neurons, are activated by hypercapnia via a HCO₃⁻ dependent pathway, involving soluble adenylyl cyclase (sAC) and elevated cAMP (Imber et al., 2014). All of these pathways will result in neuronal depolarization and increased firing rate (Fig 2; Fig 3a). These pathways thus serve as “accelerators” of the firing rate response of chemosensitive neurons to hypercapnia. It is likely that hypercapnic activation of Ca²⁺ channels leads to increased [Ca²⁺]i. This could result in activation of Ca²⁺ activated K⁺ channels by hypercapnia, which in turn allow K⁺ ions to passively flow down the electrochemical gradient and out of the cell. This would result in neuronal hyperpolarization and a decrease in the firing rate response of neurons to hypercapnia. Thus these Ca²⁺ activated K⁺ channels such as BK channels could work in concert with Ca²⁺ channels to decrease the firing rate
**Figure 2:** A prototype of the response of a single neuron to hypercapnia. CO$_2$ diffuses across the cell membrane, hydrates and then dissociates into HCO$_3^-$ and a proton to result in the acidification of pH$_i$, which inhibits K$^+$ channels. Also, decreases in pH$_o$ cause an inhibition of the Na$^+$ - H$^+$ exchanger (NHE) to prevent the recovery of pH$_i$. This causes the neuron to depolarize and increase its firing rate. This describes how a neuron increases its firing rate in response to hypercapnia and can be thought of as an “accelerator” pathway.
FIGURE 2:

A single neuron's response to hypercapnia (accelerator model).

Diagram: Diagram showing the interaction of pH, H^+, CO_2/H^+, NHE, Na^+, K^+, and neuron firing rate. The diagram includes the chemical reactions involving H_2O, CO_2, H_2CO_3, HCO_3^-, and H^+. The pathway shows how changes in pH initiate a series of reactions leading to an increase in neuron firing rate.
Figure 3 A and B: Model for the hypercapnic activation of both an accelerator and brake pathway in a central chemosensitive neuron. Left side represents a: summary of the proposed role of the accelerator pathway involving the pH modification of various cation channels, including the inhibition of K⁺ channels, depolarization and an increase in the firing rate in the chemosensitive neuron (as in the LC). Right side depicts b: a brake pathway involving the HCO₃⁻ activation (1) of Ca²⁺ channels (2) to increase BK current (5). The brake pathway may exist to control the firing rate response to hypercapnia. Activation of Ca²⁺ channels can also contribute to the accelerator pathway (3) but the resulting increase in intracellular Ca²⁺ could result in neuronal death (4). The ultimate firing rate response to hypercapnia can be seen as the balance between the accelerator (a) and the brake (b) pathways. Figure is acquired from Imber & Putnam, *J Appl Physiol* (1985). 2012 May 15; 112(10): 1715–1726.
FIGURE 3:

Figure 3A. Accelerator

Figure 3B. Brake
response to CO₂ in chemosensitive neurons such as those in the LC (Imber et al., 2014; Patrone et al., 2014) (Fig 3B). Thus, the BK channel acts as a “brake” to the chemosensitive firing rate response of LC neurons to CO₂ and therefore potentially to the HCVR of rats to CO₂. The mechanism behind this “brake” and “accelerator” model is that the actual firing rate response of chemosensitive neurons would arise from the balance between the accelerator and the brake, as might the HCVR.

In summary, the response of central chemosensitive neurons to changes in CO₂/H⁺ appears to involve multiple signaling pathways and, multiple ion channels are the targets of these various signals that result in the total neuronal response to CO₂/H⁺. Furthermore, the CO₂/H⁺-sensitive modulation of the “brake” and the “accelerator” work to regulate breathing by altering the chemosensitive-firing rate of neurons that are involved in central chemoreception.

**Fear and CO₂**

Elevated CO₂ leads to arousal from sleep and increased vigilance (Haxhiu et al. 2001; Johnson et al. 2005; Williams et al. 2007; Buchanan & Richerson, 2010). These are well known responses to warn us that either a dangerous buildup of atmospheric CO₂ has occurred, or ventilation is inadequate. Furthermore, the CO₂ sensitivity of many acid-sensitive neurons in the brain contributes to numerous pathological conditions including learning and memory disorders, and psychiatric disorders such as panic disorder (PD) and depression (Pineda & Aghajanian, 1997; Bayliss et al., 2001; Putnam et al., 2004; Wemmie et al., 2004; Coryell et al., 2009; Ziemann et al., 2009). CO₂ inhalation, a biological test and clinical indicator of PD, induces extreme fear and panic attacks in predisposed persons (Papp
et al., 1993; Bailey et al., 2003; Berridge & Waterhouse, 2003; Rassovsky et al., 2003; Jedema & Grace, 2004; Nardi et al., 2009; Colasanti et al., 2012). In spontaneous panic attacks, the most typical ailments are respiratory symptoms (Schruers, et al., 2004; Colasanti, et al., 2008). Importantly, PD patients experience more anxiety to elevated \( \text{CO}_2 \) than healthy controls (Papp et al., 1993; Nardi et al., 2009).

Based on hypercapnia resulting in both increased panic and ventilation, it seems likely that some overlap probably exists between chemosensitive neurons that control breathing and panic resulting from elevated \( \text{CO}_2 \). However, the exact identity of \( \text{CO}_2 \)-sensing structures that lead to \( \text{CO}_2 \)-evoked fear remains unclear. Furthermore, the chemosensitive neurons that are involved in the events that cause panic are likely to be a complex system involving many brain locations. Nevertheless, the LC is a brain site with \( \text{CO}_2/\text{H}^+ \)-sensitive neurons that serves both respiration (Putnam, et al., 2004) and panic and anxiety responses (Bailey, et al., 2003; Williams, et al., 2007).

**The Amygdala, ASIC1a and Fear**

For humans and other animals, some fears seem to be involuntary and innate rather than learned (e.g. being afraid of snakes or large carnivores) (Wemmie et al., 2004). Innate or instinctive fear responses can also be evoked by stimuli that produce an internal threat to homeostasis and imminent danger to survival. For example, \( \text{CO}_2 \) inhalation is a stimulus that, as stated in the last section, produces intense fear (Papp et al., 1993) and autonomic and respiratory responses. Several theories have been proposed to explain how \( \text{CO}_2 \) inhalation produces fear and panic (Drury, 1918; Sanderson et al., 1989; Klein, 1993; Gorman et al., 2000) however the amygdala is known to have neurons that monitor \( \text{CO}_2/\text{H}^+ \) (Wemmie et
al., 2004) and is also implicated in fear and anxiety behavior. The other type of fear is conditioned fear which is learned when an aversive stimulus (e.g., a shock, loud noise, or unpleasant odor) is associated with a neutral context (e.g., a room) or neutral stimulus (e.g., a tone). This eventually results in fear responses to the originally neutral stimulus or context alone (Maren, 2001). The amygdala plays a critical role for both innate and conditioned fear behavior (Bechara et al., 1995; Goosens and Maren, 2001; Adolphs, 2002; Rosen, 2004; Anglada-Figueroa & Quirk, 2005; Phelps & LeDoux, 2005; Kim & Jung, 2006; Maren, 2008; Rabinak & Maren, 2008). Furthermore, the amygdala is central for processing and directing the inputs and outputs that are key to fear behavior. Traumatic memories stored in the hippocampus can stimulate the amygdala and cause it to produce a fear reaction, which is a feature of posttraumatic stress disorder (Ziemann et al., 2009).

The acid sensing ion channel (ASIC) is made of subunits that join together as trimers (Jasti et al., 2007) to form channels. These channels are permeable to Na\(^+\) and Ca\(^{2+}\) (Waldmann et al., 1997; Xiong et al., 2004; Yermolaieva et al., 2004). Additionally, synaptic vesicles release protons (DeVries, 2001; Palmer et al., 2003), and intense neural activity decreases pH. In central neurons ASIC1a is distributed to dendritic spines where it increases Ca\(^{2+}\) concentration in reaction to low pH (Zha et al., 2006) enabling dendritic spines to respond to acid pH. Thus, synaptically released protons might activate ASIC1a.

Moreover, the amygdala can sense acid through ASIC1a (Ziemann et al., 2009) since it is particularly abundant in the amygdala and other fear circuit structures (Wemmie et al., 2003; Coryell et al., 2007). Also, the expression of ASIC1a in the amygdala is required for innate and conditioned fear responses (Wemmie et al., 2003; Wemmie, 2004; Coryell et al.,
ASIC1a in the amygdala detects a reduced pH from increased CO₂ or from a direct injection of acid, which triggers ASIC1a in the amygdala to elicit fear. Additionally, buffering with HCO₃⁻ administration attenuates fear conditioning and innate fear, indicating that ASIC1a action is also pH-dependent. Furthermore, eliminating or inhibiting ASIC1a decreases CO₂ induced fear behavior in mice caused by 10% CO₂ inhalation and reduces fear in conditioning paradigms (Wemmie et al., 2003; Wemmie, 2004; Coryell et al., 2007; Coryell et al., 2008; Ziemann et al., 2009). Also overexpressing ASIC1a increases fear conditioning (Wemmie et al., 2004), which is consistent with a function in fear learning and memory. Importantly, however, knocking out ASIC1a did not alter the ventilatory response to inspired CO₂ or arterial CO₂ levels, suggesting that there are additional sites of CO₂/pH sensors in the brain. Other pH-sensitive receptors may include K⁺ channels (Trapp et al., 2008), pH-sensitive TRP channels (Huang et al., 2006), or other pH-sensitive channels.

To summarize, in this model at least, pH induced fear and pH induced hyperventilation involve distinct pathways. The amygdala has neurons that sense and monitor CO₂/H⁺ through ASIC1a as ASIC1a action is pH-dependent. Additionally, the expression of ASIC1a in the amygdala is critical for innate and conditioned fear responses.

**Case Study**

In animal studies it has been found that amygdala ablation interferes with conditioned fear and other forms of fear and anxiety-related behaviors (Davis et al., 2010). The amygdala also detects potential danger in the external environment and physiologically prepares the organism to confront the threat (Funayama et al., 2001). Studies on humans with amygdala
damage showed they have abnormal fear reactions and a reduced experience of fear (Broks et al., 1998; Sprengelmeyer et al., 1999; Hurlemann et al., 2009). This led to the suggestion that the amygdala is essential for fear and anxiety. A case study was performed on a patient, SM, who had focal bilateral amygdala lesions (Adolphs et al., 1994). While SM was able to display other basic emotions and experience the related feelings, she never recognized feeling more than minimal levels of fear, and, failed to exhibit fear behaviors (Feinstein et al., 2011). Furthermore, studies have shown that SM did not recognize fearful faces (Adolphs & Tranel, 2001) or condition to aversive stimuli (Bechara, et al. 1995). SM had a complete absence of all fear, even in life threatening situations. However in another study of SM and two other rare patients with bilateral amygdala damage, astonishingly, it was found that inhalation of 35% CO$_2$ evoked fear and panic attacks in all three patients (Feinstein et al., 2013)! This experience of fear was the first for SM since she was a child. These results indicate that there may be an important difference between fear triggered by external threats from the environment versus fear triggered internally by CO$_2$. This may happen by CO$_2$ directly activating CO$_2$/pH-sensitive chemoreceptors, including acid-sensing ion channels in extra-amygdalar brain structures that underlie fear and panic such as the LC, RTN, NTS or SFO perhaps linking breathing and anxiety (my hypothesis, see below).

This study concludes that CO$_2$ is an internal threat detected and translated as fear and panic, which does not require an intact amygdala. **CO$_2$ is a unique and potent stimulator for breathing and fear; furthermore they share a common pathway in the LC.** In this study I want to discover if hypercapnia-induced panic and hyperventilation share common sensory pathways. To find out if there is a link between breathing and fear, two different animal models were used in my study: T-cell death-associated gene 8 (TDAG8) KO and BK
KO mice. The TDAG8 KO mice have attenuated conditioned and innate fear phenotypes (Vollmer et al., 2016) and the BK KO rats have an increase in HCVR (Patrone et al., 2014). Now I will describe more about these mice in the next two sections.

TDAG8 KO mice

The pH-sensing TDAG8 G-protein coupled receptor is located on microglia that are abundant in the SFO (circumventricular organ) (Vollmer et al., 2016). CVOs are areas that are devoid of a blood brain barrier and have access to the CNS, thus the SFO helps with the maintenance of homeostasis (Johnson, A.K. & Gross, 1993). In mouse macrophages, the TDAG8 receptor stimulates pro-inflammatory molecules and cytokine production such as interleukin 1 beta (IL1 beta) through the Gs protein/cAMP/PKA signaling pathway (Vollmer et al., 2016). Microglial TDAG8 has been shown to be a chemosensor for sensing and translating hypercarbia to fear (Fig 4). CO2-inhalation evoked freezing and contextual conditioned fear to CO2 is activated via this G protein/cAMP/PKA pathway (Fig 5) (Vollmer et al., 2016).

Thus, TDAG8 KO mice have been found to have an attenuation of the anxiety response to high CO2, (Vollmer et al., 2016) however whether the HCVR is altered in TDAG8 KO mice has not been studied. Since the most typical ailments in panic disorder are respiratory symptoms, we suggest that knocking out the TDAG8 receptor in mice will lead to a decreased HCVR. In this study, using plethysmography we examine the effect of the presence or absence of the TDAG8 receptor on the HCVR, to assess whether the TDAG8 KO mice have a change in both respiratory and anxiety responses to hypercapnia.
Figure 4: TDAG8 and CO₂-evoked fear. (1) The chemosensory signal acidosis (H⁺) generated from CO₂ activates (2) microglial acid sensing TDAG8 receptors which trigger (3) the pro-inflammatory cytokine IL-1β in the microglia. This leads to neuronal activation in the SFO (4), which has efferent projections to areas that regulate cardiovascular and behavioral responses, causing fear behaviors such as freezing and panic. This figure is acquired from Vollmer et al. *Biological Psychiatry* October 1, 2016; 80: p. 545.
FIGURE 4:

Activation of microglial acid sensing TDAG8 receptor
**Figure 5:** Fear-like behavioral responses to CO₂ inhalation are significantly attenuated in TDAG8-deficient (KO) mice. After acclimating to the setup, mice were exposed to either 5 or 10% CO₂ in which fear-like freezing behavior was scored for a 10 min breathing period. In both WT and KO TDAG8 mice, only minor freezing was observed in the air only inhalation test. In both the 5 and 10% CO₂ challenge, there was significant attenuation of CO₂-evoked freezing in TDAG8 KO mice. This figure is adapted from Vollmer et al. *Biological Psychiatry* October 1, 2016; 80: p. 545.
FIGURE 5:

TDAG8 KO attenuated fear-like behavioral response to CO₂

Freezing (%)

Air 5%CO₂ 10%CO₂

TDAG8WT TDAG8KO

*
**BK KO Mice**

It has previously been shown that inhibition of BK channels results in an increased HCVR. Inhibiting the BK channel in the LC, by bilateral injections of Paxilline (a BK channel inhibitor) into the LC resulted in increased ventilation due to hypercapnia (Patrone et al., 2014). Therefore we propose that if the BK channel is knocked out in mice, this will lead to an increased HCVR that may be due to the loss of the “braking” effect of BK channels. In this experiment we have chosen to use BK KO mice and BK WT mice to study the HCVR and anxiety. Using multiple techniques including plethysmography, open field test (OFT), acoustic startle response (ASR), elevated plus maze (EPM), and an activity test we examine if the hypercapnic ventilatory phenotype is altered in BK mice and also assess if global KO of BK channels in mice has an effect on anxiety. In this way we can assess whether the effects of hypercapnia on both the HCVR and on anxiety are altered in BK KO mice. If so, this suggests that similar CO₂ sensing pathways are involved in the control of the HCVR and in the etiology of anxiety.

In summary, TDAG8 KO and BK KO mice are two appropriate models to use to look for a potential link between the HCVR and CO₂ evoked fear/ anxiety. We know that TDAG8 KO mice have been found to have a lower anxiety phenotype, however their HCVR has not yet been looked at. Also, we know that inhibition of BK channels in the LC results in an increased HCVR however we do not yet know if this has an effect on CO₂ evoked fear.

**Significance**

This study emphasizes a potential role of the TDAG8 receptor in the HCVR and a potential role for the BK channel in both the HCVR and CO₂ evoked fear/ anxiety. The fact
that the TDAG8 receptor and the BK channel can be involved in the HCVR and PD is significant since a TDAG8 receptor blocker or BK channel activator could lead to effective treatment for PD. Similarly, an activator of TDAG8 receptor or a BK channel blocker could serve to increase ventilatory drive in response to elevated CO$_2$. Thus abnormalities in the TDAG8 receptor or the BK channel for example may contribute to PD or breathing pathology.

There is evidence linking the pathology of PD to the HCVR, including an enhanced sensitivity to hypercapnia. Also there are many acid-sensing areas that are involved with both the expression of anxiety and the control of breathing such as the NTS (Ghosal et al., 2014) and the LC (Redmond & Huang, 1979). The TDAG8 receptor has been identified as a chemosensor that contributes to the behavioral (freezing) and physiological effects of CO$_2$ inhalation (Vollmer et al., 2016). Also in rats, inhibiting the BK channel in the LC increases ventilation due to hypercapnia (Patrone et al., 2014). Accordingly, abnormalities in the TDAG8 receptor or BK channel may have direct implications for the expression of PD or the HCVR.

Thus there is a great need to better distinguish the pathways involved in CO$_2$ induced fear and the HCVR. My hypothesis is that the TDAG8 receptor and the BK channel play a significant role in the HCVR. I also hypothesize that the BK channel is important in CO$_2$ induced fear. The involvement of both TDAG8 receptors and BK channels in the expression of PD in response to the hypercarbia and the HCVR within the same animals would imply common sensory elements involved in both the HCVR and the generation of fear/ anxiety.
HYPOTHESIS & SPECIFIC AIM
Hypothesis

It has been shown (Vollmer et al., 2016) that anxiety produced by hypercapnia is reduced in TDAG8 KO mice (Fig 4). I hypothesize that TDAG8 KO mice will also have a reduced HCVR and be more active than TDAG8 WT mice due to reduced fear behavior (freezing).

Inhibition of BK channels in LC neurons has been shown to result in increased LC neuronal firing rate in response to hypercapnia (Imber et al., 2014) and an increase in the HCVR (Patrone et al., 2014). I therefore hypothesize that BK KO mice will have a larger HCVR than BK WT mice. I further hypothesize that BK KO mice will have a larger anxiety response to hypercapnia and overall decreased activity than BK WT mice.

Specific Aim 1: TDAG8 KO MICE

Specific Aim 1A: Determine the HCVR in TDAG8 KO vs. WT mice. I will measure the HCVR and the metabolism (oxygen consumption) in male TDAG8 KO and WT mice in response to breathing room air vs. hypercapnia (5% CO₂) gas. I will use whole body plethysmography to determine the HCVR and measures of \( \dot{V}O_2 \) to determine metabolic rate in mice. I expect that the HCVR will be lower in TDAG8 KO mice compared to WT mice but that the metabolic rate will be similar in both types of mice under all conditions.
Specific Aim 1B: Measure the total activity in TDAG8 KO vs. WT mice. I will measure the total distance moved and the velocity moved in male TDAG8 KO and WT mice while breathing room air. I will use a USB video camera and the ShuttleSoft software to measure the distance and velocity traveled by each mouse in a large container over a 30-minute period. *I hypothesize that the activity level of the TDAG8 KO mice will be higher than the activity level of the TDAG8 WT mice.*

Specific Aim 2: BK KO MICE

Specific Aim 2A: Determine the HCVR in BK KO vs. WT mice. I will measure the HCVR and the metabolism (oxygen consumption) in male BK KO and WT mice in response to breathing room air vs. hypercapnia (7% CO₂) gas. I will use whole body plethysmography to determine the HCVR and measures of $\dot{V}O_2$ to determine metabolic rate in mice. *I expect that the HCVR will be higher in BK KO mice compared to WT mice but that the metabolic rate will be similar in both types of mice under all conditions.*

Specific Aim 2B: Measure the total activity in BK KO vs. WT mice. I will measure the total distance moved and the velocity moved in male BK KO and WT mice while breathing room air. I will use a USB video camera and the ShuttleSoft software to measure the distance and velocity traveled by each mouse in a large container over a 30-minute period. *I hypothesize that the activity level of the BK KO mice will be lower than the activity of the BK WT mice.*
**Specific Aim 2C:** Determine the generalized anxiety level of and the CO$_2$-induced anxiety in BK KO mice compared to BK WT mice. I will use the Open Field Test (OFT) with room air to evaluate generalized anxiety levels of BK KO vs. BK WT mice. I will also use the Elevated Plus Maze (EPM) and the Acoustic Startle Response (ASR) to assess anxiety in room air in BK KO and WT mice. To measure CO$_2$-induced anxiety I will use the OFT filled with room air or with 10% CO$_2$ (balance room air) to determine the anxiety level of BK KO and WT mice. *I hypothesize that the BK KO mice will exhibit more anxiety during the 10% CO$_2$ than the BK WT mice.*
GENERAL METHODS
Animals

Mice were kept on a 12-hr light-dark cycle and received standard chow (LM-485; Teklab, Madison, Wisconsin) and water ad libitum. All procedures involving the use of animals were reviewed and approved by the Wright State University Institutional Animal Care and Use Committee. These procedures are in accord with the standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. The Association for Assessment and Accreditation of Laboratory Animal Care has accredited Wright State University, which is covered by National Institutes of Health assurance (no. A3632-01). Experiments were done on either wild type or TDAG8 knock-out adult male BALB c mice age 8 -13 weeks housed individually until used. Also experiments were done on either wild type or BK knock-out adult male C57BL6J mice age 4 -19 weeks housed individually or with 1-3 others until used.

Elevated Plus Maze (EPM)

Anxiety-like behavior was assessed in 15 male BK KO and 22 male BK WT mice using the EPM. Data acquisition and analysis were performed automatically, using the Motor Monitor software version 5.05 (Kinder Scientific, Poway, CA, USA). The elevated plus maze consists of two open arms (38 cm long × 5 cm wide) and two closed arms (38 cm long × 5 cm wide × 15 cm tall (walls of the closed arm)) with a central open intersection (5 cm × 5 cm). The entire apparatus is elevated 55 cm off the floor. For testing, each animal was placed on the center square of the maze facing the same open arm. Each mouse’s entry, movement, distance and total time was recorded and scored automatically over a five minute period with equally spaced photocells that are aligned at the bottom of the closed and opened arms of the
EPM (Hamilton-Kinder). Then after five minutes the total time and distance in each type of arm was given. Percentage of time in open arms, and distance traveled in open arms (in) were used to determine the level of anxiety in each mouse. Greater anxiety is indicated by a greater percent of time spent in the closed arms and less time spent in the open arms. Greater anxiety is also indicated by less distance traveled in the open arms and closed arms.

**Acoustic Startle Response**

The ASR test was used to test for general anxiety or possible motor deficit of BKWT and BKKO mice. Reflexive behavior was measured (n = 9 BK WT, 8 BK KO) using the Startle Monitor System (Kinder Scientific, Poway, CA, USA) and Startle Monitor software (Kinder Scientific). Startle testing was conducted in ventilated and sound attenuated startle boxes where animals were placed into small, non-restrictive, Plexiglas encasements mounted on a movement sensitive platform within the chamber. A high-frequency loudspeaker inside the chamber produced both a continuous background noise of 65 dB and the acoustic stimuli of 105db for 20ms. Data were recorded using load cell platforms, which measure actual force changes during an animal's jump. The displacement force of the animal was translated directly by a transducer mounted below the platform. This transducer transduced force into an electric signal converting vertical movements of the platform induced by the startle response of the mouse, into a voltage signal and the startle response was measured in Newtons. Each load cell was calibrated with a 100 g weight, which corresponds to 1N of force. These signals were then digitized and stored by a computer and the digitized values were normalized for body weight in all mice. Newton values were recorded from 0 to the positive peak (Fig 6A). The maximum amplitude of the signal was measured in a 250ms time
window after the acoustic stimulus onset (Fig 6B), using the Startle Monitor associated software for the stimulus presentation and recordings. The peak startle amplitude was used as a dependent variable. In this test there were 11 varying and pseudorandomized inter-trial intervals.

Before the actual testing the animals were acclimatized to the startle boxes for 5min on three consecutive days. During the acclimatization periods only the background noise (65dB sound pressure level (SPL) white noise) was presented. On the next day the animals were tested using the following protocol: the animals were acclimatized to the startle box and the background noise for 5min. Subsequently, the startle stimulus (20ms, 105dB SPL white noise) was presented 11 times with pseudorandomized inter-trial intervals on top of the background noise. Each interval time, between each startle stimuli was 4 – 22s in length. The Analog step tells the software when to begin recording and the stimulus pulse begins immediately following this Analog step. The data are recorded for a total of 250ms (Fig 6B). The larger the maximum force that was exerted during startle is interpreted as a greater level of anxiety or fear. The experimental groups in the acoustic startle response were identical to the groups in the elevated plus maze, and activity test.

**Open Field Test**

Mice were placed individually into the center of an open-field chamber with dimensions of 40.6 cm wide x 40.6 cm deep x 38.1 cm high (Hamilton-Kinder). The chamber has a photo beam frame at the bottom with 16 × 16 equally spaced photocells. Diffused light illuminated the chamber at 5.5 lux. The chamber was covered with a clear Plexiglas lid.
**Figure 6:** Measurement of anxiety of adult male BKKO and WT mice. (A) The startle reaction of a mouse. The top graph signifies the acoustic stimulus white noise pulse of 105db for 20ms. The bottom graph denotes the startle response (roughly 100ms after stimulus pulse) of the mouse, which is measured in Newtons. The arrow represents the Newton values that are recorded from 0 to the positive peak of the maximum response. The higher this max peak is, corresponds to a higher level of anxiety/fear. (B) Screenshot from the user interface of the Startle Monitor associated software used to analyze and chart startle response data of a mouse. Panel displays the startle reaction of a mouse. The maximum amplitude peak of the startle response signal (vertical red line) is measured and recorded in the 250ms time window after the acoustic stimulus onset. The stimulus pulse begins immediately following the Analog step (tells the software when to begin recording). The oscillation before the max value is most likely the animal pressing down on the plate in response to the stimuli, then the negative pulse is when the plate is being relieved of the pressure, and then the second positive pulse (max value) is the animal coming back down onto the plate with close to its full weight. The panel displays the 11 pseudorandomized inter-trial intervals (shown in the runtime display at the bottom). The runtime display indicates that trial 11 (in the display window) had a maximum startle response of 0.889 Newton, with an average of 0.219N for all of trial 11 (which is the average of all of the values recorded within that 250ms record window), and a first value in all of trial 11 of 0.035N. In trial 11, the first recorded value was near zero. This helps to show that the animal was in fact steady before the stimulus. The 1st value is the absolute first value that is recorded by the software after the Analog step. Also the display indicates that the maximum startle response occurred at 53ms from the time of the Analog step.
FIGURE 6:

A. Acoustic Stimulation Pulse
(105db, 20ms white noise)

Startle Reaction
(Newton)

B. Graph of - 20ms105db @ Trial 11

<table>
<thead>
<tr>
<th>Trial</th>
<th>Name</th>
<th>Max</th>
<th>@</th>
<th>Ave</th>
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<tbody>
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</tr>
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and pre–filled with compressed air, or CO₂ concentrations similar to those that trigger panic in humans and rodents (10%CO₂, 21%O₂, balance N₂). Behavior in the open-field chamber was assessed for 20 min, and automatically scored using the Motor Monitor software (Hamilton-Kinder) in room air for 10 BKKO and 10 BKWT mice and then in 10% CO₂ for 6 BKWT and 5 BKKO mice. The percentage of time in the center vs. the periphery was measured. The % of center (30.5 cm x 30.5 cm) beam breaks was also measured relative to total beam breaks (both center and periphery). A high level of anxiety would be indicated by a low % of time in the center and a higher % of time in the periphery and relatively few beam breaks. How long the mouse was immobile was another measurement of anxiety. A higher amount of time spent immobile indicates a higher level of anxiety. How often the mouse reared (stood on back 2 feet, an exploratory act) and time spent rearing was also assessed. A high anxiety mouse would have a low amount of time spent rearing and a low number of rearing occurrences.

**Activity data analysis and statistics**

A mouse was placed in a large 25-gal tub 28” W x 28” L x 13” H and allowed to move freely for 30 minutes. A USB video camera and the ShuttleSoft software were used to measure total activity. Two values were determined. The first was the total distance the mouse traveled in the large container over a 30-minute period. The second was how fast the mouse moved during the same time period. In this activity test, an anxious mouse moves less and “freezes” more and a less anxious mouse is more active. In order to try to capture a representative sample of behavior, I assessed each mouse on two different days. For the
TDAG8 mice there was no statistically significant effect of day of measurement on distance or average speed for either genotype (KO n=18, WT n=22). Consequently the day factor was dropped from the analysis, as the genotype differences were the ones of scientific interest. Data from both days were used (two values averaged for each mouse) to calculate both the descriptive and the inferential statistics; sample sizes and degrees of freedom reflect the inclusion of data from both days. All analyses for movement were carried out using the statistical software R version 3.1.2. For the statistics, analysis of variance (ANOVA) was used with genotype as the predictor and speed as the response and then again using genotype as the predictor and distance as the response. Since two ANOVAs were being performed to answer this research question, a Bonferroni correction was applied. This correction is a method used to offset the problem of multiple comparisons. When multiple comparisons are done the chance of making a Type I error increases (i.e., incorrectly rejecting a null hypothesis). The Bonferroni correction compensates for that increase by testing each individual hypothesis at a significance level of the newly calculated alpha. This correction gave a level of significance alpha = 0.025 that was used for all comparisons.

I also conducted this activity test on BKKO and BKWT mice and again, in order to try to capture a representative sample of behavior, I assessed each mouse (n = 12 BKKO, 10 BKWT) on two different days and the values were recorded twice for each mouse, once on the first day and again on a second day. Total distance traveled was analyzed first. There was strong evidence to suggest that the average distance traveled for the mice was significantly different for the two days. It also appeared that both the KO and WT mice were more active in terms of total distance traveled on the first day than the second day. Furthermore, there was not sufficient evidence to suggest that the average velocities of the mice were
significantly different for the two days. Since these data were collected twice, a repeated measures ANOVA was used for the analysis with day as a repeated factor. Also since two ANOVAs were being performed to answer this research question, a Bonferroni correction was again applied. This correction gave a level of significance alpha = 0.025 that was used for both comparisons.

**Whole Body Plethysmography**

Ventilation and metabolism were measured in the same experiment for each mouse. Ventilatory parameters in unrestrained, non-anesthetized mice were measured using the whole-body barometric method of plethysmography ((TDAG8 n = 8WT, 3KO) (BK n = 8WT, 6KO)). O₂ consumption (\(\dot{V}O_2\)) was measured (TDAG8 n = 8WT, 3KO) (BK n = 6WT, 4KO) simultaneously with ventilation using open-flow respirometry. Ventilation and O₂ sampling periods were after the mouse had reached steady state to establish resting ventilation and metabolism. The setup consisted of a 450ml glass chamber with ports in the top for gas inflow and outflow. In addition we sealed a temperature probe (Physitemp HT-1 general purpose) into the top of the chamber to measure chamber temperature. Calibration of the O₂ was done with dry room air (20.93% O₂, 0.03% CO₂, 78.09% N₂) and then with commercially available gas (10% CO₂ 16% O₂, balanced with N₂). Air was pumped through the inflow line by a Marina 100 Aquarium Air Pump. An air stone in a 125 ml Erlenmeyer flask containing 100ml distilled water saturated the inflow air. The inflow air then was fed to an Aalborg rotameter (GMR3-010022) for the calibration and adjustment of the rate of airflow (VE) to 500ml/min, before entering the chamber. This humidified incoming gas was then pulled through the chamber containing the animal
at VE = 500 mL/min. The outflow air passed through a Millipore in-line filter (6 cm diameter) connected to an in-line TSI 4100 Series mass flow meter. This filter removed debris from the air flowing from the chamber, protecting the mass flow meter. This TSI 4100 Series mass flow meter was used as a more accurate digital reading of the airflow. This meter also measured and digitally displayed the ambient pressure in the room. Outflow air was then drawn through a 10ml syringe filled with desiccant (DM-AR; Perma pure LLC, NJ, USA) and then through an infrared O₂ analyzer (S-3A/I; AEI Technologies Inc., PA, USA). Additionally, by way of another port in the top of the glass chamber, a tube connected the chamber to a differential pressure transducer (TSD 160A, Biopac Systems), which measured humidified, breathing induced changes in pressure. The signal from this transducer went to a differential pressure signal conditioner (DA 100C, Biopac Systems), an A/D signal converter and was stored in digital form with Biopac System’s MP100ACE data acquisition software. The sampling frequency was 200 samples s⁻¹. Biopac System’s Acknowledge (v3.8.1) data acquisition program was used for data analysis.

Ventilation and metabolism experiments were performed between the hours of 9:00am and 3:00pm. A mouse was placed individually into the chamber and was allowed to acclimate 30 minutes daily for 3 days prior to the testing. During the acclimation periods, room air flowed through the chamber continuously at ~500 ml/min. During both acclimation and testing periods, the chamber was placed in a water bath to help maintain a more constant external temperature. On the testing day, a mouse was put in the chamber and allowed to acclimate for 30 min with room air flowing through the chamber before testing began. At the end of the acclimation period, airflow was turned off for 1 min. This was followed by two periods of airflow for 5min followed by no airflow for 1 min. The inflow air
was shifted from room air to room air containing 5% CO₂ (for TDAG8) or 7% CO₂ (for BK), (derived from a tank containing gas with 5% CO₂ or 7% CO₂, 21% O₂, balance N₂) (hypercapnia). Exposure to hypercapnia consisted of 3 bouts of airflow on for 5min followed by airflow off for 1 min. After this 18min time period the mouse was exposed to hypercapnia for an additional 15min with air flow on and then air flow was turned off for another 1min period. The hypercapnia exposure was followed by a 15min exposure to room air again with airflow on followed by a 1min period with airflow off. Finally, we had two additional periods of room air exposure with airflow on for 5min and off for 1 min each. Thus, the mouse was in the chamber for a total of 105min. The airflow was checked during every airflow period and adjusted to 500ml/min when necessary. The temperature of the chamber was measured for every airflow off period, just before or just after the airflow off period, and its value was used in the calculation for each point at that time. The temperature of the water bath was measured one time for three off bouts in room air and then once in hypercapnia and then once in room air again, using the glass thermometer. These values were used as the room air temperature in the calculation for the tidal volume of the mouse breath. At the end of the experiment, we turned off inflow and outflow lines and did several volume calibration injections by injecting and withdrawing known volumes with a calibrated syringe before finally removing the mouse from the chamber and measuring the mouse’s body temperature rectally with a RET-3 Physitemp mouse rectal probe. The mouse was then weighed and returned to its housing.
**VO₂ Data analysis**

Measurement for VO₂ was taken, using an open flow system (during the flow-on period), close to when each breathing segment was measured; three times before hypercapnia, four times during hypercapnia and then three times after hypercapnia (during recovery). The fractional incurrent O₂ (FIO₂) was measured during open-flow respirometry by disconnecting the inflowing line from the chamber and connecting that inflowing line directly to the gas analyzer. Incurrent air is defined as the air that is flowing into the chamber that is measured and used to determine what amount of O₂ the mouse breaths in. Excurrent air is the air that is flowing out of the chamber and is measured and used to determine the value of the amount of O₂ the mouse breaths out. VO₂ values (TDAG8 n = 8WT, 3KO) (BK n = 6WT, 4KO) were obtained by determining fractional differences between incurrent and excurrent O₂, respectively, multiplied by the flow rate (VE), \( \dot{V}O₂ = VE * (FIO₂ - FEO₂) / (1 - FIO₂) \) (Frappell et al., 1992) and then divided by the body weight of the mouse. This value is expressed as mL/O₂/100g/min STPD. VO₂ was collected with the gas flow on. At the same time in the same animal with the gas flow off, we measured the \( \dot{V}E \), which is explained in the next section.

**Plethysmography data analysis and statistics**

The pressure records were analyzed during the periods in which the airflow was off. We looked for segments of 3 or more seconds when ventilation was regular and no gross body movements were observed. The data were analyzed during awake states. For each mouse, while initially breathing room air we analyzed 2 to 3 segments of stable breathing
with air flow off; then while breathing a hypercapnic gas mixture containing 7% CO₂, 21% O₂, balance N₂ (BK mice) or 5% CO₂, 21% O₂, balance N₂ (TDAG8 mice). We analyzed at least 1 stable breathing segment for each of the three 1 min air off periods which followed 5 min of hypercapnic air flow and for the last 1 min air off period following the 15-min period of hypercapnic air flow; upon return to breathing normocapnia we analyzed at least one stable breathing segment for each air off period. For each breathing segment, the respiratory parameters from the pressure signal (tidal volume (Vₜ), respiration frequency (Fᵣ), and \( \dot{V}_E = V_T \times F_R \)) were analyzed. In all cases, plethysmograph data for the Vₜ were normalized to the weight of the mouse except for Fᵣ. Fᵣ was calculated from the number of breathing pulses per time. Tidal volume (Vₜ) was calculated from the equation of Malan (1973):

\[
V_T = V_K \times P_T/P_K \times T_A/T_R \times (P_B - P_C)/\left[ \left( (P_B - P_C) - (T_A/T_b \times (P_B - P_R)) \right) \right]
\]

Vₚ is the volume of the calibration injection (100 µl), Pₜ is the pressure deflection associated with each breath, Pₚ is the pressure deflection associated with the injection of the calibration volume, Tₐ is the temperature in the animal chamber (average for all mice: 23.9 ± 0.3 °C), Tᵣ is the temperature in the water bath (average for all mice: 22.3 ± 0.4 °C), Pₜ is the barometric pressure, Pₙ is the water vapor pressure in the animal chamber, Tₚ is the body temperature of the mouse (average for all mice: 35.3 ± 0.3 °C; range: 34.1-36.4 °C), and Pᵣ is the water vapor pressure at Tₚ. The water vapor pressure at Tₐ and Tₚ were calculated indirectly using an appropriate table (Dejours, 1982). The calculated value for Vₜ was multiplied by our calculated value for Fᵣ for the same mouse to obtain minute ventilation. This was normalized for body mass by dividing by the mouse’s body weight and multiplying by 100 g so that the final units for our \( \dot{V}_E \) values were ((mL/min)/100 g).
Statistics

For the TDAG8KO and WT mice while comparing the ventilation ($\dot{V}_E$), metabolism ($\dot{V}O_2$) and respiration ratio ($\dot{V}_E / \dot{V}O_2$) we used independent samples t-test; differences were considered significant if $P < 0.05$. The values in the figures are expressed as mean ± S.E.M. * indicates a significant difference between the control group (WT) and the knockout group (KO).

For the activity tests on the TDAG8KO and WT mice analysis of variance (ANOVA) was used using genotype as the predictor and velocity as the response, and then again using genotype as the predictor and distance as the response. Since two ANOVAs were being performed to answer this research question, a Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for all comparisons. Differences were considered significant if $P < 0.025$. The values in the figures are expressed as mean and range. * indicates a significant difference between the control group (WT) and the knockout group (KO).

For ventilation in BKKO and WT mice we used two-way ANOVA and differences were considered significant if $P < 0.05$. The values in the figures are expressed as mean ± S.E.M. *** indicates a significant difference between the control group (WT) and the knockout group (KO) at $P < 0.001$ and ** indicates a significant difference between the control group (WT) and the knockout group (KO) at $P < 0.005$. 
For the metabolism test in the BKKO and WT mice and while comparing the respiration ratio \( (\dot{V}_E / \dot{V}O_2) \) we used a two-way ANOVA; \( P < 0.05 \). The values in the figures are expressed as mean ± S.E.M. * indicates a significant difference between the WT group and the KO group.

For the metabolism test in the BKKO and WT mice and while comparing the respiration ratio \( (\dot{V}_E / \dot{V}O_2) \) we used a two-way ANOVA; \( P < 0.05 \). The values in the figures are expressed as mean ± S.E.M. * indicates a significant difference between the WT group and the KO group.

For the BKKO and WT mice Activity tests, since these data were collected twice, a repeated measures ANOVA was used for the analysis with day as a repeated factor. Since two repeated measures ANOVAs (genotype and velocity and then genotype and distance) were being performed to answer this research question, a Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for both comparisons. The values in the figures are expressed as mean ± S.E.M. * indicates a significant difference between the WT group and the KO group.

To test for general anxiety and possible motor deficits in the BKKO and WT mice we used the Acoustic Startle Response (ASR) test. We did a two-way, repeated measures ANOVA. The values in the figures are expressed as mean + S.E.M. ** indicates a significant difference between the WT group and the KO group (\( P < 0.01 \)). The scores are normalized by the mouse’s weight.

For anxiety measures in the BKKO and WT mice we used the Elevated Plus Maze (EPM) test. We did a one-way ANOVA for mouse type. Differences were considered significant if \( P \)
< 0.05. The values in the figures are expressed as mean and range. ** indicates a significant difference between mouse type (P < 0.01; one-way ANOVA, for mouse type).

For the BKKO and WT mice Open Field Test (OFT) we used two-way ANOVAs (factor one: mouse type; factor two: air type). A Bonferroni correction was applied and the corrected level gave a significance alpha = 0.00555 (0.05/9 ANOVA tests). The values in the figures are expressed as mean and range. Given the Bonferroni correction, ** indicates a significant difference between mouse type or air type at 0.001 ≤ P ≤ 0.005. *** indicates a significant difference between mouse type or air type at P < 0.001.
RESULTS
TDAG8 in ventilatory, metabolic and activity level of adult mice.

The role of the TDAG8 receptor in ventilation and metabolism in room air and hypercapnia in non-anesthetized adult male mice.

Figure 7 represents the values of tidal volume ($V_T$), breathing frequency ($f_R$) and minute (pulmonary) ventilation ($\dot{V}_E$) in TDAG8 WT and KO mice during hypercapnia. As expected when inspired air was switched from room air to hypercapnia (5% CO$_2$), hypercapnia resulted in a significant mean increase (108% for WT, 64% for KO) in $\dot{V}_E$ (HCVR) observed in both samples of mice ($n =$ 8WT, 3KO). This increase was due to a significant mean increase in the $V_T$ values in room air vs hypercapnia in the KO mice, and in both the $f_R$ and $V_T$ in the WT mice. For both KO and WT mice this increase in $\dot{V}_E$ was not significantly different from each other. Upon return to room air, $\dot{V}_E$ was restored to initial values in both genotypes with no significant differences. Also, no significant difference was noted for minute ventilation $\dot{V}_E$ breathing frequency $f_R$ or tidal volume $V_t$ between TDAG8KO vs WT mice in hypercapnia. For $\dot{V}_E$, WT mice: 521.0 ± 42.7 vs. KO mice: 546.2 ± 26.8; values in mL / 100g / min $P > 0.05$; independent samples t-test).

In relation to the consumption of oxygen $\dot{V}O_2$, hypercapnia did not result in changes in metabolism (Fig 8A). Additionally, no significant difference was observed between the WT and KO mice when exposed to hypercapnia except a one time point about 30 minutes after the exposure to hypercapnia (Figure 8A). While comparing the respiration ratio ($\dot{V}_E / \dot{V}O_2$) there were no significant differences observed between the WT and KO mice after exposure to hypercapnia (Fig 8B) (15 minutes into hypercapnia, WT mice: 177.7 ± 51.9 vs. KO mice: 121.9 ± 3.42, $P > 0.05$; independent samples t-test). Thus, these data suggest that TDAG8 is not involved in the HCVR.
Figure 7: Measurement of ventilation. (A) Tidal volume (VT): volume of a single breath, (B) the respiratory frequency rate (fR), in breaths/min and (C) minute ventilation (VE) the product of VT and fR, of adult male TDAG8 wild type (WT) and knockout mice (KO). The start and duration of hypercapnia is shown in the graph. All values are expressed as mean ± SEM. Significant differences between means for KO vs WT mice were determined by independent samples t-tests. Differences were considered significant if P < 0.05.
FIGURE 7:

A) Measurement of Ventilation in TDAG8 WT vs. KO mice using Plethysmography. 
   A. Tidal Volume $V_T$ (ml/100g). 
   B. Breathing Frequency $f_R$ (breaths/min). 
   C. Minute Ventilation $V_E$ (ml/100g/min).

- $V_T$ (ml/100g)  
  - WT (n=8) 
  - KO (n=3)

- $f_R$ (breaths min$^{-1}$)

- $V_E$ (ml 100g$^{-1}$ min$^{-1}$)  
  - 5% CO$_2$

- Time (min)

B) 

C)
**Figure 8:** (A) Measurement of oxygen consumption ($\dot{V}O_2$) of adult male wild type (WT) and knockout (KO) mice. The start and duration of hypercapnia is signaled in the graph. The values are expressed as mean ± S.E.M. (B) Ventilation oxygen consumption ratio ($\dot{V}_E / \dot{V}O_2$) of adult male mice during hypercapnia. The start and duration of hypercapnic exposure is shown in the chart. The values are expressed as mean ± S.E.M. * indicates a significant difference between the WT group and the KO group (P < 0.05, independent samples t-test).
FIGURE 8:

A)

![Graph showing VO₂ (mL/100g/min) over time with 5% CO₂ and the difference between WT (n=8) and KO (n=3)].

* p = 0.0193

B)

![Graph showing VE/VO₂ over time with 5% CO₂ and the difference between WT (n=8) and KO (n=3)].
The role of the TDAG8 receptor on the activity level of adult male mice.

Since two ANOVAs (using genotype as the predictor and velocity as the response, and then again using genotype as the predictor and distance as the response) were being performed to answer this research question, a Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for all comparisons. Genotype had a statistically significant effect on the distance traveled in a 30 minute period (Fig 9B; F(1,38) = 6.132, P = 0.0164) with the wild-type mice moving further (mean = 10185 cm, SEM = 350 cm, n = 22) than the TDAG8 knockout mice (mean = 8008 cm, SEM = 573 cm, n = 18). Genotype also had a statistically significant effect on average speed (Fig 9A; F(1,38) = 5.47, P = 0.0247) with wild-type mice moving faster (mean = 5.66 cm/s , SEM= 0.350 cm/sec, n = 22) than the TDAG8 knockout mice (mean = 4.52 cm/s, SEM = 0.330 cm/sec, n = 18). Thus, to summarize, TDAG8 KO mice moved significantly less and significantly slower (21%) than TDAG8 WT mice.

BK channel in ventilation, metabolism and activity and anxiety level of adult mice.
The role of the BK channel in ventilation and metabolism in room air and hypercapnia in non-anesthetized adult male mice.

Figure 10 represents the values of tidal volume (\(V_T\)), respiratory rate (\(f_R\)) and minute ventilation (\(\dot{V}_E\)) in BKWT and KO mice in room air and in hypercapnia (7% CO\(_2\)).

Hypercapnia resulted in an increase in pulmonary (minute) ventilation in both groups, due to an increase in the \(f_R\) and \(V_T\) (P < 0.001, two-way ANOVA). However, BK channel KO mice showed a significantly increased \(\dot{V}_E\) (about 45%), when compared to the control group (at 10 minutes into hypercapnia (Fig 10C); control mice: 803.8 ± 66.8 vs. knockout mice:
**Figure 9A and B:** The (A) average velocity and (B) total distance that the TDAG8 WT and KO mice moved during an activity test in room air. Mouse genotype had a statistically significant effect on both average speed and distance traveled within a confined area in 30 minutes. The wild-type mice moved further and faster than the TDAG8 knockout mice. A Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for all comparisons. Differences were considered significant if P < 0.025. The values are expressed as mean and range. * indicates a significant difference between the control group (WT) and the knockout group (KO) at p < 0.025.
FIGURE 9:

A)

![Graph A: Velocity (cm sec⁻¹)]

- Velocity (cm sec⁻¹)
- TDAG8 WT
- TDAG8 KO
- WT (n=22)
- KO (n=18)

B)

![Graph B: Distance (cm)]

- Distance (cm)
- TDAG8 WT
- TDAG8 KO
**Figure 10:** Measurement of ventilation. (A) Tidal volume \( (V_T) \): the volume of a single breath adjusted for body weight, (B) the respiratory frequency \( (f_R) \) in breaths/min and (C) minute ventilation \( (\dot{V}_E) \) the product of \( V_T \) and \( f_R \), of adult male BK wild type (WT) and knockout (KO) mice in mL/100/min. The start and duration of hypercapnia is indicated in the graph. The values are expressed as mean ± S.E.M. ** indicates a significant difference between the control group (WT) and the knockout group (KO) for \( V_t \) (P< 0.01) and *** indicates a significant difference between the control group (WT) and the knockout group (KO) for \( \dot{V}_E \) (P < 0.001) two-way ANOVA. Notice the BKKO mice have a significantly higher \( \dot{V}_E \) and \( V_T \) than BKWT mice.
FIGURE 10:

A. Tidal Volume of Breath $V_T$ (mL/100g).

B. Frequency of Breath $f_R$ (breaths/min).

C. Minute Ventilation $V_E$ (mL/100g/min).
1162.9 ± 62.8; values in mL/100g/min for \( \dot{V}_E \), \( P < 0.001 \); Two-way ANOVA). It is observed that this ventilatory increase occurred due to the significant increase in \( V_T \) (Fig 10A), which was sustained throughout the period of exposure to CO\(_2\) (\( P < 0.005\), two-way ANOVA), while no significant difference was noted for \( f_R \) between WT and KO mice (Fig 10B). In relation to \( \dot{V}_O_2 \), hypercapnia did not result in metabolic changes except during the first 10 minutes in the chamber. Additionally, no significant difference was observed in \( \dot{V}_O_2 \) between the WT and KO mice during hypercapnia (Figure 11A). While comparing the respiration ratio (\( \dot{V}_E / \dot{V}_O_2 \)), note that the knockout mice showed significantly higher values compared to the wild type mice at times 10, 15, and 30 minutes during hypercapnia (at 15 minutes into hypercapnia; wild type mice: 135.5 ± 23.4 vs. knockout mice: 264.7 ± 37.9, \( P < 0.05\); Two-way ANOVA) (Figure 11B). These findings are consistent with BK channels’ limiting (i.e. braking) HCVR, which then increases when BK channels are knocked out.

*The effect of the BK channel on the activity level of BKWT and BKKO non-anesthetized adult male mice.*

Figure 12 represents the values of the velocity and the total distance traveled over a 30-minute period. Two ANOVAs were performed (one for distance and one for velocity). Based on \( P = 0.0201 \) for genotype there is strong evidence to suggest that the average velocity for BKKO mice (mean = 6.10 cm/sec, SEM = 0.70 cm/sec, \( n = 12 \)) is significantly higher than the BKWT mice (mean = 3.93 cm/sec, SEM = 0.35 cm/sec, \( n = 10 \)) (Fig 12A). Also genotype had a statistically significant effect on the distance traveled (\( P = 0.0202 \)) with the BKKO mice moving further (mean =10,948 cm, SEM = 1237 cm, \( n = 12 \)) than
**Figure 11:** (A) Measurement of oxygen consumption ($\dot{V}O_2$) of adult male wild type (WT) and knockout (KO) mice. The start and duration of hypercapnia is indicated in the graph. The values are expressed as mean ± S.E.M. (B) Ventilation: oxygen consumption ratio ($\dot{V}E / \dot{V}O_2$) of adult male mice during hypercapnia. The start and duration of exposure is indicated in the chart. The values are expressed as mean ± S.E.M. * indicates a significant difference between the WT group and the KO group (P < 0.05, two-way ANOVA). Notice the BKKO mice have a significantly higher ($\dot{V}E / \dot{V}O_2$) than the BKWT mice during exposure to hypercapnia.
FIGURE 11:

A) Measurement of oxygen consumption in BK WT vs. KO mice. 

B) $\frac{V_{E}}{V_O}$ vs. Time (min) for WT (n=4) and KO (n=6) mice.
Figure 12: BKWT and KO mice activity measured as (A) average velocity (cm sec$^{-1}$) and (B) total distance moved (cm) recorded for 30 minutes in room air. Since these data were collected twice, a repeated measures ANOVA was used for the analysis with day as a repeated factor. Since two ANOVAs (genotype and velocity and then genotype and distance) were being performed to answer this research question, a Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for both comparisons. The values in the figures are expressed as mean and range. * indicates a significant difference between the WT group and the KO group.
FIGURE 12::

A)

![Graph A: Velocity (cm sec⁻¹)]

B)

![Graph B: Distance (cm)]

WT (n=10)
KO (n=12)
the WT mice (mean = 7,358 cm, SEM = 515 cm, n = 10) (Fig 12B). A Bonferroni correction was applied. This correction gave a level of significance alpha = 0.025 that was used for both comparisons. Thus, BKKO mice move faster and further than BKWT mice over a 30-minute test period.

Assessing the effect of BK channel deletion on anxiety, in non-anesthetized adult male mice measured by the Acoustic Startle Response (ASR).

The startle response was affected significantly by genotype (Fig 13, F=12.156 (df = 1, 15), p < 0.01; two-way, repeated measures ANOVA). The data indicate that the overall acoustic startle response of the BKWT mice was larger than the response of the BKKO mice (BKWT: mean = 1.19 (N/100g), SEM = 0.17 (N/100g), n = 9; BKKO: mean = 0.623 (N/100g), SEM = 0.09 (N/100g), n = 8).

The effect of BK channel deletion on anxiety, in non-anesthetized adult male mice measured by the % time spent in the open arms of the Elevated Plus Maze.

Anxiety-like behavior was assessed in BKKO and BKWT male mice using the EPM for a five-minute test period. For testing, each animal was placed on the center square of the maze facing the same open arm. The percentage of time spent in open arms (Fig 14) were recorded and calculated during a 5-min test period. The behaviors of the mice and min-by-min scoring were recorded automatically with equally spaced photocells. Finally using a one-way ANOVA for mouse type, F = 0.049, df = (1, 37), P = 0.826, no significant difference was found between the percent of time spent in open arms for the two types of mice (WT mean = 10.78%, SEM = 2.02%, n = 21; KO mean = 10.04%, SEM = 2.83%, n =15). As
**Figure 13:** Figure 13 represents the response of BKKO and BKWT adult male mice, during the Acoustic Startle Response test, which measures anxiety level or reflects some motor impairment. The values are expressed as mean + SEM. ** indicates a significant difference between the WT group and the KO group (P < 0.01, two-way, repeated measures ANOVA). Notice the BKWT mice have a significantly larger startle response. Scores are normalized by the weight of the mouse.
FIGURE 13:

Average Baseline Startle Amplitude (N/100g)

WT (n=9)

KO (n=8)
Figure 14: The % time spent in open arms of the Elevated Plus Maze in adult male BKWT and BKKO mice. Notice the BKWT mice spent a similar fraction of time in the open arms as BKKO mice, indicating a similar level of anxiety (one-way ANOVA, for mouse type). Differences were considered significant if P < 0.05. The values in the figures are expressed as mean, (horizontal black line) and range.
FIGURE 14:

% Time in Open Arms

BKWT n=21
BKKO n=15
expected, mice spent very little time (10%) in the open arms, spending most of the five minutes (90%) in the closed arms. Thus based on the % time spent in the open arms of the EPM it appears there is no difference in the baseline anxiety in room air between BKWT and BKKO mice.

*The effect of BK channel deletion on anxiety measured by the distance traveled in the open arms of the Elevated Plus Maze.*

Anxiety-like behavior was assessed in BKKO and BKWT male mice using the distance moved in open arms (Fig 15) (measured in inches) in the EPM during a 5-min test period. There are no significant differences between the distance in the open arms of the EPM for the two types of mice (F = 0.222, df = (1,37), P = 0.640; one-way ANOVA) (BK WT total mean = 61.96 in, SEM= 10.82 in, n=22; BKKO total mean = 72.73 in, SEM = 22.78 in, n=15). This indicates that the BK WT and KO mice have similar levels of general anxiety.

*The effect of the BK channel deletion on anxiety: measured by the Open Field Test (OFT).*

There were several variables that were measured with the OFT. Each group of mice was placed in the open field apparatus in either room air or in hypercapnia (10% CO₂) and their total time spent rearing (standing on hind limbs), number of times rearing, time spent in the periphery, time spent in the center area, number of center entries, % time in the center, and the distance they traveled in the center vs. the distance traveled in the periphery of the OFT chamber was measured. Two-way ANOVAs (factor one: mouse type; factor two:
Figure 15: Distance moved in the Elevated Plus Maze Test. This figure represents the total distance moved in the open arms (measured in inches), in adult male BK WT and BK KO mice. The values are expressed as mean (horizontal black bar) and range. (P < 0.01; one-way ANOVA, for mouse type).
FIGURE 15:

Distance in Open Arms (in)

BKWT n=22
BKKO n=15
air type) were done on all of the variables. A Bonferroni correction was applied and the corrected level gave a significance of alpha = 0.00555 (0.05/9 ANOVA tests).

The effect of BK channel deletion on anxiety measured by the amount of time the mouse spent reared up on hind limbs and the number of times the mouse reared up on hind limbs.

Figure 16A represents the total amount of time the mouse spent reared up on its hind legs in room air and in hypercapnia (10% CO$_2$). Air type had a significant effect on the total time spent rearing (F = 9.98, df = (1, 27), P = 0.004), as the total time the mice spent rearing was higher in room air than in hypercapnia (room air: WT mean = 47.43 sec, SEM = 6.52 sec n = 10; KO mean = 9.31 sec, SEM = 3.17 sec, n = 10; hypercapnia: WT mean = 18.17 sec, SEM = 1.87 sec, n = 10; KO mean = 6.20 sec, SEM = 1.57 sec, n = 10). There is also a significant effect of genotype on the total time spent rearing (F = 23.899, df = (1, 27), P < 0.001, BKWT mean = 36.5 sec, SEM = 5.48 sec, n=16, BKKO mean = 8.27 sec, SEM = 2.22 sec, n=15), (there is an interaction between air type and genotype: F = 6.516, df = (1, 27), P = 0.017).

Figure 16B represents the number of times the mouse reared in either room air or in hypercapnia. Air type had a significant effect on the number of times the mouse reared (F =11.230 with df = (1, 27) and P = 0.002). The number of times rearing was higher during room air than it was during hypercapnia (room air: WT mean = 112 rearing events, SEM= 12.98 rearing events, n = 10; KO mean = 48.30 rearing events, SEM= 14.68 rearing events, n = 10; hypercapnia: WT mean = 46.33 rearing events, SEM = 6.73 rearing events n = 6; KO mean = 21.20 rearing events, SEM = 6.76 rearing events, n = 5). There is also a significant difference in the number of rearing events between the two types of mice F = 10.302, df =
**Figure 16 A and B:** Figure 16A represents the time (measured in seconds) and 16B (number of times) that the mouse spent rearing up on his hind legs in room air or hypercapnic air (10%CO₂) in the OFT. The values in the figures are expressed as mean (horizontal black line) and range. ** indicates a significant difference between mouse type or air type at 0.001 ≤ P ≤ 0.00555. A Bonferroni correction was applied and the corrected level gave a significance of alpha = 0.00555 (0.05/9 ANOVA tests). Note that the WT mice have a much higher total time rearing and number of times rearing in room air than the KO mice. In hypercapnia, the situation is similar but the times are more similar.
FIGURE 16:

A)

B)
(1,27), p =0.003, WT mean = 87.63 rearing events, SEM= 11.71 rearing events, n=16; KO mean = 39.27 rearing events, SEM = 10.41 rearing events, n=15). There is not a significant interaction between the two factors (genotype and airtype) (F=1.965, df = (1,27), p = 0.172).

In summary, in room air, BKKO mice rear significantly less frequently and for less time compared to BKWT mice. Also there are significantly less rearings in BKKO vs BKWT mice in hypercapnia. Furthermore, the time spent rearing and the number of times rearing is significantly less in BKWT mice under hypercapnic conditions compared to room air conditions, but there was not as much of a difference in the low rearing values for BKKO mice between room air and hypercapnia.

The effect of BK channel deletion on anxiety, measured by the amount of time (sec) mice stay in periphery vs. center of the OFT.

There is a significant difference in the amount of time the mice stayed in the periphery of the OFT that is due to air type. A Bonferonni correction was applied (alpha = 0.00555 (0.05/9 ANOVA tests) (F = 11.993, df = (1, 27), P = 0.005). As expected, for both mice, the time spent at the periphery (sec) was less during room air than it was during hypercapnia (BKKO: room air mean = 1085 sec, SEM= 21.22 sec, n =10; hypercapnia mean =1165 sec, SEM = 11.95 sec, n=5), (BKWT: room air mean = 1116 sec, SEM = 10.40 sec, n =10; hypercapnia mean =1159 sec, SEM = 15.82 sec, n = 6 (Fig17A)). Genotype did not make a significant difference in the time spent in the periphery during room air or hypercapnia (BKWT total mean = 1132 sec, SEM= 10.07 sec, n = 16; BKKO total mean = 1112 sec, SEM = 17.55 sec, n = 15), (F = 0.477, df = (1, 27), P = 0.496). Also there was no interaction between air type and genotype (F = 1.046, df = (1, 27) and P = 0.316). Figure
17B represents the time spent in the center of the open field chamber (sec). The time spent in the center was significantly higher during room air than it was during hypercapnia (BKKO: room air mean = 115 sec, SEM= 21.22 sec, n = 10; hypercapnia mean = 34.88 sec, SEM= 11.95 sec, n = 5; BKWT: room air mean = 84.32, SEM = 8.23 sec, n =16; hypercapnia mean = 40.80 sec, SEM = 10.01 sec, n = 15, F = 11.993, df = (1, 27)). However there is no significant difference between the two types of mice in the time spent at the center while in the OFT (BKWT total mean = 68.00 sec, SEM = 10.07 sec, n = 16; BKKO total mean = 88.21 sec, SEM = 17.55 sec, n = 15). (F = 0.477, df = (1, 27), P = 0.496 and there is no interaction between air type and mouse type (F = 1.046, df = (1, 27), P = 0.316). Bonferonni correction was applied, alpha= 0.00555 (0.05/9 ANOVA tests). Note that both WT and KO mice spent roughly the same amount of time in the center and periphery in room air and the same elevated time in the periphery and reduced time in the center in hypercapnia suggesting that WT and KO mice had similar levels of anxiety.

In all cases mice spent most of their time (> 90%) in the periphery, and it increased significantly in hypercapnia. As expected, hypercapnia significantly reduced the time in the center for both BKKO and BKWT mice.

The effect of BK channel deletion on anxiety in male mice measured by the number of center entries and the percent of time spent in the center of the (OFT) chamber.

Air type had a significant effect on the number of times the mice entered the center space in the OFT. The number of center entries was significantly higher in room air than it was in hypercapnia for both BKKO and BKWT mice (BKKO room air mean = 108 entries, SEM = 15.50 entries, n = 10, hypercapnia mean = 18.00 entries, SEM= 5.17 entries, n = 5;
**Figure 17A and B:** Figure 17 represents the time (measured in seconds) that the mouse spent in the center vs. time they spent in the periphery during room air or hypercapnic air (10% CO₂) in the OFT. The values in the figures are expressed as mean horizontal black line and range. ** indicates a significant difference between mouse type or air type at 0.001 ≤ P ≤ 0.00555, (Bonferroni correction was applied (alpha = 0.00555 (0.05/9 ANOVA tests). For both time spent in periphery and center).
FIGURE 17:

A) Time in periphery (sec).

B) Time in center (sec).
BKWT room air mean = 44.40 entries, SEM = 4.60 entries, n = 10 hypercapnia mean = 21.33 entries, SEM = 5.35 entries, n = 6, F = 24.245, df = (1, 27), p-value < 0.001, (Fig 18A)). There was not a significant difference in the number of center entries for the two types of mice (BKWT total mean = 35.75 entries, SEM = 4.45 entries, n = 16; BKKO total mean = 77.67 entries, SEM = 15.25 entries, n = 15) (F = 6.835, df = (1, 27), P = 0.014), and there was no significant interaction between air type and mouse type (F = 8.444, df = (1, 27) and P = 0.007). Hypercapnia significantly decreases the number of center entries for both WT and KO mice, to the same very low level.

The air type had a significant effect on the % of time that the mouse spent in the center of the OFT chamber (F = 11.993, df = (1, 27), P = 0.002). The percent time in center was higher for room air than it was for hypercapnic air (BKKO room air mean = 9.57%, SEM = 1.77%, n = 10, hypercapnia mean = 2.90%, SEM= 1.0%, n = 5; BKWT room air mean = 7.03%, SEM = 0.87%, n = 10, hypercapnia mean = 3.40%, SEM = 1.32%, n = 6 (Fig 18B). Genotype had no effect on the percent of time that the mouse spent in the center (F = 0.477, df = (1, 27), with P= 0.477) thus there is no significant difference between the percent of time that the mouse spent in the center for the two types of mice (BKWT total mean = 5.67%, SEM = 0.84%, n = 16; BKKO total mean = 7.35%, SEM = 1.46%, n = 15). There is also no significant interaction between the two factors. These data suggests that both BKWT and BKKO mice experience hypercapnia-induced anxiety but there was no difference between that anxiety in KO and WT mice.

*The effect of BK channel deletion on anxiety, in male mice measured by the distance traveled in the center vs, the distance traveled in the periphery of the OFT chamber.*
Figure 18A and B: Figure 18A represents the number of center entries in the OFT chamber of BKKO and BKWT mice in room air and then in hypercapnia (10% CO\textsubscript{2}). Figure 18B represents the % of time in the center of the OFT chamber of BKKO and WT mice in room air and then in hypercapnia. The values are expressed as mean and range. ** indicates a significant difference between the air type or the mouse type (0.001 ≤ P ≤ 0.005; two-way ANOVA (factor one: mouse type; factor two: air type)). A Bonferroni test gave a corrected level of significance alpha = 0.00555 (0.05/9 ANOVA tests).
FIGURE 18:

A) Center Entries (# times) vs. KO in Room air vs. Hypercapnia. A. Number of center entries. B. % time in the center.

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B) % Time in Center vs. KO in Room air vs. Hypercapnia 10% CO₂.

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<td><strong>KO (n=5)</strong></td>
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Air type had a significant effect on the distance (measured in inches) the mice traveled in the periphery of the OFT chamber (Fig 19A). The distance traveled in the periphery was significantly higher in room air than it was for hypercapnia for both BKKO and BKWT mice (BKKO room air mean = 4177 in, SEM = 263 in, n=10, hypercapnia mean = 1652 in, SEM = 317 in, n = 4; BKWT room air mean = 2725 in, SEM = 132 in, n = 10, hypercapnia mean = 1647 in, SEM = 230 in, n = 6, $F = 51.473$ with df = (1, 27), p-value < 0.001). Genotype did not have a significant effect on the distance traveled in the periphery (F = 8.412, df = (1, 27), with an associated p-value of .007, mean total BKKO = 3455 in, SEM = 376 in, n = 14, mean total BKWT = 2321 in, SEM = 177 in, n = 16). Finally, there was no interaction between the two factors mouse and air type (F = 8.310, df = (1, 27), and p = 0.008).

Air type had a significant effect on the distance (measured in inches) the mice traveled in the center of the OFT chamber (Fig 19B). The distance traveled in the center was significantly higher in room air than it was for hypercapnia for both BKKO and BKWT mice (BKKO room air mean = 847.8 in, SEM = 151 in, n=10, hypercapnia mean = 177.8 in, SEM = 39.45 in, n = 4; BKWT room air mean = 399 in, SEM = 33.20 in, n = 10, hypercapnia mean = 171 in, SEM = 48.17in, n = 6, $F = 15.185$, df = (1, 26), p-value < 0.001. There is no significant difference in the distance traveled in the center of the OFT chamber for the two types of mice (total BKWT mean = 313 in, SEM =39 in, n = 16, total BKKO mean = 656 in, SEM = 189 in, n = 14; $F = 3.922$, df = (1, 26), P = .058), (two-way ANOVA factor one: mouse type; factor two: air type). There is no significant interaction between the two factors (air type and mouse type): $F = 3.867$, df = (1, 26), p = 0.066. A Bonferroni test was applied which gave a corrected alpha = 0.00555 (0.05/9 ANOVA tests. Note that both mice traveled
**Figure 19: A and B** Figure 19 represents the distance (in/20min) BKKO and BKWT mice traveled in the periphery and the center of the OFT chamber in room air and then in hypercapnia (10% CO₂). The values are expressed as mean (horizontal black line) and range. ** indicates a significant difference between air type, 0.0001 ≤ P ≤ 0.005, two-way ANOVA, Bonferroni corrected level of significance alpha= 0.00555 (0.05/9 ANOVA tests). Note that the BKKO mice traveled more in the room air, but the two groups traveled about the same distance in hypercapnia, indicating similar levels of anxiety more in the periphery than in the center. Also, the two groups traveled roughly the same small amount of distance in hypercapnia, which was significantly less than in room air indicating similar levels of anxiety.
FIGURE 19:

A) Room Air

- WT (n=10)
- KO (n=10)

10% CO2

- WT (n=6)
- KO (n=4)

Distance in periphery (in/20min)

B) Room Air

Distance in center (in/20min)
DISCUSSION
In this study we demonstrated that ventilation and anxiety both have complex regulatory pathways. The respiratory and anxiety phenotypes in both of the TDAG8 and BK mice use a separate acid sensitive respiratory pathway and a separate acid sensitive anxiety pathway. These are different pathways that have distinct neural routes. Previous work has shown that knocking out TDAG8 in mice significantly reduces CO$_2$-induced anxiety (Vollmer et al., 2016). Specifically we found here that, in mice knocking out TDAG8: 1) does not have an effect on the hypercapnic ventilatory response; 2) has no effect on metabolism and 3) significantly decreases activity level. In contrast we found that, in mice knocking out the BK channel; 1) significantly increases the hypercapnic ventilatory response; 2) has no effect on metabolism; 3) significantly increases activity level; and 4) has no effect on generalized anxiety or CO$_2$-induced anxiety.

The role of TDAG8 on HCVR and metabolism

There are many acid-sensing areas such as the LC and NTS that are involved with both the expression of anxiety (Redmond & Huang, 1979) and the control of breathing (Dean & Putnam, 2010; Gargaglioni et al., 2010; Ghosal et al., 2014). It has been found that there is an attenuation of CO$_2$ induced anxiety in TDAG8 KO mice (reduced freezing) (Vollmer et al., 2016) (Fig 5). Since TDAG8 has been identified as a chemosensor, we examined the role of TDAG8 on the HCVR (Fig 7) of mice. Using plethysmography measurements on TDAG8 WT vs. KO mice we found that ventilation and the ventilation/metabolic ratio are the same in room air and in hypercapnia (Fig 8B), which indicates that TDAG8 WT and KO mice have a similar HCVR. In summary, these findings show that TDAG8 is involved in the CO$_2$-
sensitive anxiety pathway but not in the HCVR. These findings also suggest that the CO₂-sensitive pathways for the HCVR and anxiety differ and do not both involve TDAG8.

It’s not surprising that the CO₂ sensitive respiratory pathway and the acid sensitive anxiety pathway are different pathways given the unique CO₂ sensing pathway described for anxiety in the TDAG8 mice. Acidosis (H⁺) generated from CO₂ activates the acid sensitive TDAG8 receptors, which appears, to reside on microglia (Vollmer et al., 2016). This triggers the release of the pro-inflammatory cytokine IL-1β from microglia (Vollmer et al., 2016). This then leads to activation of neurons within the SFO, which has efferent projections to areas that regulate cardiovascular and behavioral responses, causing panic behaviors such as freezing (Vollmer et al., 2016). In contrast, neurons within the SFO have never been implicated in respiratory control, so it is not surprising that knocking out TDAG8 would have no effect on the HCVR.

Another reason why TDAG8 has no effect on ventilation may be due to its location. Chemosensitive cells have been identified in glomus cells within the carotid body (Gonzalez, 1992; Peers, 1995) and in neurons localized within various regions of the brain stem (Coates et al., 1993; Nattie et al., 1998; Nattie et al., 1999; Feldman et al., 2003). Since HA must be detected by these cells in order for ventilation to be increased, and since SFO neurons do not project to respiratory control areas it is not likely that TDAG8 would have an effect on ventilation.

These findings are similar to another study that found inhaled CO₂ reduces amygdalar pH and evokes fear behavior in mice (Ziemann et al., 2009). The amygdala expresses ASIC1a acid sensitive ion channels, which are required for normal anxiety responses. Eliminating or inhibiting ASIC1a or buffering pH in the amygdala attenuates anxiety.
behavior in response to elevated CO$_2$, whereas directly reducing pH with amygdalar microinjections reproduces the effect of CO$_2$ on anxiety (Ziemann et al., 2009). Like TDAG8, knocking out the acid sensitive protein ASIC1a decreases anxiety but not the ventilatory response to hypercapnia (Ziemann et al., 2009), demonstrating that ventilation and anxiety both have separate regulatory pathways and use distinct acid sensory molecules.

**TDAG8 effect on activity level**

Since it was found that knocking out TDAG8 significantly reduced CO$_2$-evoked anxiety (Vollmer et al., 2016), we postulated that TDAG8 KO mice would be more active (due to less anxiety-induced freezing) than TDAG8 WT mice. However we found TDAG8 WT mice move farther and faster than the KO mice (Fig 9). The TDAG8 KO mice were significantly less active defined by the distance and the velocity traveled in a large tub for 30 minutes, on two consecutive days. This is unexpected, since the lower activity does not fit with our prediction for mice with reduced anxiety. However Vollmer found that there was no difference in a 24-hour activity test between the WT and KO TDAG8 mice in their home cages (Vollmer et al., 2015). Also, there was no evidence found for a motor phenotype with TDAG8 KO mice that would reduce activity (Vollmer et al., 2015). Furthermore, the difference in activity in the TDAG8 WT vs. KO mice is statistically significant however the power is not great. For the KO mice there is just a 21% decrease in total distance and a 20% decrease in speed. Therefore this actually may not indicate much of a difference in activity. We do not know why TDAG8 WT mice move more than KO mice but the small differences in our study may reflect the way in which activity was measured and not any major differences in motor activity.
The role of BK channels in the HCVR and metabolism

In agreement with our hypothesis, we found that knocking out the BK channel causes a significant increase in the HCVR in mice (Fig 10). This finding is supported by previous findings in which the inhibition of the BK channel leads to an increase in the neuronal firing rate of LC neurons in response to hypercapnia (Imber & Putnam, 2012; Imber et al., 2014). It is also reinforced by another study whereby inhibition of the BK channel, by-way of a bilateral injection of the BK channel inhibitor paxilline into the LC of rats, resulted in a significant increase in the HCVR (Patrone et al., 2014). Increases in ventilation were not due to increases in metabolism, which was the same in both BK WT and KO mice in normoxia and in hypercapnia (Fig 11). BK KO mice did have a significantly higher respiration ratio ($\dot{V}_E / \dot{V}O_2$), during hypercapnia when compared to WT mice (Figure 11B) keeping consistent with the notion that the BK channel is a limiting factor (i.e. braking) in the HCVR, which increases when BK channels are knocked out channels.

BK effect on activity level

Since inhibiting BK channels leads to an increased firing rate in LC neurons, we hypothesized that BK KO mice would have a higher anxiety level in room air and consequently a decreased overall activity level (more freezing). However, contrary to our prediction the BK KO mice were significantly more active, defined by the velocity and distance traveled in a large tub for 30 minutes on two consecutive days (Fig 12A and B) and the distance traveled in the OFT for 20 minutes (Fig 19). It is not clear why we observed an increased activity in BK mice. However, the range of the velocity and distance that the BK KO mice traveled was quite variable. Also a previous two-hour open field activity test has
shown that there is no significant difference in the velocity or distance traveled between the two BK WT and KO mice (Typlt et al., 2013). We previously did not find an inhibition of BK channels in room air affected LC neuron firing rate (Imber & Putnam, 2012; Imber et al., 2014; Patrone et al., 2014) so BK channels may only be active in hypercapnia. Thus, our unexpected activity are still unexplained, but they do not correlate with our measurement of anxiety (see below). It is possible that the BK KO mice have a generalized activity phenotype unrelated to anxiety.

**BK effect on anxiety**

We predicted that since there are BK channels that are located in the LC, which has been shown to be linked to anxiety (Redmond & Huang, 1979), and BK channels are known to act as a brake in the HCVR and in the neuronal firing response to hypercapnia of LC neurons (Imber & Putnam, 2012; Imber et al., 2014; Patrone et al., 2014) then knocking out the BK channel would increase anxiety in response to hypercapnia. However, in most of our tests we found that there is no difference in the CO$_2$ induced anxiety or general anxiety level between the BK WT and KO mice except for a few incongruities.

**Acoustic Startle Response**

Using the ASR test we did find that the BK channel has a significant effect on the ASR of the mice in that BK KO mice have a significantly lower ASR than the BK WT mice (Fig 13). This may be due to the KO mice having an activity phenotype as proposed above in which they might display a weaker muscle force and/or sensory motor skills. However previous tests have shown that BK KO mice have normal sensory and locomotor skills and a normal ASR compared to the BK WT mice (Typlt et al., 2013).
**Elevated Plus Maze**

For general anxiety measures in the BK KO and WT mice we also used the EPM test (Figs 14 and 15). With this test the longer the mice are in the open arms and the further that they travel in the open arms indicate a lower level of anxiety. There were no significant differences in the % of time that the KO and the WT mice spent in the open arms of the EPM (Fig 14). Also there were no significant differences in the distance that the KO and WT mice traveled in the open arms of the EPM (Fig 15). Thus there is no evidence for differences in generalized anxiety behavior in the performance of BK WT and KO mice in the EPM test in room air. These findings would support our interpretation of the results from the ASR test, that the differences observed with that test may be more reflective of a motor difference than an anxiety difference in BK KO mice.

**Open Field Test**

A test used to measure the general anxiety and CO₂-induced anxiety of the BK WT and KO mice is the OFT. In general, a mouse with anxiety will spend less time in the center and do fewer crosses from the periphery of the center of the OFT chamber. There were significant genotype differences for the WT and KO mice in the total time spent rearing and the frequency of rears in the OFT (Fig 16A and B). In room air BK KO mice reared significantly less frequently and for less time compared to BK WT mice. Again this is most likely due to the BK KO mice having weaker muscle strength (Typlt et al., 2013) and correspondingly not able to rear much. In hypercapnia, the situation was similar and there was still a significant difference in genotype as the KO mice reared for a significantly less amount of time and less frequently. These findings are consistent with our previous hypothesis of a motor phenotype in BK KO mice.

The WT mice reared for a significantly less amount of total time in hypercapnia vs.
room air however the KO mice reared the same small amount of time in room air and in hypercapnia (Fig 16A). The number of rearing times (Fig 16B) for the BK WT and KO mice was significantly lower in hypercapnia vs. room air. These results again indicate similar levels of CO₂-induced anxiety. However in a previous study in room air, on average BK KO and WT mice showed similar rearing activity (Typlt et al., 2013). These differences however may reflect the way in which rearing was measured than in any major differences in rearing activity. The mice were in the OFT chamber for 2 hours in Typlts experiment whereas we tested them in the OFT for 20 minutes.

Another variable of the OFT that was used to measure general anxiety and CO₂ induced anxiety is the time the mice spent in the center vs. the time spent in the periphery of the open field chamber. Mice typically spend most of their time in the periphery due to their instinctive drive to remain invisible to prey. Therefore time spent in the center is indicative of the mouse having less fear. In this test, as expected both BK WT and KO mice spent significantly more time in the periphery and significantly less time in the center of the open field chamber during hypercapnia vs. room air (Fig 17A and B). Also, there were no significant differences between the BK WT and KO mice in room air or in hypercapnia for both time spent in the center and time spent in the periphery of the chamber. These findings that both BK WT and KO mice spent roughly the same amount of time in the center and periphery both in room air and hypercapnia indicates the mice have similar levels of general anxiety and CO₂-induced anxiety.

The number of times the mice entered the center of the open field chamber (center entries) and the % of time the mice stayed in the center, both in normoxia and hypercapnia was also used as a measurement of general anxiety and CO₂-induced anxiety (Fig 18A and
B). For both the number of center entries and the % time that the mice were in the center of the chamber, the amount was significantly less for both BK WT and KO mice in hypercapnia vs. room air. Additionally, there were no significant differences between the WT and KO mice in both room air and in hypercapnia, again suggesting similar degrees of general and CO$_2$-induced anxiety in BK WT and BK KO mice. These data suggest that knocking out BK channels does not affect either general anxiety of CO$_2$ induced anxiety.

The distance the mice traveled in the center and the periphery of the open field chamber was also measured in BK WT and KO mice in both room air and CO$_2$ to assess general and CO$_2$-induced anxiety. In this test again the air type is what caused the significant differences, as both the WT and KO mice traveled significantly less in the center and significantly more in the periphery in hypercapnia vs. room air (Fig 19A and B). Also although not significantly, the BK KO mice traveled more in room air than the WT mice. This finding parallels the results of the activity test in which the KO mice also traveled significantly further than the WT mice. The two groups traveled about the same small amount of distance in hypercapnia, which again indicates similar levels of CO$_2$-induced anxiety.

In summary, we’ve shown that the BK channel has a significant effect on the HCVR and the activity level of mice but has no effect on metabolism, general anxiety or CO$_2$-induced anxiety. We predicted that the BK channel would have an effect on CO$_2$-induced anxiety mainly because there are acid-sensing areas that are involved with both the expression of anxiety (Redmond & Huang, 1979) and the control of breathing (Dean & Putnam, 2010; Gargaglioni et al., 2010; Ghosal et al., 2014) such as the LC and NTS and the BK channel has been found to be in the LC (Imber et al., 2014; Patrone et al., 2014; Sausbier
et al., 2006). Also there is evidence that links panic disorder to the HCVR, including an enhanced sensitivity to hypercapnia (Coryell, 1997; Schmidt et al., 2007) in which the blood level of CO$_2$ exceeds the normal value. Hypercapnic hypersensitivity has also been found to be a familial marker of PD (Coryell, 1997) and also an indicator of PD vulnerability (Battaglia et al., 2007).

We predicted and we have shown that the BK channel has an effect on the HCVR in that the HCVR is significantly increased in BK KO mice compared to WT mice. These results strengthen the theory that the BK channel acts as a brake on the HCVR and in the firing rate response to hypercapnia in central chemosensitive neurons from the LC (Imber et al., 2014; Patrone et al., 2014). In response to hypercapnia this brake pathway involves the HCO$_3$- and sAC activation of Ca$^{2+}$ channels, which then activates Ca$^{2+}$ activated K+ channels (BK) (Imber et al., 2014). This BK channel activation leads to hyperpolarization and a decrease in the neuronal firing rate of LC neurons (Fig 2 and 3B) (Imber et al., 2014; Patrone et al., 2014). In contrast the accelerator model involves the inhibition of K+ channels in response to hypercapnia and results in depolarization and an increase in the neuronal firing rate (Fig 3A). Therefore the brake pathway may exist with the accelerator pathway as an ultimate control of the central chemosensitive neuronal firing rate response to hypercapnia to control the magnitude of the HCVR (Fig 3A and B).

Although we found that the BK channel has no effect on general anxiety or CO$_2$ induced fear, interestingly in a previous study it has been found that fear conditioning in mice leads to the suppression of BK channels in the lateral amygdala (LA), the brain structure that is essential in emotional disorders such as fear (Sun et al., 2015). This down regulation of the
BK channel contributes to long-term potentiation of learned fear and helps the learned fear to be retained and reinforced (Sun et al., 2015). This corresponds to the BK brake theory in that the suppression of the BK channels in the LA is attributed to the augmented excitability of LA neurons. It would be of interest to see if BK KO mice have longer retention times for learned fear and more intense learned fears.

It has also been found that the BK channel is suppressed in cingulate pyramidal cortex neurons in a mouse model of depression and this BK channel suppression increases the excitability of these neurons (Sun et al., 2011). This BK channel suppression was accompanied by a downregulation in the expression of the scaffold protein Homer1a (Sun et al., 2011). The suppression of the BK channels, Homer1a and the accompanying increased neuronal excitability in the cingulate pyramidal cells in the depressed mice, were recovered with reversal of depression-like behavior through techniques like transcranial magnetic stimulation (TMS) and electroconvulsive therapy ECT (Sun et al., 2011). Another possibility underlying the downregulation of the BK channels is that the trafficking of BK channels might be in service of the cytosolic population. Consistent with this probability, glutamate receptor trafficking could rapidly occur, which would cause onset of the depression like behavior. These possibilities correspond to the BK brake model, in that the suppression of the BK channels in the cingulate cortex is attributed to the excitability of these neurons.

Andrea Meredith made the BKKO mouse while she was a post doc in the lab of Dr. Richard Aldrich at Stanford University. The BK KO mice have been found to be smaller, have a possible learning deficit, muscular tremor, reduced grip force, impaired gait (Typlt et al., 2013) and depression (Sun et al., 2011), however knock out of BK channels does not
impair working memory or locomotion. Since the BK channel is in many different tissues and its disregulation is involved in numerous disorders, a BK channel antagonist or agonist might serve as a novel therapy. For example a BK channel agonist could facilitate suppressed BK channels and induce normalization of hyperexcitability in LA or cingulate pyramidal cortex neurons, resulting in amelioration of conditioned fear like in PTSD or the depression like phenotype.

Similarly, alterations of this braking pathway may contribute to pathological conditions such as sleep apnea and sudden infant death syndrome (SIDs). Consequently a BK channel blocker could also serve to increase ventilatory drive in response to elevated CO₂. Another therapeutic use could be for elderly patients while under analgesia after an operation. A BK channel antagonist could reverse the respiratory depression caused by analgesia (pain medicine, especially opioids) by preventing the BK channel from opening, thus eliminating the BK brake and increasing ventilation in response to CO₂.

**Conclusion**

We’ve looked at the KOs of two different CO₂ sensors. The TDAG8 CO₂ sensor effects CO₂ induced anxiety/panic but it does not affect the HCVR, and the BK channel effects the HCVR but not the CO₂ induced anxiety/panic. These combined with a previous study on ASIC1a, suggests that central CO₂ sensing for the control of breathing, and CO₂ sensing for the initiation of anxiety/panic reflect different pathways. Therein lies the question whether there are any central CO₂ sensors that could play an important role for the control of both CO₂-induced anxiety/panic and the HCVR. This does not mean that there are no CO₂ sensors that could affect both HCVR and CO₂ induced fear. These two pathways might have some points of interaction, but are too weak to manifest any phenotype. Also there may be
compensatory mechanisms that are in place to protect the HCVR.

We found some confounding evidence in BK mice in that levels in activity, ASR and rearing behavior suggests that BK channels also might be effecting locomotion, coordination or grip strength. This would bear further studies in the future. For example a behaviorist could perform an electrodiagnostic test such as electromyography (EMG) to detect the electric potential generated by muscle cells and evaluate electrical activity produced by the skeletal muscles of the BK KO mice to detect abnormalities (Kamen et al., 2004). The motor pathway of the BK KO mice could also be studied to determine any differences in gait compared to BK WT mice.

A greater understanding of how neuromodulators contribute to mechanisms that govern the HCVR may be vital to furthering our understanding of disorders in humans that are marked by ventilation system dysfunction such as obstructive sleep apnea (OSA) and analgesic-induced hypoventilation. OSA is thought to be due to an increase in the ventilatory sensitivity to CO$_2$, which is coupled with the respiratory pathology of irregular, breathing (Chapman et al., 1988; Verbraecken et al., 1995; Younes et al., 2001; Wang et al., 2007). With this we could use a BK channel agonist for OSA patients to lower the HCVR to correct the abnormalities in the chemosensitive braking pathway. In contrast, a BK channel antagonist could serve to increase the ventilatory drive in response to analgesic-induced hypoventilation. This is a dire problem especially in the elderly when prescribed analgesics after an operation.

Clearly the control of breathing involves many areas and the control and initiation of fear and anxiety appears to involve a complex network of varying receptors. The TDAG8 is a receptor that's involved in the control and initiation of fear and anxiety, that’s
wired into an unusual part of the system. The SFO neurons do not even sense CO₂, they’re secondary to the sensing and they’re in a region that nobody has shown effects ventilation. Thus, there is no reason to suspect that the TDAG8 receptor would have anything to do with the HCVR however they do provide a new systematic insight into the origin of CO₂ induced fear and PD. Abnormalities in the TDAG8 receptor in the SFO may contribute to PD in that if the TDAG8 receptor is overactive, this may lead to a larger fearful response to a stimulus or situation that would otherwise be non-threatening. Thus, a TDAG8 receptor blocker could lead to effective treatment for PD.

It was surprising that the BK channel did not contribute to CO₂ induced anxiety/fear since chemosensitive BK channels are found in the LC which projects norepinephrine to multiple areas in the brain including the basolateral amygdala, which is involved in the fear response (Asan, 1998). However the BK channel actually may contribute to CO₂ induced anxiety/fear but just be a very weak contributor. Or the lack of the BK channels involvement in CO₂ induced anxiety/fear could be due to developmental and/or other compensatory mechanisms to protect the HCVR.

The results of my work are that many CO₂ sensors in well-known CO₂ sensitive areas seem to contribute to either CO₂ induced anxiety/fear or the HCVR but not both. Consequently, further identification of chemosensitive areas of the brain that contribute to the hypercapnic ventilatory response, and CO₂ induced anxiety/panic would be invaluable to an understanding of behavior and respiratory control. Importantly, further research may provide novel mechanistic insights into the genesis of fear and panic attacks and to the many disorders in the ventilatory response to hypercapnia.
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