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INTERACTION BETWEEN DELTA OPIOID RECEPTORS AND BENZODIAZEPINES IN CO₂- INDUCED RESPIRATORY RESPONSES IN MICE

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Abstract

The false-suffocation hypothesis of panic disorder (Klein, 1993) suggested δ -opioid receptors as a possible source of the respiratory dysfunction manifested in panic attacks occurring in panic disorder (Preter and Klein, 2008). This study sought to determine if a lack of δ -opioid receptors in a mouse model affects respiratory response to elevated CO₂, and whether the response is modulated by benzodiazepines, which are widely used to treat panic disorder. In a whole-body plethysmograph, respiratory responses to 5% CO₂ were compared between δ -opioid receptor knockout mice and wild-type mice after saline, diazepam (1 mg/kg), and alprazolam (0.3 mg/kg) injection. The results show that lack of δ -opioid receptors does not affect normal response to elevated CO₂, but does prevent benzodiazepines from modulating that response. Thus, in the presence of benzodiazepine agonists, respiratory responses to elevated CO₂ were enhanced in δ -opioid receptor knockout mice compared to wild-type mice. This suggests an interplay between benzodiazepine receptors and δ -opioid receptors in regulating the respiratory effects of elevated CO₂, which might be related to CO₂ induced panic.

INTRODUCTION

A panic attack is a brief episode characterized by marked fear or discomfort accompanied by an array of symptoms that may include: dyspnea, pounding heart, sweating, shaking, chest pain, nausea/abdominal distress, dizziness, paresthesias, chills, and hot flushes (APA, 2000). Panic disorder is defined by repeated panic attacks that often seem causeless, spontaneous, "out of the blue" and involving flight to help, resulting in chronic anxiety anticipating future attacks, worry over what the attacks mean, and avoidance of circumstances that prevent access to help (APA, 2000). Acute dyspnea (air hunger) during a

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panic attack differentiates such attacks from attacks limited to palpitations, sweating and trembling. These last are typical of danger induced fear (Klein, 1993).

It has been hypothesized that an evolved suffocation alarm system may become episodically hypersensitive due to endogenous opioidergic dysfunction, predisposing to false alarms. These are experienced as dyspneic panics, inciting rapid escape (Klein, 1993). Panic attacks can be reliably induced in about 70% of those with panic disorder by lactate infusions and, in a subset of these patients, by inhalation of 5% CO₂ (Gorman et al., 1988; Klein, 1993; Papp et al., 1997). Such elicitation occurs very rarely in normal subjects or other anxiety disorders, and does not occur during infusions of such stressors as physostigmine, insulin, 5HTP, etc (Brambilla et al., 1995; den Boer and Westenberg, 1990; Di Lorenzo et al., 1987; Rapaport et al., 1991; Strawn et al., 2008). Hypersensitivity to elevated CO₂ may also help identify childhood groups at familial risk for subsequent development of panic disorder (Roberson-Nay et al., 2010; Spatola et al., 2011).

Having hypothesized this opioidergic dysfunction underlying panic disorder, it was of interest to note that reductions in delta opioid receptor (DOR) efficacy induces substantial anxiety in mice. DOR knockout mice have been reported to display enhanced anxiety on elevated plus mazes and other standardized tests compared to wild-type controls (Filliol et al., 2000). Furthermore, DOR-specific agonists produce anxiolytic effects that are blocked by DOR-specific antagonists. The same antagonists administered alone and at a higher dose produce anxiogenic effects (e.g., based on performance in an elevated-plus maze) (Perrine et al., 2006; Saitoh et al., 2004). DORs have further been implicated in normative anxiety regulation, most likely through the action of endogenous opioids (Jutkiewicz, 2007; Nieto et al., 2005). DORs also modulate the anxiolytic effects of the benzodiazepine diazepam. For example, the anxiolytic effects of diazepam in an elevated-plus maze can be reduced by the DOR antagonist naltrindole (Primeaux et al., 2006). These results seem consonant with experimental evidence in normal human subjects that show 2 mg/kg naloxone given before lactate infusions regularly elicited a tidal volume accentuation resembling those in clinical panic attacks. Lesser doses ranging from 0.5 to 1.5 mg/kg were less effective (Preter and Klein, 2008; Sinha et al., 2007). In humans it requires about 2mg/kg to block DOR while as little as 0.15 mg/kg blocks mu-opioid receptors (Preter and Klein, 2008; Sinha et al., 2007).

Given these results, we hypothesized here that DOR knock-out mice should be particularly vulnerable to hypercarbia. Two basic questions stemming from earlier hypotheses (Preter and Klein, 2008) were examined: 1) does the absence of DORs exacerbate the respiratory response to elevated CO₂, and 2) do benzodiazepines decrease this response? The results suggest that while DORs are not required for a normal respiratory response to elevated CO₂, they are required for benzodiazepine modulation of this response.

METHODS

Subjects

Male mice (age 6–7wk at shipment) were used for this experiment, 14 wild-types (Jackson Labs, C57BL/6J) and 14 delta-opioid receptor knockouts (Jackson Labs, B6.129S2-Oprd1tm1Kff/J). These mice have been previously well characterized behaviorally (Filliol et al., 2000; Ghozland et al., 2002; Nadal et al., 2006; Olmstead et al., 2009), though the anxiety behaviors that were originally reported (Filliol et al., 2000) are now reduced, perhaps due to differences in breeding background from the mice used in original characterization (see also Jackson Labs strain description <http://jaxmice.jax.org/strain/007557.html>). In addition, opioid receptor binding has been quantified and shows a selective loss of delta opioid receptors with only minor compensatory changes in mu and kappa receptor expression (Goody et al., 2002). The mice

were separated by genotype and group-housed 2 or 3 to a cage in a central vivarium, under a 12-hr light-dark cycle with *ad lib* access to food and water. On the day of testing, mice were taken in their home cages from the vivarium and held in their cages in the testing room, then returned to the vivarium after testing. All experiments were conducted in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Nathan Kline Institute.

Respiration analyses

Breathing was monitored with a Buxco whole body plethysmograph (#PLY4211), amplified and band-pass filtered, and interfaced with a digital-to-analog converter (Cambridge Electronic Design) with a digitizing rate of 5 kHz. A continuous stream of breathable air was connected to the plethysmograph chamber by way of a flow controller, and allowed to flow into the chamber at 3 LPM. This air could be switched to a mix containing 5% CO₂ (3 LPM). Respiratory rate (bpm), breath tidal volume (V_t; μ L), and minute volume (μ L/min) for the duration of each run for each mouse were collected and analyzed with Spike2 software (CED).

Each mouse was weighed to determine drug dosage just prior to being placed into the chamber under breathing air for a 15-minute habituation period. At the end of this 15 min period, a 30 sec recording of basal respiration was made for comparison with post-injection basal (non- 5% CO₂) respiration. The mouse was then removed from the chamber, briefly sedated with isoflurane, and injected with one of the two drugs or saline. It was quickly returned to the chamber and maintained under normal breathing air, with maintenance times dependent on the injected drug (see below). After habituation, a 30 sec baseline recording of normal respiration was made immediately prior to the first exposure to 5% CO₂. The 5% CO₂ exposure trial lasted 2 min and the air was then returned to normal breathing air. A total of three 2-minute 5% CO₂ trials, with 10-minute breathing air recovery periods between CO₂ trials were presented. After the last 10-minute recovery period, the mouse was removed from the plethysmograph and returned to its home cage.

Basic experimental design and pharmacology

Each animal (wild-type and knockout) was tested under all three drug conditions (saline, diazepam (1 mg/kg) and alprazolam (0.3 mg/kg)) in a counter-balanced, pseudo-random design with at least 2 weeks between manipulations. A constant injection volume of 50 μ L/ 25g body mass was maintained across all drugs. Effective drug doses and time to maximum efficacy were based on the literature (David et al., 2001; Griebel et al., 1995).

Saline consisted of 0.9% NaCl in dH₂O, injected s.c. with a 20 min. recovery period. Alprazolam (Sigma-Aldrich) was vortexed vigorously in dH₂O to a concentration of 0.15mg/mL for a dose of 0.3 mg/kg, and injected s.c. with a 30 min. recovery period (Griebel et al., 1995). Due to diazepam's extreme insolubility in pure water, an injectable stabilized solution (Hospira, 5mg/mL) was diluted in 0.9% saline to 0.5mg/mL (dose=1 mg/kg) immediately prior to each use to avoid significant precipitation and injected i.p. with a 20 min recovery period (David et al., 2001). As noted above, following the recovery period that allowed drug efficacy to approach plateau, all animals were exposed to three, 2-min exposure trials to 5% CO₂, with a 10 min inter-trial interval (see Fig. 1A).

Genotyping

Genotype was confirmed with standard PCR analysis of tail clips using primer sequences provided by Jackson Labs. All mice were confirmed to be accurately identified.

Statistical Analyses

Respiratory data were extracted from the plethysmograph recording using the *Resp_38a* script for Spike2 available online (<http://www.ced.co.uk/seu.shtml>). Tidal volume, rate, and minute volume data for each mouse per treatment were condensed to a set of two 30 sec samples per trial (pre-CO₂ and during CO₂), as well as a 30 sec pre-drug injection baseline (see Fig. 1A). Analyses of drug versus saline effects on basal respiration were determined by comparing the change in mean between the pre-CO₂ (30 sec prior to trial 1) and the 30 sec baseline prior to injection. Analysis of the respiratory response to CO₂ challenge (Δ BPM, Δ Vt, Δ μ L/min) was determined by comparing the change in mean between the 30 sec during CO₂ and 30 sec pre-CO₂ per mouse for each treatment and genotype. Respiratory responses to CO₂ were stable across the 2 min exposure, thus for statistical purposes the respiratory values during the middle 30 sec of the 2 min exposure (during CO₂) were used for comparisons.

Statistical comparisons between genotype, drug, and trial number (2 genotype X 3 drug X 3 trial) in the CO₂ exposure were performed with ANOVA. Multiple comparisons between treatments and genotypes were made with Fisher's LSD tests.

RESULTS

To record baseline differences in respiratory response under normal breathing air and 5% CO₂, WT (n=12) and DOR-KO (n=14) mice were injected with saline and placed in the whole body plethysmograph. Representative examples of the raw data traces are seen in Figure 1B. As noted in Methods, tidal volume, breathing rate, and minute volume airflow were all extracted from the raw data and quantified.

DOR knockout did not affect basal respiration

Basal respiration (tidal volume, rate, and minute volume) to normal air did not differ between genotypes (e.g., tidal volume, repeated measures ANOVA genotype X condition, main effect of genotype, $F(1,24) = 0.00$, N.S.). Wildtype mean ($\pm$ S.E.M.) basal respiration rate was 741.6 ± 24 BPM, tidal volume 87.5 ± 8.8 μ L, and minute volume 143768 ± 7480 μ L/min. DOR- KO mean basal respiration rate was 733.1 ± 17 BPM, tidal volume 89.2 ± 8.0 μ L, and minute volume 138705 ± 9069 μ L/min.

DOR knockout did not affect respiratory response to elevated CO₂

In response to elevated (5%) CO₂ (Fig. 1), both WT and DOR-KO mice showed elevated tidal volume and minute volume during initial 5% CO₂ exposure (main effect of condition, tidal volume: $F(1,24) = 240.64$, $p < 0.0001$; minute volume: $F(1,24) = 229.38$, $p < 0.0001$; tidal and minute volume Fisher test, air vs CO₂, WT $p < 0.05$ and DOR-KO $p < 0.05$). Respiration rate was significantly reduced in response to elevated CO₂ (main effect of condition, $F(1,24) = 48.18$, $p < 0.0001$; Fisher test, air vs. CO₂, WT $p < 0.05$ and DOR-KO $p < 0.05$). There was no significant difference between genotype in the respiratory tidal volume, rate or minute volume response to CO₂ (Figure 1C, genotype X condition interaction--tidal volume: $F(1,24) = 0.38$, N.S.; respiration rate: $F(1,24) = 0.68$, N.S.; minute volume: $F(1,24) = 0.85$, N.S.).

As shown in Fig. 1C, the tidal volume and minute volume respiratory response to elevated CO₂ habituated over the course of the three, 2-min trials in both WT and DOR-KO mice (main effect of trial number, tidal volume: $F(2,24) = 21.49$, $P < 0.001$; minute volume: $F(2,24) = 20.06$, $p < 0.001$). For both tidal volume and minute volume, the respiratory response in trial 1 was significantly greater than trials 2 and 3 across both genotypes and all

treatments (Fisher tests, $p < 0.05$) (Figure 1C). No significant habituation was observed in respiration rate (main effect of trial number, $F(2,24) = 0.67$, N.S.).

DOR knockout reduced the effect of benzodiazepines on response to elevated CO₂

These results suggest that in mice, DORs are not required for respiratory responses to elevated CO₂. However, response to elevated CO₂ includes both a homeostatic physiological response and an emotional, anxiety-related response (Dudley et al., 1968; Sanderson and Wetzler, 1990; Schoepp et al., 2003). Thus, we examined the effects of benzodiazepines on the mean respiratory response to CO₂ in the two genotypes. Dosages were obtained from published dose-response curves for anxiety measures in mice (David et al., 2001; Griebel et al., 1995). We tested both diazepam (1 mg/kg) and alprazolam (0.3 mg/kg). Basal respiration (rate, tidal and minute volume) to normal air was not differentially modified between genotypes by the presence of benzodiazepines (genotype X treatment interaction volume: $F(2,24) = 0.35$, N.S.; rate: $F(2,24) = 2.29$, N.S.; minute volume: $F(2,24) = 0.73$, N.S.). However, as shown in Figure 2A, benzodiazepines differentially affected tidal volume response to 5% CO₂ between genotypes (repeated measures ANOVA, genotype X treatment interaction, $F(2,24) = 3.51$, $p < 0.05$). In WT mice both alprazolam and diazepam significantly reduced the mean tidal volume response to 5% CO₂ compared to saline controls (Fisher tests, $p < 0.05$).

In contrast, within DOR-KO mice, CO₂-induced respiratory tidal volume showed no significant effect of benzodiazepine treatment (alprazolam or diazepam) versus saline. The response of DOR-KO mice to 5% CO₂ under either benzodiazepine did not differ from that observed with saline injections. Due to this differential response to benzodiazepines, comparisons across genotypes within drug treatments revealed a significantly enhanced response to 5% CO₂ in DOR-KO mice compared to WT mice (Fisher tests, $p < 0.05$). This enhancement was due to the failure of benzodiazepines to reduce the respiratory response in DOR-KO mice compared to wildtype mice. Despite the genotype-dependent effect of benzodiazepines on CO₂ response, benzodiazepine treatment did not affect the within-session significant habituation for either genotype (Figure 2B; repeated measures ANOVA, genotype X treatment X trial number interaction, $F(4,24) = 0.32$, N.S.). No significant drug effect was detected in respiration rate (repeated measures ANOVA, genotype X treatment interaction, $F(2,24) = 0.51$, N.S.).

DISCUSSION

The results suggest that DORs do not play a detectable role in basal respiration nor in the respiratory response (rate, tidal volume or minute volume) to elevated (5%) CO₂, nor do they affect habituation of this CO₂ respiratory response over repeated trials in mice. Thus, our primary hypothesis that the respiratory response to elevated CO₂ would be enhanced in DOR knockout mice was not supported. It should be noted that future testing of other concentrations of CO₂ to characterize the dose-response function in DOR knock-out mice may allow a more sensitive indicator of any potential respiratory effects. However, despite the apparently normal response to CO₂ in the absence of DOR's, DORs are required for benzodiazepine modulation of the respiratory tidal volume response to elevated CO₂. Benzodiazepines significantly reduced CO₂-evoked increases in tidal volume in wild-type mice, but had no effect in DOR-KO mice. Therefore, our secondary hypothesis that DORs play a role in the anxiolytic effects of benzodiazepines was supported in this experimental paradigm. Of course, caution must be taken given the possibility of compensatory responses to loss of DOR throughout development that could contribute to our effects. For example, some compensatory changes in mu and kappa receptors have been reported following delta-receptor knock-out (Goody et al., 2002). Furthermore, these data do not allow determination

of whether other actions of benzodiazepines beyond the respiratory responses measured here are affected by DOR loss.

Habituation of the CO₂-evoked respiratory response was unaffected by either DOR-KO or by benzodiazepine agonists. The mechanism of habituation in this paradigm is unclear, though over the period of CO₂ exposure used here, there is unlikely to be any substantial adaptation of the physiological contributions to this response, such as peripheral receptor sensitivity, oxygen carrying capacity or CO₂ clearance rates. Habituation of CO₂-induced subjective anxiety or unpleasantness has been reported in humans (Forsyth et al., 2000; van den Hout et al., 1987; Wan et al., 2009) with no corresponding change in the sensory or physiological intensity of the response (Wan et al., 2009). Thus, habituation of respiratory response to CO₂ over repeated trials may be related to the anxiety and/or cognitive/emotional response they provoke. In addition, Hu et al. (Hu et al., 2007) have demonstrated that mice can smell near-atmospheric levels of CO₂. Therefore the habituation in breathing seen across trials during CO₂ exposure could also be due in part to habituation to the odor of 5% CO₂.

The data clearly indicate a key role for DORs in benzodiazepine modulation of respiration during hypercapnic challenge. Benzodiazepines have been shown to bind with some affinity to both delta and kappa, but not mu opioid receptors (Cox and Collins, 2001). The loss of delta receptors could directly lead to suppression of benzodiazepine efficacy, though other forms of interaction are likely. The specific sites of interaction within the brain are not clear. Delta opioid receptors are expressed widely throughout the brain in the mouse (Moskowitz and Goodman, 1984) as are benzodiazepine receptors (Young and Kuhar, 1980). Based on the binding studies, opportunities for interaction include respiratory centers, as well as limbic and neocortical areas (Moskowitz and Goodman, 1984; Young and Kuhar, 1980). However, injections of the DOR antagonist naltrindole into the central nucleus of the amygdala attenuated the anxiolytic effects of diazepam in virally modified rats (Primeaux et al., 2006), suggesting the central amygdala as a potentially important locus. Nonetheless, the present results suggest that DORs play a critical role in the ability of benzodiazepines to moderate respiratory response (tidal volume) to elevated CO₂. Thus, in the presence of benzodiazepine agonists, wildtype mice showed a reduced response to elevated CO₂ – a reduction which did not occur in DOR-KO mice. This suggests an important interplay between benzodiazepine receptors and DORs in regulating the anxiogenic effects of elevated CO₂, which ultimately could contribute to CO₂ induced panic. Further work is warranted to explore the role of DOR in other benzodiazepine-related responses.

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Fig 1.

A) Experimental design of individual drug trials. Each mouse was tested in this protocol following saline, alprazolam and diazepam injections. **B)** Typical respiratory response to CO₂. Raw data trace (bottom trace) and calibrated tidal volume (top trace) in a saline-injected wild-type (WT) mouse exposed to normal breathing air and 5% CO₂ respectively. There is a marked change in breathing pattern between the two conditions. DOR-KO mice followed the same response pattern. **C)** The respiratory response to CO₂ habituated across trials. This profile is for saline, and shows that both genotypes habituate and showed no difference in their CO₂ response pattern for change in breath volume (ΔV_t) and change in minute volume ($\Delta V_L/\text{min}$) in the absence of benzodiazepine administration. Note that for breathing rate (ΔBPM) no clear habituation was observed. Asterisks signify significant difference ($p < 0.05$) from trial 1.

Fig 2.

Benzodiazepine modulation of the CO₂ response profile differed between genotypes. **A)** CO₂ induced change in tidal volume (ΔV_t) showed a significant interaction between genotypes and treatment when benzodiazepines were administered ($p < 0.05$). Both diazepam and alprazolam significantly reduced the respiratory response to CO₂ in WT, but not in DOR-KO. Furthermore, diazepam treated DOR-KO showed a significantly greater response to CO₂ than similarly treated WT. Asterisks signify significant difference ($p < 0.05$) from saline; pound sign signifies significant difference ($p < 0.05$) from WT diazepam response. **B)** Benzodiazepines did not affect habituation of the respiratory response to CO₂. For all three treatments both genotypes consistently habituated to CO₂ over repeated trials.