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Fluoxetine augments ventilatory CO₂ sensitivity in Brown Norway but not Sprague Dawley rats

Matthew R. Hodges^{1,2}, Ashley E. Echert¹, Madeleine M. Puissant¹, and Gary C. Mouradian Jr¹

¹Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

²Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Abstract

The Brown Norway (BN; BN/NHsdMcow) rat exhibits a deficit in ventilatory CO₂ sensitivity and a modest serotonin (5-HT) deficiency. Here, we tested the hypothesis that the selective serotonin reuptake inhibitor fluoxetine would augment CO₂ sensitivity in BN but not Sprague Dawley (SD) rats. Ventilation during room air or 7 % CO₂ exposure was measured before, during and after 3 weeks of daily injections of saline or fluoxetine (10 mg/kg/day) in adult male BN and SD rats. Fluoxetine had minimal effects on room air breathing in BN and SD rats ($p > 0.05$), although tidal volume (V_T) was reduced in BN rats ($p < 0.05$). There were also minimal effects of fluoxetine on CO₂ sensitivity in SD rats, but fluoxetine increased minute ventilation, breathing frequency and V_T during hypercapnia in BN rats ($p < 0.05$). The augmented CO₂ response was reversible upon withdrawal of fluoxetine. Brain levels of biogenic amines were largely unaffected, but 5-HIAA and the ratio of 5-HIAA/5-HT were reduced ($p < 0.05$) consistent with selective and effective 5-HT reuptake inhibition. Thus, fluoxetine increases ventilatory CO₂ sensitivity in BN but not SD rats, further suggesting altered 5-HT system function may contribute to the inherently low CO₂ sensitivity in the BN rat.

Keywords

CO₂ chemoreception; SSRI; control of breathing

Introduction

The inbred Brown Norway (BN; BN/NHsdMcow) rat has an inherently low ventilatory sensitivity to hypercapnia relative to other rat strains (Strohl et al. 1997; Hodges et al. 2002). Despite this, the BN rats maintain equivalent levels of resting ventilation and blood gases, and exhibit robust ventilatory responses to hypoxia and moderate exercise. The deficit in

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Correspondence: Matthew R. Hodges, PhD, Department of Physiology, BSB 504, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, mhodges@mcw.edu, Tel: (414) 955-7528.

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ventilatory CO₂ sensitivity is not likely due to altered CO₂/pH sensitivity in the peripheral chemoreceptors (carotid body), or their potential influence on central respiratory chemoreceptors (Blain et al. 2010), as carotid body denervation in BN rats had no effect on the hypercapnic ventilatory response (Mouradian et al. 2012). These data suggest that the low ventilatory sensitivity to CO₂ in BN rats is likely due to deficiencies in central CO₂/pH chemoreception, and further suggest that there are powerful genetic determinants of the hypercapnic ventilatory response.

There are several cell types in multiple brain regions that are currently hypothesized to contribute to central respiratory CO₂/pH chemoreception. These putative central chemoreceptors include serotonergic (5-HT neurons) raphe neurons. 5-HT neurons are integrated into the brainstem respiratory network and are thought to influence the control of breathing through two major mechanisms: 1) by generating a tonic output of excitatory neuromodulators like 5-HT, substance P and thyrotropin releasing hormone (TRH) to respiratory neurons and/or other central CO₂/pH chemoreceptors, and 2) through an intrinsic cellular CO₂/pH chemosensitivity (Richerson 2004). Thus, it could be hypothesized that 5-HT neurons have their greatest influence on ventilatory control *via* effects on CO₂ sensitivity, consistent with the observations from 5-HT deficient mouse models (Hodges et al. 2008b; Hodges et al. 2011). These effects likely occur through multiple post-synaptic 5-HT receptors expressed within the respiratory network, or in other sites of central CO₂/pH chemoreception including the retrotrapezoid nucleus (RTN) (Mulkey et al. 2007; Dias et al. 2008). The sum of the data indicates that alterations in 5-HT neuronal activity may have large effects on CO₂ sensitivity through multiple mechanisms at multiple levels within the respiratory control network (Hodges et al. 2010a; Hodges et al. 2010b).

It would then be predicted that increasing synaptic 5-HT by blocking 5-HT reuptake with a selective serotonin reuptake inhibitor (SSRI) would augment baseline ventilation and the respiratory system's sensitivity to inhaled CO₂. However, results from previous studies testing this hypothesis are equivocal. For example, acute intracerebroventricular (ICV) injections of fluoxetine increased baseline breathing and the ventilatory response to hypercapnia in anesthetized, carotid body denervated rabbits (Sahin et al. 2011). Others have shown that chronic SSRI treatment (5 or 15 weeks) with paroxetine increased baseline breathing (Olsson et al. 2004; Annerbrink et al. 2010), while acute treatment with either paroxetine or fluoxetine decreased breathing frequency and thus minute ventilation ($\dot{V}_E$) in a dose-dependent manner (Annerbrink et al. 2010). Isolated brainstem spinal cord preparations from neonatal mice superfused with 10 or 50 μ M fluoxetine had no effect on fictive breathing frequency and blocked the increase in frequency elicited by decreased pH (Voituron et al. 2010), suggesting an attenuated CO₂ sensitivity in this preparation. Finally, repetitive reverse microdialysis of fluoxetine (250–500 μ M) directly into the medullary raphe increased ventilatory responses to hypercapnia, whereas chronic systemic (intraperitoneal; IP) treatment had no effect on ventilatory CO₂ sensitivity in conscious adult Sprague Dawley (SD) rats (Taylor et al. 2004). Thus, it remains unclear if or how SSRI treatment affects the control of ventilation at rest or during hypercapnia, and whether or not these differential effects are due to differences in species, developmental age or perhaps genetic background.

Recently, we reported moderately lower brain tissue levels of 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) in multiple brain regions in the BN rat compared to another inbred strain, the Dahl salt-sensitive (SS; SS/JrHsdMcwi) rat (Hodges et al. 2012). This suggested a potential difference in the function of the 5-HT system in BN rats. Given the important role of 5-HT neurons in the respiratory CO₂ chemoreflex and the potential for 5-HT system dysfunction in the BN rat, we hypothesized that chronic treatment with fluoxetine would enhance the ventilatory response to CO₂ in BN rats but not SD rats.

Methods

Adult (7–13 weeks of age) male Brown Norway (BN/NHsdMcwi; n=22) rats, which are derived from a single breeder pair of SsN line of BN rats from Harlan (Alabama colony), have been inbred and maintained since 1995 (strain information available at www.rgd.mcw.edu). This colony was tested with 200 microsatellite markers and confirmed for homozygosity (Cowley et al. 2000). Commercially available adult (7–11 weeks of age) outbred Sprague Dawley (Hsd; Harlan Laboratories; n=12) rats were also used in this study. All rats were housed in the Biomedical Research Center at the Medical College of Wisconsin, allowed access to low salt chow (Dyets 0.4% NaCl) and water *ad libitum*, and maintained on a 12:12 hr light/dark cycle. All experimental protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee prior to initiation of experimental protocols.

Experimental Design

After 2 or more training sessions of 20–30 minutes to acclimate to the plethysmograph, baseline (control) ventilatory measurements were obtained using whole-body, flow-through plethysmography. Breathing was measured while breathing room air (RA; F_IO₂ = 0.21, bal. N₂) for 20 minutes, followed by a 10-minute hypercapnic challenge (F_IO₂ = 0.21, F_ICO₂ = 0.07, bal. N₂). Each rat underwent 2–3 control experiments before initiating the regimen of daily injections with either saline (0.9% NaCl IP) or fluoxetine (10 mg/kg/day IP; Tocris Biosciences). Fluoxetine doses were calculated for each animal based upon the weight on each injection day and the total injection volume was limited to a maximum of 1 ml per day. Upon completion of control experiments, ventilation in room air and during hypercapnia was studied on days 4, 10, 15 and 22 of treatment in all experimental groups (injections began on Day 0) and all injections given on days of study were given after completing the experiment for that day. To study the reversibility of any effects of fluoxetine (n=6) or saline (n=4) treatment, ventilation was further studied in a subset of BN rats on Days 25, 31, 36 and 43 after cessation of injections on Day 22. Brain tissues were collected from all fluoxetine and saline treated SD rats (n=6, 6), and a subset of fluoxetine or saline treated BN (n=6, 6) rats for HPLC analysis of 5-HT and its metabolite 5-HIAA, dopamine (DA) and its metabolite DOPAC, and norepinephrine (NE).

Physiologic and Tissue Measurements

Plethysmography—Ventilatory measurements were made using a custom-built 10 liter Plexiglass plethysmograph using methods similar to those described previously (Hodges et al. 2002; Mouradian et al. 2012). Briefly, gas inflow rate (10 L/min) was balanced at the

same or slightly lower flow rate as vacuum outflow rates to provide rapid gas exchange, avoid CO₂ accumulation, and to maintain the absolute chamber pressure at or slightly above atmospheric pressure. Chamber O₂ and CO₂ levels (O₂ Capnograph (07-0193; Mountain View, CA)), chamber pressure (Validyne differential pressure transducer), temperature (~23°C) and relative humidity ((~0–30%) HX93A; Omega) were measured continuously. The ventilatory signal was calibrated after each study similar to our previous studies (Hodges et al. 2002; Mouradian et al. 2012). All analog signals were connected to a 16-channel A/D converter and digitally recorded using data acquisition software (Windaq) sampled at 200 Hz. Animal temperature was obtained following each experimental period using a J-type rectal thermocouple probe and reader (BAT-12, Life Science Instruments).

High-performance Liquid Chromatography (HPLC) with electrochemical detection—Upon completion of the protocol, saline (n=6) and fluoxetine (n=6) treated SD, and subpopulations of saline (n=6) and fluoxetine treated (n=6) BN rats were deeply anesthetized with isoflurane in propylene glycol (20% v/v), decapitated and the brain tissues rapidly removed, separated into medulla, pons, cerebellum, hypothalamus and forebrain and frozen at –80°C. Samples were then thawed in 0.1 M perchloric acid (0.1 g/ml) and wet weights obtained before sonication and subsequent centrifugation at 10,000 rpm for 20 minutes (4°C). The supernatant was then removed for HPLC analysis of norepinephrine (NE), epinephrine, dopamine (DA), DOPAC, serotonin (5-HT), 5-HIAA and HVA. Standards were injected with DHBA (internal standard) added and 0.1 M perchloric acid to establish the quantitative chemical profile. Samples were subjected to electrochemical detection (BAS LC4C; 0.65 V, 0.1 nA, 0.1 Hz filter with Ag/AgCl reference electrode) with a Waters uBondapak column (3.9X300) at ambient temperature.

Data Analysis

All data collected were analyzed offline using a waveform browser (Windaq). Breathing frequency (breaths/minute), tidal volume (V_T), and their product minute ventilation ($\dot{V}_E$) were calculated from periods of raw data lasting more than 10–12 sequential breaths. The selected data were breathing segments devoid of animal movements, sniffing, or other behaviors, and represented quiescent breathing from the final 10 minutes of room air breathing and during the last 5 minutes of the hypercapnic ventilatory challenge. Voltage deflections from peak to valley were calibrated to a known volume and corrected for animal and chamber temperature and relative humidity to calculate the estimated V_T per breath (Drorbaugh et al. 1955; Hodges et al. 2002).

Statistics

Statistical analyses were carried out using SigmaPlot 12.0 software. We studied two groups of BN rats; one up to, and another throughout a withdrawal period. Because these groups underwent identical study protocols up to Day 22 of treatment, and when compared had few differences statistically, we combined these sets of data. A two-way ANOVA with repeated measures was employed to determine significance of effects on all measured variables, using the factors Treatment (saline or fluoxetine) $\times$ Time (Day of Treatment) for within strain comparisons, or Strain (BN or SD) $\times$ Time for within Treatment comparisons. Alternatively, we also used a two-way ANOVA with repeated measures to determine the effects of Group

(BN SSRI, BN Saline, SD SSRI, and SD Saline) on the % Control data (see also Results) and the change in the ventilatory response to hypercapnia over the repeated measure Time. HPLC data were tested for Treatment and Strain effects with a two-way ANOVA for each neurochemical within each brain region collected, with the exception of the cerebellum as there were multiple neurochemicals that failed to reach the detection threshold. In addition, there were a few HPLC samples that yielded data >4 standard deviations from the group mean, and thus all neurochemical measurements from that animal were excluded from those brain regions (see also Results). A Bonferroni post-hoc analysis was used to determine significance among multiple pairwise comparisons, and significant interaction terms between factors noted (see also Results). Significance thresholds were $p < 0.05$.

Results

Effects of fluoxetine treatment on body weight or temperature

We evaluated body weight data collected on days of ventilatory study including a pre-injection control period, and days 4, 10, 15, and 22 of treatment (Figure 1). We found significant differences among the strains within each treatment group ($p < 0.001$), where saline treated and fluoxetine treated SD rats consistently had greater body weights than BN rats and within the SD strain there were significant effects of both treatment ($p < 0.001$) and time ($p < 0.001$; Figure 1). Within the BN strain there was only a significant effect of time ($p < 0.001$) but not treatment ($p = 0.21$), as body weights increased steadily throughout the protocol but did not differ among treatment groups (Figure 1). Fluoxetine treated SD rats increased on average 3.5 ± 0.1 g/day compared to an average increase of 5.1 ± 0.2 g/day in saline treated SD rats ($p < 0.05$), suggesting an attenuated weight gain in SD rats due to fluoxetine treatment. There were no treatment or strain differences among SD or BN rats on body temperature in either saline or fluoxetine treated BN or SD rats over time ($p > 0.05$; data not shown), although mean body temperature tended to increase from the beginning to the end of the protocol in both BN and SD saline and fluoxetine treated rats ($p < 0.001$).

Effects of fluoxetine treatment on eupneic (room air) breathing

Given the differences in weight between strains and the differential effects on weight gain with fluoxetine treatment in SD rats, we chose to normalize V_T and $\dot{V}_E$ to body weight. We first assessed the effects of treatment and time on $\dot{V}_E$, breathing frequency and V_T during room air breathing (Figure 2A–B; closed symbols). There were no differences ($p > 0.05$) in breathing frequency, weight-normalized tidal volume (V_T ; ml/breath/100 g) or $\dot{V}_E$ (ml/min/100 g) among the saline or fluoxetine rats within each strain prior to initiating injections (day 0), indicating equivalent starting points among the treatment groups within each strain. In BN rats there were significant effects of treatment ($p = 0.036$) on V_T , which was greater in saline treated BN rats compared to fluoxetine treatment on days 4 and 10 of treatment ($p < 0.05$). However, there were no significant effects of treatment on either breathing frequency ($p = 0.574$) or $\dot{V}_E$ ($p = 0.124$) in saline and fluoxetine treated BN rats during room air breathing. In addition, there were no effects of treatment on $\dot{V}_E$, breathing frequency or V_T among saline or fluoxetine treated SD rats while breathing room air ($p > 0.05$). $\dot{V}_E$, V_T and breathing frequency also significantly varied with time in all groups

studied ($p = 0.002$), suggesting significant but consistent variation among groups within each strain over time in both BN and SD rats. Thus, there were few significant effects of fluoxetine on room air breathing and when noted they were only in BN rats.

Effects of fluoxetine on the ventilatory responses to hypercapnia

Prior to injections, we found no differences in $\dot{V}_E$, V_T , or breathing frequency among the saline and fluoxetine treated groups within both BN and SD strains while breathing 7% CO_2 ($p > 0.05$; Figure 2 A–B (open symbols)). In the BN rats there was a significant effect of treatment on $\dot{V}_E$ ($p < 0.001$), V_T ($p = 0.044$) and breathing frequency ($p < 0.001$) during hypercapnia (Figure 2B). Breathing frequency during hypercapnia was greater in fluoxetine treated BN rats after 4, 10, and 15 days of treatment compared to saline treated BN rats, and V_T was increased in the fluoxetine treated BN rats after 15 and 22 days of treatment. Fluoxetine treated BN rats had a greater $\dot{V}_E$ during hypercapnia compared to saline treated BN rats over all of the treatment days tested. In contrast, there was no effect of treatment on $\dot{V}_E$ ($p = 0.055$) or breathing frequency ($p = 0.295$) during hypercapnia in SD rats (Figure 2A). There was a significant effect of treatment on V_T during hypercapnia in SD rats, as V_T in SD rats treated with fluoxetine was greater after 10 and 22 days of treatment ($p < 0.05$; Figure 2A). Thus, the number of effects of fluoxetine on breathing during hypercapnia appears to be greater in BN rats compared to SD rats.

In addition to assessing the effects of fluoxetine on weight-normalized ventilation during the CO_2 challenges, we also expressed CO_2 sensitivity as a percentage of room air breathing (% control; Figure 3A). The overall results of the analyses of the % control data were consistent with the weight-normalized data (Figure 2). There were significant effects of group ($p < 0.001$), time ($p = 0.003$) and group $\times$ time ($p < 0.001$) for $\dot{V}_E$ (Figure 3A), V_T (data not shown) and breathing frequency (data not shown) when each was expressed as a % of control. The ventilatory response to CO_2 in saline and fluoxetine treated SD rats was greater than saline and fluoxetine treated BN rats at all timepoints (Figure 3A; $p < 0.05$). In addition, there were significant effects of treatment on $\dot{V}_E$ ($p < 0.001$; Figure 3A), V_T ($p < 0.001$), and breathing frequency ($p = 0.004$) when comparing the saline and fluoxetine treated BN rats during CO_2 breathing. Breathing frequency during hypercapnia was greater in fluoxetine treated BN rats after 4, 10, and 15 days of treatment ($p < 0.05$; data not shown), whereas V_T ($p < 0.05$; data not shown) and $\dot{V}_E$ ($p < 0.05$; Figure 3A) were greater in the fluoxetine group at each time point during fluoxetine treatment in BN rats. In contrast, in SD rats we only found significant effects of treatment on V_T ($p = 0.029$) after 15 and 22 days of treatment, but no effects of treatment on $\dot{V}_E$ ($p = 0.165$; Figure 3A) or breathing frequency ($p = 0.996$) in SD rats (Figure 3A and not shown). These data demonstrate that the hypercapnic ventilatory response was augmented by fluoxetine treatment in BN but not SD rats compared to saline treated controls.

To further test our hypothesis that fluoxetine would augment the ventilatory response to CO_2 in BN but not SD rats, the hypercapnic ventilatory ($\dot{V}_E$) data at 4, 10, 15 and 22 days of treatment were also expressed as a % change from pre-injection control (Figure 3B) allowing for direct comparisons of the effects of treatment on the ventilatory response to hypercapnia within each group. There were significant effects of group ($p < 0.001$) and time

($p < 0.001$), but not their interaction ($p = 0.059$). Fluoxetine treated BN rats demonstrated a significantly greater % increase in the hypercapnic ventilatory response compared to saline treated BN, saline treated SD, and fluoxetine treated SD rats ($p = 0.003$; Figure 3B). In contrast, there were no significant differences among fluoxetine treated SD compared to saline treated SD or BN rats, and no differences among saline treated SD and BN rats ($p = 0.838$). The CO_2 response in fluoxetine treated BN rats was greater than fluoxetine treated SD rats on treatment day 10 ($p < 0.001$), greater than saline treated SD rats on treatment days 10 and 15 ($p = 0.005$), and greater than that observed in saline treated BN rats at all treatment timepoints ($p < 0.05$; Figure 3B). Thus, the data support our hypothesis that fluoxetine treatment increased the ventilatory response to CO_2 in BN but not SD rats.

A subset of BN rats that were treated with either saline ($n = 4$) or fluoxetine ($n = 6$) were further studied after the last day of treatment (day 22) to determine if the effects of fluoxetine on ventilatory CO_2 sensitivity in BN rats were reversible (Figure 4). $\dot{V}_E$, V_T , and breathing frequency (expressed as a % of control) was not different ($p > 0.05$) among the saline and fluoxetine treated BN rats prior to initiating injections in this subset, as seen in the combined group of BN rats (Figure 2). During CO_2 breathing, $\dot{V}_E$ (% control) was significantly increased ($p < 0.05$) on treatment days 4, 10, and 15 of fluoxetine compared to saline treatment, and both V_T and breathing frequency (% control) were increased on treatment days 10 and 15 ($p < 0.05$; data not shown). After cessation of fluoxetine and saline treatments, $\dot{V}_E$ during CO_2 breathing in fluoxetine treated BN rats remained significantly greater ($p < 0.05$) compared to those previously treated with saline on day 25 of the protocol (day 4 of withdrawal), but was not different for the remainder of the withdrawal period ($p > 0.05$; Figure 4). V_T and breathing frequency also remained significantly greater ($p < 0.05$) in fluoxetine treated BN rats compared to saline treatment on day 25, but were no longer different for the remainder of the protocol ($p > 0.05$; data not shown). Thus, the increases in the ventilatory response to CO_2 observed with fluoxetine treatment in BN rats remained augmented at least 4 days post-treatment, and these effects were reversible over the following weeks.

Effects of fluoxetine or saline on brain tissue biogenic amines

In the BN rats, brain tissue levels of 5-HT, NE, DA, DOPAC, and the DOPAC/DA ratios were not significantly ($p > 0.05$) affected by treatment within the medulla, pons, hypothalamus and forebrain (Figure 5A and data not shown). However, as expected both 5-HIAA and the 5-HIAA/5-HT ratios were significantly affected by treatment ($p < 0.05$), where 5-HIAA and 5-HIAA/5-HT was reduced in the pons, hypothalamus and forebrain of fluoxetine compared to saline treated BN rats (Figure 5A). Within the medulla of fluoxetine treated BN rats, 5-HIAA ($p = 0.051$) and the 5-HIAA/5-HT ratio ($p = 0.074$) trended lower but were not significantly altered compared to saline treated BN rats (Figure 5A).

In SD rats there was no effect of treatment ($p > 0.05$) on 5-HT levels in all brain regions tested. However, there were significant treatment effects on both 5-HIAA and the 5-HIAA/5-HT ratio in all brain regions tested ($p < 0.05$), where fluoxetine treated SD rats demonstrated reductions in both 5-HIAA and 5-HT turnover compared to saline treated SD rats ($p < 0.05$; Figure 5B and data not shown). NE was greater in fluoxetine treated SD rats

(1318.8 ± 98.8 pg/mg) compared to saline treated SD rats (1081.1 ± 85.2 pg/mg) measured in the hypothalamus, but no different in the medulla, pons, and forebrain ($p > 0.05$; data not shown). We also found significant effects of treatment ($p < 0.05$) on pontine levels of DA, where fluoxetine treated SD rats (117.1 ± 15.0 pg/mg) showed lower DA levels than saline injected control SD rats (153.4 ± 6.6 pg/mg), but found no differences in DA levels in the medulla, hypothalamus, and forebrain in SD rats. DOPAC levels, as well as the DOPAC/DA ratio in the medulla were significantly greater ($p < 0.05$) in the saline treated SD rats (10.1 ± 1.5 pg/mg and 0.25 ± 0.04 , respectively) compared to fluoxetine treated SD rats (7.1 ± 1.0 pg/mg and 0.17 ± 0.03 , respectively), but there were no effects of treatment ($p > 0.05$) in the pons and hypothalamus (forebrain not tested).

We also noted significant effects of strain on multiple neurochemicals in multiple brain regions within each treatment group, where the levels of measured neurochemicals were consistently greater in SD rats compared to BN rats. In addition, whenever significant strain differences were detected it was in both saline and fluoxetine treated rats with very few exceptions. For example, in saline treated rats we found lower levels of 5-HT in the medulla and hypothalamus of BN rats compared to SD rats (Figure 5 and data not shown), lower NE levels in all brain regions tested in BN rats compared to SD rats (data not shown), and lower DA levels in the medulla of BN rats compared to SD rats ($p < 0.05$; data not shown). We also found a lower 5-HIAA/5-HT ratio in the pons of saline treated SD rats compared to BN rats, in addition to lower 5-HIAA levels in the medulla, pons and hypothalamus of BN rats compared to SD rats ($p < 0.05$; Figure 5 and data not shown). There were no strain differences in DOPAC or the DOPAC/DA ratio in saline treated BN and SD rats ($p > 0.05$).

Overall, the neurochemical data demonstrated: 1) consistent effects of fluoxetine treatment to reduce 5-HIAA levels and the ratio of 5-HIAA/5-HT in multiple brain regions in BN and SD rats with little or no effect on tissue 5-HT, NE and/or DA levels, and 2) significant strain effects, where BN rats showed lower levels of multiple neurochemicals in multiple brain regions compared to SD rats irrespective of treatment.

Discussion

Genetic background has clear effects on physiologic function, where phenotypic differences are thought to be derived from allelic variance among populations and/or strains. Previous studies comparing ventilatory control mechanisms among several inbred and outbred rat strains demonstrated great variation in ventilatory pattern and minute ventilation under several conditions, including breathing room air or multiple gases eliciting ventilatory chemoreflexes (Strohl et al. 1997; Hodges et al. 2002). The BN rat stands apart from most other strains in that their ventilatory CO₂ sensitivity is extremely low (slope ~ 1.5 ml/min/mmHg) compared to SD rats (~ 4.0 ml/min/mmHg) (Mouradian et al. 2012), despite having robust hypoxic and exercise ventilatory responses (Hodges et al. 2002). Thus, BN rats demonstrate a relatively specific deficit in their hypercapnic ventilatory response, likely due to altered mechanisms of central CO₂ chemoreception, and certainly due to the complement of alleles inherent to the BN strain.

Genetically modified mouse models of central 5-HT deficiency, such as *Pet-1* null and/or conditional *Lmx1b^{fl/fl}* mice which lack most or all brain 5-HT neurons, demonstrate reduced hypercapnic ventilatory responses (Hodges et al. 2008a; Hodges et al. 2008b; Hodges et al. 2011). The moderate or severe reduction in 5-HT neurons in the *Pet-1* null and conditional *Lmx1b^{fl/fl}* mice, respectively, did not alter eupneic breathing or the ventilatory responses to hypoxia, suggesting a relatively selective effect on ventilatory CO₂ sensitivity similar to the phenotype of the BN rat. There are no gross abnormalities in the numbers or distribution of 5-HT-producing (tryptophan hydroxylase-expressing) neurons among BN, SS and SD rats (Hodges et al. 2012), although BN rats had lower tissue levels of 5-HT and 5-HIAA in multiple brain regions compared to SS rats (Hodges et al. 2012). This suggested that BN rats are moderately deficient in central 5-HT or perhaps is indicative of an alteration in 5-HT system function in the BN rat.

We reasoned here that if aspects of the 5-HT system were deficient in the BN rat that inhibiting 5-HT reuptake and increasing synaptic 5-HT with fluoxetine would enhance CO₂ sensitivity in BN rats. Indeed, daily fluoxetine injections significantly increased CO₂ sensitivity in the BN rat within 4 days of treatment, which remained elevated for 3 weeks of treatment and up to 4 days after discontinuing injections. At the time of the peak effect (treatment days 10 and 15), the hypercapnic ventilatory response was increased about 35% from pre-injection controls, and increased ~50% above that observed in saline treated BN rats, while SD rats receiving the same dose of fluoxetine for the same duration demonstrated no changes in CO₂ sensitivity similar to previous reports (Taylor et al. 2004). These findings support our hypothesis that 5-HT reuptake inhibition with fluoxetine would enhance the ventilatory response to CO₂ in BN but not SD rats.

The actions of SSRIs on the 5-HT system are complex, but they are generally postulated to acutely act through enhancing synaptic levels of 5-HT by blocking reuptake through the 5-HT transporter (SERT). Inhibition of SERT consequently reduces metabolic breakdown of 5-HT by monoamine oxidase (MAO) into 5-HIAA within the neuron, and reduces 5-HT neuron firing rates. 5-HT neuron firing rates normalize during chronic treatment despite continued SERT inhibition, pointing to long-term adaptive changes of unknown mechanisms (Blier et al. 1987; Le Poul et al. 2000). SSRIs have been shown to increase the 5-HIAA/5-HT ratio, which indicates 5-HT “turnover” or activity when measured by microdialysis of extracellular fluid 5-HT and 5-HIAA. Herein, HPLC measurements of 5-HT and 5-HIAA were made from bulk tissue homogenates. Therefore our measure of the 5-HIAA/5-HT ratio is less indicative of synaptic 5-HT activity/turnover but instead indicates effective SERT inhibition and no change in overall 5-HT production in both strains (Brenes et al. 2009; Lowry et al. 2009). The consistency of the HPLC data demonstrating similar average reductions in 5-HIAA ($-32.9 \pm 1.1\%$ and $-36.9 \pm 3.6\%$) and the 5-HT turnover ratio ($-26.0 \pm 3.9\%$ and $-31.6 \pm 5.6\%$) across all brain regions tested points to a similar degree of effective and global reuptake inhibition in both SD and BN strains, respectively.

Based on the above data it appears that fluoxetine had essentially the same effect on the inhibition of 5-HT reuptake in in both BN and SD rats but only augmented the ventilatory sensitivity to CO₂ in the BN rats. The mechanisms that lead to this differential effect on CO₂ sensitivity are not readily apparent, but could result if the levels of synaptic 5-HT and/or or

post-synaptic receptors are regulated differently in these rat strains. It is possible that the combination of alleles specific and inherent to the BN genome leads to changes in the regulation of multiple aspects of 5-HT system function that manifests as moderately lower tissue concentrations of 5-HT. The brain tissue levels of 5-HT in BN rats (although lower relative to SD and SS rats) are still relatively high, and so it is unclear if synaptic 5-HT is inherently altered. This represents a limitation of our HPLC dataset as we were not able to measure synaptic 5-HT. Alterations in synaptic levels of 5-HT might induce long-term adaptive changes in post-synaptic receptor expression. Indeed, previous studies have shown lower levels of 5-HT_{2A} receptor mRNA but higher levels of 5-HT₇ receptor mRNA in the ventral cervical spinal cord of another strain of BN rat compared to Fisher 344 and Lewis rat strains (Baker-Herman et al. 2010). Moreover, early in the treatment fluoxetine tends to increase the hypercapnic ventilatory response in BN rats *via* increases in breathing frequency and later in the treatment period increases V_T . This shift in ventilatory pattern could result from acute increases in synaptic 5-HT and activation of 5-HT receptors within sites contributing to respiratory rhythm generation followed by longer-term adaptive changes in post-synaptic receptor activity at respiratory pre-motor and/or motor neurons. Given that the majority of the physiologic effects of fluoxetine were on CO₂ sensitivity (and not room air breathing) and specific to the BN strain, we predict that fluoxetine-induced changes in ventilatory CO₂ sensitivity may occur at sites thought to play a role in respiratory CO₂/pH chemoreception including but not limited to the retrotrapezoid nucleus (Mulkey et al. 2007). However, we cannot rule out possible contributions from the carotid bodies which may contribute to the augmented hypercapnic ventilatory response, are modulated by 5-HT, and express tryptophan hydroxylase and SERT (Peng et al. 2006; Forster et al. 2007; Yokoyama et al. 2012).

Alternatively, some (but not all) data show SSRI treatment in humans is associated with increases in anxiety (Thaler et al. 2012). Thus, the possibility exists that the strain-specific effects in the effects of fluoxetine on ventilatory CO₂ sensitivity could be indirectly through different levels of “stress” induced by or associated with fluoxetine treatment. Associated with the increased levels of SSRI-induced anxiety is significant weight loss (Serretti et al. 2010). Indeed, SD rats treated with fluoxetine fail to gain weight at rates similar to fluoxetine treated BN rats, or saline treated BN and SD rats, which may be indicative of a strain-related difference in SSRI-induced anxiety. Kinkead and colleagues previously investigated the effects of a specific form of stress through prolonged restraint (90 min) on resting ventilation and respiratory responses to hypoxia and hypercapnia in adult SD rats (Kinkead et al. 2001). They noted that repeated restraint had little or no effect on breathing at rest or during hypoxic challenges, but reduced the hypercapnic ventilatory response by 45%. These data suggests that fluoxetine treated SD rats may experience greater drug-induced stress levels that could mitigate or counteract any effects of fluoxetine on the CO₂ response. Conversely, fluoxetine treatment in BN rats had no effect on weight gain but significantly augmented the hypercapnic ventilatory response. Our own anecdotal observations of BN and SD rats treated with fluoxetine is consistent with this hypothesis as well, as the treated SD rats were more adverse to handling and seemingly more aggressive than treated BN rats. Overall, these data suggests that the strain-related differences in the effect of fluoxetine to augment the CO₂ response may not be through direct mechanisms on

5-HT system function or its effects on the respiratory control network, but indirectly through the potential differential effects of stress which may be greater in SD compared to BN rats.

But the question remains: are the moderately lower tissue 5-HT levels the major contributor to the low CO₂ sensitivity in BN rats? Jan and colleagues reported no difference in tissue 5-HT levels in the hippocampus of BN rats compared to SD rats using similar HPLC techniques, and significantly higher 5-HT levels in the frontal cortex of BN rats (Jans et al. 2010). These BN and SD rats are of European origin and thus it is unclear how genetically similar they are to the MCW strain of BN rats and domestic SD rats from Harlan Laboratories we studied here. It is noteworthy that upon tryptophan depletion (which in turn also depletes 5-HT) in these European strains, SD (but not BN) rats demonstrated increased anxiety- and depression-related behaviors despite similar levels of brain 5-HT and 5-HIAA compared to the BN strain. Thus, differences in tissue levels of 5-HT may instead be an indicator of altered function, activity, or regulation of the entire 5-HT system rather than the driving force behind differences in the measured responses. These differences could relate to altered expression or activity of the 5-HT biosynthetic enzymes (tryptophan hydroxylase and dopamine decarboxylase), vesicular packaging of 5-HT (VMAT2), and multiple mechanisms regulating the release, reuptake and subsequent metabolism of 5-HT. In other words, it is conceivable that the moderately reduced tissue level of 5-HT in the BN rat by itself is unlikely to directly produce the inherently reduced CO₂ sensitivity in BN rats. Rather, the relative uniformity in the bulk tissue HPLC data indicate that there are likely additional phenotypic differences in other neurotransmitter systems that contribute to the inherently low CO₂ sensitivity in naïve BN rats. Elucidating the genetic determinants of the inherently low CO₂ sensitivity in the BN rat will therefore require a more detailed approach.

Previous studies aiming to determine the general effects of SSRI's on ventilatory control have been largely equivocal, showing stimulatory or inhibitory effects on baseline breathing and/or the hypercapnic ventilatory response, or no effects on either (Olsson et al. 2004; Taylor et al. 2004; Annerbrink et al. 2010; Voituron et al. 2010; Sahin et al. 2011). While these studies employed a variety of methods to study the effects of SSRIs in multiple preparations and species, our data further suggest that fluoxetine significantly augments ventilatory CO₂ sensitivity and to a lesser extent alters room air breathing in the BN rats. However, our study also demonstrates that these effects are specific to one strain, thus the effects are likely dependent upon genetic background. While the mechanisms of such differential effects of SSRI treatment on the control of breathing remain unclear, the data highlight the potential for differential effects in the millions of patients receiving SSRI therapy depending upon the complement of allelic variants in each individual's genome and further demonstrates central effects of SSRI treatment beyond the desired effect of treating the symptoms of major depression.

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Highlights of submission entitled “Fluoxetine augments ventilatory CO₂ sensitivity in Brown Norway but not Sprague Dawley rats”

- Brown Norway (BN) rats have a specific deficit in CO₂ chemoreception and low brain serotonin (5-HT)
- Here we determined if increasing brain 5-HT with fluoxetine increases CO₂ sensitivity in BN rats
- CO₂ sensitivity was increased in BN but not Sprague Dawley rats despite equal doses of fluoxetine
- HPLC measurements of neurochemicals confirm specificity and efficacy of drug in both strains
- Thus, differential effects on CO₂ sensitivity are due to altered 5-HT system function in BN rats

Figure 1.

Body weights (g) of saline (n=10, 6) or fluoxetine (n=12, 6) treated BN and SD rats, respectively, over the pre-injection control period (day 0) up to 22 days of treatment. * indicates significant effect of treatment within SD rats ($p < 0.05$) determined by two-way RM ANOVA and Bonferroni post-hoc analyses.

Figure 2.

Ventilation ($\dot{V}_E$; ml/min/100 g), breathing frequency (breaths/min), and tidal volume (V_T ; ml/breath/100 g) while breathing room air (closed symbols) or 7% CO₂ (open symbols) in saline (n=6, 10) or fluoxetine (n=6, 11) treated SD (A) and BN (B) rats, respectively, over the pre-injection control period (day 0) up to day 22 of treatment. * indicates significant effect of treatment comparing saline to fluoxetine ($p < 0.05$) determined by two-way RM ANOVA and Bonferroni post-hoc analyses.

Figure 3.

A) Ventilation (expressed as a % of room air breathing (% Control)) during hypercapnia in saline (n=6, 10) and fluoxetine (n=6, 11) treated SD and BN rats, respectively, over the pre-injection control period (day 0) up to 22 days of treatment. **B)** The % change in the hypercapnic ventilatory response is plotted over time. * indicates significant differences between saline and fluoxetine in BN rats, # indicates significant differences between SSRI treated BN rats and saline treated SD rats, and \$ indicates significant differences between

SSRI treated BN rats and SSRI treated SD rats ($p<0.05$), as determined by two-way RM ANOVA and Bonferroni post-hoc analyses.

Figure 4.

Ventilation (expressed as a % of room air breathing (% Control)) during hypercapnia in a subpopulation of saline (n=4) and fluoxetine (n=6) treated BN rats over the pre-injection control period (day 0), through 22 days of treatment and another 21 days of no treatment (withdrawal). * indicates significant effect of treatment comparing saline and fluoxetine in BN rats ($p < 0.05$) determined by two-way RM ANOVA and Bonferroni post-hoc analyses.

Figure 5.

Medullary and pontine tissue levels of 5-HT (pg/mg) and 5-HIAA (pg/mg), and the ratio of 5-HIAA/5-HT in saline (n=6) and fluoxetine (n=6) treated BN rats (**Panel A**), and in saline (n=6) and fluoxetine (n=6) treated SD rats (**Panel B**). # indicates significant effects of strain within each treatment group ($p < 0.05$), and * indicates significant effects of treatment within each strain ($p < 0.05$) determined by two-way ANOVA and Bonferroni post-hoc analyses within each brain region.