Buprenorphine Depresses Respiratory Variability in Obese Mice with Altered Leptin Signaling

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ABSTRACT

Background: Opiate-induced respiratory depression is sexually dimorphic and associated with increased risk among the obese. The mechanisms underlying these associations are unknown. The present study evaluated the two-tailed hypothesis that sex, leptin status, and obesity modulate buprenorphine-induced changes in breathing.

Methods: Mice (n = 40 male and 40 female) comprising four congenic lines that differ in leptin signaling and body weight were injected with saline and buprenorphine (0.3 mg/kg). Whole-body plethysmography was used to quantify the effects on minute ventilation. The data were evaluated using three-way analysis of variance, regression, and Poincaré analyses.

Results: Relative to B6 mice with normal leptin, buprenorphine decreased minute ventilation in mice with diet-induced obesity (37.2%; $P < 0.0001$), ob/ob mice that lack leptin (62.6%; $P < 0.0001$), and db/db mice with dysfunctional leptin receptors (65.9%; $P < 0.0001$). Poincaré analyses showed that buprenorphine caused a significant ($P < 0.0001$) collapse in minute ventilation variability that was greatest in mice with leptin dysfunction. There was no significant effect of sex or body weight on minute ventilation.

Conclusions: The results support the interpretation that leptin status but not body weight or sex contributed to the buprenorphine-induced decrease in minute ventilation. Poincaré plots illustrate that the buprenorphine-induced decrease in minute ventilation variability was greatest in mice with impaired leptin signaling. This is relevant because normal respiratory variability is essential for martialing a compensatory response to ventilatory challenges imposed by disease, obesity, and surgical stress.
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THIS study was motivated by two imperatives. First, the ongoing epidemics of opiate abuse[1] and obesity[2] reinforce our interest in opiate research with potential for translational relevance.[3–12] Second, opiate-induced alterations in the ventilatory response to hypercapnia are sexually dimorphic,[13] consistent with evidence that respiratory control is altered by sex steroid hormones.[14] Additional data show that opiate-induced respiratory depression is associated with increased risk among subjects who are female[15] and/or obese.[16] There is consensus that gaps in knowledge regarding mechanisms by which opiates alter breathing "warrant further study of the sex-dependent and sex-independent genetic factors" and that "inbred strains of mice and rats may serve as the best model."[13] We concur, and the present study quantified the effects of buprenorphine on minute ventilation variability in obese and normal weight male and female mice. The obese mice used for this study were characterized by either disrupted leptin signaling or diet-induced obesity. This design made it possible to use a three-way, mixed effects analysis of variance model to evaluate the two-tailed hypothesis that buprenorphine causes breathing to vary significantly due to sex, leptin status, and obesity. Buprenorphine was a logical choice because it is used for pain management

What We Already Know about This Topic

- Obese patients and female patients are reported to be at increased risk for opioid-induced respiratory depression
- Respiratory variability is an essential aspect of maintaining homeostasis in response to challenges imposed by disease, obesity, surgical stress, and opioids
- Leptin is an anorexigenic hormone produced by adipocytes

What This Article Tells Us That Is New

- The hypothesis that sex, obesity, and leptin status modulate buprenorphine-induced changes in breathing was tested in male and female normal weight mice and in obese mice with disrupted leptin signaling
- Leptin status, but not body weight or sex, contributed to the buprenorphine-induced decrease in minute ventilation variability
- Mice with impaired leptin signaling had the largest buprenorphine-induced decrease in minute ventilation variability

in addicted patients[17] and because it is one of three medications approved for treating opiate use disorder.[1,18]

Materials and Methods

Animals

Experiments and procedures were reviewed and approved by the University of Tennessee Institutional Animal Care

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and Use Committee (Knoxville, Tennessee). All phases of the experiments adhered to the Guide for the Care and Use of Laboratory Animals (National Academies Press, 8th edition, Washington, D.C., 2011). Mice had *ad libitum* access to food and water and were housed under 24-h illumination to promote free-running rhythms and offset potential circadian confounds.[10] Adult mice (n = 80) were purchased from The Jackson Laboratory (USA). These congenic mice (10 males and 10 females of each line) included normal weight C57BL/6J (B6) wild-type mice that produce leptin; B6 mice with diet-induced obesity (DIO) that may lead to leptin resistance; obese B6.Cg-Lep[ob]/J mice (ob/ob) that do not make leptin; and B6.BKS(D)-Lepr[db]/J (db/db) mice that are obese, produce leptin, and lack functional leptin receptors. None of these mice are transgenic animals, and the ob/ob and db/db mice were derived from spontaneous mutations in the B6 line. These congenic lines are maintained at The Jackson Laboratory by a multigenerational breeding program that backcrosses a mutation of interest into different mice, thereby transferring the mutations of interest. The B6, ob/ob, and db/db mice were fed normal Teklad 8640 rodent chow comprised of 5% fat. The DIO mice were fed a 60% fat diet for 20 weeks before and during the testing period. Both rodent chows were purchased from Research Diets, Inc. (USA).

Congenic Mice in Relation to the Experimental Design

The four congenic lines of mice (B6, DIO, ob/ob, and db/db) are ideally suited to calls for studies "of the sex-dependent and sex-independent genetic factors"[13] contributing to opiate-induced changes in breathing. Efforts to understand the sexually dimorphic response to opiates[15,19] are scientifically sound[20] and consistent with the 2016 policy mandating that National Institutes of Health–funded studies include sex as a biologic variable.[21]

Among the four mouse lines, the obesity and leptin phenotypes were regarded as sex-independent factors that may contribute to buprenorphine-induced alterations in breathing. This possibility is suggested by the fact that leptin, produced by adipocytes, covaries with obesity and modulates breathing[22–25] and nociception.[7,10,12] Table 1 summarizes similarities and differences between the obesity and leptin phenotypes in the four congenic mouse lines studied. Use of three-way analysis of variance made it possible to evaluate the effects of buprenorphine on breathing as a function of sex, leptin status, and obesity.

Buprenorphine Administration and Measures of Breathing

Mice were injected (intraperitoneally) with saline or buprenorphine (0.3 mg/kg) (Sigma–Aldrich, USA). Buprenorphine was dissolved in saline to yield the desired concentration while holding injection volume (0.3 ml) constant. The 0.3-mg/kg dose of buprenorphine has been shown to be antinociceptive in B6 mice[26] and in males and females of the four congenic mouse lines used in this study.[12] Before starting data collection, the mice were habituated to being placed in whole body plethysmography chambers (Data Sciences International, USA), where they were free to move and groom. After 1 week of habituation, the mice were randomly assigned to receive either saline or buprenorphine injection and were placed in the plethysmography chambers where breathing was automatically recorded for 1 h. Testing occurred at the same time of day, and all mice had 1 week for recovery between injections. Breathing signals from mice in the plethysmography chambers were automatically digitized by the software, circumventing the need to blind experimenters to injection condition (saline *vs.* buprenorphine). Pioneering studies of rodent breathing across genetic lines[27] demonstrated that measures must be scaled to body mass, differences in CO_2 production, hypoxic

Table 1. Congenic Mouse Lines Differ in Body Weight and Leptin Status

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

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

and hypercapnic ventilatory responses, dead space, fat distribution, and many other factors. The present measures of the effects of buprenorphine on minute ventilation across four congenic lines of mice were therefore expressed as ml/min/g body weight, consistent with our previous studies on the respiratory effects of isoflurane[28] and sedative/hypnotics.[29]

Statistical Analyses

Sample sizes (n = 80 mice consisting of 10 males and 10 females representing each of the four congenic mouse lines) were based on previous experience.[7,10,12,29] The data were analyzed with Statistical Analysis System v9.4TS1M3 (SAS Institute, Inc., USA), using a three-way mixed-effect, repeated-measures ANOVA model with two between-subject factors (sex and mouse line) and one within subject factor of injection condition (saline *vs.* buprenorphine). Mouse nested within sex by mouse line was included as a random effect. After a log transformation was applied to minute ventilation values, the data satisfied all assumptions of the ANOVA model including normal distribution of residuals, similar variances, and no major outliers. No other transformations were required. Bonferroni adjustment was used to correct the *P* values for all *post hoc* comparisons.

Poincaré as Exploratory Data Analysis

Poincaré analysis is well established and permits novel diagnostic and prognostic insights.[30] The heuristic value of Poincaré analysis also makes it clinically relevant for diverse disorders ranging from locomotion[31] to sleep.[32] Poincaré analysis can even help identify phenotypes of metabolic syndrome.[33] Relative to anesthesiology, Poincaré analysis has been used as a weaning predictor for some postoperative patients,[34] for estimating levels of sedation analgesia,[35] and to characterize anesthesia-induced changes in electroencephalogram.[36]

By plotting a dependent measure relative to the previous dependent measure, Poincaré analysis makes it possible to quantify and visualize variability within a data set.[30] The resulting dispersion of points conveys novel information about variability in the data (fig. 1). For the present study, Poincaré analysis was used to characterize how buprenorphine alters the variability of minute ventilation. Whole-body plethysmography data were averaged every 5 min for 1 h. Poincaré analysis was used to quantify respiratory variability among male and female B6, DIO, ob/ob, and db/db mice in response to injection of saline and buprenorphine. Points in the Poincaré plots of minute ventilation represent time intervals of an inspiratory and expiratory cycle. In the present study, Poincaré short-term variability (SD1) quantified the variation in breathing during the 5-min measurement intervals. Long-term variability (SD2) quantified the overall variability in breathing throughout the 1-h measurement period. SD1 describes more rapid changes, and SD2 describes long-term changes. Short-term variability (SD1) also is described by the SD of successive differences in a breath-to-breath cycle, and long-term variability (SD2) also
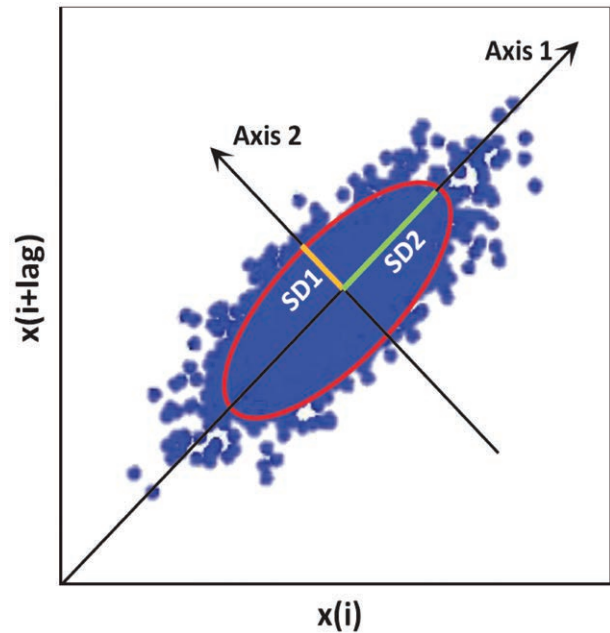
Fig. 1. Poincaré plot illustrating a distribution of points representing heart rate beat to beat (RR) interval. Similar plots can be created for any biologic rhythm. For example, breath-to-breath intervals x(i), are plotted *versus* the delay time between breaths, x(i +lag). The descriptors *SD1* and *SD2* are Poincaré metrics describing the resulting dispersion of points. SD1 (*yellow line* along *axis 2*) is the SD of successive differences between sequential events, such as breaths. SD1 is a measure of short-term (*e.g.*, breath-to-breath) variability and is illustrated as the SD of the points that are perpendicular to the line of identify. SD2 (*green line* along *axis 1*) quantifies the overall (long-term) variability during the measurement period as the distribution of points along the line x = y. Poincaré plots are widely applicable for characterizing clinically relevant biologic rhythms. The figure is reprinted from the work of Bolaños *et al.*[35] with permission.

can be described by the SD in breath-to-breath intervals.[37] Both analytic approaches were applied to the present data set.

Results

Figure 2 shows that DIO, ob/ob, and db/db mice had decreased minute ventilation under the control (saline) condition. Compared to the B6 (wild-type) mice, buprenorphine decreased average minute ventilation by 37.2% in DIO mice (t = 7.68, df = 72, *P* < 0.0001), 62.6% in ob/ob mice (t = 16.61, df = 72, *P* < 0.0001), and 65.9% in db/db mice (t = 18.21, df = 72, *P* < 0.0001). There was no significant sex main effect on minute ventilation. Regression analyses revealed that in no case did body weight alone account for a significant decrease in minute ventilation.

Buprenorphine Decreased Variability in Minute Ventilation across all Congenic Mouse Lines

Figure 3 shows Poincaré plots illustrating features of the buprenorphine-induced decrease in minute ventilation
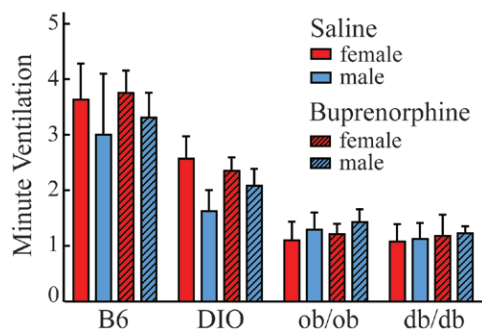
Fig. 2. Minute ventilation varied significantly among congenic mice. Mice with leptin dysfunction (ob/ob and db/db) had the lowest minute ventilation (means ± SD ml/min/g body weight). B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

variability. The collapse in short-term variability (SD1), visualized in figure 3 as the distribution of points perpendicular to x = y, is not apparent in figure 2. The Poincaré plots show that compared to saline, buprenorphine caused a collapse in minute ventilation variability across all four lines of mice. Three-way ANOVA indicated that short-term variability (SD1) was significantly decreased (F = 45.03; df = 3,72; P < 0.0001) as a function of mouse line and injection condition (saline or buprenorphine) (F = 148.98; df = 1,72; P < 0.0001). The only significant interaction was between mouse line and injection condition (F = 5.30; df = 3,72; P = 0.0023). *Post hoc* comparisons showed that buprenorphine caused a greater compression of short-term variability of minute ventilation in the DIO, ob/ob, and db/db mice than in the B6 mice. Figure 4 shows that when short-term variability was expressed as the SD of successive differences in breath-to-breath intervals (SD of successive differences), mice with disrupted leptin signaling had lower minute ventilation variability than B6 (wild-type) mice.

Long-term variability (SD2) of minute ventilation (fig. 3, dispersion of points along the line x = y) differed significantly by mouse line (F = 52.66; df = 3,72; P < 0.0001), sex (F = 9.05; df = 1,72; P = 0.0036), and injection condition (F = 184.42; df = 1,72; P < 0.0001). There was a significant (F = 4.02; df = 3,72; P = 0.0105) sex-by-mouse-line interaction for long-term changes in variability of minute ventilation. *Post hoc* analyses showed that long-term variability in minute ventilation of normal weight B6 mice differed from that of DIO, ob/ob, and db/db mice. Applying three-way ANOVA to long-term variability data calculated as the SD of breath-to-breath intervals revealed similar results (fig. 5). Considered together, the results of analyses illustrated by figures 4 and 5 support the conclusion that buprenorphine caused the greatest decrease in minute ventilation variability in mice with impaired leptin signaling.

Discussion

Three major findings emerged from this study. First, minute ventilation was significantly altered by leptin status in the
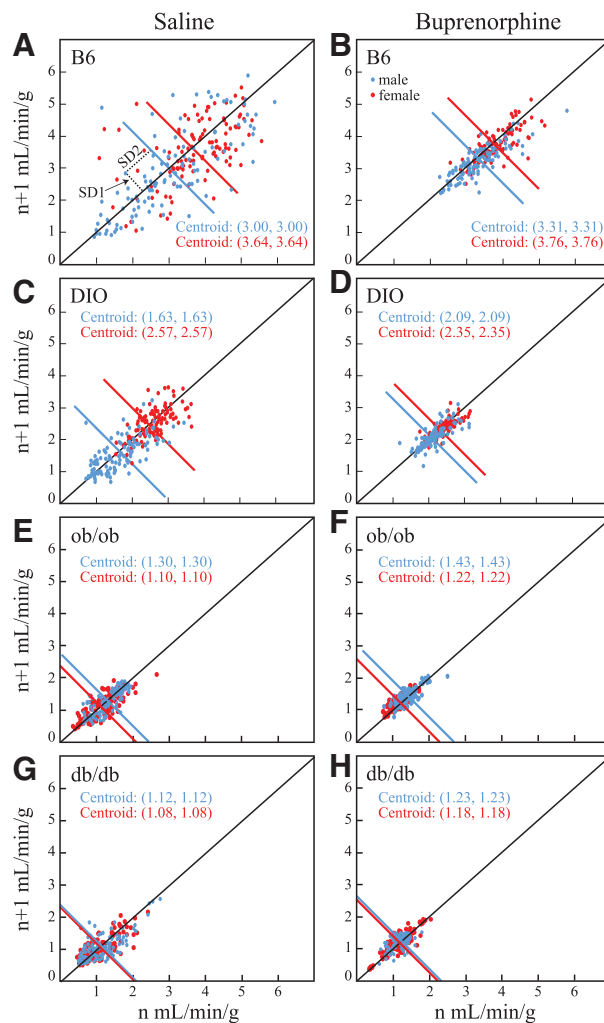
Fig. 3. Poincaré plots of buprenorphine-induced decrease in minute ventilation variability. Each frame plots data from 10 male (*blue dots*) and 10 female (*red dots*) mice for 1 h after injection of saline (*A, C, E, G*) or an antinociceptive dose of buprenorphine (0.3 mg/kg; *B, D, F, H*). Also shown in *black* for each frame is the *line of identity* (x = y) and *coordinates* (x, y) of the average position of all data points (centroid) for males (*blue type*) and females (*red type*). The *red* and *blue lines* placed perpendicular to the line of identity mark the centroid for males and females, respectively. The *dotted lines* in *A* indicate how short-term variability (SD1) and long-term variability (SD2) are calculated for a single data point. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

absence of buprenorphine. Second, minute ventilation did not vary significantly due to sex. Third, Poincaré plots illustrate that buprenorphine causes a collapse in minute ventilation variability. The findings are discussed below relative to the relationship between leptin and obesity, making the point that studies of congenic mice can provide unique insights into this relationship. Finally, we describe the previously unrecognized effect of buprenorphine to decrease respiratory variability. Limitations of the present study also are addressed.
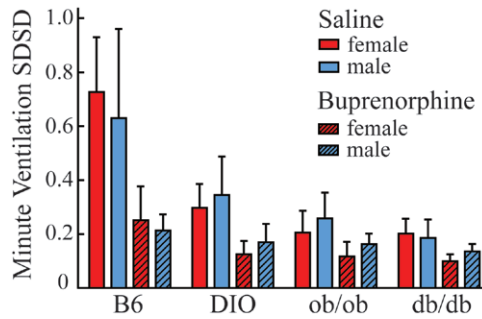
Fig. 4. SD of successive differences in breath-to-breath intervals (SDSD) indicates short-term variability, as does SD1 in figure 3. Histograms plot the means ± SD of SDSD for mouse line by sex and injection condition (saline *vs.* buprenorphine). In all mouse lines, buprenorphine caused a collapse in breath-to-breath variability of minute ventilation. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

Leptin, Obesity, and Murine Models of Disease

The perspective that obesity is a disease satisfies multiple criteria, the most stringent of which is the increased risk that obesity conveys for premature death.[38,39] Analysis of closed claims cases identified obesity as a risk factor for opiate-induced respiratory depression resulting in severe brain damage or death.[40] Leptin covaries with obesity, making it difficult to quantify the exact degree to which breathing varies as a function of obesity, leptin, or both. The present study confronted this complexity in two ways. First, the experiments were specifically designed to compare the respiratory effects of buprenorphine in normal weight B6 mice possessing normal leptin to the respiratory effects of buprenorphine in three congenic lines of obese mice, each with different obesity/leptin profiles. The four mouse lines made it possible to relate buprenorphine-induced changes in breathing (figs. 2–5) to obesity and leptin status (table 1). B6 mice provided an ideal control group because they are among the lines of healthy mice that the Mouse Phenome Committee recommend receive the highest priority for phenotyping.[41] B6 mice with diet-induced obesity are genetically identical to normal weight B6 mice. DIO mice are homologous to the vast majority of obese humans in that the mice possess both leptin and leptin receptors that, over time, may become leptin-resistant. Regression analyses provided a second approach for evaluating the degree to which buprenorphine-induced changes in breathing were altered by obesity or leptin. Those analyses showed that for none of the four mouse lines did body weight account for a significant change in minute ventilation. This finding is consistent with evidence that body weight alone does not significantly alter the antinociceptive effects of buprenorphine.[12]

Human obesity due to congenital, monogenic leptin deficiency is rare,[42] and leptin-deficient ob/ob mice do not emulate most cases of human obesity. The ob/ob mice, however, provide a valuable animal model with the potential to clarify the multiple functions of leptin. The db/db mice produce leptin but fail to express the long form of the leptin receptor.[43,44] Studies by others have used DIO, ob/ob, and db/db mice to gain
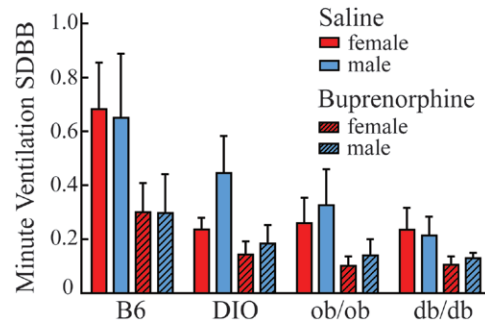
Fig. 5. Buprenorphine decreased overall (long-term) variability of minute ventilation. Changes in variability of the SD of breath-to-breath intervals (SDBB) are plotted as means ± SD. These histograms correspond to the variability along the line x = y in fig. 3. B6 = C57BL/6J; db/db = B6.BKS(D)-Lepr^{db}/J; DIO = B6 with diet-induced obesity; ob/ob = B6.Cg-Lep^{ob}/J.

insights into metabolic syndrome[45] and type 2 diabetes.[46,47] To our knowledge, the present data provide the first evidence that leptin signaling alters the effects of buprenorphine on minute ventilation by reducing minute ventilation variability.

Poincaré Analysis of Minute Ventilation Variability

Comparing figure 3 with figures 2, 4, and 5 illustrates the ability of Poincaré plots to provide a nuanced visualization of respiratory variability. In the Materials and Methods section, the description of Poincaré as exploratory data analysis refers to an "attitude, a state of flexibility, a willingness to look for those things that we believe are not there, as well as those we believe to be there."[48] The present application of Poincaré analysis demonstrates that buprenorphine significantly reduces minute ventilation variability. Figure 3 also makes clear that after saline administration, the mice with functional leptin systems (B6 and DIO[49]) had significantly greater variability in minute ventilation than the mice with dysfunctional leptin transmission (ob/ob and db/db). Consequently, the decrease in minute ventilation variability caused by buprenorphine was greatest for B6 and DIO mice that express both leptin and functional leptin receptors. Statistical analyses showed that leptin status played a central role *via* its ordinal interaction with sex and injection of buprenorphine. This finding supports the interpretation that leptin dysfunction (ob/ob and db/db), rather than obesity in the presence of leptin (DIO), conferred the greatest risk for decreased minute ventilation variability (fig. 3). The present results are consistent with evidence that leptin status rather than obesity causes an impaired ventilatory response to hypercapnia in ob/ob mice.[50]

Limitations

Potential limitations of the present study include the murine model, unknown mechanisms of leptin signaling, and the mathematics underlying the present discovery. The limitations of mice as models of human obesity[51] and obesity-related diseases derive from evolutionary differences in gene expression between mouse and human.[52] While acknowledging these limitations, the National Institutes of Health consider the

mouse as "the premier mammalian model system for genetic research" (https://www.genome.gov/10005834/background-on-mouse-as-a-model-organism/; accessed July 23, 2017). The genetics of human obesity reveal good homology with genotypes known to promote obesity in mice.[53] In addition, the mechanisms by which leptin, a protein produced by adipocytes, influences breathing remain unknown.[24,25,50,54,55] It is clear, however, that leptin function is not limited to energy homeostasis.[56] Leptin modulates neuronal excitability,[57] and long-standing evidence shows that leptin stimulates breathing.[22,23] The present results should not be misinterpreted as indicating that leptin is the only adipocyte-derived molecule modulating the buprenorphine-induced collapse in minute ventilation variability. The potential involvement of additional cell signaling proteins is suggested by the fact that adipose tissue functions as an endocrine organ and secretes hundreds of different adipokines.[58] Furthermore, inferring that only leptin from adipocytes modulates the effects of buprenorphine may be too simplistic in view of the discovery that leptin also is produced by parathyroid glands.[59] A third potential limitation is interpreting and communicating time-domain data. The present study included Poincaré methods because the analyses (fig. 3) "correspond so conveniently to time domain summary statistics."[37] The strengths and limitations of Poincaré analysis and its numeric descriptors (figs. 4 and 5) have been reviewed in detail.[30,60]

Conclusions

The results show that buprenorphine causes a collapse in minute ventilation variability, with the greatest collapse occurring in mice that lack normal leptin signaling. The results are consistent with evidence that leptin signaling alters the antinociceptive effects of buprenorphine.[12] Respiratory variability is clinically relevant because such variability is an essential aspect of homeostasis (*e.g.*, increasing tidal volume or respiratory rate) in response to challenges imposed by disease, obesity, surgical stress, and opiates. In cases where buprenorphine does cause respiratory depression, pharmacokinetic data make the cautionary point that buprenorphine-induced respiratory depression "outlasts the effects of naloxone-induced reversals from bolus injections."[61] Thus, the present findings encourage future studies to characterize the time course of the buprenorphine-induced decrease in respiratory variability. The novelty of the present findings is further illustrated by the fact that all previous clinical studies of the respiratory effects of buprenorphine have involved normal weight individuals. Maintaining ventilation within a normal range is key in the perioperative setting. However, that goal is complicated by the increasing number of obese patients and by patients who chronically use opiates or who are prescribed buprenorphine to manage their opiate use disorder.[1] Poincaré analysis of heart rate variability has a long record of diagnostic and prognostic success. The present results encourage future human studies to determine whether Poincaré plots have diagnostic or prognostic value relevant to respiratory depression caused by opiates.

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Streams of Unconsciousness II: Hypnosis Reflected in the Styx
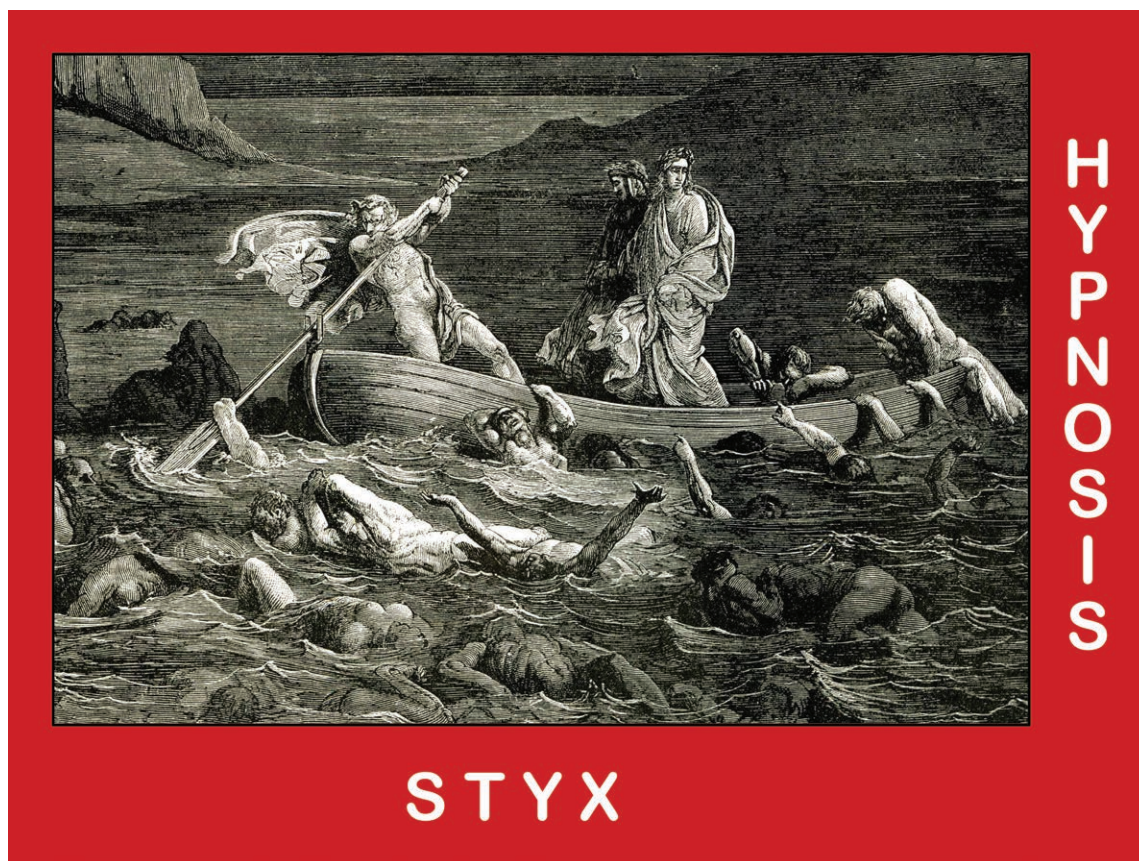
Besides the amnesia associated with the underworld's River Lethe, Greco-Roman mythology reflected the hypnosis or heavy trance induced by a different plutonic river, the Styx. The invulnerability conferred by Stygian waters—which famously had missed shielding Achilles's heel—ensured that Wrathful souls could flail into each other eternally while swimming in a ceaseless daze through the Styx. As illustrated by Gustave Dore for Dante's *Divine Comedy*, Phlegyas is ferrying Dante and the poet Virgil (all standing, left to right, *above*) through the thrashing Wrathful and across this shadowy "River of Hate." And stewing beneath the gloom of Stygian waters were the sinking souls of the Sullen, the dead whose anger was repressed. Submerging excitement into trance or even blacking out consciousness, the dark hypnotic power of the Styx was eclipsed by the river's fabled role as "the great Oath-witness of the gods." This honor was bestowed by a grateful Zeus for Styx's swift support during the Battle of the Titans. According to the Greek poet Hesiod, the "heavy trance" of Styx could even overshadow any of the immortal gods who violated an oath sworn in the name or with the water of the Styx. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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