Hypnotic Hypersensitivity to Volatile Anesthetics and Dexmedetomidine in Dopamine β-Hydroxylase Knockout Mice

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ABSTRACT

Background: Multiple lines of evidence suggest that the adrenergic system can modulate sensitivity to anesthetic-induced immobility and anesthetic-induced hypnosis as well. However, several considerations prevent the conclusion that the endogenous adrenergic ligands norepinephrine and epinephrine alter anesthetic sensitivity.

Methods: Using dopamine β-hydroxylase knockout (*Dbh*$^{-/-}$) mice genetically engineered to lack the adrenergic ligands and their siblings with normal adrenergic levels, we test the contribution of the adrenergic ligands upon volatile anesthetic induction and emergence. Moreover, we investigate the effects of intravenous dexmedetomidine in adrenergic-deficient mice and their siblings using both righting reflex and processed electroencephalographic measures of anesthetic hypnosis.

Results: We demonstrate that the loss of norepinephrine and epinephrine and not other neuromodulators co-packaged in adrenergic neurons is sufficient to cause hypersensitivity to induction of volatile anesthesia. However, the most profound effect of adrenergic deficiency is retarding emergence from anesthesia, which takes two to three times as long in *Dbh*$^{-/-}$ mice for sevoflurane, isoflurane, and halothane. Having shown that *Dbh*$^{-/-}$ mice are hypersensitive to volatile anesthetics, we further demonstrate that their hypnotic hypersensitivity persists at multiple doses of dexmedetomidine. *Dbh*$^{-/-}$ mice exhibit up to 67% shorter latencies to loss of righting reflex and up to 545% longer durations of dexmedetomidine-induced general anesthesia. Central rescue of adrenergic signaling restores control-like dexmedetomidine sensitivity. A novel continuous electroencephalographic analysis illustrates that the longer duration of dexmedetomidine-induced hypnosis is not due to a motor confound, but occurs because of impaired anesthetic emergence.

Conclusions: Adrenergic signaling is essential for normal emergence from general anesthesia. Dexmedetomidine-induced general anesthesia does not depend on inhibition of adrenergic neurotransmission.

What We Already Know about This Topic

- Modulation of adrenergic signaling inversely affects volatile anesthetic potency *in vivo*, suggesting a critical role anesthetic mechanisms
- Loss of norepinephrine and epinephrine in mutant mice deficient in dopamine β-hydroxylase increases sensitivity to isoflurane

What This Article Tells Us That Is New

- Loss of norepinephrine and epinephrine causes hypersensitivity to induction of and delayed emergence from volatile anesthetics, as well as hypersensitivity to the highly selective α2 adrenoceptor agonist dexmedetomidine
- These findings indicate that adrenergic signaling is essential for normal emergence from volatile anesthesia, but is not required for dexmedetomidine's hypnotic effects

ADRENERGIC neurons that utilize norepinephrine and epinephrine play an important role in the regulation of numerous physiological events, including thermoregulation, stress responses, attention, learning, and the sleep–wake cycle.[1] Although previous studies have shown

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that manipulations of adrenergic neurons affect the onset and dissipation of the anesthetic state, it remains unproven whether norepinephrine and epinephrine themselves are directly sufficient to alter anesthetic hypnosis. Acute administration of reserpine and α-methyldopa, agents known to reduce norepinephrine release, significantly decreases the amount of halothane required to prevent movement to noxious stimuli in animals.[2,3] Conversely, acute administration of agents such as cocaine, amphetamine, and the monoamine oxidase inhibitor, iproniazid, which generate increased norepinephrine release or prevent norepinephrine reuptake at synapses, increases the dose of halothane required to prevent movement.[2–4] Systemic delivery of dexmedetomidine, a highly selective α_2 adrenoceptor agonist, is an anesthetic on its own[5] but also decreases halothane anesthetic requirements,[6] providing support for anesthetic-induced suppression of adrenergic signaling in producing or altering an anesthetic state. Although these results consistently suggest a role for adrenergic signaling in modulating anesthetic sensitivity, inherent problems with pharmacologic specificity remain as a potential confounder.

A second class of studies supporting a role for adrenergic signaling in altering anesthetic sensitivity has been conducted using lesions. Systemic injections of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine or 6-hydroxydopamine combined with a tyrosine hydroxylase inhibitor severely deplete central nervous system norepinephrine levels and are associated with decreased halothane minimum alveolar concentration and prolonged duration of barbiturate-induced hypnosis.[6–9] Nevertheless, pharmacologic lesions cannot cleanly eliminate the entire adrenergic neuron population. Bilateral electrolytic lesions of the locus coeruleus (LC), implicated in mediating hypnosis and predominantly composed of noradrenergic neurons, significantly decrease halothane and cyclopropane anesthetic requirements.[10] However, in destroying LC neurons, the electrolytic lesions cause a loss of other cotransmitters in addition to norepinephrine, as well as a loss of nonadrenergic cells and fibers.

We previously reported that inactivation of LC neurons was not required for anesthetic action by halothane, questioning whether the removal of adrenergic signaling from the LC was the sole source behind hypnosis.[11] This finding heightens the importance of the question of whether the release of norepinephrine itself or that of co-packaged neurotransmitters, including neuropeptide Y, adenosine, and galanin, may be held responsible for modifying anesthetic sensitivity.

Using dopamine β-hydroxylase knockout (*Dbh*$^{-/-}$) mice that lack the adrenergic ligands norepinephrine and epinephrine, we previously demonstrated that loss of norepinephrine and epinephrine produced increased sensitivity to isoflurane.[12] Herein, we investigated the effects of sevoflurane and halothane on this genetically engineered model to extend our previous finding. We hypothesize that adrenergic deficiency in *Dbh*$^{-/-}$ mice accounts for hypersensitivity to induction of and delayed emergence from volatile anesthesia. Under the supposition that adrenergic signaling suffices to alter anesthetic hypnosis, we test the possibility that dexmedetomidine acts solely on adrenergic neurons.[13,14] If inhibition of adrenergic signaling were responsible for dexmedetomidine-induced hypnosis, we hypothesize that the intravenous anesthetic would produce no hypnotic effect in the norepinephrine-deficient *Dbh*$^{-/-}$ mice. As a control, we reexamined anesthetic effects upon restoring norepinephrine and epinephrine to the central nervous system using L-threo-3,4-dihydroxyphenylserine and benserazide, a peripheral L-amino acid decarboxylase inhibitor.[15,16]

Materials and Methods

Animals

All efforts were made to minimize animal suffering and reduce the number of animals used. *Dbh* heterozygous and knockout mice were maintained on a hybrid C57BL/6J × 129/SvCPJ genetic background and included equal numbers of males and females. *Dbh*$^{+/-}$ females were mated to *Dbh*$^{-/-}$ males and treated with 100 μg/ml each of phenylephrine and isoproterenol (Sigma, St. Louis, MO), from embryonic days 8.5 to 16.5 followed by 2 mg/ml L-threo-3,4-dihydroxyphenylserine (Sumitomo Pharmaceuticals, Osaka, Japan) until birth, in the drinking water to increase likelihood of fetal survival. Given that neither norepinephrine nor epinephrine is essential for postnatal survival, litters were not provided treatment after birth.[17] Heterozygous *Dbh*$^{+/-}$ siblings were used as controls as tissue samples revealed levels of norepinephrine and epinephrine indistinguishable from wild types.[16] The animals were housed under controlled conditions (12 h of light starting at 7:00 PM, 22°–24°C) in an isolated ventilated room and given food and water *ad libitum*. As *Dbh*$^{-/-}$ mice exhibit ptosis, a condition absent in their *Dbh*$^{+/-}$ siblings,[16] investigators could not be blinded to genotype. All studies were performed with approval from the Institutional Animal Care and Use Committee at the University of Pennsylvania (Philadelphia, PA) and in accordance with National Institutes of Health guidelines.

Loss and Return of Righting Reflex

Anesthetic sensitivity was assessed behaviorally using loss of righting reflex (LORR). A mouse unable to turn itself prone onto all four feet was considered to have lost its righting reflex and entered a hypnotic state. All mice were observed until they had regained the righting reflex (RORR) at which point the mouse was able to right itself two consecutive times within 1 min of each other. In single-step anesthetic wash-in and washout studies, the times to induction and emergence were recorded.

Dbh$^{+/-}$ (n = 30) and *Dbh*$^{-/-}$ (n = 36) mice ranged from 4 to 6 months in volatile anesthetic experiments. To acclimatize animals to the testing environment, mice were placed in 200-ml cylindrical open circuit chambers and exposed to 200 ml/min of fresh oxygen flow for 2 h daily in the 4 days before anesthetic testing. Mice were exposed to an average

initial concentration of 0.63% isoflurane, 0.73% halothane, or 0.96% sevoflurane in 100% oxygen. After every 15-min period, chambers were rotated 180° to assess LORR. Anesthetic gas concentrations were determined in triplicate during the last 2 min of each 15-min interval using a Riken FI-21 refractometer (AM Bickford, Wales Center, NY). The concentration of volatile anesthetic was increased incrementally by 0.05%, 0.06%, or 0.10% to peak values of 1.10%, 1.32%, and 1.92% for isoflurane, halothane, and sevoflurane, respectively. After the last mouse had lost its righting reflex, volatile anesthetic was discontinued and time until emergence as defined by the return of the righting reflex was determined. Body temperature was maintained at 36.6° ± 0.2°C by submerging the chambers in a heated water bath.

After fitting *Dbh*$^{+/-}$ and *Dbh*$^{-/-}$ LORR data with separate sigmoidal dose–response curves in Prism 4.0c (GraphPad Software, Inc., La Jolla, CA) to characterize induction in each genotype, volatile anesthetic concentrations required to elicit LORR in 95% of mice (ED_{95}) were extrapolated for both *Dbh*$^{+/-}$ and *Dbh*$^{-/-}$ mice. To control for genotype differences in volatile anesthetic induction, a second round of single-step anesthetic wash-in and washout experiments was conducted with all mice exposed to their corresponding ED_{95} induction dose in oxygen for 2 h, after which the anesthetic gas was discontinued (n = 36/genotype). Times to LORR and RORR were recorded.

In dexmedetomidine experiments, *Dbh*$^{+/-}$ (n = 20) and *Dbh*$^{-/-}$ (n = 23) mice ranged from 8 to 14 weeks. Animals received an intravenous injection in one of four possible doses: 50, 100, 200, or 400 mg/kg, administered in identical volumes of 5 ml/kg. Immediately after injection, mice were held in 200-ml cylindrical open circuit chambers under a heating lamp and above a heating pad in order to prevent hypothermia. Chambers were rotated 180° every 30 s until a mouse failed to right itself. A total of 4 of 43 mice had failed intravenous injections and were consequently excluded from the study. Rectal temperatures, both preinjection and postanesthesia were measured to ensure that the mice remained euthermic during the experiment.

Pharmacologic Rescue of Adrenergic Signaling

Rescue of adrenergic signaling in *Dbh*$^{-/-}$ mice was performed according to published protocols.[16,18] Five hours in advance of anesthetic sensitivity testing, four *Dbh*$^{-/-}$ mice received a subcutaneous injection of 20 mg/ml pH-neutralized L-threo-3,4-dihydroxyphenylserine, 2 mg/ml vitamin C (Sigma), and 1 mg/ml benserazide (Sigma), a peripheral aromatic L-amino acid decarboxylase inhibitor, in a total volume of 50 μl/g. This treatment restores norepinephrine to near-normal levels for 12 h with peaks around 5 h postinjection. As a control for injection-induced stress, four additional *Dbh*$^{-/-}$ mice received a subcutaneous injection of 50 μl/g vehicle. *Dbh*$^{+/-}$ mice were not treated with L-threo-3,4-dihydroxyphenylserine and benserazide as such therapy can cause supranormal levels of norepinephrine in these control mice.[16]

Surgical Procedure

Dbh$^{+/-}$ (*n* = 12) and *Dbh*$^{-/-}$ (*n* = 8) mice ranged in age from 5 to 9 months. Animals were deeply anesthetized with 1.5–2.0% isoflurane and warmed during surgery. Mice were treated aseptically, given cefazolin 40 mg/kg intraperitoneally (Sagent Pharmaceuticals, Schamburg, IL), placed in a Model 940 stereotaxic frame (David Kopf Instruments, Tujunga, CA), and given 0.3 ml 0.25% bupivacaine (Hospira, Inc., Lake Forest, IL) subcutaneously before opening a 3-cm vertical scalp incision. After exposing the periosteum, four burr holes were drilled into the skull over primary motor (relative to bregma, 1.5 mm anterior and ±1.6 mm lateral) and visual cortices (3.5 mm posterior, ±1.7 mm lateral) bilaterally.[19] Four miniature stainless-steel screws (Small Parts, Miami Lakes, FL) were inserted into each hole to form epidural electroencephalographic leads and secured with dental acrylic (Co-Oral-Ite Dental Manufacturing Company, Diamond Springs, CA). A fifth burr hole was drilled (1.0 mm posterior and 1.7 mm lateral) to permit placement of a 600-μm wide, calibrated 10-kΩ microthermistor (AB6E3-GC16KA103L, GE Infrastructure Sensing, St. Marys, PA) inserted to acquire continuous cortical brain temperatures[20] (T_{br}) and secured with dental acrylic. Two Teflon-coated silver wires (A-M Systems, Inc., Sequim, WA) were inserted along the neck muscles to form electromyographic leads. The four screw electrodes, two electromyographic leads, and thermistor had previously been electrically connected to an insulated 2 × 4 pin row connector (Digi-Key, Thief River Falls, MN) to form an ultra lightweight 0.6-g socket headpiece.[20] Headpieces were secured with dental acrylic. The surgical wound was treated with a triple antibiotic ointment (Neomycin and Polymyxin B sulfates and Bacitracin Zinc; E. Fougera & Co., Mellville, NY). Skin edges were reapproximated with suture. Immediately after surgery, mice were injected with 1.0 ml 0.9% sterile saline and given a single injection of buprenorphine 0.3 mg/kg intraperitoneally.

Physiological Recordings

Mice were allowed at least 7 days to recover from surgery before experimentation. All experiments were performed at room temperature (22°–24°C). Mice were connected to the recording apparatus *via* a lightweight flexible shielded tether and then back loaded into a custom-modified plexiglass tail-vein injector with free access to the mouse's head. Electroencephalographic and electromyographic analog signals were amplified (factor of 5000) and filtered (high-pass 1.0 Hz, low-pass 100 Hz, 60 Hz Notch filter for electroencephalogram; high-pass 10 Hz, low-pass 5000 Hz, 60 Hz Notch filter for electromyogram). The thermistor signal was amplified by means of a direct-current powered, custom-made bridge (Intec Associates Ltd., Surrey, United Kingdom), and had been calibrated before implantation, using a water bath and precision thermometer. All signals were converted from analog to digital with Biopac's MP150 System (BIOPAC Systems, Inc., Goleta, CA) and visualized

using Acknowledge 3.9.2 software for Mac (BIOPAC Systems, Inc.). All biopotential signals were digitally sampled at 200 Hz except for T_{br}, which was sampled at 1.5 Hz. Recordings began a minimum of 30 min before and continued for 4–6 h after a single intravenous tail injection of 50 μg/kg dexmedetomidine (Hospira, Inc.) given in a volume of 5 μl/g. Dexmedetomidine dosing was based on postoperative weight minus the weight of the headpiece. T_{br} was continuously monitored throughout the recording, whereas rectal temperatures were measured both preinjection and postanesthesia. During these physiological recording sessions, mice were actively warmed with a servo-controlled heat lamp set to shut off at 37°C.

Analysis of Electroencephalographic and Electromyographic Signals

For each mouse, three channels of electroencephalographic and one channel of electromyographic data were imported into the Somnologica 3.2 rodent sleep scoring software module (Embla Systems, Broomfield, CO). Each 10-s epoch was automatically scored for the presence of wakefulness. Automatically scored epochs were visually inspected to confirm correct assignments. Any epoch containing artifact was excluded from subsequent analyses. During the baseline period, before dexmedetomidine, sample entropy was only determined during epochs of wakefulness. Sample entropy values were calculated for all channels using a freely available module[21] written for Matlab Student 7.4 (The Mathworks, Inc., Natic, MA) with an N = 1,000, m = 2, r = 0.2. Integrated electromyography values were computed directly using AcqKnowledge 3.9.2 and were normalized so that the highest value obtained during a baseline 5-s epoch of wakefulness was defined as 100%.

Statistical Analysis

Investigators were blinded to genotype during data processing and statistical analysis and simply identified mice by ear-tag number. For volatile anesthetic experiments, dose responses for induction were fit with a sigmoidal curve (Prism 4.0c) to obtain EC_{50} and hill slopes along with their corresponding 95% confidence limits.[22,23] For analysis of times to LORR and RORR after stepwise or single-step ED_{95} volatile anesthetic experiments, a two-way ANOVA with Bonferroni posttests was used with main factors of genotype and volatile anesthetic. Using JMP (SAS Institute, Inc., Cary, NC), a log transformation was used to normalize the unequal variances in emergence times. Two-way ANOVA with Bonferroni posttests was also used for comparison of latency to and duration of dexmedetomidine-induced LORR values with main factors of genotype and dexmedetomidine dose using Prism 4.0c. An independent two-sample Student t test was used to compare the duration of and latency with LORR values between *Dbh*$^{-/-}$ mice involving rescue or sham-rescue of adrenergic signaling. LORR values are reported as mean ± standard error. Continuous temperature data were imported into Prism 4.0c and analyzed as change from average baseline wakefulness temperature. The Wilcoxon test was used for comparison of continuous temperature data between genotypes whereas the independent two-sample Student t test was used for comparison of rectal temperatures. Integrated electromyographic and sample entropy raw data were independently fit with five linear segments to approximate baseline, induction, duration at nadir values, slope of recovery, and post–dexmedetomidine recovery states using least-squares regression to the mean. A Wilcoxon test was used to assess genotypic differences both for integrated electromyographic and sample entropy values. Due to technical problems with screw implantation, 1 or 2 electroencephalographic leads in 3 of 12 *Dbh*$^{+/-}$ and 2 of 8 *Dbh*$^{-/-}$ had to be excluded from data analysis. The exact number of mice represented for each electroencephalographic lead is shown in the corresponding box plot. All comparisons were unpaired and performed using two-tailed hypothesis testing with the exception of one-tailed testing for sample entropy values. A P value of less than 0.05 was considered statistically significant.

Results

Adrenergic-deficient Mice Are Hypersensitive to Volatile Anesthetic Induction and Exhibit Delayed Emergence from Volatile Anesthetics

Using the LORR assay, *Dbh*$^{-/-}$ mice, which completely lack epinephrine and norepinephrine, exhibit hypersensitivity to the induction of isoflurane, sevoflurane, and halothane anesthesia, as compared with *Dbh*$^{+/-}$ sibling controls (fig. 1) shown previously to have normal catecholamine levels.[16] EC_{50} values (95% CI) for induction of isoflurane, sevoflurane, and halothane anesthesia are 0.79% (0.77–0.81%), 1.26% (1.23–1.29%), 0.93% (0.91–0.96%) in *Dbh*$^{-/-}$ mice and 0.92% (0.90–0.94%), 1.64% (1.61–1.68%), 1.04% (1.02–1.06%) in *Dbh*$^{+/-}$ mice, respectively. Moreover, the effects of genotype upon emergence from volatile anesthetics were significant ($F_{1,236}$ = 46.7, P value less than 0.0001). When exposed to identical concentrations of a volatile anesthetic, emergence takes two to three times as long in *Dbh*$^{-/-}$ mice relative to their sibling controls (fig. 1B, 1D, 1F). Not surprisingly, time to emerge also depended significantly upon the anesthetic drug ($F_{2,236}$ = 41.6, P value less than 0.0001). There was no significant interaction between genotype and volatile anesthetic ($F_{2,236}$ = 5.8, P = 0.06).

Because the induction sensitivity of *Dbh*$^{-/-}$ mice is left-shifted as compared with that of their sibling controls (fig. 1A, 1C, 1E), exposing both *Dbh*$^{-/-}$ mice and *Dbh*$^{+/-}$ siblings to identical concentrations of volatile anesthetics leads to a relative overdosing of the *Dbh*$^{-/-}$ mice that could affect their subsequent emergence. To avoid this confound, *Dbh*$^{+/-}$ and *Dbh*$^{-/-}$ mice were each exposed to their respective ED_{95} for LORR based on the best-fit curves generated in figure 1. Times to induction and emergence were assessed by LORR and RORR. Upon exposure to their ED_{95} dose for volatile anesthetics, *Dbh*$^{+/-}$ and *Dbh*$^{-/-}$ exhibited a significant difference

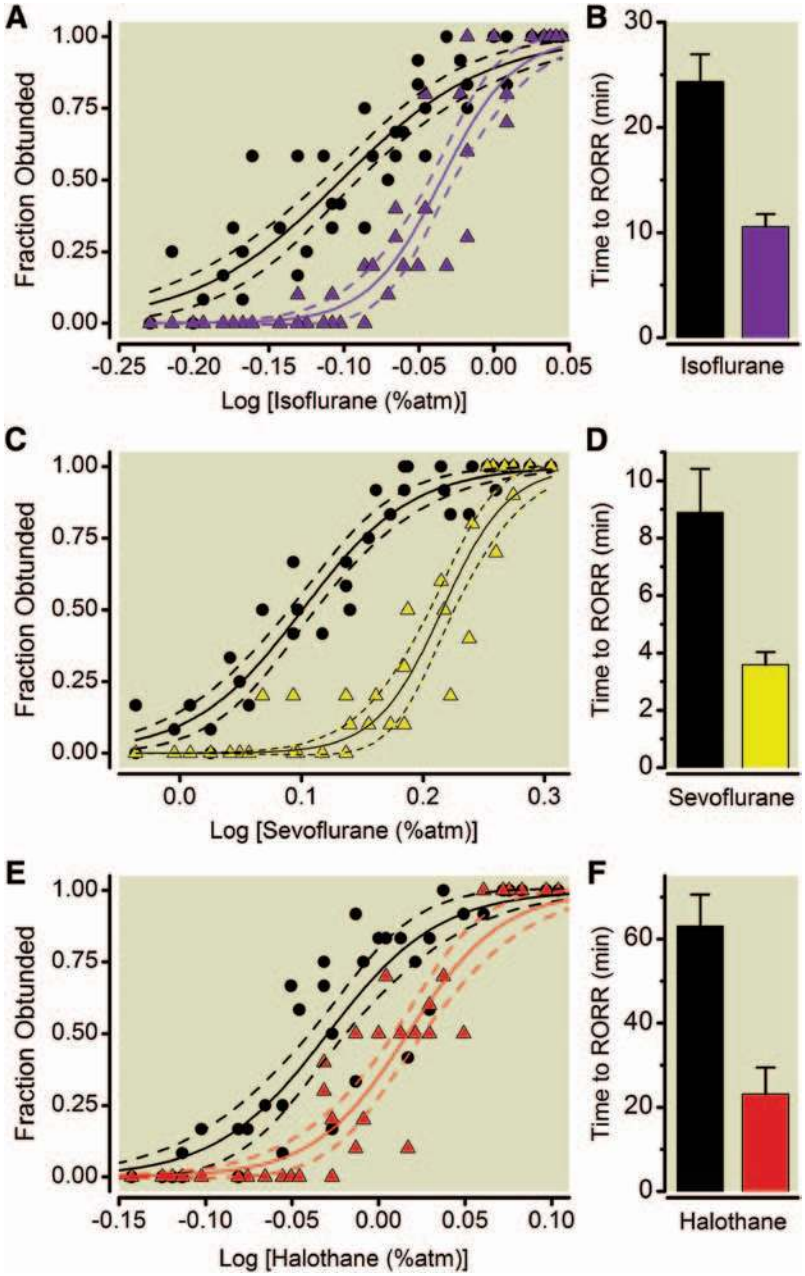

Fig. 1. Adrenergic-deficient mice exhibit volatile anesthetic hypersensitivity relative to sibling controls with normal levels of norepinephrine/epinephrine. *Symbols* respectively depict the fraction of $Dbh^{+/-}$ (n = 10, *triangles*) and $Dbh^{-/-}$ (n = 12, *squares*) mice that exhibit a loss of righting reflex (LORR) at each specified anesthetic dose for *(A)* isoflurane, *(C)* sevoflurane, and *(E)* halothane. *Solid lines* denote the best-fit curves with *dashed lines* showing 95% confidence interval bracketing the best-fit curves. *Bars* represent mean ± SEM time lapsed from the termination of anesthetic exposure (shown in *A*, *C*, *E*) until the return of righting reflex (RORR) for $Dbh^{-/-}$ (*black*) and $Dbh^{+/-}$ (*colored*) mice for *(B)* isoflurane, *(D)* sevoflurane, and *(F)* halothane. Effects of genotype are significant $F_{1,236} = 46.7$, *P* value less than 0.0001. Atm = atmosphere; *Dbh* = dopamine β-hydroxylase.

in latency to LORR with respect to anesthetic ($F_{2,66} = 35.67$, *P* value less than 0.0001), which may be explained by differences in volatile anesthetic solubility. However, no significant difference was observed with respect to genotype ($F_{1,66} = 0.14$, $P = 0.7132$), and no significant interaction ($F_{2,66} = 2.20$, $P = 0.1193$) occurred between anesthetic and genotype, suggesting an identical rate of volatile anesthetic induction (fig. 2A). Upon discontinuation of anesthetic gases after 2 h, $Dbh^{+/-}$ and $Dbh^{-/-}$ mice similarly exhibited a significant difference in time to emergence with a main effect of the anesthetic ($F_{2,66} = 27.04$, *P* value less than 0.0001). Crucially, a significant difference was observed with respect to genotype ($F_{1,66} = 12.33$, *P* value less than 0.0003) while no significant interaction ($F_{2,66} = 0.51$, P = 0.6046) occurred between anesthetic and

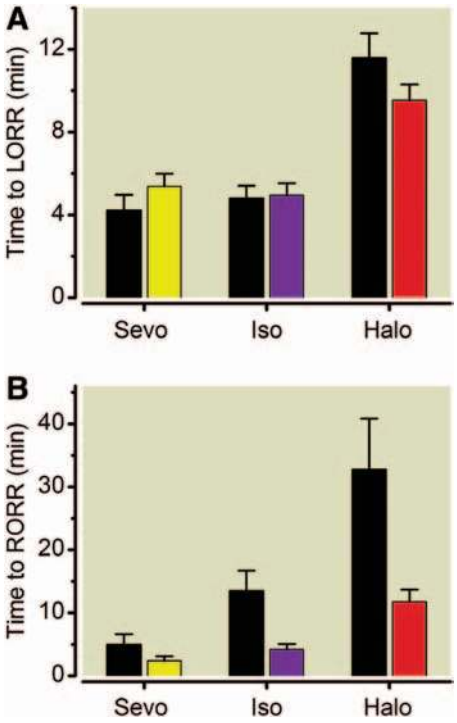

Fig. 2. Delayed emergence in *Dbh*$^{-/-}$ mice is not due to a relative volatile anesthetic overdose. *(A)* Time to LORR in *Dbh*$^{-/-}$ and *Dbh*$^{+/-}$ mice when each group is exposed to their respective ED_{95} dose for eliciting LORR. *(B)* Time until RORR in *Dbh*$^{-/-}$ (*black*) and *Dbh*$^{+/-}$ (*colored*) mice after a 2-h exposure to each group's respective volatile anesthetic ED_{95} (n = 12/group). There is a significant effect of genotype on emergence $F_{1,66}$ = 12.33, P value less than 0.0003, but not upon induction $F_{1,66}$ = 0.14, P = 0.7132. *Bars* show mean ± SEM. *Dbh* = dopamine β-hydroxylase; Halo = halothane; Iso = isoflurane; LORR = loss of righting reflex; RORR: return of righting reflex; Sevo = sevoflurane.

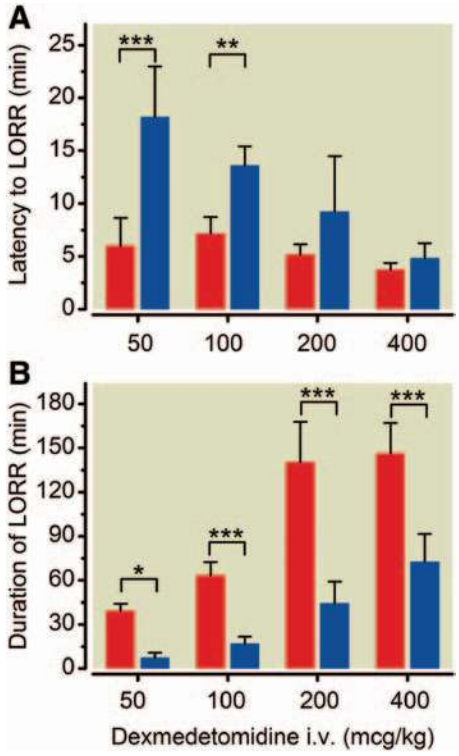

Fig. 3. Loss of adrenergic ligands is sufficient to cause hypersensitivity to dexmedetomidine as assessed by LORR. *Dbh*$^{-/-}$ mice illustrate hypersensitivity relative to *Dbh*$^{+/-}$ mice in response to varying intravenous doses of dexmedetomidine. *(A)* Latency to loss of righting reflex and *(B)* duration of LORR in *Dbh*$^{-/-}$ (*red*) and *Dbh*$^{+/-}$ (*blue*) mice (n = 5–6/group). Significant genotypic effects were found for both latency to $F_{1,35}$ = 53.40, P value less than 0.0001 and duration of $F_{1,35}$ = 158.77, P value less than 0.0001 dexmedetomidine-induced hypnosis. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to *Dbh*$^{+/-}$ mouse latency to LORR or duration of LORR times. *Bars* show mean ± SEM. *Dbh* = dopamine β-hydroxylase; LORR = loss of righting reflex.

genotype (fig. 2B). These results emphasize that despite correcting for intrinsic changes in sensitivity to anesthetic induction, the *Dbh*$^{-/-}$ mice continue to exhibit significant impairments in their ability to emerge after volatile anesthetic exposures.

Cumulatively, these results suggest that loss of norepinephrine and epinephrine in *Dbh*$^{-/-}$ mice is sufficient to cause hypersensitivity to volatile anesthetic induction as well as delayed emergence after volatile anesthetic exposure. Consequently, we proceeded to test the hypothesis that *Dbh*$^{-/-}$ mice would be insensitive to dexmedetomidine, an intravenous anesthetic that inhibits noradrenergic neurons in the LC.

Adrenergic-deficient Mice Are Hypersensitive to Dexmedetomidine

Surprisingly, as with the volatile anesthetics, the *Dbh*$^{-/-}$ mice illustrated hypersensitivity to dexmedetomidine-induced hypnosis at all tested doses. With intravenous dexmedetomidine, there were significant differences in latency to LORR with respect to both genotype ($F_{1,35}$ = 53.40, P value less than 0.0001) and dose ($F_{3,35}$ = 18.07, P value less than 0.0001), with a significant interaction between genotype and dose ($F_{3,35}$ = 8.37, P = 0.0003; fig. 3A). Moreover, two-way ANOVA revealed significant differences in the duration of LORR with respect to both genotype ($F_{1,35}$ = 158.77, P value less than 0.0001) and dose ($F_{3,35}$ = 69.87, P value less than 0.0001), and the interaction between the two factors is significant ($F_{3,35}$ = 8.32, P = 0.0003; fig. 3B). Larger doses of dexmedetomidine were associated with decreased latency to LORR and longer duration of LORR. At the highest tested intravenous dose of dexmedetomidine, 400 μg/kg, *Dbh*$^{-/-}$ mice showed significant mortality with four of six mice dying. All deaths occurred within 120 min of dexmedetomidine administration, and these data were excluded. Meanwhile, all sibling control mice survived.

To address whether this apparent hypersensitivity to dexmedetomidine in *Dbh*$^{-/-}$ mice could be the result of a developmental compensation, we rescued adrenergic signaling specifically in the central nervous system of adult *Dbh*$^{-/-}$ mice. Five hours after treatment with vehicle or L-threo-3,4-dihydroxyphenylserine plus benserazide, *Dbh*$^{-/-}$ mice

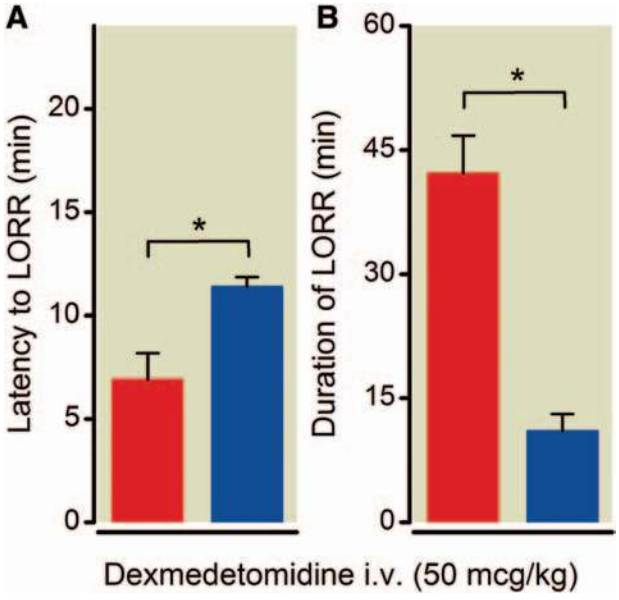

Fig. 4. Central nervous system–specific rescue of norepinephrine and ephinephrine in adrenergic-deficient mice restores duration of dexmedetomidine-induced hypnosis to control levels. Both the *(A)* latency to LORR after 50 μg/kg of intravenous dexmedetomidine and *(B)* the duration of LORR are rescued in *Dbh*$^{-/-}$ mice receiving L-threo-3,4-dihydroxyphenylserine and benserazide rescue treatment (*blue*) as compared with *Dbh*$^{-/-}$ mice that received vehicle treatment (*red*) (n = 4/group). * $P < 0.05$. *Bars* show mean ± SEM. *Dbh* = dopamine β-hydroxylase; LORR = loss of righting reflex.

received 50 μg/kg dexmedetomidine intravenously. Restoration of adrenergic signaling partially, but significantly restored the latency to dexmedetomidine-induced LORR (P = 0.021) (fig. 4A). However, central rescue of adrenergic signaling in *Dbh*$^{-/-}$ mice fully rescued the duration of dexmedetomidine anesthesia (P = 0.016) (fig. 4B). Rescued *Dbh*$^{-/-}$ averaged 11.0 ± 2.1 min for duration of LORR as compared with 42.2 ± 4.6 min in sham rescued *Dbh*$^{-/-}$ mice. These values are respectively identical to untreated *Dbh*$^{-/-}$ animals, that averaged 39.3 ± 2.1 min and to *Dbh*$^{+/-}$ mice, which averaged 7.2 ± 1.6 min after 50 μg/kg intravenous dosing (fig. 3).

Electroencephalographic Evidence of Dexmedetomidine Hypersensitivity in Adrenergic-Deficient Mice Is Not Accompanied by Corresponding Motor Atonic Hypersensitivity

To avoid a potential motor confound and to isolate the hypnotic properties of anesthetics, we measured sample entropy of the electroencephalogram in multiple leads acquired simultaneously from in *Dbh*$^{-/-}$ mice and their sibling *Dbh*$^{+/-}$ controls. A typical example depicting how sample entropy changes after administration of dexmedetomidine is shown (fig. 5A). In practice, no significant allelic effects at the *Dbh* locus were found in the bifrontal, bioccipital, or left-occipital to left-frontal leads for (1) baseline entropy level prior to dexmedetomidine injection (P = 0.06, P = 0.10, P = 0.18); (2) minimum entropy level immediately after intravenous dexmedetomidine (P = 0.60, P = 0.70, P = 0.20); (3) the duration of time spent at the minimum entropy level (P = 0.84, P = 0.57, P = 0.70); or (4) post–dexmedetomidine emergence entropy levels (P = 0.09, P = 0.48, P = 0.08). However, *Dbh*$^{-/-}$ mice showed a significantly reduced rate of emergence based on the entropy recovery slope as compared with *Dbh*$^{+/-}$ mice in the bifrontal (P = 0.008), bioccipital (P = 0.044) and left frontal to left occipital (P = 0.028) leads (fig. 5B). *Dbh*$^{-/-}$ mice demonstrated no significant differences from *Dbh*$^{+/-}$ mice with regard to slope from minimum integrated electromyogram to postanesthesia integrated electromyogram (P = 1.00) or in any other measure of motor activity save for baseline muscle tone (P = 0.042) where integrated electromyographic tone during wakefulness in the knockouts exceeded that of their siblings (fig. 6). Finally, no significant effects of genotype were found for changes in temperature after dexmedetomidine administration either for continuously recorded T_{br} (P = 0.1905) or for baseline rectal temperature minus that recorded immediately after emergence (P = 0.07; data not shown).

Discussion

Loss of the adrenergic ligands norepinephrine and epinephrine and not of neurotransmitters co-packaged in adrenergic neurons is sufficient to cause hypersensitivity to induction of anesthesia. Impairing central adrenergic signaling left-shifts induction dose–response curves for many anesthetics,[2,3,7,12,24,25] including isoflurane, but our work extends this association to other volatile anesthetics and dexmedetomidine as well. Moreover, our results address a lingering question about the role of dopamine in modulating anesthetic induction. Although pharmacological treatments that deplete norepinephrine may also deplete other monoamines such as dopamine,[2] in the *Dbh*$^{-/-}$ model dopamine levels increase;[17] we demonstrate induction hypersensitivity indicating that modulation of dopamine may not predominate as has been implied.[26]

Adrenergic deficits also affect anesthetic emergence, causing significant temporal delays in RORR, our surrogate marker for recovery of consciousness in mice. By comparing the magnitude of this temporal effect with our previously published study on isoflurane,[12] we assert that the most profound effect in the *Dbh*$^{-/-}$ mice occurs during emergence. In support of this conclusion, when adrenergic-deficient mice and their sibling controls are exposed to their respective equipotent induction doses of isoflurane, sevoflurane, or halothane, we find no differences in the time to LORR. Although much is known about the cardiorespiratory physiology in adrenergic-deficient mice,[27–29] formal evaluation of their cardiac output and minute ventilation remains lacking. As anesthetic uptake and distribution depend on cardiac output and minute ventilation, it is reasonable to assume that such values may differ between *Dbh*$^{-/-}$ and sibling control mice. However, within the limits of sensitivity for righting-reflex assay,

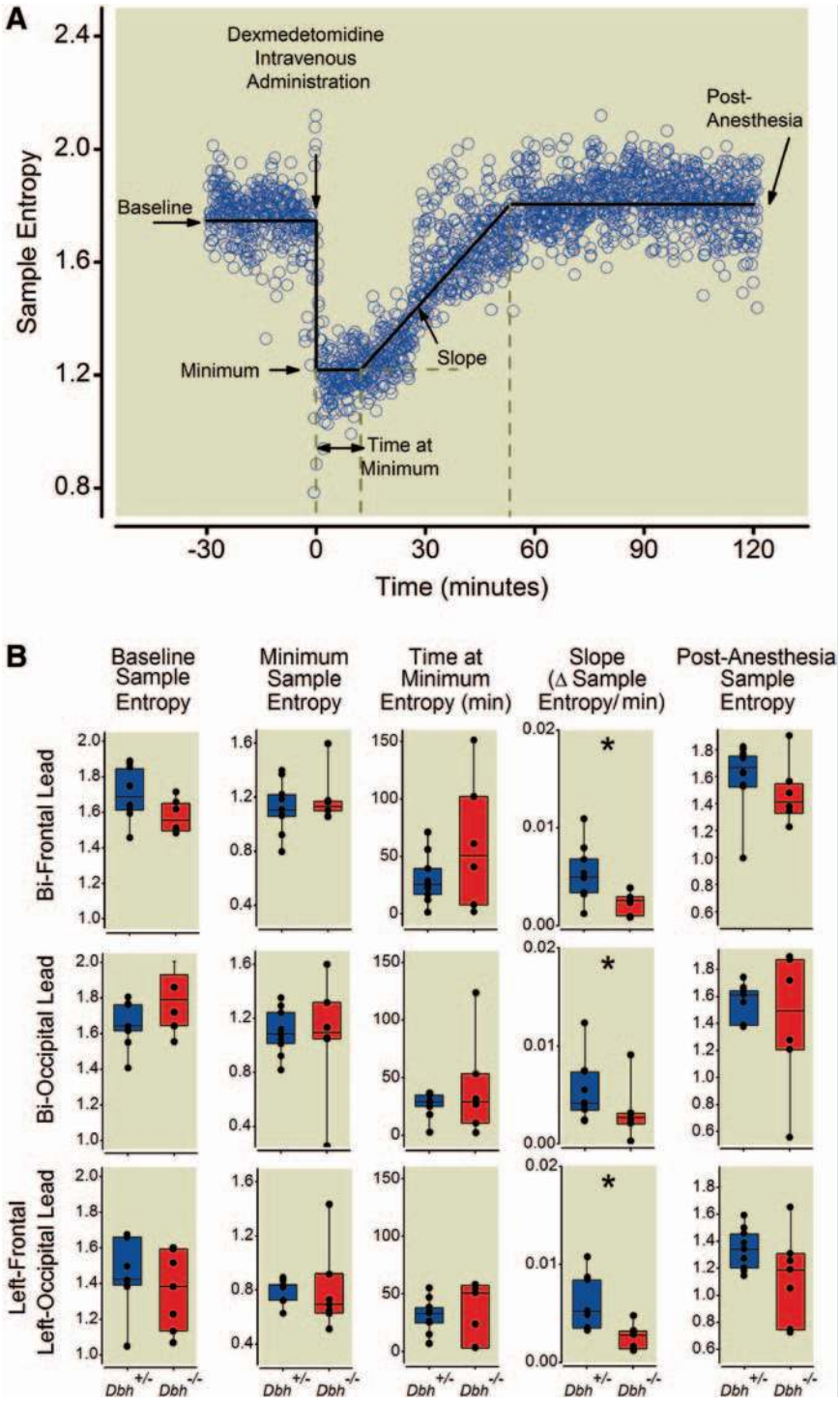

Fig. 5. *Dbh*$^{-/-}$ mice show delayed emergence from dexmedetomidine relative to *Dbh*$^{+/-}$ mice using a motor-independent electroencephalographic measure. *(A)* Panel shows a characteristic segmental best-fit (*solid line*) analysis of sample entropy values calculated from raw electroencephalogram in a control mouse before, during, and after intravenous dexmedetomidine. Raw sample entropy values (*circles*) were calculated from the electroencephalogram, which were fit with five linear segments to approximate five variables as labeled. Dexmedetomidine was administered intravenously at time = 0 as denoted by the *arrow*. *(B) Box plots* illustrate sample entropy values along with lower quartile, upper quartile, group minimum and group maximum for predrug baseline wakefulness, minimum entropy level, the duration of time at the minimum entropy level, the slope of sample entropy recovery that defines emergence, and sample entropy post–dexmedetomidine emergence for *Dbh*$^{+/-}$ (n = 12) and *Dbh*$^{-/-}$ (n = 8) mice as computed from right frontal–left frontal, right occipital–left occipital, and left frontal–left occipital leads. *$P < 0.05$ in *Dbh*$^{-/-}$ mice relative to *Dbh*$^{+/-}$ mice. *Dbh* = dopamine β-hydroxylase.

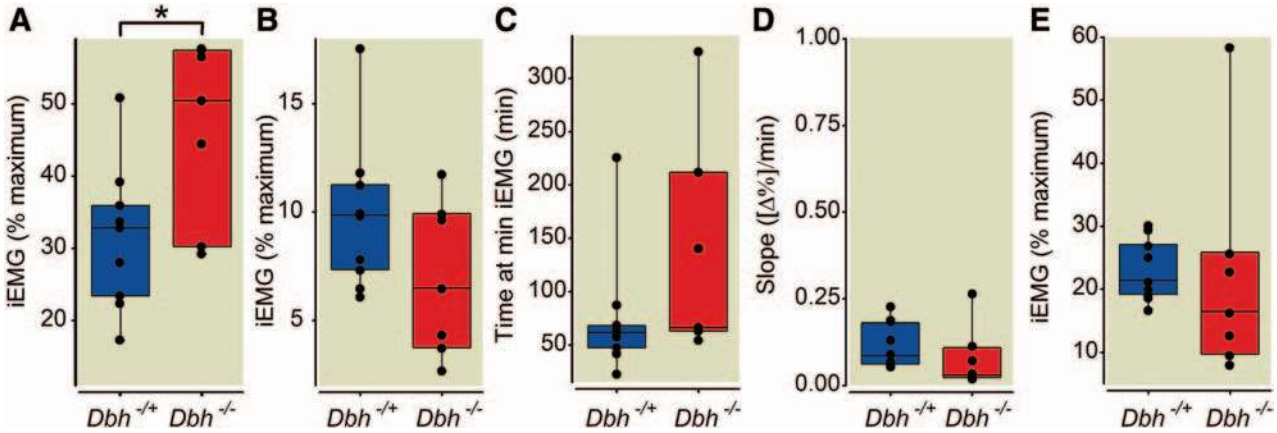

Fig. 6. Adrenergic-deficient mice show no significant differences from sibling controls with respect to motor tone after intravenous dexmedetomidine administration. *Box plots* depict integrated electromyographic intensity (normalized to percentage of maximum during wakefulness) along with lower quartile, upper quartile, group minimum, and group maximum for *(A)* predrug baseline wakefulness, *(B)* minimum integrated electromyogram values, *(C)* the duration of time at the minimum integrated electromyogram values, *(D)* the slope of integrated electromyogram recovery, and *(E)* stabilized integrated electromyogram values post–dexmedetomidine emergence in $Dbh^{+/-}$ (n = 12) and $Dbh^{-/-}$ (n = 8) mice. *$P < 0.05$ in $Dbh^{-/-}$ mice relative to $Dbh^{+/-}$ mice. *Dbh* = dopamine β-hydroxylase; iEMG = integrated electromyogram.

and by using each genotype's respective ED_{95} as a driving dose, we are unable to detect differences between $Dbh^{-/-}$ and sibling controls in the times to induction. This places a small upper limit on the degree to which anesthetic uptake and distribution might differ between $Dbh^{-/-}$ and their siblings. Nonetheless, under identical methodological constraints, we find significant differences for emergence in $Dbh^{-/-}$ mice (fig. 1). Even when corrected for intrinsic differences in volatile anesthetic sensitivity, adrenergic-deficient mice still take two to three times longer to emerge from volatile anesthetics than sibling controls do (fig. 2). Considering that metabolism of isoflurane, sevoflurane, and halothane varies over 100-fold and that elimination also depends upon cardiac output and minute ventilation, these results point to large and important differences in the pharmacodynamic effects of volatile anesthetics in adrenergic-deficient $Dbh^{-/-}$ mice.

Adrenergic Signaling and Emergence from Volatile Anesthesia

The loss of norepinephrine and epinephrine is associated with a protracted RORR, signifying delayed emergence. These studies reveal a prominent role for norepinephrine and epinephrine and more broadly, a necessary reactivation of adrenergic signaling, in facilitating anesthetic emergence. During emergence from halothane, LC neurons exhibit burst-like firing patterns,[30] causing endogenous release of norepinephrine. Microdialysis studies confirm surges in norepinephrine in the preoptic area of the hypothalamus during emergence from sevoflurane and isoflurane.[31] Within basal forebrain, norepinephrine can directly promote transient arousals.[32] If released upon sleep-active ventrolateral preoptic hypothalamus (VLPO) and median preoptic hypothalamic neurons, norepinephrine's hyperpolarizing actions should inhibit activity in these groups, which would release inhibition of other wake-active systems, thus enhancing arousal.[33,34] Studies conducted in humans demonstrate an arousal-promoting effect of ephedrine upon processed electroencephalographic measures of anesthetic hypnosis.[35,36] As a mixed indirect adrenergic agonist, ephedrine could affect dopamine levels in addition to norepinephrine. There is accumulating evidence to suggest that facilitation of dopaminergic signaling itself may enhance emergence from anesthesia *via* multiple mechanisms.[37,38] However, the relative contributions of noradrenergic *versus* dopaminergic mechanisms merit additional clarification.[37,39]

Mechanism of Action of Dexmedetomidine

Despite the studies supporting the LC as the key site of action underlying the hypnotic actions of dexmedetomidine,[13,14] we find that inhibition of adrenergic neurons within LC or elsewhere is unlikely to mediate the hypnotic properties of dexmedetomidine. Given that the loss of adrenergic ligands (and not a co-packaged neuromodulator) is sufficient for hypnotic hypersensitivity to and delayed emergence from volatile anesthetics, the causal association of dexmedetomidine-induced hypnosis proceeding *via* inhibition of the LC and ensuing disinhibition of the VLPO must be challenged.[13] We demonstrate significantly reduced latencies and prolonged duration of dexmedetomidine action in $Dbh^{-/-}$ mice, as compared with sibling controls (fig. 3). These changes are unlikely to arise from potential developmental compensation because postnatal central nervous system–specific rescue of adrenergic signaling reverted the hypnotic duration in $Dbh^{-/-}$ mice to levels indistinguishable from controls. Rather, the rescue of norepinephrine and epinephrine suggests that adrenergic signaling restoration is sufficient for

normal emergence.[12] Although dexmedetomidine still should inhibit LC neuronal activity *via* α_{2A} adrenoceptors in *Dbh*$^{-/-}$ mice,[40] these mice have no norepinephrine to release at the synapse. Consequently LC targets normally inhibited by norepinephrine, such as the VLPO,[41–43] hypothetically should not be affected unless dopamine or another co-packaged neurotransmitter could substitute for norepinephrine. If inhibiting release of co-packaged neuromodulators from the LC and other adrenergic neurons such as galanin, neuropeptide Y, or adenosine were sufficient to cause anesthesia, then *Dbh*$^{-/-}$ mice should not have had altered responses to volatile anesthetics. This leaves two viable alternative explanations that might still rescue the Nelson model[13] for dexmedetomidine or the possibility that the model requires revision. Dopamine accumulates in adrenergic neurons of *Dbh*$^{-/-}$ mice.[17] Although the exact adrenoceptors mediating the hyperpolarizing effects of norepinephrine upon VLPO remain unknown, presynaptic α_{2A} adrenoceptors abutting putative sleep-promoting VLPO neurons are involved.[44] Dopamine is more than 1,000-fold less potent than norepinephrine at α_{2A} adrenoceptors and 10- to 10,000-fold less potent than norepinephrine at other adrenoceptors.[45] Although this does not formally exclude dopamine released from adrenergic neurons in *Dbh*$^{-/-}$ mice, two studies demonstrate that adrenergic neurons themselves are not required for α_2 agonist-induced hypnosis. After pharmacologic depletion of catecholamines, dexmedetomidine retains an additional profound volatile anesthetic-sparing effect.[6] Moreover, medetomidine retains its hypnotic properties in mice that lack α_{2A} adrenoceptors on adrenergic neurons,[46] proving that inhibition of adrenergic neurons, including those in the LC, is not necessary. Finally, it could be possible that the apparent interpretation of hypnosis in *Dbh*$^{-/-}$ mice given either medetomidine[46] or dexmedetomidine (fig. 3) could simply be loss of postural muscle tone leading to LORR with preserved consciousness. It is known that exhaustion of norepinephrine from its terminals is sufficient to mimic cataplexy, inhibiting movement.[47] Here, our studies of electroencephalographic effects of dexmedetomidine in *Dbh*$^{-/-}$ mice and sibling controls are particularly informative. We demonstrate no difference in the onset, depth, duration, or recovery from motor inhibition in both groups of mice given intravenous dexmedetomidine (fig. 6). Although we detect an increased baseline resting motor tone in *Dbh*$^{-/-}$ mice, the presence of significantly higher delta power during wakefulness in these animals[18] may have confounded our scoring of wakefulness, with a greater fraction of active, high-motor tone wakeful epochs selected for *Dbh*$^{-/-}$ mice than in control siblings. Nevertheless, even if *Dbh*$^{-/-}$ mice have heightened resting motor tone, this finding would not confound our righting-reflex results because only muscle weakness might cloud the interpretation of LORR. By applying a novel segmental best-fit algorithm to continuous sample entropy measurements, we confirm a processed electroencephalographic parameter that conclusively demonstrates delayed rate of emergence in *Dbh*$^{-/-}$ mice given dexmedetomidine (fig. 5). Together with the LORR studies, we demonstrate that *Dbh*$^{-/-}$ mice are hypersensitive to anesthetics using methodology that permits a dissociation between the myorelaxant and hypnotic effects of anesthetic drugs. It is possible that we were underpowered to detect differences in awake baseline sample entropy between *Dbh*$^{-/-}$ and *Dbh*$^{+/-}$ siblings. Increased sleepiness in *Dbh*$^{-/-}$ mice[18] would be predicted to result in lower sample entropy values.

One issue that remains is the question of whether modulation of adrenergic signaling mechanistically underlies anesthetic hypnosis. Proving a mechanistic link would require demonstrating that all anesthetics inhibit adrenergic signaling as a requirement for induction and that direct inhibition (enhancement) of adrenergic signaling is sufficient to facilitate (retard) induction. In the case of volatile anesthetic induction, this study and others demonstrate the latter. However, although many anesthetics do inhibit adrenergic output or downstream adrenoceptor-coupled signal transduction, this is not uniformly true.[2,3,6,7,9,11–13,24,48–51] With respect to anesthetic emergence, a parallel analysis of necessity and sufficiency would require the demonstration both 1) surges in adrenergic signaling that precede emergence and 2) the association that increased (decreased) adrenergic signaling is sufficient to facilitate (retard) emergence.[12,30–32,37] Formally meeting such strict requirements would require detailed understanding of the anesthetic effects on every step from adrenergic neuronal activity, synaptic release of adrenergic ligands, receptor–ligand binding, post–receptor signal transduction, and modulation of ligand metabolism or reuptake. Complete characterization for each and every step in the presence of distinct anesthetics is lacking. Hence, we cannot formally exclude the possibility that modulation of adrenergic signaling independently affects arousal parallel to but independent of anesthetic drug effects.

Concluding Remarks

Our results with the α_2 selective agonist, dexmedetomidine, are consistent with those obtained with volatile anesthetics in which adrenergic-deficient mice also exhibit delayed emergence. Cumulatively, our results suggest that current understanding of the mechanisms through which dexmedetomidine works are incomplete. Although dexmedetomidine may indeed inhibit the LC and subsequently disinhibit the VLPO, the initial step of LC inhibition is neither required[11] nor sufficient[46] to explain anesthetic hypnosis. Dexmedetomidine's hypnotic effects must arise *via* actions on postsynpatic α_{2A} adrenoceptors located on nonadrenergic neurons. Determining the identity of the nonadrenergic neurons should become a priority for subsequent studies, as targeted inhibition of this neural substrate may prove important for existing as well as novel therapeutics.

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