Bidirectional Regulation of Intravenous General Anesthetic Actions by α3-containing γ-aminobutyric Acid$_A$ Receptors

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ABSTRACT

Background: γ-aminobutyric acid$_A$ (GABA$_A$) receptors mediate the actions of several intravenous general anesthetics. However, the contribution of α3-containing GABA$_A$ receptors to the action of these drugs is unknown.

Methods: The authors compared anesthetic endpoints (hypnosis, immobility, hypothermia) in response to various intravenous anesthetics in mice lacking the α3 subunit of the GABA$_A$ receptor (α3 knockout) and in wild-type mice. Furthermore, the authors generated and analyzed conditional mutant mice expressing the GABA$_A$ receptor α3 subunit exclusively in noradrenergic neurons.

Results: α3 knockout mice displayed decreased hypnotic and hypothermic responses to etomidate and midazolam, but an increased response to pentobarbital. The hypnotic response to ketamine was unaltered, whereas the hypothermic response was increased. In contrast, the hypnotic but not the hypothermic response to medetomidine was increased. The combination of ketamine/xylazine displayed increased hypnotic, immobilizing, and hypothermic effects in α3 knockout mice. Mice expressing the α3 subunit exclusively in noradrenergic neurons were generated to assess whether the lack of α3 subunits on noradrenergic neurons may be responsible for this effect. In these mice, the increases of the hypnotic and immobilizing actions induced by ketamine/xylazine were largely absent, whereas the increase in the hypothermic action was still present.

Conclusion: α3-containing GABA$_A$ receptors bidirectionally regulate essential anesthetic actions: they mediate anesthetic actions of etomidate and midazolam, known to selectively act at GABA$_A$ receptors, and they negatively constrain anesthetic actions of compounds with targets partly or exclusively distinct from GABA$_A$ receptors such as medetomidine, ketamine, and pentobarbital. Furthermore, our results indicate that α3-containing GABA$_A$ receptors on noradrenergic neurons may contribute to this constraint.

What We Already Know about This Topic

- The γ-aminobutyric acid$_A$ (GABA$_A$) receptor system mediates actions of many general anesthetics in the central nervous system. However, the contributions of the different GABA$_A$ receptor subtypes and the role of noradrenergic neurons require further clarification

What This Article Tells Us That Is New

- GABA$_A$ receptors containing the α3 subunit mediate anesthetic responses to etomidate and midazolam. Lack of the α3 subunit results in enhanced responses to the combination ketamine/xylazine, an effect that is rescued in part by expression of the α3 subunit selectively in noradrenergic neurons, indicating an involvement of noradrenergic neurons in modulating hypnosis

γ-AMINOBUTYRIC acid$_A$ (GABA$_A$) receptors are chloride channels, which mediate fast synaptic inhibition and tonic inhibition in the central nervous system. They are composed of five subunits drawn from a repertoire of eight

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different subunit families comprising at least 19 different genes (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3).[1–4] Depending on the exact subunit composition $GABA_A$ receptors possess different physiological and pharmacological properties.[1,2] Many general anesthetics potentiate the activity of $GABA_A$ receptors, and the role of several $GABA_A$ receptors subtypes in mediating anesthetic effects has been described.[3,4] We have previously shown that β3(N265M) knock-in mice display dramatically reduced hypnotic responses to etomidate and pentobarbital and cannot be immobilized by these compounds, demonstrating that a subpopulation of $GABA_A$ receptors is essential for the anesthetic actions of these anesthetic agents.[5] Most frequently, the $GABA_A$ receptors are classified by the α subunit that is present. On the basis of studies in global knockout (KO) mice, the α1 subunit,[6] but not the α5[7] and α6 subunits,[8] contribute to the hypnotic actions of intravenous general anesthetics. However, the significance of α3 subunit–containing $GABA_A$ receptors for anesthetic effects remained to be clarified.

Performing stereotaxic surgeries we made the unexpected and surprising observation that a combination of ketamine, an *N*-methyl-D-aspartate receptor antagonist and xylazine, an α2-adrenergic agonist, induces remarkably prolonged anesthesia in α3KO mice compared with wild-type (WT) mice. The α3 subunit of the $GABA_A$ receptor is present in 10–15% of all $GABA_A$ receptors in the central nervous system and $GABA_A$ receptors containing the α3 subunit are expressed widely in the brain.[1,2] However, in monoaminergic neurons the α3 subunit seems to be the major α subunit expressed. High expression levels in noradrenergic neurons[9–11] suggest that α3 subunit–containing $GABA_A$ receptors might be important modulators of the noradrenergic system.

The noradrenergic system (cell groups A1–A10), which includes the locus coeruleus (A6) whose neurons project widely throughout the neuraxis and other noradrenergic nuclei (*e.g.*, A1 and A2, which project mainly to the hypothalamus) plays an important role in the regulation of sleep and arousal as well as thermoregulation.[12–14] Activity of the noradrenaline-containing locus coeruleus neurons depends on the behavioral state (*e.g.*, sleeping or waking) of the organism and this system is likely involved in switching between these different states.[15] Moreover, α2-adrenoceptor agonists such as dexmedetomidine have been shown to exert their sedative/hypnotic effects through endogenous sleep pathways,[16] at least in part through inhibition of the locus coeruleus.[17]

In order to obtain insights into the role of α3-containing $GABA_A$ receptors in general anesthetic actions, we investigated (1) in α3KO mice whether α3-containing $GABA_A$ receptors mediate the action of intravenous general anesthetics known to act at least in part *via* $GABA_A$ receptors such as etomidate, midazolam, and pentobarbital; (2) whether α3-containing $GABA_A$ receptors play a role in the actions of the *N*-methyl-D-aspartate receptor antagonist ketamine and of the α2-adrenergic agonist medetomidine, either alone or in combination, using α3KO mice; and (3) whether α3-containing $GABA_A$ receptors on noradrenergic neurons are responsible for the increased sensitivity to ketamine/xylazine. To this end, using conditional cre-*lox*P-mediated recombination limited to noradrenergic neurons, we generated α3KO mice in which the α3 subunit was reintroduced exclusively in noradrenergic neurons.

Materials and Methods

Animals

Male mice on the C57BL/6J background between 8 and 18 weeks of age at beginning of experiments were used. Biochemical and morphological studies were performed in Zurich (Switzerland) with approval of the Cantonal Veterinary Office of Zurich and performed in accordance with international guidelines on animal use and care (European Community Council Directive 86/609/EEC). All behavioral experiments were conducted in Belmont in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals, and were approved by the McLean Hospital Institutional Animal Care and Use Committee, Belmont, Massachusetts. Mice lacking the α3 subunit of the $GABA_A$ receptor (α3KO mice) have been described previously.[18] For behavioral testing mice were group-housed (3–4 per cage) and kept under a reversed 12/12 h light–dark cycle (lights on from 9:00 PM to 9:00 AM) for at least 2 weeks before beginning of experimental testing and throughout the study.

Generation of Global Rescue Mice

The generation of mice lacking the α3 subunit of the $GABA_A$ receptor (α3KO mice) has been described in detail.[18] The absence of α3 messenger RNA was demonstrated by reverse transcription polymerase chain reaction, and the absence of the α3 protein by Western blot and immunohistochemistry.[18] As the nomenclature of exons in the *Gabra3* gene has changed over time, exon 4 in the study by Yee *et al.*[18] is identical to exon 5 as described here. These mice carry an artificial exon flanked by *lox*P sites in the *Gabra3* gene, which is located on the X-chromosome. In mice that carry both the mutant gene and express the Cre recombinase this artificial exon is removed, thereby restoring α3 subunit expression. Mice in which the α3 subunit is rescued globally have been generated by crossing α3KO mice with EIIa-Cre mice, which express the Cre recombinase in oocytes and preimplantation stages of the embryo.[19] Mice carrying both the mutant gene and the Cre recombinase gene have been selected by polymerase chain reaction genotyping. These mice were crossed with C57BL/6J WT mice. Hemizygote male mice carrying only the mutant gene were then selected for experiments to ensure that the rescue of the α3 subunit had been present in the germline.

Generation of Neuron-specific Rescue Mice

Mice expressing the α3 subunit of the $GABA_A$ receptor exclusively in noradrenergic (dopamine β hydroxylase [DBH]-rescue mice) and dopaminergic (dopamine

transporter [DAT]-rescue mice) neurons, respectively, have been generated by crossing female α3KO mice with male mice expressing the improved Cre (iCre) recombinase specifically in (nor)adrenergic (DBH-iCre mice) or dopaminergic neurons (DAT-iCre mice). DBH-iCre mice express the iCre recombinase under the control of the promoter of the DBH gene and have been described previously.[20,21] DAT-iCre mice express the iCre recombinase under the control of the promoter of the DAT gene and have been described previously.[22] Male mice carrying both the α3KO gene and the respective iCre transgene have been selected by polymerase chain reaction genotyping.

Immunoperoxidase Staining

Four WT and four global rescue mice (9 weeks of age) were deeply anaesthetized with pentobarbital (Nembutal®, Abbott, Chicago, IL; 50 mg/kg, intraperitoneally) and perfused through the ascending aorta with fixative solution (4% paraformaldehyde in 0.15 M phosphate buffer and 15% saturated picric acid solution; pH 7.4). Brains were postfixed in fixative solution for 3 h and incubated in citrate buffer (0.2 M Na_2HPO_4 dihydrate, 0.1 M citric acid; pH 4.5) overnight. The next day brains were cut parasagittally into approximately 8-mm thick blocks and irradiated in citrate buffer at 650 watt in the microwave for 90 s for antigen retrieval.[23] Brains were cryoprotected in 10% dimethyl sulfoxide in phosphate buffered saline (PBS) for at least 2 h before sectioning them transversally at 40 μm with a sliding microtome. The sections were collected in PBS. Free-floating sections were incubated overnight at 4°C with a primary antibody directed against the α3 subunit of the $GABA_A$ receptor (1:5,000; antibody was developed in-house and is described in detail)[9] in TrisTriton buffer (0.05 M Tris, 0.15 M NaCl, 0.2% Triton X-100; pH 7.4) containing 2% normal goat serum. The next day sections were washed three times in Tris-Triton buffer and incubated for 30 min at room temperature in biotinylated secondary antibody (1:300; Jackson Immunoresearch, West Grove, PA) in Tris-Triton buffer containing 2% normal goat serum. After three washes in Tris-Triton buffer, sections were incubated in the Avidin-Peroxidase (ABC)-Solution (1:100 in TrisTriton buffer) for 30 min (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) and after another wash finally reacted with diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) in Tris-Triton buffer (pH 7.7) containing 0.015% hydrogen peroxide. After 15 min the color reaction was stopped with ice-cold PBS. After two washes in PBS at room temperature, sections were mounted on gelatin-coated slides and air-dried. Finally, they were dehydrated with an ascending series of ethanol, cleared in xylene, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). Brains and sections from WT and global rescue mice were processed in parallel under identical conditions to minimize variability in staining intensity. Images were taken with a stereo microscope (Zeiss, Jena, Germany).

Immunofluorescence Staining

DAT-Rescue (n = 4), DBH-Rescue (n = 3), WT (n = 3), and α3KO (n =2) mice (8–14 weeks of age) were examined to confirm selective expression of the α3 subunit in dopaminergic and noradrenergic neurons, respectively. For optimal and simultaneous detection of tyrosine hydroxylase, a neuronal marker for dopaminergic and noradrenergic neurons, and the α3 subunit of the $GABA_A$ receptor a novel protocol was used, which has been described in detail previously.[24,25] In brief, mice were anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (composition in mM: NaCl, 125; $NaHCO_3$, 26; NaH_2PO_4, 1.25; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 2.5; and glucose, 11; oxygenated with 95% O_2–5% CO_2). The brain was affixed to a vibratome stage with cyanoacrylate and kept in the ice-cold artificial cerebrospinal fluid for slicing. Parasagittal 300 μm-thick slices were prepared and incubated at 33°C for 20 min in oxygenated artificial cerebrospinal fluid. They were then fixed by immersion for 10 min in 4% paraformaldehyde in 0.15 M phosphate buffer, washed with PBS, cryoprotected overnight in 30% sucrose in PBS, and frozen on a flat surface in the cryostat. Parasagittal sections were cut at a thickness of 16 μm, mounted onto gelatin-coated glass slides, air-dried at room temperature for 30 s, and stored at 20°C for at least 1 h. Sections were preincubated for 1 h at room temperature in PBS containing 10% normal goat serum and 0.2% Triton X-100, followed by overnight incubation at 4°C with primary antibodies against tyrosine hydroxylase raised in rabbit (1:5,000; Merck Millipore, Billerica, MA) and against the α3 subunit of the $GABA_A$ receptor raised in guinea pig (1:3,000)[9] diluted in the same solution. Sections were then washed extensively in PBS and incubated for 1 h at room temperature in corresponding affinity-purified goat immunoglobulin G coupled to Cy3 (1:500) or Alexa 488 (1:1000; Jackson Immunoresearch). Sections were washed again, and coverslipped with aqueous mounting medium (Dako, Carpinteria, CA). Immunofluorescence staining was visualized by confocal laser scanning microscopy (LSM-510 Meta; Zeiss) with a 40× objective (N.A. 1.3) and sequential acquisition of separate color channels. Image-acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. The pinhole size was set to 1.0 Airy units for each channel, and stacks of 12 sections (512 × 512) spaced by 0.4 μm were acquired. For display, images were processed with the image-analysis program Imaris (Bitplane, Zurich, Switzerland). Images from both channels were overlaid, background was subtracted, and a low-pass filter ("edge preserving" filter) was applied. Images are displayed as extended projection of the entire stack.

Western Blotting

Membrane preparation and Western blotting have been conducted as described previously.[26] Fresh frozen brains from five WT and five global rescue mice (10 weeks of age) were homogenized in 5 ml sucrose buffer (0.32 M sucrose,

5 mM ethylenediaminetetraacetic acid, 10 mM Tris pH 7.5, 0.02% NaN_3, phenylmethanesulfonyl fluoride 1:500, complete, mini protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]) and centrifuged for 10 min at 1,000*g*. The supernatant was collected and the step repeated. Both supernatants were combined and centrifuged for 20 min at 40,000*g* to obtain the crude membrane fraction. The pellet was resuspended in 3 ml sucrose buffer. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Samples were incubated for 5 min at 95°C with an equal volume of 125 mM Tris-HCl pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate and stored at −20°C. Before use samples were incubated for 10 min at 60°C and diluted to a concentration of 1 mg/ml. Aliquots with increasing protein content (2.5, 5, 7.5, and 10 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% minigels (Mini protean II; Bio-Rad, Hercules, CA). Proteins were transferred onto nitrocellulose membranes using a Trans Blot Mini Cell (Bio-Rad). The blots were blocked for 1–2 h in 10 mM Tris-HCl pH 8, 0.15 M NaCl, 0.05% Tween 20 containing 5% nonfat dry milk at room temperature, followed by incubation with affinity-purified antiserum against the α3 subunit of the $GABA_A$ receptor (1:500)[9] together with a monoclonal antibody directed against β-actin (1:20,000, Merck Millipore) overnight at 4°C in 10 mM Tris-HCl pH 8, 0.15 M NaCl, 0.05% Tween 20/5% nonfat dry milk. The blots were washed once with 20 mM Tris pH 7.5, 60 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.4% SDS, 0.4% Triton-X 100, and 0.4% deoxycholate and four times with 10 mM Tris-HCl pH 8, 0.15 M NaCl, 0.05% Tween 20. Incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies was carried out for 1 h at room temperature. After extensive washing immunoreactivity was detected by the enhanced chemoluminescence method (Super Signal West Pico chemoluminescence, Pierce), images were captured using a Fujifilm (Tokyo, Japan) LAS-1000 Plus Gel Documentation System, and immunoreactive bands were quantified with the AIDA software (Version 3.25, Raytest, Pforzheim, Germany). Actin immunoreactivity was used to monitor equal sample loading. The specificity of the antibody against the α3 subunit has been confirmed previously using α3KO mice.[18]

Drugs

Ketamine-HCl/Xylazine-HCl solution (Sigma-Aldrich) was administered at a volume of 20 ml/kg. Ketamine-HCl (Sigma-Aldrich), medetomidine-HCl solution (Domitor®, Pfizer Animal Health, New York, NY) and pentobarbital-Na (Sigma-Aldrich) were administered at a volume of 10 ml/kg. Etomidate (Amidate®, Hospira, Lake Forest, IL) was administered at a volume of 15 ml/kg. Midazolam-HCl solution (Bedford Laboratories, Bedford, OH) was administered at a volume of 16.5 ml/kg. All drugs were dissolved or diluted in physiological (0.9%) saline where necessary and drug doses were calculated as mg/kg base. All drugs were administered by intraperitoneal injection. The doses administered are reported in the Results section.

Behavioral Testing

Tests were conducted during the dark phase (9:00 AM–9:00 PM) and each test was conducted at approximately the same time of day. The tester was blind to the genotype of the animals. Animals were tested in three cohorts. Cohort 1 containing WT, α3KO, DBH-Rescue, and DBH-iCre mice received a single injection of ketamine/xylazine and anesthetic properties were observed. Cohort 2 containing WT and α3KO mice were first tested for medetomidine-induced sedation in the locomotor activity test, 3 days later for medetomidine-induced hypothermia, and 4–6 days later for anesthetic properties of ketamine, medetomidine, or pentobarbital. Animals received increasing doses of ketamine, medetomidine, or pentobarbital every 7 days. Mice were counterbalanced for drug experience. Cohort 3 containing WT and α3KO mice were first tested for anesthetic properties of midazolam and 7 days later for anesthetic properties of etomidate.

Locomotor Activity

Animals were moved into the testing room at least 1 h before testing. Animals were injected with medetomidine (30 μg/kg or 60 μg/kg) or vehicle immediately before testing. The testing apparatus consisted of four evenly illuminated (approximately 130 lux) clear plexiglas (J. Freeman, Inc., Dorchester, MA) boxes (41 × 41 × 31 cm) separated by white cardboard. Boxes were cleaned with 70% ethanol, between animals. Locomotor activity was recorded for 60 min using the video-tracking system EthoVisionXT (Noldus Information Technology, Wageningen, The Netherlands).

Medetomidine-induced Hypothermia

Animals were moved into the testing room directly before testing. Body temperature was determined using a TH-5 digital thermometer (Physitemp Instruments, Clifton, NJ) with a RET3-Iso thermal probe for mice (inserted 2 cm deep) immediately before drug injection (vehicle, 60 μg/kg, 120 μg/kg medetomidine) and 30, 60, 90, and 120 min thereafter. Mice were kept together in their home cage throughout the experiment.

Anesthetic Endpoints

The assessment of anesthetic end points was adapted from published protocols.[27–29] Animals were moved into the testing room directly before testing. Each animal was placed in a clean holding cage without bedding material immediately after drug injection. Body temperature was determined using a TH-5 digital thermometer (Physitemp Instruments) with an RET3-Iso thermal probe for mice (inserted 2 cm deep) immediately before drug injection and every 30 min thereafter. Once the animal stopped moving it was turned on its back. Beginning of loss of righting reflex (LORR) was defined as the time-point when the animal

was placed on its back. End of LORR was defined as the time-point when the animal righted itself spontaneously from dorsal to sternal recumbency with all four extremities touching the ground. The time between beginning and end of LORR was defined as duration of LORR. After regaining the righting reflex animals were turned on their back every minute and time to righting was scored: less than 2 s (score = 1), between 2 and 10 s (score = 2), more than 10 s (score = 3). Animals were defined as having fully recovered when they were able to right themselves three times consecutively within 2 s. Time to recovery was defined as the time between beginning of LORR and full recovery. A total LORR score was calculated by adding the score values for every minute from beginning of LORR until full recovery. The loss of hind limb withdrawal reflex (LHWR) was determined every 5 min by pinching the paw firmly with an atraumatic forceps alternating between the left and right hind paw until animals regained the righting reflex. LHWR was defined as present if no signs of reaction to the pinch were observed.

Anesthetic properties of medetomidine were assessed using a different protocol. Animals were moved into the testing room directly before testing and each animal was placed in a clean holding cage without bedding material immediately after drug injection. Animals were gently rolled on their side 30, 60, 90, and 120 min after injection and time to righting defined as completely returning to the prone position with all four extremities touching the ground was noted. LORR was defined as present if animals were not able to roll back to the prone position within 10 s.

Statistical Analysis

Data were analyzed using the appropriate statistical test (Student *t* test, ANOVA followed by *post hoc* Dunnett *t* test, ANOVA with repeated measures followed by *post hoc* *t* tests with Bonferroni correction) or Sheffé *post hoc* test. Hypothesis testing was two-tailed. Data for medetomidine-induced LORR were analyzed by fitting standard least-squares mixed models with subject as a random effect. Drug dose and genotype were included as fixed effects. All effects were assessed by F tests based on the fitted regression models. This analysis was followed by *post hoc* Mann–Whitney U tests. Data are expressed as the mean ± SEM. *P* value less than 0.05 was considered to be statistically significant. Statistical analysis was performed using the SPSS (SPSS, Inc., Chicago, IL) and GraphPad (GraphPad Software, Inc., La Jolla, CA) software packages.

Results

When performing stereotaxic surgeries for implantation of electrodes into the medial forebrain bundle under ketamine/xylazine (139/21 mg/kg intraperitoneally) anesthesia, we unexpectedly observed that using the same doses of anesthetics α3KO mice were anesthetized longer than WT mice (approximately 130 min compared with 80 min), prompting us to investigate the role of α3-containing $GABA_A$ receptors in the action of intravenous general anesthetics.

Anesthetic Endpoints with Etomidate, Midazolam, and Pentobarbital

The anesthetic effects of etomidate, midazolam and pentobarbital, three compounds known to act at least in part *via* $GABA_A$ receptors, were assessed by determining the following endpoints: duration of LORR, time to recovery and total LORR score as a measure of hypnosis, LHWR as a measure of immobility, and body temperature as a measure of hypothermic effects of the drug (fig. 1 and table 1).

Etomidate

Etomidate (30 mg/kg) was less effective in α3KO mice compared with WT mice. Duration of LORR (WT: 88.3 ± 4.3 min, α3KO: 72.7 ± 5.8 min) and total LORR score (WT: 273.7 ± 10.3, α3KO: 224.9 ± 17.5) were significantly decreased in α3KO mice (unpaired *t* test, $P = 0.0439$ and $P = 0.0263$, respectively), whereas time to recovery was not different (WT: 91.1 ± 4.5 min, α3KO: 77.2 ± 5.8 min, unpaired *t* test, $P = 0.0710$). Anesthetic-induced decrease in body temperature was smaller in α3KO mice compared with WT controls (two-way ANOVA with repeated measures of time: significant effect of time $F(2,40)$, $P < 0.0001$; significant effect of genotype $F(1,20) = 4.90$, $P = 0.0386$; significant time × genotype interaction $F(2,40) = 8.12$, $P = 0.0011$). *Post hoc t* tests with Bonferroni correction showed that the temperature differed significantly 90 min after injection ($P < 0.01$).

Midazolam

The hypnotic action of midazolam (75 mg/kg) was decreased in α3KO mice compared with WT mice. Although the difference in the duration of LORR failed to reach significance (unpaired *t* test, $P = 0.0691$), the time to recovery (WT: 101.1 ± 7.5 min, α3KO: 53.8 ± 9.9 min) and the total LORR score (WT: 298.8 ± 23.6, α3KO: 159.4 ± 27.3) were significantly lower in α3KO mice (unpaired *t* test, $P = 0.0011$ and $P = 0.001$, respectively). Similarly, the reduction in body temperature was smaller in α3KO mice (two-way ANOVA with repeated measures of time: significant effect of time $F(2,40) = 17.65$, $P < 0.0001$; significant effect of genotype $F(1,20)$, $P = 0.0395$ and significant time × genotype interaction $F(2,40)$, $P = 0.0008$). *Post hoc t* tests with Bonferroni correction showed that the temperature difference was significant 90 min after injection ($P < 0.01$).

Pentobarbital

For pentobarbital two-way ANOVAs using drug dose and genotype as factors revealed main effects of drug and genotype on LORR (drug $F(2,32) = 160.92$, $P < 0.0001$; genotype $F(1,16) = 5.64$, $P = 0.304$), on time to recovery (drug $F(2,32) = 163.15$, $P < 0.0001$; genotype $F(1,16) = 5.51$, $P = 0.0321$) and on total LORR score (drug $F(2,32) = 158.06$,

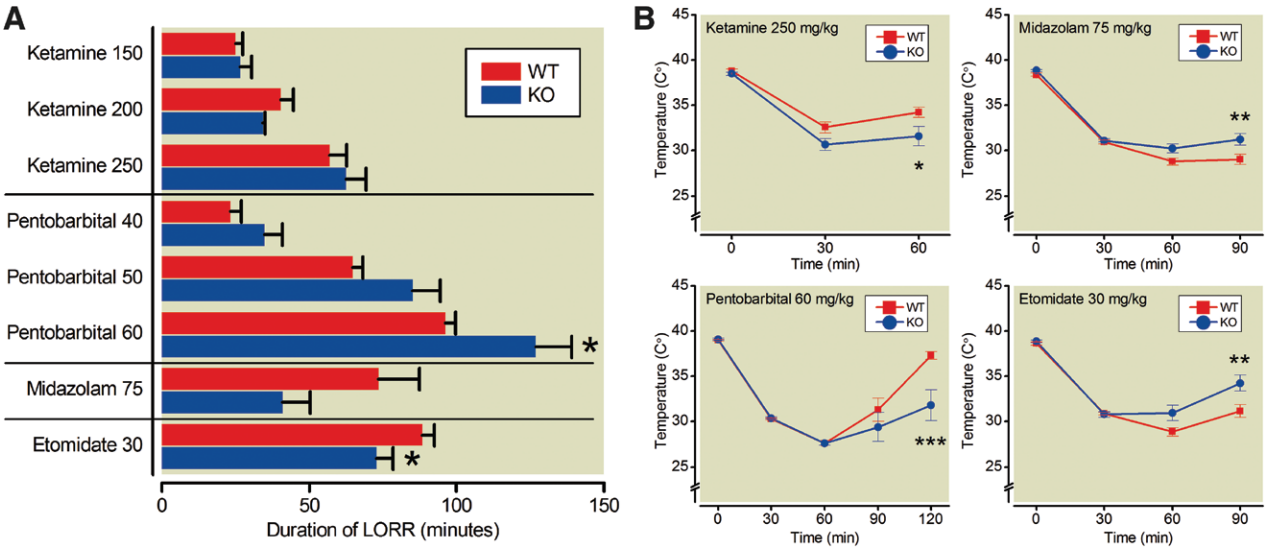

Fig. 1. Effect of ketamine, pentobarbital, midazolam, and etomidate on (*A*) duration of LORR and (*B*) core body temperature. (*A*) Duration of LORR was significantly increased in α3KO mice by 60 mg/kg pentobarbital (repeated measures ANOVA followed by *t* tests with Bonferroni correction) and significantly decreased by 30 mg/kg etomidate (unpaired *t* test). (*B*) Repeated measures ANOVA showed a mean effect of genotype on core body temperature for 250 mg/kg ketamine, 75 mg/kg midazolam, and 30 mg/kg etomidate. *t* Tests with Bonferroni correction revealed that the temperature decrease was more pronounced in α3KO mice compared with WT mice 60 min and 120 min after administration of ketamine and pentobarbital, respectively, and that the temperature decrease was less pronounced in α3KO mice compared with WT mice 90 min after administration of midazolam or etomidate. Data represent the mean ± SEM; **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, n = 9 WT and n = 7 α3KO for ketamine; n = 9 for pentobarbital; n = 11 for etomidate and midazolam. KO = knockout; LORR = loss of righting reflex; WT = wild type.

Table 1. Anesthetic Properties (Ketamine, Pentobarbital, Midazolam, Etomidate)

Drug	Dose (mg/kg)	Genotype	n	Duration of LORR (min)	Time to Recovery (min)	Total LORR Score	LHWR (min)
Ketamine	150	WT	9	25.0 ± 2.4	36.1 ± 2.4	94.8 ± 5.6	0.0 ± 0.0
		α3KO	7	26.7 ± 4.4	42.0 ± 5.3	110.1 ± 14.4	0.0 ± 0.0
	200	WT	9	40.3 ± 4.4	61.7 ± 3.7	168.3 ± 10.3	0.0 ± 0.0
		α3KO	7	34.1 ± 1.1	61.3 ± 3.0	161.6 ± 7.1	0.0 ± 0.0
	250	WT	9	56.8 ± 5.9	83.0 ± 4.1	231.3 ± 11.7	0.0 ± 0.0
		α3KO	7	62.6 ± 6.7	97.3 ± 5.8	269.6 ± 18.3	0.0 ± 0.0
Pentobarbital	40	WT	9	23.2 ± 3.7	26.3 ± 3.5	75.2 ± 10.6	0.0 ± 0.0
		α3KO	9	34.8 ± 6.0	36.8 ± 5.7	107.6 ± 17.0	0.0 ± 0.0
	50	WT	9	64.7 ± 3.6	65.9 ± 3.5	194.4 ± 10.6	0.0 ± 0.0
		α3KO	9	85.0 ± 9.5	86.0 ± 9.5	255.2 ± 28.5	0.0 ± 0.0
	60	WT	9	96.2 ± 3.6	98.2 ± 3.6	290.3 ± 10.7	0.0 ± 0.0
		α3KO	9	126.8 ± 12.2*	129.1 ± 12.3*	382.1 ± 36.7*	25.0 ± 11.6
Midazolam	75	WT	11	73.5 ± 14.1	101.1 ± 7.5	298.8 ± 23.6	0.0 ± 0.0
		α3KO	11	40.8 ± 9.4	53.8 ± 9.9**	159.4 ± 27.3***	0.0 ± 0.0
Etomidate	30	WT	11	88.3 ± 4.3	91.1 ± 4.5	273.7 ± 10.3	0.0 ± 0.0
		α3KO	11	72.7 ± 5.8*	77.2 ± 5.8	224.9 ± 17.6*	0.0 ± 0.0

Data represent the mean ± SEM. Duration of LORR, time to recovery and the global anesthetic score were significantly increased in α3KO mice compared with WT mice after 60 mg/kg pentobarbital (two-way ANOVA with repeated measures followed by *post hoc t* tests with Bonferroni correction). Time to recovery and the global anesthetic score were significantly decreased after 75 mg/kg midazolam and the duration of LORR and the global anesthetic score were significantly decreased after 30 mg/kg etomidate in α3KO mice compared with WT mice (unpaired *t* test).

* *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.001.

LHWR = loss of hind limb withdrawal reflex; LORR = loss of righting reflex; KO = knockout; WT = wild type.

$P < 0.0001$, genotype $F(1,16) = 5.63$, $P = 0.305$). Furthermore, four out of nine α3KO mice lost the hind limb withdrawal reflex at the highest dose of pentobarbital (60 mg/kg), whereas none of the WT mice lost the reflex. This difference was not statistically significant (Fisher's exact probability test: $P = 0.0824$). At the highest dose of pentobarbital (60 mg/kg), two-way ANOVA with repeated measures of time showed a significant effect of time $F(3,48) = 27.19$, $P < 0.0001$ but no effect of genotype $F(1,16) = 3.60$, $P = 0.0761$). However, with respect to body temperature, there was a significant time x genotype interaction ($F(3,48) = 5.56$, $P = 0.0023$) and *t* tests with Bonferroni correction showed that body temperature was significantly lower in α3KO mice 120 min after injection ($P < 0.001$).

Anesthetic Endpoints with Ketamine and Medetomidine

Ketamine

There were no differences in duration of LORR, time to recovery, total LORR score or LHWR between WT and α3KO mice at any dose of ketamine, although two α3KO mice died after administration of 150 and 250 mg/kg, respectively. However, the reduction in body temperature was significantly larger in α3KO mice compared with WT mice by 250 mg/kg ketamine (ANOVA with repeated measures of time: significant effect of time $F(1,14) = 7.99$ $P = 0.0134$; significant effect of genotype $F(1,14) = 7.99$ $P = 0.025$). *Post hoc t* tests with Bonferroni correction showed that body temperature was significantly different 60 min after injection ($P < 0.05$).

Medetomidine

Sedative Effects of Medetomidine

The sedative effects of the α2-adrenergic agonist medetomidine in α3KO and WT mice were assessed by measuring the locomotor activity in a novel open field over 60 min following drug injection. The total distance traveled was decreased by medetomidine (30 or 60 μg/kg) (fig. 2A) but there were no differences between α3KO and WT mice (two-way ANOVA: significant effect of drug ($F(2,48) = 32.25$, $P < 0.0001$) (insert in fig. 2A). Analysis with two-way ANOVA with repeated measures of 5-min time bins also did not reveal any difference between genotypes, although a general reduction in activity over time was observed in both genotypes (Vehicle: significant effect of time $F(11,176) = 32.41$, $P < 0.0001$; 30 μg/kg: significant effect of time $F(11,176) = 41.85$, $P < 0.0001$; 60 μg/kg: significant effect of time $F(11,176) = 47.54$, $P < 0.0001$) (fig. 2A).

Hypnotic Effects of Medetomidine

The hypnotic effects of medetomidine in α3KO and WT mice were assessed 60 min after injection by rolling the mice on their side and noting the time to righting (fig. 2B). When righting did not occur within 30 min the observation was stopped. Data were analyzed by fitting standard least-squares mixed models with subject as a random effect.

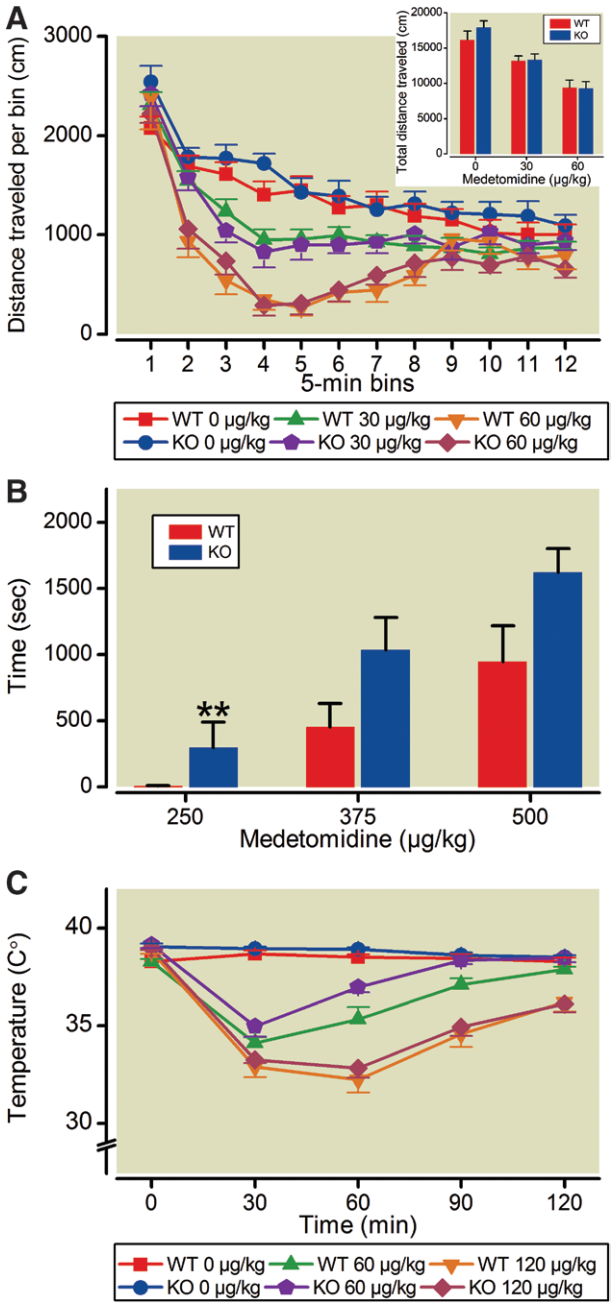

Fig. 2. Effects of medetomidine on locomotor activity (*A*), duration of LORR (*B*) and body temperature (*C*) in α3KO and WT mice. (*A*) Locomotor activity was comparable in both genotypes at all medetomidine doses over the whole 60 min time period (insert) and looking at 5-min time bins. (*B*) Duration of LORR was significantly increased in α3KO mice by 250 μg/kg medetomidine compared with WT mice (Sheffé *post hoc* tests). (*C*) Body temperature was comparable between α3KO mice and WT mice. Data represent the mean ± SEM; n = 9 per genotype. KO = knockout; LORR = loss of righting reflex; WT = wild type.

Drug dose and genotype were included as fixed effects. Effects were assessed by F tests based on the fitted regression models. This revealed a significant overall effect of genotype

(F[1,16] = 8.90, $P = 0.0088$) and dose (F[1,34] = 36.96, $P < 0.0001$). *Post hoc* Sheffé tests showed that the LORR was significantly increased in α3KO mice (298 ± 192 s) compared with WT mice (6 ± 4 s) by 250 μg/kg medetomidine ($P = 0.0098$). At this dose, two of nine WT mice lost the righting reflex (loss of righting reflex defined as inability to right within 10 s) in contrast to seven of nine α3KO mice.

Hypothermic Effects of Medetomidine

The hypothermic effects of medetomidine in α3KO and WT mice were assessed by determining the rectal body temperature immediately before drug or vehicle injection and every 30 min thereafter over a period of 120 min (fig. 2C).

The baseline body temperature before injection was increased in α3KO mice compared with WT mice in the groups that received vehicle (WT = 38.29 ± 0.11°C, α3KO = 39.01 ± 0.18°C, unpaired *t* test, $P < 0.01$) and 60 μg/kg medetomidine (WT = 38.297 ± 0.15°C, α3KO = 39.13 ± 0.17°C, unpaired *t* test, $P < 0.01$) in α3KO mice (39.0 ± 0.2°C), but not in the group that received 120 μg/kg medetomidine (WT = 38.82 ± 0.14°C, α3KO = 38.97 ± 0.09°C, independent samples *t* test, $P = 0.398$). This precluded direct statistical analysis of the hypothermic effects of 60 μg/kg medetomidine. For the 120 μg/kg medetomidine dose, a genotype × time two-way ANOVA did not reveal a significant genotype main

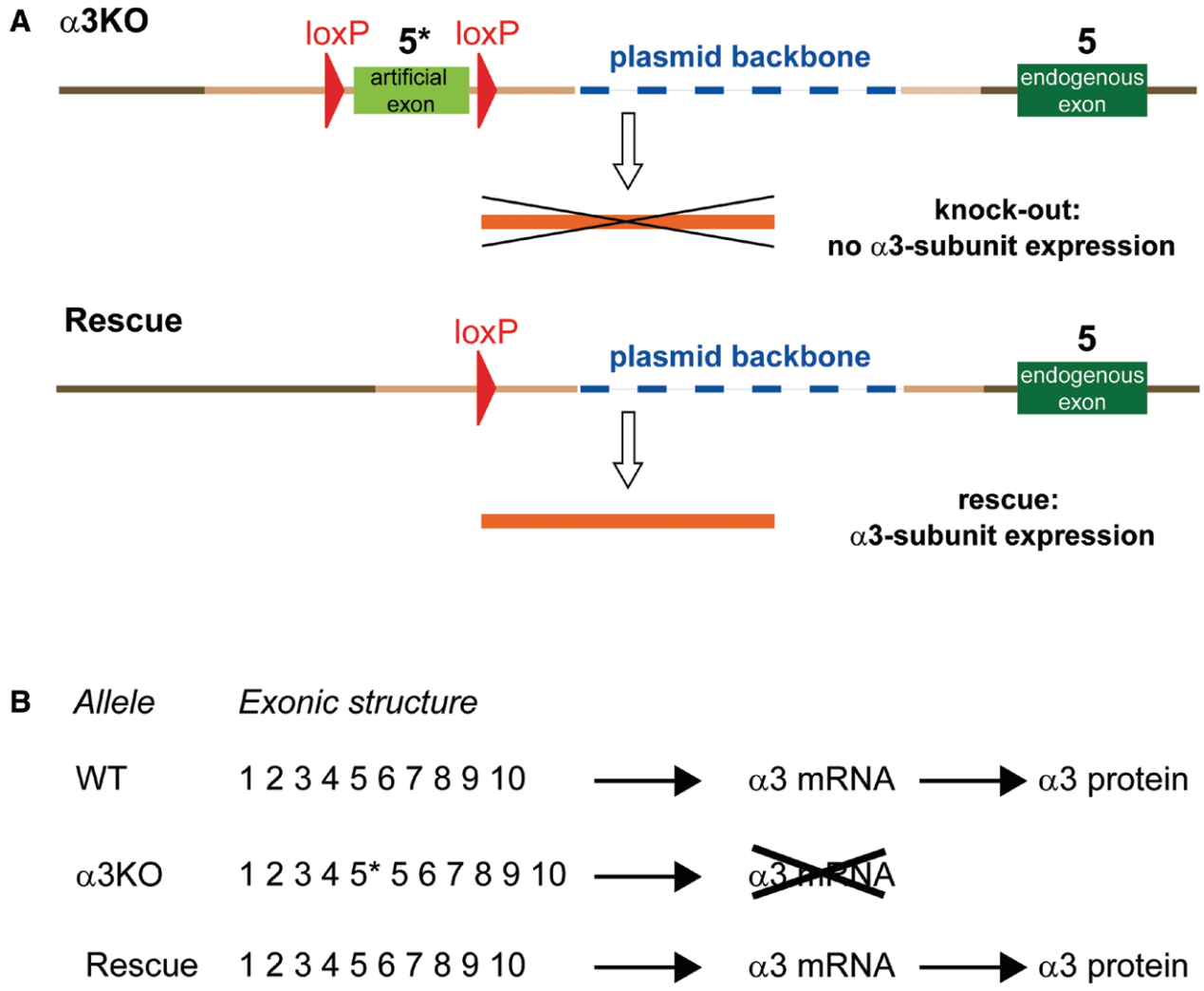

Fig. 3. Schematic of genetic rescue of the α3KO in noradrenergic neurons. (*A*) α3KO and rescue alleles. The α3KO allele (*top*) has integrated the insertion-type gene targeting vector for single reciprocal recombination in its entirety.[18] It carries an artificial exon (*light green*, 5*) flanked by *lox*P sites (*red*), targeting vector-derived homologous genomic sequences (*light brown*), and the plasmid backbone of the targeting vector (*blue*). Genomic sequences that were not included in the targeting vector are shown as *dark brown*. The most relevant feature is that the α3KO allele contains the endogenous exon 5 (*dark green*, 5) and an artificial exon 5 (*light green*, 5*), which results in mRNA degradation, which is the likely mechanism of the α3KO.[18] In the rescue allele, elimination of the artificial exon by Cre-*lox*P-mediated recombination results in α3 mRNA (*orange*) being made and thus the expression of the α3 subunit being restored. (*B*) Exonic structure of predicted gene products from WT, α3KO, and rescue allele. Presence of the artificial exon 5, 5*, in the α3KO results in mRNA breakdown and thus a functional KO. The exonic structure of WT and rescue alleles is identical. KO = knockout; mRNA = messenger RNA; WT = wild type.

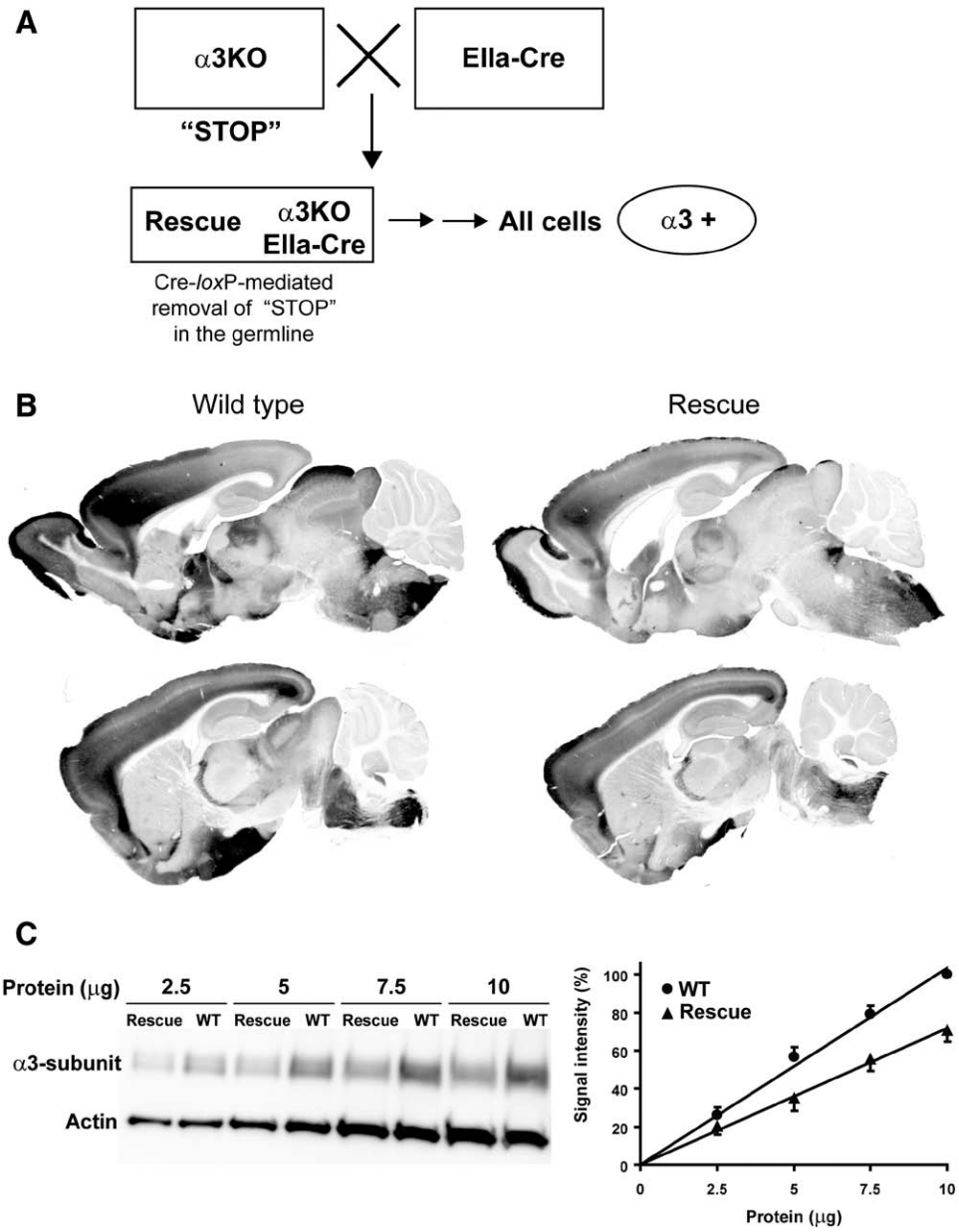

Fig. 4. Localization and quantification of the α3KO in global rescue mice. (*A*) Generation of global rescue mice. An α3KO mouse (with artificial exon 5, the "STOP" signal) is bred with an Ella-Cre mouse.[19] Some of the offspring will carry both the α3KO allele and the Ella-Cre transgene. This mouse is bred with a WT mouse to breed out the Ella-Cre transgene (depicted by 2 sequential *horizontal arrows*) to confirm that the artificial exon 5 (the "STOP" signal) has been removed from the germline, so that the α3 subunit is expected to be expressed in all cells in which it is naturally expressed. (*B*) Immunoperoxidase staining of perfusion-fixed parasagittal brain sections: α3 subunit distribution pattern is equivalent in WT and global rescue mice. Representative sections from two WT and two global rescue mice are shown. (*C*) Left panel: Representative Western Blot: α3 subunit expression level is decreased in global rescue mice compared with WT mice. Right panel: Quantification of Western blot signal: signals were normalized to the α3 subunit signal at 10 μg protein in WT mice (100%). α3 subunit expression level in global rescue mice is 70 ± 7% of WT expression. Data represent the mean ± SEM of five experiments. KO = knockout; WT = wild type.

effect or genotype × time interaction. In the entire study, a difference in baseline temperature was only present in 2 of the 11 experiments performed in total with midazolam, pentobarbital, and ketamine. We therefore assume that extraneous undefined factors specific to the experiments with medetomidine are responsible for this baseline difference, and that overall WT mice and α3KO mice do not display a difference in baseline body temperature. When we analyzed the change in body temperature from baseline, we still did not obtain any significant differences between genotypes both for 60 μg/kg and for 120 μg/kg medetomidine (not shown).

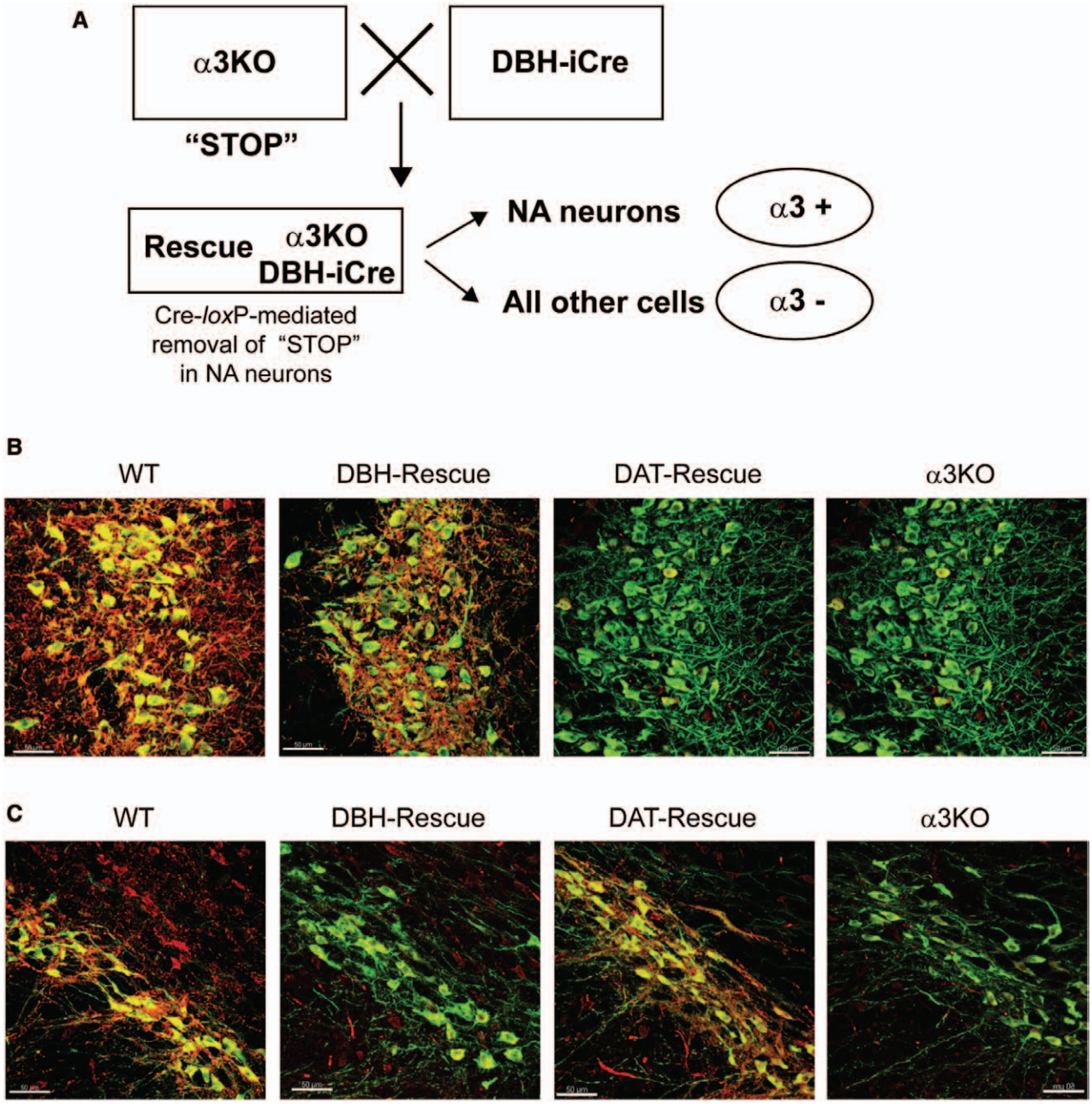

Fig. 5. Recue of α3 expression in defined neuronal cell types. (*A*) An α3KO mouse is crossed with a DBH-iCre mouse to obtain DBH-iCre Rescue mice (in our studies hemizygous males) containing both the α3 knockout allele and the DBH-iCre transgene. The artificial exon 5 in the α3 KO allele functions as a "STOP" signal, resulting in messenger RNA degradation.[18] In noradrenergic neurons of the Rescue mouse, this "STOP" signal is removed by cre-*lox*P-mediated recombination. Thus, in the Rescue mice, the α3 subunit is expressed only in the noradrenergic neurons. Similar considerations apply to the generation of the DAT-Rescue mice using the DAT-iCre transgene expressing iCre specifically in dopaminergic neurons. (*B*) Neuron-specific rescue of α3 subunit expression in the locus coeruleus: Immunofluorescence double-labeling of the α3 subunit (*red*) and tyrosine hydroxylase (*green*) shows that the α3 subunit is highly expressed in the LC in WT mice. In α3KO mice expressing the iCre recombinase selectively in noradrenergic neurons (DBH-Rescue) α3 subunit expression is restricted to noradrenergic neurons. It is not detectable in the locus coeruleus of α3KO mice expressing the iCre recombinase exclusively in dopaminergic neurons (DAT-Rescue), nor in α3KO mice. Scale bar: 50 μm. (*C*) Neuron-specific rescue of α3 subunit expression in the SNpc: Immunofluorescence double-labeling of the α3 subunit (*red*) and tyrosine hydroxylase (*green*) shows that α3 subunit expression is found in dopaminergic neurons of the SNpc of WT mice and in α3KO mice expressing the iCre recombinase selectively in dopaminergic neurons (DAT-Rescue). It is not detectable in the SNpc of α3KO mice expressing the iCre recombinase selectively in noradrenergic neurons (DBH-Rescue), nor in α3KO mice. Scale bar: 50 μm. DBH-iCre = dopamine β hydroxylase impoved Cre; DAT = dopamine transporter; WT = wild type; LC, locus coeruleus; SNpc = substantia nigra pars compacta.

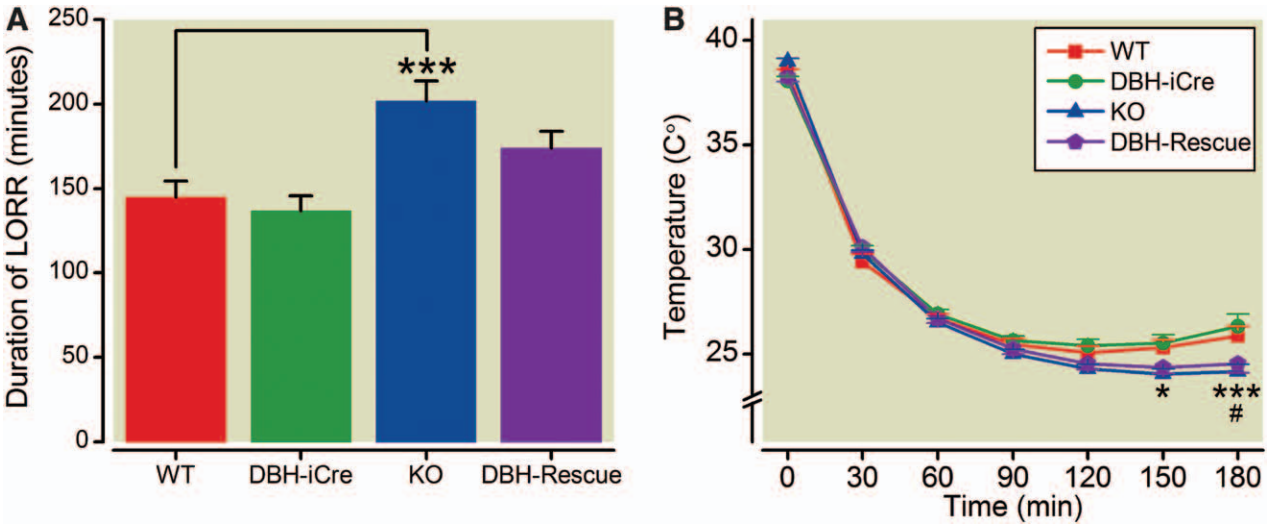

Fig. 6. Effect of ketamine (139 mg/kg)/xylazine (21 mg/kg) on (*A*) duration of LORR and (*B*) core body temperature. (*A*) Duration of LORR was significantly increased in α3KO mice compared with WT mice, whereas mice expressing the α3 subunit exclusively in noradrenergic neurons (DBH-Rescue) and mice carrying the iCre transgene (DBH-iCre) did not differ from WT mice (one-way ANOVA followed by *post hoc* Dunnett *t* tests). (*B*) Body temperature was significantly lower in α3KO mice compared with WT mice 150 min and 180 min after injection. Body temperature in DBH-Rescue mice was significantly lower 180 min after injection compared with WT mice (two-way repeated measures ANOVA followed by *post hoc t* tests with Bonferroni correction). Data represent the mean ± SEM; ***$P < 0.001$ (α3KO compared with WT), *$P < 0.05$ (α3KO compared with WT), #$P < 0.05$ (DBH-Rescue compared with WT); n = 15 WT and DBH-iCre, n = 14 α3KO and DBH-Rescue. LORR = loss of righting reflex; WT = wild type; DBH = dopamine β hydroxylase; iCre = improved cre.

Generation of Mice Expressing the $GABA_A$ Receptor α3 Subunit Exclusively in Noradrenergic Neurons

α3KO mice used in this study have been generated using an insertion targeting strategy (fig. 1 in reference 18) with an insertion-type targeting vector containing exon 5 of the *Gabra3* gene flanked by two *lox*P sites. This vector was inserted into the target locus in its entirety by single reciprocal recombination, resulting in a duplication of exon 5 (*i.e.,* exon 4 in fig. 1 in the study by Yee *et al.*).[18] The resulting α3KO mice carry both an endogenous exon 5 and an artificial exon 5* (5* in fig. 3A). This artificial exon 5* provides a "STOP" signal functionally disrupting the *Gabra3* gene (fig. 3, A and B). We predicted that a cre-*lox*P-mediated excision of this *lox*P-flanked artificial exon 5* would functionally restore the *Gabra3* gene. In a proof-of-principle experiment, α3KO mice were crossed with EIIa-cre mice, which express the cre transgene at early embryonic stages[19] (fig. 4A). As predicted, cre-*lox*P-mediated recombination resulted in the elimination of the *lox*P-flanked artificial exon 5*. Subsequently, the Ella-Cre transgene was bred out to ensure that the rescue of the α3 subunit had been present in the germline. These global rescue mice were examined for α3 subunit expression. Immunohistochemical analysis revealed that α3 subunit expression is restored in these global rescue mice and the distribution pattern of α3 subunit expression is indistinguishable from WT mice (fig. 4B). Western blot analysis showed that α3 subunit expression levels in global rescue mice are 70 ± 7% compared with WT mice (fig. 4C). Thus, the genomic rearrangement in the rescue allele, which includes the presence of a plasmid backbone, reduces the expression of the α3 subunit by approximately 30% without affecting its regional distribution. The precise mechanisms of this reduction are unknown.

Table 2. Anesthetic Endpoints with Ketamine (139 mg/kg)/ Xylazine (21 mg/kg)

Genotype	n	Duration of LORR (min)	Time to Recovery (min)	Total LORR Score	LHWR (min)
WT	15	144.7 ± 9.5	159.9 ± 9.7	466.3 ± 28.7	95.0 ± 7.8
DBH-iCre	15	136.9 ± 8.5	153.9 ± 9.4	448.3 ± 27.5	83.3 ± 5.0
α3KO	14	201.7 ± 11.9***	218.2 ± 11.2***	641.3 ± 33.9***	137.1 ± 8.3***
DBH-Rescue	14	174.0 ± 9.8	201.9 ± 10.6*	588.4 ± 31.5*	113.2 ± 7.1

All anesthetic parameters were significantly increased in α3KO mice compared with WT. Time to recovery and total anesthetic score were significantly increased in DBH-Rescue mice compared with WT (one-way ANOVAs followed by *post hoc* Dunnett *t* tests). Data represent the mean ± SEM.

* $P < 0.05$ and *** $P < 0.001$ (compared with WT).

DBH = dopamine β hydroxylase, iCre = improved Cre; KO = knockout; LHWR = loss of hind limb withdrawal reflex; LORR = loss of righting reflex; WT = wild type.

In order to generate mice expressing the α3 subunit exclusively in noradrenergic neurons, mice carrying the α3 knockout allele and a DBH-iCre transgene (DBH-iCre) were generated (fig. 5A). In addition, mice carrying the α3 KO allele and a DAT-iCre transgene (DAT-iCre) were generated to demonstrate the specificity of the rescue approach.

Immunofluorescence analysis confirmed that the α3 subunit is exclusively expressed in noradrenergic neurons in α3KO/DBH-iCre (DBH-Rescue) mice (fig. 5B), whereas it is exclusively expressed in dopaminergic neurons in α3KO/DAT-iCre (DAT-Rescue) mice (fig. 5C). WT mice show intense expression of the α3 subunit on noradrenergic neurons of the locus coeruleus and on unidentified neurons in the proximity, whereas DBH-Rescue mice express the α3 subunit only on noradrenergic neurons (fig. 5B). α3 subunit expression is not detectable in α3KO mice and DAT-Rescue mice in this brain region (fig. 5B). To confirm the specificity of the rescue approach α3 subunit expression in DAT-Rescue mice was examined further. In the dopaminergic neurons of the substantia nigra pars compacta the α3 subunit protein is absent in α3KO and DBH-Rescue mice, whereas high levels of α3 subunit expression are found in WT mice, and expression is specifically restored in dopaminergic neurons in DAT-Rescue mice (fig. 5C). In other brain regions where the α3 subunit is normally expressed, such as the cerebral cortex or the hippocampus but where no noradrenergic or dopaminergic neurons are present, no α3 subunit protein was detectable in DBH- or DAT-rescue mice (not shown). Overall, these data demonstrate that the α3 subunit can be rescued specifically and to a large extent in noradrenergic neurons.

Anesthetic Endpoints with Ketamine/Xylazine and Rescue of the Phenotype in Mice Expressing the $GABA_A$ Receptor α3 Subunit Exclusively in Noradrenergic Neurons

In order to confirm the unexpected observation that ketamine/xylazine-induced anesthesia is significantly prolonged in α3KO mice and to address the question whether α3 subunit–containing $GABA_A$ receptors on noradrenergic neurons modulate these anesthetic effects, we studied several anesthetic endpoints and the body temperature decrease during anesthesia in α3KO mice, WT mice, and DBH-Rescue mice (fig. 6 and table 2). To exclude the possibility that the iCre transgene, which is also present in the DBH-Rescue mice, causes any effects by itself (also referred to as potential "Cre toxicity"), control mice expressing the iCre transgene in noradrenergic neurons (DBH-iCre) were included.

One-way ANOVA showed a significant effect of genotype for all anesthetic parameters: duration of LORR ($F[3,54] = 8.81$, $P < 0.0001$), time to recovery ($F[3,54] = 9.52$, $P < 0.0001$), total LORR score ($F[3,54] = 9.51$, $P < 0.0001$) and LHWR ($F[3,54] = 10.76$, $P < 0.0001$) (table 2). *Post hoc* Dunnett tests revealed that α3KO mice show significantly increased measures on all anesthetic parameters (LORR, total LORR score, LHWR, $P < 0.001$ and time to recovery $P < 0.01$) compared with WT mice. In contrast, DBH-Rescue mice showed an increase only in the time to recovery and the total LORR score ($P < 0.05$) compared with WT mice; they were not different from WTs in terms of LORR or LHWR times. In fact, the rescue mice were not different from either WT or α3KO mice on these parameters, suggesting that they rank in between the two genotypes, leading to a "partial rescue" of the α3KO phenotype. The body temperature decrease caused by ketamine/xylazine was analyzed by a two-way repeated measures ANOVA. A significant effect of genotype ($F[3,54] = 2.85$, $P = 0.0456$), significant effect of time ($F[5,270] = 475.04$, $P < 0.0001$) and a significant time × genotype interaction ($F[15,270] = 5.69$, $P < 0.0001$) were observed. *Post hoc t* tests with Bonferroni correction showed that the temperature was significantly lower in α3KO mice 150 min ($P < 0.05$) and 180 min ($P < 0.001$) after injection compared with WT mice. DBH-Rescue mice showed a significantly lower temperature only 180 min ($P < 0.05$) after injection compared with WT mice. DBH-iCre mice did not differ from WT mice in any anesthetic endpoint or in the body temperature, indicating that the iCre transgene itself has no effect on the parameters measured. Overall, our results indicate that the hypnotic phenotype present in α3KO mice can be partially rescued by expression of the α3 subunit in noradrenergic neurons in DBH-Rescue mice, whereas the hypothermic phenotype seems to be independent of the α3 subunit in noradrenergic neurons.

Discussion

Clinically used intravenous general anesthetics are a structurally highly diverse class of drugs mediating their anesthetic effects through a variety of molecular targets.[12] In this study, we evaluated the role of α3-containing $GABA_A$ receptors in the actions of intravenous anesthetics and found that although actions of GABAergic drugs like etomidate and midazolam are in part mediated by this receptor subtype, α3-containing $GABA_A$ receptors also constrain the actions of anesthetics that have partially (*e.g.,* pentobarbital) or exclusively (ketamine/xylazine, medetomidine) non-GABAergic targets. We also demonstrate that α3-containing $GABA_A$ receptors on noradrenergic neurons may constrain the hypnotic but not the hypothermic action of ketamine/xylazine action.

Studies using mice carrying β2(N265S) or β3(N265M) point mutations rendering the mutated subunit insensitive to etomidate or etomidate and propofol, respectively, have revealed that $GABA_A$ receptors containing the β2 or β3 subunits mediate the immobilizing (β3), hypnotic (β2 and β3), and hypothermic actions (mainly β2, minor role of β3 shown for etomidate and pentobarbital, but not for propofol) of etomidate, propofol, and pentobarbital.[30–32] In line with previous reports showing that mice lacking the β3 subunit[33] or the α1 subunit[6] are less sensitive to the hypnotic effects of midazolam and etomidate, we found that α3KO mice are likewise less sensitive to these agents, collectively

indicating that the α1, α3, and β3 subunits are involved in mediating the hypnotic response to GABAergic anesthetics. In contrast, α3KO mice showed an increase in the sensitivity to the hypnotic, immobilizing, and hypothermic effects of pentobarbital. This is astonishing because $GABA_A$ receptors are a major target for pentobarbital.[34] Consistent with this assumption α1KO mice[6] and β3(N265M) point-mutant mice[32] are less sensitive to the hypnotic effects of pentobarbital; moreover, the immobilizing response to pentobarbital is completely absent in β3(N265M) point-mutant mice.[32] However, the $GABA_A$ receptor antagonist gabazine attenuates the hypnotic response to pentobarbital to a much lesser extent than the hypnotic response to muscimol and propofol, pointing to the potential relevance of non-$GABA_A$ receptor targets for pentobarbital actions.[35] Furthermore, in contrast to etomidate some effects of pentobarbital such as respiratory depression are similar in WT and β3(N265M) point-mutant mice.[32] Together with the fact that pentobarbital additionally acts on other drug targets such as nicotinic acetylcholine receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, kainate receptors, and glycine receptors[36] it seems likely that at least some of the anesthetic effects of pentobarbital are mediated through non-GABAergic mechanisms. It is therefore possible that the increased sensitivity to pentobarbital in α3KO mice results from alterations in non-GABAergic pathways such as the cholinergic system where α3 subunit expression has been described[37] or potentially from up-regulation of non-GABAergic targets of pentobarbital or other compensatory mechanisms.

On the basis of the initial observation that ketamine/xylazine anesthesia is prolonged in α3KO mice and actions of such compounds would normally be expected to be unaltered in a $GABA_A$ receptor subunit KO mouse, investigating the contribution of *N*-methyl-D-aspartate receptor antagonism and of α2-adrenergic receptor agonism to this phenomenon was a major goal in this study. We therefore examined the action of ketamine and of the α2-adrenergic agonist medetomidine. Medetomidine was chosen for two reasons: (1) Essential pharmacologically relevant data, for example, with respect to binding to α2-adrenergic receptor subtypes were not available for xylazine, and (2) there is a significant body of experimental studies on the sedative, hypnotic, and hypothermic actions with medetomidine.[38] Strikingly, our studies revealed a clear dissociation between the sedative, hypothermic, and hypnotic effects induced by medetomidine. Although the sedative and hypothermic effects were indistinguishable between WT and α3KO mice, α3KO mice were more sensitive to the hypnotic effects of medetomidine. However, it is interesting to note that the unspecific blockade of all $GABA_A$ receptor subtypes by the antagonist gabazine attenuates the hypnotic effects of medetomidine.[16] This indicates that the hypnotic response to medetomidine may be modulated in different directions by different $GABA_A$ receptor subtypes. Interestingly, α3KO mice did not differ from WT mice in their hypnotic response to ketamine. However, the decrease in body temperature was more pronounced. Even though ketamine does not typically exhibit synergistic effects in combination with other anesthetic agents,[39] our findings suggest that the increased sensitivity to the hypnotic effects of α2-adrenergic agents combined with the increased sensitivity to the hypothermic effects of ketamine might cause the significant phenotype observed in α3KO mice in response to the drug combination ketamine/xylazine in a synergistic manner.

Another objective of this study was to evaluate whether α3-containing $GABA_A$ receptors on noradrenergic neurons constrain ketamine/xylazine action. For this purpose, we generated "rescue" mice expressing the α3 subunit exclusively in noradrenergic neurons. In these mice, the α3 subunit is expressed under the control of its endogenous promoter, thus limiting expression to cells in which expression occurs naturally. Compared with the WT allele the expression level of the rescue allele was approximately 70%. We assume that transcriptional processes may be partially disrupted due to the presence of foreign DNA (*i.e.*, plasmid backbone of the targeting vector) in the allele. Presumably α3 expression levels in noradrenergic neurons will not reach WT expression levels in the α3-DBH-Rescue mice either, although this has not been formally examined. As a consequence a rescue of the α3KO phenotype may not restore function to WT levels. Indeed, this is what we observed in this study: the presence of α3-containing $GABA_A$ receptors on noradrenergic neurons partially abolishes the increased hypnotic and immobilizing actions of ketamine/xylazine observed in the α3KO mice. The increased hypothermic actions, however, were not affected. This indicates that although noradrenergic neurons are involved in modulating the hypnotic and immobilizing actions of ketamine/xylazine, they may not be involved in modulating their hypothermic action.

Our findings are consistent with a model in which the noradrenergic neurons naturally express both α2-adrenergic receptors and α3-containing $GABA_A$ receptors.[11] Activation of both receptors would be predicted to decrease the activity of the noradrenergic neurons. Similar to previous findings in the reticular nucleus of the thalamus,[40] currently unexplained compensations to preserve inhibitory function may be present in α3KO mice. At the same time, increased sensitivity to α2-adrenergic agonists might occur in α3KO mice to compensate for the lack of GABAergic inhibition and designed to keep the inhibitory modulation of these neurons constant. This would explain why the hypnotic and hypothermic actions of compounds such as midazolam and etomidate, which act mainly or exclusively *via* $GABA_A$ receptors without any effect on α2-adrenergic receptors, are reduced in the α3KO mice, whereas the hypnotic action of medetomidine, which has no effect on $GABA_A$ receptors, is increased.

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