0026-895X/07/7103-852–859$20.00
MOLECULAR PHARMACOLOGY
Vol. 71, No. 3
30049/3181803
Mol Pharmacol 71:852–859, 2007
Printed in U.S.A.

Identification of a Molecular Target Mediating the General Anesthetic Actions of Pentobarbital

Anja Zeller, Margarete Arras, Rachel Jurd, and Uwe Rudolph

Institute of Pharmacology and Toxicology (A.Z., R.J., U.R.) and Institute of Laboratory Animal Science (M.A.), University of Zürich, Zürich, Switzerland; and Laboratory of Genetic Neuropharmacology, McLean Hospital, Department of Psychiatry, Harvard Medical School, Belmont, Massachusetts (U.R.)

Received August 23, 2006; accepted December 12, 2006

ABSTRACT

Barbiturates were introduced into medical practice in 1934. They are widely used today as general anesthetics. Although in vitro studies revealed that the activity of a variety of ligand-gated channels is modulated by barbiturates, the target(s) mediating the anesthetic actions of barbiturates in vivo are unknown. Studying pentobarbital action in β3(N265M) mice harboring β3-containing $GABA_A$ receptors insensitive to a variety of general anesthetic agents, we found that the immobilizing action of pentobarbital is mediated fully, and the hypnotic action is mediated in part by this receptor subtype. It was surprising that the respiratory depressant action of pentobarbital is indistinguishable between β3(N265M) and wild-type mice and thus is mediated by other as-yet-unidentified targets. Whereas the target for the immobilizing and hypnotic actions of pentobarbital seems to be the same as for etomidate and propofol, these latter agents' respiratory depressant actions are mediated by β3-containing $GABA_A$ receptors. Thus, in contrast to etomidate and propofol, pentobarbital can elicit respiratory depression by a β3-independent pathway. Pentobarbital reduced heart rate and body temperature to a slightly smaller extent in β3(N265M) mice compared with wild-type mice, indicating that these actions are largely mediated by other targets. Pentobarbital-induced increase of heart rate variability and prolongation of ECG intervals are seen in both β3(N265M) mice and wild-type mice, suggesting that they are not dependent on β3-containing $GABA_A$ receptors. In summary, we show a clear pharmacological dissociation of the immobilizing/hypnotic and respiratory/cardiovascular actions of pentobarbital.

The introduction of general anesthetics into medical practice 160 years ago has revolutionized surgery. However, the mechanisms of action of this class of drugs are still only poorly understood. Although general anesthetics have been shown to modulate the activity of a number of proteins (e.g., ligand-gated ion channels; Krasowski and Harrison, 1999) and two-pore domain potassium channels in vitro (Franks and Honore, 2004), the identification of targets mediating specific actions of general anesthetics in vivo has only just begun.

$GABA_A$ receptors are pentameric ligand-gated ion channels, the majority of them containing two α, two β, and one γ subunit (Backus et al., 1993; Chang et al., 1996). Mutagenesis studies have identified amino acid residues in $GABA_A$ receptor β subunits that are crucial for the actions of the general anesthetics etomidate and propofol in vitro (Belelli et al., 1997; Mihic et al., 1997; Krasowski et al., 1998; Siegwart et al., 2002, 2003).

It has been shown that β3(N265M) mice are insensitive to the immobilizing and respiratory depressant action of etomidate and propofol and have a reduced sensitivity to the hypnotic action of these drugs, suggesting that β3-containing $GABA_A$ receptors mediate these actions, whereas etomidate retains its sedative (motor depressant) action at subanesthetic doses (Jurd et al., 2003; Zeller et al., 2005). In line with these findings, β2(N265S) mice are still sensitive to the immobilizing and hypnotic actions of etomidate but lack the sedative response to low doses of etomidate (Reynolds et al., 2003). Furthermore, the hypothermic response to etomidate is strongly decreased in β2(N265S) mice (Cirone et al., 2004) and only moderately decreased in β3(N265M) mice (Zeller et al., 2005), indicating that the hypothermic response to etomidate is mediated in large part by β2-containing $GABA_A$ receptors and to a more limited degree by β3-containing $GABA_A$ receptors.

In contrast to etomidate and propofol, which exert most if not all of their clinically relevant actions via β2- and β3-

This work was supported by a grant from the Swiss National Science Foundation.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.030049.

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; LORR, loss of righting reflex; LHWR, loss of hindlimb withdrawal reflex; HR, heart rate; CBT, core body temperature; HRV, heart rate variability.

containing $GABA_A$ receptors, the barbiturate pentobarbital has a wider range of targets, modulating the activity not only of $GABA_A$ receptors (Thompson et al., 1996) but also of nicotinic acetylcholine receptors, AMPA receptors, kainate receptors, and glycine receptors (Krasowski and Harrison, 1999).

In this study, we investigated in wild-type and β3(N265M) mice the following actions of pentobarbital: loss of righting reflex (LORR) as a measure of the hypnotic activity, loss of the hindlimb withdrawal reflex (LHWR) as a measure of the immobilizing activity, respiratory depression, heart rate, core body temperature, and ECG. We show that some clinically important but not all of the actions of pentobarbital are mediated by β3-containing $GABA_A$ receptors and that there are striking differences compared with the β3 subtype-dependence of etomidate and propofol actions.

Materials and Methods

Animals. Generation, characterization, and breeding of β3(N265M) mice has been described previously (Jurd et al., 2003). Mice used for telemetry were 3 months old at the time of surgery, 4 months old at the beginning of the experiments, and 10 months at the end of the telemetry experiments. Mice used for blood gas analysis and reflex tests were 5 to 8 weeks old. Mice were of a mixed background [129/Sv (12.5%) × 129/SvJ (87.5%) mice]. Mice were all female.

Behavioral Analysis of Intravenous Anesthetics. Female mice were treated with increasing doses of pentobarbital (Nembutal 50, 62.5, and 75 mg/kg, Abbott AG, Baar, Switzerland/Abbott Laboratories, Chicago, IL) administered intravenously into the tail vein in a volume of 4 μl/kg body weight. The duration of the LORR and LHWR was recorded as described previously (Arras et al., 2001). In brief, the LORR was assessed by measuring the time a mouse remains on its back on a flat surface. The LHWR, which is always shorter than the LORR and starts after onset of LORR and stops before LORR is regained, was determined by pinching a mouse with a pair of tweezers into the interdigital skin of the hindlimb. The reflex was rated as being present when a mouse retracts its hindlimb upon pinching. Each mouse was tested only once.

Blood Gas Measurements. Arterial blood samples were taken from the carotid artery 130 s (range, 110–140 s) after injection of 75 mg/kg pentobarbital i.v. or 30 mg/kg alphaxalone i.v. after the procedure described by Arras et al. (2001). In brief, the ventral aspect of the neck was incised, the right common carotid artery was dissected, and a small hole was cut in the artery using a fine-bladed pair of scissors. Arterial blood was collected in a heparinized syringe. Oxygen partial pressure (pAO_2, millimeters of mercury), carbon dioxide partial pressure ($pACO_2$, millimeters of mercury), acid-base balance (pH value), and standard bicarbonate concentrations (HCO_3^-, millimolar) were determined immediately by use of a blood gas analyser (AVL Compact 3; AVL List, Graz, Austria). For ethical reasons, no arterial blood samples were taken from nonanesthetized mice. Instead, data obtained previously from wild-type mice (strain HanIbm: NMRI), male, obtained from Research and Consulting Company (Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland) and reported in Arras et al. (2001) were used for comparison.

Surgery. Sixteen female mice [8 β3(N265M) mice and 8 wild-type controls] were implanted under isoflurane anesthesia (3–5% in oxygen) with intraperitoneal radiotelemetry transmitters for measuring core body temperature and ECG (model ETA-F20; Data Sciences International, St. Paul, MN). The transmitter body was implanted under sterile conditions in the abdominal cavity, and the sensing leads were positioned as described previously (Späni et al., 2003). Mice received postoperative antibiotics once [20 mg/kg sulfadoxin/5 mg/kg trimethoprim (Borgal 7.5%); Hoechst Roussel Vet, Provet AG, Lyssach, Switzerland] and postoperative pain treatment for 5 days (2.5 mg/kg flunixin s.c.; Finadyne, BERNA Veterinärprodukte AG, Berne, Switzerland). Mice were allowed to recover for 4 weeks before the first experiment. To ascertain full recovery after surgery, we measured core body temperature and heart rate over 72 h before starting the experiments.

Experimental Conditions. Mice implanted with telemetry transmitters were singly housed in standard laboratory conditions with a 12-h light/dark schedule (lights on at 8:00 AM, lights off at 8:00 PM) and free access to food and water. Experiments were performed between 9 AM and 12 PM. Mice used for reflex tests and blood gas analysis were group-housed, and experiments were performed between 8 AM and 5 PM.

Effect of Anesthetics on Core Body Temperature, Heart Rate, and ECG Parameters (PQ and QT). For drug and vehicle administration experiments, a baseline was recorded between 0 and 2 h after lights on, and drugs were administered immediately afterward. Drug effects were compared with vehicle effects, which did not differ significantly from baseline. Before injection of pentobarbital, 60 mg/kg i.p. (Nembutal), mice were already treated with other anesthetics (Zeller et al., 2005). Vehicle solutions were as follows: alphaxalone [Saffan (3:1 mixture of alphaxalone/alphadolone); Schering Plough Animal Health, Welwyn Garden City, Herts, UK)] 14% Cremophor EL, pentobarbital 10% EtOH, and 40% propylene glycol. The time interval between single injections was 7 days. Half of the mice in each group were injected first with vehicle and then with the corresponding anesthetic, the other half vice versa. Mice were used for several experiments because the transmitters are very expensive and their implantation is time-consuming. After turning on the transmitters with a magnet, a 1-h baseline was measured with data sampling for 30 s every 3 min. Five minutes before injection, the sampling schedule was switched to continuous ECG recording, and body temperature and heart rate were sampled every 30 s. Two hours after the return of righting reflex, the continuous sampling was switched to a data sampling for 30 s every 3 min and then continued for another 15 h. Data were acquired with the Dataquest ART 3.0 acquisition system (DataSciences International). All signals [core body temperature (CBT), heart rate (HR), and ECG parameters] were recorded simultaneously in the same experiment. CBT and HR were calculated by the acquisition software (Dataquest A.R.T. 3.01; DataSciences International). The ECG signal was further processed to derive time domain parameters (PQ, QT) with the Physiostat ECG Analysis 4.00 (DataSciences International) software.

Statistical Analysis. Results are expressed as mean ± S.E.M. For analysis of reflex and blood gas data the unpaired Student's *t* test was used. For analysis of telemetry data, statistical differences were assessed by using the paired Student's *t* test for testing whether the effect of anesthetic is significant compared with the vehicle and the unpaired Student's *t* test for determining potential differences between wild-type and mutant mice. The minimum CBT or HR after injection of anesthetic and the mean of vehicle values over a time period of 2 h after injection were determined and compared with the mean of 1 h baseline before injection.

Results

β3(N265M) Mice Are Resistant to Pentobarbital-Induced Hypnosis and Immobility. We measured two different endpoints to assess the anesthetic action of pentobarbital in wild-type and β3(N265M) mice. The LORR was taken as a measure of hypnosis (loss of consciousness), and the LHWR was taken as a measure of immobility (surgical tolerance, loss of response to a noxious stimulus). Both reflexes are used widely in animal research to assess the effectiveness

of anesthetics. The dose range of pentobarbital that could be used was very small (50–75 mg/kg pentobarbital, i.v.). At lower doses, neither genotype showed a reliable loss of reflexes, and at higher doses all animals died (data not shown). Pentobarbital at doses of 50, 62.5, and 75 mg/kg i.v. induced a LORR in both wild-type and β3(N265M) mice, and the duration of LORR was significantly reduced in β3(N265M) mice compared with wild type (62, 57, and 64% of the duration of LORR in wild-type mice) (Fig. 1). The LHWR was very short in wild-type mice treated with 50 mg/kg pentobarbital but robust at the higher doses. LHWR was completely abolished in almost all β3(N265M) mice at all doses tested. At 62.5 and 75 mg/kg, all wild-type mice lost the hind-limb withdrawal reflex, whereas at 62.5 mg/kg, none of 11 of the β3(N265M) mice and at 75 mg/kg, one of nine β3(N265M) mice lost the hind-limb withdrawal reflex (Fig. 1). At 75 mg/kg, 2 of 12 wild-type and 2 of 11 β3(N265M) mice died. At 62.5 mg/kg, 1 of 12 wild-type mice died, and at 50 mg/kg, 1 of 17 β3(N265M) mice died. In contrast to what was observed for etomidate and propofol, where at the highest dose 50% of wild-type mice but none of the β3(N265M) mice died (Jurd et al., 2003), after injection of pentobarbital, no genotype difference in lethality was observed.

In summary, β3(N265M) mice are completely resistant to pentobarbital-induced loss of hindlimb withdrawal reflex and are partially resistant to pentobarbital-induced loss of righting reflex compared with wild-type mice. These results are very similar to those obtained previously for etomidate and propofol in these mice (Jurd et al., 2003) and indicate that the immobilizing action and in part the hypnotic action of pentobarbital are mediated by β3-containing $GABA_A$ receptors.

β3(N265M) Mice Are Susceptible to Pentobarbital-Induced Respiratory Depression. To assess respiratory depression induced by the general anesthetic pentobarbital, arterial blood gases and pH values were determined after intravenous injection in β3(N265M) and wild-type mice (Fig. 2). After injection of 75 mg/kg pentobarbital i.v., both genotypes showed a marked respiratory depression. The pAO_2 was 53 ± 7 mm Hg in wild-type mice and 64 ± 5 mm Hg in β3(N265M) mice. The normal range for pAO_2 in awake mice is 101 ± 3 mm Hg (Arras et al., 2001). The $pACO_2$ was 54 ± 4 mm Hg in wild-type mice and 45 ± 2 mm Hg in β3(N265M) mice. The normal range for $pACO_2$ in wild-type mice is 25 ± 1 mm Hg (Arras et al., 2001). The pH was 7.15 ± 0.02 in wild-type mice and 7.14 ± 0.02 in β3(N265M) mice (normal value in mice, pH 7.44 ± 0.01) (Arras et al., 2001). The unpaired Student's *t* test reveals a significant decrease of the oxygen partial pressure, an increase in carbon dioxide partial pressure, and a decrease in pH after pentobarbital in both β3(N265M) and wild-type mice compared with blood gas parameters in awake mice ($p < 0.001$ for both genotypes and all parameters measured) (Arras et al., 2001) but no genotype difference (pentobarbital: pAO_2, $p = 0.435$; $pACO_2$, $p = 0.144$, pH $p = 0.475$). The results are similar to those re-

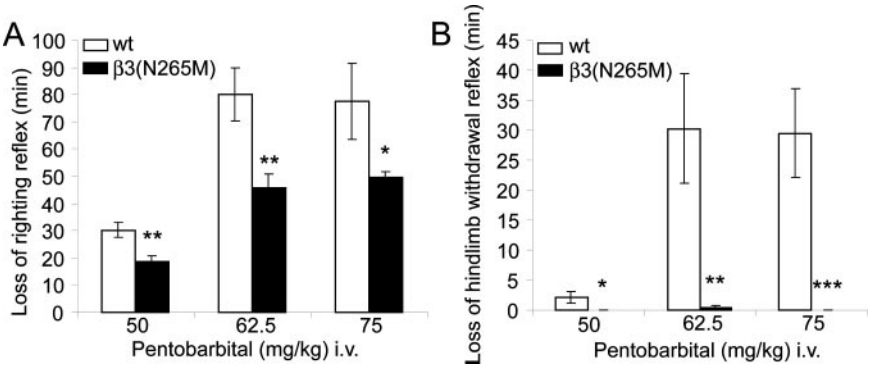

Fig. 1. Behavioral responses to pentobarbital in β3(N265M) and wild-type mice. A, reduction in the duration of the LORR induced by pentobarbital in β3(N265M) mice compared with wild-type mice. B, pentobarbital failed to induce loss of hindlimb withdrawal reflex in β3(N265M) mice in contrast to wild-type mice; $n = 7$ to 17. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

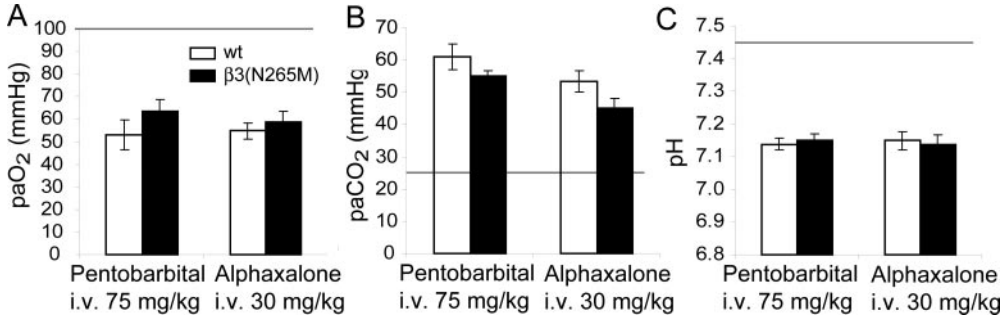

Fig. 2. Assessment of pentobarbital-induced respiratory depression by blood gas analysis. A and B, in β3(N265M) mice injected with pentobarbital, pAO_2 was decreased and $pACO_2$ was increased similar to wild-type mice, indicating the independence of the respiratory depressant effect of pentobarbital on β3-containing $GABA_A$ receptors. For comparison, the values for the neurosteroidal anesthetic alphaxalone, which have already been published (Zeller et al., 2005), are displayed as well. Alphaxalone, whose action is not affected by the β3(N265M) mutation in vitro, elicits changes in blood gases without a difference between genotypes. C, after pentobarbital and alphaxalone, pH was decreased in both β3(N265M) mice and wild-type mice; $n = 10$ to 17. For all three parameters, there was no genotype difference (pentobarbital: pAO_2, $p = 0.435$; $pACO_2$, $p = 0.144$; pH, $p = 0.475$; alphaxalone: pAO_2, $p = 0.515$; $pACO_2$, $p = 0.183$; pH, $p = 0.757$). The light gray lines indicate normal values in awake mice (taken from Arras et al., 2001).

ported previously for the neurosteroidal anesthetic alphaxalone/alphadolone mixture (Zeller et al., 2005), whose actions are not affected by the β3(N265M) point mutation (Belelli et al., 1999; Siegwart et al., 2002). These results indicate that the respiratory depressant action of pentobarbital is not dependent on β3-containing $GABA_A$ receptors in contrast to the respiratory depressant effects of etomidate and propofol.

The Heart Rate Depressant Effect of Pentobarbital Is Present but Reduced in β3(N265M) Mice. To determine the cardiac depressant effect of pentobarbital in β3(N265M) mice, HR was measured using a radiotelemetry system in unrestrained animals (Zeller et al., 2005). The baseline heart rate is similar for both genotypes without any handling stress [561 ± 19 bpm for wild-type mice, 554 ± 24 bpm for β3(N265M) mice; data not shown]. After injection of 60 mg/kg pentobarbital i.p., HR decreases in wild-type mice from 620 ± 53 bpm to 220 ± 17 bpm (−65%, $p < 0.01$) and in β3(N265M) mice from 636 ± 15 to 363 ± 21 bpm (−43%, $p < 0.01$) (Fig. 3). HR after vehicle injection was slightly increased in both genotypes compared with the baseline (Fig. 3A), probably because of handling stress. The HR decrease induced by pentobarbital is significantly less pronounced in β3(N265M) mice compared with wild-type mice (Fig. 3B, $p < 0.01$, maximum HR decrease after injection compared with vehicle). Our results suggest that there is a minor contribution of β3-containing $GABA_A$ receptors to the heart rate depressant action of pentobarbital.

The Hypothermic Effect of Pentobarbital Is Present but Reduced in β3(N265M) Mice. Most general anesthetics induce hypothermia. We therefore measured the changes in CBT after injection of pentobarbital. After injection of 60 mg/kg pentobarbital i.p., the CBT decreased significantly in both genotypes, from 36.5 ± 0.3 and 36.8 ± 0.2 to 28.9 ± 0.3°C (−21%, $p < 0.01$) and 30.9 ± 1.1°C (−16%, $p < 0.01$) in wild-type and β3(N265M) mice, respectively (Fig. 4). The decrease of CBT is pronounced in both genotypes after pentobarbital application but is significantly less in β3(N265M) mice compared with wild-type mice ($p < 0.05$). Thus, whereas the decrease in CBT seems to be largely mediated by other targets, presumably β2-containing $GABA_A$ receptors, there is clearly a minor component of hypothermia mediated by β3-containing $GABA_A$ receptors.

Effects of Pentobarbital on ECG Parameters. General anesthetics are known to change the duration of various intervals of the ECG in humans. To our knowledge, with the exception of ketamine (Mitchell et al., 1998), this has not been demonstrated in mice. We investigated the actions of pentobarbital on the ECG in wild-type and β3(N265M) mice. Pentobarbital prolonged the PQ, QRS, and QT intervals from 31.8 ± 1.3, 12.2 ± 0.5, and 23.5 ± 0.6 ms to 45.5 ± 1.2 ($p <$

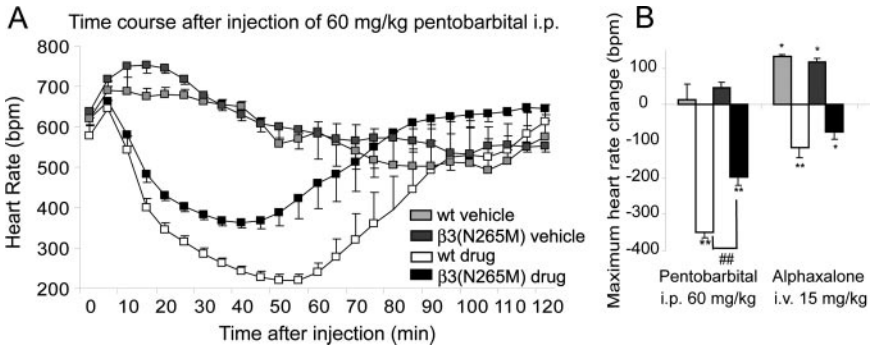

Fig. 3. Pentobarbital-induced heart rate depression. A, after injection of pentobarbital, HR decreases in both wild-type and β3(N265M) mice. B, maximum HR change after injection of anesthetic or vehicle compared with 1 h baseline before injection. A value of 0 would designate no deviation from baseline, whereas a positive value designates an increase of heart rate compared with the baseline and a negative value designates a decrease of heart rate compared with the baseline. For comparison, values for alphaxalone, a neurosteroid whose action at the $GABA_A$ receptor is not influenced by the β3(N265M) point mutation, are displayed as well (Zeller et al., 2005). Pentobarbital: $n = 5$ for wild type, $n = 7$ for β3(N265M); intravenous alphaxalone: wild type, $n = 6$; β3(N265M), $n = 6$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when the effect of the vehicle or anesthetic is compared with the baseline; ##, $p < 0.01$ for genotype difference.

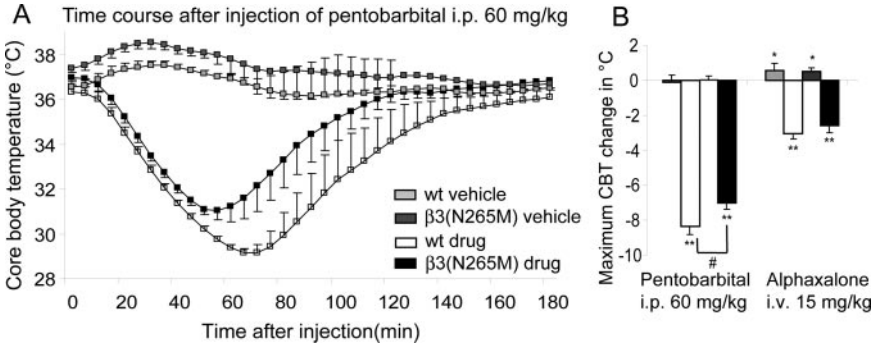

Fig. 4. Pentobarbital-induced hypothermia. A, after injection of pentobarbital, CBT decreases in both wild-type and β3(N265M) mice. B, maximum CBT change after injection of anesthetic or vehicle compared with 1 h baseline before injection. For comparison, values for alphaxalone are displayed as well. Pentobarbital: $n = 5$ for wild type, $n = 7$ for β3(N265M); intravenous alphaxalone: wild-type, $n = 6$; β3(N265M), $n = 6$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when the effect of the vehicle or anesthetic is compared with the baseline; #, $p < 0.05$ for genotype difference.

0.01 versus vehicle), 16 ± 2 (p = 0.113 versus vehicle), and 27.6 ± 1.3 ms ($p < 0.05$ versus vehicle) in wild-type mice and from 32.2 ± 0.7, 11.2 ± 0.5, and 19.7 ± 0.9 ms to 35.1 ± 1.4 (p = 0.217 versus vehicle), 13.8 ± 5 ($p < 0.05$ versus vehicle), and 25.6 ± 1 ms ($p < 0.01$ versus vehicle) in β3(N2565M) mice. In wild-type mice, PQ and QT interval were increased significantly compared with vehicle, and the QRS interval was not significantly increased. In β3(N2565M) mice, the QRS and QT intervals were increased, whereas the PQ interval was not significantly different from baseline, presumably because of a high variability (Table 1 and Fig. 5). There is no significant genotype difference for the ECG intervals. Heart rate variability (HRV) is measured as the standard deviation of the interbeat interval. A dose of 60 mg/kg pentobarbital i.p. increases HRV 8-fold in wild-type and 4-fold in β3(N265M) mice [$p < 0.01$ in wild type, $p < 0.05$ in β3(N265M) versus vehicle, $p < 0.05$ between genotypes after drug]. For comparison purposes, we also studied alphaxalone, whose action is not influenced by the β3(N265M) point mutation. Alphaxalone induces similar changes of all analyzed ECG parameters in wild-type and β3(N265M) mice. HRV increases in wild-type mice 5-fold and in β3(N265M) mice 3.5-fold ($p < 0.05$ versus vehicle in both genotypes, p = 0.317 between genotypes). QT, QRS, and PQ are prolonged from 23.5 ± 0.6, 12.2 ± 0.5, and 31.8 ± 1.3 ms to 26.6 ± 0.8, 14 ± 0.8, and 44.6 ± 1 ms (p = 0.186, 0.181, and <0.001 for QT, QRS, and PQ, respectively, versus vehicle) in wild-type mice and from 19.7 ± 0.9, 11.2 ± 0.5, and 32.2 ± 0.7 ms to 24.9 ± 1, 13 ± 1.2, and 43.9 ± 0.8 ms (p = 0.113, 0.811, and <0.05 for QT, QRS, and PQ, respectively) versus vehicle in β3(N265M) mice (p = 0.644, 0.964, and 0.139 between genotypes).

Thus, HRV is slightly less increased in β3(N265M) mice compared with wild type after injection of pentobarbital,

TABLE 1

Effects of pentobarbital on baseline ECG parameters

All values are mean ± S.E.M. Group sizes: pentobarbital: wt, n = 5; β3(N265M), n = 7; intravenous alphaxalone: wt, n = 6; β3(N265M), n = 6. If not indicated, the deviation from baseline or the genotype difference was not statistically significant.

	Baseline		Pentobarbital Vehicle		Pentobarbital 60 mg/kg i.p.		Alphaxalone Vehicle		Alphaxalone 15 mg/kg i.v.	
	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)
					ms					
RR	107 ± 3.6	108 ± 4.7	130 ± 29	117 ± 3.5	285 ± 13.6**	138 ± 7.6*#	87 ± 0.5	93 ± 2	147 ± 9.7**	132 ± 2.9*
HRV	5.2 ± 0.8	5.4 ± 0.8	6.4 ± 2.8	3.2 ± 0.7	40.4 ± 5.8**	25.7 ± 6.2*#	1.8 ± 0.2	1.9 ± 0.3	18.1 ± 4.1*	12.3 ± 1.6*
QT	23.5 ± 0.6	19.7 ± 0.9	26 ± 1	24.1 ± 0.9	27.6 ± 1.3**	25.6 ± 1**	24.7 ± 0.7	26.5 ± 1	26.6 ± 0.8	24.9 ± 1
QRS	12.2 ± 0.5	11.2 ± 0.5	11.8 ± 0.6	11.1 ± 0.6	16 ± 2	13.8 ± 5*	11.3 ± 0.4	11.2 ± 0.5	14 ± 0.8	13 ± 1.2
PQ	31.8 ± 1.3	32.2 ± 0.7	33.8 ± 2.1	36.4 ± 2.7	43.8 ± 1.2***	43.1 ± 1.5***	32.7 ± 1.3	31.3 ± 0.9	44.6 ± 1*	43.9 ± 0.8*

RR, interbeat interval; wt, wild type.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with baseline.

# $p < 0.05$ wild-type compared with β3(N265M) mice

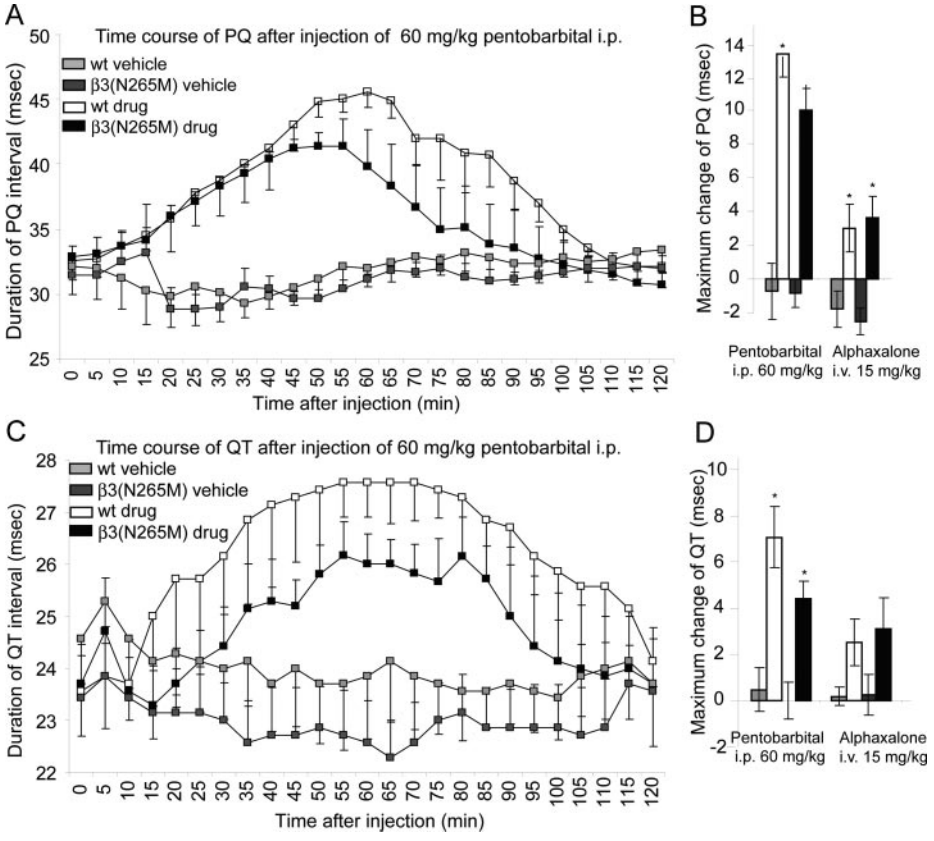

Fig. 5. Pentobarbital-induced changes of ECG intervals. A and C, with injection of 60 mg/kg pentobarbital i.p., PQ and QT intervals are prolonged. The prolongation is slightly less pronounced in β3(N265M) mice compared with wild type. B and D, maximum change of PQ and QT after injection of pentobarbital compared with 1-h baseline before application. For comparison, values for alphaxalone are displayed as well. Pentobarbital: n = 5 for wild type, n = 7 for β3(N265M); intravenous alphaxalone: wild-type, n = 6; β3(N265M), n = 6. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

whereas the HRV increase is similar in both genotypes after alphaxalone, suggesting that the β3(N265M) mice respond normally to anesthetic-induced ECG changes and that the pentobarbital-induced increase in HRV is mediated by β3-containing $GABA_A$ receptors. Both anesthetics induced prolongation of QT, QRS, and particularly PQ intervals, but these changes, presumably because of high variability, only partly reached statistical significance. Most importantly, there was no genotype difference both for pentobarbital and alphaxalone, indicating that β3-containing $GABA_A$ receptors do not play a role in pentobarbital-induced ECG interval prolongation.

Discussion

We studied the effects of general anesthetics in mice harboring an asparagine-to-methionine point mutation in position 265 of the β3 subunit of the $GABA_A$ receptor. This point mutation renders recombinant β3-containing $GABA_A$ receptors insensitive to the actions of the general anesthetics etomidate and propofol but not to the neurosteroidal anesthetic alphaxalone (Pistis et al., 1999; Siegwart et al., 2002). The β3(N265M) mutation completely abolishes the direct (i.e., GABA-independent) action of pentobarbital and shifts the concentration-response curve for the modulatory action of pentobarbital to the right (Pistis et al., 1999). In β3(N265M) mice the suppression of noxious-evoked movements in response to the anesthetics propofol and etomidate was completely abolished, and the hypnotic response was also decreased significantly (Jurd et al., 2003). In addition, the respiratory depressant action of etomidate and propofol was strongly reduced. The β3(N265M) mice also show a slightly reduced hypothermia in response to etomidate but not to propofol (Zeller et al., 2005).

We investigated the actions of pentobarbital in β3(N265M) mice by assessing different anesthetic endpoints like immobility (suppression of noxious-evoked movements), hypnosis, respiratory depression, hypothermia, heart rate depression, and influence on ECG. The β3(N265M) mice show a strongly reduced duration of the loss of righting reflex in response to pentobarbital and a complete absence of the loss of the hindlimb withdrawal reflex. This reduction or loss of response to pentobarbital is similar to the altered response of the β3(N265M) mice to etomidate and propofol. The suppression of noxious-evoked withdrawal reflexes (immobility) is believed to be mediated by spinal cord circuits (Antognini and Schwartz, 1993; Rampil et al., 1993; Rampil, 1994). β3-Containing $GABA_A$ receptors are indeed the predominant $GABA_A$ receptor subtype expressed in dorsal root ganglia, the superficial dorsal horn of the spinal cord and motor neurons (Persohn et al., 1991; Ma et al., 1993). Our data now indicate that the action of pentobarbital on spinal cord-mediated reflexes occurs via β3-containing $GABA_A$ receptors. Generation of respiratory rhythms occurs in a network of neurons originating from the preBötzinger complex (Richter et al., 2003). Synaptic interactions involving AMPA, *N*-methyl-D-aspartic acid, $GABA_A$, $GABA_B$, and glycine receptors are believed to play a major role in regulating this network. We have shown previously that etomidate and propofol-induced respiratory depression is mediated by β3-containing $GABA_A$ receptors. Which neurons specifically mediate this effect is currently unknown. We investigated in this study whether pentobarbital-induced respiratory depression is also mediated by β3-containing $GABA_A$ receptors. After injection of pentobarbital, β3(N265M) mice show a very pronounced respiratory depression similar to wild-type mice. This indicates that pentobarbital-induced respiratory depression is not mediated by β3-containing $GABA_A$ receptors or, if it is, to some degree, that pentobarbital can also induce respiratory depression via other targets. This anesthetic endpoint is therefore mediated by different receptors or circuits in etomidate- and propofol-induced anesthesia compared with pentobarbital-induced anesthesia.

Respiratory depression can be achieved by either inhibition of the overall glutamatergic drive or an enhanced overall GABAergic inhibitory drive to the neurons of the preBötzinger complex or a combination of decreased excitation and enhanced inhibition (Stucke et al., 2005a,b). Sevoflurane, for example, has both effects (Stucke et al., 2005a). Etomidate and propofol apparently bind quite exclusively to $GABA_A$ receptors and might therefore induce respiratory depression mostly by increasing GABAergic inhibition. Pentobarbital modulates the activity of additional targets (e.g., it negatively modulates the activity of neuronal nACh, AMPA, and Kainate receptors) (Krasowski and Harrison, 1999; Petrenko et al., 2004) and might therefore have effects on both the excitatory and the inhibitory drive of the neurons of the preBötzinger complex. The increase of inhibitory drive might be abolished in β3(N265M) mice, but the remaining excitatory drive may be sufficient to induce respiratory depression. Pentobarbital might therefore induce respiratory depression exclusively by decreasing the excitatory drive of the neurons of the preBötzinger complex, and for that reason, β3(N265M) mice are still susceptible to pentobarbital-induced respiratory depression. It is tempting to speculate that this essential difference underlies the significantly smaller therapeutic range of barbiturates compared with etomidate and propofol. The propensity of barbiturates to cause potentially lethal respiratory depression is also exploited in assisted suicide, and it is used as a euthanizing agent in veterinary medicine.

Hypothermia is a common side effect of anesthesia. It has been shown that etomidate-induced hypothermia is largely mediated by both β2- and β3-containing $GABA_A$ receptors, with the β2-containing $GABA_A$ receptors playing a dominant role (Cirone et al., 2004). We now measured the effect of pentobarbital on core body temperature. We show that pentobarbital-induced hypothermia is mediated to a limited degree by β3-containing $GABA_A$ receptors. Other targets mediating the majority of pentobarbital-induced hypothermia might be β2-containing $GABA_A$ receptors but also other receptors.

General anesthetics are known to reduce heart rate in both mice and humans (Mitchell et al., 1998; Zeller et al., 2005). The heart rate depression is much less pronounced in humans where, for example, thiopental and low doses of propofol slightly increase heart rate, whereas higher doses of propofol and etomidate depress the heart rate (Kienbaum and Peters, 2001). However, in mice, heart rate depression is usually stronger, because of either different regulation of the cardiac system in mice and humans or the higher dosages usually used in experimental research (Mitchell et al., 1998; Appleton et al., 2004). We have shown previously that etomidate, propofol, and alphaxalone depress the heart rate strongly in both β3(N265M) and wild-type mice. Heart rate

depression is slightly reduced in β3(N265M) mice after etomidate. In this report, we show that pentobarbital depresses heart rate less in β3(N265M) mice compared with wild-type mice. This shows that pentobarbital-induced heart rate depression is partly mediated by β3-containing $GABA_A$ receptors but mainly by other targets. The cardiovascular effects of general anesthetics are probably mediated not only by targets in the central nervous system but also by peripheral targets. Pentobarbital has additional targets in the central nervous system like Na^+ channels (Lingamaneni and Hemmings, 2003), voltage-sensitive Ca^{2+} channels (Hirota et al., 2000), diverse potassium channels (Friederich and Urban, 1999; Wan et al., 2003), L-type calcium channels (Guertin and Hounsgaard, 1999), P-type calcium channels (Hall et al., 1994; Kitayama et al., 2002), and AMPA receptors (Krasowski and Harrison, 1999). Potassium channels are peripheral targets of pentobarbital especially in the heart (Bachmann et al., 2002).

General anesthetic agents also alter ECG intervals, and they decrease heart rate variability in humans (Ledowski et al., 2005). Here, we report that HRV is increased by pentobarbital in mice. HRV is considered to be an indicator of cardiac vagal control, and drugs increasing HRV have been shown to reduce mortality and sudden death in patients with several chronic cardiac conditions in clinical trials (Routledge et al., 2002). This might indicate that general anesthetics induce a sympathetic blockade in mice that results in prolongation of time domain intervals such as QT, QRS, and PQ and in an increase in HRV (Gehrmann et al., 2000). The increase in HRV after pentobarbital is slightly but significantly reduced in β3(N265M) compared with wild-type mice. Prolongation of QT, QRS, and PQ intervals is similar and not statistically different in β3(N265M) and wild-type mice. Although β-adrenergic receptors are believed to regulate HRV (Ecker et al., 2006), HRV might also be influenced by central nervous system mechanisms. Our results suggest that β3-containing $GABA_A$ receptors might play a role in this latter regulation.

In summary, in this study, we provide evidence that some anesthesia-related endpoints of pentobarbital, in particular LHWR and in part LORR, are mediated by β3-containing $GABA_A$ receptors. Particularly striking is that the respiratory depressing action of pentobarbital is independent of this receptor subtype, whereas the respiratory-depressing actions of etomidate and propofol are mediated by this receptor subtype, consistent with a wider spectrum of relevant targets for pentobarbital. Our results show that it is possible to separate the immobilizing and the respiratory-depressing action of general anesthetics.

Acknowledgments

We thank Dr. Bernd Antkowiak (Tübingen) for critically reading the manuscript and Isabelle Camenisch for genotyping mice.

References

Antognini JF and Schwartz K (1993) Exaggerated anesthetic requirements in the preferentially anesthetized brain. *Anesthesiology* **79:**1244–1249.

Appleton GO, Li Y, Taffet GE, Hartley CJ, Michael LH, Entman ML, Roberts R, and Khoury DS (2004) Determinants of cardiac electrophysiological properties in mice. *J Interv Card Electrophysiol* **11:**5–14.

Arras M, Autenried P, Rettich A, Spaeni D, and Rulicke TR (2001) Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth. *Comp Med* **51:**443–456.

Bachmann A, Mueller S, Kopp K, Brueggemann A, Suessbrich H, Gerlach U, and Busch AE (2002) Inhibition of cardiac potassium currents by pentobarbital. *Naunyn-Schmiedeberg's Arch Pharmacol* **365:**29–37.

Backus KH, Arigoni M, Drescher U, Scheurer L, Malherbe P, Mohler H, and Benson JA (1993) Stoichiometry of a recombinant $GABA_A$ receptor deduced from mutation-induced rectification. *Neuroreport* **5:**285–288.

Belelli D, Lambert JJ, Peters JA, Wafford K, and Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. *Proc Natl Acad Sci USA* **94:**11031–11036.

Belelli D, Pau D, Cabras G, Peters JA, and Lambert JJ (1999) A single amino acid confers barbiturate sensitivity upon the GABA rho 1 receptor. *Br J Pharmacol* **127:**601–604.

Chang Y, Wang R, Barot S, and Weiss DS (1996) Stoichiometry of a recombinant $GABA_A$ receptor. *J Neurosci* **16:**5415–5424.

Cirone J, Rosahl TW, Reynolds DS, Newman RJ, O'Meara GF, Hutson PH, and Wafford KA (2004) Gamma-aminobutyric acid type A receptor beta 2 subunit mediates the hypothermic effect of etomidate in mice. *Anesthesiology* **100:**1438–1445.

Ecker PM, Lin CC, Powers J, Kobilka BK, Dubin AM, and Bernstein D (2006) Effect of targeted deletions of beta1- and beta2-adrenergic-receptor subtypes on heart rate variability. *Am J Physiol* **290:**H192–H199.

Franks NP and Honore E (2004) The TREK K2P channels and their role in general anaesthesia and neuroprotection. *Trends Pharmacol Sci* **25:**601–608.

Friederich P and Urban BW (1999) Interaction of intravenous anesthetics with human neuronal potassium currents in relation to clinical concentrations. *Anesthesiology* **91:**1853–1860.

Gehrmann J, Hammer PE, Maguire CT, Wakimoto H, Triedman JK, and Berul CI (2000) Phenotypic screening for heart rate variability in the mouse. *Am J Physiol* **279:**H733–H740.

Guertin PA and Hounsgaard J (1999) Non-volatile general anaesthetics reduce spinal activity by suppressing plateau potentials. *Neuroscience* **88:**353–358.

Hall AC, Lieb WR, and Franks NP (1994) Insensitivity of P-type calcium channels to inhalational and intravenous general anesthetics. *Anesthesiology* **81:**117–123.

Hirota K, Kudo M, Kudo T, Kitayama M, Kushikata T, Lambert DG, and Matsuki A (2000) Barbiturates inhibit K^+-evoked noradrenaline and dopamine release from rat striatal slices—involvement of voltage sensitive Ca^{2+} channels. *Neurosci Lett* **291:**175–178.

Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, et al. (2003) General anesthetic actions in vivo strongly attenuated by a point mutation in the GABA(A) receptor beta3 subunit. *FASEB J* **17:**250–252.

Kienbaum P and Peters J (2001) Sympathetic control mechanisms during general anesthesia. *Anasthesiol Intensivmed Notfallmed Schmerzther* **36:**268–275.

Kitayama M, Hirota K, Kudo M, Kudo T, Ishihara H, and Matsuki A (2002) Inhibitory effects of intravenous anaesthetic agents on K^+-evoked glutamate release from rat cerebrocortical slices. Involvement of voltage-sensitive Ca^{2+} channels and $GABA_A$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **366:**246–253.

Krasowski MD and Harrison NL (1999) General anaesthetic actions on ligand-gated ion channels. *Cell Mol Life Sci* **55:**1278–1303.

Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, and Harrison NL (1998) Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid type A receptor distinct from that for isoflurane. *Mol Pharmacol* **53:**530–538.

Ledowski T, Bein B, Hanss R, Paris A, Fudickar W, Scholz J, and Tonner PH (2005) Neuroendocrine stress response and heart rate variability: a comparison of total intravenous versus balanced anesthesia. *Anesth Analg* **101:**1700–1705.

Lingamaneni R and Hemmings HC Jr (2003) Differential interaction of anaesthetics and antiepileptic drugs with neuronal Na+ channels, Ca^{2+} channels, and $GABA_A$ receptors. *Br J Anaesth* **90:**199–211.

Ma W, Saunders PA, Somogyi R, Poulter MO, and Barker JL (1993) Ontogeny of GABAA receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. *J Comp Neurol* **338:**337–359.

Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, et al. (1997) Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature (Lond)* **389:**385–389.

Mitchell GF, Jeron A, and Koren G (1998) Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol* **274 (3 Pt 2):**H747–H751.

Persohn E, Malherbe P, and Richards JG (1991) In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. *Neuroscience* **42:**497–507.

Petrenko AB, Yamakura T, Fujiwara N, Askalany AR, Baba H, and Sakimura K (2004) Reduced sensitivity to ketamine and pentobarbital in mice lacking the *N*-methyl-D-aspartate receptor GluRepsilon1 subunit. *Anesth Analg* **99:**1136–1140.

Pistis M, Belelli D, McGurk K, Peters JA, and Lambert JJ (1999) Complementary regulation of anaesthetic activation of human (alpha6beta3gamma2L) and *Drosophila* (RDL) GABA receptors by a single amino acid residue. *J Physiol* **515 (Pt 1):**3–18.

Rampil IJ (1994) Anesthetic potency is not altered after hypothermic spinal cord transection in rats. *Anesthesiology* **80:**606–610.

Rampil IJ, Mason P, and Singh H (1993) Anesthetic potency (MAC) is independent of forebrain structures in the rat. *Anesthesiology* **78:**707–712.

Reynolds DS, Rosahl TW, Cirone J, O'Meara GF, Haythornthwaite A, Newman RJ, Myers J, Sur C, Howell O, Rutter AR, et al. (2003) Sedation and anesthesia mediated by distinct $GABA_A$ receptor isoforms. *J Neurosci* **23:**8608–8617.

Richter DW, Manzke T, Wilken B, and Ponimaskin E (2003) Serotonin receptors: guardians of stable breathing. *Trends Mol Med* **9:**542–548.

Routledge HC, Chowdhary S, and Townend JN (2002) Heart rate variability—a therapeutic target? *J Clin Pharm Ther* **27:**85–92.

Siegwart R, Jurd R, and Rudolph U (2002) Molecular determinants for the action of

general anesthetics at recombinant alpha$_2$beta$_3$gamma$_2$gamma-aminobutyric acid$_A$ receptors. *J Neurochem* **80:**140–148.
Siegwart R, Krahenbuhl K, Lambert S, and Rudolph U (2003) Mutational analysis of molecular requirements for the actions of general anaesthetics at the gamma-aminobutyric acidA receptor subtype, alpha1beta2gamma2. *BMC Pharmacol* **3:**13.
Späni D, Arras M, König B, and Rülicke T (2003) Higher heart rate of laboratory mice housed individually vs in pairs. *Lab Anim* **37:**54–62.
Stucke AG, Zuperku EJ, Krolo M, Brandes IF, Hopp FA, Kampine JP, and Stuth EA (2005a) Sevoflurane enhances gamma-aminobutyric acid type A receptor function and overall inhibition of inspiratory premotor neurons in a decerebrate dog model. *Anesthesiology* **103:**57–64.
Stucke AG, Zuperku EJ, Tonkovic-Capin V, Krolo M, Hopp FA, Kampine JP, and Stuth EA (2005b) Sevoflurane depresses glutamatergic neurotransmission to brainstem inspiratory premotor neurons but not postsynaptic receptor function in a decerebrate dog model. *Anesthesiology* **103:**50–56.
Thompson SA, Whiting PJ, and Wafford KA (1996) Barbiturate interactions at the human GABA-A receptor: dependence on receptor subunit combination. *Br J Pharmacol* **117:**521–527.
Wan X, Mathers DA, and Puil E (2003) Pentobarbital modulates intrinsic and GABA-receptor conductances in thalamocortical inhibition. *Neuroscience* **121:**947–958.
Zeller A, Arras M, Lazaris A, Jurd R, and Rudolph U (2005) Distinct molecular targets for the central respiratory and cardiac actions of the general anesthetics etomidate and propofol. *FASEB J* **19:**1677–1679.

Address correspondence to: Dr. Uwe Rudolph, Laboratory of Genetic Neuropharmacology, McLean Hospital, Harvard Medical School, Belmont, MA 02478. E-mail: urudolph@mclean.harvard.edu