Molecular Pharmacology Fast Forward. Published on December 12, 2006 as doi:10.1124/mol.106.030049

MOL #30049

Identification of a molecular target mediating the general anesthetic actions of pentobarbital

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Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.030049
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Running title page

Running title
Targets mediating the anesthetic actions of pentobarbital

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List of non-standard abbreviations

GABA	γ- aminobutyric acid
$GABA_A$ receptor	GABA type A receptor
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA	N-methyl-D-aspartic acid
GluR2	an AMPA subunit type of the glutamate receptor
GluRε1	an NMDA subunit type of the glutamate receptor
LORR	loss of righting reflex
LHWR	loss of hindlimb withdrawal reflex
HR	heart rate
CBT	core body temperature
ECG	electrocardiogram
HRV	heart rate variability
QT, QRS, PQ	ECG intervals

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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. Surprisingly, 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. While the target for the immobilizing and hypnotic actions of pentobarbital appears 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 as compared to 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.

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Introduction

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; Krasowski et al., 1998; Mihic et al., 1997; Siegwart et al., 2002; Siegwart et al., 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, while 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

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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-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 the electrocardiogram (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 to the β3 subtype dependence of etomidate and propofol actions.

Material 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

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of a mixed background (129/Sv (12.5%)x 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, 75 mg/kg, Abbott AG, Baar, Switzerland/Abbott Laboratories, Chicago, USA) administered intravenously (i.v.) into the tail vein in a volume of 4µl/kg body weight. The duration of the LORR and LHWR was recorded as described previously (Arras et al., 2001). Briefly, 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 seconds (range 110 to 140 seconds) after injection of 75 mg/kg pentobarbital i.v. or 30 mg/kg alphaxalone i.v. following the procedure described by Arras et. al. (Arras et al., 2001). Briefly, 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 heparinised syringe. Oxygen partial pressure (paO_2, mmHg), carbon dioxide partial pressure ($paCO_2$, mmHg), acid-base balance (pH value), and standard

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bicarbonate concentrations (HCO_3^-, mmol/L) 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 non-anesthetized mice. Instead, data previously obtained from wild type mice (strain HanIbm:NMRI), male, obtained from RCC (Research and Consulting Company, Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland)) and reported in Arras et al. (2001) were used for comparison.

Surgery

16 female mice (8 β3(N265M) mice and 8 wild type controls) were implanted under isoflurane anaesthesia (3-5% in oxygen) with intraperitoneal radiotelemetry transmitters for measuring core body temperature and ECG (model No. ETA-F20, Data Sciences International (DSI), 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

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Mice implanted with telemetry transmitters were singly housed in standard laboratory conditions with a 12 h light/dark schedule (lights on 8:00 am, lights off 8:00 pm) and free access to food and water. Experiments were performed between 9 am and 12 am. 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 (CBT), Heart Rate (HR) and ECG Parameters (PQ, QT)

For drug and vehicle administration experiments, a baseline was recorded between 0 and 2 hours after lights on and drugs were administered immediately afterwards. Drug effects were compared to vehicle effects, which did not differ significantly from baseline. Before injection of pentobarbital 60 mg/kg i.p. (Nembutal, Abbott AG, Baar, Switzerland / Abbott Laboratories, Chicago, USA), mice were already treated with other anesthetics (see also (Zeller et al., 2005)). Vehicle solutions were as follows: alphaxalone (Saffan®) 14 % Cremophor EL, pentobarbital 10 % EtOH, 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 transmitter are very expensive and their implantation is time-consuming. After turning on the transmitters with a magnet, a one hour baseline was measured with data sampling for 30 s every 3 minutes. Five minutes before injection the sampling schedule was switched to continuous ECG recording and body temperature and heart rate were sampled every 30

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s. Two hours after the return of righting reflex the continuous sampling was switched to a data sampling for 30 s every 3 minutes and then continued for another 15 hours. Data were acquired with the Dataquest ART 3.0 acquisition system (DataSciences International, St. Paul, MN, USA). All signals (CBT, 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±SEM. 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 to 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 two hours after injection were determined and compared to the mean of one hour baseline before injection.

Results

β3(N265M) mice are resistant to pentobarbital-induced hypnosis and immobility

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We measured two different endpoints to assess the anesthetic action of pentobarbital in wild type and β3(N265M) mice. The loss of righting reflex (LORR) was taken as a measure of hypnosis (loss of consciousness) and the loss of hindlimb withdrawal reflex (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 to 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 to 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 mg/kg and 75 mg/kg all wild type mice lost the hind-limb withdrawal reflex, whereas at 62.5 mg/kg none out of 11 of the β3(N265M) mice and at 75 mg/kg one out of 9 β3(N265M) mice lost the hindlimb withdrawal reflex (Fig. 1). At 75 mg/kg, 2 out of 12 wild type and 2 out of 11 β3(N265M) mice died. At 62.5 mg/kg one out of 12 wild type mice died and at 50 mg/kg one out 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.

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In summary, β3(N265M) mice are completely resistant to pentobarbital-induced loss of hind-limb withdrawal reflex, and are partially resistant to pentobarbital-induced loss of righting reflex, compared to 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 i.v. injection of 75 mg/kg pentobarbital, both genotypes showed a marked respiratory depression. The oxygen partial pressure (paO_2) was 53±7 mmHg in wild type mice and 64±5 mmHg in β3(N265M) mice. The normal range for paO_2 in awake mice is 101±3 mmHg (Arras et al., 2001). The carbon dioxide partial pressure ($paCO_2$) was 54±4 mmHg in wild type mice and 45±2 mmHg in β3(N265M) mice. The normal range for $paCO_2$ in wild-type mice is 25±1 mmHg (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 to blood gas parameters in awake mice ($p<0.001$ for both

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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 previously reported for the neurosteroidal anesthetic alphaxalone/alphadolone (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, heart rate (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 beats per minute (bpm) to 220±17 bpm (-65%, p<0.01) and in β3(N265M) mice from 636±15 bpm to 363 ± 21 bpm (-43%, p<0.01) (Fig. 3). HR after vehicle injection is slightly increased in both genotypes compared to the baseline (Fig. 3A), probably due to handling stress. The HR decrease induced by pentobarbital is significantly less pronounced in β3(N265M) mice compared to wild type mice (Fig. 3B, p<0.01, maximum HR decrease after injection compared to vehicle). Our results suggests that there is a minor contribution

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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 core body temperature (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°C and 36.8±0.2°C 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 significantly less in β3(N265M) mice compared to wild type mice ($p<0.05$). Thus, while the decrease in CBT appears 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 ms, 12.2±0.5 ms, 23.5±0.6 ms to 45.5±1.2 ms ($p<0.01$ versus vehicle), 16±2 ms ($p=0.113$ versus vehicle), 27.6±1.3 ms ($p<0.05$ versus

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vehicle) in wild type mice and from 32.2±0.7 ms, 11.2±0.5 ms, 19.7±0.9 ms to 35.1±1.4 ms ($p=0.217$ versus vehicle), 13.8±5 ms ($p<0.05$ versus vehicle), 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 to vehicle, the QRS interval was not significantly increased. In β3(N2565M) mice, the QRS and QT intervals were increased, while the PQ interval was not significantly different from baseline, presumably due to a high variability (Table 1, Figure 5). There is no significant genotype difference for the ECG intervals. Heart rate variability (HRV) is measured as the standard deviation of the inter-beat-interval (RR interval). 60 mg/kg pentobarbital i.p. increases HRV 8-fold in wild type and 4-fold in β3(N265M) mice ($p<0.01$ in wt, $p<0.05$ in β3(N265M) versus vehicle, $p<0.05$ between genotypes after drug). For comparison purposes, we also studied an alphaxalone/alphadolone mixture, subsequently referred to as 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±0.6ms, 12.2±0.5 ms and 31.8±1.3 ms to 26.6±0.8 ms, 14±0.8 ms and 44.6±1 ms ($p=0.186$, $p=0.181$, $p<0.001$ for QT, QRS, PQ, respectively, versus vehicle) in wild type mice and from 19.7±0.9 ms, 11.2±0.5 ms and 32.2±0.7 ms to 24.9±1 ms, 13±1.2 ms and 43.9±0.8 ms ($p=0.113$, $p=0.811$, $p<0.05$ for QT, QRS, PQ, respectively, versus vehicle in β3(N265M) mice ($p=0.644$, $p=0.964$, $p=0.139$ between genotypes).

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Thus, HRV is slightly less increased in β3(N265M) mice compared to wild type after injection of pentobarbital, 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 in particular PQ intervals, but these changes, presumably due to 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 harbouring 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

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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 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 thought to be mediated by spinal cord circuits (Antognini and Schwartz, 1993; Rampil, 1994; Rampil et al., 1993). 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 (Ma et al., 1993; Persohn et al., 1991). 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 pre-Bötzinger complex (Richter et al., 2003). Synaptic interactions involving AMPA, NMDA, $GABA_A$, $GABA_B$ and glycine receptors are thought to play a major role in regulating this network. We have shown previously that etomidate and propofol-induced respiratory depression is mediated by β3-

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containing $GABA_A$ receptors. It is currently unknown which neurons specifically mediate this effect. 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 to 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 pre-Bötzinger complex or a combination of decreased excitation and enhanced inhibition (Stucke et al., 2005a; Stucke et al., 2005b). 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 more 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 pre-Bötzinger 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

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induce respiratory depression exclusively by decreasing the excitatory drive of the neurons of the pre-Bötzinger 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 to etomidate and propofol. The propensity of barbiturates to cause potentially lethal respiratory depression is also exploited in assisted suicide and as an 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 e.g. 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, either due to different regulation of the

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cardiac system in mice and humans or due to the higher dosages usually used in experimental research (Appleton et al., 2004; Mitchell et al., 1998). 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 to 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 cardiavascular effects of general anesthetics are likely mediated not only by targets in the CNS, but also by peripheral targets. Pentobarbital has additional targets in the CNS 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 electrocardiography (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 induces a sympathetic blockade in mice

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which 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 to 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 thought 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.

Acknowledgements

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

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Footnotes

This work was supported by a grant from the Swiss National Science Foundation.

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Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.030049
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Figure Legends

Figure 1. Behavioural responses to pentobarbital in β3(N265M) and wild type mice. A. Reduction in the duration of the loss of righting reflex (LORR) induced by pentobarbital in β3(N265M) mice compared to wild type mice. **B.** Pentobarbital failed to induce loss of hindlimb withdrawal reflex in β3(N265M) mice in contrast to wild type mice. n=7–17. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2. Assessment of pentobarbital-induced respiratory depression by blood gas analysis. A,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. As a comparison, the values for the neurosteroidal anesthetic alphaxalone which have already been published before (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=10-17. 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 grey lines inducate normal values in awake mice (taken from Arras et.al. (Arras et al., 2001)).

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Figure 3. Pentobarbital-induced heart rate depression. A. After injection of pentobarbital heart rate (HR) decreases in both wild type and β3(N265M) mice. **B.** Maximum HR change after injection of anesthetic or vehicle compared to 1 hour baseline before injection. A value of zero would designate no deviation from baseline, whereas a positive value designates an increase of heart rate compared to the baseline and a negative value designates a decrease of heart rate compared to 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 wt, n=7 for β3(N265M); alphaxalone i.v.: wt 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 to the baseline; ## $p < 0.01$ for genotype difference.

Figure 4. Pentobarbital-induced hypothermia. A. After injection of pentobarbital, core body temperature (CBT) decreases in both wild type and β3(N265M) mice. **B.** Maximum CBT change after injection of anesthetic or vehicle compared to 1 hour baseline before injection. For comparison, values for alphaxalone are displayed as well. Pentobarbital: n=5 for wt, n=7 for β3(N265M); alphaxalone i.v.: wt 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 to the baseline; # $p < 0.05$ for genotype difference.

Figure 5. Pentobarbital-induced changes of ECG intervals. A,C. With injection of 60 mg/kg pentobarbital i.p., PQ and QT intervals are prolonged.

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The prolongation is slightly less pronounced in β3(N265M) mice compared to wild type. **B,D.** Maximum change of PQ and QT after injection of pentobarbital compared to 1 hour baseline before application. For comparison, values for alphaxalone are displayed as well. Pentobarbital: n=5 for wt, n=7 for β3(N265M); alphaxalone i.v.: wt n=6, β3(N265M) n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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Tables

	Baseline		Pentobarbital Vehicle		Pentobarbital 60 mg/kg i.p.		Alphaxalone Vehicle		Alphaxalone 15 mg/kg i.v.	
(msec)	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)	wt	β3(N265M)
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*

Table 1. Effects of pentobarbital on baseline ECG parameters. All values are mean±SEM. RR inter-beat-interval. Group sizes: pentobarbital: wt n=5, β3(N265M)n=7; alphaxalone i.v.: wt n=6, β3(N265M) n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to baseline, # $p<0.05$ wild type compared to β3(N265M) mice. If not indicated, the deviation from baseline or the genotype difference is statistically not significant.

Figure 1

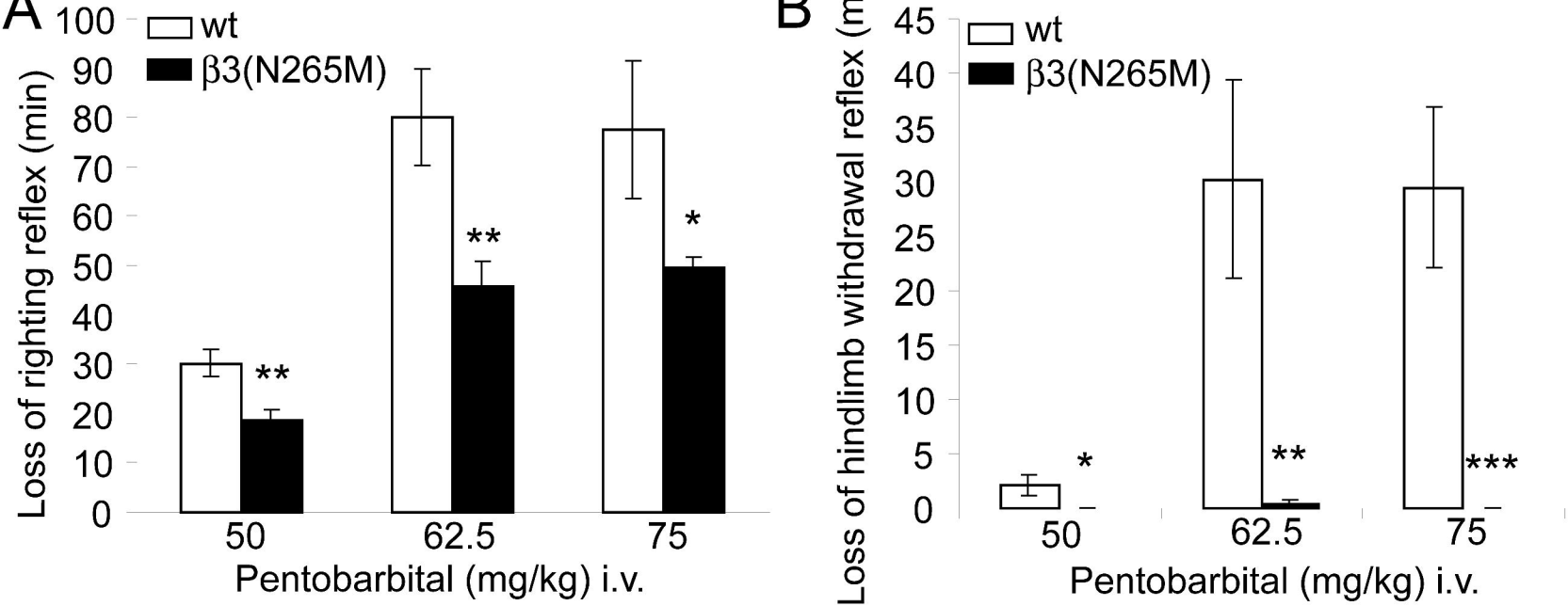


Figure 2

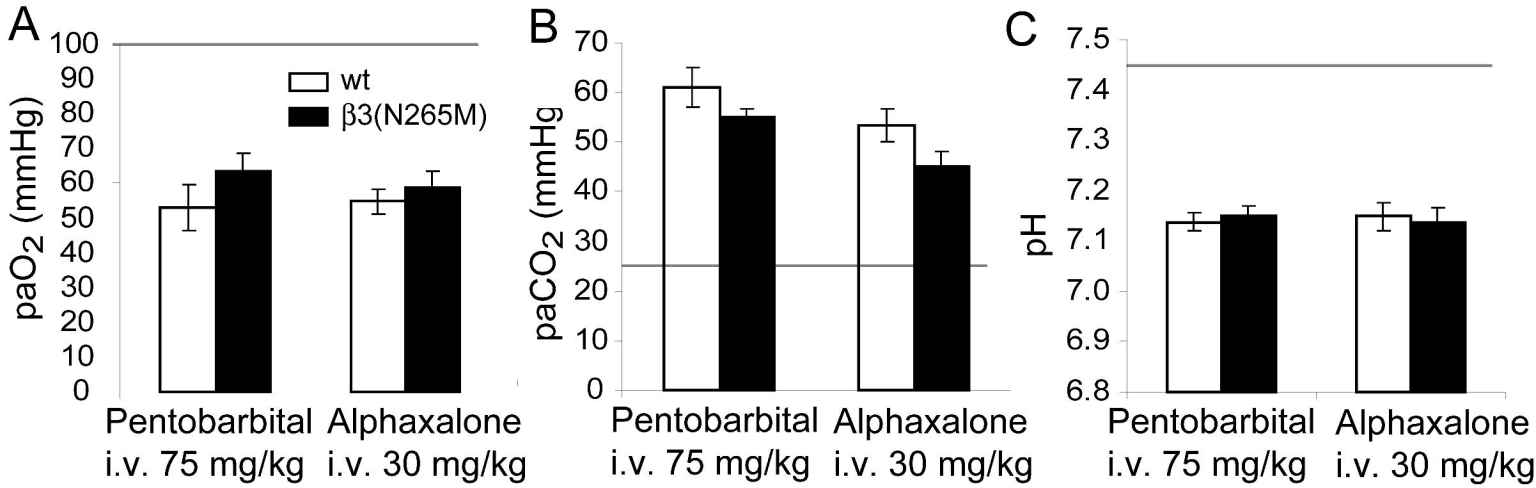
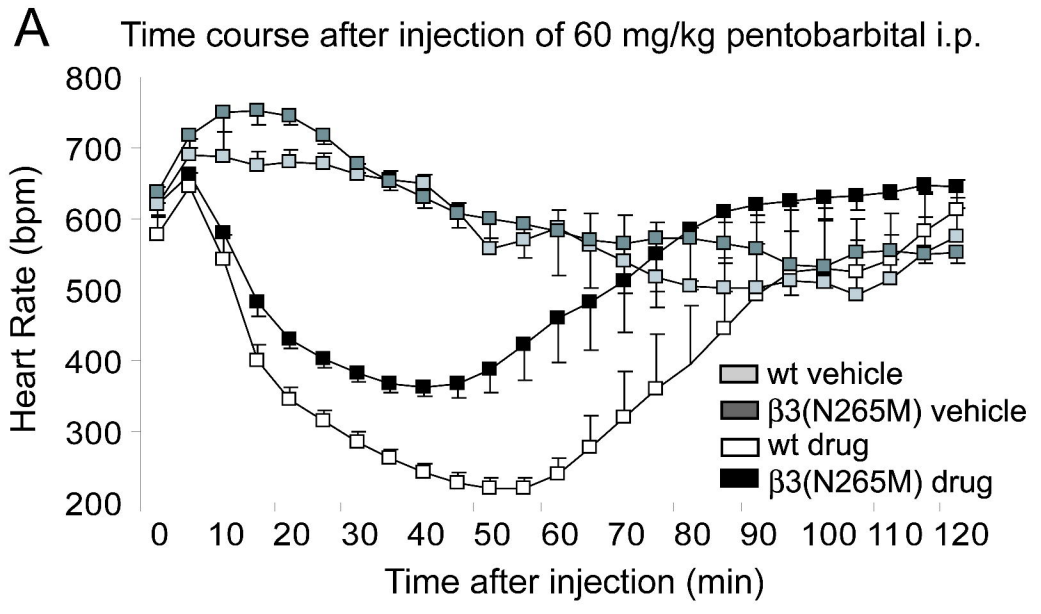

A
100
90
80
70
60
50
40
30
20
10
0
paO2 (mmHg)
wt
β3(N265M)
Pentobarbital i.v. 75 mg/kg
Alphaxalone i.v. 30 mg/kg
B
70
60
50
40
30
20
10
0
paCO2 (mmHg
Pentobarbital i.v. 75 mg/kg
Alphaxalone i.v. 30 mg/kg
C
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
pH
Pentobarbital i.v. 75 mg/kg
Alphaxalone i.v. 30 mg/kg

Figure 3


A
Time course after injection of 60 mg/kg pentobarbital i.p.
Heart Rate (bpm)
800
700
600
500
400
300
200
0 10 20 30 40 50 60 70 80 90 100 110 120
Time after injection (min)
wt vehicle
β3(N265M) vehicle
wt drug
β3(N265M) drug

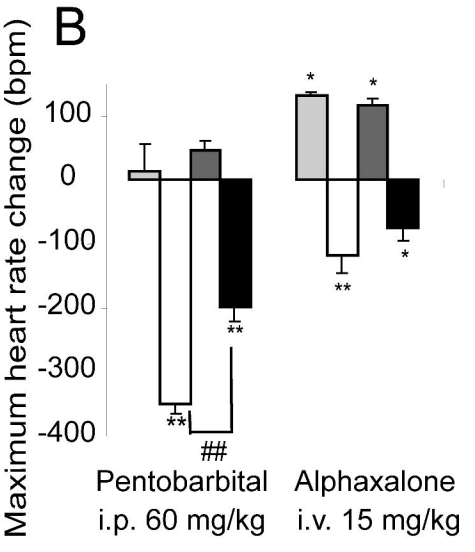

B
Maximum heart rate change (bpm)
100
0
-100
-200
-300
-400
**
**
##
*
**
*
*
Pentobarbital
i.p. 60 mg/kg
Alphaxalone
i.v. 15 mg/kg

Figure 4

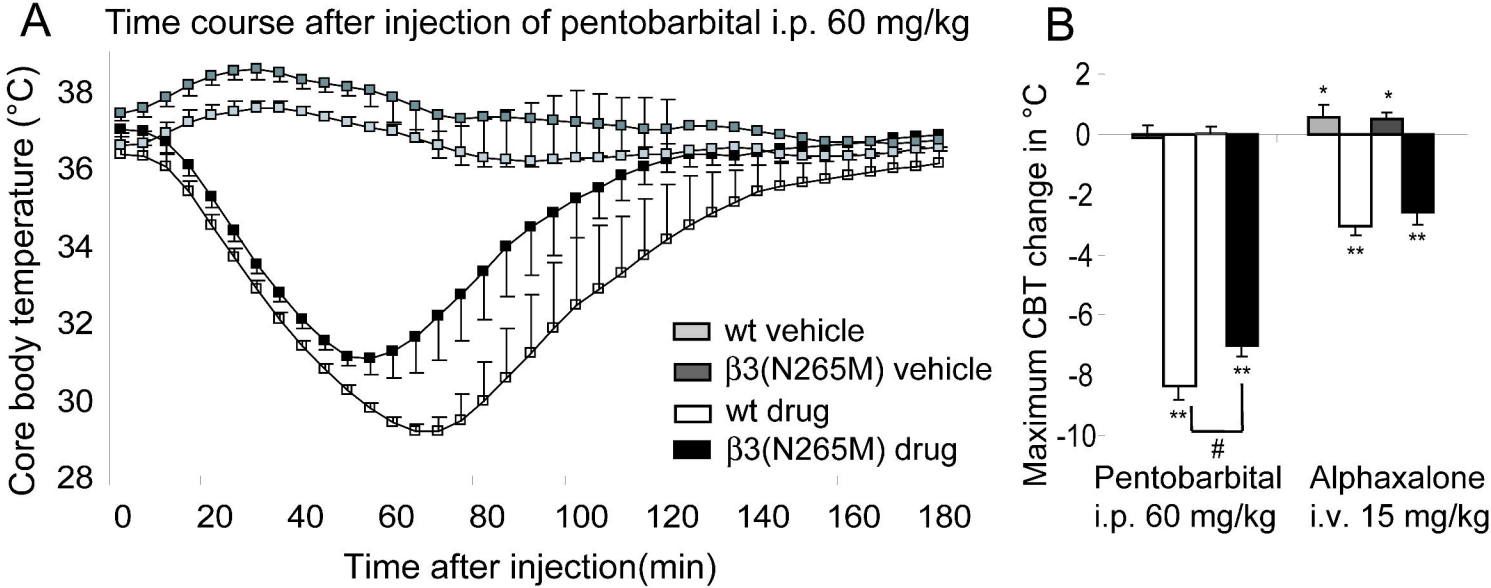
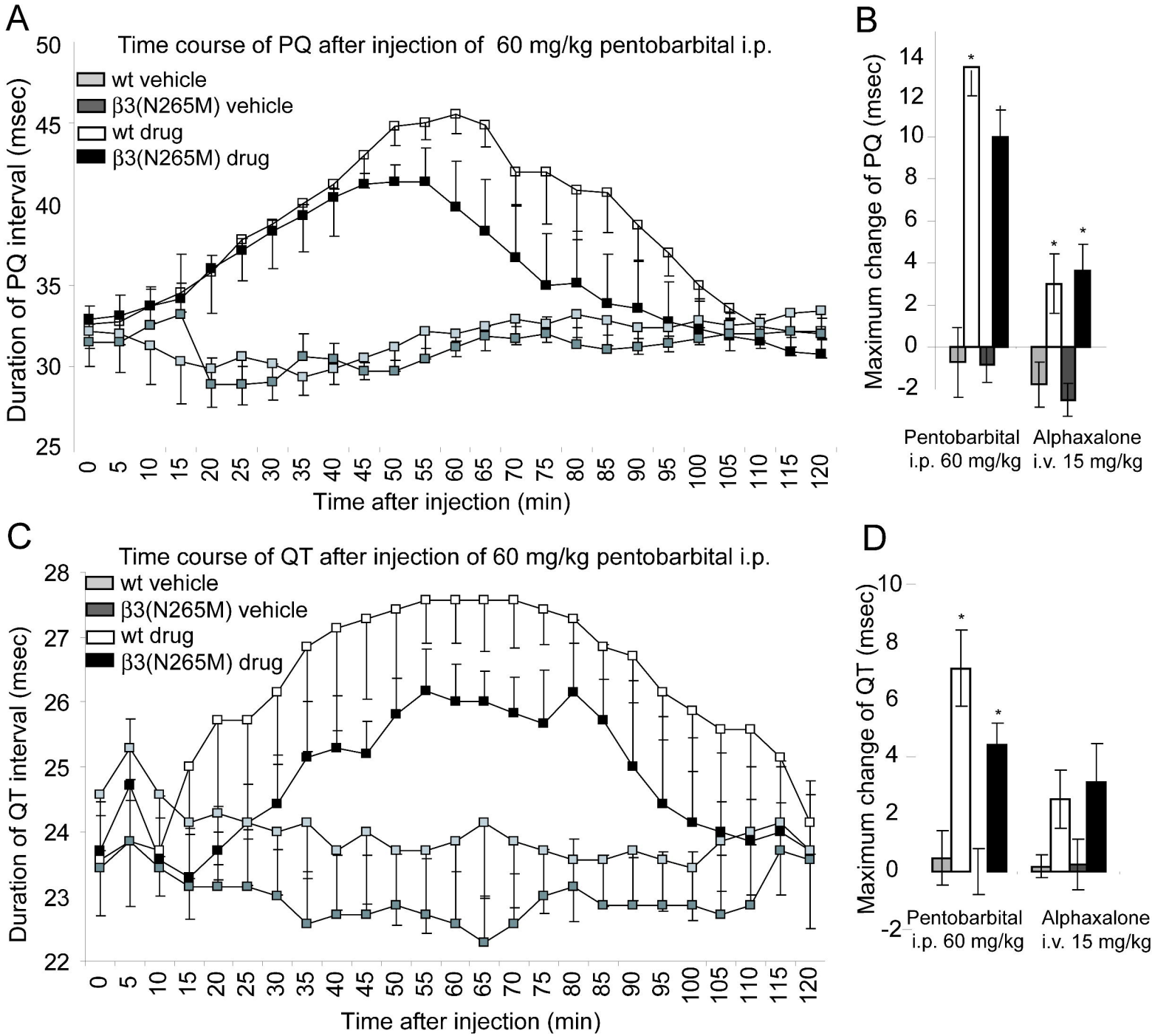

A
Time course after injection of pentobarbital i.p. 60 mg/kg
Core body temperature (°C)
38
36
34
32
30
28
0
20
40
60
80
100
120
140
160
180
Time after injection(min)
wt vehicle
β3(N265M) vehicle
wt drug
β3(N265M) drug
B
Maximum CBT change in °C
2
0
-2
-4
-6
-8
-10
*
**
#
Pentobarbital
i.p. 60 mg/kg
Alphaxalone
i.v. 15 mg/kg

Figure 5


A
Time course of PQ after injection of 60 mg/kg pentobarbital i.p.
wt vehicle
β3(N265M) vehicle
wt drug
β3(N265M) drug
Duration of PQ interval (msec)
Time after injection (min)
B
Maximum change of PQ (msec)
Pentobarbital i.p. 60 mg/kg
Alphaxalone i.v. 15 mg/kg
C
Time course of QT after injection of 60 mg/kg pentobarbital i.p.
wt vehicle
β3(N265M) vehicle
wt drug
β3(N265M) drug
Duration of QT interval (msec)
Time after injection (min)
D
Maximum change of QT (msec)
Pentobarbital i.p. 60 mg/kg
Alphaxalone i.v. 15 mg/kg