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Dual Actions of Enflurane on Postsynaptic Currents Abolished by the γ-Aminobutyric Acid Type A Receptor β_3(N265M) Point Mutation

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Background: **At concentrations close to 1 minimum alveolar concentration (MAC)-immobility, volatile anesthetics display blocking and prolonging effects on γ-aminobutyric acid type A receptor–mediated postsynaptic currents. It has been proposed that distinct molecular mechanisms underlie these dual actions. The authors investigated whether the blocking or the prolonging effect of enflurane is altered by a point mutation (N265M) in the β_3 subunit of the γ-aminobutyric acid type A receptor. Furthermore, the role of the β_3 subunit in producing the depressant actions of enflurane on neocortical neurons was elucidated.**

Methods: **Spontaneous inhibitory postsynaptic currents were sampled from neocortical neurons in cultured slices derived from wild-type and β_3(N265M) mutant mice. The effects of 0.3 and 0.6 mM enflurane on decay kinetics, peak amplitude, and charge transfer were quantified. Furthermore, the impact of enflurane-induced changes in spontaneous action potential firing was evaluated by extracellular recordings in slices from wild-type and mutant mice.**

Results: **In slices derived from wild-type mice, enflurane prolonged inhibitory postsynaptic current decays and decreased peak amplitudes. Both effects were almost absent in slices from β_3(N265M) mutant mice. At clinically relevant concentrations between MAC-awake and MAC-immobility, the anesthetic was less effective in depressing spontaneous action potential firing in slices from β_3(N265M) mutant mice compared with wild-type mice.**

Conclusion: **At concentrations between MAC-awake and MAC-immobility, β_3-containing γ-aminobutyric acid type A receptors contribute to the depressant actions of enflurane in the neocortex. The β_3(N265M) mutation affects both the prolonging and blocking effects of enflurane on γ-aminobutyric acid type A receptor–mediated inhibitory postsynaptic currents in neocortical neurons.**

MICE harboring an N265M point mutation in the β_3 subunit of the γ-aminobutyric acid type A ($GABA_A$) receptor are useful tools for investigating the role of this subunit in general anesthesia.[1,2] Recently, the effects of several volatile anesthetics have been examined in these mice to answer the question of whether the β_3 subunit is involved in mediating amnesia, hypnosis, or immobility.[3–5] Conclusions drawn from these behavioral studies are based on the assumption that, on the molecular level, the N265M mutation significantly attenuates the effects of volatile anesthetics at β_3-containing $GABA_A$ receptors. However, experimental evidence supporting this assumption is limited.

Siegwart *et al.*[6] reported that enflurane augments GABA-evoked currents in wild-type but significantly less in mutated $\alpha_2\beta_3\gamma_2$ recombinant receptors expressed in human embryonic kidney 293 cells. In this work, effects of enflurane were evaluated in the presence of 3 μM GABA in wild-type and 30 μM GABA in mutant receptors corresponding to EC_5 values. However, these findings do not predict how the mutation affects $GABA_A$ receptor–mediated synaptic transmission. At intact central synapses, the GABA concentration within the synaptic cleft rises into the millimolar range.[7] Furthermore, effects of volatile anesthetics at $GABA_A$ receptors substantially depend on the concentration of GABA. In the case that this concentration is approximately 1 mM, volatile anesthetics not only enhance $GABA_A$ receptor function but also induce receptor blockade.[8,9] Hapfelmeier *et al.*[10] and Neumahr *et al.*[11] studied the effects of isoflurane and sevoflurane on currents evoked by 1 mM GABA at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Using an ultrafast application method, they discovered that anesthetic-induced blockade of $GABA_A$ receptors results in a reduced peak current. At an intact central synapse, receptor blockade by volatile anesthetics decreases inhibitory postsynaptic current (IPSC) peak amplitudes, whereas potentiation of $GABA_A$ receptor function manifests as a prolongation of current decays. The latter effect increases $GABA_A$ receptor–mediated synaptic currents, whereas the former effect decreases synaptic currents. Because the potentiating action dominates, the overall effect of volatile anesthetics is enhancement of $GABA_A$ receptor function. Banks and Pearce[9] have carefully analyzed these dual actions. The concentration–response curves revealed a dissociation of the effects on the amplitude and time course of IPSCs, suggesting the involvement of distinct molecular mechanisms. This conclusion is corroborated by the recent finding that a point mutation in the α subunit of the $GABA_A$ receptor abolishes the enhancing but not blocking effect of isoflurane in a recombinant system.[12]

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Taken together, these observations raise the question of whether the β_3(N265M) mutation exclusively modulates the potentiating action or both the blocking and potentiating actions of volatile anesthetics. So far, experimental data demonstrating how volatile anesthetics modulate GABAergic synaptic transmission at intact synapses in preparations derived from β_3(N265M) mutant mice are absent. In the case that the mutation exclusively alters anesthetic-induced prolongation of IPSC decay times, while leaving the blocking action unchanged, the mutation should turn the overall potentiating effect of volatile anesthetics into a blocking one. This hypothetical mechanism would greatly complicate the interpretation of data originating from comparative behavioral studies on wild-type and β_3(N265M) mutant mice.[3–5]

In the current work, we first explore the effects of enflurane on $GABA_A$ receptor–mediated IPSCs recorded from neurons in cultured neocortical brain slices, derived from wild-type and β_3(N265M) mutant mice. Enflurane was chosen for this investigation because its dual actions on $GABA_A$ receptors have been characterized in great detail previously.[9,13] In a previous study, the action of enflurane on spontaneous action potential firing of neocortical neurons in brain slices from wild-type and β_3(N265M) mutant mice has been examined only for one single small concentration.[3] Therefore, here we compare the effects of this volatile anesthetic over a variety of concentrations to test whether β_3-containing $GABA_A$ receptors are involved in the depressant effects of volatile anesthetics on neocortical neurons reported recently.[14]

Materials and Methods

Animals

Mice of both sexes homozygous for an asparagine to methionine point mutation at position 265 of the $GABA_A$ receptor β_3 subunit (N265M) and homozygous wild-type controls on the same genetic background as described previously (statistically 87.5% 129/SvJ, 12.5% 129/Sv) were used for this study.[3] All procedures were approved by the animal care committee (Eberhard-Karls-University, Tuebingen, Germany) and were in accordance with German law on animal experimentation.

Organotypic Slice Cultures

Neocortical slice cultures were prepared from 2- to 5-day-old mice as described by Gähwiler *et al.*[15,16] In brief, for the preparation of somatosensory cortex, animals were deeply anesthetized with halothane and decapitated. Cortical hemispheres were aseptically removed and stored in ice-cold Gey solution. After removal of the meninges, 300-μm-thick coronal slices were cut. Slices were transferred onto clean glass coverslips and embedded in a plasma clot. The coverslips were transferred into plastic tubes (Nunc, Roskilde, Denmark) containing 750 μl nutrition medium and incubated in a roller drum at 37°C. After 1 day in culture, antimitotics were added. The suspension and the antimitotics were renewed twice a week. Cultures were used after 2 weeks *in vitro*.

Preparation and Application of Test Solutions

Test solutions were prepared by dissolving enflurane in artificial cerebrospinal fluid (ACSF) to yield the desired concentration as described previously.[13] A closed, air-free system was used to prevent evaporation of the anesthetic.

Enflurane (Abbott, Wiesbaden, Germany) was applied *via* bath perfusion using syringe pumps (ZAK, Marktheidenfeld, Germany), connected to the experimental chamber *via* Teflon tubing (Lee, Frankfurt, Germany). The flow rate was approximately 1 ml/min. When switching from ACSF to drug-containing solutions, the medium in the experimental chamber was replaced by at least 95% within 2 min. Effects of the anesthetic were stable approximately 5 min later. To ensure steady state conditions, recordings during anesthetic treatment were performed 10 min after commencing the change of the perfusate.

Electrophysiology

Extracellular network recordings were performed in a recording chamber mounted on an inverted microscope. Slices were perfused with ACSF consisting of 120 mM NaCl, 3.3 mM KCl, 1.13 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 11 mM glucose. ACSF was bubbled with 95% oxygen and 5% carbon dioxide. ACSF-filled glass electrodes with a resistance of approximately 3–5 MΩ were positioned on the surface of the slices and advanced into the tissue until extracellular spikes exceeding 100 μV in amplitude were visible. All experiments were conducted at 34°–36°C. Data were acquired on a personal computer with the Digidata 1200 AD/DA interface and Axoscope 9 software (Axon Instruments, Foster City, CA).

Whole cell patch clamp recordings were performed with an EPC 7 amplifier (List, Darmstadt, Germany) at room temperature. Extracellular medium consisted of 120 mM NaCl, 3.3 mM KCl, 1.13 mM NaH_2PO_4, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 11 mM glucose. To block glutamatergic currents, 6-cyano-7nitroquinoxaline-2,3-dione and DL-2-amino-5-phosphonopentanoic acid (50 μm each) were added to the extracellular medium. Cells in neocortical slices were identified as pyramidal neurons according to their morphologic appearance on a television monitor using infrared illumination and a 40× water immersion objective. Patch pipettes were fabricated from borosilicate glass using a P-2000 laser puller (Sutter Instruments, Novato, CA), fire

polished, and coated with Sylgard (Dow Corning, Seneffe, Belgium) to reduce electrode capacitance. When filled with recording solution containing 145 mM CsCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, and 4 mM ATP at pH 7.2, patch pipettes had a resistance of 1.5–3.5 MΩ. Neurons were held at −70 mV. IPSCs were sampled at 10 kHz with the Digidata 1200 AD/DA interface and Clampex 9.0 software (Axon Instruments) for a recording period of 180 s.

Data Analysis

Extracellular recorded spikes were counted offline using custom routines in OriginPro7 (OriginLab Corporation, Northampton, MA). The mean of spikes occurring during a recording period of 180 s was used as the average spike rate.

Inhibitory postsynaptic current data were analyzed offline using self-written programs in OriginPro7. Spontaneous events were counted using an automated event detection algorithm. Events were discarded if the next event occurred during the decay tail ("stacked" events). Decay phases were best fit by two exponential components, using a biexponential equation in the form

$$I(t) = Af \exp(-t/\tau fast) + As \exp(-t/\tau slow) + c,$$

where I(t) is the current amplitude at any given time t; c is the baseline current; τfast and τslow are the fast and slow time constants of current decay, respectively; and Af and As are the estimated fast and slow intercepts of the components at time zero.[17] Drug-induced changes in the net charge transferred during $GABA_A$ receptor–mediated synaptic events were estimated by calculating the area under the curve of averaged IPSCs. Furthermore, the frequency and amplitudes of IPSCs were evaluated in the absence and presence of the anesthetic.

We used the Student *t* test for statistical testing. *P* values less than 0.05 were considered significant. All results are given as mean ± SEM.

Results

Spontaneous Inhibitory Postsynaptic Currents from Wild-type and β_3(N265M) Mutant Mice Do Not Differ under Control Conditions

We sampled spontaneous IPSCs from pyramidal cells in cultured brain slices derived from the somatosensory cortex of wild-type and β_3(N265M) mutant mice by performing whole cell patch clamp recordings in the voltage clamp mode. Spontaneous IPSCs were characterized by frequency of occurrence, amplitude, the rapid and slow phases of current decay, and net charge transfer. IPSC characteristics as obtained in the absence of enflurane are summarized in figure 1. The frequency of occurrence was 2.01 ± 0.14 Hz (n = 44) in the wild-type slices and 1.91 ± 0.16 Hz (n = 33) in the β_3(N265M) mutant slices. These mean values were not statistically different. Similarly, peak amplitudes of averaged IPSCs did not differ between slices from wild-type (61.34 ± 3.25 pA, n = 43) and β_3(N265M) mutant mice (60.15 ± 3.79 pA, n = 32). Current decays were fitted by two time constants (τfast and τslow) using a biexponential model. The fast component of the decay time was 10.81 ± 0.63 ms (n = 41, 73.5 ± 2.2% of amplitude) in the wild-type and 10.17 ± 0.60 ms (n = 33, 75.7 ± 3.4% of amplitude) in β_3(N265M) mutant slices. Regarding the slow phase of the current decay, the β_3(N265M) mutant showed

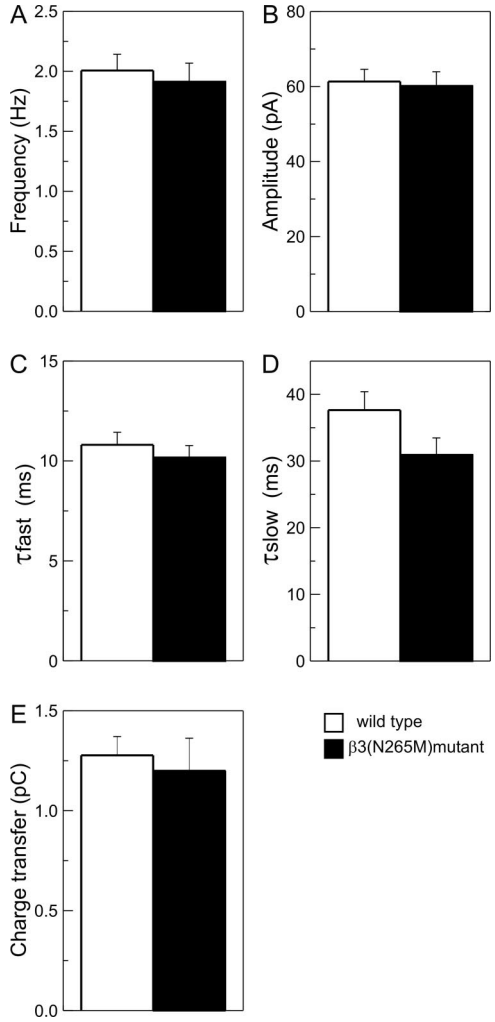

Fig. 1. Characteristics of spontaneous inhibitory postsynaptic potentials (IPSCs) in cultured neocortical neurons of wild-type (WT, *white*) and β_3(N265M) mutant (MU, *black*) mice as obtained under control conditions. There are no significant differences between the two preparations regarding (*A*) frequency (WT 2.01 ± 0.14 Hz, n = 44, and MU 1.91 ± 0.16 Hz, n = 33; *P* > 0.5), (*B*) amplitude of the mean IPSC (WT 61.34 ± 3.25 pA, n = 43, and MU 60.15 ± 3.79 pA, n = 32; *P* > 0.5), (*C*) fast component of the current decay (τfast; WT 10.81 ± 0.63 ms, n = 41, and MU 10.17 ± 0.60 ms, n = 33; *P* > 0.1), (*D*) slow component of the current decay (τslow; WT 37.65 ± 2.76 ms, n = 42, and MU 30.92 ± 2.55 ms, n = 32; *P* > 0.05), and (*E*) the charge transfer, measured as the area under the curve of averaged IPSCs (WT 1.28 ± 0.09 pC, n = 41, and MU 1.20 ± 0.16 pC, n = 34; *P* > 0.5).

smaller values (30.92 ± 2.55 ms, n = 32) than the wild type (37.65 ± 2.76 ms, n = 42). However, this difference did not reach statistical significance. Previous investigators have reported similar values for neocortical pyramidal neurons.[18] Finally, we calculated the area under the curve as a measure of charge transfer. These values were also not different between wild-type (1.28 ± 0.09 pC, n = 41) and β_3(N265M) mutant (1.20 ± 0.16 pC, n = 34) mice. In summary, IPSC parameters did not differ between wild-type and β_3(N265M) mutant mice in the absence of enflurane.

Different Actions of Enflurane on Spontaneous Inhibitory Postsynaptic Currents in Wild-type and β_3(N265M) Mutant Neurons at Concentrations Below 1 MAC

Representative recordings of spontaneous IPSCs in slices from wild-type mice, performed in the absence and presence of enflurane (0.6 mM), are shown in figure 2A. The anesthetic reduced IPSC amplitudes and prolonged current decays. In figures 2B–D, averaged IPSCs are displayed. The current decay was prolonged by 0.3 mM enflurane, and the amplitude was slightly decreased (fig. 2C). Both effects were stronger in the presence of 0.6 mM enflurane (fig. 2D).

In figure 2E, representative traces obtained from experiments on β_3(N265M) mutant slices are presented. Enflurane at 0.3 mM did not attenuate the amplitude of the averaged IPSC, and the current decay was only marginally prolonged (figs. 2F–G). The latter effect was enhanced at 0.6 mM enflurane but still less pronounced compared with the IPSCs monitored in slices from wild-type mice (fig. 2H). Even at this high concentration, enflurane did not reduce the amplitude of the mean IPSC in the β_3(N265M) mutant.

Figure 3 summarizes the effects of enflurane on IPSCs monitored in wild-type and β_3(N265M) mutant slices. The effects of the anesthetic on the amplitudes of averaged IPSCs are displayed in figure 3A: At 0.3 mM, enflurane did not change the IPSC amplitudes significantly, but there was a trend to smaller values in the wild type (50.08 ± 3.97 pA, n = 14) compared with mutant (63.72 ± 6.24 pA, n = 9). At 0.6 mM enflurane, however, the amplitude of the averaged IPSC was significantly reduced in the wild type (37.15 ± 2.64 pA, n = 13), whereas no such effect could be observed in the β_3(N265M) mutant (66.59 ± 6.35 pA, n = 7). Figure 3B shows the actions of enflurane on the rapid phase of the current decay (τfast). This parameter was prolonged by enflurane in a concentration dependent manner in the wild type (0.3 mM enflurane 17.42 ± 1.07 ms, n = 14; 0.6 mM enflurane 23.49 ± 2.26 ms, n = 12) but not in the β_3(N265M) mutant (0.3 mM enflurane 9.59 ± 0.42 ms, n = 8; 0.6 mM enflurane 12.36 ± 0.96 ms, n = 8). Prolongation of τfast in the wild-type was significantly different from the β_3(N265M) mutant and from control conditions. The corresponding effects of enflurane on the slow phase of the current decay (τslow) are presented in figure 3C. Again, enflurane led to longer decay times in the wild type but not in the β_3(N265M) mutant. In the wild type, enflurane (0.3 mM) increased τslow to 66.14 ± 3.87 ms (n = 14), an effect that was further enhanced at 0.6 mM enflurane to 117.45 ± 12.86 ms (n = 12). In contrast, in the β_3(N265M) mutant, there was only a minor prolongation to 37.16 ± 4.66 ms (n = 8) at 0.3 mM enflurane and to 35.74 ± 3.74 ms (n = 8) at 0.6 mM.

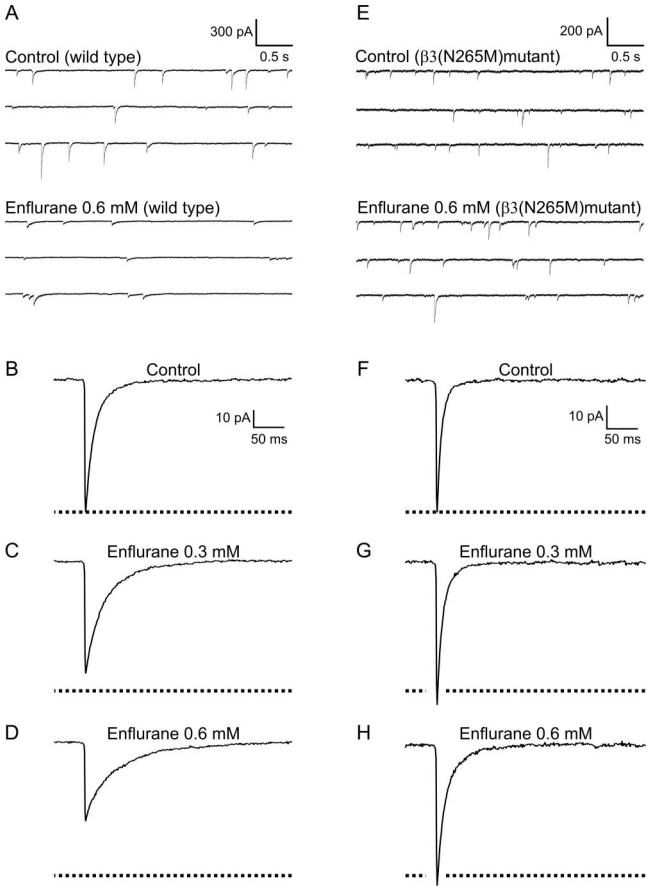

Fig. 2. Effects of enflurane on spontaneous inhibitory postsynaptic currents (IPSCs) recorded from neocortical neurons of wild-type and β_3(N265M) mutant mice. (*A–D*) Data obtained from wild-type mice. (*A*) Continuous recordings (12 s) under control conditions (*upper three traces*) and in the presence of 0.6 mM enflurane (*bottom three traces*). (*B*) Mean IPSC calculated from synaptic events occurring during a 180-s recording under control conditions. (*C*) In the presence of 0.3 mM enflurane (approximately 0.5 minimum alveolar concentration [MAC]), the amplitude of the averaged IPSC is only slightly reduced, whereas the decay time is prolonged. (*D*) These dual actions of enflurane are further enhanced at 0.6 mM enflurane (approximately 1 MAC). (*E–H*) Actions of enflurane on IPSCs recorded from β_3(N265M) mutant mice neurons. (*E*) Representative trace (12 s) in the absence (*upper three traces*) and in the presence of 0.6 mM enflurane (*bottom three traces*). (*F*) Mean IPSC obtained from a 180-s recording under control conditions. (*G*) In the presence of 0.3 mM enflurane, amplitude and current decay of the averaged IPSC are almost unchanged. (*H*) At 0.6 mM enflurane (approximately 1 MAC), the amplitude of the mean IPSC remains unchanged, whereas the decay time is only moderately prolonged in the β_3(N265M) mutant neuron.

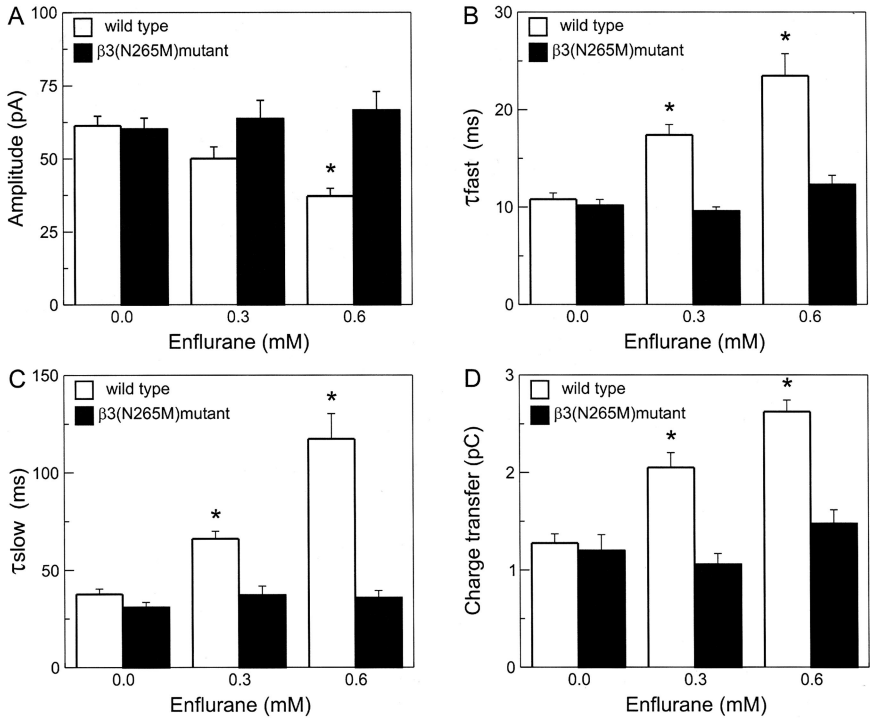

Fig. 3. Enflurane-induced changes in characteristics of inhibitory postsynaptic currents calculated from recordings on wild-type (*white*) and β_3(N265M) mutant mice (*black*) neocortical neurons. (*A*) Enflurane (0.6 mM) causes a significant reduction of the amplitude of the mean inhibitory postsynaptic current in wild-type ($P < 0.05$) but not in β_3(N265M) mutant mice neurons. (*B*) Enflurane prolongs the fast component of the decay time (τfast) in a dose-dependent manner in the wild-type ($P < 0.05$). In the β_3(N265M) mutant, however, the values in the presence of 0.3 and 0.6 mM enflurane are not significantly different from control condition. (*C*) Regarding the slow component of the current decay (τslow), enflurane again only causes a significant prolongation in the wild-type ($P < 0.05$) but not in neurons from β_3(N265M) mutant mice. (*D*) In wild-type neurons, enflurane enhances the charge transfer in a dose-dependent manner ($P < 0.05$), whereas there is only a slight increase in the presence of 0.6 mM enflurane in the β_3(N265M) mutant. * $P < 0.05$ compared to control condition and to β_3(N265M) mutant.

Again, the effect of enflurane on the wild-type preparation was significantly different from β_3(N265M) mutant preparations and from control conditions.

To draw conclusions about the overall effect of enflurane on IPSCs, we calculated the net charge transferred by averaged IPSCs (fig. 3D). In wild-type preparations, this parameter was increased by enflurane in a concentration-dependent manner (0.3 mM enflurane 2.05 ± 0.15 pC, n = 13; 0.6 mM enflurane 2.62 ± 0.12 pC, n = 12). In the β_3(N265M) mutant, however, only a small and nonsignificant effect was observed (0.3 mM enflurane 1.06 ± 0.11 pC, n = 9; 0.6 mM enflurane 1.48 ± 0.14 pC, n = 7). The net charge transferred during IPSCs as determined in the presence of enflurane in wild-type slices was significantly different from control conditions as well as from the charge transferred in β_3(N265M) mutant slices in the presence of the anesthetic.

The frequency of spontaneous IPSCs was decreased by enflurane in a concentration-dependent manner in both preparations (table 1), but no significant difference was observed between wild-type and β_3(N265M) mutant slices.

At Sedative and Hypnotic Concentrations Enflurane Exerts Stronger Depression of Spontaneous Action Potential Firing in Wild-type Than in β_3(N265M) Mutant Neocortical Slices

The effects of enflurane on spontaneous action potential firing of neocortical neurons were investigated. As in our previous studies, spontaneous neuronal activity was reinforced by removing Mg^{2+} ions from the extracellular solution.[19] Under control conditions, action potential firing occurred typically in bursts (episode of ongoing activity). In slices from wild-type mice, episodes of ongoing activity showed a frequency of 0.16 ± 0.01 Hz (n = 51). In β_3(N265M) mutant cultures, this frequency was 0.15 ± 0.01 Hz (n = 53). These mean values were not statistically different and lie in the range of previously reported values.[19] Mean action potential rates were slightly higher in slices from wild-type than in β_3(N265M) mutant preparations (13.62 ± 2.03 *vs.* 9.93 ± 0.86 Hz, n = 65/64), but this did not reach statistical significance. In summary, activity patterns monitored in slices from wild-type and β_3(N265M) mutant mice did not differ under control conditions.

Table 1. Effects of Enflurane on IPSC Frequency

IPSC Frequency	Wild Type	β_3(N265M) Mutant
Control	2.01 ± 0.14 Hz (n = 44)	1.91 ± 0.16 Hz (n = 33)
0.3 mM enflurane	1.16 ± 0.18 Hz (n = 13)*	1.28 ± 0.22 Hz (n = 9)
0.6 mM enflurane	0.94 ± 0.16 Hz (n = 12)*	0.93 ± 0.18 Hz (n = 7)*

Frequency of spontaneous inhibitory postsynaptic currents (IPSCs) monitored in wild-type and in β_3(N265M) mutant cortical slice cultures.

* $P < 0.05$ compared with control condition.

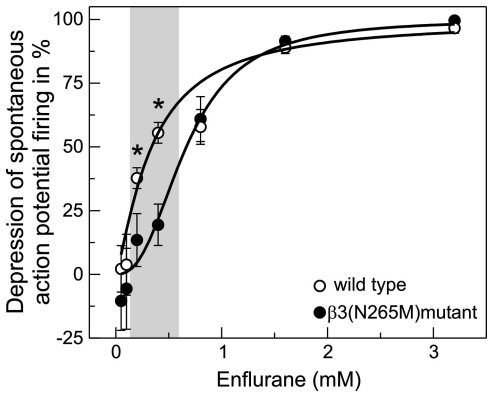

Fig. 4. Concentration-dependent depression of spontaneous action potential firing in cultured neocortical slices by enflurane. The anesthetic reduces action potential activity in both wild-type (*white*) and β_3(N265M) mutant mice (*black*) preparations. At concentrations between minimum alveolar concentration (MAC)-awake and MAC-immobility (*light gray rectangle*), this depression is significantly smaller in the β_3(N265M) mutant. The curves were fitted with Hill equations. The calculated median effective concentrations (EC_{50} values) were 0.34 mM for the wild-type and 0.67 mM for β_3(N265M) mutant mice preparations. * $P < 0.05$ compared to β_3(N265M) mutant.

Enflurane depressed spontaneous action potential firing in a concentration-dependent manner in cultures from both wild-type and β_3(N265M) mutant mice (fig. 4). At concentrations of 0.2 and 0.4 mM, enflurane depressed action potential firing in the wild type to a significantly greater extent than in the β_3(N265M) mutant (0.2 mM enflurane: WT 37.75 ± 4.06%, n = 25, MU 13.45 ± 10.4%, n = 18; 0.4 mM enflurane: WT 55.53 ± 4.08%, n = 20, MU 19.44 ± 8.13%, n = 15). However, at higher concentrations, enflurane was similarly effective in slices from wild-type and β_3(N265M) mutant mice.

Discussion

Characteristics of Spontaneous IPSCs under Drug-free Conditions

In recombinant $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors expressed in human embryonic kidney 293 cells, the β_3(N265M) mutation may alter channel gating, agonist binding, or both. Support for the latter conclusion is derived from the finding that EC_{50} values for activation of $\alpha_2\beta_3\gamma_2$ receptors by GABA were 47 μM in wild-type and 122 μM in $\alpha_2\beta_3$(N265M)γ_2 receptors.[6] This prompts the question of whether the change in GABA efficacy, introduced by the mutation, is mirrored in the time course of IPSCs. In our study, IPSCs from wild-type and β_3(N265M) mutant mice did not differ under drug-free conditions regarding frequency, amplitude, current decay, and charge transfer (fig. 1). This observation is based on 44 recordings in neocortical slices prepared from wild-type and 33 recordings in slices from β_3(N265M) mutant mice. By monitoring from a single neuron, 200–300 IPSCs were sampled and used for further analysis. The finding that under drug-free conditions IPSCs did not differ in slices from wild-type and mutant mice implies that $GABA_A$ receptors involved in these IPSCs were exposed to almost saturating agonist concentrations.[7] At first glance, this explanation seems to be at variance with the data published by Liao *et al.*[4] These authors report that in the absence of anesthetics, β_3(N265M) mutant mice display less freezing in a pavlovian fear conditioning paradigm compared with wild-type mice. One of a number of possibilities to explain this difference is that extrasynaptic $GABA_A$ receptors in the hippocampus, cerebellum, or neocortex may be playing an important role in such a behavioral outcome. Because of their location, these extrasynaptic receptors are exposed to GABA concentrations much lower than in the synaptic cleft,[7,20] possibly close to the EC_{50} values cited above.

Actions of Enflurane on Spontaneous IPSCs

When recording miniature IPSCs from hippocampal pyramidal cells, Banks and Pearce[9] observed that at concentrations close to 1 MAC-immobility (0.58 mM) enflurane depressed IPSC amplitudes by 40% and increased the time constant of current decay by 3.5-fold. These findings are consistent with results obtained with cerebellar Purkinje cells, which express almost exclusively $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_2$ subunits.[13] In the latter study, enflurane (0.64 mM) attenuated amplitudes of miniature and spontaneous IPSCs by 55% and increased current decay times by 5.1-fold. This indicates that these $GABA_A$ receptors are also sensitive to enflurane and that the blocking effects are not subtype specific.

In the current work, effects of enflurane on spontaneous IPSCs recorded from neocortical neurons in brain slices prepared from wild-type mice were comparable with the results mentioned above. At 0.6 mM, IPSC amplitudes were decreased by 39%, and decay times were prolonged by 117% (τfast) and 211% (τslow), respectively.

How is this blocking and potentiating action of enflurane altered by the β_3(N265M) mutation? The results summarized in figure 3 indicate that the mutation eliminates both effects. This stands in contrast to the S270H mutation, which was introduced in the α_1 subunit of recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, which exclusively affects the potentiating action of the volatile anesthetic isoflurane, leaving anesthetic-induced receptor blockade unaltered.[12]

Because the β_3(N265M) mutation abolished both (*i.e.*, the prolongation of current decay and the blocking action of enflurane), the different effects of the anesthetic on spontaneous action potential activity monitored in slices from wild-type and β_3(N265M) mutant mice can be explained as follows: The weaker depression by enflurane in the β_3(N265M) mutant at concentrations between MAC-awake and MAC-immobility (fig. 4) does not involve blockade of mutated $GABA_A$ receptors but is

produced by the lack of an effect of enflurane on mutated β_3-containing $GABA_A$ receptors in this concentration range.

How can we explain that the β_3(N265M) mutation abolishes both the blocking and potentiating effects of enflurane? One possibility is that both potentiation and inhibition arise from occupation of a molecular site that is disrupted by the mutation. Another possibility is that the mutation is distant from the enflurane binding site but produces a conformational change, thereby affecting both the prolonging and blocking action of the anesthetic.

The finding that the effects of enflurane on IPSCs were largely abolished by the β_3(N265M) mutation indicates that a major fraction of IPSCs represented currents carried by β_3-containing $GABA_A$ receptors. This was unexpected, because only approximately 15–20% of all $GABA_A$ receptors in the neocortex contain β_3 subunits.[21] However, neocortical pyramidal cells are innervated by different classes of GABAergic interneurons.[22] Furthermore, specific synaptic contacts, as defined by the identity of the presynaptic cell, frequently show specific $GABA_A$ receptor subtypes. For example, α_1 subunits, which most commonly coassemble with β_2 subunits, are enriched at synapses between fast spiking basket cells and pyramidal cells.[23] In contrast, α_2 subunits, which frequently coassemble with β_3 subunits, are enriched at synapses between cholecystokinin-positive basket cells and pyramidal cells. It seems unlikely that under our recording conditions (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and *N*-methyl-D-aspartate receptors were blocked to isolate $GABA_A$ receptor–mediated events) fast spiking basket cells were active, because these neurons exhibit a rather negative membrane resting potential.[24] Therefore, IPSCs monitored in the course of our experiments may predominantly originate from a yet unidentified subpopulation of interneurons, namely those displaying the most positive membrane resting potential.

At 0.6 mM, we observed that enflurane decreased the frequency of spontaneous IPSCs by approximately 30%. This effect probably results from a decrease in spontaneous action potential firing of presynaptic neurons, because at the concentrations tested in this study, the anesthetic does not depress action potential–independent GABA release.[9] Because GABAergic interneurons express $GABA_A$ receptors, these may also have been the mediators of enflurane-induced depression of presynaptic activity.[25] Interestingly, attenuation of presynaptic activity by enflurane did not differ between wild-type and β_3(N265M) mutant mice. This finding is consistent with the observation that β_3 subunits are mostly expressed in pyramidal cells and not in GABAergic interneurons.[26]

Are β_3-Containing $GABA_A$ Receptors in the Neocortex Relevant Targets for Volatile Anesthetics?

By combining *in vivo* and *in vitro* recordings, Hentschke *et al.*[14] have recently identified the neocortex as a major target of subhypnotic concentrations of enflurane. The authors showed that concentration-dependent depression of spontaneous action potential firing of neurons in the somatosensory cortex caused by enflurane is almost identical *in vivo* and in isolated cortical brain slices, indicating a minor contribution of subcortical structures. In the same study, $GABA_A$ receptors, located on cortical pyramidal cells, were identified as a prime target of the anesthetic.

Here we report that at clinically relevant concentrations between MAC-awake and MAC-immobility, enflurane depresses spontaneous action potential firing in cortical slices from β_3(N265M) mutant mice to a lesser extent than in slices from wild-type mice, suggesting that β_3-containing $GABA_A$ receptors significantly contribute to this effect.

However, β_3-containing $GABA_A$ receptors are not the exclusive molecular target of enflurane, because at concentrations exceeding MAC-immobility, full suppression of neuronal activity also occurred in slices from β_3(N265M) mutant mice.

How do these findings on cortical neurons *in vitro* relate to the observation that enflurane concentrations causing loss of righting reflexes do not differ in wild-type and β_3(N265M) mutant mice?[5] The latter result clearly indicates that ablation of righting reflexes does not involve effects of enflurane on neocortical neurons. Righting reflexes are mediated by brainstem centers including the vestibular nuclei in the medulla and pontine reticular nuclei, integrating mechanoreceptor input into motor commands. In fact, focal application of barbiturates into the brainstem mesopontine tegmentum ablates the righting reflex.[27,28] Furthermore, discrete injections of $GABA_A$ receptor agonists in the tuberomammillary nucleus produce loss of righting reflexes.[29] Therefore, enflurane possibly abolishes righting reflexes *via* these routes and not *via* depressing neocortical neurons. In addition, the data discussed so far suggest that the molecular targets by which enflurane depresses cortical neurons and abolishes righting reflexes are distinct. Assuming that righting reflexes involve anesthetic actions in brainstem nuclei, but not in neocortex, enflurane-sensitive ion channels in the neocortex and brainstem should be different molecular entities. What channels are abundant in only one of these brain regions? Strychnine-sensitive glycine receptors are expressed in the brainstem, but not in neocortical networks.[30] Glycine and $GABA_A$ receptors are equally sensitive to volatile anesthetics.[31] Therefore, we speculate that enflurane ablates righting reflexes largely *via* molecular targets located in subcortical structures. These may include gly-

cine receptors, non-β_3 subunit-containing $GABA_A$ receptors, and possibly yet unidentified molecular targets.

Taken together, our results provide further evidence for the hypothesis that the sedative and hypnotic properties of anesthetic agents are related to drug actions in different parts of the brain.[2] While sedation involves targets in the neocortex, hypnosis, as defined by the absence of righting reflexes, seems to be mediated predominantly by subcortical structures.

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