Cellular/Molecular

Extrasynaptic $GABA_A$ Receptors of Thalamocortical Neurons: A Molecular Target for Hypnotics

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Among hypnotic agents that enhance $GABA_A$ receptor function, etomidate is unusual because it is selective for β_2/β_3 compared with β_1 subunit-containing $GABA_A$ receptors. Mice incorporating an etomidate-insensitive β_2 subunit (β_{2N265S}) revealed that β_2 subunit-containing receptors mediate the enhancement of slow-wave activity (SWA) by etomidate, are required for the sedative, and contribute to the hypnotic actions of this anesthetic. Although the anatomical location of the β_2-containing receptors that mediate these actions is unknown, the thalamus is implicated.

We have characterized $GABA_A$ receptor-mediated neurotransmission in thalamic nucleus reticularis (nRT) and ventrobasalis complex (VB) neurons of wild-type, $\beta_2^{-/-}$, and β_{2N265S} mice. VB but not nRT neurons exhibit a large GABA-mediated tonic conductance that contributes ~80% of the total $GABA_A$ receptor-mediated transmission. Consequently, although etomidate enhances inhibition in both neuronal types, the effect of this anesthetic on the tonic conductance of VB neurons is dominant. The GABA-enhancing actions of etomidate in VB but not nRT neurons are greatly suppressed by the β_{2N265S} mutation. The hypnotic THIP (Gaboxadol) induces SWA and at low, clinically relevant concentrations (30 nM to 3 μM) increases the tonic conductance of VB neurons, with no effect on VB or nRT miniature IPSCs (mIPSCs) or on the holding current of nRT neurons. Zolpidem, which has no effect on SWA, prolongs VB mIPSCs but is ineffective on the phasic and tonic conductance of nRT and VB neurons, respectively. Collectively, these findings suggest that enhancement of extrasynaptic inhibition in the thalamus may contribute to the distinct sleep EEG profiles of etomidate and THIP compared with zolpidem.

Key words: **thalamus; $GABA_A$ receptor; hypnotics; etomidate; tonic inhibition; EEG**

Introduction

The $GABA_A$ receptor is an important target for certain sedatives and hypnotics including etomidate, benzodiazepines, and neurosteroids (Belelli et al., 1999). Etomidate is of interest because, at clinically relevant concentrations, it is selective for $GABA_A$ receptors that incorporate the β_2 or β_3 versus the β_1 subunit, a specificity conferred by a single amino acid (asparagine: β_2 and β_3 subunits vs serine: β_1 subunit) (Belelli et al., 1997; Hill-Venning et al., 1997). We generated a mouse in which the β_2 subunit is replaced by an etomidate-insensitive β_2 subunit (β_{2N265S}) and demonstrated the sedative and a component of the hypnotic action of this anesthetic to be mediated by β_2 subunit-containing receptors (Reynolds et al., 2003). In agreement, specific modifications of the sleep electroencephalogram (EEG) pattern [e.g., enhancement by etomidate of slow-wave activity (SWA) during slow-wave sleep (SWS)] are reduced in β_{2N265S} mice (Reynolds et al., 2003). These findings implicate $GABA_A$ receptors in the generation of the rhythmic activities that underlie sleep.

The neuronal location of the β_2 subunit-containing receptors that mediate the hypnotic actions of etomidate and their contribution to distinct sleep oscillatory patterns are not known. The thalamus is implicated in the generation of sleep. The synchronous activity in thalamocortical neurons [in the ventrobasalis complex (VB)] is influenced by inhibitory GABAergic inputs from the nucleus reticularis (nRT) to neurons of the VB (Jones, 2002; Steriade, 2005). Specifically, the transition from the "relay mode" typical of the awake state or rapid eye movement (REM) sleep to the "spindle" and "delta" modes, which characterize the light and deep stages of sleep, respectively, is accompanied by a progressive hyperpolarization (Steriade et al., 1991; Steriade, 2003). However, the role of $GABA_A$ receptors in this process, particularly in VB neurons, is not known. Interestingly, immunohistochemistry has demonstrated a high density of α_4 and δ subunit expression in VB neurons (Pirker et al., 2000). In cerebellar and dentate gyrus granule cells, the δ subunit is associated with a tonic inhibitory conductance mediated by extrasynaptic or perisynaptic $GABA_A$ receptors (Stell et al., 2003; Farrant and Nusser, 2005). Extrasynaptic receptors are proposed to be an important target for certain general anesthetics, neurosteroids, and alcohol (Wallner et al., 2003; Caraiscos et al., 2004; Wei et al., 2004; Belelli and Lambert, 2005). These observations raise the

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Table 1. Summary of the properties of the tonic and phasic (i.e., mIPSCs) $GABA_A$ receptor-mediated transmission in VB and nRT neurons of WT, β_{2N265S}, and $\beta_2^{-/-}$ mice

	VB			nRT		
	WT	β_{2N265S}	$\beta_2^{-/-}$ [a]	WT	β_{2N265S}	$\beta_2^{-/-}$
mIPSC amplitude (pA)	74 ± 4	82 ± 6	30 ± 3**	59 ± 3	68 ± 4	64 ± 6
mIPSC τ_W (ms)	4.1 ± 0.2	3.5 ± 0.1	ND	14.5 ± 1	12.1 ± 1	13.7 ± 1
mIPSC charge transfer (fC)	308 ± 20	317 ± 25	136 ± 20**	736 ± 39	713 ± 39	790 ± 63
mIPSC frequency (Hz)	52.6 ± 4	47.6 ± 8	20.8 ± 2**	12.4 ± 1	16.6 ± 3	14.6 ± 1
Phasic charge (fC/s)	16,201	15,090	2829	9126	11,836	11,534
Tonic current (pA)	62 ± 12	70 ± 13	28 ± 2*	5 ± 6	ND	ND
Tonic charge (fC/s)	62,000	70,000	28,000	ND	ND	ND
Total charge (fC/s)	78,201	85,090	30,829	ND	ND	ND

*$p < 0.05$, **$p < 0.001$ versus wild type ($n = 9–42$ neurons). ND, Not determined.

[a]mIPSCs were evident only in 12 of 20 VB cells in $\beta_2^{-/-}$ mice.

intriguing prospect that a similar form of tonic inhibition in VB neurons contributes to the specific modifications of sleep EEG patterns induced by etomidate and other hypnotics.

Here, we characterized $GABA_A$ receptor-mediated neurotransmission in thalamic nRT and VB neurons of wild-type (WT), $\beta_2^{-/-}$, and β_{2N265S} mice (Sur et al., 2001; Reynolds et al., 2003). We report that, in this circuit, $GABA_A$ receptor-mediated transmission is dominated by the tonic, extrasynaptic conductance of VB neurons. Zolpidem does not induce SWS, and this hypnotic had no effect on the tonic conductance, whereas 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP or Gaboxadol) and etomidate both induce SWS, and in common these hypnotics greatly increased the tonic conductance. These findings suggest that enhancement of extrasynaptic inhibition in the thalamus may underlie the sleep EEG profile of THIP and etomidate.

Materials and Methods

Thalamic slice preparation and electrophysiology. Thalamic slices were prepared from mice of either sex [postnatal day 16 (P16) to P24] according to standard protocols (Belelli and Herd, 2003; Reynolds et al., 2003). The animals were killed by cervical dislocation in accordance with Schedule 1 of the United Kingdom Government Animals (Scientific Procedures) Act 1986. The brain was rapidly removed and placed in oxygenated ice-cold artificial CSF (aCSF) solution containing the following (in mM): 225 sucrose, 2.95 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 glucose, 1 ascorbic acid, and 3 pyruvic acid, pH 7.4, 330–340 mOsm. The tissue was maintained in ice-cold aCSF while horizontal 300 μm slices were cut using a Vibratome (Intracel; Royston, Hertfordshire, UK). The slices were incubated at 32°C for 1 h in an oxygenated extracellular solution (ECS) containing the following (in mM): 126 NaCl, 2.95 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-glucose, and 2 $MgCl_2$, pH 7.4, 300–310 mOsm. Subsequently, slices were maintained at room temperature before being used for recordings. Whole-cell patch-clamp recordings were performed at 35°C from thalamic nRT and VB neurons visually identified with an Olympus (Southall, UK) BX51 microscope equipped with infrared-differential interference contrast optics as described previously (Belelli and Herd, 2003; Reynolds et al., 2003). Patch pipettes were prepared from thick-walled borosilicate glass (Garner Glass Company, Claremont, CA) and had open tip resistances of 3–5 MΩ when filled with an intracellular solution that contained the following (in mM): 140 CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 1 $CaCl_2$, and 5 QX-314 [*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide], pH 7.3 with CsOH, 300–305 mOsm. Miniature IPSCs (mIPSCs) were recorded using an Axopatch 1D or 200B amplifier (Molecular Devices, Union City, CA) at a holding potential of −60 mV in ECS that additionally contained 2 mM kynurenic acid (Sigma-Aldrich, Poole, UK) and 0.5 μM tetrodotoxin (Tocris Bioscience, Bristol, UK) to block ionotropic glutamate receptors and sodium-dependent action potentials, respectively.

Drug application. Etomidate, THIP, pentobarbital, picrotoxin, and bicuculline methobromide (10^{-2} M) were dissolved in water, whereas zolpidem was prepared as a concentrated (1000×) stock solution in DMSO. These stock solutions were diluted in ECS to the desired concentration. The final maximum DMSO concentration (0.1%) had no effect on mIPSCs or the tonic current. All modulatory agents were applied via the perfusion system (2–4 ml/min) and allowed to infiltrate the slice for a minimum of 10 min while recordings were acquired. With the exception of THIP, which was a generous gift from B. Ebert (Lundbeck, Copenhagen, Denmark), all drugs tested were obtained from either Sigma-Aldrich or Tocris UK (Bristol, UK).

Data analysis. Data were recorded onto a digital audiotape using a Biologic DTR 1200 recorder and analyzed off-line using the Strathclyde Electrophysiology Software, WinEDR/WinWCP (J. Dempster, University of Strathclyde, Glasgow, UK). Individual mIPSCs were detected using a −4 pA amplitude threshold detection algorithm and visually inspected for validity. Accepted events were analyzed for peak amplitude, 10–90% rise time, charge transfer, and time for events to decay by 50% (T50) and 90% (T90). To minimize the contribution of dendritically generated currents, which are subject to cable filtering, analysis was restricted to events with a rise time ≤1 ms. A minimum of 100 accepted events were digitally averaged by alignment at the mid-point of the rising phase, and the mIPSC decay was fitted by either monoexponential [$y(t) = Ae^{(-t/\tau)}$] or biexponential [$y(t) = A_1e^{(-t/\tau 1)} + A_2e^{(-t/\tau 2)}$] functions using the least-squares method, where A is amplitude, t is time, and τ is the decay time constant. Analysis of the SD of residuals and use of the F test to compare goodness of fit revealed that the average mIPSC decay was always best fit with the sum of two exponential components. Thus, a weighted decay time constant (τ_w) was also calculated according to the following equation: $\tau_w = \tau_1P_1 + \tau_2P_2$, where τ_1 and τ_2 are the decay time constants of the first and second exponential functions and P_1 and P_2 are the proportions of the synaptic current decay described by each component. The mIPSC frequency was determined over 10 s bins for 2 min with the EDR program using a detection method based on the rate of rise of events (35–40 pA/ms) and visual scrutiny. The tonic current was calculated as the difference between the holding current before and after application of 30 μM bicuculline methobromide or 100 μM picrotoxin (Brickley et al., 1996; Belelli and Herd, 2003; Caraiscos et al., 2004). All results are reported as the arithmetic mean ± SEM. Statistical significance of mean data was assessed with the unpaired or paired Student's t test or repeated-measures ANOVA, followed *post hoc* by the Newman–Keuls test as appropriate, using the SigmaStat (SPSS, Chicago, IL) software package.

Results

A comparison of inhibitory transmission in nRT and VB neurons

The properties of nRT and VB mIPSCs were clearly distinct (Table 1). The frequency of mIPSCs was approximately fourfold greater in VB compared with nRT neurons, but mIPSC amplitude was similar. VB mIPSCs decayed ~3.5-fold more rapidly than those of nRT, and therefore the average total charge transferred per mIPSC was greater for nRT mIPSCs (Fig. 1*A*, Table 1). However, considering both the frequency and the total charge transferred by each event, the total charge transferred by mIPSCs in the VB is ~1.8-fold that occurring in the nRT (Table 1). The

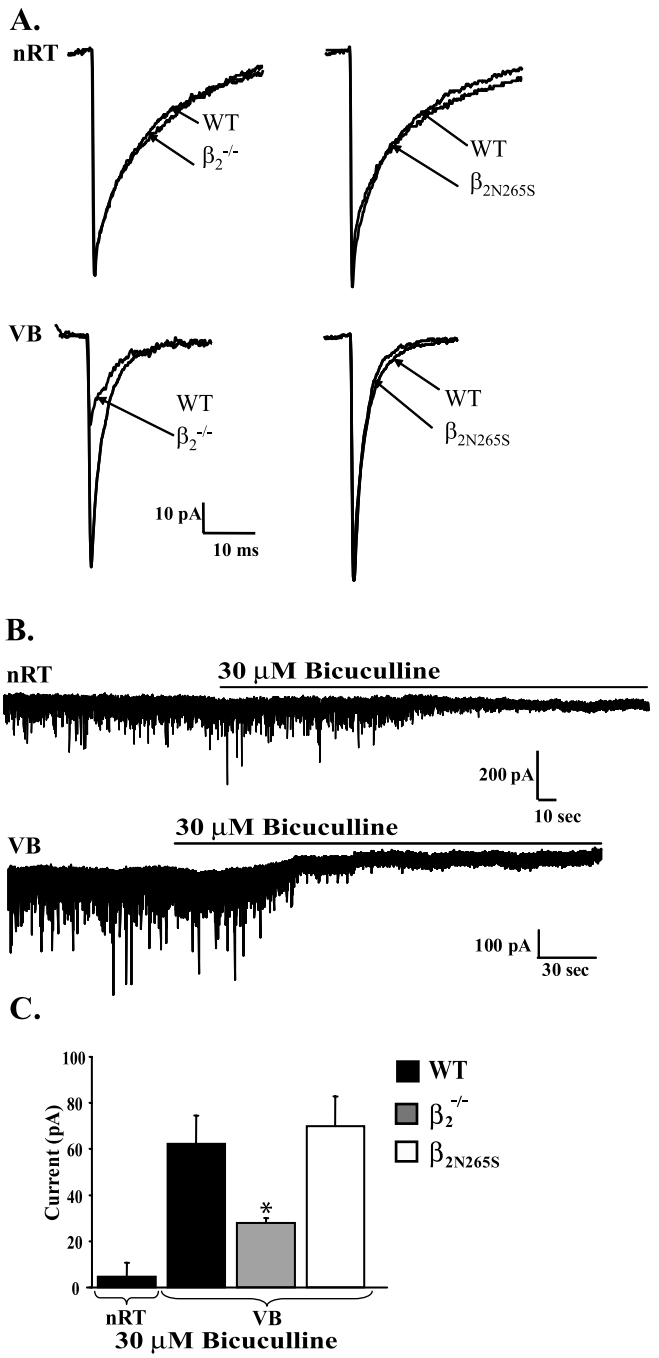

Figure 1. The properties of synaptic and extrasynaptic $GABA_A$ receptors of nRT and VB neurons. ***A***, Overlayed averaged mIPSCs recorded from exemplar nRT and VB cells of WT, β_{2N265S}, and $\beta_2{}^{-/-}$ mice. Note that the deletion of the β_2 subunit greatly decreases the amplitude of VB mIPSCs (of those neurons for which mIPSCs were detected), but with no effect on nRT mIPSCs. The β_{2N265S} mutation had no effect on VB or nRT mIPSCs. ***B***, Bicuculline (30 μM) has no effect on the holding current recorded from an exemplar nRT neuron but reveals a large tonic current in a VB neuron from a WT mouse. Note that the mIPSCs of both nRT and VB neurons are abolished by this antagonist. ***C***, Bar graph showing the lack of a tonic current in nRT neurons and the influence of deleting ($\beta_2{}^{-/-}$) or mutating (β_{2N265S}) the β_2 subunit compared with wild type on the tonic current of VB neurons. Data are obtained from three to nine cells. Note that the deletion, but not the mutation, of the β_2 subunit significantly reduces the VB tonic current. *$p < 0.05$ versus wild type (unpaired Student's *t* test). Error bars indicate the SE of the arithmetic mean.

$GABA_A$ receptor antagonist bicuculline (30 μM), in a reversible manner, completely abolished nRT mIPSCs (Fig. 1*B*) but had no effect on the holding current (4.5 ± 6.1 pA; $n = 3$) (Fig. 1*C*). Similarly, bicuculline (30 μM), or picrotoxin (100 μM), completely abolished VB neuron mIPSCs, but in contrast to the nRT, these antagonists induced a decrease in membrane noise and an outward current (62 ± 12 pA, $n = 9$ and 73 ± 9 pA, $n = 4$, respectively) (Fig. 1*B*,*C*; Table 1). Under these conditions, in VB neurons, the charge transfer mediated by these extrasynaptic receptors is ~3.8-fold greater than that produced by the synaptic receptors (Table 1).

The influence of the deletion or mutation (β_{2N265S}) of the β_2 subunit on thalamic inhibitory transmission

The thalamic expression of the β subunit isoforms is distinctive, with the β_3 and β_2 subunit predominantly expressed in nRT and VB neurons, respectively (Pirker et al., 2000). Deletion of the β_3 subunit greatly reduced the amplitude and frequency of nRT mIPSCs, with no effect on VB mIPSCs (Huntsman et al., 1999). In contrast, the frequency, amplitude, and decay kinetics of mIPSCs recorded from nRT neurons of $\beta_2{}^{-/-}$ mice were not significantly different from their WT counterparts ($p > 0.05$) (Fig. 1*A*, Table 1). However, inhibitory synaptic transmission was severely disrupted in the VB neurons of $\beta_2{}^{-/-}$ mice with no mIPSCs present in 40% (8 of 20) of cells and mIPSCs of a reduced amplitude and frequency evident in the remaining neurons ($p < 0.001$) (Fig. 1*A*, Table 1). In VB neurons, the outward current induced by bicuculline (30 μM) was significantly reduced ($p < 0.05$) (Fig. 1*C*, Table 1) by the deletion of the β_2 subunit, suggesting that extrasynaptic receptors also incorporate this subunit. The frequency, amplitude, and kinetics of mIPSCs recorded from nRT and VB neurons of β_{2N265S} mice were indistinguishable from wild type (Fig. 1*A*, Table 1). Similarly, the magnitude of the tonic current induced by bicuculline (30 μM) in VB neurons of β_{2N265S} mice was not significantly different from that of WT neurons ($p > 0.05$) (Fig. 1*C*, Table 1). Therefore, importantly, this mutation appears to be silent.

Etomidate

Etomidate (3 μM) had no effect on the rise time or amplitude of mIPSCs recorded from WT or β_{2N265S} nRT neurons but was equieffective in prolonging their decay [control τ_W, 11.8 ± 0.9 ms; etomidate τ_W, 21.9 ± 2 ms; $p < 0.05$ (i.e., an 85 ± 8% increase), $n = 5$; β_{2N265S} control τ_W, 11.6 ± 0.8 ms; etomidate τ_W, 23 ± 3 ms; $p < 0.05$ (i.e., an 82 ± 18% increase), $n = 4$; $p > 0.1$; wild type vs β_{2N265S}] (Fig. 2*A*). Etomidate (3 μM) did not directly induce an inward current in WT or β_{2N265S} nRT neurons (Fig. 2*A*).

Etomidate (3 μM) had no effect on the rise time or amplitude of mIPSCs recorded from WT VB neurons ($p > 0.05$) but significantly prolonged their decay (194 ± 34% increase; $p < 0.001$) (Fig. 2*B*,*D*; Table 2), an effect greatly reduced by the β_2 subunit mutation (68 ± 9% increase; $p < 0.05$) (Fig. 2*B*,*D*; Table 2) ($p <$ 0.001 vs wild type). Hence, the mutation reduced the effects of this anesthetic on synaptic $GABA_A$ receptors of VB but not nRT neurons. The β subunit selectivity of etomidate is not shared by other intravenous general anesthetics such as pentobarbital (Hill-Venning et al., 1997; Belelli et al., 1999). In agreement, the effect of pentobarbital to prolong the decay of mIPSCs of VB neurons of WT mice [control τ_W, 4.1 ± 0.4 ms; 100 μM pentobarbital τ_W, 18.2 ± 2.2 ms; $p < 0.01$ (i.e., 359 ± 62% increase); $n = 6$] and β_{2N265S} mice [control τ_W, 3.3 ± 0.2 ms; 100 μM pentobarbital τ_W, 15.4 ± 3.4; $p < 0.05$ (i.e., 364 ± 99% increase); $n = 6$] was not significantly different ($p > 0.1$; wild type vs β_{2N265S}) (Fig. 2*C*,*D*). Therefore, the mutation is selective, having disrupted the allosteric effects of etomidate but not those of the barbiturate.

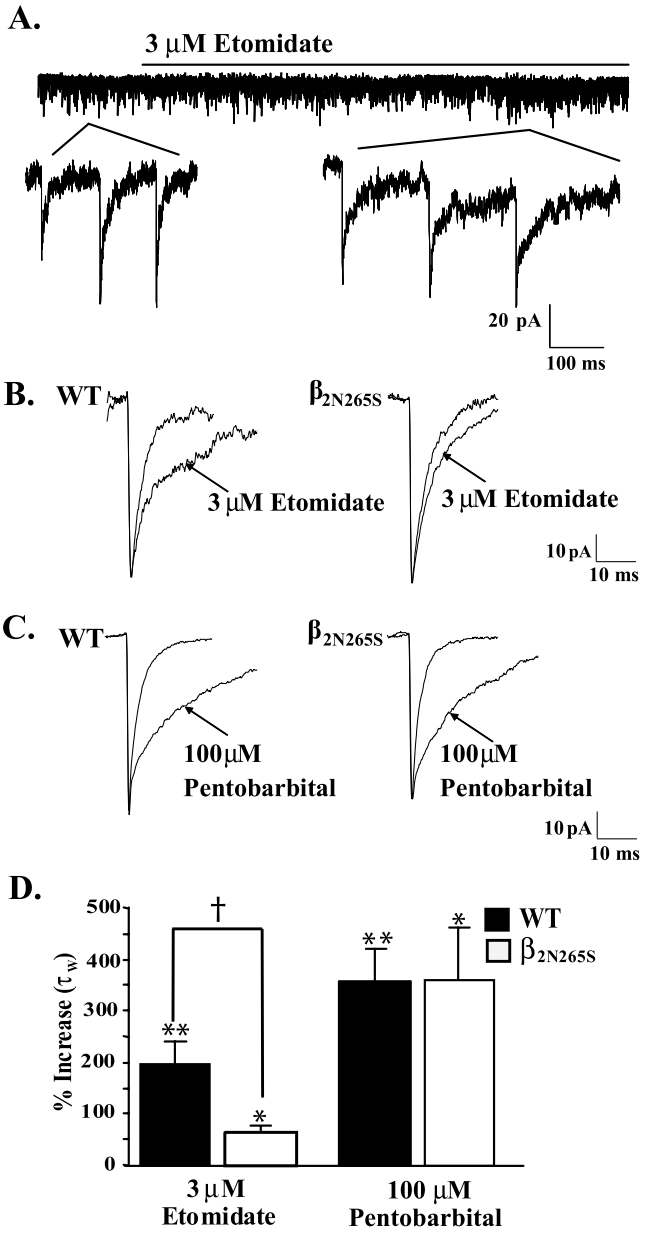

Figure 2. The effect of etomidate on the mIPSCs and the tonic current of nRT and VB neurons. ***A***, Etomidate (3 μM) has no effect on the holding current of the exemplar WT nRT neuron but prolongs the mIPSC decay (see expanded traces). ***B***, ***C***, Overlayed averaged mIPSCs recorded from exemplar VB neurons of WT and β_{2N265S} mice showing the effect of 3 μM etomidate (***B***) and 100 μM pentobarbital (***C***). Note that etomidate-induced but not pentobarbital-induced prolongation of VB mIPSCs is reduced by the β_{2N265S} mutation. ***D***, Bar graph showing the percentage of increase in the decay time constant τ_w produced by etomidate (3 μM) and pentobarbital (100 μM) in VB neurons from WT (■) and β_{2N265S} (□) mice. Data were obtained from six cells. *$p < 0.05$ versus control; **$p < 0.001$ versus control; $^{\dagger}p < 0.01$, wild type versus β_{2N265S} (two-way, repeated-measures ANOVA). Error bars indicate the SE of the arithmetic mean.

In contrast to nRT neurons, the bath application of etomidate (3 μM) induced a large bicuculline-sensitive inward current (152 ± 15 pA) (Table 2), presumably by enhancing the actions of ambient levels of GABA on extrasynaptic receptors (Fig. 3). The reduced tonic current evident in the VB neurons of $\beta_2^{-/-}$ mice suggests that a substantial number of these extrasynaptic $GABA_A$ receptors incorporate the β_2 subunit. Furthermore, the current induced by 3 μM etomidate was significantly reduced by the β_2 subunit deletion ($p < 0.001$) (Fig. 3*C*, Table 2). However, even taking into account the reduced control tonic current in $\beta_2^{-/-}$ VB neurons, the remaining extrasynaptic receptors are less sensitive to the anesthetic ($\beta_2^{-/-}$, ~150% increase of tonic; wild type, ~245% increase of tonic). The inward current induced by etomidate (3 μM) for VB neurons of β_{2N265S} mice was significantly reduced compared with WT neurons ($p < 0.001$) (Fig. 3*B*,*C*; Table 2), although the mutation did not influence the magnitude of the bicuculline-sensitive tonic current per se (Fig. 1*C*, Table 1). This observation confirms the incorporation of the β_2 subunit into extrasynaptic receptors. Mirroring the situation for the synaptic $GABA_A$ receptors, the enhancement of the tonic current by 100 μM pentobarbital was unaffected by this mutation (wild type: 195 ± 21 pA, $n = 6$; β_{2N265S}: 175 ± 29 pA, $n = 6$; $p > 0.05$) (Fig. 3*C*).

THIP

The $GABA_A$ receptors that mediate the tonic current in VB neurons are proposed to contain α_4, β, and δ subunits (Porcello et al., 2003), receptor isoforms that are highly sensitive to the $GABA_A$ receptor agonist THIP (Brown et al., 2002). Consistent with recombinant receptor studies, relatively low concentrations of THIP (30 nM to 3 μM), induced a concentration-dependent, well maintained inward current in VB neurons (30 nM THIP: 15 ± 3 pA, $n = 3$; 1 μM THIP: 310 ± 23 pA, $n = 6$) (Fig. 4*A*,*B*,*D*). This effect was neuron selective because 3 μM THIP did not induce an inward current (5.8 ± 8.4 pA; $n = 4$) in nRT neurons and, importantly, as highlighted above, these neurons compared with VB neurons do not exhibit a tonic conductance (Fig. 4*C*,*D*). Furthermore, 1 and 3 μM THIP had no significant effect on the amplitude or decay of VB and nRT mIPSCs, respectively. Therefore, in the thalamus, at these relatively low concentrations, this agonist appears selective for the extrasynaptic $GABA_A$ receptors of VB neurons. As highlighted above, deletion of the β_2 subunit reduced the tonic current in VB neurons presumably reflecting a decreased expression of extrasynaptic $GABA_A$ receptors. In agreement, although THIP (1 μM) still induced an inward current (100 ± 14 pA; $n = 5$; data not shown) in VB neurons of $\beta_2^{-/-}$ mice, this response was significantly reduced ($p < 0.001$) compared with control neurons (310 ± 23 pA; $n = 6$).

Zolpidem

Low concentrations of zolpidem are selective for $GABA_A$ receptors incorporating the α_1 subunit. For VB neurons, zolpidem (100 nM and 1 μM) had little or no effect on mIPSC frequency, rise time, or amplitude (data not shown) but caused a significant prolongation of the mIPSC decay (control τ_W, 3.2 ± 0.2 ms; 100 nM zolpidem τ_W, 5 ± 0.3 ms; $p < 0.001$; 1 μM zolpidem τ_W, 5.8 ± 0.3 ms; $p < 0.005$; $n = 6$). Hence, these data are consistent with VB neurons expressing synaptic $GABA_A$ receptors incorporating the α_1 and γ_2 subunits. In contrast to etomidate and THIP, zolpidem (100 nM to 1 μM) had no effect on the holding current of VB neurons (Fig. 4*D*), reinforcing the concept that these extrasynaptic receptors are pharmacologically distinct from their synaptic counterparts.

The synaptic receptors of nRT neurons incorporate the α_3 compared with the α_1 subunit (Pirker et al., 2000; Sohal et al., 2003). In agreement with the notion that low nanomolar concentrations of zolpidem are selective for α_1 subunit-containing receptors, this hypnotic (100 nM) had no effect on nRT mIPSCs (data not shown).

Discussion

The thalamus is crucial in the generation of the thalamocortical oscillations across the sleep–wake cycle (McCormick and Con-

Table 2. The effect of 3 μM etomidate on the properties of mIPSCs and the tonic current of VB neurons of WT, β_{2N265S}, and $\beta_2^{-/-}$ mice

	Control			+3 μM Etomidate		
VB neurons	WT	β_{2N265S}	$\beta_2^{-/-}$	WT	β_{2N265S}	$\beta_2^{-/-}$
mIPSC amplitude (pA)	69 ± 5	94 ± 8	20 ± 2	73 ± 7	79 ± 10	22 ± 1.9
mIPSC τ_W (ms)	5 ± 0.8	3.5 ± 0.1	ND	14.4 ± 2.3*	5.9 ± 0.4*	ND
mIPSC charge (fC)	357 ± 30	435 ± 70	66 ± 11	834 ± 101*	531 ± 42*	82 ± 6
Phasic charge (fC/s)	18,779	22,881	1373	43,869	27,931	1706
Tonic current (pA)	62 ± 12	70 ± 13	28 ± 2	+152 ± 15	+60 ± 7	+42 ± 6
Tonic charge (fC/s)	62,000	70,000	28,000	+152,000	+60,000	+42,000
Total charge (fC/s)	80,779	92,881	29,373	+177,090	+65,050	+42,333

The effect of etomidate on the tonic current is expressed as the change in the holding current (i.e., the current induced) by the anesthetic. Total inhibitory charge induced by etomidate is calculated as the sum of the etomidate-induced change in the phasic charge and the etomidate-induced increase in the tonic current.*$p < 0.01$ versus control (paired Student's t test; $n = 4–6$ neurons). ND, Not determined

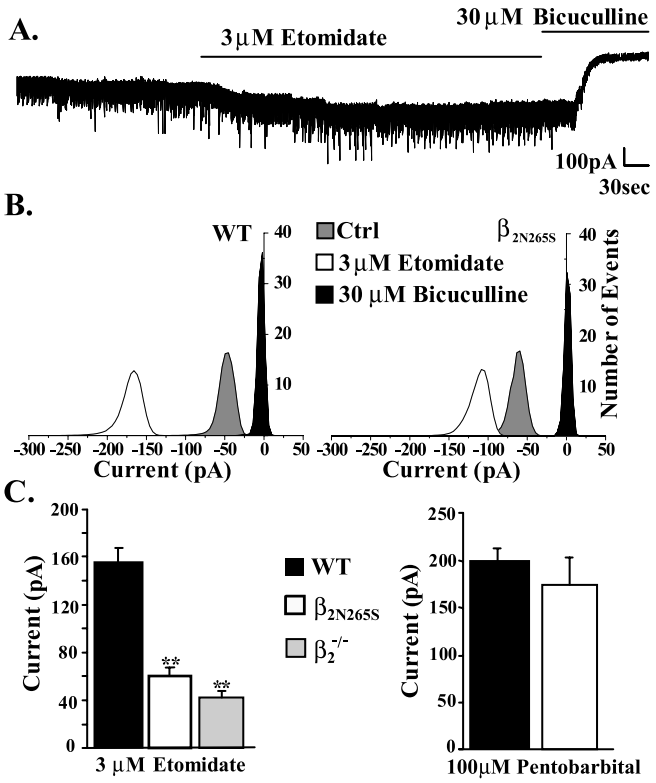

Figure 3. The etomidate-induced but not the pentobarbital-induced enhancement of the VB tonic current is reduced by the β_{2N265S} mutation. ***A***, The tonic current (calculated as the difference between the holding current (picoamperes) in the presence and absence of 30 μM bicuculline (see Materials and Methods) recorded from an exemplar VB neuron of a WT mouse is greatly enhanced by etomidate (3 μM). ***B***, All-points histograms illustrating the amplitude of the holding current (normalized to the current in the presence of bicuculline) under control (Ctrl) conditions (gray), in the presence of etomidate (3 μM; white), and after the application of bicuculline (30 μM; black) recorded from exemplar VB neurons of a WT (left) and β_{2N265S} (right) mouse. ***C***, Bar graph showing the inward current induced by 3 μM etomidate (left) and 100 μM pentobarbital (right) in VB neurons of WT, β_{2N265S}, and $\beta_2^{-/-}$ mice. Data were obtained from five to six cells. **$p < 0.001$ versus wild type (unpaired Student's t test). Error bars indicate the SE of the arithmetic mean.

treras, 2001; Steriade, 2003). Sensory information is routed to the somatosensory cortex through the thalamus and is disrupted by hypnotics (Rudolph and Antkowiak, 2004). Human brain imaging studies reveal propofol-induced changes in the level of consciousness to correlate with thalamic function (Fiset et al., 1999) and the thalamus and midbrain reticular formation to be selectively suppressed by volatile anesthetics (Alkire et al., 2000). Although sleep states and general anesthesia are distinct, they share certain EEG and behavioral properties (Keifer, 2003). Indeed, the thalamocortical cells, implicated in the generation of the cortical δ rhythms (1–4 Hz, characteristic of stage III/IV sleep, non-REM sleep, or SWS), may also be the locus of the δ activity induced by anesthetics, and the generation of these waveforms is critically dependent on GABAergic transmission (Alkire et al., 2000). The purpose of our study was to identify and characterize the properties of the thalamic $GABA_A$ receptor isoforms involved in this crucial neuronal pathway and to determine the effect of the hypnotics etomidate, THIP, and zolpidem on these receptors.

In agreement with reports on spontaneous IPSCs (Huntsman et al.,1999; Huntsman and Huguenard, 2000), nRT and VB mIPSCs exhibited similar amplitudes but distinct kinetics and frequency of occurrence. $GABA_A$ receptor antagonists blocked the mIPSCs and revealed in VB but not in nRT neurons a large tonic conductance. The tonic current is approximately six and three times greater than that of cerebellar and dentate granule cells, respectively (Brickley at al., 1996; Nusser and Mody, 2002). In part, this difference results from the larger cell size of VB neurons. When normalized to whole-cell capacitance, the VB tonic conductance (38.3 ± 7.6 pS pF^{-1}; $n = 9$) was similar to that of dentate (44 pS pF^{-1}) and cerebellar granule (50 pS pF^{-1}) cells (Stell et al., 2003). However, the dentate data were obtained from adult neurons (compared with P16–P24) and in the presence of 5 μM GABA, whereas the thalamic experiments were performed without exogenous agonist or treatments that enhance this conductance (Stell and Mody, 2002; Belelli and Herd, 2003; Caraiscos et al., 2004).

There is a restricted repertoire of $GABA_A$ receptor subunits in the thalamus (Pirker et al., 2000). In nRT neurons, the sensitivity of IPSCs to benzodiazepines is lost in α_{3H126R} mice, suggesting the predominant receptor isoform to incorporate α_3 and γ_2 subunits (Sohal et al., 2003). β_3 subunit deletion almost abolishes inhibitory synaptic transmission in nRT neurons, with no effect on VB mIPSCs (Huntsman et al., 1999). In contrast, we found that the properties of nRT mIPSCs are not influenced by the β_2 subunit deletion. Furthermore, the effect of etomidate (β_3/β_2 subunit selective) on nRT mIPSCs is not changed by the β_{2N265S} subunit mutation. These studies suggest the presence of $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors at nRT inhibitory synapses.

Immunohistochemistry reveals expression of α_1, α_4, β_2, γ_2, and δ subunits in the VB (Pirker et al., 2000). The relatively fast decay kinetics of VB mIPSCs, their sensitivity to low concentrations of the α_1 subunit-selective zolpidem, and the benzodiazepine insensitivity of IPSCs of VB neurons from α_{1H101R} mice (Sohal et al., 2003) suggest synaptic receptors incorporating α_1 and γ_2 subunits. In contrast to nRT, β_2 subunit deletion decreased the amplitude and frequency of VB mIPSCs. This observation, coupled with the reduced effect of etomidate on VB mIPSCs from β_{2N265S} mice identifies the β_2

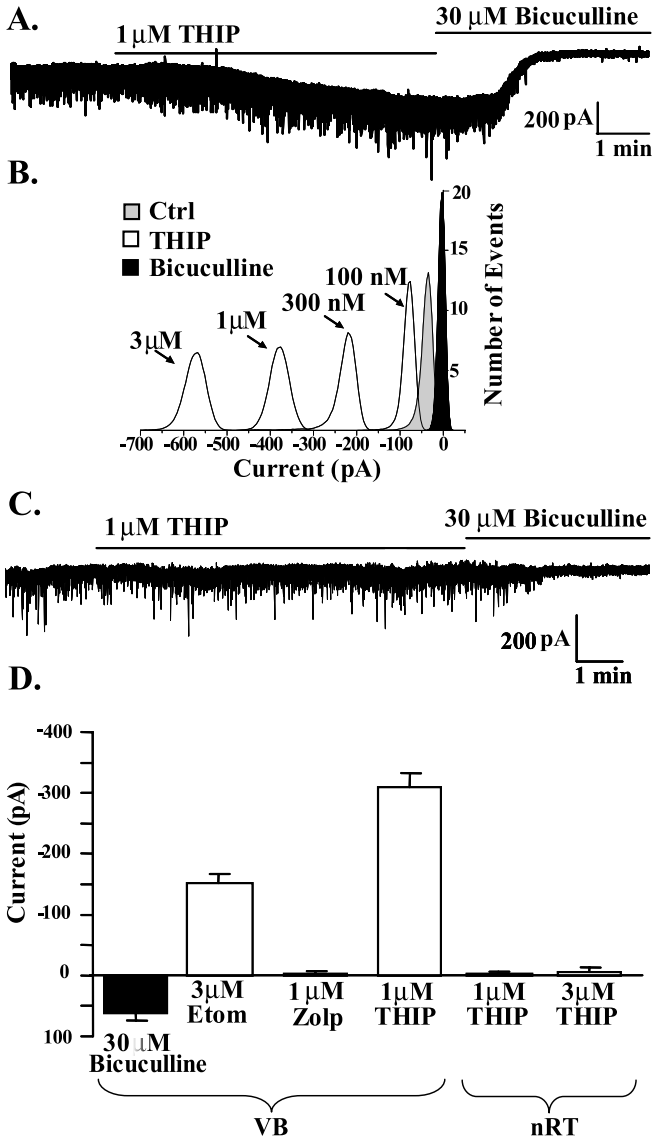

Figure 4. THIP potently activates a tonic current in VB but not nRT neurons. ***A***, The tonic current (calculated as the difference between the holding current (picoamperes) in the presence and absence of 30 μM bicuculline (see Materials and Methods) recorded from an exemplar VB neuron of a WT mouse is greatly increased by THIP (1 μM). ***B***, A corresponding all-points histogram illustrating the amplitude of the holding current (normalized to the current in the presence of bicuculline) under control (Ctrl) conditions (gray), in the presence of THIP (100 nM to 3 μM; white), and after the application of 30 μM bicuculline (black) recorded from an exemplar VB neuron of a WT mouse. ***C***, Neither THIP (1 μM) nor bicuculline (30 μM) has any effect on the holding current recorded from an exemplar nRT neuron of a WT mouse. ***D***, Bar graph showing the current induced by 30 μM bicuculline (i.e., tonic current), 3 μM etomidate (Etom), 1 μM zolpidem (Zolp), and 1–3 μM THIP in VB and nRT neurons of WT mice. Data were obtained from four to nine cells. Error bars indicate the SE of the arithmetic mean.

subunit to be synaptically located. Therefore, VB synaptic $GABA_A$ receptors are composed of α_1, β_2, and γ_2 subunits. Receptors assembled from α_4, β, and δ subunits mediate the inhibitory tonic conductance of dentate granule cells (Nusser and Mody, 2002; Stell and Mody, 2002; Farrant and Nusser, 2005), and a similar receptor isoform serves this function in VB neurons. In $\delta^{-/-}$ mice, the baseline noise of VB neurons is reduced (Porcello et al., 2003). The VB tonic current is greatly increased by concentrations of THIP that selectively enhance $\alpha_4\beta_3\delta$ compared with $\alpha_4\beta_3\gamma_2$ receptors (Brown et al., 2002; Krogsgaard-Larsen et al., 2004) but is insensitive to a high concentration of zolpidem. The reduction of the tonic conductance by β_2 subunit deletion and the decreased effect of etomidate on this conductance in β_{2N265S} mice indicates that in VB neurons, the β_2 subunit contributes to both synaptic and extrasynaptic receptors. Therefore, the $\alpha_4\beta_2\delta$ isoform mediates the extrasynaptic conductance in VB neurons.

The sensitivity of $\beta_2^{-/-}$ VB neurons to low concentrations of THIP suggests that, like wild type, the receptors mediating the tonic conductance incorporate the δ subunit. These remaining receptors are sensitive to etomidate. However, the effect of this anesthetic on the tonic current of $\beta_2^{-/-}$ VB neurons (~150% increase) is intermediate between that produced by etomidate on WT (~250%) and β_{2N265S} (~86%) neurons. Therefore, the isoform of the β subunit (β_1, β_3, or coexpression) expressed in $\beta_2^{-/-}$ VB neurons remains to be clarified.

The effects of the hypnotics on nRT and VB $GABA_A$ receptors were quite distinct. Zolpidem (100 nM) exclusively enhanced the phasic conductance of VB neurons. In contrast, low concentrations of THIP (≥30 nM) selectively activated the VB tonic conductance with much greater concentrations (1–3 μM), having no effect on the holding current of nRT neurons or on VB and nRT mIPSCs. Similarly, THIP (5 μM) activates extrasynaptic δ subunit-containing receptors in dentate granule cells, with no effect on mIPSCs (Maguire et al., 2005). In agreement, we found low micromolar concentrations of THIP to have no effect on mIPSCs of dentate granule cells or cortical neurons (maintained in culture; our unpublished observations). Therefore, THIP seems to selectively activate δ subunit-containing extrasynaptic receptors, although whether all inhibitory synapses are similarly insensitive to low concentrations of this agonist is not known. Furthermore, higher concentrations of THIP would be expected to interact with synaptic $GABA_A$ receptors. Etomidate enhanced synaptic inhibition in both nRT and VB neurons and increased the tonic current of the latter. Although the synaptic and extrasynaptic receptors of VB neurons are both sensitive to etomidate, given the dominant influence of the tonic versus phasic conductance on charge transfer, the overall effect of this hypnotic on inhibition in the VB is primarily mediated (~86%) by facilitation of the tonic conductance.

What is the relevance of these effects to the hypnotic action of these compounds? As described above, the thalamic nRT–VB circuitry is implicated in the generation of sleep. Spindle oscillations require the pacemaker activity of nRT neurons and occur in VB neurons near their resting membrane potential, whereas slow oscillations, or δ waves, are generated in VB neurons at more hyperpolarized potentials (Pare et al., 1991; Steriade et al., 1991; Steriade, 2003, 2005). $GABA_A$ receptors play an important role in this circuitry. Deletion of the β_3 subunit (selectively localized to the intra-nRT inhibitory circuit) blunts (12–16 Hz) spindle activity and augments (1–4 Hz) δ EEG power (Wisor et al., 2002). Selective $GABA_A$ receptor agonists (muscimol, THIP) or modulators (etomidate) augment the 1–4 Hz EEG power spectra (Lancel and Faulhaber, 1996; Lancel et al., 1996; Lancel and Stiger, 1999; Reynolds et al., 2003; cf. Vyazovskiy et al., 2005). In β_{2N265S} mice, the enhancement of SWA by etomidate is reduced (Reynolds et al., 2003). Implicating the VB extrasynaptic receptors, in the thalamus this mutation primarily reduces the effects of this hypnotic on the VB tonic current. Furthermore, in the thalamus, hypnotic concentrations (~1 μM) (Madsen et al., 1983) of THIP selectively activate the VB extrasynaptic $GABA_A$ receptors, and in $\delta^{-/-}$ mice, both the ability of this hypnotic to induce a tonic current in dentate gyrus granule cells (Maguire et al., 2005) and to

cause a loss of the righting reflex is greatly reduced (R. A. Harris, personal communication). In contrast to THIP and etomidate, zolpidem has no effect on the VB tonic conductance, but interestingly, this hypnotic either decreases or has no effect on the δ activity of the EEG in man and rodents, respectively (Borbely et al., 1985; Lancel and Steiger, 1999; Kopp et al., 2004). Importantly, the diazepam-induced changes of the sleep EEG, including suppression of δ activity during SWS, are not mediated by α_1 or α_3 subunit-containing receptors [although receptors containing these subunits are abundantly expressed in the corticothalamic network (Tobler et al., 2001; Kopp et al., 2003)] but require α_2-containing $GABA_A$ receptors, which are localized in neuronal circuits (e.g., hypothalamus) out with the nRT–VB network (Kopp et al., 2004).

In conclusion, the effects of zolpidem on the EEG and on thalamic $GABA_A$ receptors are quite distinct from those of etomidate and THIP. The established role of the thalamus in sleep, together with the data reported here, implicates the extrasynaptic $GABA_A$ receptors of VB neurons in the hypnotic actions of THIP and etomidate. However, additional experimentation combining the use of isoform-selective drugs and mice in which the function of specific $GABA_A$ receptors are compromised (e.g., VB-specific knock-in/out β_{2N265S}, $\delta^{-/-}$) is required to categorically evaluate the importance of this target. This approach should permit a better understanding of the molecular mechanisms that govern the generation of specific sleep rhythms and aid the future development of sleep-restorative therapeutics.

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