

Etomidate blocks LTP and impairs learning but does not enhance tonic inhibition in mice carrying the N265M point mutation in the beta3 subunit of the GABA_A receptor

E.D. Zarnowska^{a,*,1}, F.C. Rodgers^{a,b,1}, I. Oh^c, V. Rau^c, C. Lor^a, K.T. Laha^a, R. Jurd^{d,2}, U. Rudolph^{d,e,f}, E.I. Eger 2nd^c, R.A. Pearce^{a,*}

^a School of Medicine and Public Health, Department of Anesthesiology, University of Wisconsin–Madison, USA

^b Neuroscience Training Program, University of Wisconsin–Madison, USA

^c Department of Anesthesia, University of California–San Francisco, USA

^d Institute of Pharmacology and Toxicology, University of Zurich, Switzerland

^e Laboratory of Genetic Neuropharmacology, McLean Hospital, Belmont, USA

^f Department of Psychiatry, Harvard Medical School University, Belmont, USA

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ABSTRACT

Enhancement of tonic inhibition mediated by extrasynaptic $\alpha 5$ -subunit containing GABA_A receptors (GABA_ARs) has been proposed as the mechanism by which a variety of anesthetics, including the general anesthetic etomidate, impair learning and memory. Since $\alpha 5$ subunits preferentially partner with $\beta 3$ subunits, we tested the hypothesis that etomidate acts through $\beta 3$ -subunit containing GABA_ARs to enhance tonic inhibition, block LTP, and impair memory. We measured the effects of etomidate in wild type mice and in mice carrying a point mutation in the GABA_A $\beta 3$ -subunit ($\beta 3$ -N265M) that renders these receptors insensitive to etomidate. Etomidate enhanced tonic inhibition in CA1 pyramidal cells of the hippocampus in wild type but not in mutant mice, demonstrating that tonic inhibition is mediated by $\beta 3$ -subunit containing GABA_ARs. However, despite its inability to enhance tonic inhibition, etomidate did block LTP in brain slices from mutant mice as well as in those from wild type mice. Etomidate also impaired fear conditioning to context, with no differences between genotypes. In studies of recombinant receptors expressed in HEK293 cells, $\alpha 5\beta 1\gamma 2L$ GABA_ARs were insensitive to amnestic concentrations of etomidate (1 μ M and below), whereas $\alpha 5\beta 2\gamma 2L$ and $\alpha 5\beta 3\gamma 2L$ GABA_ARs were enhanced. We conclude that etomidate enhances tonic inhibition in pyramidal cells through its action on $\alpha 5\beta 3$ -containing GABA_A receptors, but blocks LTP and impairs learning by other means – most likely by modulating $\alpha 5\beta 2$ -containing GABA_A receptors. The critical anesthetic targets underlying amnesia might include other forms of inhibition imposed on pyramidal neurons (e.g. slow phasic inhibition), or inhibitory processes on non-pyramidal cells (e.g. interneurons).

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1. Introduction

The ability of general anesthetics to cause sedation, amnesia, and immobility has been a subject of interest and intense study for

many years. There is now an emerging recognition that these different anesthetic end-points may reflect different anesthetic actions at the molecular, cellular and network levels (Rudolph and Antkowiak, 2004). The present study addresses the mechanism by which the general anesthetic etomidate impairs learning and memory.

GABA_A receptors (GABA_ARs) are heteropentameric ligand-gated anion channels responsible for the majority of inhibitory synaptic transmission in the brain. Functional GABA_A receptors most commonly incorporate two α -subunits, two β -subunits, and one γ -subunit (Olsen and Sieghart, 2008). GABA_ARs are considered to be

* Corresponding authors. University of Wisconsin, 1111 Highland Ave, WIMR II, Madison, WI 53705, USA. Tel.: +1 608 263 4429.

E-mail addresses: edzaska@gmail.com (E.D. Zarnowska), rapearce@wisc.edu (R.A. Pearce).

¹ These authors contributed equally to this work.

² Present address: Department of Neurology, NYU Langone Medical Center, USA.

important targets of a variety of agents, including etomidate (Jones et al., 1992; Jones and Harrison, 1993; Uchida et al., 1995). Because of its favorable hemodynamic profile, etomidate is used in the clinical setting to induce anesthesia in patients at risk for cardiovascular compromise, and in other select circumstances such as electroconvulsive therapy (Forman, 2011). It has also become an important experimental drug because its receptor sensitivity can be controlled by genetic manipulation: the discovery that receptors that incorporate $\beta 1$ -subunits (together with $\alpha 1$ -subunits) are markedly less sensitive to etomidate compared to those that incorporate $\beta 2$ - or $\beta 3$ -subunits (Sanna et al., 1997) led to the identification of a single amino acid residue in the pore-forming transmembrane domain that controls etomidate sensitivity (Belelli et al., 1997). Mice carrying single point mutations in these subunits ($\beta 2$ -N265S or $\beta 3$ -N265M) were found to resist etomidate's action in studies of sedation and immobility (Jurd et al., 2003; Reynolds et al., 2003). The impact of these mutations on etomidate-induced amnesia was not tested.

It was shown previously that genetic and pharmacologic manipulations that reduce or eliminate inhibitory current carried by GABA_A $\alpha 5$ -subunits ($\alpha 5$ -KO) are resistant to etomidate's suppression of long term potentiation (LTP) *in vitro* and learning and memory *in vivo* (Cheng et al., 2006). Because the majority of $\alpha 5$ -GABA_ARs are located extrasynaptically on pyramidal cells, where they mediate a persistent conductance termed "tonic inhibition" (Caraiscos et al., 2004), and tonic inhibition is strongly enhanced by amnestic drugs, it was proposed that the effect of etomidate on synaptic plasticity is due to its enhancement of tonic inhibition (Cheng et al., 2006; Orser, 2007; Martin et al., 2009). Since $\alpha 5$ -subunits preferentially partner with $\beta 3$ -subunits (Luddens et al., 1994; Sur et al., 1998), we hypothesized that mice carrying the N265M mutation in the $\beta 3$ -subunit would similarly resist etomidate's enhancement of tonic inhibition and suppression of LTP *in vitro* and learning *in vivo*.

We found that tonic inhibition in the $\beta 3$ -N265M mice was indeed insensitive to etomidate, showing that this form of inhibition is mediated by $\beta 3$ -subunit containing GABA_ARs. Surprisingly, although etomidate did not enhance tonic inhibition in these mice, it did suppress synaptic plasticity *in vitro* and learning *in vivo*. Studies of expressed recombinant receptors showed that GABA_ARs incorporating $\beta 1$ -subunits together with $\alpha 5$ -subunits were insensitive to amnestic concentrations of etomidate, mirroring previous results from studies of $\alpha 1$ -containing GABA_ARs (Sanna et al., 1997). We conclude that tonic inhibition in CA1 pyramidal cells is mediated by $\alpha 5\beta 3$ -subunit containing GABA_ARs, but that this form of inhibition does not play a key role in anesthetic suppression of synaptic plasticity in these neurons. Rather, etomidate appears to control LTP of Shaffer collateral synapses, and fear conditioning to context, by modulating $\alpha 5\beta 2$ -GABA_ARs.

2. Materials and methods

All experiments were performed in accordance with the National Institutes of Health guide the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the University of Wisconsin Institutional Animal Care and Use Committee, Madison, Wisconsin, or by the University of California Institutional Animal Care and Use Committee, San Francisco, California. All efforts were made to minimize animals suffering and reduce the number of animals used.

2.1. Mice

The male offspring of heterozygous breeding pairs homozygous for an asparagine-to-methionine point mutation at position 265 of the GABA_A receptor $\beta 3$ subunit ($\beta 3$ -N265M), and homozygous wild-type controls, were used for this study. The strain background of the $\beta 3$ -N265M mice was 129X1/SvJ. Mice were genotyped using DNA template from tail tips, amplified by PCR using the specific primers: *RJM-8* (5'-GTT CAG CTT CCA TTC TCA CTG-3') and *RJM-24* (5'-GCT ATG GCT TTC TGG TGG AG-3'). Animals were housed in the animal care facility under

12-h cycles of light and dark and had continuous access to standard mouse chow and water.

2.2. Brain slice preparation

2.2.1. LTP

Hippocampal brain slices were prepared from mice aged 42–77 days (57 ± 9 , $n = 31$). Before decapitation mice were anesthetized with 2.5% isoflurane (Novaplu, Hospira, Inc., Lake Forest, IL), then the brain was removed, blocked by removing the cerebellum and olfactory cortex, glued to a microtome slice tray with cyanoacrylate glue (Krazy Glue Instant, Westerville, OH), and placed for slicing in ice-cold cutting artificial cerebrospinal fluid ("cutting aCSF") containing (in mM) 127 NaCl, 1.9 KCl, 2.7 $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.9 $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 26 NaHCO_3 , 1.2 KH_2PO_4 , 1 ascorbic acid, 15 glucose, bubbled with 95% O_2 –5% CO_2 ("carbogen"). Coronal slices 500 μm thick were cut using a vibratome (Leica VT 100S, Leica Microsystems Nussloch GmbH, Nussloch, Germany). A portion of the slice including the hippocampus was trimmed with a scalpel to ensure proper fit within a custom-manufactured microfluidic recording chamber (Blake et al., 2010). Brain slices recovered in a holding chamber filled with carbogenated recording aCSF containing (in mM) 127 NaCl, 1.9 KCl, 26 NaHCO_3 , 1.2 KH_2PO_4 , 1.4 $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 2.2 $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 15 glucose, 1 ascorbic acid for at least 60 min at room temperature (20–22 °C). This same solution was used for recording ("recording aCSF").

2.2.2. Tonic and synaptic currents

Hippocampal brain slices were prepared from mice aged 40–50 days (44 ± 1 , $n = 7$). Before decapitation mice were anesthetized with 2.5–3% isoflurane then the brain was removed and placed in ice-cold N-methyl-D-glucamine (NMDG)-based cutting solution containing (in mM): 2.5 KCl, 1.25 Na_2HPO_4 , 25 NaHCO_3 , 10 $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.5 $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 25 glucose, 110 NMDG, 2.5 sodium ascorbate, bubbled with carbogen (pH adjusted with 6N HCl to 7.3, 300–310 mOsm) (Ting et al., 2014). Horizontal slices 350 μm thick were cut with oscillating blade microtome 7000 smz2 vibratome (Campden Instruments, Loughborough, England). Thereafter slices recovered while submerged in warmed (35 °C), carbogenated cutting solution which was next slowly exchanged (at a rate of 5 ml/min) with warmed (35 °C), carbogenated recording aCSF containing (in mM): 130 NaCl, 2.5 KCl, 1.25 Na_2HPO_4 , 25 NaHCO_3 , 2 $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 2 $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 10 glucose, 2.5 sodium ascorbate (pH 7.3, 300–310 mOsm). The exchange process was completed in 30 min. The slices were maintained at room temperature until they were transferred to the recording chamber.

2.2.3. Cell culture and recombinant receptor expression

Human embryonic kidney cells (HEK-293T, American Type Culture Collection, Manassas, VA) were cultured in minimum essential medium with L-glutamine and Earle's salts (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MA) and penicillin-streptomycin (Sigma–Aldrich, St. Louis, MO) in at 37 °C under a 5% CO_2 atmosphere. Cells were initially plated onto 60 mm dishes and co-transfected 24 h later with pUNIV vectors (Venkatachalan et al., 2007) containing appropriate rat GABA_A subunits ($\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 2\text{L}$) or eGFP cDNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The ratio of α vs. β vs. γ subunits was 1:1:3, and a total of 0.6 μg of cDNA was used for transfections. After 24 h cells were re-plated onto 12 mm glass coverslips, and electrophysiological studies were conducted 48–72 h post-transfection.

2.3. Data acquisition

2.3.1. LTP

Brain slices were transferred to a microfluidic recording chamber (Blake et al., 2010) perfused with recording aCSF at a flow rate of 2.5 ml/min. The bath temperature was maintained at 30 ± 0.5 °C using an in-line temperature controller (Warner Instruments Corp., Hamden, CT). A 16-channel linear recording electrode (50 μm separating recording sites; NeuroNexus Technologies, Ann Arbor, MI) was inserted orthogonal to the hippocampal layers, in the middle of CA1, at a depth (along the rostral–caudal axis) of 200 μm beneath the surface of the tissue. Field excitatory postsynaptic potentials (fEPSPs) were electrically evoked by a tungsten stereotrode stimulating electrode (0.5 M Ω , World Precision Instruments, Sarasota, FL) placed in stratum radiatum for activation of the Schaffer collateral/commissural path (SC). Recorded signals were amplified 1000 \times , band-pass filtered between 1 and 3000 Hz (model LYNX-8 amplifiers, Neuralynx Inc., Tucson, AZ), digitized at 10 kHz using an analog-to-digital converter (Digidata 1440A, Molecular Devices, Sunnyvale, CA), and acquired using pClamp software (Version 10.2, Molecular Devices). Stimuli of 0.1 ms duration were delivered using a constant current stimulus isolator (model A365D, World Precision Instruments, Sarasota, FL). SC axons were stimulated at 0.03 Hz, using stimulus intensity ("baseline") adjusted to evoke responses below half-maximal fEPSP amplitude. Baseline stimulus amplitude was typically between 30 and 90 μV . LTP protocols consisted of a 30 min stable baseline recording period in which evoked fEPSP slope changed by less than 10%, followed by a theta burst stimulus (TBS), and then an additional 60 min recording period. The LTP-inducing TBS ("40 \times 5Hz") consisted of 10 bursts delivered every 200 ms (i.e. 5 Hz inter-burst interval) with each burst consisting of 4 pulses separated by 10 ms (i.e. 100 Hz inter-stimulus interval). The stimulus intensity during the burst was adjusted

to evoke half-maximal population spike amplitude, judged by measuring the amplitude of the downward-going negative voltage peak in the pyramidal channel (see below) online during recordings.

2.3.2. Tonic and synaptic currents

Brain slices were transferred to a submersion-style recording chamber perfused at a flow rate of 2.5–3 ml/min with recording aCSF containing kynurenic acid (KA, 3 mM). The bath temperature was maintained at $30 \pm 0.5^\circ\text{C}$ using an in-line temperature controller (Warner Instruments Corp.). Pyramidal cells in the CA1 region were visualized with a $40\times$ water immersion objective and IR-DIC video camera installed on BX50WI microscope (Olympus America Inc., Center Valley, PA). Patch clamp recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices), low pass filtered at 4 kHz, digitized at 10 kHz using an analog-to-digital converter (Digidata 1322A, Molecular Devices), and acquired using pClamp software (Version 10.3, Molecular Devices). Borosilicate glass pipettes (O.D. 1.5 mm \times I.D. 0.86 mm, Sutter Instruments, Novato, CA) were pulled to tip diameters of $\sim 1\ \mu\text{m}$ using a horizontal puller (P-97, Sutter Instruments) then filled with an intracellular solution containing (in mM) 90 CsCl, 30 KCl, 5 NaCl, 10 NaHEPES, 5 EGTA, 4 Mg_2ATP 2, 0.4 Na_3GTP , 10 $\text{Na}_2\text{phosphocreatine}$, 4 QX-314, pH adjusted with 1M CsOH to 7.3 (290 ± 5 mOsm). The resistances of filled pipettes were 5–7 M Ω . Whole-cell, voltage-clamp recordings were performed at a -60 mV holding potential. Cell capacitances and membrane resistances were measured using the software membrane test algorithm. Series resistances were not compensated but were monitored, and recordings were discontinued if resistance increased more than 25%. Uncompensated series resistances were 10–20 M Ω .

2.3.3. Recombinant receptors

Recordings were performed at room temperature (21°C) on the stage of an inverted microscope equipped with DIC optics and epifluorescence (Leica DM IRB, Leica Microsystems, Wetzlar GmbH, Germany). The recording chamber was perfused with recording solution containing (in mM): 145 NaCl, 5 KCl, 10 HEPES, and 1.8 CaCl_2 , pH adjusted with 10 M NaOH to 7.4 (305 mOsm). Transfected cells were identified by expression of eGFP. Outside-out excised patch recordings were made using borosilicate glass pipettes filled with (in mM): 130 KCl, 10 HEPES, 5 EGTA, 1 MgCl_2 , and 5 Mg_2ATP , pH adjusted with 10 M KOH to 7.4 (310 mOsm). Patches were held at -40 mV. Currents were low-pass-filtered at 2 kHz with a four-pole Bessel filter, and data were collected at 20 kHz using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), a Digidata 1200 (Axon Instruments), and Clampex 10.4 (Axon Instruments). Solutions were applied using a custom gravity-fed multibarreled device fabricated with polyimide tubes with OD 0.0142"/ID 0.0122" (Cole–Parmer, Vernon Hills, IL), and attached to a stepper motor-based microscope translation stage (ITK Dr. Kassen GmbH, Lahnau, Germany). Open tip exchange rates of 20 ms (10–90% rise time) were achieved. GABA was dissolved fresh in bath solution. Etomidate was prepared as stock (50 mM in DMSO), stored at -20°C , and diluted in recording solution the day of an experiment.

2.4. Behavioral testing

Experiments were conducted at the University of California, San Francisco. Three to 10 male mice, between 60 and 80 days old, of each genotype (7 on average per group) were assessed at 0, 7.5, 9.38, 10.3, 11.25 and 15 mg/kg etomidate. These doses were chosen based on a previous study indicating that etomidate's ED_{50} for conditional freezing for a similar fear conditioning protocol in 129/SvJ \times C57BL/6J mice was 11 mg/kg (Benkowitz et al., 2007). A 35% propylene glycol solution (Sigma, St. Louis, MO) was used for all vehicle injections. The intraperitoneal injection of etomidate was given 30 min before training, and all mice were injected at 7.7 ml/kg body weight.

Thirty minutes after the injection, groups of four mice at a time were transported from their home cage and transferred to fear conditioning chambers (27 cm L \times 24.5 cm W \times 20 cm H) constructed of clear acrylic. The chamber floor was made of 31 stainless steel bars (3 mm in diameter, spaced 7 mm center to center)

and was connected to a shock delivery system (San Diego Instruments, San Diego, CA). Before and after each session, the chambers walls were cleaned with 5% Pine Scented Disinfectant (Midland, Inc., Sweetwater, TN). Pine solution was placed in a dish underneath each chamber as well. Room lights were left on and white noise (65 db) was played in the background. The odor, tactile, auditory and visual stimuli of the chamber comprised the training “context”.

After a 3-min baseline period, mice received 6 shocks (1 mA, 2 s), separated by 1 min. They were removed from the chamber 30 s after the last shock. Mice were tested for fear to the training context the following day by placement back in their training chamber in the absence of shock for a period of 8 min. The context tested was identical to the training context.

Freezing, the absence of all movement except that necessary for respiration, is an innate defensive response in rodents and is a reliable measure of learned fear (Fanselow, 1980). A camera installed in an experimental room transmitted a video to the monitor placed in adjoining room where animals were scored online by the observer. The observer scoring the behavior was blinded to the genotypes of the mice. Each animal's behavior was scored every 8 s during the test. If the animal showed freezing, it was given a score of “1” for that observation; if the animal showed movement, it was given a score of “0” for that observation. A percentage was calculated by dividing the number of freezing observations a mouse had by the total number possible during the observation period. This number represented the animal's freezing score.

To derive ED_{50} values for etomidate suppression of fear conditioning, data were fit by a logistic dose response curve: $y = A2 + (A1 - A2)/(1 + (x/x0)^p)$, where A1 (max value) was fixed at 1 and A2 (min value) was fixed at 0, and $\text{ED}_{50} = x0$.

2.5. Data analysis

Electrophysiological data were analyzed using Clampfit 10.2 and 10.3 (Molecular Devices), Origin 9.0 (Microcal Software Inc., Northampton, MA), GraphPad Prism 6.05 (GraphPad Software Inc., La Jolla, CA), custom-written R programming language scripts (R Foundation for Statistical Computing, Vienna, Austria), and Mini Analysis Program 6 (Synaptosoft Inc., Decatur, GA).

2.5.1. LTP

The recording electrode site used for LTP analysis was the site with the largest amplitude fEPSP in response to the baseline stimulus (“dendritic channel”). Field EPSP slopes were analyzed using Clampfit (10.2, Molecular Devices) to determine the maximum rate of rise at the dendritic channel. The electrode site closest to the pyramidal cell layer (“pyramidal channel”), used for population spike analysis, was chosen by examining the evoked waveform in response to a stimulus that was suprathreshold for population spike generation. Slices without population spikes of at least 0.5 mV prior to TBS, or that showed unstable responses before or after TBS, were excluded from analysis. The coefficient of variation of the pre-tetanus baseline for the slices that met these inclusion criteria was 0.09. LTP was calculated as the percentage change of the fEPSP slope collected 10 min pre-TBS and the last 10 min (51–60 min) post-TBS of the LTP experiment.

2.5.2. Tonic inhibition

The level of tonic inhibition present in CA1 pyramidal cells was measured by adding the non-competitive GABA_A receptor antagonist picrotoxin (PTX, 200 μM) to the aCSF superfusate during whole cell recording. The amount of tonic current was calculated as the difference in the baseline current before and after the addition of PTX. The mean current values were obtained from Gaussian fits to all point amplitude histograms. Histograms (1 pA bin-width) were constructed using 1 min of data before and 20 s of data after the PTX effect had stabilized, which required ~ 2 min. The histogram generated from data before PTX application had skewed distribution towards larger negative value. To ensure that sIPSCs were not included into the measurement of tonic inhibition, the Gaussian fit was applied to the unskewed (outward current) portion of the distribution. The resulting parameters were used to

Table 1

Characteristics of spontaneous inhibitory postsynaptic currents recorded from CA1 pyramidal cells in WT and $\beta 3\text{-N265M}$ mice, in the absence (Ctrl) and presence of 1 μM etomidate (Etom). RT – 10–90% rise time, $\tau 1$ – fast component of deactivation, $\tau 2$ – slow component of deactivation, A1 – fractional contribution of fast component, τw – weighted time constant of deactivation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 7$ cells for each condition.

GABA _{A,fast}		Frequency (s ⁻¹)	10–90% RT (ms)	Amplitude (pA)	$\tau 1$ (ms)	$\tau 2$ (ms)	A1 (%)	τw (ms)
WT	Ctrl	21.1 \pm 2.7	0.61 \pm 0.03	-37.8 \pm 2.4	7.0 \pm 0.6	13.7 \pm 2.3	73 \pm 6	8.5 \pm 0.6
	Etom	14.9 \pm 2.0	0.59 \pm 0.04	-34.1 \pm 3.6	** 4.1 \pm 0.4	21.7 \pm 3.1	** 38 \pm 3	*** 14.9 \pm 2.0
$\beta 3\text{-N265M}$	Ctrl	19.3 \pm 1.7	0.60 \pm 0.04	-39.6 \pm 3.6	* 4.9 \pm 0.4	15.2 \pm 2.7	67 \pm 7	7.7 \pm 0.5
	Etom	17.2 \pm 1.5	0.60 \pm 0.04	-34.6 \pm 2.5	4.3 \pm 0.6	17.6 \pm 2.0	55 \pm 5	9.8 \pm 0.2

simulate a symmetric Gaussian curve. The difference between peak values of two simulated Gaussians was used as the measure of the baseline tonic current.

2.5.3. Synaptic currents

To detect sIPSCs, the search protocol threshold was set at 3 times the root mean square (RMS) noise level, which typically was 3–6 pA. For each cell, the averaged frequency and amplitude characteristics of sIPSCs were computed by the software. For each cell, at least 40 sIPSCs were averaged, normalized, and characterized by their 10–90% rise, rates of fast (τ_1) and slow (τ_2) components, and weighted (τ_w) decay times, and fraction of the fast component of deactivation (A1) (Table 1). The sIPSCs used for averaging were selected based on the presence of a stable baseline level and the lack of spontaneous events during the deactivation phase. These events were aligned at the time of half-maximal amplitude of the rising phase. The decay phases of averaged fast sIPSCs were fitted to bi-exponential functions using a Simplex fitting algorithm Mini Analysis Program 6 (Synaptosoft Inc.). Weighted decay time constant was calculated using the formula: $\tau_w = ((A1/(A1 + A2) * \tau_1) + ((A2/(A1 + A2) * \tau_2))$.

2.5.4. Recombinant receptors

For each excised-patch, 1–5 responses were averaged. Peak current amplitude was identified visually and the mean value of the surrounding 200 data points (10 ms) was measured. GABA concentration-response data were fit using GraphPad Prism software to the sigmoidal dose–response equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log EC_{50} - X) * \text{HillSlope}))})$, where Y is the peak response to a given GABA concentration, Top is the maximal amplitude of current, X is GABA concentration, and Bottom was constrained to 0. $EC_{10-GABA}$ was calculated using the equation $\log EC_{50} = \log EC_F - (1/\text{HillSlope}) * \log(F/(100-F))$, where F was set to 10. The calculated $EC_{10-GABA}$ derived from these fits was used in experiments to evaluate modulation by etomidate. The percent potentiation of GABA-evoked responses by etomidate was expressed as $((\text{Amplitude Drug}) / ((\text{Amplitude Control} + \text{Amplitude Drug}) / 2) - 1) * 100$.

2.6. Statistics

Data are presented as mean \pm SEM, with *n* specifying the number of mice, slices or recorded cells. Unless noted otherwise, statistical comparisons were performed using two-way ANOVA, with Tukey's post-hoc test for pairwise comparisons and *p*-values adjusted for multiple comparisons. For experiments assessing tonic inhibitory current under control conditions, a one-sample *t*-test was used to test the null hypothesis that the shift in baseline current upon PTX application was equal to zero. For experiments on expressed recombinant receptors, a one-sample *t*-test was used to test the null hypothesis that potentiation of the GABA-evoked response was equal to zero. For all experiments, the critical value for statistical significance was set at *p* < 0.05.

Variance in the ratio between independent estimates was calculated by the formula

$$\sigma_{A/B} \approx \left| \frac{A}{B} \right| \sqrt{\left(\frac{\sigma_A}{A} \right)^2 + \left(\frac{\sigma_B}{B} \right)^2}$$

where $\sigma_{A/B}$ is the standard deviation of the ratio, A is the mean of A, B is the mean of B, σ_A is the standard deviation of A, and σ_B is the standard deviation of B.

2.7. Chemicals

For experiments *in vitro*, etomidate ((*R*)-1-(1-phenylethyl)-1*H*-imidazole-5-carboxylic acid ethyl ester) was purchased from Tocris (Bristol, UK) as a powder that was dissolved in DMSO and kept in aliquots at 50 mM concentration. For each experiment, an aliquot was thawed and diluted appropriately in recording aCSF. The final concentration of DMSO did not exceed 0.1%, which in previous studies was found to have no effect on GABA_ARs (Harney et al., 2003). Effects of etomidate were quantified in slices that had been pre-incubated in etomidate for at least 1 h (Benkowitz et al., 2007). For behavioral experiments *in vivo*, etomidate was purchased pre-dissolved in a 35% propylene glycol solution at a concentration of 2 mg etomidate/ml solution (Hospira, Inc; Lake Forest, IL). All salts were obtained from Sigma–Aldrich, and kynurenic acid from Abcam Biochemicals®, NaHEPES from ChemCruz™ Biochemicals, and CaCl₂ × 2H₂O from Fisher Scientific.

3. Results

3.1. Etomidate enhances tonic inhibition in WT but not $\beta 3$ -N265M mice

To test whether tonic inhibition is mediated by GABA_A receptors that contain $\beta 3$ -subunits, we measured and compared the amplitudes of tonic currents in wild type and $\beta 3$ -N265M mice, under control conditions and in the presence of 1 μ M etomidate (Fig. 1). Under control conditions, the amplitude of tonic current (measured

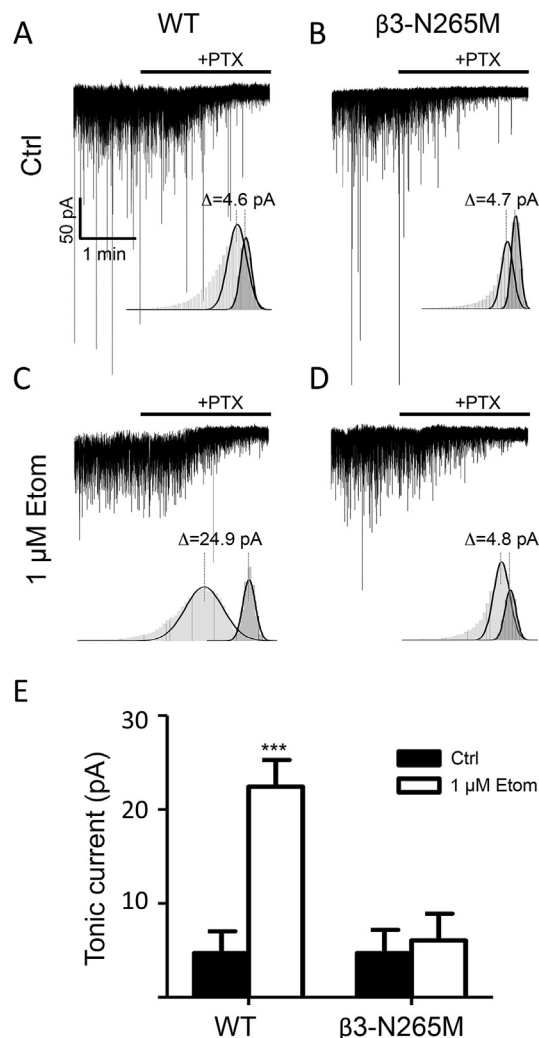


Fig. 1. Tonic inhibition is mediated by $\beta 3$ -subunit containing GABA_A receptors. (A and C) Representative traces containing spontaneous inhibitory currents recorded in WT mice in the absence (Ctrl) and presence of etomidate (1 μ M Etom). (B and D) Representative traces containing spontaneous inhibitory currents recorded in $\beta 3$ -N265M mice in the absence (Ctrl) and presence of etomidate (1 μ M Etom). Insets show frequency histograms (light and dark grey) with overlaid Gaussian fits (black). The peaks of the fits were used as a measure of the amplitude of tonic currents before and after application of the non-competitive GABA_ARs channel-blocker, picrotoxin (PTX, 200 μ M). (E) Summary bar plot of average amplitudes of tonic inhibition measured in the two genotypes under different conditions, ****p* < 0.001, *n* = 7 cells for each condition.

as the change in the baseline current upon addition of picrotoxin) approached, but did not reach, statistical significance (WT 4.7 ± 2.3 pA, *n* = 7, *p* = 0.09; $\beta 3$ -N265M 4.7 ± 2.5 pA, *n* = 7, *p* = 0.1; one-sample *t*-test). These values did not differ between genotypes (*p* = 0.99). Etomidate increased the amplitude of tonic current in WT mice (22.45 ± 2.8 pA; *p* = 0.005 vs. WT control) but not in $\beta 3$ -N265M mice (6.05 ± 2.8 pA; *p* = 0.98 vs. mutant control).

We conclude from these results that tonic inhibition in CA1 pyramidal neurons arises mostly or entirely from receptors that incorporate $\beta 3$ -subunits.

3.2. Etomidate modulates fast phasic inhibition in WT but not $\beta 3$ -N265M mice

Spontaneous inhibitory postsynaptic currents (sIPSCs) were evident in our whole cell recordings before PTX was applied. We characterized and compared the properties of these “GABA_{A, fast}”

sIPSCs in WT and $\beta 3$ -N265M mice, in the absence and presence of 1 μ M etomidate (Table 1).

Under control conditions, the only characteristic of sIPSCs that differed between genotypes was the fast component of decay (τ_1 , $p = 0.033$; for all others $p > 0.8$). Etomidate increased the weighted decay time constant (τ_w) in WT mice ($175 \pm 26\%$, $p = 0.005$), but not in $\beta 3$ -N265M mice ($127 \pm 8\%$, $p = 0.7225$). This change was driven by an increase in the relative proportion of the slow component of decay (A_2 , $p = 0.0005$), a reduction in the time constant of the fast component of decay (τ_1 , $p = 0.0025$), and a trend toward an increase in the time constant of the slow component (τ_2 , $p = 0.15$). None of these effects were observed in mutant mice, indicating that GABA_A, fast IPSCs are mediated largely by $\beta 3$ -containing GABA_ARs. There was also a trend toward reduced sIPSC frequency produced by etomidate in WT ($p = 0.16$) but not in mutant mice ($p = 0.88$). This finding suggests that the cells that give rise to fast spontaneous IPSCs are themselves inhibited via $\beta 3$ -GABA_ARs.

3.3. Etomidate blocks LTP in WT and $\beta 3$ -N265M mice

It has been proposed that enhancement of tonic inhibition in CA1 pyramidal cells by etomidate accounts for its ability to block synaptic plasticity *in vitro* and learning and memory *in vivo* (Cheng et al., 2006). Thus, we hypothesized that etomidate would fail to block LTP or impair memory in $\beta 3$ -N265M mice, since it did not potentiate tonic inhibition. To test this hypothesis, we induced LTP of the fEPSP using TBS in slices from WT and $\beta 3$ -N265M mice, under control conditions and in the presence of 0.25 μ M etomidate (Fig. 2).

Theta burst stimulation induced LTP under drug-free conditions in both genotypes (WT $165 \pm 17\%$, $n = 7$; $\beta 3$ -N265M $160 \pm 10\%$, $n = 7$). Etomidate reduced the amplitude of LTP (WT $129 \pm 11\%$, $n = 8$; $\beta 3$ -N265M $122 \pm 7\%$, $n = 7$), by an amount that did not differ between genotypes (genotype $F(1,25) = 0.4$, $p = 0.5$; drug $F(1,25) = 10.24$, $p < 0.01$; interaction $F(1,25) = 0.01$, $p = 0.9$; two-way ANOVA).

From these results we conclude that, contrary to our expectation, etomidate is able to block synaptic plasticity *in vitro* in $\beta 3$ -N265M mice as well as in WT mice. The dissociation between the effect of etomidate on tonic inhibition and on LTP suggests that enhanced tonic inhibition is not the mechanism by which etomidate blocks TBS-induced synaptic plasticity.

3.4. Etomidate impairs fear conditioning to context in WT and $\beta 3$ -N265M mice

To test whether etomidate impairs learning and memory *in vivo* by modulating GABA_A receptors that incorporate $\beta 3$ -subunits, we compared effects of different doses of etomidate on fear conditioning to context in WT and $\beta 3$ -N265M mice (Fig. 3).

Under drug-free conditions, freezing scores did not differ in WT vs. $\beta 3$ -N265M mice ($55.7 \pm 9.5\%$, $n = 10$ vs. $35 \pm 11.5\%$, $n = 8$; $p = 0.861$). Increasing doses of etomidate (7.5–15 mg/kg) reduced freezing scores in both genotypes (Fig. 3), with an ED₅₀ dose of 8.2 ± 1.1 mg/kg, 95% confidence interval [CI] 4.5–9.6 mg/kg in WT, and 8.8 ± 0.8 mg/kg, 95% confidence interval [CI] 6.6–10.7 mg/kg in $\beta 3$ -N265M mice.

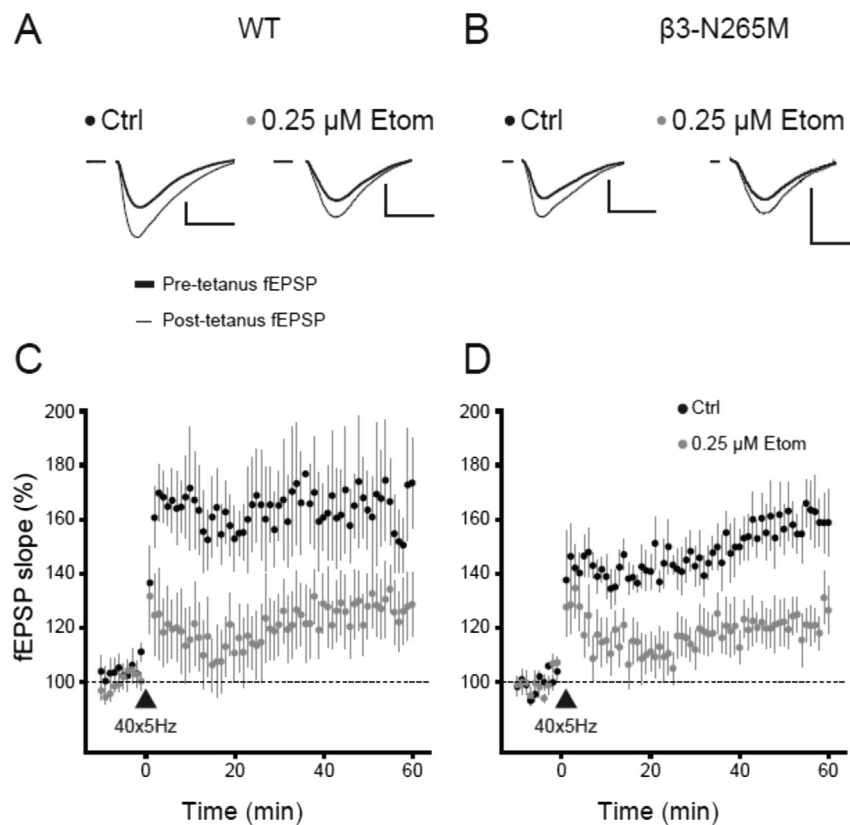


Fig. 2. Effect of etomidate on long-term potentiation does not depend on $\beta 3$ -subunit containing GABA_A receptors. (A and B) Representative traces showing field excitatory post-synaptic potentials (fEPSPs) recorded from stratum radiatum of the CA1 region in slices from WT (A) and $\beta 3$ -N265M mice (B) in the absence (Ctrl) and presence of etomidate (0.25 μ M Etom). Traces are the averages of fEPSPs acquired 10 min pre-TBS (thick line) and the last 10 min (51–60 min) post-TBS (thin line). Scale bars indicate 0.5 mV and 10 ms. (C and D) Summary time series of the fEPSPs slope changes following 40×5 Hz TBS (triangle) in WT (C) and $\beta 3$ -N265M mice (D) in the absence (black circles, Ctrl) and presence of etomidate (grey circles, 0.25 μ M Etom), $n = 7$ –8 slices per group.

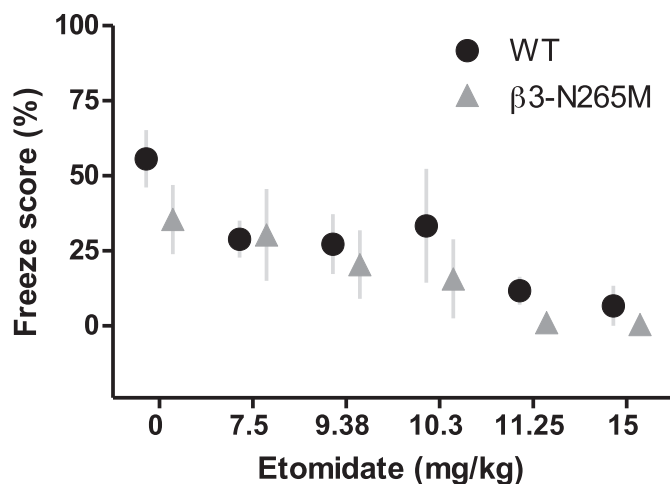


Fig. 3. Effect of etomidate on contextual fear conditioning does not depend on $\beta 3$ -subunit containing GABA_A receptors. Learning was assessed as % time spent freezing when re-exposed to context in WT (black circles) and $\beta 3$ -N265M (grey triangles) mice. Increasing doses of etomidate reduced freezing scores equally in both genotypes, $n = 3$ –10 mice per group, 7 mice on average.

From these results we conclude that etomidate is able to block learning and memory *in vivo* in $\beta 3$ -N265M mice as in WT mice, thus mirroring our findings above for TBS-induced LTP. Taken together, our findings indicate that neither tonic inhibition nor $\beta 3$ -GABA_ARs contribute critically to the amnestic effect of etomidate.

3.5. Amnestic concentrations of etomidate do not modulate $\alpha 5\beta 1\gamma 2L$ GABA_A receptors

Previous studies of recombinant $\alpha 1\beta\gamma 2S$ receptors expressed in *Xenopus* oocytes showed that etomidate modulates receptors that incorporate $\beta 2$ - or $\beta 3$ -subunits, but not $\beta 1$ -subunits (Sanna et al., 1997). This result would seem to indicate that the target of etomidate in $\beta 3$ -N265M mice must be receptors that incorporate $\beta 2$ -subunits. However, a recent report that 3 μM etomidate potentiates GABA-evoked responses of $\alpha 5\beta 1\gamma 2$ -GABA_ARs to similar extent as $\alpha 5\beta 3\gamma 2$ -GABA_ARs (Janssen et al., 2009) caused us to re-examine the selectivity of etomidate for $\beta 2$ and $\beta 3$ versus $\beta 1$ -subunits when expressed in combination with $\alpha 5$ subunits.

A summary of results is presented graphically in Fig. 4. At a concentration of 0.25 μM , which corresponds to ED50-amnesia (Benkowitz et al., 2007), etomidate potentiated responses of $\alpha 5\beta 2\gamma 2L$ - and $\alpha 5\beta 3\gamma 2L$ -GABA_ARs, but not $\alpha 5\beta 1\gamma 2L$ -GABA_ARs. Similarly, 1 μM etomidate potentiated $\alpha 5\beta 2\gamma 2L$ - and $\alpha 5\beta 3\gamma 2L$ -GABA_ARs, but not $\alpha 5\beta 1\gamma 2L$ -GABA_ARs. Only in the presence of 4 μM etomidate, which corresponds to 16 times the ED50-amnesia concentration, were $\alpha 5\beta 1\gamma 2L$ -GABA_ARs potentiated ($27 \pm 14\%$, $p = 0.0002$, one-sample t-test). However, the amount of potentiation was significantly smaller than for $\alpha 5\beta 2\gamma 2L$ - and $\alpha 5\beta 3\gamma 2L$ -GABA_ARs ($130 \pm 74.5\%$, $p < 0.0001$ and $266 \pm 102\%$, $p < 0.0001$, respectively).

From these results we conclude that amnestic concentrations of etomidate do selectively target $\beta 2$ - and $\beta 3$ -GABA_ARs. Therefore, effects of etomidate on LTP and learning *in vivo* arose most likely from modulation of $\alpha 5\beta 2$ -GABA_ARs.

4. Discussion

The data presented here support the hypothesis that $\alpha 5\beta 3$ -GABA_ARs underlie tonic inhibition in hippocampal CA1 pyramidal cells. However, they also show that impairment of learning *in vivo*

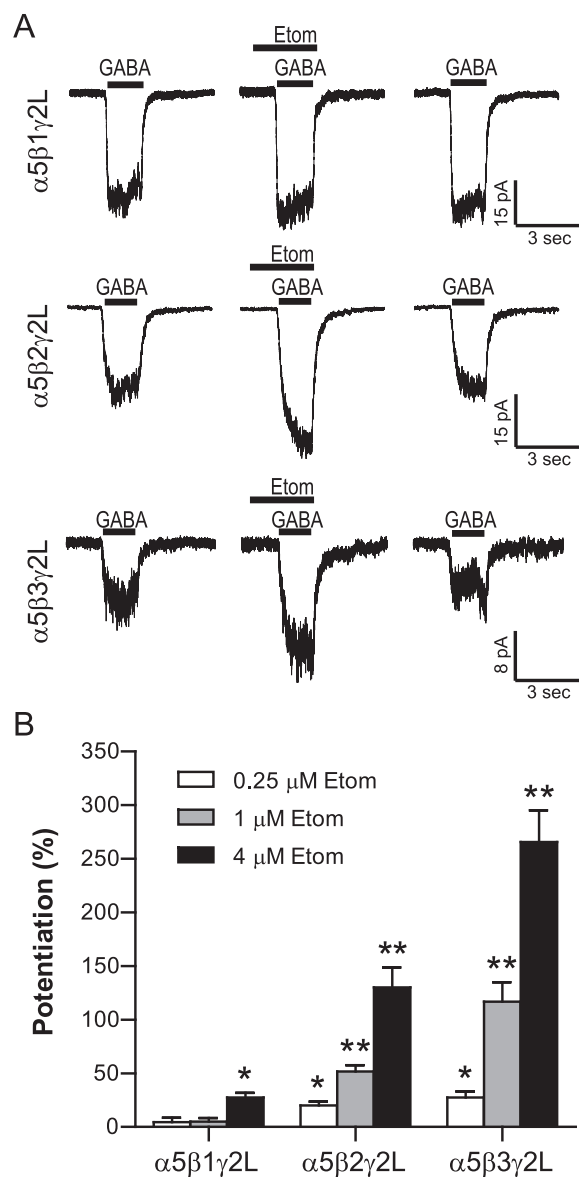


Fig. 4. $\alpha 5\beta 1\gamma 2$ GABA_ARs resist potentiation by etomidate. (A) Responses of recombinant receptors in excised outside-out patches in the absence and presence of 1 μM etomidate. Receptors were activated by EC₁₀ concentrations of GABA, which were 16 μM for $\alpha 5\beta 1\gamma 2L$, 6.2 μM for $\alpha 5\beta 2\gamma 2L$, and 1.3 μM for $\alpha 5\beta 3\gamma 2L$. (B) Summary bar plot of average potentiating effect of 0.25 μM , 1 μM and 4 μM etomidate on GABA-evoked responses. * $p < 0.001$, ** $p < 0.0001$, $n = 11$ –19 patches for each condition.

and LTP *in vitro* by etomidate is independent of $\beta 3$ -subunit containing GABA_ARs. This dissociation between enhanced tonic inhibition and impaired plasticity and memory challenges the notion that tonic inhibition mediates the amnestic effect of etomidate, as derived from studies of $\alpha 5$ -KO mice (Cheng et al., 2006; Martin et al., 2009).

To arrive at these conclusions we used mice carrying a point mutation in the $\beta 3$ -subunit of the GABA_AR that renders these receptors insensitive to etomidate (Jurd et al., 2003). This model allowed us to dissect the roles of different β -subunits in etomidate enhancement of tonic inhibition and suppression of learning and memory. Our results are consistent with previous reports that $\alpha 5$ -GABA_ARs mediate tonic inhibition in CA1 pyramidal neurons (Caraiscos et al., 2004), and that $\alpha 5$ -GABA_ARs are important for learning and memory (Collinson et al., 2002; Crestani et al., 2002). However our data indicate that $\alpha 5$ -subunits do so in partnership

with $\beta 2$ -subunits. One caveat is that we have not measured $\alpha 5$ -subunit expression directly, so it is possible that associated changes in these subunits may have occurred in these mice. However, substantial changes in $\alpha 5$ subunit expression seems unlikely, since the knock-in mutation does not alter expression of the $\beta 3$ subunit itself, nor of any of the other subunits that have been measured ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 2/3$, $\gamma 2$) (Jurd et al., 2003). Moreover, reduction in the expression of $\alpha 5$ -GABA_ARs would itself be expected to prevent etomidate from impairing LTP (Cheng et al., 2006), which did not occur in these mice (Fig. 3).

The finding that etomidate achieves its amnesic effects through selective modulation of $\alpha 5\beta 2$ -containing GABA_ARs is unexpected. Previous investigators showed that $\alpha 5$ subunits are essential for etomidate-induced amnesia (Cheng et al., 2006), that $\alpha 5$ subunits preferentially co-assemble with $\beta 3$ subunits (Luddens et al., 1994; Sur et al., 1998), that properties of recombinant $\alpha 5\beta 3\gamma 2$ receptors are similar to those of native receptors in CA1 pyramidal cells, which are enriched in $\alpha 5$ and $\beta 3$ subunits (Burgard et al., 1996; Sur et al., 1998; Caraiscos et al., 2004), and that $\alpha 5$ and $\beta 3$ subunits are co-depleted in mice lacking either the $\beta 3$ or $\alpha 5$ subunits (Olsen and Homanics, 2000). Nevertheless, there is evidence that native $\alpha 5\beta 2$ receptors do exist, though they constitute a minority of $\alpha 5$ -containing receptors (Ju et al., 2009).

Our findings support behavioral results obtained in forebrain specific $\beta 3$ -KO mice showing that $\beta 3$ -GABA_ARs do not mediate etomidate-induced amnesia (Rau et al., 2011). Previous experiments in $\beta 3$ -N265M mice similarly showed that propofol-induced amnesia is independent of $\beta 3$ -GABA_ARs (Zeller et al., 2007). Experiments with isoflurane produced conflicting results: studies in $\beta 3$ -N265M knock-in mice indicated no contribution of $\beta 3$ subunits (Liao et al., 2005) but studies in forebrain-specific knockout suggested a partial contribution (Rau et al., 2011). Because of the lower likelihood for compensatory changes in receptor expression in knock-in vs. knock-out mice, the present results strengthen the evidence that etomidate acts through receptors that lack $\beta 3$ -subunits.

At doses that impair memory, etomidate also causes sedation, as indicated by a decrease in exploratory activity, (Benkowitz et al., 2007). Since sedation has been attributed to modulation of $\beta 2$ -containing GABA_ARs (Jurd et al., 2003; Reynolds et al., 2003), this effect may well have contributed to the $\beta 2$ -GABA_AR-mediated learning impairment that we observed *in vivo*. However, this consideration does not apply to experiments *in vitro*. Therefore, the finding that etomidate suppresses LTP in $\beta 3$ -N265M mice implicates $\beta 2$ -GABA_ARs in etomidate-induced amnesia specifically, rather than indirectly by causing sedation.

Since $\beta 3$ -GABA_AR-mediated tonic inhibition appears not to be the means by which etomidate impairs synaptic plasticity and learning, what inhibitory processes might be involved? Our analysis of spontaneous GABA_A, fast IPSCs showed that they are mediated primarily by $\beta 3$ -GABA_ARs. Also, fast inhibitory currents are relatively insensitive to amnesic concentrations of etomidate (Dai et al., 2009). Taken together, these findings indicate that GABA_A, fast inhibition is unlikely to contribute substantially. By contrast, GABA_A, slow, an inhibitory current that overlaps anatomically with excitatory receptors on the dendrites (Pearce, 1993), was shown previously to engage $\alpha 5$ -GABA_ARs (Zarnowska et al., 2009), and to be particularly sensitive to amnesic concentrations of etomidate (Dai et al., 2009). This form of inhibition thus appears to be well suited to anesthetic control of synaptic plasticity and memory.

It is also possible that etomidate controls synaptic plasticity and memory by targeting interneurons. GABA_A, slow synapses have been observed in interneurons (Banks et al., 2000), and activation of synaptic $\alpha 5$ -GABA_ARs significantly reduces the excitability of *oriens lacunosum-moleculare* (OLM) interneurons (Salesse et al., 2011). In

addition, OLM interneurons have been shown to impact mnemonic processes in the hippocampus by inhibiting other interneurons located in *stratum radiatum* of the hippocampus (Leao et al., 2012). Thus, etomidate may target non- $\beta 3$ -subunit containing GABA_ARs on interneurons to impair learning and memory and synaptic plasticity through “disinhibition” (Freund and Gulyas, 1997; Pi et al., 2013; Wolff et al., 2014). Experiments that restrict changes in receptor expression or anesthetic sensitivity to specific classes of neurons (Lee and Maguire, 2013) will be useful for testing these possibilities.

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