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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

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ABSTRACT

Enhancement of tonic inhibition mediated by extrasynaptic α5-subunit containing $GABA_A$ receptors ($GABA_A$Rs) has been proposed as the mechanism by which a variety of anesthetics, including the general anesthetic etomidate, impair learning and memory. Since α5 subunits preferentially partner with β3 subunits, we tested the hypothesis that etomidate acts through β3-subunit containing $GABA_A$Rs 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 $GABA_A$R β3-subunit (β3-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 β3-subunit containing $GABA_A$Rs. 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, α5β1γ2L $GABA_A$Rs were insensitive to amnestic concentrations of etomidate (1 μM and below), whereas α5β2γ2L and α5β3γ2L $GABA_A$Rs were enhanced. We conclude that etomidate enhances tonic inhibition in pyramidal cells through its action on α5β3-containing $GABA_A$ receptors, but blocks LTP and impairs learning by other means - most likely by modulating α5β2-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).

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 ($GABA_A$Rs) 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). $GABA_A$Rs are considered to be

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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 β1-subunits (together with α1-subunits) are markedly less sensitive to etomidate compared to those that incorporate β2-or β3-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 (β2-N265S or β3-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 $GABA_AR$ α5-subunits (α5-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 α5-$GABA_ARs$ 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 α5-subunits preferentially partner with β3-subunits (Luddens et al., 1994; Sur et al., 1998), we hypothesized that mice carrying the N265M mutation in the β3-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 β3-N265M mice was indeed insensitive to etomidate, showing that this form of inhibition is mediated by β3-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 β1-subunits together with α5-subunits were insensitive to amnestic concentrations of etomidate, mirroring previous results from studies of α1-containing $GABA_ARs$ (Sanna et al., 1997). We conclude that tonic inhibition in CA1 pyramidal cells is mediated by α5β3-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 α5β2-$GABA_ARs$.

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 β3 subunit (β3-N265M), and homozygous wild-type controls, were used for this study. The strain background of the β3-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 (Novaplus, 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 $MgSO_4 \times 7H_2O$, 0.9 $CaCl_2 \times 2H_2O$, 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_2PO4, 1.4 $MgSO_4 \times 7H_2O$, 2.2 $CaCl_2 \times 2H_2O$, 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 $MgSO_4 \times 7H_2O$, 0.5 $CaCl_2 \times 2H_2O$, 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 $MgSO_4 \times 7H_2O$, 2 $CaCl_2 \times 2H_2O$, 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 $GABA_AR$ subunits (α5,β1,β2,β3,γ2L) 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 MΩ, World Precision Instruments, Sarasota, FL) placed in *stratum radiatum* for activation of the Schaffer collateral/commissural path (SC). Recorded signals were amplified 1000×, 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 μA. 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 × 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