

Etomidate Targets α_5 γ-Aminobutyric Acid Subtype A Receptors to Regulate Synaptic Plasticity and Memory Blockade

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Background: **The memory-blocking properties of general anesthetics have recently received considerable attention because of concerns related to intraoperative awareness and postoperative cognitive dysfunction. The goal of this study was to identify the mechanisms by which γ-aminobutyric acid subtype A receptors that contain the α_5 subunit (α_5GABA$_A$Rs) induce memory-blockade by etomidate and a pharmacologic strategy to reverse this impairment.**

Methods: **The effects of etomidate and the α_5GABA$_A$R-preferring inverse agonist L-655,708 on the plasticity of glutamatergic excitatory transmission in hippocampal slices and behavioral memory for spatial navigational and fear-associated memory tasks were studied in wild-type and null mutant mice for the gene that encodes the α_5 subunit (*Gabra5*−/− mice). Long-term potentiation of field excitatory postsynaptic potentials was induced in CA1 pyramidal neurons following high-frequency stimulation of Schaffer collaterals. Memory performance was studied in contextual, cued, and trace fear conditioning assays and the Morris water maze.**

Results: **Robust synaptic plasticity induced by high-frequency stimulation and memory performance for contextual fear and spatial navigational memory were not influenced by a decrease in the function of α_5GABA$_A$Rs. Nevertheless, etomidate, *via* an increase in α_5GABA$_A$R activity, completely blocked long-term potentiation and impaired memory performance, and these effects were reversed by pretreatment with L-655,708.**

Conclusions: **The results provide the first proof of concept that memory blockade by a general anesthetic can be reversed by inhibiting the function of α_5GABA$_A$Rs. The findings suggest a mechanism and model for awareness during anesthesia.**

THE single most common fear expressed by patients who are about to undergo surgery is that they will remember traumatic surgical events.[1] Unfortunately, 1 in 1,000 patients who undergo general anesthesia do experience some form of awareness during surgical procedures,[2] and the incidence may be even higher among children.[3] Despite the disturbing frequency of this problem, the mechanisms underlying insufficient amnesia during surgery remain elusive. While the "memory disorders" associated with general anesthesia, including awareness and persistent undesirable memory deficits after anesthesia, likely result from complex cellular processes, specific targets of interest have recently been identified.[4] In particular, many of the behavioral endpoints associated with the anesthetic state are mediated, at least in part, by positive allosteric modulation of γ-aminobutyric acid type A receptors (GABA$_A$Rs).[5] GABA$_A$Rs are heteropentameric ion channels that form from a combination of different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε , π, θ, ρ_{1-3}). The regional and cell-specific distributions of these subunits present the possibility that GABA$_A$R subtypes can be selectively targeted to alter activity in specific neuronal networks and behaviors.[6] For example, the α_5 subunit of the GABA$_A$R has been strongly implicated in mediating the memory-blocking properties of inhaled and intravenous anesthetics.[7–9] A high proportion of these receptors are expressed in CA1 and CA3 pyramidal neurons of the hippocampus, a structure that is critically involved in the encoding, consolidation, and retrieval of episodic memories.[10] Electrophysiologic studies have shown that α_5GABA$_A$Rs generate a tonic inhibitory conductance in CA1 pyramidal neurons in the hippocampus of rodents,[8] and this tonic conductance is enhanced by low, memory-blocking concentrations of anesthetics.[7,9] More importantly, genetically engineered null mutant mice that lack the α_5 subunit (*Gabra5*−/− mice) exhibit resistance to the amnestic properties of etomidate through mechanisms that are poorly understood.[9]

A molecular process that is thought to be essential to the storage of information involving the hippocampus is the long-term modification of excitatory glutamatergic transmission, which is known as long-term potentiation (LTP).[11] LTP is the most widely studied *in vitro* model for memory and is evoked by repetitive stimulation of relevant afferent pathways. Similar changes in glutamatergic synaptic strength occur *in vivo* during memory formation.[12] Etomidate, studied at a concentration that occurs *in vivo* during memory impairment, abolished LTP induced by high-frequency stimulation in hippocampal slices from wild-type (WT) but not *Gabra5*−/− mice.[9] The authors and others have shown that genetic deletion of α_5GABA$_A$Rs does not alter the strength of LTP evoked by high-frequency stimulation in hippocampal slices.[9,13] Others report that paired pulse facilitation was enhanced and the inhibitory postsynaptic currents were

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decreased in slices prepared from *Gabra5−/−* mice.[13] The mechanisms by which α_5GABA$_A$Rs regulate synaptic plasticity and the memory-blocking property of etomidate remain unclear. It is plausible that etomidate increases the activity of α_5GABA$_A$Rs, even under conditions where these receptors do not play a dominant physiologic role, and thereby attenuates synaptic plasticity and memory. To test this hypothesis, studies were designed to determine whether pharmacologically inhibiting the activity of α_5GABA$_A$Rs by pretreatment with L-655,708 altered etomidate blockade of synaptic plasticity and behavioral memory. L-655,708 is a imidazobenzodiazepine inverse agonist that preferentially reduces both the function of human recombinant α_5GABA$_A$Rs[14] and a tonic inhibitory conductance in CA1 pyramidal neurons.[8,15] Animal studies suggest that memory blockade, but neither hypnosis nor immobility, is influenced by α_5GABA$_A$R activity.[9] If correct, the model could account for why subjects with a reduced complement of functional α_5GABA$_A$Rs who exhibit normal memory performance for hippocampus-dependent learning tasks are at risk for awareness during general anesthesia.

Materials and Methods

Experimental Animals

All experimental procedures and protocols were approved by the Animal Care Committee of the University of Toronto (Toronto, Ontario, Canada). Mice were obtained from two sources. Male mice (postnatal age [P] P90–P120) were purchased from Taconic Laboratories (Germantown, NY) or were obtained from the authors' breeding colony. The commercially purchased mice had the same hybrid genetic background (50% C57Bl/6 and 50% Sv129Ev) as *Gabra5−/−* mice.[13] WT and *Gabra5−/−* mice were bred in the University of Toronto animal care facilities. The generation, genotyping, and characterization of *Gabra5−/−* mice have been previously described.[13] Behavioral studies of *Gabra5−/−* mice used aged-matched male WT controls. All mice were handled in 5-min epochs every day, for 1 week before their use in behavioral experiments. The experimenter was blind to the drug treatment and genotype of the mice for all studies.

Synaptic Plasticity in Hippocampus Slices

The LTP of excitatory potentials was studied with hippocampal slices prepared from P90–P120 mice. Mice were decapitated during isoflurane anesthesia, and their brains were quickly removed and placed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (composition: 124 mM NaCl, 3 mM KCl, 1.3 mM $MgCl_2$, 2.6 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM d-glucose), with the osmolarity adjusted to 300–310 mOsm. Transverse brain slices (350 μm thick) were prepared with a VT1000E tissue slicer (Leica, Deerfield, IL). After a recovery period of 1 h in the oxygenated artificial cerebrospinal fluid, the slices were transferred to a submersion-type recording chamber. The residual concentration of isoflurane is assumed to be negligible under the above conditions. Field excitatory postsynaptic potentials (fEPSPs) were recorded at room temperature (21°–23°C) as previously described.[9] Baseline stimulation frequency was 0.05 Hz, and the stimulus intensity was adjusted to evoke a half-maximal fEPSP amplitude. LTP was induced in the slices by stimulating with a theta burst stimulation (TBS) protocol, which consisted of 10 stimulus trains at 5 Hz, with each train including 4 pulses at 100 Hz.

Vehicle (dimethyl sulfoxide), etomidate (1 μM), L-655,708 (20 nM), or both etomidate and L-655,708 were perfused into the recording chamber for 15 min before the induction of LTP. The fEPSPs were monitored before and 60 min after TBS. L-655,708 is an inverse agonist, which is a compound that binds to the same receptor binding site as the agonist but has an opposite pharmacologic effect. It has a 100-fold higher functional affinity for α_5GABA$_A$Rs than for GABA$_A$Rs that contain the α_1, α_2, or α_3 subunits.[14,16,17] The concentration of L-655,708 selected for use in this study binds preferentially to α_5GABA$_A$Rs in tissue slices,[17] whereas the concentration of etomidate was selected because it occurs in the brains of mice injected with an amnestic dose of etomidate.[9] Others have suggested that the free aqueous concentration of etomidate that corresponds to amnesia *in vivo* is 0.25 μM.[18] This value was based on an estimate of the brain:artificial cerebral spinal fluid partition coefficient (3.35). However, to achieve an appropriate steady state concentration at the depth of the recording electrode, perfusion of the slices for up to several hours may be required. Given the time-dependent decline in the integrity of the hippocampus slices, studies of plasticity were performed approximately 15–20 min after application of the drug, and a higher concentration of etomidate was added to the extracellular solution (1 μM).

Voltage Clamp Recordings

The extracellular recording solution contained 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM) and (2R)-amino-5-phosphonovaleric acid (10 μM) to block ionotropic glutamate receptors and tetrodotoxin (0.3 μM) to block voltage-dependent sodium channels. Patch pipettes had open tip resistances of 3–5 MΩ when filled with an intracellular solution that contained mM CsCl (140), 10 mM HEPES, 10 mM EGTA, 2 mM MgATP, and 1 mM $CaCl_2$ (pH 7.3 with CsOH, 295–305 mOsm). Currents were sampled at 10 kHz and filtered at 2 kHz by using an eight-pole low-pass Bessel filter. All cells were recorded at a holding potential of −60 mV. A stable baseline current (< 20% change) was confirmed before the application of drugs. The amplitude of the tonic current under control conditions was measured as the difference in the holding current before and during the application of etomidate (1 μM), L-655,708 (20 nM), bicuculline (10 μM), or a combination of these drugs.

Fear-conditioned Learning

In the pavlovian fear conditioning tasks, mice were exposed to a tone, which was subsequently paired with a foot shock in a novel conditioning context, with either no time delay (0 s for cued conditioning) or an interval of 20 s between the tone and the foot shock (trace conditioning).[19,20] Several different associative memory tests were conducted to determine the contribution of certain brain regions for which the extent of expression of α_5GABA$_A$Rs differs. More specifically, the hippocampus, which has a high expression of α_5GABA$_A$Rs, is known to play a key role in contextual and trace fear conditioning.[19,21] In contrast, the expression of α_5GABA$_A$Rs is relatively low in the amygdala.[22] Cued fear conditioning, which requires the basal lateral nucleus of the amygdala, served as a control.[23]

Thirty minutes before being placed in the fear conditioning chamber, mice were randomly assigned to receive an intraperitoneal injection (2 ml/kg) of the vehicle (35% propylene glycol, 10% dimethyl sulfoxide), etomidate (4 mg/kg), L-655,708 (0.7 mg/kg), or the combination of etomidate and L-655,708 (administered together). For these experiments, the dose of etomidate[9] was carefully selected to cause conscious amnesia, a state characterized by minimal sedation (which confounds the study of learning and memory) combined with loss of explicit or episodic memory.[24] In addition, the dose of L-655,708 was selected to modify learning behaviors *via* preferential modulation of α_5GABA$_A$Rs, as previously determined.[25] On day 1, single animals were allowed to explore the chamber for 180 s. An 800-Hz tone, created by a frequency generator, amplified to 70 dB, and lasting 20 s, was then presented. For cued fear conditioning, the last 2 s of each auditory tone was paired with an electric foot shock (2 s, 1 mA); for trace fear conditioning, the auditory stimulus and foot shock (2 s, 0.5 mA) were separated by 20 s. Each of these sequences was presented three times, separated by 60 s (for cued fear conditioning) or 240 s (for trace fear conditioning). For contextual fear conditioning, either a strong (2 s, 1 mA) or weak (2 s, 0.5 mA) foot shock was applied, depending on the protocol. On day 2, 24 h after the conditioning session, each mouse was assessed for a freezing response by placing it in the original context and scoring every 8 s for a total of 8 min to determine contextual fear. On day 3, the conditioning chamber was modified to measure the freezing response to the tone to study either cued or trace fear conditioning. Mice were monitored for 180 s for freezing to the modified context, to rule out contextual influences. After the monitoring period, the auditory tone was presented continuously for 300 s, and the freezing response was measured every 8 s.

Water Maze Learning

The water maze is a hippocampus-dependent spatial navigation task that requires the mouse to use visual cues positioned around the room to locate a hidden platform in a circular tub of opaque water and has been previously described.[26] Briefly, a circular pool of diameter 1.2 m was filled with tap water (25° ± 2°C), which was made opaque by the addition of a white nontoxic paint. Mice were pretrained for 10 days, with four trials on each day to locate a hidden platform. In the match-to-place paradigm, the location of the platform was changed daily, and the first trial of each day was used as a comparator or reference trial to determine learning on trials 2, 3, and 4. During the acquisition phase of the probe trial, each mouse was randomly assigned to receive an intraperitoneal injection of vehicle, etomidate (4 mg/kg), L-655,708 (0.7 mg/kg), or both etomidate and L-655,708 (administered together) 30 min before the experiment. The next day, a probe trial was performed to test the ability of the mice to recall the correct spatial location that previously contained the hidden platform. Data records were stored with HVS Water 2020 software (VHS Image, Hampton, United Kingdom) for off-line analysis. The time, swim path, and latency of each mouse were recorded during each trial, and the percentage of time spent in the correct region was calculated by the software during analysis.

Visible platform trials were also performed to test for possible differences in motivational factors, perceptual and motor abilities, and any possible nonspecific effects of etomidate and L-655,708 as previously described.[9] The mice were injected 30 min before the visible platform trial, similar to the treatment during the learning acquisition phase before the probe trial.

Elevated Plus Maze

The elevated plus maze is designed to measure the anxiety levels of the mice.[27] The test hinges on the natural tendency of rodents to explore a novel environment and their aversion to open, elevated, and brightly lit areas. The elevated plus maze consisted of four arms (5 cm × 27.5 cm) that were joined by a central area (5 cm × 5 cm). Two opposite arms were enclosed by 30-cm-high walls, and the other two arms were open.

Mice were injected intraperitoneally with vehicle, etomidate (4 mg/kg), L-655,708 (0.7 mg/kg), or both etomidate and L-655,708 (administered together) 30 min before the experiment. Mice were placed in the central area of the maze facing an open arm and were scored for the amount of time they spent in the central area, the open arms, or the closed arms. The number of entries into the open and closed arms was also monitored. All mice were allowed to explore the maze for 5 min.

Statistical Analysis

Statistical analyses for the electrophysiologic and behavioral data were completed with GraphPad Prism Version 4.0c (San Diego, CA). All pooled data are presented as mean ± SEM. Electrophysiologic and behavioral statistical comparisons were completed using a one-way (*i.e.*, drug treatment only) or two-way (drug treatment *vs.* genotype) analysis of variance with two-tailed infer-

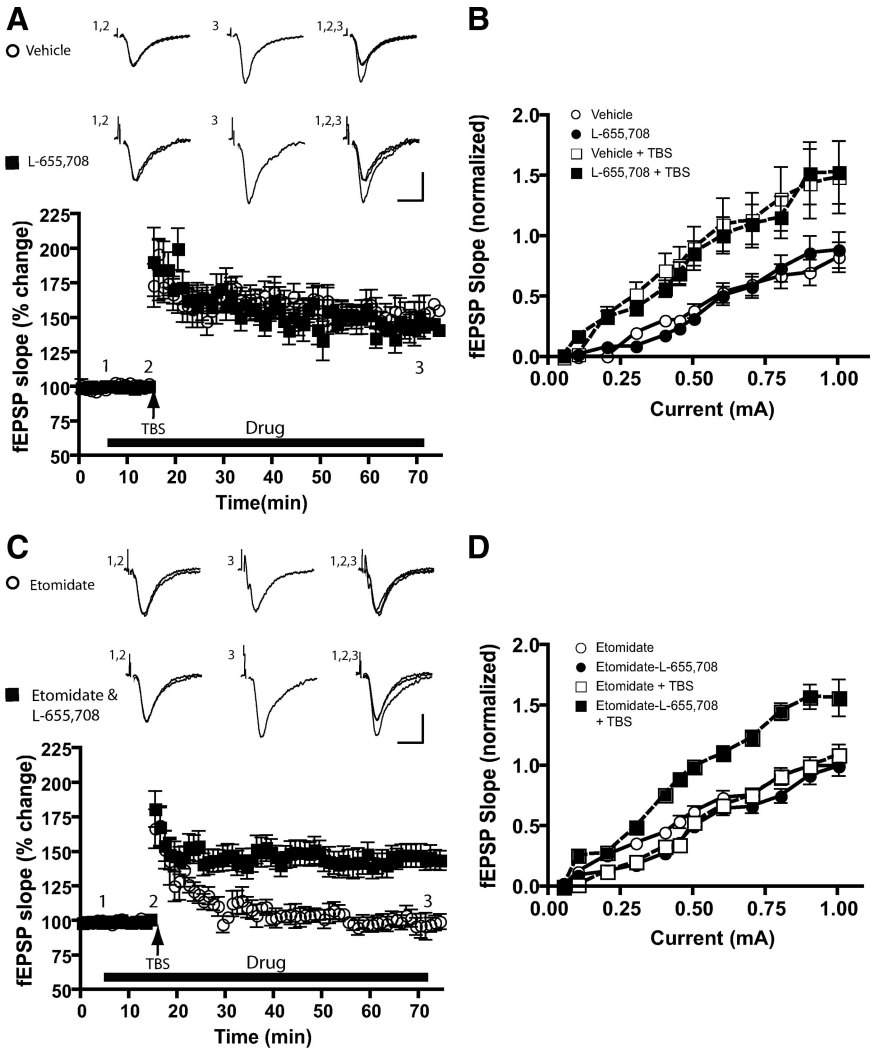

Fig. 1. The activity of α_5-containing γ-aminobutyric acid subtype A receptors does not modify long-term potentiation (LTP) evoked by high frequency stimulation but mediates etomidate-induced LTP impairment. (*A*) L-655,708 does not potentiate LTP above control levels. This suggests minimal involvement for α_5-containing γ-aminobutyric acid subtype A receptors in theta burst stimulation (TBS) LTP. (*B*) Input–output curves before and after TBS in the presence and absence of L-655,708. There were no differences between vehicle-treated and L-655,708-treated slices, although excitability increased after TBS in both groups. (*C*) Etomidate blocked LTP, and this effect was reversed by applying both L-655,708 and etomidate. (*D*) Input–output curves before and after TBS with etomidate application in the presence and absence of L-655,708. There were no differences between vehicle-treated and L-655,708-treated slices. Raw traces presented above the LTP plots represent the no-drug baseline field excitatory postsynaptic potential (fEPSP) and drug baseline fEPSP (1 and 2) and the drug post-TBS fEPSP.[3] *Calibration bars*: 0.5 mV, 10 ms.

ence testing. *Post hoc* analyses were conducted using the Tukey–Kramer method, which accounted for both equal and unequal sample size comparisons. For the plots of LTP, the data points (slope of the fEPSP measured between 25% and 70% of the rising phase) were binned in 1-min increments to facilitate readability. The extent of LTP was quantified for statistical comparisons by averaging the slope of the fEPSPs during the final 5 min of each experiment and normalizing to baseline values. $P < 0.05$ was considered statistically significant.

Results

L-655,708 Reverses Etomidate Blockade of Long-term Potentiation

First, to determine whether a reduction in α_5GABA$_A$R activity modifies synaptic plasticity induced by TBS, L-655,708 (20 nM) was applied at a concentration that selectively blocks the tonic inhibitory conductance in CA1 pyramidal neurons without substantially altering inhibitory synaptic transmission.[15] In vehicle-treated slices, robust LTP was induced following a 1-s presentation of TBS, such that the slope of the fEPSP was significantly increased ($P = 0.03$ *vs.* baseline; n = 8; fig. 1A). The application of L-655,708 did not modify the strength of LTP ($P = 0.02$ *vs.* control slices; n = 8; fig. 1A). Next, the effect of L-655,708 on synaptic excitability was studied by comparing the slope of the input–output relation, where current intensity was plotted against the slope of the fEPSP (fig. 1B). The application of L-655,708 after TBS did not further enhance synaptic excitability. Therefore, a decrease in α_5GABA$_A$R activity, similar to a reduction in the expression of α_5GABA$_A$Rs, did not modify synaptic plasticity or neuronal excitability under baseline conditions.

Next, slices from WT mice were cotreated with the same concentration of L-655,708 and etomidate to determine whether inhibiting α_5GABA$_A$Rs can reverse etomidate-induced blockade of LTP. Etomidate inhibited LTP in WT slices stimulated with the TBS protocol ($P = 0.01$ *vs.* control LTP; n = 8; fig. 1C).[9] This effect of etomidate was reversed by the coapplication of L-655,708 ($P = 0.02$ *vs.* etomidate-treated slices; n = 8; fig. 1C). There-

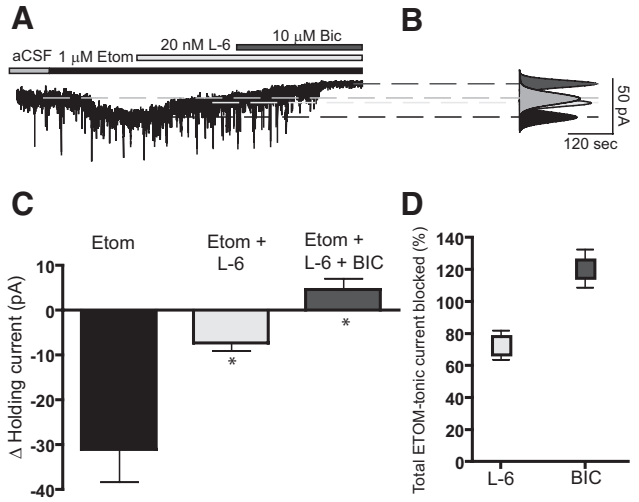

Fig. 2. Etomidate-induced increase in the holding current in CA1 pyramidal neurons. (*A*) Current traces indicate an inward tonic current with etomidate application. The etomidate increase in the holding current is reduced by 73.18 ± 9.05% with L-655,708 application. Bicuculline was applied at the end of the recording to reveal the total tonic conductance. (*B*) The all-point histograms for the current traces and the shifts in the holding current are shown. (*C*) Pooled data showing the relative changes in the holding current with etomidate, L-655,708, and bicuculline application. (*D*) The percentage of the block by L-655,708 and bicuculline on the total etomidate-induced tonic current is shown. *Short forms* in the figure refer to artificial cerebral spinal fluid (aCSF), etomidate (Etom), L-655,708 (L-6), and bicuculline (Bic). * Significantly different from the etomidate group.

fore, inhibition of α_5GABA$_A$Rs reverses the LTP-blocking property of etomidate. In addition, etomidate blocked an increase in synaptic excitability after TBS; this effect was not observed in slices treated with both etomidate and L-655,708 (fig. 1D).

L-655,708 Blocks Etomidate Potentiation of a Tonic Inhibitory Conductance in CA1 Pyramidal Neurons

Voltage clamp experiments were performed to determine whether L-655,708 reversed the enhancement of the tonic conductance by etomidate. Etomidate caused a significant increase in the tonic current, as evidenced by an inward shift in the holding current (I_{Hold}; n = 5; figs. 2A–C), as previously reported in studies of pyramidal neurons grown in dissociated cell cultures.[9] To determine the proportion of the etomidate-potentiated tonic current that was attributed to α_5GABA$_A$Rs, L-655,708 (20 nM) was applied. L-655,708 caused a 73 ± 9.04% reduction in the I_{Hold} (n = 5; figs. 2A–C), suggesting that a large proportion of the etomidate-enhanced tonic current is mediated by α_5GABA$_A$Rs. The L-655,708-treated tonic current was not significantly different from control (P = 0.93; n = 5). In addition, the coapplication of bicuculline, L-655,708, and etomidate caused a reduction in I_{Hold} by 120 ± 11.98% (fig. 2D). This reduction in I_{Hold} beyond the baseline indicates that a tonic conductance is present in the absence of etomidate or interventions intended to increase the extracellular concentration of γ-aminobutyric acid.[28] Interestingly, etomidate and L-655,708, at the concentrations tested, did not influence the kinetics of miniature inhibitory postsynaptic currents (table 1), suggesting that these drugs mediate their effects predominantly by modifying extrasynaptic GABA$_A$R activity.

Table 1. Effects of Etomidate and L-655,708 on Spontaneous mIPSCs

	CA1 Pyramidal Neurons, n = 5			
	aCSF	1 μM Etomidate	1 μM Etomidate + 20 nM L6	20 nM L6
Peak amplitude, pA	49.7 ± 4.3	53.6 ± 3.4	51.5 ± 5.2	50.4 ± 3.2
Rise time, ms	1.3 ± 0.2	1.1 ± 0.3	1.2 ± 0.4	1.3 ± 0.6
Weighted decay τ, ms	8.5 ± 1.9	9.5 ± 1.5	8.4 ± 2.1	8.8 ± 1.3
Frequency, Hz	2.9 ± 1.1	3.1 ± 0.7	2.6 ± 1.6	2.8 ± 0.9

Values are mean ± SEM. There were no statistically significant effects between the different conditions ($P > 0.05$ for all comparisons).

aCSF = artificial cerebrospinal fluid; L6 = L-655,708; mIPSC = miniature inhibitory postsynaptic current.

Memory Blockade by Etomidate Is Reversed by L-655,708

The general procedure and the training protocols used to assess fear conditioning are shown in figures 3A and B, respectively. In contextual fear conditioning, mice pretreated with L-655,708 exhibited robust freezing that was similar to that of vehicle-injected control mice ($P <$ 0.01; n = 8/group; fig. 3C). These results suggest that either α_5GABA$_A$Rs are not important for contextual fear memory or the fear conditioning protocol produced a saturating response under the experimental conditions, such that L-655,708 could produce no further enhancement. The strong contextual fear response was considerably reduced in mice that had been injected with etomidate ($P < 0.01$ *vs.* control; n = 8; fig. 3C). Mice treated with both etomidate and L-655,708 displayed freezing levels comparable to those injected with the vehicle control (P = 0.62; n = 8; fig. 2C). This latter result indicates that decreasing α_5GABA$_A$R activity completely reversed the impairment of memory by etomidate.

To address the concern that the initial experimental conditions used to study contextual fear memory produced a saturated freezing response (*i.e.*, a ceiling effect), the level of the foot shock was reduced (2 s, 0.5 mA). Under these new conditions, the effects of L-655,708 and etomidate were studied in WT and *Gabra5*−/− mice. The presentation of the weaker foot shock significantly reduced the baseline freezing in control mice when compared with mice trained with the stronger (2 s, 1 mA) foot shock (freezing with strong foot shock, fig. 3C *vs.* weak foot shock, fig. 3D; $P < 0.01$). Despite a weak contextual fear

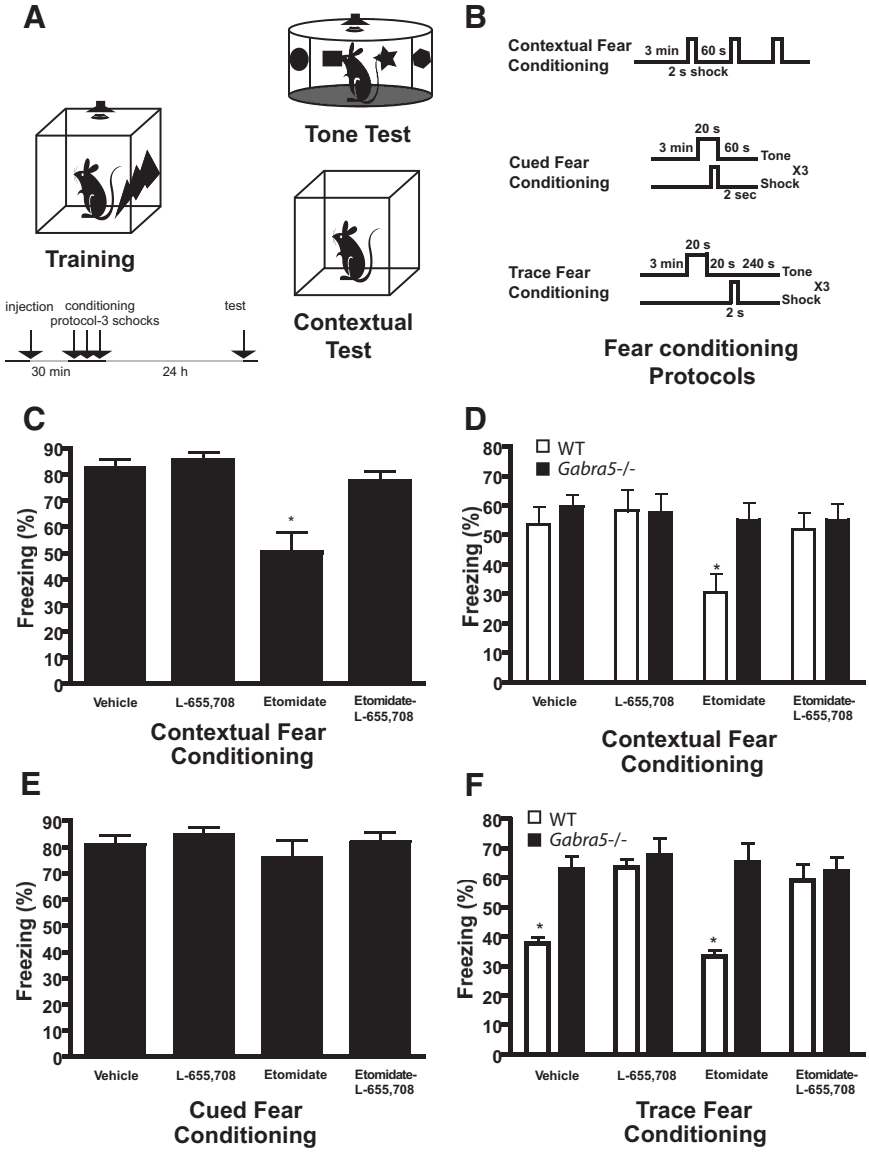

Fig. 3. The expression and activity of α_5-containing γ-aminobutyric acid subtype A receptors modify trace fear conditioning. (*A*) The basic procedure used for the fear conditioning protocol is illustrated. Injection of the drug was followed 30 min later by the fear conditioning protocol, which always consisted of three consecutive foot shocks. During the conditioning, three foot shocks were paired with a 20-s tone (see *B* or the Materials and Methods section for details of the fear conditioning protocols). After the fear conditioning protocol, the mice were tested 24 h later for freezing to context or 48 h later for freezing to the tone. For the assessment of freezing to the tone, the conditioning chamber was modified such that the shape was circular, a rubber mat covered the shock grid, and visual cues were located on the walls surrounding the chamber. See the Materials and Methods section for a more detailed description. (*B*) A schematic representation illustrating the timing for all three fear conditioning protocols is shown. In all protocols, a baseline activity period of 3 min preceded the conditioning procedure. (*C*) L-655,708 did not enhance contextual fear conditioning when a strong foot shock was used during training. Etomidate impaired performance in contextual fear conditioning, and L-655,708 restored freezing to control values when the two drugs were coadministered. (*D*) When a weaker foot shock was used for the unconditioned stimulus, contextual freezing scores were not enhanced by pretreatment with L-655,708 or in α_5 subunit null mutant (*Gabra5*−/−) mice. Etomidate impaired contextual fear conditioning in wild type (WT) but not *Gabra5*−/−, and L-655,708 occluded this effect. (*E*) Etomidate and L-655,708 did not influence performance in amygdala-dependent cued fear conditioning. (*F*) The performance of *Gabra5*−/− mice was enhanced in trace fear conditioning (a weak associative task), relative to the effect in vehicle-treated WT mice; in addition, inhibiting α_5-containing γ-aminobutyric acid subtype A receptors with L-655,708 improved the performance of WT mice to the level observed in *Gabra5*−/− mice. Etomidate did not significantly reduce freezing scores in WT mice and *Gabra5*−/− mice in trace fear conditioning. * Etomidate group is different from the other groups.

conditioning protocol, there were no differences between WT and *Gabra5*−/− mice (n = 10 and n = 9, respectively; $P = 0.53$; fig. 3D). Notably, L-655,708 did not further enhance freezing in WT mice or *Gabra5*−/− mice (n = 10 and n = 9, respectively; $P = 0.58$; fig. 3D). Injections of etomidate reduced the freezing scores for WT but not *Gabra5*−/− mice (n = 11 and n = 10, respectively; $P = 0.04$). The ability of etomidate to reduce freezing scores was reversed by L-655,708 ($P = 0.03$ compared with control mice; fig. 3D). Consistent with the changes in synaptic plasticity, these findings indicate that α_5GABA_AR activity is not important for baseline contextual fear conditioning, but these receptors can be activated by etomidate to impair memory performance in this task. Finally, the effects of etomidate and L-655,708 were studied on basolateral amygdala-dependent cued fear conditioning. Neither drug had a significant effect (one-way analysis of variance, $P = 0.52$; fig. 3E).

α_5GABA_AR Activity Impairs Performance for Trace Fear Conditioning

In trace fear conditioning, the strength of classic conditioning can be reduced by introducing a time interval (or "trace") between the tone and the foot shock. For trace fear conditioning, *Gabra5-/-* mice treated with vehicle outperformed their WT littermates, as indicated by significantly higher freezing scores (n = 10 and n = 11, respectively; $P = 0.03$; fig. 3F). To confirm that the difference between the genotypes was attributable to a reduction in α_5GABA_AR activity, mice were injected with L-655,708, which consid-

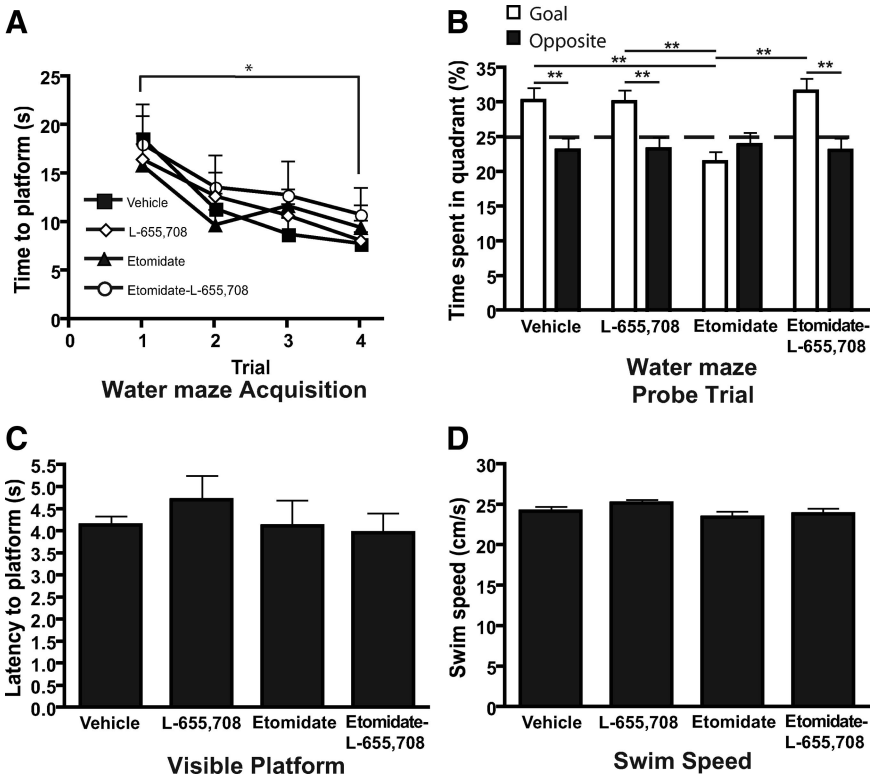

Fig. 4. Normal acquisition of the matching to place version of the Morris water maze but impaired recall with etomidate treatment. (*A*) Injections of either L-655,708 or etomidate do not influence the acquisition of the matching to place version of the Morris water maze. All injections were performed on day 11 after 10 days of naive training in the water maze. (*B*) L-655,708 did not enhance free recall of the platform location, 24 h after injection, in the probe trial of the water maze. Etomidate impaired performance in the water maze, but coapplication with L-655,708 returned performance to control levels. The percentage of time spent swimming in the target quadrant *versus* the average time spent in the other quadrants (nontarget) was calculated during the probe trial. The drug treatments did not influence the swim speed (*C*) or the visible platform trial (*D*) of the mice in the water maze. * Statistically significantly difference from the control group at $P < 0.05$.

erably improved freezing scores for WT mice but had no effect on *Gabra5*−/− mice (n = 8/group; $P = 0.34$; fig. 3F). Interestingly, etomidate did not significantly decrease freezing in WT and *Gabra5*−/− mice (n = 10/group) because the freezing scores were similar to those of vehicle-injected mice ($P = 0.26$; fig. 3F). However, WT mice that received both etomidate and L-655,708 displayed a high level of freezing, which was no different from *Gabra5*−/− mice (n = 10 and n = 9, respectively; $P = 0.23$; fig. 3F).

Etomidate Impairment of Spatial Memory Is Reversed with L-655,708

Next, the Morris water maze was used as an independent measure of hippocampus-dependent learning.[26] Notably, regardless of drug treatment, the performance of the mice was similar for the acquisition trials of the water maze task (fig. 4A). The mean time savings (time to locate platform during trial 4 minus time required during trial 1) was used to quantify immediate memory. There were no significant differences in the mean time savings to locate the hidden platform between treatment groups (7.2 ± 2.0 s for vehicle-injected mice, 5.7 ± 2.6 s for L-655,708-treated mice, 6.1 ± 3.1 s for etomidate-treated mice, 5.4 ± 3.5 s for etomidate- and L-655,708-treated mice; n = 28/group; $P = 0.01$; fig. 4A). The results suggest that neither the up- nor down-regulation of α_5GABA$_A$R activity influenced the ability of the mice to initially learn and complete the task.

To study long-term memory performance, mice underwent a probe trial 24 h after the acquisition of the task. L-655,708 did not enhance the recall of the hidden platform location, as shown by the percentage of time spent swimming in the correct quadrant of the water maze (n = 28/group; $P = 0.35$; fig. 4B). In contrast, etomidate decreased the total amount of time spent swimming in the correct quadrant of the pool during the probe trial (n = 28; $P = 0.02$; fig. 4B). This reduction in performance was not exhibited by the mice that were injected with both etomidate and L-655,708 (n = 28; $P = 0.29$). Therefore, the mice demonstrated equal learning during the acquisition phase of the water maze task, but etomidate impaired recall of the task 24 h later, and this effect that could be reversed by L-655,708.

The visible platform studies revealed that there were no differences among treatment groups in the latency to locate the platform in the presence of any drug combination (n = 28; $P = 0.4$; fig. 4C). There were also no differences among the groups in terms of mean swimming speed during the acquisition trial (n = 28; $P = 0.87$; fig. 4D). The lack of an effect on swimming speed confirmed the procedural ability of the mice to perform the tasks.

α_5GABA$_A$Rs Do Not Contribute to Anxiety-like Behaviors

The inhibition of α_5GABA$_A$Rs with L-655,708 has been shown to increase anxiety-like behaviors in the elevated plus maze.[17,29] However, this anxiogenic effect has been attributed to inhibition of GABA$_A$R subtypes other than

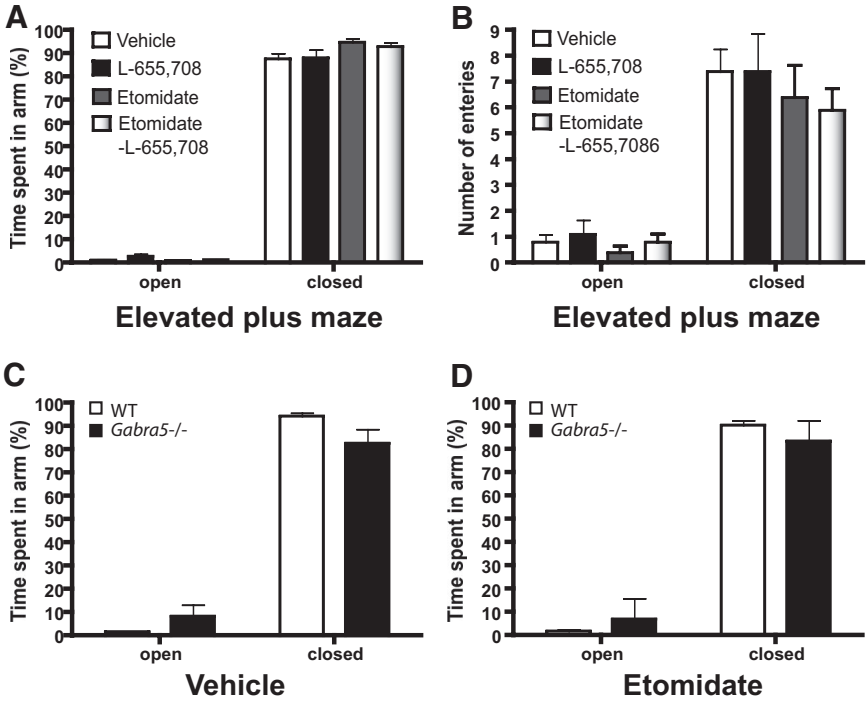

Fig. 5. L-655,708 and etomidate do not contribute to anxiety-like behaviors in the elevated plus maze. (*A*) L-655,708 and etomidate did not change the time spent by mice in the open or closed arms of the elevated plus maze. (*B*) L-655,708 and etomidate also did not change the total number of entries into the open and closed arms of the elevated plus maze. (*C*) There were no differences between wild-type (WT) and α_5 subunit null mutant (*Gabra5*−/−) mice for the amount of time spent in the open or closed arms of the elevated plus maze. This confirms that α_5GABA$_A$Rs do not readily contribute to anxiety-like behaviors. (*D*) Etomidate did not influence the amount of time spent in either the open or closed arms of the elevated plus maze in WT or *Gabra5*−/− mice.

α_5GABA$_A$R because nonselective doses were used.[29] Nevertheless, to determine whether the activity of α_5GABA$_A$Rs contributed to anxiety-like behaviors that would confound studies of performance in the fear conditioning and Morris water maze tasks, etomidate and L-655,708 were tested in the elevated plus maze at the same doses as used for the memory assays. There were no significant differences in the time spent in the open arms (n = 8/group; $P = 0.47$; fig. 5A) and the closed arms (n = 8/group; $P = 0.31$; fig. 5A) of the elevated plus maze. Similarly, there was no difference in the frequency of entries into the open arms ($P = 0.21$; fig. 5B) and closed arms ($P = 0.53$; fig. 5C) and closed arms ($P = 0.27$; fig. 5C), and the frequency of entries into the open and closed arms (data not presented; $P = 0.58$) were not significantly different. Furthermore, etomidate did not alter the time spent in the open arms (n = 8/group; $P = 0.56$; fig. 5D) or the closed arms ($P > 0.36$; fig. 5D), nor did it affect the frequency of entry into either type of arm (data not shown; $P = 0.62$).

Discussion

This study supports a pharmacogenetic mechanism to account for resistance to the memory-blocking properties of etomidate. The results show that α_5GABA$_A$R activity does not regulate baseline synaptic plasticity evoked by high-frequency stimulation in an *in vitro* mouse hippocampus slice model or behavioral performance for contextual fear and spatial navigation memory; nevertheless, etomidate increases α_5GABA$_A$R activity and thereby impairs plasticity and memory. These memory-blocking effects of etomidate can be completely reversed by pretreatment with L-655,708.

γ-Aminobutyric acid type A receptors play a critical role in orchestrating neuronal activities by altering spike timing in neurons and synchronized rhythms in neuronal circuits. Etomidate, as well as most inhaled anesthetics, increases the activity of GABA$_A$Rs.[7,30] This facilitation typically causes membrane hyperpolarization and the shunting of excitatory currents, which reduce neuronal excitability.[31] The propensity for general anesthetics to block the induction of LTP by increasing GABA$_A$R activity has been widely reported.[32,33] Etomidate, at the concentration used for this study blocked LTP through selective potentiation of α_5GABA$_A$Rs rather than through nonselective enhancement of γ-aminobutyric acid–mediated neurotransmission. Furthermore, nonselectively inhibiting all GABA$_A$Rs with antagonists such as picrotoxin and bicuculline is known to enhance plasticity evoked by high-frequency stimulation[34] and reverse anesthetic blockade of LTP.[32,33] This study shows that pretreatment with the α_5GABA$_A$R-preferring agent L-655,708 is sufficient to reverse etomidate impairment of LTP and memory blockade.

Etomidate preferentially enhances the tonic rather than synaptic inhibitory conductance in CA1 pyramidal neurons,[9] and this action can be attenuated by L-655,708. Higher concentrations of anesthetics, which are less selective, may increase both tonic and synaptic inhibition; however, the increase in inhibitory charge mediated by the tonic current is typically many times greater than that mediated by synaptic inhibition.[28,35] Consequently, we attribute etomidate effects on plastic-

ity and behavior primarily to an increase in the tonic inhibitory conductance, but recognize that inhibitory postsynaptic currents might also contribute. Immunocytoimaging[36] and electronmicroscopy[37] studies have shown that α_5 subunits are also expressed in the synaptic regions of hippocampal pyramidal neurons. These synaptic receptors do not appreciably contribute to fast inhibitory postsynaptic currents,[38] but they may generate a subset of slow synaptic currents that are termed *slow inhibitory postsynaptic currents*.[38] The slow inhibitory postsynaptic current contributes to less than 1% of synaptic γ-aminobutyric acid–mediated inhibition but may powerfully regulate plasticity due to its position in the neuronal circuitry and its temporal association with *N*-methyl-D-aspartate receptor activation.[39] The influence of low concentrations of etomidate on this slow inhibitory postsynaptic current remains to be studied.

The activity or expression of α_5GABA_ARs did not seem to influence baseline synaptic plasticity, as was previously reported.[9,13] Consistent with this result, others have shown that the strength of LTP induced by high-frequency stimulation was similar in hippocampal slices from α_5 (H105R) point mutant and WT mice.[40] However, another benzodiazepine inverse agonist selective for α_5GABA_ARs enhanced synaptic plastic in hippocampal slices.[41] Several factors could account for such discordant results. Notably, although many populations of $GABA_ARs$ are expressed in the neuronal network and within the same cells, only some types may be active during any given experimental condition. With changes in experimental conditions, such as the intensity of the network stimulation, different subpopulations of $GABA_ARs$ may be recruited. Therefore, the contribution of particular $GABA_AR$ populations to synaptic plasticity is critically dependent on the *in vitro* experimental protocol. For example, we found that L-655,708 did not alter the baseline synaptic plasticity induced by high-frequency stimulation, whereas others have shown that L-655,708 enhanced plasticity induced by TBS. In the latter study, plasticity was induced by a brief priming stimulus (10 stimuli at 100 Hz) followed 30 min later by TBS.[41] Notably, after the priming stimulus, synaptic strength was increased to 200% in both the L-655,708-treated and control slices, suggesting that α_5GABA_ARs do not play a critical role. However, after the second phase of stimulation, L-655,708-treated slices showed greater plasticity. Together, the studies indicate that the stimulation protocol dramatically influences the subpopulation of $GABA_ARs$ that modify plasticity.

The above results also suggest that caution must be exercised when making direct comparisons between studies aimed at understanding the role of $GABA_AR$ subtypes that used different animal species, behavioral protocols, and electrophysiologic measurements. We observed that baseline freezing for fear-associated learning and the Morris water maze task was similar in WT and *Gabra5−/−* mice. This result is seemingly at odds with studies showing that a reduction in α_5GABA_AR activity enhances learning performance.[42] Others have shown that L-655,708 increased the performance of rats in a water maze probe trial; however, for these experiments, the probe trial was performed 15 min after completion of a series of rigorous training trials.[17] We studied the probe trial 24 h after training.

Because the strength of associated learning also depends on the strength of the aversive stimulus and the number of presentations,[43] we sought to determine whether the experimental conditions contributed to the inability to demonstrate improved contextual learning (*i.e.*, a ceiling effect), and a weaker foot shock was used in some studies. Even under these modified conditions where baseline freezing scores were reduced, L-655,708 did not strengthen contextual learning. Therefore, α_5GABA_ARs play a minimal role in processes that elicit strong and even moderate contextual memory. In contrast, the performance in trace fear conditioning was greater in *Gabra5−/−* mice and WT mice treated with L-655,708 than in WT controls. This result is consistent with studies of α_5 (H105R) point mutant mice, which have a partial deficit of α_5GABA_ARs.[40] The α_5H105R mice exhibit higher baseline freezing scores for trace fear conditioning compared with WT littermates. Trace fear conditioning adds complexity to the delay conditions, because the time interval requires the formation of a temporal relation between the two stimuli. The reasons for differences in trace fear learning but not contextual fear in *Gabra5−/− versus* WT mice remains to be determined because the hippocampus is required for tone-shock association in rodents and humans during contextual learning[44] and spatial navigation.[26]

The concentration of an anesthetic that is required to disrupt behavior is critically dependent on the specific behavioral endpoint under consideration and the methods used to probe behavior. For example, the concentration of inhaled anesthetic that disrupts learning and memory depends on the specific memory tasks used, with hippocampus-dependent learning being particularly vulnerable to disruption by anesthetic drugs.[45] We showed that memory was impaired during the probe trial; however, during the acquisition tasks, working memory seemed to be intact. Interestingly, working memory is thought to involve the prefrontal cortex. Although early studies suggested that α_5 subunit levels are low in the cortex, a tonic inhibitory conductance is generated by α_5GABA_ARs in layer 5 of the neocortex.[46] Also, α_5GABA_ARs may contribute to inhibitory synaptic transmission in the neocortex.[46] It is possible that memory tests of higher difficulty or those designed to specifically probe neocortical function may reveal that etomidate modulates these populations of α_5GABA_ARs.

To reconcile our *in vitro* and behavioral data, we developed a schematic model (fig. 6). LTP is mediated, in

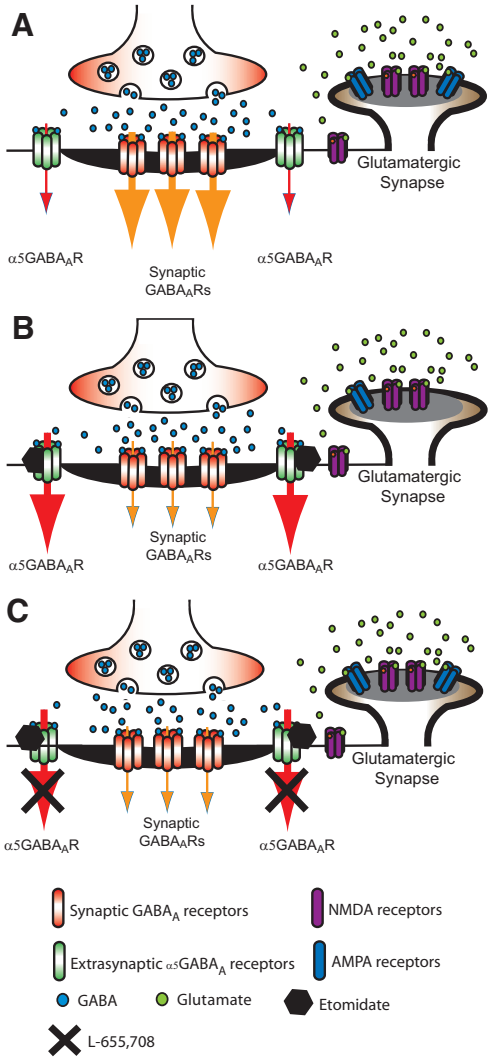

Fig. 6. A model illustrating the regulation of synaptic plasticity in the hippocampus by extrasynaptic α_5-containing γ-aminobutyric acid subtype A receptors (α_5GABA$_A$Rs) and blockade of long-term potentiation (LTP) by etomidate. (*A*) High-frequency stimulation leads to intense inhibitory drive and activation of postsynaptic glutamate and γ-aminobutyric acid subtype A receptors. LTP is present under these conditions because of the preferentially stronger activation of glutamatergic synapses. (*B*) Despite strong activation of glutamate synapses, LTP is not generated during the application of a general anesthetic etomidate. This model proposes that general anesthetics selectively and robustly activate α_5GABA$_A$Rs, which may override glutamatergic activation and LTP because of dramatic increases in a α_5GABA$_A$R-associated shunting conductance. (*C*) L655,708 inhibits α_5GABA$_A$R activity and thereby reverses etomidate blockade of LTP. AMPA = α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; GABA = γ-aminobutyric acid; GABA$_A$ = γ-aminobutyric acid subtype A; NMDA = *N*-methyl-D-aspartate.

part, by an increase in the surface expression and function of glutamate receptors in the postsynaptic neuron, and high-intensity stimulation stimulates GABA$_A$Rs, to regulate the induction of LTP. The model proposes the following: (1) α_5GABA$_A$Rs are expressed in neuronal circuits that normally regulate memory behavior. However, (2) α_5GABA$_A$Rs regulate network activity in a constrained manner. High-frequency stimulation recruits both high- and low-affinity GABA$_A$Rs, such that the contribution of the α_5GABA$_A$Rs is overshadowed or obscured by other GABA$_A$R subtypes. (3) Etomidate preferentially targets α_5GABA$_A$Rs and "supraactivates" these receptors, causing them to function beyond their normal physiologic limits. Under such conditions, α_5GABA$_A$Rs exert a dominant role in attenuating plasticity. (4) L-655,708 reverses the effects of etomidate on α_5GABA$_A$Rs.

The above results provide a compelling foundation for further work, but the studies have important limitations that deserve mention. First, the dose of etomidate was selected to cause amnesia and not general anesthesia. At higher doses, etomidate may modify the activity of other GABA$_A$R subtypes and other neurotransmitter systems.[47,48] Second, high-efficacy and affinity-selective α_5GABA$_A$Rs compounds such as L-655,708 or similar compounds such as α_5IA, at higher doses, may reduce the activity of α_1, α_2, and α_3 subunit-containing GABA$_A$R subtypes causing anxiogenic and proconvulsant effects that limit their clinical utility.[17,29] Third, the behavioral paradigms used to study hippocampus-dependent memory do not mimic the clinical scenarios involving etomidate anesthesia. Fourth, α_5GABA$_A$Rs may play a predominant role under different conditions that evoke plasticity. Finally, it remains to be determined whether α_5GABA$_A$R-preferring agents reverse memory impairment by inhaled anesthetics.[7]

Preclinical studies show that pathologic conditions, including epilepsy and chronic alcohol abuse, alter expression of the α_5 subunit.[49,50] Also, polymorphisms of the human *Gabra5* gene occur, although their functional significance is still unknown. Mouse models might be useful in developing strategies to treat persistent postanesthetic memory impairment and predict awareness. Finally, the implications of the study extend well beyond the purview of anesthesiology, because the results further implicate α_5GABA$_A$Rs as targets for the development of memory-modifying drugs.

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