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Anesthetics Discriminate Between Tonic and Phasic Gamma-Aminobutyric Acid Receptors on Hippocampal CA1 Neurons

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Abstract

Background—Anesthesia is produced by a depression of neuronal signaling in the central nervous system (CNS), however, the mechanism(s) of action underlying this depression remain unclear. Recent studies have indicated that anesthetics can enhance inhibition of CNS neurons by increasing current flow through tonic gamma-aminobutyric acid (GABA_A) receptor gated chloride channels in their membranes. Enhanced tonic inhibition would contribute to CNS depression produced by anesthetics, but it remains to be determined to what extent anesthetic actions at these receptors contribute to CNS depression. In the present study, we compared and contrasted the involvement of tonic versus synaptic GABA_A receptors in the functional depression of CNS neurons produced by isoflurane and thiopental.

Methods—In rat hippocampal slices, whole cell patch clamp recordings were used to study anesthetic effects on CA1 neuron intrinsic excitability, and population spike recordings were used to investigate effects on synaptically evoked discharge. These responses were chosen to test whether anesthetic effects on GABA receptors alter single neuron discharge and/or circuit level synaptic functioning. Phasic (synaptic) GABA receptors were selectively blocked using the GABA_A antagonist gabazine and tonic responses were blocked using the chloride channel blocker picrotoxin.

Results—Clinically relevant and equi-effective concentrations of thiopental and isoflurane depressed CA1 neuron synaptically evoked discharge. This depression was partially reversed by blocking synaptic GABA_A receptors with gabazine (20 μM). The thiopental-induced depression was reversed by ~ 60 %, but the isoflurane-induced depression was reversed by only ~ 20%. Blocking tonic GABA_A receptors with the addition of 100 μM picrotoxin produced an additional 40 % reversal of the thiopental-induced depression, but no additional reversal was seen for isoflurane-depressed responses. In response to direct DC current injection, CA1 neuron discharge was depressed by thiopental and membrane conductance was increased. Both of these effects were reversed by picrotoxin, but not by gabazine. Isoflurane, in contrast, neither depressed current-evoked discharge, nor altered the membrane conductance of CA1 neurons.

Conclusions—These results indicate that general anesthetics discriminate between synaptic and tonic GABA_A receptors. Effects on both phasic and tonic receptors combined to depress circuit responses produced by thiopental, whereas only effects on synaptic GABA receptors appeared to play an important role for isoflurane. Together with the other known sites of action for these 2 anesthetics, our results support a multi-site, agent-specific mechanism for anesthetic actions.

Keywords

GABA_A receptors; tonic current; extrasynaptic; perisynaptic; Anesthetic (Anaesthetic); thiopental; thiopentone; barbiturate; IV; isoflurane; volatile; inhalational; Anesthesia (Anaesthesia); chloride channel; synapse; synaptic; depression; excitability; action potential; population spike; cortex

INTRODUCTION

General anesthetics have been shown to depress neuronal responses throughout the central nervous system and some of this depression appears to result from actions at gamma-aminobutyric acid (GABA_A)-mediated inhibitory synapses via postsynaptic ionotropic chloride channels (1,2). GABA_A receptor/channels (GABARs) exist at synapses, clustered beneath active zones of nerve terminals that release GABA, and also in perisynaptic locations where they can sense the spillover of GABA from nearby synapses (3,4). There appear to be many types of GABA_A receptors expressed, made up of different combinations of subunits which impart distinct biophysical, physiological and pharmacological characteristics to each receptor/channel (5). In hippocampal CA1 neurons, for example, there are traditional fast GABARs found at synapses on the cell bodies of pyramidal cells and these are thought to be comprised of alpha 1–2, beta 2, and gamma 2 subunits.(6) They exhibit rapid activation (~ 1 ms) and fast desensitizing responses to GABA and likely function to control rapid feedback inhibition of pyramidal neurons (7,8). Slow synaptic GABA_A receptors are localized to dendritic synapses and are thought to include beta 3 subunits and exhibit slower onset (> 3 ms) and longer decay time kinetics (9,10). These slow synaptic receptors are ideally suited to modulate synaptic plasticity and dendritic integration of CA1 neurons (11). A third type of receptor appears to be more diffusely distributed, responding to low ambient levels of GABA not associated with synapses, perhaps located in extrasynaptic cellular regions (12). These receptors generate tonic chloride currents that show little evidence of desensitizing (5). Tonic receptors expressed by CA1 neurons respond to low μ M concentrations of GABA and appear to be comprised of receptors that include alpha 5 subunits (13,14).

Several recent studies suggest that tonic GABARs could be important targets for general anesthetics and alcohols (13,15–20). The purpose of the present study was to compare the involvement of tonic versus synaptic GABA_A receptors in the depressant effect produced by a barbiturate, thiopental and an inhaled anesthetic, isoflurane. Synaptic GABA_A receptors were selectively blocked using gabazine (15,16,21), and any additional involvement of tonic receptors was determined by blocking remaining chloride channels with picrotoxin (Figure 1). Two measures of neuronal depression were used. The first tested individual nerve cell responses to depolarizing current injection, as a measure of effects on intrinsic excitability. The second measured effects on population spike responses to assess whether synaptic and circuit level depression involved anesthetic-induced tonic GABA_A receptor activation.

METHODS

Brain slice preparation methods have been described in detail (22,23). In short, standard transverse hippocampal slices (0.4 μ m) from mature Sprague-Dawley rats (P28–P40, most P33–36) were prepared using a vibratome. All procedures conformed to National Institute of Health guidelines for the care and use of animals and were approved by Stanford University's animal use IRB.

Electrophysiology

Standard visualized slice procedures were used. All whole-cell recordings were from CA1 pyramidal neurons in the cell body layer (stratum pyramidale) with seal resistances of at least

3 GOhm. Input resistances ranged between 150 to 400 Mohm, and there were no obvious differences between cells that responded to thiopental or isoflurane. Every neuron displayed stable spiking and input resistance during control recordings (± 2 to 4 %). Throughout current clamp experiments, sets of current steps were applied repetitively at fixed intervals (typically, 6 steps/set: 2 hyperpolarizing, 1 at 0 pA, and 3 depolarizing); 1 set/minute). In long duration (> 2 hour) current clamp experiments, we found that neurons tolerated protocols using small numbers of depolarizing current steps repetitively applied (3/set) at these low repetition rates. We did not apply tonic currents to adjust resting potentials. All experiments were conducted at room temperature (22–24°C) using a submersion chamber and > 95 % of tubing was Teflon to minimize drug binding or permeation loss. Using continuous perfusion of artificial cerebrospinal fluid (ACSF) at 2–3 mL/minute, complete bath replacement took ~2 minutes, as measured by dye exchange. Each slice was used for only a single experiment. The following external (ACSF) was used (in mM): 124 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, 10 glucose; it was bubbled with 95% O₂/5% CO₂ to reach pH 7.4.

We used standard whole-cell methods (pipette resistance 4–8 Mohm). A potassium gluconate-based internal solution was used (in mM): 100 K-gluconate, 10 EGTA, 5 MgCl₂, 40 HEPES, 2 Na₂ATP, 0.3 NaGTP (pH 7.2 with KOH). All voltage values were corrected for electrode offset and junction potentials. All recordings were established for > 15 minutes before recording baseline data and recordings showing unstable response properties (> 4 % variability) were not used.

Whole-cell DC current-evoked spiking data were collected using PClamp 8.0 (Axon Instruments, Union City, CA) and analyzed using custom procedures written in Igor Pro 5.0 (Wavemetrics, Oswego, OR).

Population spike responses were generated by stimulating Schaffer-collateral fibers using bipolar tungsten microelectrodes (10 MOhm FHC Inc., Bowdoin, ME) driven by a Grass s48 stimulator (0.22 to 0.25 ms pulse width, 5 to 8 V). Glass micropipette recording electrodes filled with ACSF (1.0 to 2.0 KOhm) were placed at the border of stratum oriens and stratum pyramidale to monitor CA1 neuron population spike discharge amplitudes. Only slices that exhibited population spike responses of at least 10 mV were used for experiments. Signals were amplified (1000 x, Brownlee Co. Santa Clara, CA), digitized (National Instruments USB 6009) and stored (MacBook Pro, IgorPro, Wavemetrics, Lake Oswego, OR). Analysis was performed using IgorPro and two-tailed Student's t-test, or repeated measures ANOVA with Tukey test were used to evaluate statistical significance ($p < 0.05$ or below; GraphPad software, Prism Inc., San Diego, CA, USA).

Compounds

All chemicals for ACSF were reagent grade or better and obtained from Sigma/RBI. Thiopental was obtained from Sigma-Aldrich (Saint Louis, MO) and made fresh daily as a 12.5 mM stock in distilled water and NaOH. Isoflurane was obtained from Hospira Inc (Novaplus, Lake Forest, IL) and applied using a calibrated vaporizer (Isotec, SurgiVet Inc., Waukesha, WI). Gas phase isoflurane concentrations were measured using a Datex 254 agent monitor (Puriton-Bennett, Pleasanton, CA), and solution concentrations were verified using a custom electrochemical measure as previously described. (22)

RESULTS

Thiopental depressed CA1 neuron action potential discharge via tonic GABA_A receptors

The barbiturate anesthetic, thiopental, produced a rapid and reversible depression of CA1 neuron discharge activity (Figure 2). Action potential responses to low intensity current

injection (40 pA) were completely blocked and responses to higher intensity current pulses were strongly depressed. When synaptic GABA receptors were antagonized with the competitive receptor antagonist gabazine, there was no apparent reversal of thiopental-induced depression. In contrast, application of picrotoxin in the continued presence of thiopental and gabazine produced a full reversal of the barbiturate-induced depression. These findings were highly significant, comparing thiopental-induced depression to control discharge levels and for the picrotoxin-induced reversal of thiopental's effect (Figure 2).

The barbiturate-induced depression of action potential discharge was accompanied by an increase in membrane conductance, measured using hyperpolarizing current pulse injections (Figure 2). Thiopental increased membrane conductance by approximately 100 %, and this increase was not antagonized by blocking synaptic GABA receptors with gabazine. The increase in membrane conductance, however, was reversed by blocking tonic GABA-gated chloride channels with picrotoxin.

Isoflurane-induced depression of CA1 neuron excitability

In contrast to the strong depressant effects produced by thiopental, isoflurane did not alter action potential discharge responses to depolarizing current in CA1 neurons (Figure 3). Even in the presence of high concentrations (1.0 rat MAC; 350 μ M) sufficient for surgical levels of anesthesia, there was little change evident for low stimulus intensity responses (40 pA) and no apparent effect for responses to higher stimulation levels. Similarly, there was no apparent change in membrane conductance produced by isoflurane.

Synaptic and tonic GABA receptor inhibition of CA1 neurons

Gabazine alone produced a small, nonsignificant at $n = 5$, increase in action potential discharge frequency (1.4 ± 1.1 Hz; $p > 0.1$). Picrotoxin applied in the continuing presence of gabazine ($n = 5$) or alone ($n = 5$) produced a significant, but modest, increase in discharge frequency (1.5 ± 0.6 Hz; $p < 0.01$, pooled data). Thus, under resting conditions, there was only a small influence of synaptic or tonic GABA-mediated inhibition on the excitability of CA1 neurons. A more pronounced involvement of GABA inhibition was evident for synaptically driven excitation, measured using the Schaffer-collateral to CA1 neuron population spike response (Figure 4). Blocking synaptic GABA receptors with gabazine produced a significant increase in population spike amplitudes (116 ± 9 % of control; $p < 0.005$, $n = 5$). Subsequent addition of picrotoxin produced a small, but nonsignificant, further increase in population spike amplitudes, indicating little additional tonic GABA-mediated inhibition. The most dramatic effect was the appearance of additional population spikes after block of synaptically mediated inhibition with gabazine (Figure 4), with no apparent change with the addition of picrotoxin. Thus, under synaptically driven excitation, both feedforward and feedback GABA-mediated inhibition appears to limit the discharge of CA1 neurons, with little involvement of tonic GABA receptors.

Thiopental-induced depression of synaptically evoked discharge

Thiopental produced a brief increase in population spike amplitudes followed by a profound depression of discharge (Figure 5). Blocking synaptic GABA receptors with gabazine significantly reversed the thiopental-induced depression (from 0 to 66 ± 8 % of control; p , 0.001, $n = 5$). Subsequent application of picrotoxin fully reversed the remaining thiopental-induced depression (to 111 ± 10 % of control, $p < 0.001$, $n = 5$). This indicated that both synaptic and tonic GABA receptors contribute to the depressant effects of thiopental for synaptically driven CA1 neuron discharge.

Isoflurane-induced depression of synaptically evoked discharge

Isoflurane produced only a depression of population spike discharge of CA1 neurons (Figure 6). The onset kinetics for depression was considerably faster than that produced by thiopental (Tau 1/2 of 3.8 min for isoflurane vs 14.6 min for thiopental). A slower component of depression was also evident for isoflurane, that was only revealed after application of the GABA antagonists. This indicates that 1 minimum alveolar concentration (MAC) isoflurane acts at an additional, non-GABA, site which exhibits much slower onset kinetics. Gabazine reversed the isoflurane-induced depression to almost 40 % of control population spike values, indicating that a significant enhancement of synaptic GABAR-mediated inhibition contributes to this depression ($p < 0.001$; $n = 5$). The addition of picrotoxin, to block residual tonic GABAR-mediated inhibition, had no effect (Figure 6). Previous studies suggested that tonic GABARs might be more sensitive to low concentrations of isoflurane, in the amnesic range of 0.25 to 0.3 rat MAC (17). To test this, we repeated the population spike depression experiments using 90 μM of isoflurane (~ 0.25 MAC). This low concentration of isoflurane depressed responses to 60 % of control amplitudes with a Tau 1/2 of 9.3 min. Blocking synaptic GABARs with gabazine produced a 11 ± 7.3 % reversal of the isoflurane-induced population spike depression (Figure 6, bottom, $p < 0.01$). Blocking residual tonic GABARs with picrotoxin did not produce any further reversal. Thus, tonic GABARs do not appear to play a role in depression of synaptically evoked CA1 neuron discharge for either high or low concentrations of isoflurane, but synaptic GABARs contribute significantly to population spike depression at both concentrations. These results, together with the effects produced by thiopental, are summarized in Figure 7.

DISCUSSION

Thiopental acted mainly to enhance phasic GABA_A receptor-mediated inhibition, but significant effects were also seen on tonic receptors. Isoflurane, in contrast, enhanced phasic GABA_A inhibition but did not appear to enhance tonic GABA_A receptors to depress CA1 neuron synaptically evoked discharge. Thus, these 2 anesthetics can readily discriminate between tonic and phasic GABA receptors. This adds an additional level of complexity to the multiple sites of action previously shown to exist for these 2 anesthetics by demonstrating that selective interactions occur even among tonic versus phasic GABA receptors (1,6,13).

Our results confirm earlier work indicating that GABA_A receptors are important targets for both IV and volatile anesthetics (1) acting on CA1 neurons (23) and other neuron types throughout the brain (24). Recent studies have suggested that tonic receptors may be more important mediators of anesthetic-induced neuronal depression than are phasic receptors (13–18). This has been based largely on observations that tonic receptors appear to be quite sensitive to low concentrations of anesthetics and could be expected to contribute a larger charge transfer or shunting inhibition due to the continuous, non-desensitizing nature of tonic receptors (13). We provide the first quantitative comparison of anesthetic effects at phasic and tonic GABA_A receptors using functional measures of neuronal depression in a well characterized synaptic circuit, the Schaffer-collateral to CA1 neuron pathway. Surprisingly, even though isoflurane can enhance tonic GABA_A-mediated currents in these neurons (14,15,17), this did not manifest as a major effect contributing to either direct current-evoked discharge (Fig. 3) or synaptically evoked discharge of CA1 neurons (Figs. 6 and 7). Instead, isoflurane appeared to depress the Schaffer-collateral to CA1 neuron circuit through a combination of effects on phasic GABA_A receptors (Fig. 7) and by depressing glutamate-mediated excitatory synaptic inputs, together with additional enhancement non-GABA-mediated inhibitory currents as previously reported (23). Apparently, the isoflurane-induced enhancement of tonic currents in hippocampal cells is not strong enough to alter the discharge properties of these neurons. Perhaps this is because volatile anesthetics do not directly activate the receptors like IV

anesthetics do, therefore, in the absence of high ambient GABA concentrations, these drugs are relatively ineffective. If this is true, then tonic GABA_A receptors could play a larger role *in vivo* where higher levels of background GABA might pertain. The IV drug thiopental, in contrast, clearly acted through enhancing both tonic and phasic GABA_A receptors to depress CA1 neuron discharge (Fig. 7), and a major component of this depression appeared to involve postsynaptic tonic GABA_A receptors (Fig. 2). This is similar to the effect produced by propofol using direct current-evoked discharge as a functional measure of anesthetic-induced depression of discharge (16,25). Of course, direct current-evoked discharge only provides a functional measure for possible tonic receptor contributions at or near the cell body/spike initiation region of neurons. This is why we also tested for a functional depression of synaptically evoked discharge using the population spike measure. The population spike response should be sensitive to tonic GABA_A current activation throughout the dendritic region, where shunting of excitatory currents could occur, as well as other membrane regions associated with spike initiation.

It is likely that anesthetic actions at tonic GABA_A receptors will contribute more or less to functional depression of neuronal discharge depending on the particular neuron type, receptor subtypes expressed, and/or subcellular distribution of tonic receptors. For example, Jia et al. have recently found that isoflurane can significantly depress direct current-evoked discharge of ventrobasal thalamic neurons expressing alpha-4 containing tonic GABA_A receptors (25). Although this depression was only partial (~25 %), it appeared to add to enhanced synaptic GABA_A currents in these cells to inhibit firing activity. We may yet discover neurons that can be entirely depressed by isoflurane-induced enhanced tonic currents, but even ventrobasal thalamic neurons appear to be depressed by actions involving several additional sites (24,25).

In summary, we provide the first quantitative comparison of anesthetic actions on synaptic and tonic GABA_A receptors to demonstrate an agent-specific ability to discriminate between these sites of action.

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Figure 1.

Diagram showing the experimental protocol used to test anesthetic effects on synaptic vs tonic gamma-aminobutyric acid (GABA) receptor gated ion channels. Microelectrodes were used to record either direct current-evoked discharge of CA1 pyramidal neurons or synaptically evoked population spike responses. If CA1 neuron discharge were depressed via anesthetic-induced enhancement of synaptic (PHASIC) GABA receptors, then this depression should be reversed with the synaptic receptor selective antagonist gabazine (SR 95531). If extrasynaptic (TONIC) GABA receptors were involved in neuronal depression, then any remaining anesthetic effect should be reversed with the chloride channel blocker, picrotoxin. Phasic receptors are thought to contain alpha 1, 2 and 3 subunits, while tonic receptors contain alpha 5 subunits. Gabazine at 20 μM selectively blocks synaptic GABA receptors that generate inhibitory postsynaptic currents (IPSCs) in CA1 neurons, while picrotoxin at 100 μM blocks both synaptic and tonic receptors (16). In the presence of an anesthetic, gabazine was applied to test for effects on synaptic receptors, followed by picrotoxin (in the continued presence of anesthetic + gabazine) to test for any residual effect occurring via tonic GABA receptors.

Figure 2.

Thiopental produced a marked depression of CA1 action potential discharge by enhancing tonic gamma-aminobutyric acid (GABA) currents. A) Action potential discharge was generated by injecting depolarizing current pulses through a whole cell electrode. Three levels of current (pA) were used to generate increasing discharge frequencies in control conditions (CON). In the presence of thiopental (+TP 120 μ M) discharge activity was reduced at all stimulus levels. Addition of the synaptic receptor antagonist, gabazine (+SR, 20 μ M), in the continued presence of thiopental, failed to reverse the anesthetic-induced depression. Blocking tonic GABA receptors with picrotoxin (+Pic, 100 μ M) in the continued presence of thiopental and gabazine completely reversed the thiopental-induced depression. Calibration bars: 20 mV and 200 ms. B) Hyperpolarizing current pulses were used to monitor effects on membrane resistance. Thiopental produced a decrease in membrane resistance, consistent with an increased GABA-mediated chloride conductance produced by thiopental. This increased membrane resistance was not reversed by gabazine, but was completely blocked by picrotoxin, indicating that an increased tonic GABA-mediated chloride current was responsible for the decrease in membrane resistance. Calibration bars: 20 mV and 50 ms. C) Grouped data from at least 6 experimental repeats indicated that thiopental produced a significant ($p < 0.001$; **) depression of discharge frequency (Hz) and increased conductance (G; nS), that were not reversed by blocking synaptic GABA receptors with gabazine, but were reversed using picrotoxin to block tonic GABA currents. Each bar represents the mean $\pm$ SD.

Figure 3.

Isoflurane did not alter CA1 neuron discharge activity produced by direct depolarizing current injection. A) Three stimulus intensities were tested in control conditions (CON) and in the presence of isoflurane (+ISO; 350 μ M) and no significant effect was observed ($p > 0.1$) for 6 experimental repeats of cells recorded in brain slices from separate animals. Calibration bars: 20 mV and 200 ms. B) Isoflurane did not alter membrane potential responses to hyperpolarizing current pulses, indicating no significant ($p > 0.1$) effect of this anesthetic on membrane conductance. Calibration bars: 20 mV and 100 ms.

Figure 4.

Population spike (PS) responses were used to test anesthetic effects on CA1 neuron synaptically evoked discharge. PS were produced by stimulating Schaffer-collateral fibers with a bipolar tungsten electrode placed in Stratum radiatum. Blocking synaptic (phasic) gamma-aminobutyric acid (GABA) receptors with gabazine enhanced PS responses, evident in recordings shown on top, and in the PS amplitude (AMP) vs time graph on the bottom. Time of application for each drug is indicated by the bars below the PS AMP graph. Each graph point represents the mean $\pm$ SD from 5 time-aligned experiments and measurements were normalized to 100 % of control responses for each experiment. Blocking tonic GABA receptors with picrotoxin, in the continued presence of gabazine, produced only a small additional increase in PS responses. This indicates that GABA-mediated circuit inhibition involves mainly synaptic receptors and that tonic receptors contribute relatively little to inhibition of synaptically evoked CA1 neuron discharge.

Figure 5.

Thiopental markedly depressed population spike (PS) responses, evident in the recordings shown on top and in the amplitude vs time graph on the bottom. Time of application for each drug is indicated by the bars above the amplitude graph. Each graph point represents the mean $\pm$ SD from 5 time-aligned experiments and measurements were normalized to 100 % of control responses. The thiopental-induced depression was partially reversed using a synaptic gamma-aminobutyric acid (GABA) receptor antagonist, gabazine, and completely reversed with the chloride channel blocker picrotoxin. This indicates that the thiopental induced circuit depression involves actions at both synaptic and tonic GABA receptors to inhibit CA1 neuron discharge.

Figure 6.

Isoflurane-induced depression of population spike (PS) responses appeared to involve only synaptic gamma-aminobutyric acid (GABA) receptors. The anesthetic produced a marked depression of synaptically evoked discharge, in stark contrast to the lack of effect observed for direct current evoked discharge of CA1 neurons (Figure 3), and evident in the recordings shown on top, and in the amplitude vs time graphs on the bottom. Time of application for each drug is indicated by the bars above the amplitude graphs. A lower concentration of isoflurane (90 μM) was tested to determine whether anesthetic concentrations would produce a relatively greater activation of phasic or tonic GABA inhibition. Each graph point represents the mean $\pm$ SD from 5 time-aligned experiments and measurements were normalized to 100 % of control responses. The 1 MAC isoflurane-induced depression was significantly reversed by gabazine ($\sim 35\%$; $p < 0.001$; top graph), but the addition of picrotoxin failed to provide any further reversal of the depression. This indicates that isoflurane acts, in part, to enhance synaptic GABA-mediated inhibition, but with little or no effect evident on tonic receptors. Phasic and tonic GABA-mediated inhibition appeared to play even less of a role for the lower concentration of isoflurane.

Figure 7.

Bar graph comparing phasic and tonic gamma-aminobutyric acid (GABA) receptor involvement for control and anesthetic experiments. The graph compares contributions to the overall effect on population spikes, after subtracting baseline activities for each drug. Each bar represents the mean and SD from 5 experiments, from separate slices/animals. Under control conditions, phasic receptors contribute a significant amount to baseline population spike (PS) response amplitudes, tested with ANOVA; however, with the small number of experiments here, tonic receptor contribution was measurable but not significant (NS). Phasic receptors clearly play an important role for the thiopental-induced depression, but less so for isoflurane. Tonic GABA receptors also contribute substantially to thiopental's effect, but were not involved in the depression produced by isoflurane.