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Effects of Intravenous Anesthetic Agents on Glutamate Release

A Role for $GABA_A$ Receptor-Mediated Inhibition

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Background: **Many anesthetic agents are known to enhance the $\alpha_1\beta_2\gamma_{2S}$ γ-aminobutyric acid type A ($GABA_A$) chloride current; however, they also depress excitatory neurotransmission. The authors evaluated two hypotheses: intravenous anesthetic agents inhibit glutamate release and any observed inhibition may be secondary to $GABA_A$ receptor activation.**

Methods: **Cerebrocortical slices were prepared from Wistar rats. After perfusion in oxygenated Krebs buffer for 60 min at 37°C, samples for glutamate assay were obtained at 2-min intervals. After 6 min, a 2-min pulse of 46 mM K^+ was applied to the slices (S_1); this was repeated after 30 min (S_2). Bicuculline (1–100 μM) was applied when the S_1 response returned to basal level, and 10 min later, thiopental (1–300 μM), propofol (10 μM), or ketamine (30 μM) were also applied until the end of S_2. Perfusate glutamate concentrations were measured fluorometrically, and the area under the glutamate release curves was expressed as a ratio (S_2/S_1).**

Results: **Potassium (46 mM) evoked a monophasic release of glutamate during S_1 and S_2, with a mean control S_2/S_1 ratio of 1.07 ± 0.33 (mean ± SD, n = 96). Ketamine and thiopental produced a concentration-dependent inhibition of K^+-evoked glutamate release with half-maximum inhibition of release values of 18.2 and 10.9 μM, respectively. Release was also inhibited by propofol. Bicuculline produced a concentration dependent reversal of thiopental inhibition of glutamate release with a half-maximum reversal of the agonist effect of 10.3 μM. Bicuculline also reversed the effects of propofol but not those of ketamine.**

Conclusions: **The authors' data indicate that thiopental, propofol, and ketamine inhibit K^+-evoked glutamate release from rat cerebrocortical slices. The inhibition produced by thiopental and propofol is mediated by activation of $GABA_A$ receptors, revealing a subtle interplay between GABA-releasing (GABAergic) and glutamatergic transmission in anesthetic action. (Key words: Depolarization; $GABA_A$ antagonist; mechanisms of anesthesia; neurotransmitter release; rat cerebrocortical slices.)**

GLUTAMATE is the predominant excitatory amino acid neurotransmitter in the mammalian central nervous system acting on *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4isoxazolepropionic acid (AMPA), kainate, and metabotropic receptors.[1,2] Glutamatergic transmission may have a role in learning and memory, central pain transduction, and the pathophysiology of neuronal death after brain injury.[2] Although little is known about the precise molecular target site or sites for anesthetic agents, inhibition of excitatory neurotransmission and activation of inhibitory neurotransmission have been proposed as logical modes of action.[3] There is broad agreement that general anesthetics have a greater effect on synaptic transmission than on an axonal impulse propagation, and a range of anesthetic agents are known to inhibit the release of a number of neurotransmitters.[4-6]

Potentiation of inhibitory neurotransmission *via* enhancement of $\alpha_1\beta_2\gamma_{2S}$ γ-aminobutyric acid type A ($GABA_A$)-mediated chloride conductance, prolonging and enhancing inhibitory postsynaptic potentials, is a theory of anesthetic action backed by considerable evidence.[6,7] However, there is growing evidence that general anesthetics also attenuate excitatory neurotransmission in the central nervous system, particularly at glutamate synapses.[1-3,5,8,9] Glutamatergic neurotransmission and glutamate receptor-activated responses

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have been shown to be inhibited by both intravenous and inhalational agents both.[9-11] Net synaptic concentrations of glutamate are a balance between reuptake and release, and we have previously shown that clinically relevant concentrations of most commonly used anesthetic agents did not affect reuptake of [^{3}H]L-glutamate into rat cerebrocortical and cerebellar synaptosomes.[12]

Our objectives in this series of studies were to evaluate two hypotheses: that intravenous anesthetic agents inhibit glutamate release and whether this inhibition is a direct effect on glutamatergic neurons or an indirect effect *via* an action at the $GABA_A$ receptor. We studied the interaction of thiopental, propofol, and ketamine with bicuculline (a $GABA_A$ antagonist) on 46-mM K^+-evoked glutamate release from rat cerebrocortical slices.

Materials and Methods

Preparation of Cerebrocortical Slices and Perfusion Protocol

Female Wistar rats (250–300 g) were killed by cervical dislocation and decapitation. The brain was rapidly removed and placed in ice-cold oxygenated (95% O_2, 5% CO_2) bicarbonate buffer, pH 7.4, of the following composition: 115 mM NaCl; 4.7 mM KCl; 2.0 mM $CaCl_2$; 1.2 mM $MgCl_2$; 25 mM $NaHCO_3$; and 8.8 mM glucose. The cortex was dissected from the other cerebral structures, cut into 350 × 350 μM slices using a MacIlwain Tissue Chopper (Mickle Lab Engineering Co. Ltd., Surrey, UK), and suspended in bicarbonate buffer. After being washed three times in fresh bicarbonate buffer, slices were agitated in a shaking water bath at 37°C for 40 min. Approximately 1 ml gravity-packed slices (protein not determined) were pipetted into a perfusion chamber and held in place by a greased diffuser. The perfusion chamber consisted of a 2-ml syringe barrel cut at approximately 2 cm and packed at the needle end with a 0.5-cm-thick layer of glass wool. The chamber was sealed around the diffuser with two O-rings. The perfusate entered the diffuser *via* a peristaltic pump, which provided a continuous, steady flow to the cerebrocortical slices at the diffuser end and exited at the needle end of the syringe, with the eluate being collected by a fraction collector. Slices were perfused at 37°C at 1 ml/min for 60 min to allow for stabilization before collection of 2-min fractions for the estimation of glutamate concentrations. After 6 min of perfusion, 46 mM K^+ (Na^+ adjusted to maintain tonicity) was applied (total K^+ was therefore 46 + 4.7 mM = 50.7 mM) for 2 min (S_1). Slices were perfused for an additional 30 min prior to the second application of a 2-min pulse of 46 mM K^+ (S_2). Fractions were collected for 8 min after S_2.

Measurement of Endogenous Glutamate Release

The fractions collected were analyzed for glutamate using an adaptation of the fluorescence method previously described.[13] Glutamate dehydrogenase catalytically reduces any glutamate present to 2-oxoglutarate, accompanied by the reduction of NAD^+/NADP (nicotinamide adenine nucleotides) to NADH/NADPH. As NADH undergoes reoxidation, $NADP^+$ is used.[13] Assay volume was 500 μl and consisted of 15 μl of glutamate dehydrogenase (final concentration, 30 U), 5 μl of NADP (final concentration, 1 mM), and 480 μl of perfusate. The mixture was incubated at 37°C for 10 min, and fluorescence intensity was determined using a Perkin-Elmer LS50B (Beaconsfield, Bucks, UK) spectrofluorometer with excitation and emission wavelengths set at 366 and 430 nm, respectively. Perfusate sample fluorescence intensity was then compared with a known set (0.5–20 pmol) of glutamate standards.

Introduction of Anesthetic Agents and Bicuculline

Thiopental (3–300 μM), propofol (10 μM), and ketamine (3–300 μM) were introduced to the buffer immediately after S_1 until the end of the experiment (thus including S_2). In some experiments, 100 μM pentobarbital and 300 μM barbituric acid were included as additional active and inactive barbiturates, and 1 μM dizocilpine (MK-801) was included as a noncompetitive NMDA receptor antagonist. In experiments using 1–100 μM bicuculline, it was introduced when S_1 response had returned to the basal level by perfusing the slices with a solution of the relevant concentration of bicuculline. After 10 min, coincubation of bicuculline with 100 μM thiopental, 10 μM propofol, or 30 μM ketamine took place until the end of S_2. Control response with and without a high and low concentration of test agent were obtained from a single rat (*i.e.*, three conditions per animal), and these data were combined to produce a full concentration response curve.

Data Analysis

Time course of glutamate release is presented relative to the mean of the first three basal samples collected during S_1 or S_2. This measurement both confirms slice

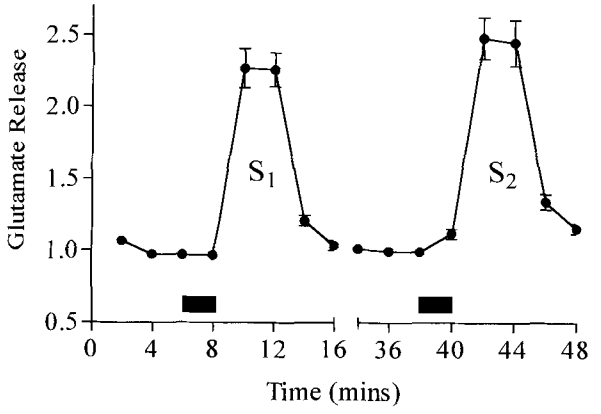

Fig. 1. Effects of 46 mM K^+ (solid bar) on glutamate release from perfused rat cerebrocortical slices. Slices were challenged twice (S_1 and S_2); challenges were separated by 30 min. Release is expressed relative to the mean of the first three fractions collected during S_1 or S_2. The effects of anesthetic agents and bicuculline added for various times after S_1 on S_2 were investigated. S_2/S_1 ratios are calculated from the area under both stimulation profiles. Data are mean ± SEM and are from the 96 control experiments performed during the entire study.

responsiveness (S_1) and normalizes for differences in protein content among experiments. The areas under the stimulation curves (S_1 and S_2) were then calculated and data presented as S_2/S_1 ratio. In some experiments, the inhibition of this ratio is presented. Statistical analysis was performed using Student paired *t* test for paired comparisons of S_2/S_1 ratios and analysis of variance when a series of comparisons was necessary. $P < 0.05$ was taken as an indication of statistical significance. The analysis of concentration-response curves in release and release-inhibition studies to yield half-maximum inhibition of release (EC_{50}; *i.e.*, an agonist effect), and half-maximum reversal of the agonist effect (IC_{50}) was performed using computer-assisted curve fitting (sigmoid concentration response curve-variable slope) on GraphPad Prism 2.0. (GraphPad Software, San Diego, CA). Because each concentration response curve is produced from data collected from multiple experiments, it is not possible to derive error estimates for these values.

Results

K^+-evoked Release

Addition of 46 mM K^+ produced monophasic releases of glutamate during both S_1 and S_2 from perfused rat cerebrocortical slices. Over the entire study, the control (n = 96) S_2/S_1 ratio was 1.07 ± 0.33 (mean ± SD; fig. 1). There was considerable variation in the S_2/S_1 ratio (95% confidence interval, 1.00–1.13; range, 0.47–1.88) between animals, but each animal acted as its own control. Glutamate release from rat cerebrocortical slices was dependent on extracellular Ca^{2+} (data not shown), indicating exocytotic release of vesicular glutamate and not reversal of the glutamate uptake transporter.

Effects of Intravenous Agents

Thiopental (fig. 2) and ketamine (fig. 3) produced a concentration-dependent inhibition of 46 mM K^+-evoked

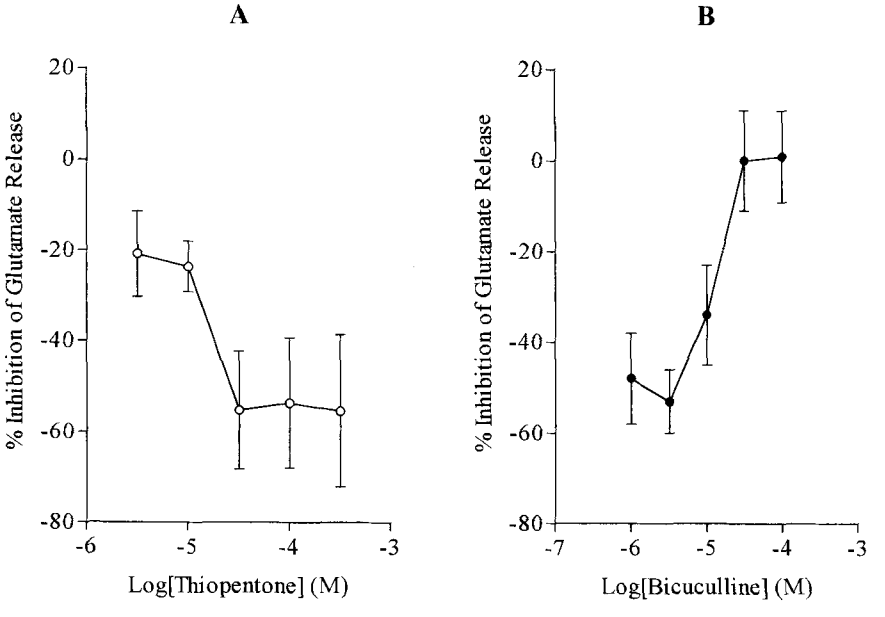

Fig. 2. (*A*) K^+-evoked (46 mM) glutamate release from perfused rat cerebrocortical slices was inhibited in a concentration-dependent manner by thiopental. (*B*) Thiopental (100 μM) inhibition of glutamate release was reversed in a concentration-dependent manner by bicuculline. Percent inhibition of release was calculated from the S_2/S_1 ratios obtained as shown in figure 1, and inhibition is denoted by a downward deflection in the concentration response curve (*i.e.*, 0 = no inhibition; −60 = 60% inhibition). Data are mean ± SEM (n ≥ 5). Estimated half-maximum inhibition of release for thiopental occurred at 10.9 μM.

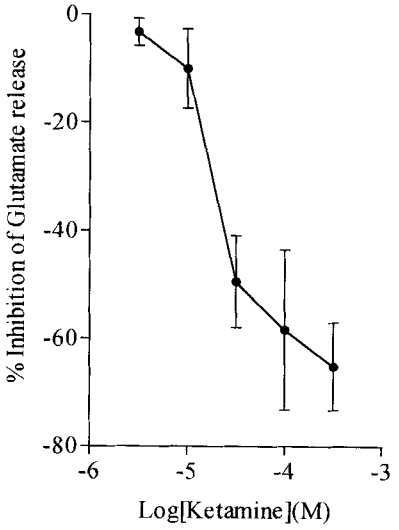

Fig. 3. K^+-evoked (46 mM) glutamate release from perfused rat cerebrocortical slices was inhibited in a concentration-dependent manner by ketamine. Percent inhibition of release was calculated from the S_2/S_1 ratios obtained as shown in figure 1, and inhibition is denoted by a downward deflection in the concentration response curve (*i.e.*, 0 = no inhibition; −60 = 60% inhibition). Data are mean ± SEM (n ≥ 5). The estimated half-maximum inhibition of release value for thiopental was 18.2 μM.

glutamate release from rat cerebrocortical slices with maximum inhibition of 55.4 ± 16.8% and 65.1 ± 8.1%, respectively, produced at 100 μM. The EC_{50} for this inhibition was 18.2 and 10.9 μM, respectively (figs. 2A and 3). In addition, 100 μM pentobarbital also significantly inhibited glutamate release by 60.4 ± 14.4% ($P < 0.05$). In contrast, the inactive barbiturate barbituric acid was ineffective. MK-801, a noncompetitive NMDA antagonist, inhibited release by 81.2 ± 7.5% compared with control ($P < 0.05$). At 10 μM, propofol inhibited glutamate release by 74.8 ± 8.2% ($P < 0.05$).

Effect of Bicuculline on Anesthetic-induced Inhibition

In a separate series of pilot studies, we showed that bicuculline 100 μM alone had no effect on 46 mM K^+-evoked glutamate release (S_2/S_1 ratio control, 1.01 ± 0.10; bicuculline, 0.96 ± 0.04; $P > 0.05$; n = 5) and that this concentration also fully reversed 100-μM thiopental-induced inhibition of release. We further probed this inhibitory effect of bicuculline by constructing a full concentration-response curve. Bicuculline produced a complete reversal of 100 μM thiopental inhibition of release with an estimated IC_{50} of 10.9 μM (fig. 2B).

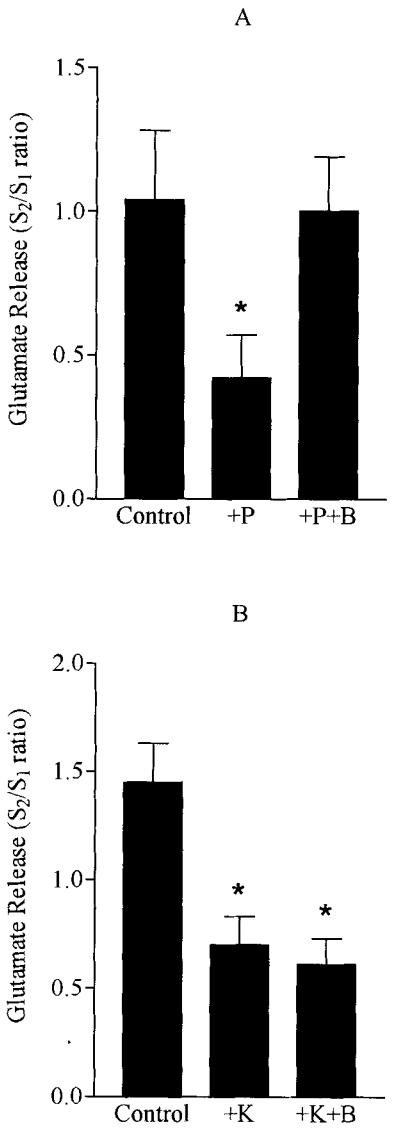

Fig. 4. (*A*) Propofol (P) (10 μM), but not (*B*) ketamine (K) (30 μM) inhibition of glutamate release from perfused rat cerebrocortical slices was reversed by bicuculline (B) 100 μM. S_2/S_1 ratios are presented as the mean ± SEM (n ≥ 5). *$P < 0.05$ reduced compared with control.

Bicuculline (100 μM) also fully reversed the inhibition produced by propofol (fig. 4A). In contrast, the inhibition produced by 30 μM ketamine was completely unaffected by bicuculline (fig. 4B).

Discussion

We report a reliable monophasic release of glutamate from perfused oxygenated rat cerebrocortical slices in

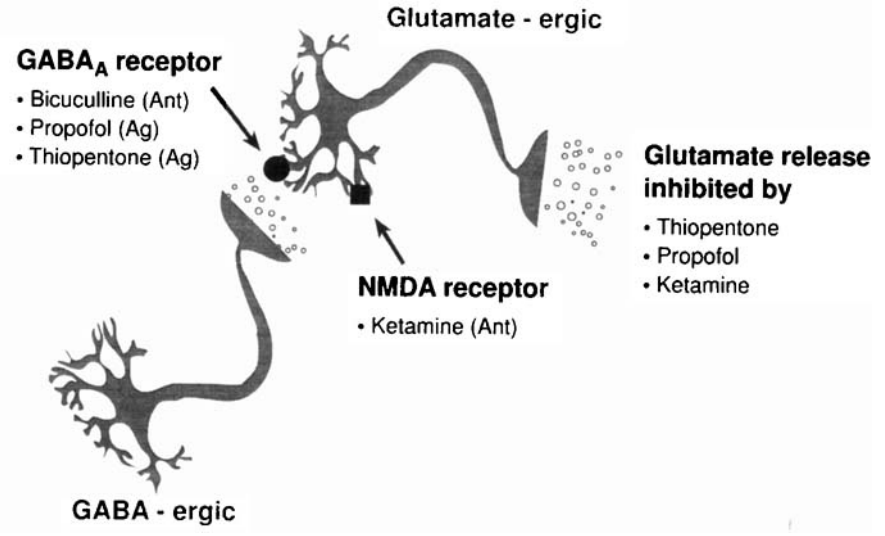

Fig. 5. Schematic representation of the presumed interplay between GABAergic and glutamatergic transmission in the actions of thiopental, propofol, and ketamine. In this model, GABA released from GABAergic neurons reduces the release of glutamate from glutamatergic neurons. Thiopental, propofol, and ketamine inhibit the release of glutamate. Our data suggest that for thiopental and propofol, the inhibition is secondary to $GABA_A$ (filled circle) receptor activation-because the inhibition of glutamate release is reversed by the $GABA_A$ antagonist bicuculline. Ketamine reduced glutamate release *via* an action at the NMDA (filled square) receptor. Precise receptor locations are hypothetical. Ag = agonist; Ant = antagonist.

response to 46 mM K^+ depolarization. We used this model system to probe the effects of a range of intravenous anesthetic agents on glutamate release. We have clearly showed that ketamine, thiopental, and propofol markedly reduce glutamate release. In addition, MK-801 (a noncompetitive NMDA antagonist) and pentobarbital, but not barbituric acid, also significantly inhibit glutamate release. These data confirm our initial hypothesis that glutamate release is a target for intravenous anesthetic agents. Bicuculline, a $GABA_A$ antagonist, reversed the inhibition of 46 mM K^+-evoked glutamate release produced by thiopental and propofol, suggesting that this inhibition of glutamate release is mediated indirectly *via* an agonist action at the $GABA_A$ receptor. As a negative control, the inhibition of release produced by the NMDA antagonist ketamine was unaffected by bicuculline. Collectively, our data indicate that there is a subtle interaction between glutamatergic and GABA-releasing (GABAergic) transmission in the production of the anesthetized state (fig. 5). In this model, it is assumed that $GABA_A$ receptor activation of glutamatergic neurons by thiopental and propofol results in hyperpolarization, which reduces the amount of glutamate released. *In vivo*, we envisage an interplay between GABAergic and glutamatergic transmission in the control of cortical activity, with release of transmitter from the former affecting glutamate release from the latter.

Enhancement of $GABA_A$ receptor activity has been championed by many authors as the principal target site for anesthetic agents.[6,7,14-17] Certainly, barbiturates have been shown to enhance the affinity of GABA for the $GABA_A$ receptor and to slow the dissociation of GABA from its receptor.[14,15] Propofol has also been shown to enhance the inhibitory actions of GABA.[14,16] Recent work using chimeric receptor constructs has identified a 45-amino acid sequence on the $GABA_A$ receptor that is both necessary and sufficient to enhance receptor function, supporting the notion that ethanol and the volatile anesthetic agent enflurane act at this site to exert a specific effect on this important ion-channel protein.[17]

Our finding of reversal of an anesthetic effect by bicuculline (and hence an action at $GABA_A$ receptors) is supported by two electrophysiologic studies. In the first, using rat hippocampal CA1 slices, inhibition of electrically evoked population spikes by pentobarbital and propofol were completely reversed by bicuculline, but ketamine had no such effect[18]; however, glutamate release was not measured. Another study, also using rat hippocampal CA1 slices, found that halothane and isoflurane reduced glutamate transmission (excitatory postsynaptic potentials), but the reduction was completely insensitive to bicuculline 10 μM.[19] The underlying mechanism of the discrepancy between intravenous agents and volatile agents in their susceptibility to reversal by bicuculline is unclear but clearly warrants further investigation.

The IC_{50} of 10.9 μM for the reversal of thiopental inhibition of K^+-evoked glutamate release by bicuculline is consistent with other studies.[20-22] In these studies, GABA-evoked responses in the vagus nerve *in vitro* were inhibited with IC_{50} values of 6.1 and 1.2 μM, respectively. Although it has been suggested that bicuculline acts on more than the $GABA_A$ receptor within the central nervous system, perhaps on dopaminergic midbrain sys-

tems (IC_{50} of 26 μM),[22] we feel it is unlikely that the effects we observed in this study are explained by non-$GABA_A$ actions of bicuculline.

Total peak serum concentrations of anesthetic agents vary considerably between studies and among different species. Peak serum concentrations for ketamine, propofol, thiopental, and pentobarbital of 9–94, 35, 380, and 200 μM, respectively, have been reported.[23,24] Protein binding variably reduces these concentrations. In addition, these may not represent target site (brain) concentrations because propofol has been shown to be concentrated approximately eightfold in the brain.[25] The clinical relevance of *in vitro* studies can always be questioned; however, the concentrations used in our study represent those that are clinically achievable. EC_{50} values for ketamine and thiopental of 18.2 and 10.9 μM fall well within the range of values noted previously, even when corrected for protein binding.

Not all barbiturates inhibit glutamate release. Wei *et al.*[26] reported that [5-(2-cyclohexylidene-ethyl barbituric acid] (CHEB), a convulsant barbiturate, stimulates glutamate release from rat cerebrocortical synaptosomes with an EC_{50} of 14.2 μM. In addition, pentobarbital and phenobarbital inhibited this CHEB-evoked release. The inhibitory effect of ketamine on glutamate release is well recognized as an NMDA receptor-mediated event. In a model of neonatal rat spinal cord, Brockmeyer and Kendig[27] showed a dose-dependent inhibitory effect of ketamine on glutamate release and ventral root potential, which constitute an NMDA receptor-mediated phenomenon. Importantly, dorsal root potential, a GABA receptor-mediated phenomenon, was unaffected by ketamine. However, the action of ketamine on glutamatergic transmission is more complex than simple inhibition. A study of glutamatergic neurotransmission in the prefrontal cortex of conscious rats indicated that low doses of ketamine increased glutamatergic transmission by an action of non-NMDA postsynaptic receptors, but that higher anesthetic doses similar to those used in our study decreased glutamate release.[28] More recently, it has been shown that thiopental and methohexital reduce glutamate release from and Ca^{2+} entry into cultured neurons.[29]

We have shown that 10 μM propofol produces a substantial inhibition of 46 mM K^+-evoked glutamate release. However, in a study of hypoxemia-induced glutamate release from rat brain slices. Bickler *et al.*[11] failed to detect an inhibition of glutamate release with propofol. The variability of glutamate release in response to propofol is further illustrated in the findings of Ratnakumari and Hemmings,[30] who reported that propofol inhibited veratridine- and 4-aminopyridine-evoked glutamate release (both Na^+ channel-dependent processes). However, propofol did not inhibit K^+-evoked glutamate release (a Na^+ channel-independent process) up to 100 μM. The reason for the difference between these results and our findings of inhibition of K^+-evoked glutamate release by propofol is unclear but may be related to the different experimental models. These investigations used a rat cerebrocortical synaptosome preparation, which may not have contained the complete circuitry of excitatory and inhibitory interneurons, in contrast to our model using rat cerebrocortical slices.

The precise role of glutamate release in the mechanism of general anesthesia remains to be fully elucidated. Although most studies support a role for glutamate release in the mechanism of volatile agent anesthesia,[31–33] a recent study using cyclobutane derivatives with anesthetic and nonanesthetic properties found that both anesthetic and nonanesthetic compounds strongly inhibited glutamate release, but only the nonanesthetic compound inhibited K^+-evoked GABA release, suggesting that inhibition of excitatory neurotransmission may be less important than activation of inhibitory neurotransmission in the primary action of general anesthetics.[34] This is consistent with our observation in the current study that inhibition of K^+-evoked glutamate release by thiopental and propofol is reversed by bicuculline, a $GABA_A$ antagonist. In humans, clinical anesthesia induced by NMDA antagonists such as ketamine differs in some qualitative respects from that induced by thiopental and propofol. Ketamine anesthesia is characterized by "dissociative anesthesia," with analgesia, sedation, and detachment. Thiopental and propofol are noted for profound depression of consciousness and lack of analgesic effects.[2,3] This is consistent with our laboratory finding that ketamine inhibits glutamate release in a manner unrelated to that of thiopental and propofol. Further studies are warranted to determine the neuronal circuitry involved in GABA-mediated inhibition of glutamate release. Using this model, it will be interesting to determine whether volatile agent inhibition of glutamate release is bicuculline sensitive.

A variety of commonly used intravenous anesthetic agents have been shown to depress the K^+-evoked release of the excitatory neurotransmitter glutamate. The inhibition produced by thiopental and propofol is probably secondary to activation of $GABA_A$ receptors.

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