Enhancement of Cortical GABAergic Function does not Account for the Anticonvulsant Effects of Midazolam, Isoflurane or Etomidate

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Abstract: *Background*: Enhancement of cerebrocortical GABAergic inhibition is postulated as the main mechanism by which anesthetics inhibit seizures; however this has not been directly tested under controlled conditions. In this study we utilized the isolated cerebral cortex slice preparation and tested the anticonvulsant properties of three anesthetics with differing specificities for GABAergic activation and with various clinical and experimental anticonvulsant efficacy; midazolam, isoflurane and etomidate.

Methods: Two interictal-like models were investigated; low-magnesium artificial cerebrospinal fluid (ACSF) and low dose (230nM) aconitine in low-magnesium ACSF. The former generates interictal-like events by facilitating NMDA activation and is well described in the cortical slice preparation; the latter generates a unique pattern of interictal-like activity by opening voltage-gated sodium channels.

Results: We found that at anesthetic concentrations and above (15μM midazolam, 480μM isoflurane and 4μM etomidate), only isoflurane had an anticonvulsant effect. The isoflurane effect was seen as a reduction in event frequency in both models (56% reduction in low-magnesium and 26% reduction in aconitine, $p<0.005$ and $p<0.05$, respectively). Picrotoxin (100μM) was ineffective at blocking the isoflurane anticonvulsant effect (56% reduction in event frequency, $p<0.01$). Picrotoxin (100μM) and bicuculline (25μM) administered on their own increased event frequency (107% and 75%, respectively, $p<0.005$), indicating the GABAergic system was functional in the slice preparation.

Conclusion: These results call into question the view that enhancement of *cortical* GABAergic function is the *primary* mechanism by which anesthetics suppress seizure activity.

Keywords: Anesthetics, anticonvulsant, neocortical slice, GABA.

INTRODUCTION

The anticonvulsant properties of many anesthetics are well recognised both experimentally [1] and clinically [2, 3]. At the receptor level, enhancement of GABAergic inhibition is postulated as the principal mechanism by which anesthetics reduce seizures [1, 4]. Furthermore, because most seizures have a strong cortical component, as evidenced by characteristic seizure-induced electroencephalogram changes, the anticonvulsant effects of anesthetics are thought to reside predominantly within the cerebral cortex. However, there is evidence for strong subcortical-mediated anticonvulsant effects for some anesthetic drugs, particularly benzodiazepines [5-7].

In this study, we have investigated the role of cortical GABAergic processes in mediating the anticonvulsant properties of three anesthetics with differing specificities for $GABA_A$ receptors; midazolam, isoflurane and etomidate. Both midazolam and etomidate have a high degree of specificity for the $GABA_A$ receptor [8, 9], while isoflurane is a relatively non-specific drug [10, 11]. We utilized the isolated neocortical slice model because this preparation is void of all subcortical connections; it is therefore a good model for investigating cortical drug effects without the added complexity of subcortical influences. We tested the effect of each drug on two interictal-like models, both of which generate unique patterns of interictal-like epileptiform activity: 1) low-magnesium artificial cerebrospinal fluid (ACSF), a model which has been extensively studied in the cortical slice preparation [12, 13]. Low-magnesium interictal-like activity is thought to be primarily of glutamatergic origin [14] and is sensitive to NMDA antagonism [14]; and 2) aconitine perfusion in low-magnesium ACSF. Aconitine is a potent neurotoxin that increases neuronal excitability by increasing intracellular calcium independent of NMDA and non-NMDA receptors [15]. It is thought to prevent synaptic voltage-gated sodium channel inactivation, causing cell depolarization and a prolonged increase in intracellular calcium [16]. We have shown recently that nano-molar concentrations of aconitine induce a unique pattern of interictal-like activity in the cortical slice preparation [17].

We hypothesized that, if the anesthetics tested act primarily *via cerebrocortical* $GABA_A$ potentiation to reduce seizures, then all three agents should inhibit interictal-like events in the neocortical slice model at anesthetic concentrations.

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MATERIALS AND METHODOLOGY

Slice Preparation

Ethical approval for the study was obtained from the Waikato Ethics Committee. Neocortical slices were prepared from 3-6 week old Sprague-Dawley rats of either sex. The rats were decapitated while anesthetised with carbon dioxide, in accordance with local animal ethics guidelines. The brain was rapidly removed and cooled in ice-cold artificial cerebrospinal fluid (ACSF), modified for cerebral protection according to Nowak and Bullier [18], with composition (in mM): NaCl 92.7; $NaHCO_3$ 24; NaH_2PO_4 1.2; KCl 3; $MgCl_2$ 19; $CaCl_2$ 0; D-glucose 25; bubbled with carbogen (95% O_2; 5% CO_2). Coronal slices (400 μm) were cut between bregma –3mm to –4mm on a vibratome (Campden Instruments, UK) in ice-cold ACSF as above and transferred to a holding chamber containing carbogenated, low-magnesium ACSF of composition: NaCl 124; $NaHCO_3$ 26; NaH_2PO_4 1.25; KCl 5; $MgCl_2$ 0; $CaCl_2$ 2; D-glucose 10. The holding chamber was maintained at room temperature (18-20°C) where the slices were held for at least one hour before being transferred to the recording chamber. Submerged slices were perfused with carbogenated ACSF at a flow rate of 2.5mL/min.

Electrical Recording

Spontaneous local field potential activity was recorded using a single 50μm teflon-coated tungsten wire positioned in the middle to outer layer of the somatosensory cortex. A silver/silver chloride disc electrode served as a common reference/bath ground. The signal was amplified (1000x, A-M Systems, USA) and bandpass filtered (1 and 3000Hz) before analog-digital conversion (Power 1401, CED, UK) and recording on computer for later analysis (Spike2, CED, UK). The entire recording set-up was enclosed within a grounded Faraday cage to reduce electrical noise.

Drug Dosage

Isoflurane was delivered to achieve a level in the perfusion bath of approximately 480μM (confirmed using gas chromatography-mass spectrometry), slightly higher than 1 MAC for rodents (350-400μM) [19, 20]. Etomidate was perfused at a dose of 4μM, equating to a deep anesthetic concentration for rodents, taking into consideration protein binding and the rate of diffusion of etomidate into brain slice tissue [21, 22]. The midazolam concentration used was 15μM, which equates approximately to an anesthetic dose [23-25] with consideration given to diffusion into the slice tissue (see the Discussion section for clarification of drug concentrations).

Experimental Protocols

For all experiments, carbogenated ACSF was perfused continuously *via* a sealed 50mL syringe, driven by an infusion pump at a rate of 2.5mL/min. Drugs were delivered *via* the same system, by adding the appropriate amount directly to the ACSF-filled syringe. All drugs were perfused for 20mins (i.e. one 50mL syringe), followed by washout with drug-free ACSF for at least 40mins. If interictal-like activity was eliminated, drug-free ACSF was continued until activity returned. This perfusion sequence achieved a gradual increase and decrease in drug concentration. The following testing regimes were carried out:

1. For testing in low-magnesium ACSF, a stable pattern of interictal-like activity was established and recorded for at least 10 minutes. Thereafter, either midazolam (15μM), isoflurane (480μM) or etomidate (4μM) were perfused for 20mins followed by washout as described above.
2. For testing the effect of anesthetics on interictal-like activity induced by aconitine, the protocol described by Voss *et al.* [17] was followed, except that aconitine was perfused in low-magnesium ACSF. The aconitine-induced activity in low-magnesium ACSF is the same as when delivered in "normal" ACSF [17], except that the sequence of changes occurs somewhat more quickly when magnesium is absent from the solution. Once the slices were positioned in the recording bath and a stable pattern of low-magnesium interictal-like activity established, aconitine (230nM) was added to the low-magnesium perfusate and run continuously until the end of the experiment. The anesthetic drugs were not added to the perfusate until the full effect of aconitine had been established, usually after 30-40 minutes.
3. The results obtained in 1) and 2) above showed that of the three agents tested, only isoflurane had a significant anticonvulsant effect. To test whether this effect was *via* a GABA-dependent mechanism, the isoflurane experiments were repeated in low-magnesium ACSF during perfusion with the $GABA_A$ antagonist picrotoxin (100μM).
4. To clarify that the GABAergic system was intact and functioning in the slices, we tested in a separate series of slices the effect of two GABA antagonists, bicuculline (25μM) and picrotoxin (100μM), on low-magnesium interictal-like activity.

Statistical Analysis

For statistical comparison of drug effects during low-magnesium perfusion, the interictal-like event frequency/amplitude was averaged over the 5min immediately prior to drug delivery and compared to the average value during the 20min window beginning 10mins after the start of drug infusion. This time window was chosen because it coincided with the greatest drug effect on interictal-like activity. This window was shifted 10mins later for the aconitine analysis because the isoflurane effect was delayed slightly. These effects were analysed using the paired t-test. The Kolmogorov-Smirnov test was used to confirm the data were normally distributed. Unless otherwise stated, the data are expressed as mean (SD) and $p<0.05$ was considered statistically significant.

RESULTS

Interictal-Like Activity Induced by Low-Magnesium ACSF and Aconitine

Perfusion of cortical slices with low-magnesium ACSF induced a consistent pattern of interictal-like activity (Fig. **1a** and **1b**). The interictal-like events typically consisted of a large depolarisation, followed immediately by a 4-7Hz oscillation of varying length, but no longer than 3s. The ampli-

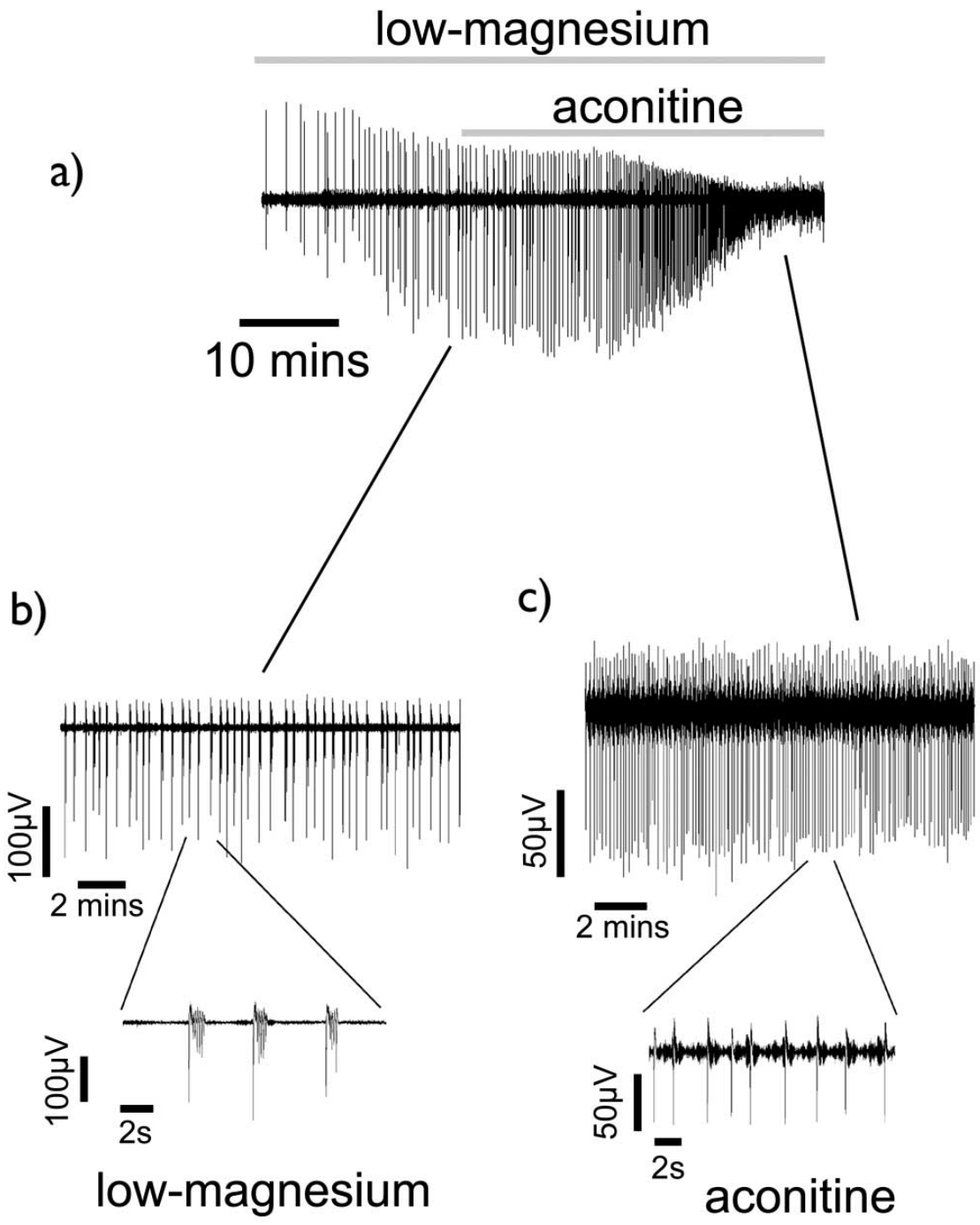

Fig. (1). Profile of interictal-like activity induced in a single cortical slice while perfusing with low-magnesium and aconitine ACSF. Each of the high amplitude lines is a single interictal-like event, several of which are expanded in the bottom graphs for low-magnesium events (**b**) and aconitine (**c**). Note the different amplitude scales.

tude of the initial depolarisation was usually larger than the oscillation that followed, and ranged from 50-300µV. Interictal-like events occurred with variable frequency, from 0.5-5 events per minute. The pattern of activity was ongoing and stable during two hours of low-magnesium ACSF infusion (results not shown).

The effect of addition of aconitine to the low-magnesium perfusate is shown in Fig. (**1a**) and (**1c**); and described in detail in Voss *et al.* [17]. Briefly, the low-magnesium pattern described above is transformed into single-spike events that recur at a frequency of 0.5-1Hz. The amplitude of the aconitine events is usually lower than low-magnesium events obtained from the same slice.

Effect of Anesthetic Agents on Low-Magnesium and Aconitine Interictal-Like Activity

For the purposes of this study, a reduction in the frequency of interictal-like events was considered equivalent to

an anticonvulsant effect. This is consistent with the clinical profiling of anticonvulsant drugs, where suppression of interictal spikes is used as a measure of anticonvulsant effectiveness [26-29]. Isoflurane had an anticonvulsive effect at an anesthetic dose in both the low-magnesium (mean 56% reduction in event frequency, $p<0.005$ n=10) and aconitine (26% reduction in event frequency, $p<0.05$ n=10) models. No change in event amplitude was observed (Table **1** and **2**). An example of the effect of isoflurane on interictal-like events during low-magnesium perfusion is shown in Fig. (**2**). Neither midazolam (15μM), nor etomidate (4μM) significantly altered event frequency or amplitude, in either model (Table **1** and **2**). Because midazolam relies on pH-induced modification of its molecular structure to act, we repeated the experiment using a 10μM concentration of diazepam (n=6, 2 animals), which showed an identical lack-of-effect on the interictal-like events (data not shown).

Table 1. Low-Magnesium Interictal-Like Event Frequency and Amplitude Statistics for Midazolam, Isoflurane and Etomidate. Data are Mean (SD)

	Interictal Frequency (events/min)	
	Baseline (low-magnesium)	After drug infusion
Midazolam 15μM (n=10, 3 animals)	2.7(1.7)	3.4(1.4) ns
Isoflurane 480μM (n=10 2 animals)	2.5(1.9)	1.1(0.8)*
Etomidate 4μM (n=9, 2 animals)	2.6(1.2)	2.6(1.1) ns

*p<0.005, compared with baseline (paired t-test).
ns, not significant.

	Interictal Amplitude (μV)	
	Baseline (low-magnesium)	After drug infusion
Midazolam 15μM (n=10, 3 animals)	167(63)	173(82) ns
Isoflurane 480μM (n=10, 2 animals)	130(49)	141(68) ns
Etomidate 4μM (n=9, 2 animals)	183(85)	195(101) ns

ns, not significant.

Table 2. Aconitine Interictal-like Event Frequency and Amplitude Statistics for Midazolam, Isoflurane and Etomidate. Data are Mean (SD)

	Interictal Frequency (events/min)	
	Baseline (low-magnesium + aconitine)	After drug infusion
Midazolam 15μM (n=8, 2 animals)	16.2(8.0)	16.7(6.5) ns
Isoflurane 480μM (n=10, 2 animals)	13.8(6.6)	10.9(7.7) *
Etomidate 4μM (n=10, 2 animals)	17.8(11.5)	15.2(5.9) ns

*p<0.05, compared with baseline (paired t-test).
ns, not significant.

	Interictal Amplitude (μV)	
	Baseline (low-magnesium + aconitine)	After drug infusion
Midazolam 15μM (n=8, 2 animals)	60(27)	57(28) ns
Isoflurane 480μM (n=10, 2 animals)	72(31)	65(26) ns
Etomidate 4μM (n=10, 2 animals)	72(21)	82(30) ns

ns, not significant.

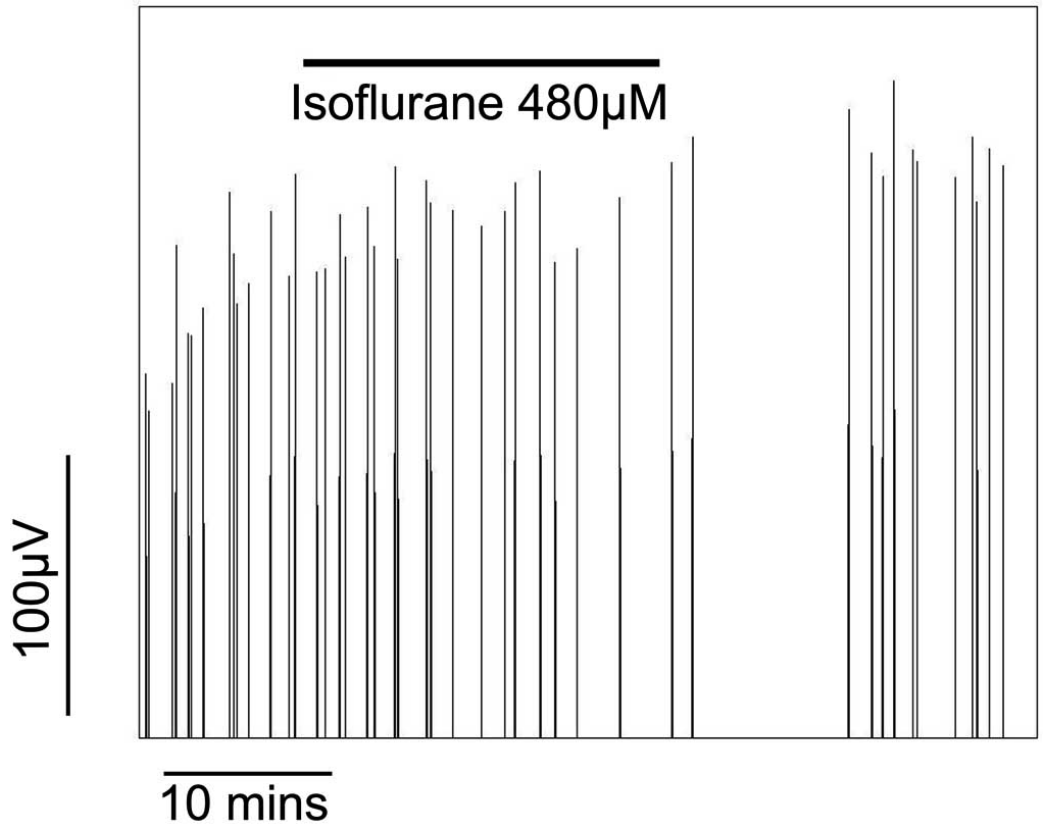

Fig. (2). Graph showing the effect of isoflurane (480μM) on low-magnesium interictal-like events.

Effect of GABA Antagonists/Agonists on Low-Magnesium Interictal-Like Activity

To determine whether the anticonvulsant effect of isoflurane could be attributed to its GABAergic action, a separate group of slices (n=6 from 1 animal) were pre-treated with picrotoxin (100μM) for 20mins, followed by combined picrotoxin (100μM) and isoflurane (480μM) for a further 20mins. A significant reduction in interictal-like event frequency was still observed (56% reduction, $p<0.01$), a reduction that was not significantly different compared to isoflurane perfusion on its own.

To test the integrity of endogenous GABAergic activity in the slice model, the effect of GABA antagonists bicuculline and picrotoxin on low-magnesium activity was investigated. Both consistently increased interictal-like event frequency (mean increase of 75%, $p<0.005$, n=6 from 2 animals and 107% $p<0.005$, n=6 from 1 animal, respectively), with no effect on event amplitude. An example of the effect of picrotoxin is shown in Fig. (**3**).

DISCUSSION

The anticonvulsant properties of anesthetics are well recognized [1], although the site and mechanism of their inhibitory effect on seizure activity has not been thoroughly investigated. Because most seizures have a strong cortical component, the anticonvulsant effects of anesthetics are thought to reside predominantly within the cerebral cortex. Furthermore, enhancement of GABAergic activity is thought to underpin the anticonvulsant actions of many anesthetics [1, 4]. In this study we tested these ideas by generating interictal-like activity within isolated neocortical slice tissue (thus eliminating the effect of subcortical interactions and drug effects) and compared the anticonvulsant properties of anesthetic drugs with differing specificities for the $GABA_A$ receptor. Our main finding was that the $GABA_A$-specific agents midazolam and etomidate were ineffective at controlling interictal-like activity of neocortical origin; even in doses that were higher than would be used clinically for seizure control. Furthermore, the anticonvulsant effect of isoflurane could not be explained by enhanced $GABA_A$ activity. These results call into question the importance of cortical GABAergic modulation in mediating anesthetic anticonvulsant activity.

An important question in addressing these findings is whether the cortical slice model is a valid proxy for the intact cortex? The main functional difference between a "healthy" cortical slice and an intact cortex is the reduction in connectedness; subcortical connections are eliminated and cortical-cortical connections drastically reduced. Isolation of the cerebral cortex from subcortical structures has the important benefit of allowing cortical drug effects to be investigated without the complication of subcortical-cortical interactions. However, the resulting reduction in subcortical "drive" has the additional effect of rendering the tissue quiescent, a state not dissimilar to that seen during certain stages of natural sleep [30]. Benzodiazepines do not directly activate $GABA_A$ receptor chloride channels and their efficacy is therefore determined by endogenous GABA levels [31]. A marked reduction in prevailing GABA activity might be expected in a quiescent cortical slice and this may explain the lack of effect of midazolam in this model. We tested this

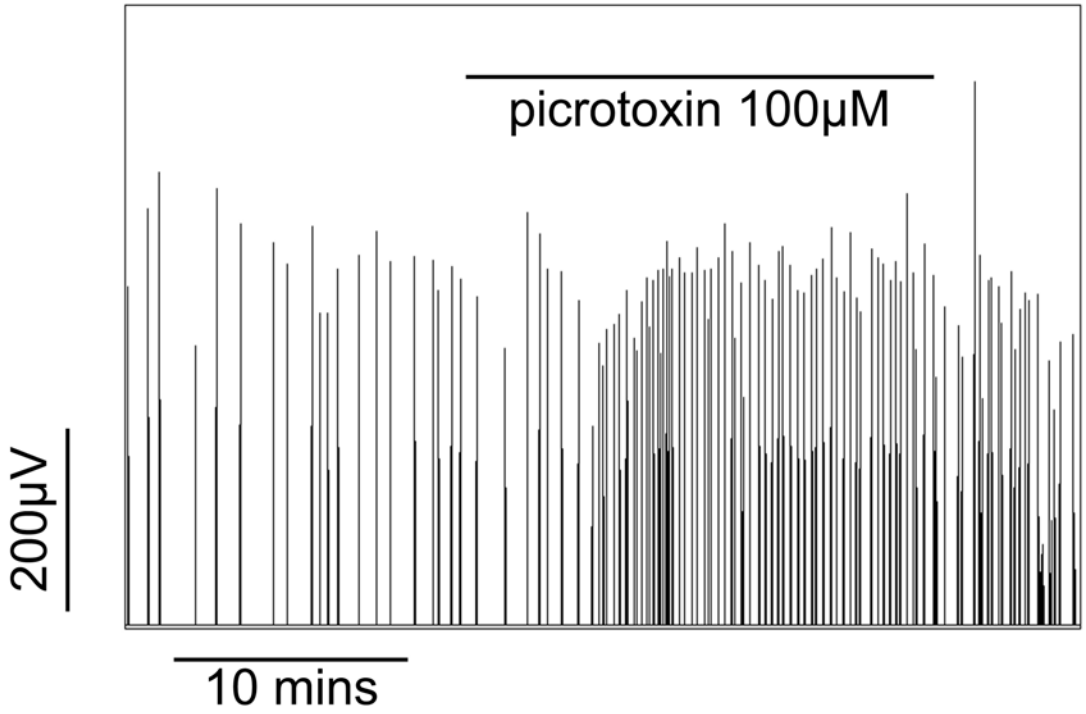

Fig. (3). Graph showing the effect of picrotoxin (100µM) on low-magnesium interictal-like events.

possibility by blocking endogenous GABAergic activity with the GABA antagonists bicuculline and picrotoxin. Both consistently increased interictal-like event frequency, confirming the presence of a background level of GABA activity in our slices. While the GABAergic system was clearly functional *and* functioning in the slice model, it would be interesting to investigate further whether changes in ambient and/or synaptic GABAergic function could explain the lack of effect of midazolam and etomidate in this study. Even so, if midazolam's anticonvulsant effects were mediated by a cortical site of action, one would have expected to see some suppressive effect on interictal-like activity. However, none was observed.

GABA function can also be altered by subtle changes in the chloride reversal potential. For example, elevated intracellular chloride can reduce the GABA hyperpolarizing effect, or even render GABA excitatory. This could occur through failure of the potassium-chloride cotransporter (KCC2), which maintains low intracellular chloride levels. The KCC2 transporter will function less effectively when extracellular potassium is elevated (i.e. because of the reduced potassium concentration gradient). In these experiments a potassium concentration of 5mM was used; thus a small effect on KCC2 activity is conceivable. However, in mouse work not presented here, midazolam had no effect on low-magnesium interictal-like activity when experiments were run with lower (2.5mM) potassium (n=5, c57bl6 mouse cortical slice, results not shown). When taken together with the pro-epileptiform effects observed with GABA antagonism (bicuculline and picrotoxin) in this study, it is difficult to explain the lack of anesthetic drug effect on anomalous GABAergic function.

These findings support the idea that the anticonvulsant effects of some anesthetics may be mediated by subcortical effects. While not investigated in this study, this hypothesis could be further investigated in slices where cortical-subcortical connections are preserved. There is evidence for strong subcortical-mediated anticonvulsant effects for some anesthetic drugs, particularly benzodiazepines. For example, Zhang *et al.* [5] have shown that benzodiazepines ablate pentylenetetrazol seizures when injected directly into the pars reticulata substantia nigra, supporting a subcortical site of action. The barbiturate anesthetic pentobarbital also suppresses pars reticulata firing over a dose range similar to that which suppresses seizures [32]. Furthermore, a distinct pharmacological class of high affinity benzodiazepine receptor has been localized exclusively to subcortical limbic structures [6] and benzodiazepine control of local anesthetic-induced seizures is presumed to reflect its activity within local anesthetic-sensitive limbic structures [7]. If midazolam inhibits seizures *via* a subcortical mechanism, expression of benzodiazepine-insensitive GABA receptors in the cortex may explain the lack of effect of midazolam observed in our cortical slice preparation. A subpopulation of $GABA_A$ receptors (α4 and α6 subtypes) are insensitive to benzodiazepines [33], and different $GABA_A$ heteromers may be unequally distributed throughout the brain [34]. However, the α6 subtype is not expressed in the cortex and the α4 subtype is no more highly expressed than the other subtypes [35]. Differential distribution of $GABA_A$ receptor subunits is an unlikely explanation for the lack of etomidate effect, because the β2 and β3 subunits which etomidate target are distributed throughout the rat brain, including the cerebral cortex [36].

Isoflurane has well-documented anticonvulsant properties and in experimental models is probably the most potent anticonvulsant of the volatile agents [1, 37]. Isoflurane is typically the volatile utilised in the clinical setting when the first choice anticonvulsants such as midazolam and/or phenytoin fail to control seizure activity. In this study, the anticonvulsant effect of isoflurane was preserved during co-

administration of the $GABA_A$ antagonist picrotoxin, implicating non-GABAergic mechanisms in this effect. Isoflurane has a number of non-GABAergic effects, including inhibition of NMDA and non-NMDA excitation [10] and suppression of cortical acetylcholine release [38]. It may also have some gap junction blocking effects [39]. Any one or a combination of these may have been responsible for the extra-GABAergic anticonvulsant effect observed.

The relevance to clinical seizurogenesis of the epileptiform models utilized in this study may be questioned on the basis that they do not address the induction of full ictal seizures. Certainly, the relationship between interictal activity and full ictal seizures is multifaceted and controversial [40]. However, counting interictal spike frequency is a common method for assessing the effectiveness of clinical anticonvulsants [26, 27]. Furthermore, some of the most clinically useful anticonvulsants effectively suppress interictal activity *in vivo* [28, 29]. That anticonvulsant drugs suppress both interictal events and full ictal seizures strongly supports the premise in this study that a reduction in interictal event frequency is a clinically meaningful measure of anticonvulsant effectiveness.

The results and interpretations of the present study rely heavily on posology. The drug doses were chosen to be at least equivalent to those required to induce anesthesia clinically. The etomidate free ACSF concentration used in this study (4 μM) equates to a deep anesthetic dose, taking into consideration the rate of diffusion of etomidate into brain slice tissue [22]. That is, anesthesia in the rat occurs at an effect-site etomidate concentration greater than 0.44μg/mL in 50% of animals [21]. At our recording depth of 200μm, etomidate tissue concentration is calculated to reach approximately 60-70% of its maximum after 20 minutes [22], giving a tissue concentration at the recording site in our experiments of 2.4 to 2.8 μM, just below the concentration required for burst suppression [21].

In the case of midazolam, we are not aware of any studies that have documented experimentally either the rate of drug diffusion into isolated brain tissue, or the brain tissue concentration equating to an anesthetic dose. A bolus intravenous midazolam dose of approximately 0.3mg/kg equates to an anesthetic dose in sheep [23] and humans [24], which according to modeling studies is likely to translate into a peak brain concentration of approximately 6μM [23, 41]. The brain:plasma coefficient for midazolam is similar to that of etomidate at 3.3 [42]; if we assume similar diffusion characteristics, then we would expect the tissue concentration to be approximately one half to two thirds that in the ACSF after 20 minutes, a concentration of 7-10μM. This data indicates that our bath concentration of 15μM would have resulted in a tissue concentration approximately equivalent to that for clinical anesthesia. The lack of anticonvulsant effect of midazolam in this study is therefore unlikely to be attributable to an inadequate dose.

CONCLUSION

In conclusion, we have shown in this study that the potent anticonvulsant midazolam is ineffective at inhibiting interictal-like activity in the neocortical slice. The anticonvulsant effect of isoflurane could not be attributed to its GABAergic action. Based on these findings, one must question the importance of cortical GABAergic modulation in mediating anesthetic anticonvulsant activity.

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