Tranexamic acid concentrations associated with human seizures inhibit glycine receptors

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Antifibrinolytic drugs are widely used to reduce blood loss during surgery. One serious adverse effect of these drugs is convulsive seizures; however, the mechanisms underlying such seizures remain poorly understood. The antifibrinolytic drugs tranexamic acid (TXA) and ε-aminocaproic acid (EACA) are structurally similar to the inhibitory neurotransmitter glycine. Since reduced function of glycine receptors causes seizures, we hypothesized that TXA and EACA inhibit the activity of glycine receptors. Here we demonstrate that TXA and EACA are competitive antagonists of glycine receptors in mice. We also showed that the general anesthetic isoflurane, and to a lesser extent propofol, reverses TXA inhibition of glycine receptor–mediated current, suggesting that these drugs could potentially be used to treat TXA-induced seizures. Finally, we measured the concentration of TXA in the cerebrospinal fluid (CSF) of patients undergoing major cardiovascular surgery. Surprisingly, peak TXA concentration in the CSF occurred after termination of drug infusion and in one patient coincided with the onset of seizures. Collectively, these results show that concentrations of TXA equivalent to those measured in the CSF of patients inhibited glycine receptors. Furthermore, isoflurane or propofol may prevent or reverse TXA-induced seizures.

Introduction

Antifibrinolytic drugs are widely used to reduce blood loss in a variety of hemorrhagic conditions, including severe trauma (1), cardiac and non-cardiac surgery (2–5), and maternal hemorrhage (6). Safe and effective pharmacological blood conservation strategies are needed, as the risks and costs associated with allogeneic blood transfusions continue to increase (7, 8). Such pharmacological treatments for hemorrhage are particularly important in developing nations, where blood banking facilities are limited, the risk of blood-borne infection is high, and the number of trauma-related deaths is rapidly rising (9).

Tranexamic acid (TXA) and ε-aminocaproic acid (EACA) are widely used antifibrinolytics (10). These two lysine analogs exert their antifibrinolytic effects by inhibiting the activation of plasminogen, thereby preventing degradation of fibrin and dissolution of clots (11). Another commonly used antifibrinolytic drug, aprotinin (a serine protease inhibitor derived from bovine lung), is structurally different from TXA and EACA. Aprotinin prevents blood loss by directly antagonizing plasmin (12). Aprotinin was suspended from the market because of reports of a higher incidence of death and renal dysfunction (13–15). Consequently, reliance on TXA and EACA will remain high for the foreseeable future.

TXA and EACA evoke seizures in both laboratory animals and patients, but the mechanisms underlying these seizures have not been clearly elucidated. Direct application of TXA to the cortex of cats (16) and intrathecal and intravenous administration of this drug to rats evoke convulsive and proconvulsive behaviors (17, 18). In patients, generalized tonic-clonic seizures have occurred after inadvertent intrathecal injection of TXA (19–21) and after intravenous administrations of EACA (22).

More recently, TXA has been associated with an increased incidence of postoperative seizures in cardiac patients (23, 24). Historically, postoperative seizures have occurred in about 0.5%–1% of cardiac patients (25, 26), but the use of higher doses of TXA has been associated with a higher incidence of seizures (2.7%–7.6%) (27, 28), primarily of the grand mal type (23, 29). The frequency of seizures is higher among patients with preoperative renal failure, patients undergoing open heart surgery, and older patients (30). These seizures typically occur within hours of the patient being transferred from the operating room to the intensive care unit, when the concentrations of anesthetic are declining rapidly and TXA levels remain high (29). Such postoperative seizures constitute a serious adverse effect, because they may be associated with an increased incidence of neurological complications (including delirium and stroke) (31), prolongation of recovery times, and higher mortality rates (25, 31). Currently, there are no mechanism-based treatments or prevention strategies for seizures associated with TXA or EACA.

Both TXA and EACA are structural analogs of the amino acid glycine, a major inhibitory neurotransmitter in the brain and spinal cord (Figure 1). Analogs of glycine may act as competitive antagonists, occupying the glycine-binding site and preventing glycine from binding to and activating its receptor. Glycine receptors are predominantly expressed in the spinal cord and brainstem but are also widely expressed in the prefrontal cortex, the hippocampus, and the amygdala (32). These receptors are pentameric chloride ion channels that are composed of α1 and β subunits (33). The subunit composition of each receptor determines its pharmacological properties, as well as its expression patterns in the CNS and the subcellular regions of neurons (34).

Glycine receptors in the CNS mediate two distinct forms of inhibition: postsynaptic and tonic (32). Postsynaptic inhibitory currents are generated by glycine receptors clustered in the postsynaptic ter-
minal, which are activated by the synchronous release of high concentrations of glycine (35). Tonic glycine currents mediate a “paracrine” form of inhibition that is generated by extrasynaptic glycine receptors, which are activated by low, ambient concentrations of glycine spilling over from the synaptic cleft or by glycine that is released by non-vesicular mechanisms (35). A tonic glycinergetic conductance has been identified in the hypothalamus, the hippocampus, and the dorsal horn of the spinal cord (36). The pharmacological and physiological properties of glycine receptors that generate postsynaptic and tonic inhibition may differ considerably (37).

Since drugs that inhibit glycine receptors are known to induce seizures, we tested the hypothesis that TXA and EACA, but not aprotinin, competitively inhibit glycine receptor function. Of the 3 antifibrinolytic drugs, we most thoroughly investigated the properties of TXA, as it is widely used. As predicted for a competitive antagonist, we anticipated that tonic inhibitory current, evoked by low concentrations of extracellular glycine, would be more sensitive than postsynaptic currents to TXA blockade. In addition, while the primary focus of this study was on TXA modulation of glycine receptors, we also studied the potency of TXA inhibition of γ-aminobutyric acid type A (GABA_A) receptors. GABA_A receptors are major mediators of inhibition in the CNS and were previously shown to be competitively inhibited by TXA when expressed recombinantly in human embryonic kidney cells (38). Here, we directly compare TXA blockade of native glycine and GABA_A receptors in cortical and spinal cord neurons.

We also sought to identify a strategy to reverse the inhibitory effect of TXA on glycine receptors. Given that anesthetics, including isoflurane and propofol, allosterically potentiate glycine receptor activity (39–41), and seizures frequently occur during emergence from anesthesia (29), we hypothesized that these drugs would reverse or attenuate inhibition of glycine receptors by TXA. We also studied the proconvulsant properties of TXA in neocortical slices and anticipated that anesthetics would attenuate TXA-induced enhancement of network excitability.

Finally, to determine whether glycine receptors represent clinically relevant targets for TXA, we measured the concentration of TXA in the cerebrospinal fluid (CSF) of patients. CSF samples were obtained at different surgical time points from patients undergoing surgical repair of thoraco-abdominal aortic pathology. This surgical procedure was selected because it involves insertion of a catheter into the lumbar intrathecal space, to drain the CSF and thereby improve blood flow in the spinal cord and reduce neurological damage (42, 43). The results show that glycine receptors, particularly those activated by a low concentration of glycine, are inhibited by concentrations of TXA that occur in the CSF of patients.

Results

TXA is a competitive antagonist of glycine receptors. TXA is a structural analog of glycine, which suggests that it may compete for the glycine-binding site and thereby inhibit glycine receptor function. The effects of TXA on glycine receptors were studied using mouse neurons grown in dissociated cell cultures. Primary cultures of mouse neurons are a suitable experimental model, because the glycine receptors of mice are remarkably similar to those of humans (32). All glycine-evoked currents were recorded in the presence of the GABA_A receptor antagonist bicuculline (10 μM). First, the concentration of glycine that evoked the half-maximal current (EC_{50}) in cortical neurons was determined to be 95.7 ± 16.2 μM (n = 4–6; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI63375DS1). Next, the effect of TXA on current evoked by a glycine concentration near the EC_{50} (100 μM) was studied. Coapplication of TXA (0.01–30 mM) and glycine caused a rapid and reversible decrease in current amplitude (Figure 2A). The half-maximal inhibitory concentration of TXA (IC_{50}) was 1.1 ± 0.1 mM (estimated Hill coefficient [nH] value = 1.4 ± 0.04, n = 6–8; Figure 2A).

TXA inhibition of glycine current was similar whether TXA was pre- or coapplied with glycine (Supplemental Figure 2). Also, TXA alone did not elicit any current (data not shown), which indicates that TXA does not act as an agonist at glycine receptors. TXA caused a slight reduction in the rate and extent of current desensitization (Supplemental Figure 3). However, desensitization in the presence of TXA was similar to that observed when glycine receptors were activated by a lower concentration of glycine alone. This latter observation suggests that TXA does not directly modulate glycine receptor desensitization.

To further determine whether TXA acts as a competitive antagonist, glycine concentration response plots were generated for currents recorded in the absence or presence of TXA (Figure 2B). TXA (1 and 10 mM) shifted the glycine concentration response plot to the right without reducing the maximal response. More specifically, TXA (1 mM) increased the glycine EC_{50} from 93.9 ± 6.8 μM to 207.1 ± 38.9 μM (n = 6–7). TXA (10 mM) further increased the glycine EC_{50} to 788.6 ± 13.1 μM (n = 6–7). Schild regression analysis yielded a slope similar to unity (slope = 0.81, n = 6–7; Figure 2B, inset), which indicates that TXA is a competitive antagonist.

We next tested whether TXA inhibition of glycine receptors exhibited use or voltage dependence, as observed for noncompetitive antagonists that preferentially bind to the open state of the channel (44). The onset of TXA inhibition was not use dependent, and recovery from blockade was reversed immediately after TXA washout (Figure 3A). The degree of TXA inhibition was only slightly greater at negative holding potentials than positive holding potentials, as evidenced by the outward rectification of the current-voltage plot in the presence of TXA (Figure 3B). This apparent voltage sensitivity of TXA inhibition was attributed to the voltage sensitivity of glycine receptors being activated by lower concentrations of the agonist (45), because in the absence of TXA, a low concentration of glycine (30 μM) also produced a slight outward rectification of the current-voltage plot (Figure 3B). These results are consistent with the interpretation that TXA is a competitive antagonist of glycine receptors.

EACA, but not aprotinin, inhibits glycine receptors. Like TXA, EACA may also antagonize glycine receptors. Coapplication of EACA (10 mM) and glycine (100 μM) rapidly and reversibly inhibited the glycine-evoked current (Figure 4A). The concentration-response
The effect of aprotinin on glycine receptor function was also investigated. Aprotinin was studied at lower concentrations, as its potency in terms of antifibrinolytic effect is greater than that of TXA or EACA (46). Aprotinin applied at concentrations as high as 100 μM did not inhibit glycine receptor function (Figure 4C).

TXA inhibits glycine receptors in spinal cord neurons. Glycine receptors expressed in the spinal cord may exhibit pharmacological properties different from those of glycine receptors expressed in the cortex (35). Therefore, we next investigated the effect of TXA on glycine receptors in spinal cord neurons. In addition, cultures of spinal cord neurons offer the experimental advantage of a relatively high-throughput model to study the effects of TXA on postsynaptic glycinergic currents as well as currents activated by low and high concentrations of exogenous glycine (37). The EC50 of glycine in spinal cord neurons was determined to be 34.9 ± 14.6 μM (n = 5, Supplemental Figure 1A). TXA rapidly and reversibly inhibited the current evoked by glycine (30 μM) (IC50 = 1.4 ± 0.1 mM, nH = 1.5 ± 0.03, n = 6; Supplemental Figure 4).

Next, we investigated the effects of TXA on currents evoked by a low concentration of glycine (EC6 = 10 μM) under conditions intended to mimic the tonic glycine current recorded in spinal cord neurons. The competitive glycine receptor antagonist strychnine (1 μM) completely abolished all mIPSCs (n = 6; Figure 5A), confirming that the currents were generated by glycine receptors, as shown previously (47). Relatively low concentrations of TXA (10 and 100 μM) did not inhibit the mIPSCs, whereas a higher concentration of TXA (1 mM) decreased the amplitude, decay time, area, and charge transfer of these currents (Figure 5 and Table 1).

TXA inhibition of glycine receptors is not use or voltage dependent. (A) Repeated application of TXA (1 mM) does not cause an increasing block of glycine (100 μM)–evoked currents. The bar graph shows mean peak amplitude of the current recorded with and without coapplication of TXA. Current recorded during the sequential application of TXA is identified in the bars as 1, 2, and 3 (n = 6). (B) Currents evoked by glycine (100 and 30 μM) in the absence and presence of TXA (1 mM) were recorded at two holding potentials. The current-voltage plot shows that TXA causes an outward rectification of glycine (100 μM) current-voltage plot and that glycine (30 μM) produced a similar modest outward rectification (n = 5–6). All values were normalized to glycine current activated by 100 μM at –60 mV. Data are mean ± SEM.
slices (33, 48, 49). Current amplitude was studied by applying strychnine (1 μM) and measuring the reduction in holding current, as described previously (36, 50). In the absence of TXA, the amplitude of the glycine-evoked current was 187.2 ± 54.3 pA (n = 12; Figure 6A). TXA caused a rapid, reversible, concentration-dependent decrease in the current (IC₅₀ = 93.1 ± 2.7 μM, n = 6–8; Figure 6, A and B).

Also, the variance of the baseline current noise regulates network excitability (51). Thus, we examined the effects of TXA on the baseline noise. TXA at concentrations as low as 10 μM reduced the variance of the noise (baseline: 20.2 ± 2.1 pA versus TXA: 16.1 ± 1.9 pA, n = 5–7; Figure 6C). In some recordings, a transient, small-amplitude, inward “rebound” current was observed after termination of

Figure 5
TXA inhibits glycinergic mIPSCs at higher concentrations. (A) Glycinergic mIPSCs recorded from a spinal cord neuron, in the absence and presence of TXA. Application of strychnine abolished all glycinergic mIPSCs. PC stands for picocoloumbs. (B) Cumulative frequency plots for mIPSCs show the effect of TXA on the amplitude and inter-event interval. TXA (1 mM) significantly decreased the amplitude of the mIPSCs (left panel) without affecting the inter-event interval (right panel). (C) Average traces from 100 individual mIPSCs before and after application of TXA (1 mM). Data for A–C were obtained from the same spinal cord neuron. (D) TXA (1 mM) significantly decreased charge transfer of glycinergic mIPSCs, whereas lower concentrations had no significant effect (n = 5). *P < 0.05, **P < 0.01 versus 0 TXA. Data are mean ± SEM.

Figure 4
EACA competitively inhibits glycine receptors, whereas aprotinin does not. (A) Inhibition of glycine (100 μM)—activated current by EACA (10 mM) in cortical neurons. The corresponding concentration-response plot shows IC₅₀ = 12.3 ± 0.9 mM (n = 5–6). (B) Plots for current recorded in the absence and presence of EACA (10 mM) show a rightward shift in the presence of EACA. The EC₅₀ values and Hill coefficients for EACA were 134.1 ± 4.7 μM and 1.7 ± 0.2, respectively (n = 5–6). All values were normalized to currents evoked by glycine (100 μM). (C) Aprotinin (100 μM) has no effect on glycine (100 μM)—evoked current in cortical neurons. The graph shows that aprotinin (1–100 μM) failed to inhibit glycine-evoked current (n = 5). Data are mean ± SEM.
TXA application. This rebound current was attributed to glycine receptors that were recovering from desensitization (52), as a similar rebound phenomenon has been reported for competitive antagonists of GABA<sub>A</sub> receptors (53). Thus, the current amplitude and the variance of the noise of the tonic glycine current were highly sensitive to TXA inhibition with the highest potency for glycine current evoked by low concentrations of agonist.

**TXA inhibits GABA<sub>A</sub> receptors in cortical and spinal cord neurons.** Since TXA was previously shown to inhibit recombinant GABA<sub>A</sub> receptors expressed in human embryonic kidney cells (IC<sub>50</sub> = 7.1 mM) (38), we next examined the effect of TXA on native GABA<sub>A</sub> receptors in both cortical and spinal cord neurons. First, the EC<sub>50</sub> of GABA was determined to be 19.7 ± 2.3 μM (n = 4–6) in cortical neurons and 23.9 ± 2.4 μM (n = 5–7) in spinal cord neurons (Supplemental Figure 1B). Next, the IC<sub>50</sub> for TXA inhibition of GABA currents evoked by concentrations near EC<sub>50</sub> values were found to be 1.5 ± 0.1 mM (n = 4–6) in both cortical and spinal cord neurons (Figure 7A).

We next investigated the effect of TXA on GABA<sub>A</sub> receptors activated by a low concentration of GABA (EC<sub>4</sub> = 1 μM), since a tonic GABA<sub>A</sub> receptor-mediated current has been described in the spinal cord (54), cortex (55), and several other regions in the CNS (56). This low concentration of GABA was selected because it is near the extracellular or ambient concentration of GABA measured previously (57). The amplitude of the “tonic current” was studied by applying bicuculline (100 μM) and measuring the reduction in holding current (56, 58). TXA inhibited currents evoked by a low concentration of GABA in both cortical neurons (IC<sub>50</sub> = 1.0 ± 0.1 mM, n = 5–7; Figure 7B) and spinal cord neurons (IC<sub>50</sub> = 0.9 ± 0.1 mM, n = 5–7; Figure 7B).

The potency of TXA for inhibition of GABA<sub>A</sub> receptors and glycine receptors in cortical and spinal cord neurons is summarized in Table 2. Notably, the potency of TXA for glycine-evoked current activated by a low concentration of glycine (EC<sub>4</sub>) was 10-fold higher than that for glycine- and GABA-evoked currents studied under all the other experimental conditions (F<sub>6,38</sub> = 6.68, P < 0.0001, Table 2).

**Isoflurane and propofol attenuate TXA inhibition of glycine receptors.** TXA-associated seizures occur most frequently as patients emerge from general anesthesia in the early postoperative period (23, 24, 27, 59). Thus, we postulated that the prototypic general anesthetics isoflurane (60) and propofol (39) would attenuate TXA inhibition of glycine receptors. Also, the benzodiazepine midazolam is frequently used as an anticonvulsant in the intensive care unit (61). Hence, the effect of midazolam, which selectively upregulates GABA<sub>A</sub> receptor activity but has no effect on glycine receptors (62), was studied as a negative control.

TXA (0.1 mM) reduced the current evoked by a low concentration of glycine to 59.7% ± 2.8% of control (n = 7; Figure 8B). Coapplication of isoflurane, at clinically relevant concentrations (150 and 250 μM), reduced TXA inhibition as the current increased to 130.6% ± 4.5% and 176.2% ± 17.3% (n = 6–7) of control, respectively, whereas isoflurane at a lower concentration (50 μM; Figure 8C) had no effect. In the absence of TXA, isoflurane (150 μM and 250 μM) potentiated glycine current, whereas isoflurane (50 μM) had no effect on current amplitude (Figure 8, A and C).

Coapplication of propofol at a concentration approximating that occurring during anesthesia (1 μM) (63) had no significant effect on TXA inhibition (Figure 8, B and D), while a higher concentration
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[Abstract of research article]

TXA increases the frequency of seizure-like events and enhances evoked field potentials in cortical slices. Previous studies have shown that disinhibition, secondary to reduced glycine receptor function, can generate spontaneous epileptiform field potentials or seizure-like events (SLEs) in cortical slices (64, 65). We therefore studied whether TXA would increase the frequency of SLEs, as has been shown for other pro-epileptic drugs (66–68). We employed a widely used seizure model, wherein neocortical slices are perfused with a solution containing no extracellular magnesium (0 Mg²⁺) (69, 70). Under these conditions, extracellular recordings in layers II or III revealed SLEs that consisted primarily of “inter-ictal” discharges (Figure 9A). The slices were then perfused with a solution containing TXA at a concentration similar to that measured in the CSF of patients (200 μM, see below). Notably, protein binding of TXA is minimal (3%) (71); thus, the effective concentration of TXA measured in the CSF of patients was equivalent to that used to perfuse the slices. We found that TXA (200 μM) doubled the frequency of SLEs (control: 1.5 Hz ± 0.4 Hz versus TXA: 3.7 Hz ± 0.1 Hz, n = 4 slices; Figure 9A), which is consistent with the interpretation that TXA increases network excitability.

The proconvulsive properties of drugs can also be studied in cortical slices by recording field potentials in response to an excitatory electrical stimulus (72). Evoked field responses represent the integrated electrical activity of a large number of neurons, reflecting a combination of synchronous synaptic potentials and action potentials (73). This model provides useful information about the link between single neuron recordings and larger-scale neurophysiological signals such as electroencephalography (74). Increases in the amplitude or area of field responses in cortical networks are correlated with increased excitatory synaptic drive (73).

We investigated whether TXA enhanced the evoked field response in neocortical slices by recording field potential in layers II or III in response to electrical stimuli in layer VI. Under baseline conditions, the evoked field response was characterized by an initial stimulation artefact, followed by a brief negatively

**Table 2**

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<th>Cortex High (EC₅₀)</th>
<th>Cortex Low</th>
<th>Spinal cord High (EC₅₀)</th>
<th>Spinal cord Low</th>
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<tr>
<td>Glycine</td>
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<td>NA</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>GABA</td>
<td>1.5 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.1</td>
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Data are mean ± SEM (mM). *P < 0.001 compared with all other IC₅₀ values.
directed peak (Figure 9B), as described previously (73). Perfusion of the slice with TXA (200 \( \mu \)M, see below) increased the amplitude and area of the peak responses by 59.1% ± 8.0% and 85.9% ± 14.9%, respectively (\( n = 6 \); Figure 9B). TXA at 1 mM further increased the amplitude and the area of the evoked response (Figure 9B). The evoked response gradually returned to baseline after TXA washout. These results suggest that TXA causes an increase in excitatory synaptic drive.

Since glycine and GABA\(_A\) receptors interact in a synchronized manner to regulate the excitability of neuronal networks, it is not possible to clearly delineate the specific contribution of each inhibitory receptor to the TXA-induced increase in network excitability. Nevertheless, we attempted to probe the relative role of glycine receptors versus GABA\(_A\) receptors by perfusing the slices with either strychnine or bicuculline in the absence and presence of TXA.

**Figure 8**
Isoflurane and propofol attenuate TXA inhibition of current evoked by a low concentration of glycine.

(A and B) The effects of isoflurane (ISO, 150 and 250 \( \mu \)M) and propofol (Prop, 1 and 3 \( \mu \)M) on current evoked by glycine (10 \( \mu \)M) in the presence and absence of TXA (0.1 mM). (C and D) Isoflurane (150 and 250 \( \mu \)M) and propofol (3 and 10 \( \mu \)M) attenuated TXA-mediated inhibition of glycine current (\( n = 6–7 \)). In the absence of TXA, isoflurane (150 and 250 \( \mu \)M) increased current amplitude to 182.2% ± 13.8% and 258.7 ± 21.8% of control, respectively. Similarly, in the absence of TXA, propofol (3 and 10 \( \mu \)M) increased current amplitude to 138.0% ± 2.6% and 260.1% ± 11.2% of control, respectively (\( n = 6–7 \)). Lower concentrations of isoflurane (50 \( \mu \)M) or propofol (1 \( \mu \)M) had no effect on current amplitude. **\( P < 0.01 \), ***\( P < 0.001 \) versus control. Data are mean ± SEM.

**Figure 9**
TXA enhances SLEs and evoked field potentials in cortical slices. (A) SLEs recorded with 0 Mg in the extracellular fluid, in the absence and presence of TXA (200 \( \mu \)M). The bar graph shows that TXA increases the frequency but not the amplitude (control: 0.12 ± 0.001 mV, TXA: 0.13 ± 0.007 mV) of the SLEs (\( n = 4 \)). (B) Evoked field potentials recorded in regular ACSF in the absence and presence of TXA (200 \( \mu \)M and 1 mM). The graphs show that both concentrations of TXA significantly increase the amplitude and the area of the evoked field responses (\( n = 6 \)). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) versus control. Data are mean ± SEM.
First, to study the effect of TXA inhibition of glycine receptors on network excitability, we recorded evoked field responses in slices that were perfused with bicuculline (10 μM) to block GABA<sub>A</sub> receptors. Bicuculline alone increased the amplitude to 234.2% ± 5.3% and the area to 6074.0% ± 214.4% of control (n = 5; Supplemental Figure 6A). Subsequent coapplication of TXA (200 μM and 1 mM) further increased the peak amplitude of the field potentials by 10.7% ± 5.4% and 29.1% ± 3.7%, respectively (n = 5; Supplemental Figure 6A), whereas the area was unchanged.

To study the contribution of GABA<sub>A</sub> receptors, we recorded evoked field responses in slices that were perfused with strychnine (10 μM) to block glycine receptors. Strychnine increased the amplitude to 313.8% ± 7.9% and the area to 1082.2% ± 89.2% of control (n = 5; Supplemental Figure 6B). Coapplication of TXA (200 μM and 1 mM) further increased the area of the evoked field potentials by 190.6% ± 58.4% and 213.8% ± 35.7% of control, respectively (n = 5; Supplemental Figure 6B). The amplitude of the evoked response was not affected further by TXA. These results suggest that TXA-mediated inhibition of both glycine receptors and GABA<sub>A</sub> receptors increases excitatory synaptic drive in the cortex.

Isoflurane is more effective than propofol at reversing TXA enhancement of evoked field potentials in cortical slices. To determine whether these anesthetics attenuate the increase in excitatory synaptic drive induced by TXA, we examined the effects of isoflurane and propofol on evoked field responses. Coapplication of isoflurane (250 μM) with TXA (200 μM) fully restored the amplitude and reduced the area by 61.8% ± 6.7% (n = 5; Figure 10A). Coapplication of propofol (1 μM) with TXA (200 μM) did not restore the amplitude of the evoked response but reversed the TXA-induced increase in area by 19.2% ± 5.5% (n = 5; Figure 10B). Perfusion of slices with isoflurane or propofol alone reduced both the amplitude and the area of the evoked field response (Supplemental Figure 7), as demonstrated previously (75, 76). Together these results suggest that isoflurane may be more effective than propofol at reversing TXA-induced increases in excitatory synaptic drive. However, the reversal of TXA-mediated excitability...
TXA concentrations in the serum and CSF of patients. Only receptors that are sensitive to clinically relevant concentrations of TXA are plausible mediators of the adverse effects of this drug. Consequently, we sought to determine whether concentrations of TXA occurring in the CNS of patients who have received this drug would be sufficient to inhibit glycine receptors and GABA<sub>A</sub> receptors. Serum and CSF samples were obtained from patients who were undergoing repair of thoraco-abdominal pathology at certain times corresponding to procedural milestones. Serum and CSF concentrations of TXA for one patient who experienced a seizure are shown in Figure 11A. The average concentrations from the sample population are summarized in Figure 11B. The mean time points at which the serum and CSF samples were obtained and the total dose of TXA are presented in Table 3.

Surprisingly, the temporal profiles for TXA concentrations in the serum and CSF were substantially different, as indicated by the samples from a single patient (Figure 11A). The peak concentration in the serum (1.9 ± 0.4 mM, n = 4; Figure 11B) occurred after cardiopulmonary bypass (CPB), whereas the peak concentration in the CSF (220.8 ± 116.7 μM, n = 4, Figure 11B) occurred after discontinuation of TXA infusion. Given that less than 3% of TXA is bound to protein (71), peak concentrations in the CSF were within the range required to inhibit glycine current evoked by a low concentration of agonist (IC<sub>50</sub> = 93.1 ± 2.7 μM).

Discussion

To our knowledge, the study presented here is the first to identify glycine receptors as novel targets for inhibition by TXA and EACA, but not aprotinin. Because TXA is a competitive antagonist, inhibition mediated by this drug was expected to be highly dependent on the concentration of glycine applied, and this expectation was corroborated by the results. The potency of TXA was 10-fold higher for current evoked by a low concentration of glycine (IC<sub>50</sub> = 93.1 μM) in spinal cord neurons than for synaptic glycinergic currents (IC<sub>50</sub> = 1 mM) or currents activated by the EC<sub>50</sub> of glycine in cortical neurons (IC<sub>50</sub> = 1.1 mM) and spinal cord neurons (IC<sub>50</sub> = 1.4 mM). Interestingly, the potency of TXA inhibition was similar for glycine receptors and GABA<sub>A</sub> receptors activated by the EC<sub>50</sub> of agonist in cortical neurons and spinal cord neurons (Table 2). We also show that TXA inhibition of glycine receptors activated by a low concentration of glycine (EC<sub>a</sub>) was reversed by isoflurane at clinically relevant concentrations. Propofol also reversed TXA-mediated glycine blockade, but only at concentrations 3-fold higher than those that occur during anesthesia (63). Consistent with these results, TXA-induced enhancement of excitatory synaptic drive in cortical slices was more effectively reversed by isoflurane than by propofol. Finally, we showed that the peak concentration of TXA in the CSF of patients undergoing major cardiovascular surgery (220.8 μM) occurred after CPB and termination of drug infusion. A similar concentration of TXA increased the frequency of SLEs and enhanced evoked field responses in cortical slices, which suggests that TXA has proconvulsive properties.

Inhibitors of glycine receptors are known to induce convulsions and proconvulsive behavior (78). For example, strychnine causes myoclonic spasms (79), twitching of muscles, convulsions, and hyperreactivity (78). Genetic disorders characterized by reduced expression of glycine receptors cause hyperreflexia, convulsions, and startle disorders in infants (80). In animals with naturally occurring mutations of the glycine receptor, the magnitude of the reduction in glycine receptor function directly correlates with the severity of the convulsive symptoms (81, 82). Thus, it is plausible that the reduction in function of these receptors caused by TXA leads to disinhibition and proconvulsive effects.

The potency of TXA was much greater for currents evoked by a low concentration of glycine than for synaptic glycinergic mIPSCs. The demonstration that TXA attenuates tonic current is of great interest, since a reduction in tonic current enhances neuronal excitability (36, 58). Tonic current reduces neuronal excitability via two mechanisms. First, this type of current allows for the transfer of large quantities of negative charge, a process that hyperpolarizes the cell membrane (36). Second, tonic conductance can attenuate the excitatory drive to generate action potentials by decreasing membrane resistance, thereby reducing the amplitude of excitatory postsynaptic potentials (83).

As anticipated for a competitive antagonist, the potency of TXA inhibition of glycine receptors was highly dependent on the concentration of agonist used in each experiment. However, this difference in potency might also have been due, at least in part, to the subunit composition of glycine receptors. Glycine receptors are composed of multiple α<sub>1</sub>–<sub>4</sub> and β subunits, which form homomeric (5α) or heteromeric (2α3β) ion channels (32). Synaptic glyciner currents are generated by heteromeric receptors that are clustered at the postsynaptic sites (35). Tonic glyciner currents are mediated by homomeric receptors, which are located predominantly extrasynaptically (35). Glycine receptor antagonists, such as picrotoxin, show higher potency for homomeric than heteromeric glycine receptors (37). Post-transitional factors such as glycine receptor phosphorylation and the clustering of receptors in the postsynaptic domain can also alter the morphological properties of glycine receptors (52, 84). TXA potency in relation to various combinations of glycine receptor subunits as well as the effects of cytosolic regulatory factors on TXA potency are topics worthy of future study.

Our results and those of others (38) showed that TXA is a competitive antagonist of GABA<sub>A</sub> receptors in both cortical and spinal cord neurons (IC<sub>50</sub> = 1.5 ± 0.1 mM for both). Interestingly, the potency of TXA for current evoked by a low concentration of GABA (EC<sub>a</sub>) in cortical (IC<sub>50</sub> = 1.0 mM) and spinal cord (IC<sub>50</sub> = 0.9 mM) was also lower than that of TXA for current evoked by a high concentration of GABA (EC<sub>50</sub> = 1.0 mM) in cortical (IC<sub>50</sub> = 1.0 mM) and spinal cord (IC<sub>50</sub> = 0.9 mM).
neurons was similar to its potency for current evoked by higher concentrations of GABA (EC₅₀). This result was unexpected and may reflect differences in the subunit composition of receptors activated by the various concentrations of GABA (56, 57, 85, 86). In addition, our results showed that TXA inhibition of GABAᵦ receptors increase evoked field responses in neocortical slices in the presence of strychnine. Thus, TXA inhibition of GABAᵦ receptors may increase network excitability.

A previous study showed that TXA has an IC₅₀ value of 7.1 ± 3.1 mM for recombinant GABAᵦ receptors (α₁β₂γ₂), transfected into human embryonic kidney cells when receptors are activated by GABA (EC₅₀) (38). Also, binding assays for TXA with [³H]muscimol showed that the IC₅₀ value for TXA binding to GABAᵦ receptors was 2.1 ± 0.2 mM (38). In our study, the potency of TXA for GABA currents evoked by EC₅₀ concentrations of agonist was lower. This difference may be attributable to our use of cell cultures with native receptors rather than recombinantly expressed receptors (87).

The peak TXA concentration in the CSF as measured in the current study was higher than that measured in the CSF of 4 patients undergoing CPB in a previous study (220.8 μM versus 31 μM) (88). In the earlier study, the TXA dose was significantly lower, and samples were taken at only two time points: 15 and 90 minutes after the start of TXA infusion (88). The discrepancy in results is probably related to two findings from the current study, specifically, that serum concentrations of TXA do not correlate well with CSF concentrations and that TXA concentrations peak in the CSF long after the start of drug infusion. Thus, the previous study may not have detected the true peak concentrations of TXA in the CSF.

The timing and magnitude of peak TXA concentrations after CPB may be attributable to increased permeability of the blood-brain barrier during and after surgery (89). In a study that used a protein marker (S100) to measure damage to the blood-brain barrier during CPB, the highest levels of the marker occurred after termination of the bypass (90). The blood-brain barrier appears to be particularly “leaky” at the end of CPB, which could account for the delayed rise of TXA concentrations in the CSF. Thus, the CSF may act as a slow compartment, which could explain the observed time course of TXA concentrations. A detailed pharmacokinetic analysis of TXA in the plasma and CSF would be of interest in future studies.

The results of this translational preclinical study have important clinical implications. First, we have shown that TXA is a competitive antagonist of both glycine and GABAᵦ receptors. Thus, higher brain concentrations of TXA likely increase the risk of seizure. Indeed a higher incidence of seizures occurs in patients with preoperative renal failure (91) and those receiving higher doses of TXA (30). Currently, there is no consensus regarding optimum TXA dosing, and studies are urgently needed to determine the minimal effective dose of this drug (92).

Second, our results also suggest that isoflurane or propofol may prevent or treat TXA-induced seizures in the early postoperative period. Given the variable clinical presentation and incidence of TXA-associated seizures, clinical trials comparing the efficacy of various anticonvulsant treatments are unlikely to be feasible. Our results suggest that it may be possible to use isoflurane or propofol to prevent or treat TXA-induced seizures in the early postoperative period. Inhaled anesthetics, including isoflurane, are already being used for sedation of intubated patients in the intensive care unit (93). It must be emphasized, however, that anticonvulsants with no direct effects on glycine receptors, such as midazolam, may nonetheless be effective for treating TXA-associated seizures.

Third, because glycine receptors regulate numerous functions in the CNS, their inhibition by TXA could have adverse behavioral consequences other than seizures. One potential implication is that TXA and EACA may have pro-nociceptive properties, since antagonists of glycine receptors increase nociceptive responses in both laboratory animals and patients (94). Consistent with this prediction, patients with subarachnoid hemorrhage who were treated with TXA and EACA required higher doses of analgesics than placebo-treated patients (95). Also, patients with menorrhagia treated with TXA experienced a higher incidence of headaches, abdominal pain, and back pain than placebo-treated patients (96). Furthermore, inhibition of glycine receptors causes excessive or involuntary motor activity (hyperkinesia) in laboratory animals (97) and patients (98). Myoclonic activity observed in some patients treated with TXA may result from inhibition of glycine receptors (99).

In summary, these results show that TXA and EACA inhibit glycine receptors and suggest a novel mechanism for seizures associated with cardiovascular surgery. Isoflurane may be effective for preventing or treating TXA-induced excitability in the early postoperative period. As the indications for TXA and EACA increase, we hope that these results will aid in the prevention and management of serious neurological side effects associated with these drugs.

Methods

Whole-cell patch clamp recording in cell culture. Primary cultures of embryonic cortical and spinal cord neurons were prepared from Swiss white mice. Briefly, fetal pups (E18) were removed from mice sacrificed by cervical dislocation. The cortex or spinal cord of each fetus was collected and placed in an ice-cooled culture dish. The neurons were then dissociated by mechanical trituration using two Pasteur pipettes (tip diameter, 150–200 μm) and plated on 35-mm culture dishes at a density of about 1 × 10⁶ cells/ml. The culture dishes were coated with collagen or poly-D-lysine (Sigma-Aldrich). For the first 5 days in vitro, cells were maintained in MEM supplemented with 10% fetal bovine serum and 10% horse serum (both from Life Technologies). The neurons were cultured at 37°C in a 5% CO₂/95% air environment. After the background cells had grown to confluence, 0.1 ml of a mixture of 4 mg 5-fluorodeoxyuridine and 10 mg uridine in 20 ml MEM was added to the extracellular solution to reduce the number of dividing cells. The medium was further supplemented with 10% horse serum and was changed every 3 or 4 days. Cells were maintained in culture for 12–16 days before use. For all reported results, data were acquired from cells from at least 3 different dissections.

These experiments were performed at room temperature. Whole-cell currents were recorded under voltage clamp (–60 mV) conditions using an Axopatch 1D amplifier (Molecular Devices) controlled with pClamp 8.0 software (Molecular Devices) via a Digidata 1322 interface (Molecular Devices). Patch pipettes were pulled from thin-walled borosilicate glass capillary tubes; these had open tip resistances of 3–5 MΩ. Patch electrodes were filled with an intracellular solution containing (in mM) 140 CsCl, 4.0 MgCl₂, 1.0 CaCl₂, 4 MgATP, and 2.0 MgCl₂ (pH 7.3 with CsOH, 285–295 mOsm). The extracellular solution contained (in mM) 140 NaCl, 1.3 CaCl₂, 2 KCl, 1 MgCl₂, 25 HEPES, and 28 glucose. pH was adjusted to 7.4 with NaOH, and osmolarity was adjusted to 320–330 mOsm. The extracellular solution was applied to neurons by a computer-controlled, multi-barrel perfusion system (SF-77B, Warner Instruments). Membrane capacitance was measured according to the membrane test protocol in the pClamp 8.0 software. Access resistance was monitored throughout the experiments by applying a brief 10-mV hyperpolarization during the experiments. Cells were eliminated from further analysis if the access resistance changed by about 20% or more. Tetrodotoxin (TTX, 300 nM) was added
to the extracellular solution to block voltage-sensitive sodium channels, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and 2-amino-4-phosphonovaleric acid (D-AP5, 40 μM) were added to inhibit the ionotropic glutamate receptors. Bicuculline (10 μM) was added to block GABA<sub>A</sub> receptors when glycine current was recorded. Strychnine (1 μM) was added to block glycine receptors when GABA current was measured.

To study synaptic glycine receptor-mediated currents, we added sucrose to the extracellular solution to stimulate glycine release and increase the frequency of glycinergic mIPSCs recorded from cultured spinal cord neurons. To increase the amplitude of tonic current and to facilitate its pharmacological characterization, we added glycine (EC<sub>50</sub> = 10 μM) or GABA (EC<sub>50</sub> = 1 μM) to the extracellular solution. These concentrations are similar to extracellular levels of glycine (48) and GABA (57) in the CNS.

**Extracellular field potentials recorded in neocortical slices.** Mice (C57BL/6 and Sv129Ev cross) at P21–P28 were anesthetized with isoflurane and decapitated. Coronal neocortical slices (400 μm) were prepared using a vibratome (Leica VT 1200S) and placed in ice-cold, oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 1.3 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 d-glucose, with osmolarity adjusted to 300–310 mOsm. After a recovery period of 1 hour in the oxygenated ACSF, the slices were transferred to a submersion recording chamber and perfused with oxygenated ACSF, maintained at 33–34°C, at a perfusion rate of 3–4 ml/min.

To demonstrate that TXA exhibits proconvulsant properties, we used the widely accepted 0 Mg<sup>2+</sup>-seizure model (69, 70). Slices were perfused with ACSF from which MgCl<sub>2</sub> was omitted (designated 0 Mg<sup>2+</sup>). Recording electrodes were filled with ACSF and placed in the cortex. SLEs, or spontaneous high-frequency rhythmic potentials, which are typically observed in this model (100), were recorded under gap-free conditions using a Multiclamp 700A amplifier (Molecular Devices) in the current-clamp configuration. SLEs are traditionally divided into “ictal” states, defined as the onset of high-frequency rhythmic field discharges, and “interictal” states, defined as the low-frequency discharges that occur in the period between ictal states (100, 101). It is not uncommon for SLEs recorded in the neocortex to lack full ictal events (101). Indeed, we were only able to record interictal events in this preparation.

Evoked field responses were recorded in layers II or III of the neocortex with recording electrodes filled with standard ACSF and were monitored in each slice for 20 minutes or until the response stabilized. A concentric bipolar stimulating electrode (Rhodes Medical Instruments) was used to stimulate layer VI of the neocortex. Responses evoked at 0.05 Hz at an intensity that yielded a half-maximal field potential amplitude. Evoked field responses were recorded in standard ACSF.

**Data analysis.** Currents were analyzed with pClamp 10 software (Molecular Devices). The glycine concentration-response plot was fitted to the modified Michaelis-Menten equation: \( I = I_{\text{max}} / (1 + (\text{EC}_{50}/c)^n) \), where \( I \) is the current amplitude, \( c \) is the concentration of agonist, EC<sub>50</sub> is the concentration of agonist that produces currents with 50% of maximal amplitude, and \( n \) is the estimated Hill coefficient. The reversal potential for Cl<sup>-</sup> was calculated with the Nernst equation: \( E_{\text{Cl}} = 58 \log([\text{Cl}]/[\text{Cl}]) \). Schild regression analysis was performed by calculating the dose-response ratios of antagonist EC<sub>50</sub> to agonist EC<sub>50</sub> for each concentration of TXA; the log of the concentration-response ratio was then plotted against the log of TXA concentration. The slope of the Schild plot was used to determine the nature of antagonism, given that competitive antagonists would generate a slope of 1. The x-intercept of the fitted regression line was used to calculate the equilibrium dissociation constant, which estimates the affinity of TXA for the glycine receptor (102).

To assess the effect of the drugs on synaptic glycinergic conductance, mIPSCs were analyzed with Mini Analysis software (Synaptosoft). For detection of mIPSCs, the threshold was set at approximately 3 times the level of baseline noise (~3.6 pA). However, compound events (i.e., those with multiple peaks) were excluded from the analysis. Manual inspection of each file was also performed, to allow rejection of false events caused by noise and inclusion of events that were not detected automatically. At least 100 individual mIPSC events were detected under each experimental condition. The peak amplitude, frequency, rise time, decay time (τ), area, and charge transfer were analyzed. Peak amplitude refers to the maximum height of the mIPSC, and the frequency was calculated by determining the inter-event interval. The rise time refers to the time elapsed between 10% and 90% of the peak amplitude response. The decay τ was determined using the biexponential equation \( I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \), where \( I \) is the current amplitude at any given time (t); \( A_1 \) and \( A_2 \) are the amplitudes of the fast and slow decay components, respectively, and \( \tau_1 \) and \( \tau_2 \) are their respective decay time constants. The weighted time constant of current decay was determined by the equation \( \tau_{\text{decay}} = \Sigma \tau_i / \Sigma A_i \). Area was determined by integrating the area under the mIPSC curve. Charge transfer was determined by multiplying the frequency by the area of the mIPSCs (51). Cumulative distribution plots of mIPSC amplitudes and frequency were generated in GraphPad Prism 5 (GraphPad Software Inc.).

The amplitude of tonic glycine current was measured as the difference in the holding current before and during application of strychnine (1 μM), while the amplitude of tonic GABA current was measured by application of bicuculline (100 μM). Drug efficacy was reported as percentage of control, where control was equal to the amplitude of the tonic current revealed by application of either strychnine or bicuculline. The variance of baseline noise, measured for tonic glycine current, was determined by the root mean square deviation from the recording segments that lacked mIPSCs, as previously described (51, 58).

SLEs and evoked field potentials were analyzed using GraphPad Prism 5. The frequency of SLEs was determined using pClamp 10 data acquisition and analysis software (Molecular Devices). The threshold for event detection was set at ~0.5 mV.

**Drugs and chemicals.** TXA and EACA were obtained from Sigma-Aldrich, while aprotinin (Trasylol) was purchased from Bayer. Propofol (Diprivan) and midazolam were purchased from AstraZeneca, while isoflurane was obtained from Abbott Laboratories. Tetrodotoxin was purchased from Alomone Laboratories; CNQX, D-AP5, bicuculline, strychnine, and glycine were obtained from Sigma-Aldrich. Propofol and midazolam were each diluted in extracellular solution as previously described (103). Isoflurane solutions were prepared and applied as previously described (104). The highest concentration of isoflurane in the undiluted aqueous solution was 2,500 μM. The aqueous phase concentration of isoflurane equivalent to minimal alveolar concentrations in vitro studies at room temperature is 253–280 μM (105, 106).

Stock solutions of all other reagents were prepared with distilled water.

**Patients and surgical procedures.** Patients included in the study were ages 19–85, inclusive, and scheduled for open thoraco-abdominal aortic surgery, with planned placement of a lumbar CSF drain and intraoperative use of TXA. This is a complex procedure that is performed in a selected number of specialized surgical centers. Due to the known variability in duration of the procedure and operative techniques, and since the amount of CSF available for study is limited, samples were obtained at predetermined procedural milestones. TXA dosing was in accordance with recommended protocols (13), with a loading dose of 30 mg/kg plus additional 2 mg/kg added to the CPB prime followed by 16 mg/kg/h infusion until chest closure. TXA concentrations in serum and CSF were quantified using a modified liquid chromatography and a tandem mass spectrometry assay (88, 107). At a functional coefficient of variance of 20%, the minimum detectable level of TXA is 0.05 μg/ml.

**Statistics.** GraphPad Prism 5 was used to generate the bar graphs. Results are presented as mean ± SEM. Differences between groups were determined using a 1-tailed Student t test, 1-way ANOVA with a Dunnett mult...
tiple comparison post hoc test, or 2-way ANOVA with Bonferroni post hoc test. Cumulative distribution plots of mIPSCs were analyzed with the Kolmogorov-Smirnov test. A P-value of less than 0.05 was considered significant.

Study approval. All animal experiments were approved by the Animal Care Committee of the University of Toronto. The human study protocol was approved by the Research Ethics Board of St. Michael's Hospital, Toronto, Ontario, Canada. Written and informed consent was obtained from all patients.

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