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Carbenoxolone modifies spontaneous inhibitory and excitatory synaptic transmission in rat somatosensory cortex

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Abstract

Gap junction (GJ) coupling between neocortical GABAergic interneurons plays a critical role in the synchronization of activity in cortical networks in physiological and pathophysiological states, e.g., seizures. Past studies have shown that GJ blockers exert anticonvulsant actions in both in vivo and in vitro models of epilepsy. However, the precise mechanisms underlying these antiepileptic effects have not been fully elucidated. This is due, in part, to a lack of information of the influence of GJ blockade on network activity in the absence of convulsant agents or enhanced neuronal excitation. One key question is whether GJ blockers act on excitatory or inhibitory systems, or both. To address this issue, we examined the effects of the GJ blocker carbenoxolone (CarbX, 150 μM) on spontaneous inhibitory postsynaptic currents (sIPSCs) and excitatory postsynaptic currents (sEPSCs) in acute slices of rat somatosensory cortex. Results showed that CarbX decreased the amplitude and frequency of sIPSCs by 30.2% and 25.7%, respectively. CarbX increased the mean frequency of sEPSCs by 24.1%, but had no effect on sEPSC amplitude. During blockade of $GABA_A$-mediated events with picrotoxin (20 μM), CarbX induced only a small increase in sEPSC frequency that was not statistically different from control, indicating CarbX enhancement of sEPECs was secondary to the depression of synaptic inhibition. These findings suggest that in neocortex, blockade of GJs leads to an increase in spontaneous excitation by uncoupling GABAergic interneurons, and that electronic communication between inhibitory cells plays a significant role in regulating tonic synaptic excitation.

Keywords: Carbenoxolone; Gap junction; Neocortex; EPSC; IPSC; In vitro

Neural communication through gap junctions (GJs) plays an important role in the generation and maintenance of synchronized neuronal network activity in both physiological and pathophysiological conditions, e.g., seizures [26]. Morphological and electrophysiological evidence indicate that electrotonic coupling between GABAergic interneurons exists widely in the cortex [6], and plays a critical role in the synchronization of network activity. Repetitive stimulation of hippocampal circuits induces GABAergic synchronization which is blocked by carbenoxolone (CarbX), a putative GJ uncoupler [9]. In hippocampus [2,23] and neocortex [3,13], 4-aminopyridine (4-AP) induces synchronized GABAergic network activity that is independent of ionotropic glutamate receptor activation, but is suppressed by GJ blockade. Putative GJ blockers, such as octanol, halothane, heptanol, and CarbX, exert anticonvulsant actions in a variety of experimental seizure models [10,11,17,21,29]. For example, CarbX suppresses spontaneous epileptiform activity caused by enhanced neuronal excitability in area CA3 of hippocampus [21]. Past studies have shown that epileptiform activity up-regulates GJ proteins [19,29] and increases electrotonic coupling [5], which would further facilitate the propagation and maintenance of synchronous network activity. GJ blockers presumably counter such enhancements to electrotonic communication, which may partly explain their anticonvulsant actions. However, the precise mechanisms underlying the antiepileptic effects of these agents are still unclear, due, in part, to a lack of detailed information about their influence on network activity in the absence of heightened neuronal excitation or convulsant agents. One key question is whether GJ blockers affect excitatory or inhibitory systems, or both. We have addressed this issue by examining the actions of CarbX on sIPSCs and sEPSCs recorded from layer II/III pyramidal cells in acute slices of rat neocortex.

Cortical slices were prepared from Sprague-Dawley rats (postnatal day 17–22), using procedures previously described [20]. Briefly, coronal slices (350 μm) of somatosensory cortex

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were cut from isolated brains using a Vibratome tissue sectioner (Vibratome Co., St. Louis, MO). Slices were transferred to an antechamber and incubated in low-Ca^{2+}, high-Mg^{2+} saline for 45 min at 35 ± 0.5 °C and then in standard saline for >1 h at room temperature (22–24 °C). Standard saline was composed of (in mM): 124 NaCl, 5 KCl, 1.6 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose, and was continuously oxygenated with 95% O_2 and 5% CO_2 (pH between 7.35 and 7.40). Potassium concentration was modestly elevated (i.e., 5 mM) to enhance spontaneous synaptic activity. Low-Ca^{2+}, high-Mg^{2+} saline had the same composition except for adjustments to $CaCl_2$ (1.0 mM) and $MgCl_2$ (10.0 mM). Nominally acidic saline included 25 mM sodium propionate, with NaCl adjusted to 99 mM (pH between 7.05 and 7.15). For recordings, a single slice was submerged and perfused with warm (32–33 °C) saline at 2.0–2.5 ml/min. Whole-cell recordings were obtained from layer II/III pyramidal cells visually identified by the shape of the cell soma and apical dendrites using infrared differential interference contrast (IR-DIC) optics. Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm OD, World Precision Instrument Inc., Sarasota, FL) and had tip resistances of 3–5 MΩ when filled with an intracellular solution composed of (in mM): 130 *Cs*-methanesulfonate, 0.15 $CaCl_2$, 2.0 $MgCl_2$, 2 EGTA, 10 HEPES, 2 Na_2-ATP, 0.25 $Na_3GTP.3H_2O$, and 10 QX-314 (to block sodium currents), pH 7.2–7.3, adjusted with CsOH. Cesium blocked potassium currents, including $GABA_B$ IPSCs. Currents were recorded under voltage-clamp with a Warner PC-501A patch clamp amplifier (Warner Instrument Corp., Hamden, CT). Cells studied had a mean input resistance of 310.0 ± 30.5 MΩ (mean ± S.E., $n = 25$) and mean access resistance of 17.6 ± 0.7 MΩ ($n = 25$). If access resistance increased by >20% during experiments, the recording was terminated and data discarded. Signals were digitized at 47 kHz via a 14-bit PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY) and stored on VHS videotape for post-hoc analysis or recorded directly to computer hard disk using pCLAMP 9.0 software (Axon Instruments Inc., Foster City, CA). Data were filtered at 2 kHz (4 pole, −3 dB Bessel filter) and digitally sampled at 20 kHz. Spontaneous synaptic events were detected and measured using Mini Analysis software (Synpatosoft, Inc., Decatur, GA). As a check, all events detected by the software were visually inspected and any which did not exhibit the general shape expected for synaptic currents were rejected. Background noise, which was measured from quiescent sections of records (i.e., devoid of spontaneous activity), had a peak-to-peak range of 2–5 pA. Data throughout this report are expressed as means ± S.E.M. Paired *t*-tests were used to determine significance at the 0.05 level.

Isolated sIPSCs were recorded at the empirical EPSC reversal potential (0–5 mV), while sEPSCs were recorded at IPSC reversal (−75 mV). Once whole cell access was achieved and determined to be stable (≥5 min), control events were recorded for 10–15 min. Slices were then exposed to CarbX (150 μM) via the bathing perfusate for 30–45 min while spontaneous events were continuously monitored, after which CarbX was washed out. Reversal of CarbX-mediated effects was not consistently achieved (see [17]) and as such, these data were not included in pooled data analyses.

Examination of sIPSCs revealed that exposure of slices to CarbX decreased both the peak amplitude and frequency of spontaneous inhibitory events, as illustrated by the representative response shown in Fig. 1. For this cell, the mean sIPSC amplitude decreased from 13.2 ± 0.2 pA ($n = 1617$ events) in control saline to 10.2 ± 0.1 pA ($n = 1333$ events) in CarbX. Wash out of CarbX from the recording chamber restored the magnitude of sIPSCs to 13.1 ± 0.2 pA ($n = 1742$ events). These effects were reflected in sIPSC amplitude histograms and cumulative distribution plots, which showed that CarbX reduced the proportion of larger amplitude sIPSCs (Fig. 1D–G). Assessment of event discharge rate showed that CarbX also decreased the mean sIPSC frequency, from 8.6 ± 0.1 Hz in control to 7.5 ± 0.1 Hz. However, sIPSC frequency did not return to control values following wash out of CarbX (7.5 ± 0.1 Hz). Interevent interval histograms and cumulative plots showed that the number of longer interevent intervals was slightly increased in CarbX and did not return to control levels following drug wash out (Fig. 1H–K). Analyses of data pooled from 12 cells (12 slices) confirmed these findings (Fig. 3A and B). For all cells, CarbX decreased the mean sIPSC amplitude by 30.2% (from 17.9 ± 2.0 to 12.5 ± 1.0 pA, $p < 0.05$) and sIPSC frequency by 25.5% (from 10.9 ± 1.1 to 8.1 ± 0.8 Hz, $p < 0.05$), which is consistent with a presynaptic site of action for CarbX, i.e., inhibitory interneurons.

The effects CarbX on sEPSCs were also examined. As shown by the representative recording in Fig. 2, CarbX had no effect on sEPSC amplitude, but did increase sEPSC frequency. For this cell, the mean sEPSC amplitude was 11.2 ± 0.1 pA ($n = 2309$ events) in control, 11.1 ± 0.1 pA ($n = 2714$ events) in CarbX, and 10.5 ± 0.1 pA ($n = 2371$ events) following wash out of CarbX. Amplitude histograms and cumulative distributions plots indicated no significant difference in the overall distribution of sEPSC amplitude values for events recorded in control and CarbX (Fig. 2D–G). However, exposure to CarbX increased the mean sEPSC frequency from 12.7 ± 0.3 Hz in control to 15.1 ± 0.3 Hz in CarbX. Following wash out of CarbX from the recording chamber, sEPSC frequency returned to 3.9% above control (13.2 ± 0.3 Hz). CarbX enhancement of event frequency was reflected in the corresponding interevent interval histograms and cumulative plots, which showed a reduction in the number of longer interevent interval values during CarbX exposure and nearly full recovery following CarbX wash out (Fig. 2H–K). Examinations of sEPSC data pooled from eight cells (eight slices; Fig. 3C and D) confirmed these findings. For all cells, there was no significant difference in the mean peak amplitude of sEPSCs recorded in control saline (10.6 ± 0.4 pA) and those obtained in CarbX (10.5 ± 0.9 pA; $p > 0.05$). However, the mean frequency of events obtained in CarbX (16.9 ± 1.4 Hz) was 23.4% greater than that of control (13.7 ± 1.4 Hz; $p < 0.05$).

To determine whether CarbX enhancement of sEPSC frequency was due to the inhibition of GABAergic processes or to direct actions on excitatory inputs, we examined the effects of CarbX on sEPSCs recorded during blockade of $GABA_A$ receptors. In the presence of the $GABA_A$ antagonist picrotoxin (20 μM), exposure of slices to CarbX had no significant effect

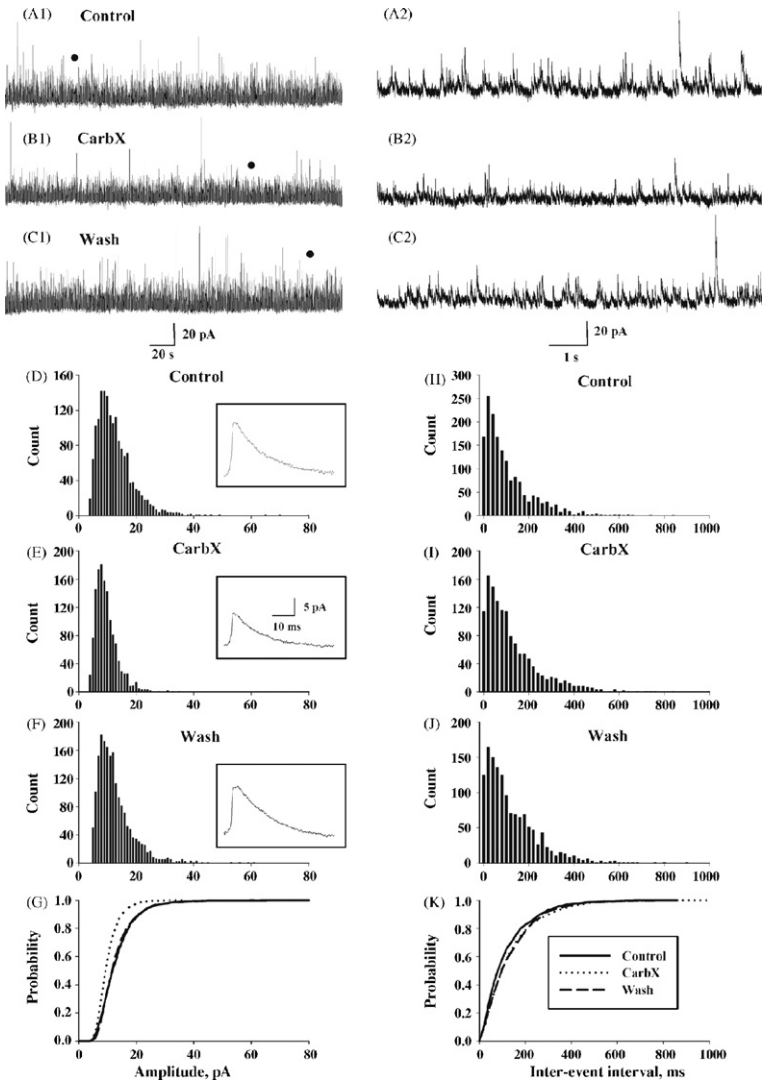

Fig. 1. Actions of CarbX on sIPSCs in layer II–III pyramidal cell. (A–C) Traces of sIPSCs recorded under voltage-clamp in control saline (A), CarbX (B), and following wash out of CarbX (C). Panels A2, B2, and C2 show expanded sections of the traces displayed in A1, B1, and C1 (indicated by dots). (D–F) Amplitude histograms of sIPSCs recorded in control saline (D), CarbX (E), and following CarbX wash out (F). Inset panels show the averaged traces of 30 randomly selected events recorded under each condition. (G) Cumulative probability distributions of sIPSC amplitudes. (H–J): Inter-event interval histograms of sIPSCs recorded in control saline (H), CarbX (I), and following CarbX wash out (J). (K) Cumulative probability distributions of sIPSC inter-event interval values. The figure legend in (K) applies to both (G) and (K).

on either sEPSC amplitude or frequency. The mean sEPSC amplitude was 11.8 ± 0.7 pA in control saline and 11.6 ± 0.8 pA in CarbX (data pooled from 15 cells (15 slices), $p > 0.05$, Fig. 3E). The mean frequency of sEPSCs recorded in CarbX (13.3 ± 1.3 Hz) was slightly higher than those in control media (11.4 ± 1.0 Hz), but this difference was not significant (Fig. 3F, $p > 0.05$). These findings suggest that CarbX enhancement of sEPSC frequency is due primarily to the inhibition of $GABA_A$-mediated processes, and not to direct effects on excitatory synaptic inputs.

To verify that CarbX-induced alterations in spontaneous synaptic transmission stemmed from the suppression of electrotonic coupling rather than direct actions on synapse function [30], we examined sIPSCs and sEPSCs in slices exposed to nominally acidic saline containing the weak organic acid, sodium propionate (25 mM), which blocks GJs via intracellular acidification [27]. Similar to CarbX, sodium propionate caused reductions in both the magnitude and frequency of sIPSCs, decreasing mean sIPSC amplitude by 9.7% (from 11.9 ± 1.0 to 10.3 ± 0.85 pA, $n = 9$ cells (6 slices), $p < 0.05$, Fig. 3G) and sIPSC frequency by 29.5% (from 7.8 ± 0.3 to 5.5 ± 0.4 Hz, $p < 0.05$, Fig. 3H). Unlike CarbX, sodium propionate attenuated sEPSCs, reducing mean amplitude by 3.2% (from 9.3 ± 0.6 to 8.9 ± 0.5 pA, $n = 8$ cells (5 slices), Fig. 3I) and mean frequency by 24.8% (from 7.7 ± 1.0 to 5.8 ± 0.9 Hz, Fig. 3J). However, past studies have shown that acidosis directly suppresses ionotropic glutamate receptor-mediated synaptic transmission [15], which would likely mask any secondary enhancement of sEPSCs consequent to reductions in synaptic inhibition. Taken together, these data suggest that GJ blockade decreases spontaneous inhibitory synaptic transmission in neocortex, presumably by uncoupling inhibitory interneurons.

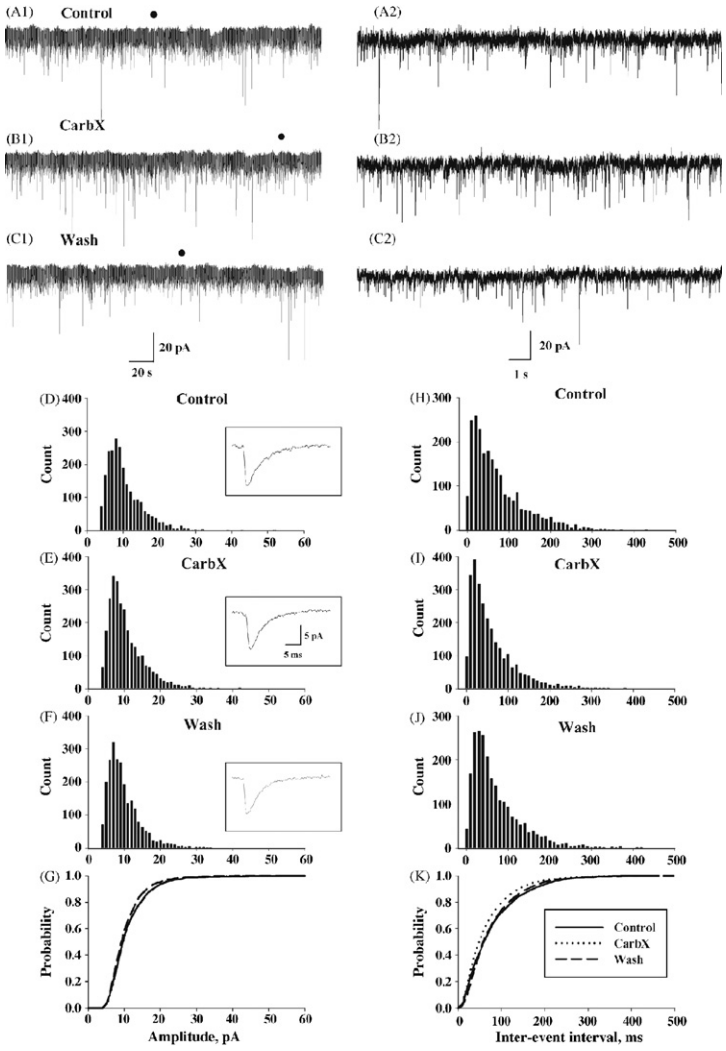

Fig. 2. Actions of CarbX on sEPSCs in layer II–III pyramidal cell. (A–C) Recordings of sEPSCs obtained under voltage-clamp in control saline (A), CarbX (B) and following CarbX wash out (C). Panels A2, B2 and C2 show expanded segments of the traces displayed in A1, B1 and C1 (indicated by dots). (D–F) Amplitude histograms of sEPSCs recorded in control saline (D), CarbX (E), and following wash out of CarbX (F). Inset panels show the averages of 30 randomly selected sEPSCs recorded under each condition. (G) Cumulative probability distributions of sEPSC amplitudes. (H–J) Inter-event interval histograms of sEPSCs recorded in control saline (H), CarbX (I) and after CarbX wash-out (J). (K) Cumulative probability distributions of sEPSC inter-event interval values. The legend in (K) applies to both (G) and (K).

A delicate balance between excitation and inhibition maintains normal physiological function in the central nervous system. Spontaneous neurotransmitter release drives persistent neuronal activity which participates in sustaining the excitation–inhibition balance. Minor modifications to synaptic transmission may change neurotransmitter release processes, leading to downstream effects on the excitability of entire networks. For example, in hippocampus [24] and neocortex [28], low concentration of the $GABA_A$ receptor antagonist bicuculline (0.5–5 μM) barely alters evoked inhibitory postsynaptic potentials but completely abolishes sIPSPs [1,4], allowing generation and propagation of evoked epileptiform activity. In the present study, we examined the effects of CarbX on spontaneous synaptic events in neocortical pyramidal cells to determine whether blockade of electrotonic coupling alters spontaneous synaptic excitation or inhibition. We found that CarbX attenuated spontaneous $GABA_A$-mediated inhibitory events, causing decreases in both sIPSC magnitude and frequency. Sodium propionate produced a similar depression of sIPSCs, causing reductions in event frequency that closely matched those induced by CarbX. Although sodium propionate caused a smaller decrease in sIPSC amplitude than CarbX, this may be the result of acidosis-induced enhancement of postsynaptic $GABA_A$ receptors [16], which would counter the attenuating effect of GJ blockade on sIPSC magnitude. Together, these findings suggest that the uncoupling of GJs attenuates spontaneous synaptic inhibition in neocortical circuits. In addition, we found that CarbX increased the frequency, but not the amplitude, of spontaneous glutamatergic responses, which was primarily attributable to CarbX-induced reduction of $GABA_A$ergic inhibition.

It is well established that electronic coupling between GABAergic interneurons via GJs promotes synchronized firing within inhibitory cell networks [7,12]. Such synchronous

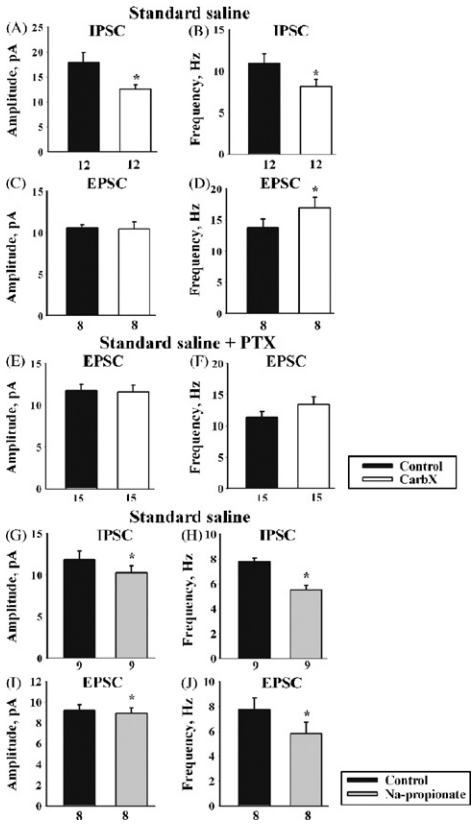

Fig. 3. Enhancement of sEPSC frequency by CarbX is due to the attenuation of $GABA_A$ergic inhibition consequent to the blockade of electrotonic coupling. (A–B) Summary data pooled from 12 cells (12 slices) of CarbX effects on sIPSC amplitude and frequency in standard saline. (C–D) Summary data pooled from 8 cells (8 slices) of CarbX effects on sEPSCs in standard saline. (E–F) Effects of CarbX on sEPSCs during blockade of $GABA_A$-mediated inhibition with 20 μM picrotoxin (PTX); data pooled from 15 cells (15 slices). (G–H) Exposure of slices to 25 mM sodium propionate (Na-propionate) reduced sIPSC amplitude and frequency, mimicking the actions of CarbX. Data was pooled from 9 cells (6 slices). (I–J): Na-propionate also reduced sEPSC amplitude and frequency, which is likely due to the direct suppressive effects of acidosis on excitatory synaptic transmission. Data shown was pooled from 8 cells (5 slices). The legend in E applies to panels (A–E), and the legend in (I) applies to panels (G–J), $^*p < 0.05$.

firing leads to a strengthening of synaptic inhibition in postsynaptic pyramidal cells. Blockade of GJ communication may diminish interneuron synchronization and, in turn, attenuate the frequency and magnitude of spontaneous inhibitory events. Although sIPSC amplitude is primarily determined by postsynaptic GABA receptors, presynaptic factors can also affect the magnitude of inhibitory events. For example, the elimination of action potential-dependent events via blockade of presynaptic sodium channels reduces both sIPSC frequency and amplitude [24]. Our findings indicate that CarbX caused a decrease in mean sIPSC amplitude by reducing the proportion of large amplitude sIPSCs. By blocking interneuron coupling, CarbX would inhibit synchronized firing of presynaptic interneurons, leading to decreased summation of unitary IPSCs in pyramidal cells and, in turn, smaller ensemble sIPSCs. The decline in spontaneous inhibition would shift the excitation–inhibition balance towards a state favoring excitation and result in heightened excitatory drive, as evidenced by the CarbX-induced increase in sEPSC frequency observed in this study. This is further supported by our finding that low doses of picrotoxin prevented CarbX enhancment of sEPSC frequency. Together, these suggest that the modifications in spontaneous synaptic transmission by CarbX appear to be principally mediated through actions on interneuronal GJs.

However, effects on GJs between other neocortical cell types could also contribute to CarbX-induced enhancement of synaptic excitation. GJs have been shown to exist between neocortical glial cells and have been implicated in spatial buffering of extracellular K^+ [22]. Uncoupling of glial cell GJs could lead to a rise in extracellular K^+ and, in turn, depolarization of neurons and heightened pyramidal cell excitability. K^+-induced depolarization would likely have a less pronounced effect on interneuron firing frequency, since interneurons generally fire action potentials with higher frequencies and larger afterhyperpolarizations than pyramidal cells. Moreover, by suppressing synchronous firing throughout inhibitory networks, blockade of interneuronal GJ communication would tend to offset any increased excitation of individual inhibitory cells. Thus, the enhancement of pyramidal cell excitability by GJ uncoupling agents may not only be mediated through actions on interneurons, but glial cells as well.

Past studies suggest that $GABA_A$ receptor activity is essential for the synchronizing mechanisms that support some [2], but not all [25] forms of seizure activity. In 4-AP or low Mg^{2+}-induced epileptiform activity, GABAergic interneuron synchronization plays a vital role in the initiation and maintenance of ictal discharges [2,18]. Treatment with GJ blockers abolishes ictal activity induced by low Mg^{2+} [18] or 4-AP [14], suggesting electrotonic communication critically supports the synchronization of inhibitory cell networks essential to these seizure-like discharges. In this study, we found that in the absence of convulsant agents or enhanced excitation, blockade of electrotonic coupling in neocortex attenuates spontaneous GABAergic inhibition, leading to an increase in tonic synaptic excitation. This could, in turn, increase excitability of individual neurons by depolarizing cell membranes [8]. However, given the established anticonvulsant effects of GJ uncouplers, it is likely that during states of heightened excitation, the suppression of interneuron synchronization by GJ blockers predominates over their downstream effects on synaptic excitation. Uncoupling inhibitory cells would desynchronize interneuron firing, causing temporal disruptions of the coordinated processes required for the initiation and maintenance of certain types of epileptiform activity.

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