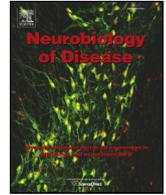




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Q1 KCC2 function modulates in vitro ictogenesis

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A B S T R A C T

GABA_A receptor-mediated inhibition is active and may contribute to epileptiform synchronization. The efficacy of inhibition relies on low levels of intracellular Cl⁻, which are controlled by KCC2 activity. This evidence has led us to analyze with field potential recordings the effects induced by the KCC2 blockers VU0240551 (10 μM) or bumetanide (50 μM) and by the KCC2 enhancer CLP257 (100 μM) on the epileptiform discharges generated by piriform and entorhinal cortices (PC and EC, respectively) in an in vitro brain slice preparation. Ictal- and interictal-like discharges along with high-frequency oscillations (HFOs, ripples: 80–200 Hz, fast ripples: 250–500 Hz) were recorded from these two regions during application of 4-aminopyridine (4AP, 50 μM). Blocking KCC2 activity with either VU0240551 or high doses of bumetanide abolished ictal discharge in both PC and EC; in addition, these experimental procedures decreased the interval of occurrence and duration of interictal discharges. In contrast, enhancing KCC2 activity with CLP257 increased ictal discharge duration in both regions. Finally, blocking KCC2 activity decreased the duration and amplitude of pharmacologically isolated synchronous GABAergic events whereas enhancing KCC2 activity led to an increase in their duration. Our data demonstrate that in vitro ictogenesis is abolished or facilitated by inhibiting or enhancing KCC2 activity, respectively. We propose that these effects may result from the reduction of GABA_A receptor-dependent increases in extracellular K⁺ that are known to rest on KCC2 function.

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30 Introduction

31 In the adult brain, γ-Aminobutyric acid (GABA) acts as the main inhibitory neurotransmitter and its failure has been often cited as a contributing factor to the generation of epileptic seizures. Indeed, early work in the in vitro hippocampal slice preparation demonstrated that interictal-like (hereafter referred to as *interictal*) epileptiform activity results from weakened inhibition (Johnston and Brown, 1981; Schwartzkroin and Prince, 1978, 1980) but later, by employing experimental procedures that enhance GABAergic signaling such as the K⁺ channel blocker 4-aminopyridine (4AP) or Mg²⁺ free medium, we and other investigators have reported that ictal-like (hereafter referred to as *ictal*) discharges in adult rodents are paradoxically contributed by GABA_A receptor-mediated signaling (see for review Avoli and de Curtis, 2011). In addition, under specific pharmacological manipulations, GABA_A receptor-mediated currents are the sole synaptic mechanism contributing to ictogenesis in the hippocampus (Uusisaari et al., 2002).

32 GABA_A receptor activation opens channels that are permeable to Cl⁻ and to a lesser extent to HCO₃⁻. In adulthood, GABA_A receptor-mediated current is hyperpolarizing due to the low intracellular [Cl⁻] that is

33 maintained by the activity of cation chloride cotransporters (CCCs). CCCs are secondarily active transporters that use cation gradient for transporting Cl⁻ (Rinehart et al. 2011) and two important CCCs in controlling neuronal [Cl⁻] are KCC2 and NKCC1. KCC2 is exclusively expressed in the central nervous system in mature brain and is the main extruder of Cl⁻ from neurons (Viitanen et al., 2010). On the other hand, NKCC1 is responsible for the uptake of Cl⁻ and renders GABA depolarizing in the immature brain (see for review Ben-Ari et al., 2012).

34 Some studies have linked failure in GABAergic inhibition to the Cl⁻ accumulation in the postsynaptic neurons thus leading to GABA_A receptor-mediated depolarization and even excitation (Ben-Ari and Holmes, 2005; Lillis et al., 2012; Staley et al., 1995). Also, downregulation of KCC2 is often observed in several pathological conditions associated with enhanced excitation such as epilepsy (Huberfeld et al., 2007; Rivera et al., 2002, 2004). Therefore, it has been proposed that the initiation and maintenance of ictal activity rest on the inability of KCC2 to control intracellular Cl⁻ thus disclosing depolarizing (and potentially excitatory) GABA_A receptor-mediated currents along with HCO₃⁻-mediated currents (Avoli and de Curtis, 2011; Jefferys et al., 2012). However, other studies have suggested that excessive activity of KCC2 during prolonged activation of GABA_A receptor and accumulation of Cl⁻ may result in an increase in extracellular [K⁺] (Viitanen et al., 2010), which in turn can lead to depolarization of neighboring neurons and excitation (see for review Avoli and de Curtis, 2011).

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To date the role played by the GABA_A receptor-mediated signaling in epileptiform synchronization and in particular its participation to ictogenesis remains unclear. Therefore, in this study we investigated the effects induced by the KCC2 inhibitor VU0240551 and the KCC2 enhancer, CLP257 on the epileptiform discharges and associated HFO that are generated by rat olfactory (PC) and limbic (EC) cortical networks maintained *in vitro* during 4AP treatment.

Methods

Brain slice preparation and maintenance—Male, adult Sprague-Dawley rats (250–275 g) were decapitated under isoflurane anesthesia according to the procedures established by the Canadian Council of Animal Care. The brain was quickly removed and placed in cold (1–3 °C), oxygenated artificial cerebrospinal fluid (ACSF) with the following composition (mM): 124 NaCl, 2 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 KH₂PO₄, 26 NaHCO₃, 10 D-glucose. Horizontal brain slices (450 μm) containing PC and EC were cut from this brain block using a vibratome. Slices were then transferred to an interface tissue chamber where they were superfused with ACSF and humidified gas (95% O₂, 5% CO₂) at a temperature of 31–32 °C and a pH of 7.4. 4AP (50 μM), VU0240551 (10 μM), bumetanide (10 μM or 50 μM), CLP257 (100 μM), 3, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 10 μM), and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM) were bath applied. Chemicals were acquired from Sigma-Aldrich Canada (Oakville, Ontario, Canada) except for CLP257 that was generously provided by Dr. Yves De Koninck from Laval University (Quebec City, Que., Canada). VU0240551 and CLP257 were dissolved in DMSO and control experiments were performed to

rule out any possible interference of this solvent on the epileptiform field activity induced by 4AP.

Electrophysiological recordings—Field potential recordings were obtained with ACSF-filled, glass pipettes (1B150F-4; World Precision Instruments, Sarasota, Florida, USA; tip diameter <10 μm, resistance 5–10 MΩ) that were connected to high-impedance amplifiers. The recording electrodes were positioned in the deep layers of the posterior PC and of the lateral EC (Fig. 1A). Field potential signals were fed to a computer interface (Digidata 1322A, Molecular Devices, and Palo Alto, CA, USA), acquired and stored using the PCLAMP 9.2 software (Molecular Devices). Subsequent data analyses were performed with CLAMPFIT 9.2 (Molecular Devices).

Detection of high-frequency oscillatory events—Time-periods containing interictal discharges recorded from the PC and EC were extracted. To identify oscillations in each frequency range (80–200 Hz and 250–500 Hz), a multi-parametric algorithm was employed using routines based on standardized functions (Matlab Signal Processing Toolbox) as described in detail in Hamidi et al. (2014) and Salami et al. (2012).

To be considered as HFOs, oscillatory events in each frequency range had to show at least four consecutive cycles, having amplitude of 3 SD above the mean of the reference period. The time lag between two consecutive cycles had to be between 5 and 12.5 ms for ripples (80–200 Hz) and between 2 and 4 ms for fast ripples (250–500 Hz). Oscillatory events containing overlapping ripples and fast ripples were excluded from the analysis (Béнар et al., 2010).

Statistical analysis—We used CLAMPFIT 9.2 (Molecular Devices) for offline analysis of the duration and interval of occurrence of ictal and interictal discharges. To segregate ictal from interictal discharges, we

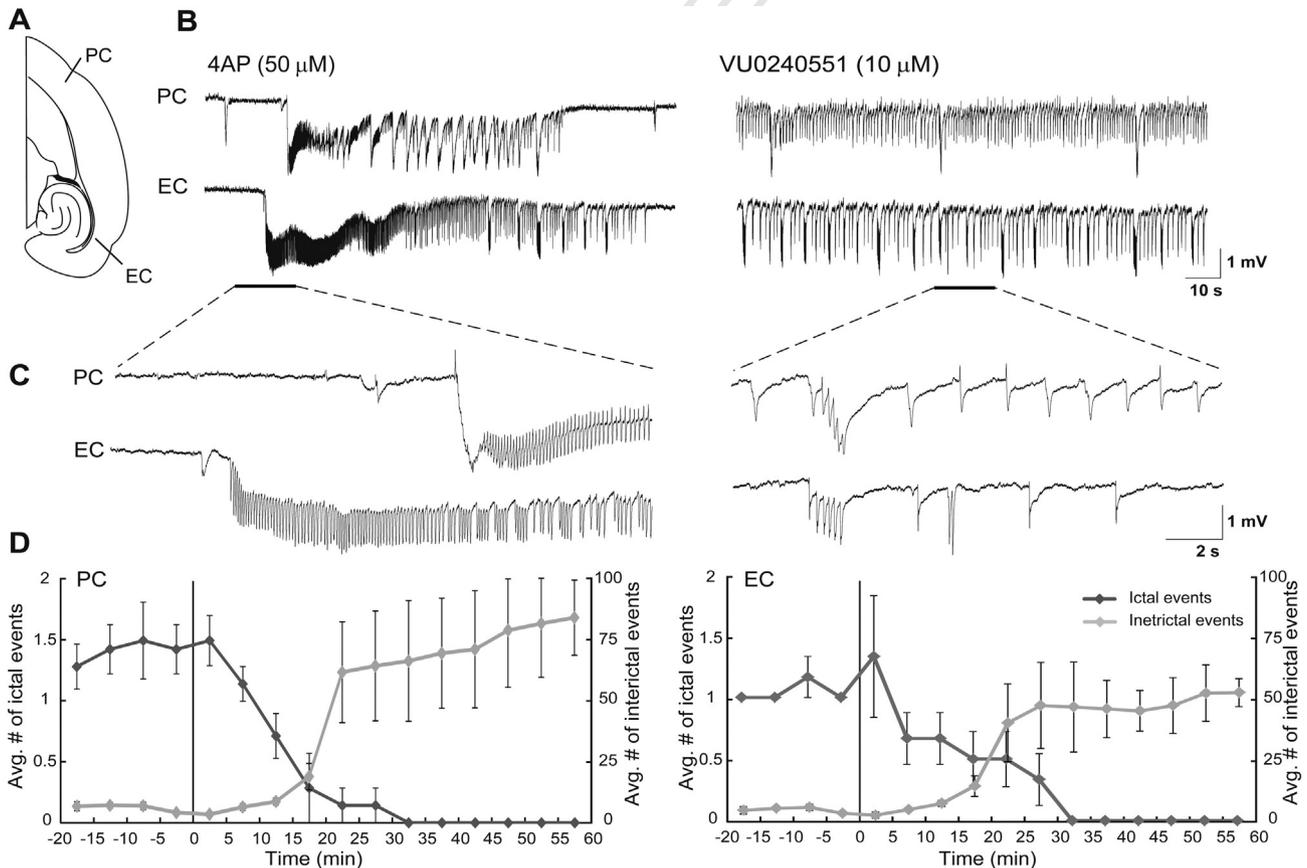


Fig. 1. A: Schematic drawing of a horizontal slice containing PC and EC. B: Field potential recordings obtained from PC and EC areas during application of 4AP and VU0240551 (10 μM). Note that blocking KCC2 with VU0240551 virtually abolishes ictal discharges in both brain regions whereas interictal discharges continue to occur. Enlarged portion of the recordings is shown in insets with traces that are further expanded at the start of an ictal discharge during 4AP and during interictal activity after application of VU0240551. C: Plots showing the average number of ictal and interictal discharges in PC and EC. Vertical lines in both graphs indicate the application of VU0240551. Note that 30 min application of VU0240551 blocked the occurrence of ictal discharges in both PC and EC while it increased the average number of interictal events in both regions.

140 applied the k-means clustering algorithm with squared euclidean distances on the duration of all recorded events and a cutoff at 3 s was chosen to segregate ictal and interictal events. These values are similar to those “arbitrarily” chosen by Traub et al. (1996). The duration of both ictal and interictal discharges were defined as the time between the first deflections of the discharge from baseline to its return to baseline. The interval of occurrence of both ictal and interictal discharges were defined as the time between the onsets of two consecutive discharges. Amplitude of epileptiform events was measured from peak to peak. Since the kurtosis and skewness measures showed that values were not normally distributed, we transformed the raw data to Z-scores and performed one-way ANOVAs followed by Tukey post-hoc tests to identify differences between experimental conditions (i.e., 4AP alone, 4AP + VU0240551, 4AP + bumetanide and 4AP + CLP257) in each region (PC and EC) regarding the rate and duration of ictal events and the rate, duration and amplitude of interictal discharges and isolated GABAergic events. Statistical tests were performed in Matlab R2012b (Matworks, Natick, MA) and the level of significance was set at $p < 0.05$. Results are expressed as mean \pm SEM and n indicates the number of slices used for analysis.

160 Results

161 Effects of KCC2 blockers on 4AP-induced epileptiform activity

162 Field potential recordings—which were obtained simultaneously from PC and EC during 4AP application—revealed ictal and interictal discharges that occurred in both structures (Hamidi et al., 2014) (Fig. 1B). Ictal discharges recorded from PC lasted 96.43 ± 5.56 s, and recurred every 155.03 ± 9.71 s (90 events, $n = 20$ slices) whereas those

occurring in EC lasted 127.62 ± 6.57 s, and recurred every 180.16 ± 167 8.65 s (78 events, $n = 20$ slices). Interictal discharges recorded from PC (375 events, $n = 20$ slices) and EC (392 events, $n = 20$ slices) in these experiments lasted 1.33 ± 0.05 s and 1.69 ± 0.04 s, occurred every 34.4 ± 3.1 s and 35.9 ± 4.4 s, and had amplitudes of 1.12 ± 171 0.03 mV and 1.21 ± 0.06 mV, respectively. 172

We studied the effect of VU0240551 (10 μ M) on the epileptiform discharges recorded from PC and EC. As illustrated in Fig. 1B, we found that 30 min application of VU0240551 (10 μ M) completely abolished the occurrence of ictal event in both PC and EC ($n = 8$ slices). In Fig. 1C we plotted the average number of ictal and interictal events over time during 4AP (50 μ M) and application of VU0240551 (10 μ M). We found that application of VU0240551 (10 μ M) resulted in reduction in the average number of ictal events while it increased simultaneously the average number of interictal events in both PC and EC. 181

We also used bumetanide that has high affinity for NKCC1 and a low affinity for KCC2; accordingly, it has been reported that at low doses (2–10 μ M), bumetanide blocks NKCC1 whereas at higher concentrations (50 μ M) it inhibits both NKCC1 and KCC2 (Löscher et al., 2013). In both PC and EC, low doses of bumetanide (10 μ M) did not significantly modulate the rate of occurrence of ictal discharges or their duration (Fig. 2A, $n = 6$ slices). In contrast, 30 min application of higher doses of bumetanide (50 μ M) completely abolished ictal discharges in both PC and EC (Figs. 2B, $n = 6$ slices). The change induced by bumetanide in the average number of ictal events over time is plotted in Fig. 2C. 191

In both PC and EC, blocking KCC2 with VU0240551 (10 μ M; $n = 8$ slices) or high concentrations of bumetanide (50 μ M; $n = 6$ slices) significantly ($p < 0.0001$) reduced the interval of occurrence of interictal events to 4.2 ± 0.9 s and 9.8 ± 0.7 s, respectively, in PC and to 3.8 ± 195 0.07 s and 9.1 ± 0.06 s, respectively, in EC (Fig. 3A). Application of 196

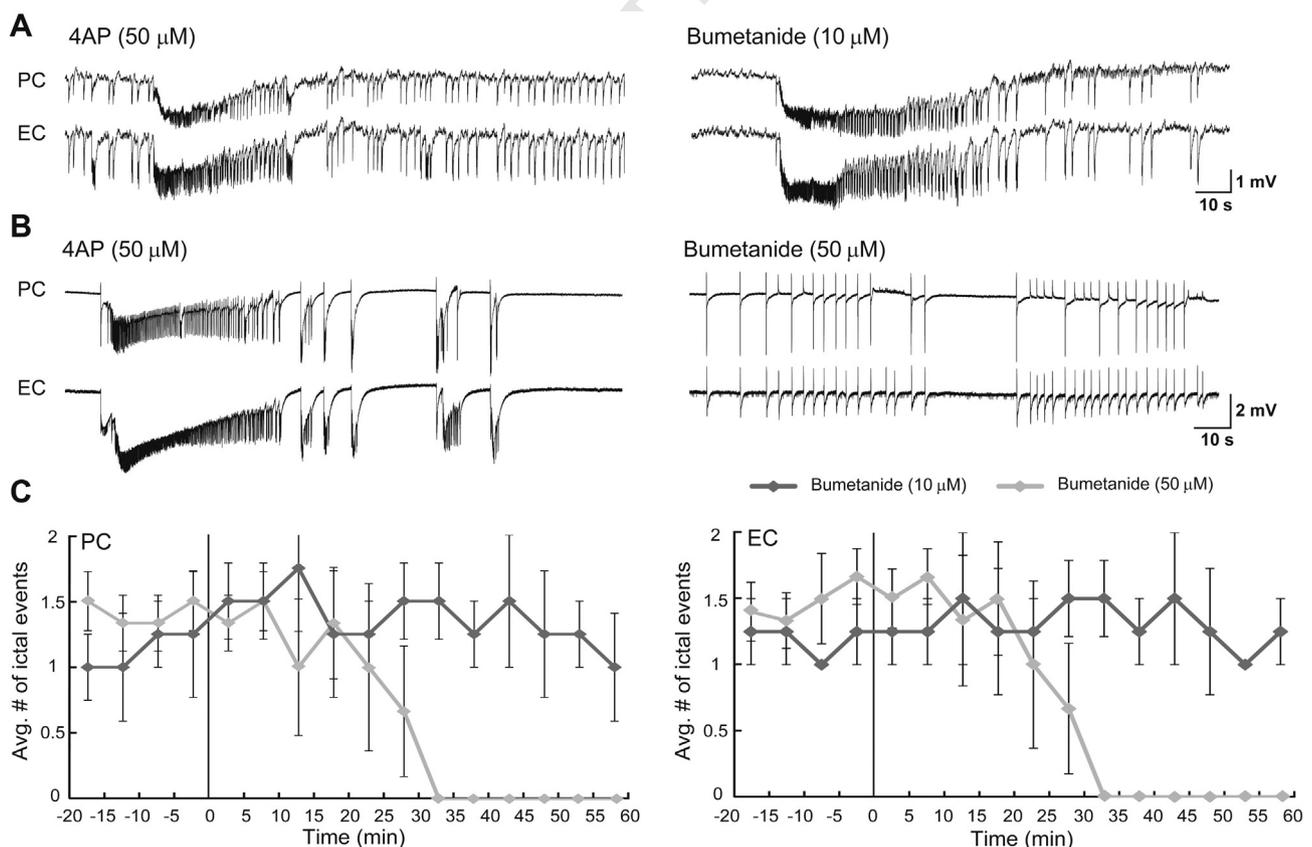


Fig. 2. A and B: Effects induced by 10 μ M (A) and 50 μ M (B) bumetanide on the epileptiform discharges recorded from the PC and EC during bath application of 4AP. Note that low dosage of bumetanide does not have any effect on the epileptiform discharges while blocking KCC2 with 50 μ M bumetanide virtually abolishes ictal discharges and discloses interictal discharges in both regions. C: Plots showing the average number of ictal discharges in PC and EC before and after the application of low and high dosage of bumetanide. Vertical lines in both graphs indicate the application of the drugs. Note that 30 min application of 50 μ M bumetanide blocked the occurrence of ictal discharges in both regions.

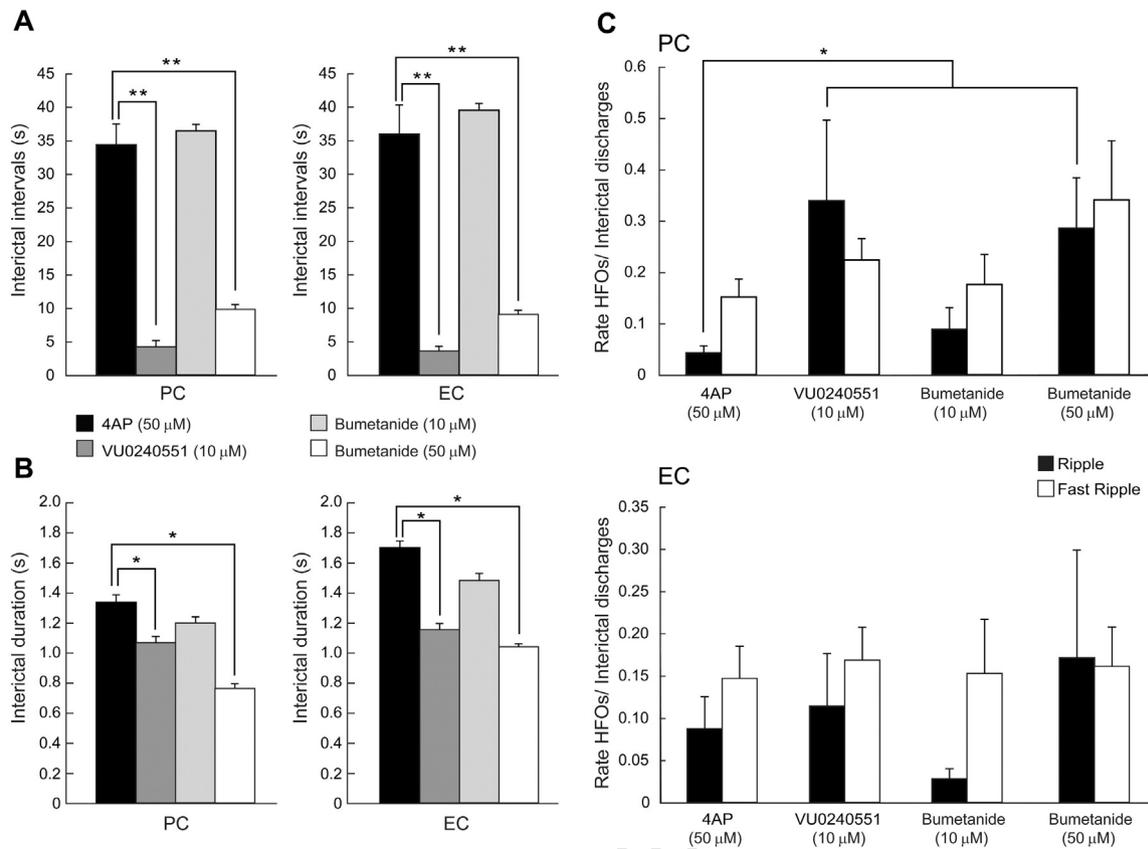


Fig. 3. A and B: Bar graphs showing the average interval of occurrence (A) and duration (B) of interictal discharges recorded from PC and EC under control conditions (i.e., 4AP) and during application of 10 μM VU0240551, 10 μM bumetanide and 50 μM bumetanide. Note that blocking KCC2 decreases the interval of occurrence and the duration of interictal discharges in both regions whereas blocking NKCC1 with bumetanide (10 μM) does not have a significant effect on interval of occurrence and duration of interictal discharges. C: Bar graphs showing the proportion of interictal events, in PC and EC, co-occurring with ripples and fast ripples under control conditions (i.e., 4AP), and during application of 10 μM VU0240551, 10 μM bumetanide and 50 μM bumetanide. Note that application of VU0240551 or bumetanide (50 μM) induces a significant increase in the proportion of interictal spikes co-occurring with ripples in the PC. * $p < 0.05$ and ** $p < 0.0001$.

VU0240551 (10 μM) or high concentrations of bumetanide (50 μM) also induced a significant reduction in the duration of the interictal discharges to 1.06 ± 0.04 s and 0.76 ± 0.03 s, respectively, in PC and to 1.15 ± 0.04 s and 1.03 ± 0.02 s, respectively, in EC (Fig. 3B, $p < 0.05$). Low concentrations of bumetanide (10 μM; $n = 6$ slices) did not influence the interval of occurrence and duration of interictal discharges in PC and EC.

We also analyzed the occurrence of HFOs in both the ripple (80–200 Hz) and fast ripple (250–500 Hz) frequency range that were associated to interictal discharges recorded from the PC and EC during 4AP (50 μM), VU0240551 (10 μM) and bumetanide (10 μM or 50 μM). Application of VU0240551 (10 μM) induced a significant increase in the occurrence of ripples associated with interictal discharges in the PC ($p < 0.05$) whereas fast ripple occurrence did not change significantly (Fig. 3C, PC). In contrast, VU0240551 (10 μM) did not change the occurrence of ripples and fast ripples in the EC (Fig. 3C, EC). Following the application of a high concentration of bumetanide (50 μM), ripple occurrence significantly increased in PC ($p < 0.05$) whereas fast ripple occurrence did not change (Fig. 3C, PC). Also, application of bumetanide (50 μM) did not change the occurrence of ripples and fast ripples in EC (Fig. 3C, EC). Application of low doses of bumetanide did not influence the rate of occurrence of ripples and fast ripples in both PC and EC (Fig. 3C). These findings indicate that blocking KCC2 activity induces a significant increase in the proportion of ripples associated to interictal discharges in the PC but not in the EC.

Effects of KCC2 enhancer on 4AP-induced epileptiform activity

Next, we studied the effect of a KCC2 enhancer, CLP257 (100 μM; $n = 5$ slices) on the 4AP-induced epileptiform discharges recorded

from PC and EC (Fig. 4A). As illustrated in Fig. 4B, we found that CLP257 (100 μM) did not affect the rate of occurrence of ictal events since the average number of ictal discharges over the time did not change after application of CLP257 (100 μM) in both PC and EC. Interestingly, CLP257 (100 μM) resulted in an increase in the duration of ictal events from 64.19 ± 6.10 s to 88.07 ± 9.46 s in PC, and from 91.51 ± 9.95 s to 126.54 ± 15.24 s in EC (Fig. 4C, $p < 0.05$). CLP257 (100 μM; $n = 5$ slices) did not influence the interval of occurrence of interictal discharges (Fig. 4D) in PC and EC but shortened the duration of interictal discharges significantly ($p < 0.01$) only in PC from 0.65 ± 0.01 s to 0.48 ± 0.02 s (Fig. 4E). This procedure did not induce any change in the rate of occurrence of ripples and fast ripples associated with ictal and interictal events.

KCC2 blockers and pharmacologically isolated synchronous events

Finally, we pharmacologically blocked glutamatergic transmission with CPP (10 μM) and CNQX (10 μM) to analyze the effects of VU0240551, bumetanide (50 μM) and CLP257 on the 4AP-induced isolated GABAergic events that are recorded under these conditions (Hamidi et al., 2014). As shown in Figs. 5A and B, CPP (10 μM) and CNQX (10 μM) abolished ictal discharges revealing the recurrence of slow events that occurred in the both PC and EC at intervals of 41.2 ± 1.9 s and 38.7 ± 1.3 s with the duration of 2.38 ± 0.17 s and 2.37 ± 0.15 s and amplitude of 0.72 ± 0.05 mV and 0.70 ± 0.05 mV, respectively. HFOs were virtually abolished by CPP and CNQX application. Adding VU0240551 (10 μM, $n = 5$ slices) or bumetanide (50 μM, $n = 5$ slices) to medium containing 4AP, CPP, and CNQX decreased the duration of the isolated slow events significantly ($p < 0.05$) in both PC (to

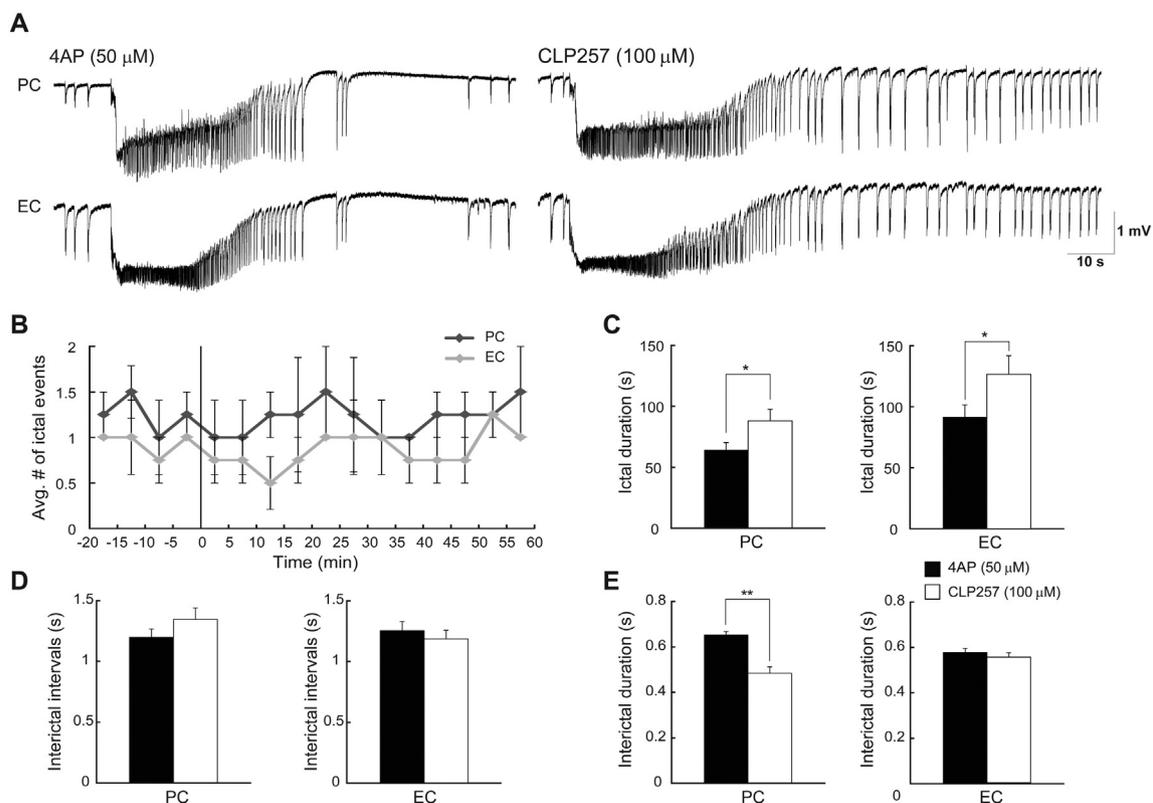


Fig. 4. A: Effects induced by 100 μM CLP257 on the epileptiform discharges recorded from the PC and EC during bath application of 4AP. B: Plot showing the average number of ictal discharges in PC and EC before and after the application of CLP257. Vertical line indicates the application of the drug. C: Bar graphs showing the duration of ictal discharges recorded from PC and EC under control conditions (i.e., 4AP) and during application of 100 μM CLP257. Note that enhancing KCC2 activity increases the duration of ictal discharges in both region without affecting their rate of occurrence. D and E: Bar graphs showing the average interval of occurrence (D) and duration (E) of interictal discharges recorded from PC and EC under control conditions and during application of CLP257. Note that CLP257 only decreases the duration of interictal discharges only in PC. * $p < 0.05$ and ** $p < 0.01$.

251 1.69 ± 0.06 s and 1.28 ± 0.14 s, respectively) and EC (to 1.86 ± 0.16 s
 252 and 1.80 ± 0.04 s, respectively) (Fig. 5C). Interestingly, application of
 253 CLP257 (100 μM , $n = 5$ slices) induced an increase in the duration of
 254 these events in both PC and EC to 3.11 ± 0.09 s and 2.89 ± 0.09 s,
 255 respectively. Also, blocking KCC2 with VU0240551 (10 μM) or bumetanide
 256 (50 μM) decreased the amplitude of the isolated slow events significant-
 257 ly ($p < 0.05$) in both PC (to 0.41 ± 0.05 mV and 0.34 ± 0.03 mV, respec-
 258 tively) and EC (to 0.26 ± 0.02 mV and 0.28 ± 0.02 mV, respec-
 259 tively) while CLP257 did not have any significant effect on the amplitude of
 260 these events (Fig. 5D). Blocking or enhancing KCC2 failed to induce a
 261 change in the interval of occurrence of these slow field events.

262 Discussion

263 The main findings of our study can be summarized as follows. First,
 264 blocking KCC2 activity with either VU0240551 or bumetanide (50 μM)
 265 abolished the occurrence of ictal discharges in both PC and EC. Second,
 266 application of KCC2 blocker increased the rate of occurrence of interictal
 267 discharges and decreased their duration in both PC and EC, and incre-
 268 ased the occurrence of ripples associated to interictal discharges in
 269 the PC. Third, enhancing KCC2 activity with CLP257 increased the dura-
 270 tion of ictal discharges in both PC and EC and decreased the duration of
 271 interictal discharges only in PC. Finally, blocking KCC2 activity induced a
 272 decrease in the duration of pharmacologically isolated synchronous
 273 GABAergic events whereas enhancing KCC2 activity had a completely
 274 opposite effect on the duration of these events.

275 Modulation of epileptiform activity by blocking KCC2 activity

276 GABA_A receptor activation sustains neuronal depolarizations that
 277 occur during epileptiform discharges in the 4AP model (Avoli and de

Curtis, 2011). It has also been shown in the human subiculum that
 278 GABA_A receptor-mediated depolarizations are associated to decreased
 279 KCC2 activity (Huberfeld et al., 2007). Therefore, we expected that
 280 blocking KCC2 activity would shift the reversal potential of GABA_A
 281 receptor-mediated currents to less negative values thus increasing
 282 both ictal and interictal epileptiform activity. Contrary to our expecta-
 283 tions, we found that ictal activity was abolished in both PC and EC dur-
 284 ing bath application of VU0240551 or bumetanide (50 μM). It has been
 285 shown that excessive activation of GABA_A receptors leads to accumula-
 286 tion of Cl⁻ inside the postsynaptic cells and to a subsequent increase in
 287 KCC2 activity that causes K⁺ and Cl⁻ efflux (Viitanen et al., 2010).
 288 In line with this evidence we have previously shown that epileptiform
 289 and in particular ictal discharges induced by 4AP are accompanied by el-
 290 evations in extracellular K⁺ (reviewed in Avoli and de Curtis, 2011).
 291 Hence, we are inclined to interpret the ability of both VU0240551 or
 292 high doses of bumetanide to abolish ictal activity as the result of the in-
 293 ability of GABA_A receptor activation to cause transient, KCC2 dependent
 294 increases in extracellular K⁺.
 295

We also found that blocking KCC2 activity with either VU0240551 or
 296 bumetanide (50 μM) induced an increase in the rate of occurrence of
 297 interictal discharges while decreasing their duration. Blocking KCC2 ac-
 298 tivity may change Cl⁻ homeostasis that may contribute to generation of
 299 interictal events by affecting GABA_A receptor-mediated signaling. In
 300 fact, Huberfeld et al. (2007) have shown that expression of KCC2 and
 301 Cl⁻ homeostasis is perturbed in 20% of subicular pyramidal cell tissue
 302 from epileptic patients. In these 20% subicular pyramidal neurons,
 303 GABA_A-mediated IPSPs reversed at depolarizing potentials and contrib-
 304 uted to the generation of interictal events (Huberfeld et al., 2007).
 305

We have discovered that blocking KCC2 activity with either
 306 VU0240551 or high doses of bumetanide induces an increase in the oc-
 307 currence of ripples during interictal discharges. This is presumably due
 308

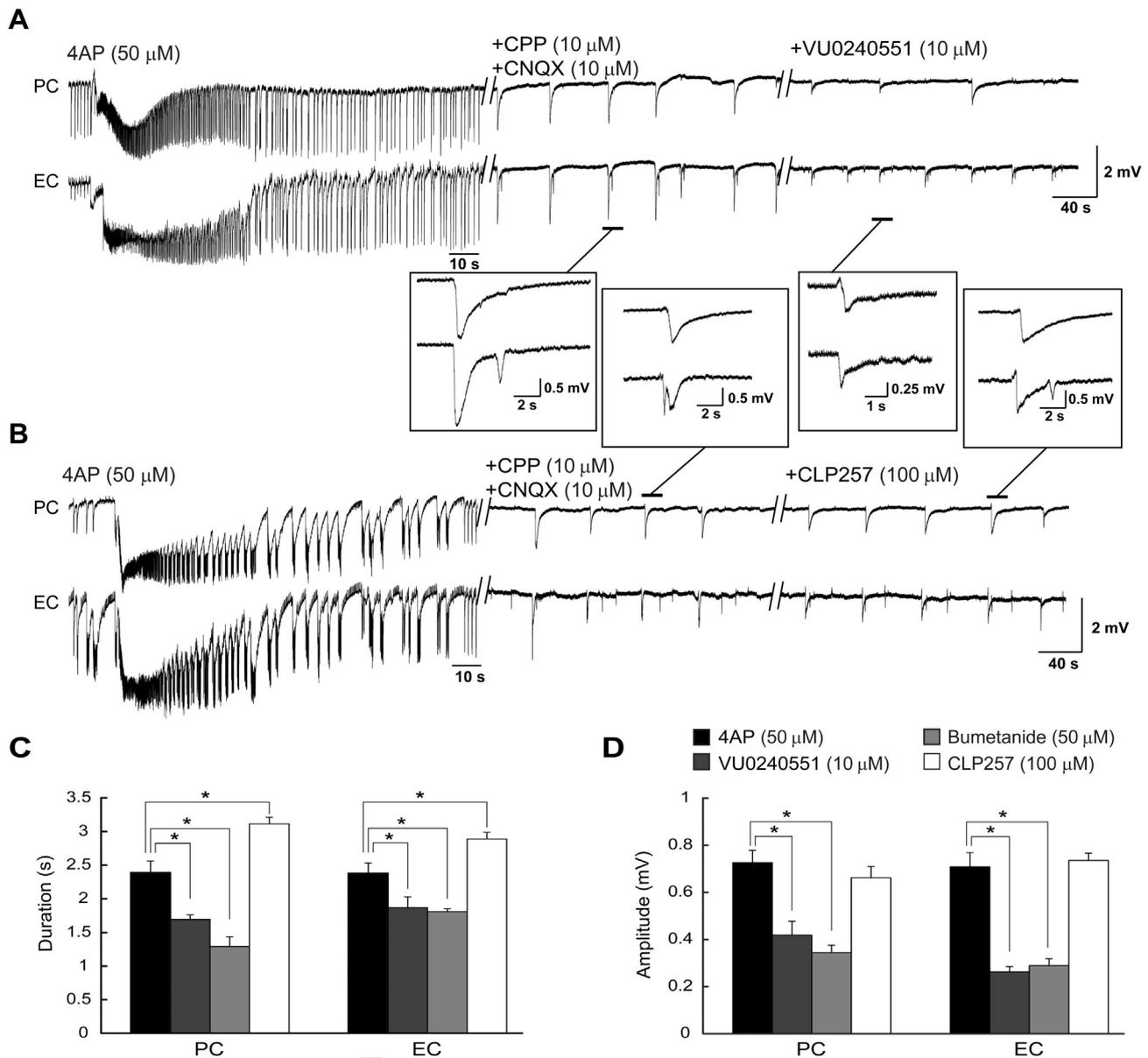


Fig. 5. A and B: Effects induced by VU0240551 (A) or CLP257 (B) on the slow field events recorded during concomitant application of 4AP, CPP (10 μM) and CNQX (10 μM). Insets show examples of the slow events at an expanded time scale. C and D: Bar graphs summarizing the change in duration (C) and amplitude (D) of the slow events under control conditions (i.e., 4AP + CPP + CNQX) and during further application of VU0240551 (10 μM), bumetanide (50 μM) and CLP257 (100 μM). Note that application of KCC2 blockers decreases the duration and amplitude of the slow events whereas KCC2 enhancer increases the duration of isolated GABAergic events. * $p < 0.05$.

to the fact that blocking KCC2 may weaken inhibition by changing $[Cl^-]_i$, thus increasing the excitability of interneurons (Viitanen et al., 2010). Interneuron activity has been indeed proposed to contribute to the generation of ripples (Buzsáki et al., 2007; Ylinen et al., 1995).

A recent, short study has identified that VU0240551, in addition to its inhibitory action on KCC2 (Delpire et al., 2009), might also interfere with G protein-coupled receptors (GPCRs) and ion channels (Delpire et al., 2012). We are however inclined to interpret the effects of this compound identified in our present study as resulting from a decrease in KCC2 activity since similar findings could be obtained with high concentrations of bumetanide (50 μM) that are known to block both KCC2 and NKCC1 (Löscher et al., 2013). Interestingly, low doses of bumetanide (10 μM), which only block NKCC1 activity, did not influence the patterns of epileptiform discharges recorded from the PC and EC; these negative findings are indeed in line with the view that NKCC1 is down regulated in the adult brain (Kahle and Staley, 2008; Wahab et al., 2011). Also one study by Wahab et al. (2011) has shown that the effects induced by low doses of bumetanide are age dependent

since 10 μM bumetanide can block epileptiform activity induced by 4AP in the immature but not in the adult EC region.

Enhancing KCC2 activity modulates epileptiform activity

Epilepsy has often been linked to inefficient GABAergic signaling derived from downregulation of KCC2 and elevated internal $[Cl^-]$ (Huberfeld et al., 2007; Payne et al., 2003; Rivera et al., 2004). Expression and function of KCC2 are tightly regulated by neuronal activity. Suppression of KCC2 during enhanced excitation is thought to alter the balance of excitation and inhibition that in turn may lead to anomalous activity such as seizures (Huberfeld et al., 2007; Rivera et al., 2002, 2004). Surprisingly, we have found that enhancing KCC2 activity with CLP257 (Gagnon et al., 2013) increased the duration of ictal events without affecting their interval of occurrence. These results suggest that downregulation of KCC2 might not be the cause of seizures in epilepsy but rather an adaptive mechanism to prevent the increase in

342 extracellular $[K^+]$ due to the excessive activation of GABA_A receptors
343 and KCC2 activity.

344 *Modulation of isolated GABAergic events by KCC2 blockers and enhancer*

345 We have confirmed here that ionotropic glutamatergic receptor-
346 mediated signaling is required for the generation of ictal discharges in
347 both PC and EC (Avoli et al., 2013; Hamidi et al., 2014; Herrington
348 et al., 2014). In keeping with evidence obtained in these previous stud-
349 ies, we have found that blockade of glutamatergic signaling using
350 NMDA and non-NMDA receptor antagonists led to the occurrence of
351 isolated, slow GABA receptor-dependent potentials during application
352 of 4AP. These glutamatergic independent field potentials result from
353 the synchronous firing of interneurons that leads to GABA release and
354 consequent postsynaptic activation of principal cells (Avoli and de
355 Curtis, 2011).

356 We have found here that blocking KCC2 activity shortened the dura-
357 tion of these synchronous events, and reduced their amplitude in both
358 PC and EC; in addition, enhancing KCC2 activity led to an increase in
359 the duration of these events in both regions. These findings, therefore,
360 demonstrate that GABA_A receptor-dependent hyperpolarization,
361 which is reduced and perhaps blocked by VU0240551 or high doses of
362 bumetanide, contributes the generation of the synchronous GABAergic
363 events induced by 4AP. Indeed, these field events are blocked by
364 GABA_A receptor antagonists and correspond to transient depolariza-
365 tions of principal cells in several limbic structures (see for review
366 Avoli and de Curtis, 2011). Blocking KCC2 activity should increase intra-
367 cellular $[Cl^-]$ but Viitanen et al. (2010) have reported that this change
368 does not influence GABA_A receptor-mediated hyperpolarizations and
369 slightly shifts their reversal potential which remains more negative
370 than the resting membrane potential. The lack of meaningful effects
371 on GABA_A receptor-mediated hyperpolarizations could be due to the
372 contribution of the Na⁺ dependent anion exchanger (NDAE), which ex-
373 trudes Cl^- and H⁺ and loads HCO_3^- and Na⁺ (Payne et al., 2003; Hübner
374 and Holthoff, 2013). Activation of NDAE can promote the inhibitory ac-
375 tion of GABA by maintaining Cl^- reversal potential negative to the rest-
376 ing membrane potential (Hübner and Holthoff, 2013). Although GABA_A
377 receptor-mediated signaling remains hyperpolarizing, KCC2 blockers
378 may influence the duration of the pharmacologically isolated GABAergic
379 events by slightly shifting the E_{GABA} and altering the ability of postsyn-
380 aptic neurons to accumulate Cl^- . In addition, expression and function
381 of KCC2 are tightly regulated by neuronal activity in a way that en-
382 hanced excitation results in suppression of KCC2 activity (Huberfeld
383 et al., 2007; Rivera et al., 2002, 2004). Indeed, restoring KCC2 activity
384 by application of CLP257 increased the duration of isolated GABAergic
385 events (Gagnon et al., 2013).

386 **Conclusions**

387 In conclusion, our results show that reducing or blocking KCC2 activ-
388 ity abolishes the occurrence of 4AP-induced ictal discharges that are
389 known to be contributed by the activation of GABA_A receptor and to
390 be associated to transient elevations in extracellular K^+ . These findings
391 were unexpected since increasing the intracellular concentration of Cl^-
392 should cause a shift of the reversal potential of inhibitory currents in the
393 positive direction and thus an enhancement of GABA_A receptor depend-
394 ent depolarizations which should reinforce ictal synchronization.
395 However, our findings can be explained by taking into consideration
396 the fact that GABA_A receptor activation leads to increases in extracellu-
397 lar K^+ that depend on KCC2 activity.

398 **Conflicts of interest**

399 None of the authors has any conflict of interest to disclose.

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405

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