

Is connexin36 critical for GABAergic hypersynchronization in the hippocampus?

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Non-technical summary In some forms of incurable epilepsies, GABAergic interneurons, which physiologically mediate inhibition in the brain, are thought to mediate excitation. The presence of a specific form of electrical communication between these cells, which is mediated by structures called gap junctions, has been proposed to be involved in the generation of synchronized epileptiform discharges. In support of this hypothesis is the repeated finding in the literature that the drug carbenoxolone, which is an effective blocker of gap junction function, decreases epileptiform activity in models of epilepsy both *in vivo* and *in vitro*. Our work challenges this view and highlights additional side-effects of carbenoxolone, which are unrelated to gap junctions, but seem to contribute to its reported antiepileptic activity. A full knowledge of these additional mechanisms is important for the rational development of new molecules to be used in the therapy of epilepsy.

Abstract Synchronous bursting of cortical GABAergic interneurons is important in epilepsies associated with excitatory GABAergic signalling. If electrical coupling was critical for the generation of this pathological activity, then the development of selective blockers of connexin36-based interneuronal gap junctions could be of therapeutic value. We have addressed this issue in the 4-aminopyridine model of epilepsy *in vitro* by comparing GABAergic epileptiform currents and their sensitivity to gap junction blockers in wild-type *vs.* connexin36 knockout mice. Although electrical coupling was abolished in stratum lacunosum-moleculare interneurons from knockout animals, epileptiform currents were not eliminated. Furthermore, epileptiform currents propagated similarly across hippocampal layers in the two genotypic groups. Blockade of electrical coupling with carbenoxolone suppressed amplitude, frequency and half-width of the epileptiform currents both in wild-type and in knockout animals, whereas mefloquine had no effects. Carbenoxolone also depressed responses to exogenous and synaptic GABA application onto interneurons. We conclude that, in the 4-aminopyridine model of epilepsy *in vitro*, connexin36 is not critical for the generation of epileptiform discharges in GABAergic networks and that the observed antiepileptic effects of carbenoxolone are likely to be due to blockade of GABA_A receptors and not of connexin36-based gap junctions. Lastly, because of its chemical structure and its effects on amplitude and kinetics of GABAergic currents, we tested the hypothesis that carbenoxolone acted via specific sites on GABA_A receptors, such as the one mediating the effects of the neurosteroid pregnenolone sulfate, or the allosteric regulatory site of benzodiazepines/ β -carbolines. Our results suggest that neither of these is involved.

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Introduction

Synchronous firing of populations of cortical GABAergic interneurons (Galarreta & Hestrin, 2001) generating depolarizing responses (Staley, 2004) has been implicated in epileptiform discharges *in vivo* and *in vitro* (Avoli, 1996). Studies on tissue obtained after surgery from patients suffering from intractable epilepsy have identified depolarizing GABAergic signalling as a mechanism sustaining electroencephalographic spikes in temporal lobe epilepsy (Cohen *et al.* 2002; Huberfeld *et al.* 2007), ictal-interictal transitions in Taylor's type focal cortical dysplasia (D'Antuono *et al.* 2004), and possibly seizures in autosomal dominant nocturnal frontal lobe epilepsy (Klaassen *et al.* 2006). Developmentally regulated depolarizing *vs.* hyperpolarizing GABAergic signalling in cortical *vs.* subcortical networks has been proposed as a mechanism of electroclinical uncoupling of neonatal seizures (Glykys *et al.* 2009), further highlighting the importance of synchronized activity of cortical interneurons for some forms of epilepsy.

GABAergic interneurons are electrically coupled by gap junctions (Galarreta & Hestrin, 2001), which depend primarily on connexin36 (Venance *et al.* 2000; Deans *et al.* 2001; Hormuzdi *et al.* 2001). Direct paired recordings have shown that coupling between interneurons promotes synchronous firing under specific conditions (Mancilla *et al.* 2007), and propagates GABAergic excitatory potentials within interneuronal networks (Zsiros *et al.* 2007). If these mechanisms were operational in the aforementioned forms of epilepsies, then a strong rationale could be made for the development of connexin36-selective gap junction blockers as anti-epileptic drugs (Margineanu & Klitgaard, 2001; Meldrum & Rogawski, 2007).

Several studies have taken advantage of the convulsive properties of the potassium channel blocker 4-aminopyridine (4-AP) to investigate seizures *in vivo* (Szente & Pongrácz, 1979) and epileptiform synchronization *in vitro* (Rutecki *et al.* 1987, see review by Avoli *et al.* 2002). In particular, 4-AP is very effective in triggering synchronized discharges in cortical interneurons (Lamsa & Kaila, 1997; Ziburkus *et al.* 2006; Zsiros *et al.* 2007). This activity depends on depolarizing GABAergic transmission (Lamsa & Taira, 2003; Kantrowitz *et al.* 2005; Zsiros *et al.* 2007) and can be conveniently isolated *in vitro* following blockade of ionotropic glutamate receptors (Perrault & Avoli, 1992). Application of gap junction blockers such as carbenoxolone, has been consistently reported to reduce 4-AP-dependent synchronization both *in vivo* (Szente *et al.* 2002; Gajda *et al.* 2003, 2005, 2006; Medina-Ceja *et al.* 2008) and *in vitro* (Ross *et al.* 2000; Yang & Michelson, 2001; Traub *et al.* 2001; Lamsa & Taira, 2003; Gigout *et al.* 2006; Zsiros *et al.* 2007). Unfortunately,

currently available blockers (reviewed by Juszczak & Swiergiel, 2009) bind to several targets other than gap junctions, making a straightforward interpretation of pharmacological experiments problematic (Tovar *et al.* 2009). Furthermore, only a limited number of studies have compared 4-AP-induced activity in wild-type *vs.* connexin36 knockout mice (Cx36 KO), and contrasting observations have been reported (see Maier *et al.* 2002, but also Buhl *et al.* 2003 and Pais *et al.* 2003). We have re-examined this issue by taking advantage of recordings from interneurons in wild-type and Cx36 KO mice. Our results indicate that carbenoxolone possesses antiepileptic activity independent of its blockade of connexin36-based gap junctions.

Methods

Ethical approval

All experimental procedures described in the present work were in accordance with the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals*, followed Northwestern University Institutional Animal Care and Use Committee (IACUC) approved protocols, and complied with the policies of *The Journal of Physiology* (Drummond, 2009) and UK regulations on animal experimentation.

Slice preparation

Slices were prepared from young (P14–P21) C57/B6 wild-type ($n = 58$) and C57BL6/129sv connexin36 knockout mice ($n = 47$, generous gift of Prof. David Paul, Harvard University) generated as described in Deans *et al.* (2001). Animals were first deeply anaesthetized using isoflurane in an induction chamber, in compliance with the guidelines provided by the IACUC of Northwestern University and the NIH. The level of anaesthesia was assessed by monitoring the pedal withdrawal reflex and by pinching the tail and ears. Following deep anaesthesia, mice were quickly decapitated and the brain removed from the skull in a small container filled with chilled solution of the following composition (in mM): 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 1 CaCl₂, 2 MgCl₂, 10 glucose saturated with 95% O₂–5% CO₂ at pH 7.4. Both hemispheres of the brain were glued on the stage of a vibrating microtome (Leica, VT1000S) and submerged in chilled artificial cerebrospinal fluid (ACSF), and sections of 400 μ m were cut and stored in an incubation chamber at 34–35°C for at least 30 min, then stored at room temperature until use. Throughout the study, polymerase chain reaction testing was used (Center for Comparative Medicine, Northwestern University via Transnetyx, Cordova, TN, USA) to confirm the genotype

of connexin36 knockout mice. Thirty-nine animals were tested and genotype was confirmed in 100% of them.

Whole-cell recordings

Conventional patch-clamp recordings were performed. Slices were superfused with preheated ACSF maintained at a constant temperature (32–35°C). ACSF was of the following composition (in mM): 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, 10 glucose saturated with 95% O₂–5% CO₂ at pH 7.4. Cells were observed and selected for recording by means of 40× IR immersion DIC objective applied to a direct microscope (Zeiss, Germany) equipped with an infrared camera system (DAGE-MTI, Michigan City, IN, USA). Interneurons were selected in the CA1 stratum lacunosum-moleculare according to the same criteria as in Zsiros & Maccaferri (2005). Pipettes were pulled from borosilicate thin glass capillaries (MTW150F-3, WPI) and filled with the appropriate filtered intracellular solution to a ~3 MΩ final resistance, as detailed below. Recordings were carried out using a Multiclamp 700 amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 3 kHz and digitized at 10–20 kHz using a Digidata A/D board and the Clampex 9 program suite (Molecular Devices). The holding potential for voltage-clamp experiments was set to –60 mV apart from a limited set of initial experiments where cells were held at –40 mV. GABA was puffed (>10 psi, 50 ms) on the recorded cells via a picospritzer device (TooheySpritzer, Toohey Company, Fairfield, NJ, USA).

The solution for current-clamp recordings was (in mM): 115 potassium methylsulfate, 4 ATP-Mg₂, 30 NaCl, 0.3 GTP, 16 KHCO₃ equilibrated with 95% O₂–5% CO₂ to give pH 7.3.

The solution for voltage-clamp recordings was (in mM): 125 KCl, 4 ATP-Mg₂, 10 NaCl, 0.3 GTP, 16 KHCO₃, 10 *N*-2(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314-Cl) equilibrated with 95% O₂–5% CO₂ to give pH 7.3. Estimated $E_{\text{GABA(A)}}$ was ~0 mV (Zsiros *et al.* 2007). QX-314 was included in the intracellular solution at high concentration in order to block voltage-dependent conductances.

Evaluation of electrical coupling in paired current-clamp experiments

We tested for the presence of gap junctions between pairs by repetitively injecting a 500 ms current step of –50/–100 pA. Cells were considered coupled if they had a coupling coefficient (ratio of the voltage deflection in the non-injected cell to the voltage deflection in the injected cell measured during the last 50 ms of the stimulus) value higher than 0.005 and the shape of the electrotonic

response was recognizable in the recording from the non-injected cell. For these experiments, synaptic blockers were added to ACSF (SR-95531 (gabazine; 12.5 μM), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX; 20 μM), D(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 50 μM), CGP55845 (5 μM)).

Drug preparation and application in slices

All the following drugs were from Tocris Biosciences. Gabazine was used at 12.5 μM. The powder was dissolved in water as a stock solution at 25 mM, aliquoted in 100 μl vials, and frozen at –20°C. D-AP5 was dissolved in water, stocked frozen in 100 μl aliquots at 50 mM and used at a 50 μM. Stock solutions of NBQX were dissolved in DMSO at 100 mM, aliquoted in 40 μl vials and frozen at –20°C. The final concentration used was 20 μM. CGP55845 was dissolved in DMSO in stock solutions at 100 mM, then aliquoted and used at a final concentration of 5 μM. Carbenoxolone was obtained from Sigma as a disodium salt and was dissolved directly into the recording solution to a final concentration of 100 μM. 4-Aminopyridine was from Sigma and was also dissolved directly into the recording solution to its final concentration of 100 μM. Pregnenolone sulfate, mefloquine and flumazenil were also obtained from Sigma. Pregnenolone sulfate (sodium salt) and mefloquine hydrochloride were dissolved directly into the recording solution to a final concentration of 100 μM. Flumazenil was dissolved in DMSO, stored as frozen 1 ml aliquots at 25 mM, and used at a final concentration of 5 μM.

Analysis of network-driven events in slices exposed to 4-aminopyridine

Spontaneous epileptiform currents recorded from single neurons were analysed using the Clampfit 9.0 (Molecular Devices), OriginPro7.0 (OriginLab Corp., Northampton, MA, USA), and Microsoft Excel suites of programs. Events were first collected using the threshold-based analysis feature of Clampfit, reviewed by visual inspection, and aligned by their peaks. Analysis of the time course of the effect of various drugs (or stability) was performed first by averaging the amplitudes of spontaneous events within the same neuron in 1 min bins and then by averaging each bin across different cells. Cross-correlations were performed on traces digitally filtered at 30 Hz.

Digital summation of the effects of carbenoxolone and pregnenolone sulfate

The digital summation of the effects of the individual applications of carbenoxolone and pregnenolone sulfate

was obtained by multiplying the averaged normalized values of peak and frequency for every bin during the time course of the effect (Fig. 14A) and the averaged normalized values during the last 5 min of the experiment in the presence of the drug (Fig. 14B).

Statistical methods

Statistical analysis was performed using the following software packages: Clampfit 9.0, Excel, Origin, Prism (GraphPad Software Inc., La Jolla, CA, USA). Significance was set at 0.05. Fisher's exact test or Student's *t* test for paired or unpaired data was used as appropriate. Because multiple comparisons were often made, the Bonferroni correction was used (Motulsky, 2010). The Bonferroni correction was implemented by multiplying the extracted value of *P* by the *m* number of comparisons. The Bonferroni correction was also applied to multiple confidence intervals by using $t_{0.025/m}$ in place of $t_{0.025}$ when constructing *m* 95% confidence intervals. Data are shown as means \pm SEM unless stated otherwise in the legend.

Results

If the antiepileptic actions of carbenoxolone *in vitro* (Zsiros *et al.* 2007) depended on the blockade of connexin36 gap junction channels, then we would expect

to see differences in 4-AP-induced epileptiform discharges recorded in hippocampal slices from wild-type *vs.* Cx36 KO mice. When glutamatergic transmission is blocked in this model of epilepsy *in vitro*, GABAergic epileptiform events are thought to be initiated in the stratum moleculare of the dentate gyrus (Gonzalez-Sulser *et al.* 2011) and propagate to the CA1 region starting from stratum lacunosum-moleculare (Sinha & Saggau, 2001; Perkins, 2002). Therefore, we decided to measure and compare 4-AP-induced epileptiform currents recorded from stratum lacunosum-moleculare interneurons, which are frequently electrically coupled (Price *et al.* 2005; Zsiros & Maccaferri, 2005).

However, as a preliminary step, we verified that electrical coupling between stratum lacunosum-moleculare interneurons was indeed abolished in slices from Cx36 animals. As shown in Figs 1 and 2, electrical coupling was present in pairs of interneurons from control slices, but absent in paired recordings from tissue obtained from Cx36 KO mice. The probability of finding connected pairs in wild-type animals was \sim 30% (5 coupled pairs out of 15 tested), similar to what was previously reported for interneurons of the same layer in rat slices (Zsiros & Maccaferri, 2005). In contrast, no coupled pairs were found in slices from Cx36 KO mice (0 out of 25 pairs tested, $P < 0.05$). Therefore, we concluded that stratum

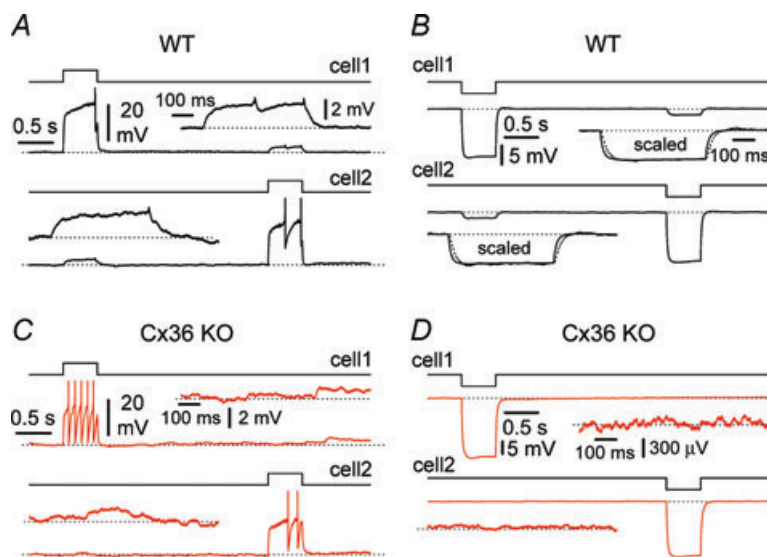


Figure 1. Electrical coupling between CA1 stratum lacunosum-moleculare interneurons in wild-type (WT, black traces) and connexin36 knockout (Cx36 KO, red traces) mice

A, sequential injection of a depolarizing current step (90 pA, black steps) to interneurons of WT mice generates an attenuated response in the non-injected cell. Insets show the attenuated responses at enhanced magnification: notice the presence of spikelets. B, similar protocol as in A, but hyperpolarizing current steps of -50 pA (black steps) are injected. Notice the propagation of the voltage response to the non-injected neuron. Also notice, in the insets, after scaling, the slower kinetics of the propagated response (dotted line) *vs.* the original one (continuous trace). C and D illustrate the same type of experiments as A and B, respectively, performed on interneurons in a slice obtained from a Cx36 KO mouse. Notice the lack of a propagated response in the non-injected neuron. Current pulses were 50 pA in C (black steps) and -50 pA in D (black steps). Insets in C and D show the lack of a voltage response in the non-injected cell at a magnified scale. Gabazine ($12.5 \mu\text{M}$), NBQX ($20 \mu\text{M}$), D-AP5 ($50 \mu\text{M}$) and CGP55845 ($1\text{--}5 \mu\text{M}$) present throughout in A–D.

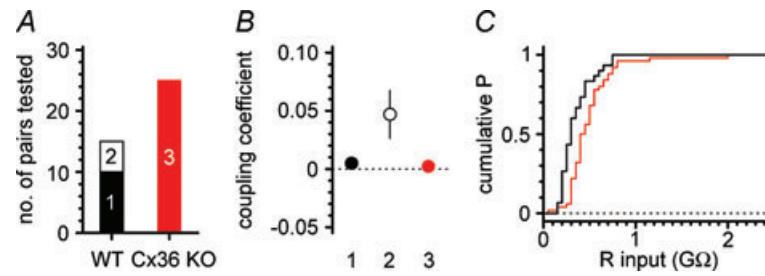


Figure 2. Network and membrane differences in interneurons of wild-type (WT) vs. connexin36 knockout (Cx36 KO) mouse

A, summary plot showing the different probability of finding electrically coupled pairs in the two types of animals. The white part of the bar (2) represents coupled pairs, in contrast to the black and red portions (1, 3), representing non-connected neurons in wild-type and Cx36 KO mice, respectively. *B*, summary graph showing the value of coupling coefficients for the uncoupled (1, black circle) and coupled (2, white circle) pairs of WT animals and for uncoupled (3, red circle) cells of Cx36 KO mice. *C*, cumulative probability distributions of resting membrane input resistances in WT (black line) vs. Cx36 KO animals (red line). Notice the shift towards higher values in the Cx36 KO mice.

lacunosum-moleculare interneurons of wild-type animals are electrically coupled via gap junctions that depend on connexin36, and that obvious compensatory mechanisms re-establishing coupling are absent in interneurons of Cx36 KO animals. It is also interesting to note that the membrane input resistance of interneurons from Cx36 KO mice was significantly higher from the one observed in control animals ($372 \pm 32 \text{ M}\Omega$ in wild-type, $n = 30$ vs. $520 \pm 41 \text{ M}\Omega$ in Cx36 KO, $n = 50$, $P < 0.05$), consistent with the idea of the loss of a resting membrane conductance, and in agreement with what was previously reported in neocortical interneurons of the same mouse (Deans *et al.* 2001).

Therefore, as mentioned above, if gap junctions played a critical role in the generation of epileptiform GABAergic activity, we would expect to see this reflected in a dramatic reduction of the amplitude and/or frequency of 4-AP-induced epileptiform currents in the animals lacking connexin36.

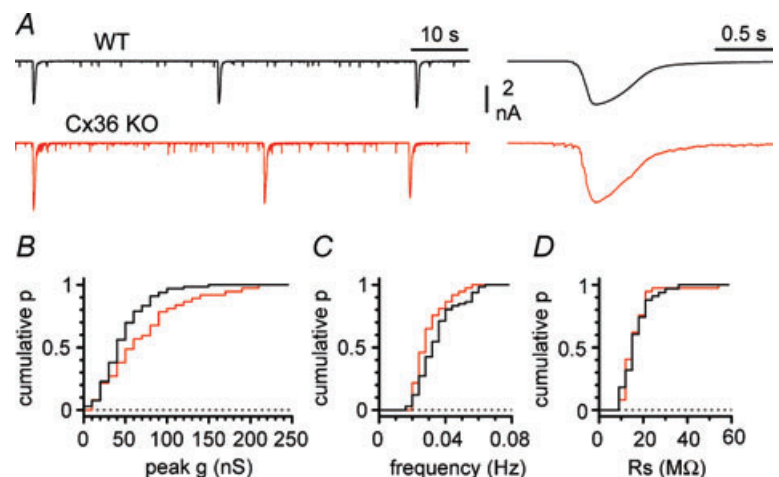
In the constant presence of blockers of ionotropic glutamatergic transmission and GABA_B receptors (NBQX

$20 \mu\text{M}$, D-AP5 $50 \mu\text{M}$ and CGP55845 $5 \mu\text{M}$), we recorded spontaneous epileptiform currents following exposure of the slices to 4-AP ($100 \mu\text{M}$, Fig. 3). Surprisingly, epileptiform events in Cx36 KO mice had a larger peak conductance than the ones observed in control animals ($74 \pm 8 \text{ nS}$ in Cx36 KO, $n = 37$ vs. $52 \pm 3 \text{ nS}$ in wild-type mice, $n = 66$, $P < 0.05$), and no difference was found in their frequency ($31 \pm 1 \text{ mHz}$ in Cx36 KO, $n = 37$ vs. $36 \pm 1 \text{ mHz}$, $n = 66$, $P > 0.05$). The difference in peak conductance could not be explained by voltage-clamp errors due to different series resistance values for the recordings in the two datasets (series resistance was $18 \pm 1 \text{ M}\Omega$ in Cx36 KO, $n = 37$ vs. $18 \pm 1 \text{ M}\Omega$, $n = 66$ in wild-type, $P > 0.05$). This result suggests that the expression of connexin36 and the presence of functional coupling between stratum lacunosum-moleculare interneurons are not an absolute requirement for the generation of GABAergic epileptiform discharges *in vitro*.

Next, we verified that epileptiform events in the interneurons of the two types of animals were sustained by GABA_A receptors and similarly sensitive to

Figure 3. Properties of 4-AP-induced epileptiform currents and recording conditions in WT (black traces) vs. Cx36 KO mice (red traces)

A, examples of voltage-clamp recording traces from stratum lacunosum-moleculare interneurons in WT and Cx36 KO mice shown at different temporal magnifications (left and right panels). *B*, cumulative probability plot of the peak conductance during epileptiform events in the two genotypes (black line, WT; red line, Cx36 KO). Notice the presence of larger conductance events in the Cx36 KO genotype. *C*, summary graph showing the similarity of the frequency of the epileptiform events in the two groups of animals. *D*, cumulative probability plot of the series resistance for the recordings in WT and Cx36 KO mice: notice the overlapping distributions. *A–D*, ionotropic glutamatergic synaptic transmission and GABA_B receptors blocked throughout the experiments.



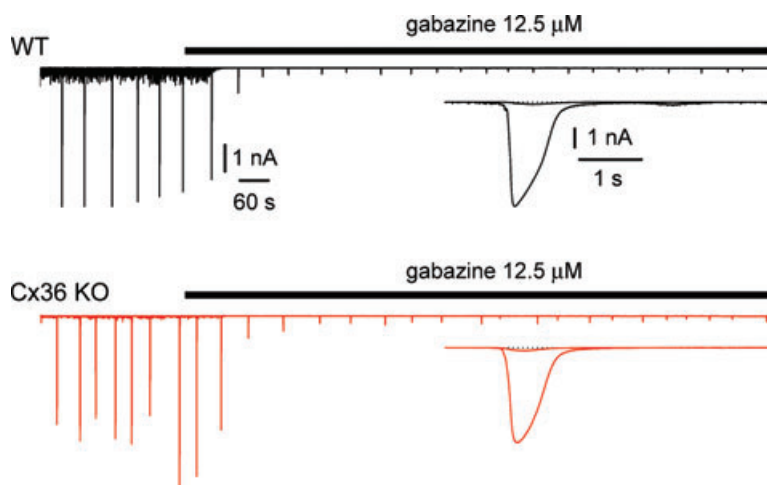


Figure 4. Pharmacological demonstration that 4-AP-induced epileptiform currents are mediated by GABA_A receptors in both WT (black traces) and Cx36 KO mice (red traces)

Application of the GABA_A receptor blocker gabazine (12.5 μM) powerfully reduces epileptiform currents in interneurons recorded in slices obtained from the two different genotypes. Upper traces show wild-type and lower traces connexin36 knockout. Insets show superimposed averaged events in control and in the presence of the drug.

gabazine (Fig. 4), as previously reported for 4-AP-induced epileptiform activity in wild-type rats (Zsiros *et al.* 2007). As expected, application of gabazine (12.5 μM) blocked epileptiform currents in both wild-type (from 100 ± 11 nS in control to 5 ± 1 nS in the presence of the drug, $n = 3$, $P < 0.05$) and Cx36 KO mice (from 66 ± 9 nS in control to 1 ± 1 nS in the presence of gabazine, $n = 3$, $P < 0.05$) to a similar degree (residual currents in the presence of gabazine were $5 \pm 1\%$ of control values in wild-type and $2 \pm 1\%$ of control values in Cx36 KO).

Despite the fact that the peak of the epileptiform events did not appear reduced in Cx36 KO mice, we thought that the absence of functional gap junctions might nevertheless impact their propagation to other hippocampal layers. Previous work by Perrault & Avoli

(1992) has indeed shown that the physical separation of different hippocampal layers via surgical cuts does not stop the occurrence of epileptiform GABAergic events in slices, but impairs their synchronization. Therefore, if the surgical cuts of Perrault & Avoli (1992) prevented synchronization of the different hippocampal layers by physically separating GABAergic networks connected via gap-junctions, we would expect to observe non-synchronized events in different hippocampal layers of slices from the Cx36 KO mice. We tested this possibility by performing simultaneous double recordings from interneurons located in stratum lacunosum-moleculare and stratum oriens of both wild-type animals and Cx36 KO mice (Fig. 5). Contrary to our expectations, synchronized epileptiform currents were observed in

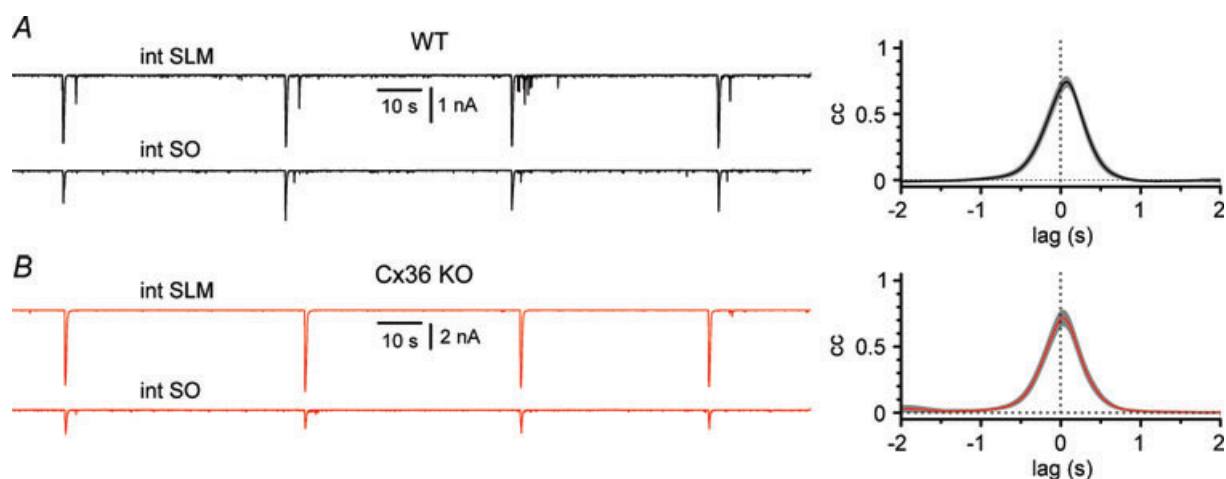


Figure 5. Propagation of 4-AP-induced epileptiform currents across hippocampal layers in WT (black traces) vs. Cx36 KO animals (red traces)

A, left panel, simultaneous recordings from a stratum lacunosum-moleculare and a stratum oriens interneuron from a WT mouse (int SLM and int SO, respectively). Notice the presence of synchronous events. Right panel, summary cross-correlogram of the activity in 10 SLM-SO WT interneuron pairs. Grey bands indicate \pm SEM. *B*, identical experiments and analysis as in *A*, but performed in 10 pairs of interneurons from Cx36 KO animals. Notice the similarity of the results. NBQX (20 μM), D-AP5 (50 μM) and CGP55845 (5 μM) present throughout in *A* and *B*.

the two layers in slices obtained from both wild-type and Cx36 KO animals. Neither the peak (peak cross correlation was 0.76 ± 0.03 in wild-type, $n = 10$ double recordings *vs.* 0.74 ± 0.05 in Cx36 KO, $n = 10$ double recordings, $P > 0.05$) nor the position of the peak ($+78 \pm 16$ ms in wild-type, $n = 10$ double recordings, $+40 \pm 19$ ms, $n = 10$ ms in Cx36 KO, $P > 0.05$) of the cross correlations were different in the two types of animals. This result confirms the reports of Sinha & Saggau (2001) and Perkins (2002) that, in the CA1 subfield, these experimental conditions trigger GABAergic epileptiform activity initiated in stratum lacunosum-moleculare. However, it shows that connexin36 and electrical coupling between interneurons are not required for propagation across layers.

Taken together, the observations presented so far suggest that the reported effects of carbenoxolone on GABAergic discharges induced by 4-AP (Ross *et al.* 2000; Yang & Michelson, 2001; Traub *et al.* 2001; Lamsa & Kaila, 1997; Gigout *et al.* 2006; Zsiros *et al.* 2007) are not due to the blockade of connexin36-dependent gap junctions between interneurons, but are likely to rely on different mechanism(s).

We decided to test this hypothesis directly by comparing the effect of carbenoxolone on epileptiform currents in wild-type *vs.* Cx36 KO animals. However, as a control experiment, we first verified that the absence of connexin36 in the KO animals does not impair the stability of epileptiform activity in prolonged recordings (Rouach *et al.* 2008). The amplitude and frequency of synchronized GABAergic currents were not different at the beginning compared to the end of 30 min recordings either in wild-type or in Cx36 KO mice, (Fig. 6). For wild-type

animals the amplitude and frequency of the events was 3928 ± 454 pA and 29 ± 2 mHz calculated for the initial 5 min of the recording *vs.* 3376 ± 268 pA and 28 ± 2 mHz in the last 5 min ($P > 0.05$, $n = 13$). Similarly, neither the amplitude nor the frequency of the epileptiform events changed in slices from Cx36 KO mice (4189 ± 734 pA and 22 ± 1 mHz at the beginning *vs.* 3884 ± 663 pA and 26 ± 2 mHz at the end of the recording, $P > 0.05$, $n = 8$).

When the effect of carbenoxolone was tested (Fig. 7), we observed that epileptiform currents were reduced in wild-type animals from 3099 ± 395 pA to 1434 ± 171 pA (first 5 min in control *vs.* last 5 min in carbenoxolone, $n = 16$) and from 5016 ± 457 pA to 2457 ± 351 pA ($n = 22$, $P < 0.05$) in Cx36 KO mice. The frequency of the events was also slightly reduced in both cases (from 35 ± 3 mHz to 21 ± 3 mHz in wild-type ($n = 16$, $P < 0.05$), and from 28 ± 2 mHz to 22 ± 2 mHz in Cx36 KO mice ($n = 22$, $P < 0.05$)). In conclusion, carbenoxolone reduced the amplitudes of epileptiform currents to $43 \pm 4\%$ in wild-type animals and to $48 \pm 5\%$ in Cx36 KO mice, and simultaneously decreased their frequency to $70 \pm 7\%$ and $76 \pm 6\%$, respectively. When we examined the kinetics of epileptiform currents (Fig. 8), we also noticed that carbenoxolone reduced the duration of the events. In particular, the half-width of the events was reduced from 339 ± 21 ms to 226 ± 26 ms in wild-type animals ($n = 16$, $P < 0.05$) and from 350 ± 20 ms to 239 ± 19 ms in Cx36 KO mice ($n = 22$, $P < 0.05$). Overall, these results show that carbenoxolone has similar effects on epileptiform events recorded in tissue from wild-type and Cx36 KO mice, thus suggesting that blockade of connexin36 is unlikely to mediate its observed antiepileptic actions. As an alternative,

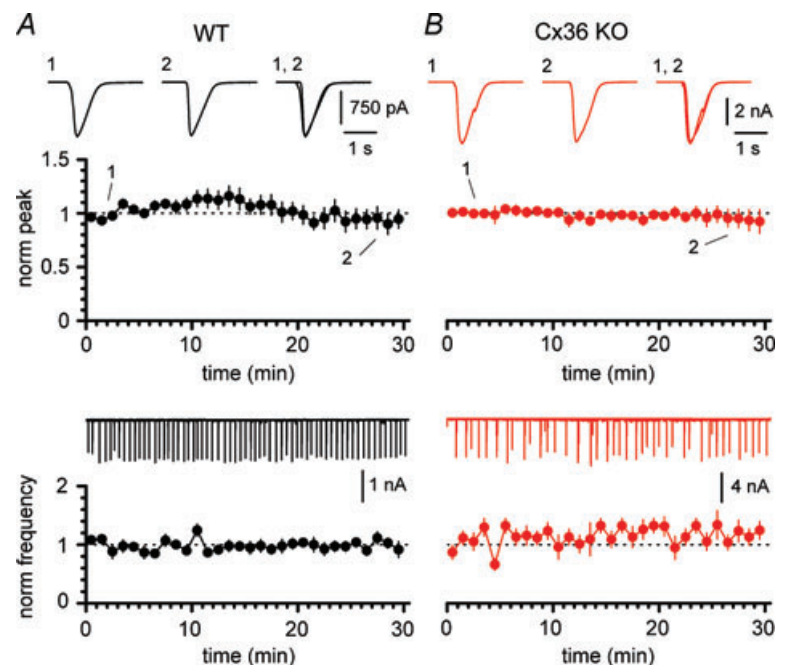


Figure 6. 4-AP-induced epileptiform activity can be maintained for long recording times in both WT (black circles and traces) and Cx36 KO mice (red circles and traces)

A, notice the stability of the amplitude (upper panel) and frequency (lower panel) of the events. Insets in the upper panel represent averaged events during the first (1) and last (2) 5 min of recording, and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. B, as in A, but experiments were performed in slices from Cx36 KO animals. Note that epileptiform activity is similarly maintained for 30 min in both genotypes. NBQX ($20 \mu\text{M}$), D-AP5 ($50 \mu\text{M}$) and CGP55845 ($5 \mu\text{M}$) present throughout in A and B.

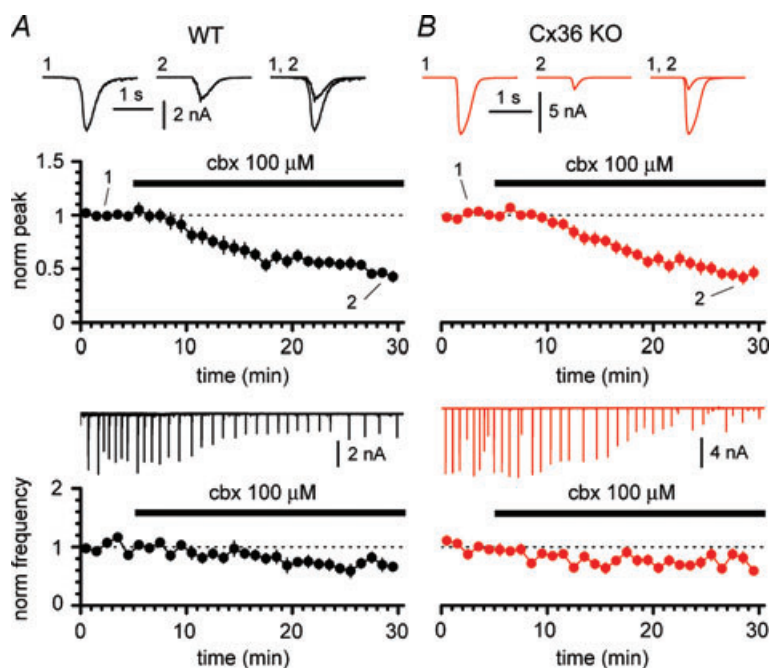


Figure 7. Carbenoxolone depresses GABAergic epileptiform events in both WT (black circles and traces) and Cx36 KO animals (red circles and traces)

A, time course of normalized peak (upper panel) and frequency (lower panel) during application of carbenoxolone (cbx, black bar) to slices from WT mice. Notice the strong decrease in the amplitude of the events and also the reduction in their frequency. Insets in the upper panel are averaged events in control (1), in the presence of carbenoxolone (2), and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. **B**, as in **A**, but experiments were performed on Cx36 KO animals. Notice that carbenoxolone also has an effect on both amplitude and frequency in this genotype. Ionotropic glutamatergic synaptic transmission and GABA_B receptors blocked throughout the experiments both in **A** and **B**.

direct suppression of GABA_A-mediated synaptic transmission could potentially explain our results. Data in the literature, however, appear contradictory. Although Traub *et al.* (2001) found that carbenoxolone at 100 μM had no effect on pharmacologically isolated depolarizing GABAergic potentials evoked by tetanic stimulation in slices, Tovar and colleagues (2009) have recently reported that carbenoxolone, at the same concentration, reduces the amplitude of IPSC evoked in autaptic cultures by $\sim 50\%$.

We designed an experiment to test the blocking effect of carbenoxolone on both exogenous and endogenous GABAergic events recorded from stratum

lacunosum-moleculare interneurons (bathed in standard ACSF plus blockers of glutamatergic ionotropic synaptic transmission and GABA_B receptor antagonists, but no 4-AP added). In order to completely exclude effects due to blockade of connexin36, slices were prepared from Cx36 KO mice. While interneurons were held in voltage-clamp at -60 mV, we first puffed GABA (50 μM , 50 ms duration) onto their soma, and then, after a delay of 42 s, an inhibitory postsynaptic current was evoked on the same cell by extracellular stimulation. As shown in Fig. 9, carbenoxolone depressed responses to exogenous GABA application and reduced inhibitory

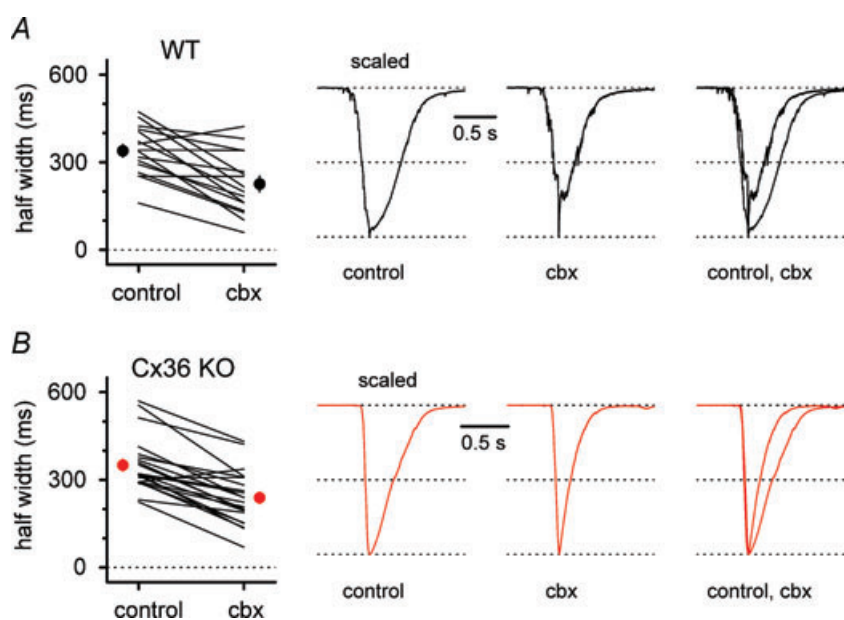


Figure 8. Carbenoxolone reduces the half-width of GABAergic epileptiform events in both WT (black circles and traces) and Cx36 KO animals (red circles and traces)

A, left, summary graph comparing half-widths in control and in the presence of carbenoxolone (cbx) for individual experiments (lines) and for their overall average (circle). Traces in control, in the presence of carbenoxolone, and superimposed are shown in the right panel. **B**, as in **A**, but experiments were performed on slices from Cx36 KO animals. Notice that carbenoxolone has a similar effect irrespective of the genotype. NBQX (20 μM), D-AP5 (50 μM) and CGP55845 (5 μM) present throughout in **A** and **B**.

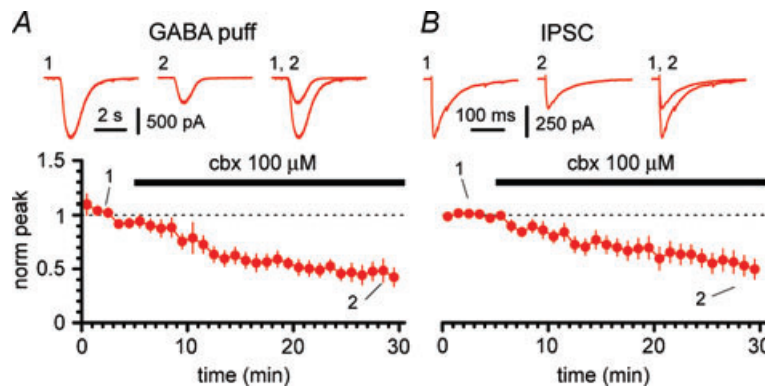


Figure 9. Carbenoxolone reduces direct responses both to exogenous and synaptic GABA applications
 A, summary plot of the time course of the effect of carbenoxolone (cbx, black bar) on currents evoked by GABA puffs onto stratum lacunosum-moleculare interneurons of the Cx36 KO mouse. Insets show averaged responses to GABA application in control (1), in the presence of carbenoxolone (2) and superimposed (1, 2). B, time course summary graph for the effect of carbenoxolone (black bar) on evoked inhibitory postsynaptic currents (IPSCs) recorded from the same cells in A. Notice that IPSCs are also depressed by carbenoxolone, similarly to responses to exogenous GABA. All experiments in A and B were performed on slices from Cx36 KO animals and ionotropic glutamatergic synaptic transmission and GABA_B receptors were pharmacologically blocked.

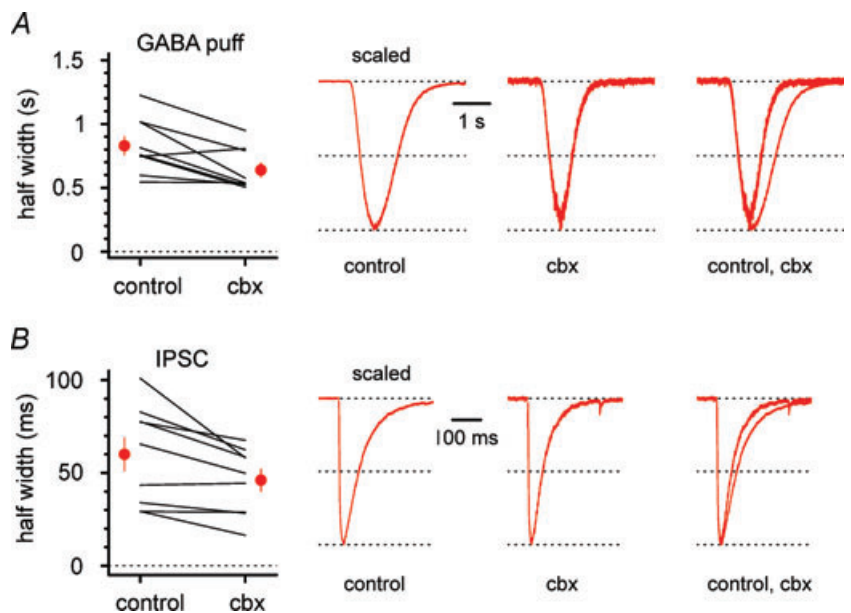
postsynaptic currents in 9 out of 10 cells tested. Currents generated by exogenous GABA application decreased from 645 ± 152 pA to 237 ± 59 pA ($n = 9, P < 0.05$), and inhibitory postsynaptic currents from 1020 ± 211 pA to 582 ± 181 pA ($n = 9, P < 0.05$). The relative effects of carbenoxolone on GABA puffs and synaptic currents in the presence of the drug were $43 \pm 9\%$ and $54 \pm 10\%$, respectively), thus suggesting that, under our experimental conditions, the major impact of carbenoxolone is on postsynaptic GABA_A receptors and not on presynaptic mechanisms. If this was the case, an additional prediction would be that carbenoxolone should also affect the kinetics of GABA puffs and evoked synaptic transmission

(Fig. 10). The half-width of the GABA puff was reduced from 829 ± 73 ms in control conditions to 639 ± 55 ms in the presence of the drug ($n = 9, P < 0.05$), and, similarly, the half-width of the evoked synaptic current decreased from 60 ± 9 ms to 46 ± 6 ms ($n = 9, P < 0.05$). Thus, our data suggest that direct blockade of GABA_A receptors, and not blockade of connexin36-based gap junctions, is the major mechanism underlying the antiepileptic effect of carbenoxolone in this model *in vitro*.

We decided to further confirm this interpretation by measuring the effect of a different gap junction blocker, mefloquine, on GABAergic epileptiform currents. The advantage of mefloquine is that this compound has been reported to lack non-specific effects on GABAergic

Figure 10. Effect of carbenoxolone on the half-width of responses to exogenous GABAergic applications and of inhibitory postsynaptic potentials in interneurons from Cx36 KO animals

A, left, summary graph comparing half-widths of voltage-clamp responses to GABA puffs in control and in the presence of carbenoxolone (cbx) for individual experiments (lines) and for their overall average (red circles). Traces in control, in the presence of carbenoxolone and superimposed are shown in the right panel. B, as in A, but data and analysis regards evoked inhibitory postsynaptic currents (IPSCs). Notice that carbenoxolone has a similar effect on GABA puffs and IPSCs. NBQX ($20 \mu\text{M}$), D-AP5 ($50 \mu\text{M}$) and CGP55845 ($5 \mu\text{M}$) present throughout in A and B.



synaptic transmission (Cruikshank *et al.* 2004). As shown in Fig. 11, application of mefloquine did not affect either the amplitude or the frequency of synchronized currents recorded from either wild-type or Cx36 KO mice. The peak of epileptiform currents in wild-type animals was 3971 ± 383 pA during the first 5 min in control conditions vs. 3545 ± 421 pA during the last 5 min in mefloquine ($n = 11$, $P > 0.05$). In Cx36 KOs, the amplitude of the events was 4112 ± 857 pA in control and 4248 ± 745 pA ($n = 5$, $P > 0.05$) in the presence of the drug. The frequency of the epileptiform currents did not change in either case (from 15 ± 1 mHz to 14 ± 2 mHz in wild-type ($n = 11$, $P > 0.05$), and from 17 ± 3 mHz to 15 ± 1 mHz in Cx36 KO mice ($n = 5$, $P > 0.05$)). No changes either were observed on the kinetics of the events as indicated by half-width measurements (from 473 ± 28 ms to 543 ± 32 ms in wild-type ($n = 11$, $P > 0.05$), and from 376 ± 23 ms to 466 ± 28 ms in Cx36 KO mice ($n = 5$, $P > 0.05$)).

Taken together, these experiments exclude connexin36 as the molecular target of carbenoxolone mediating the decrease in GABAergic epileptiform activity, and support the hypothesis that a direct effect on GABA_A receptors is involved.

GABA_A receptors are the target of several drugs and endogenous modulators. In particular, endogenous neurosteroids can affect both the amplitude and the duration of GABA_A receptor-mediated currents (Belelli & Lambert, 2005). Because carbenoxolone (Pubchem CID 636403, Davidson & Baumgarten, 1988) shares some structural similarities with steroids, we next tested the possibility that the observed negative regulation of GABA_A receptor activity following exposure to carbenoxolone shared a

common mechanism with one of the best characterized GABA_A receptor negative modulating neurosteroids, pregnenolone sulfate (Majewska *et al.* 1988; Mienville & Vicini, 1989; Shen *et al.* 2000; Eisenman *et al.* 2003). This hypothesis would predict that pregnenolone sulfate should reduce the amplitude, frequency and half-width of GABAergic epileptiform currents. As shown in Figs 12 and 13, application of pregnenolone sulfate ($100 \mu\text{M}$) on slices from wild-type animals fulfilled these predictions. The amplitude of epileptiform currents was reduced from a value of 2584 ± 330 pA (measured during the initial 5 min in the absence of the drug) to 1372 ± 148 pA (during the last 5 min in the presence of pregnenolone sulfate, $n = 8$, $P < 0.05$). Similarly, the frequency of the events decreased from 22 ± 2 mHz to 14 ± 2 mHz ($n = 8$, $P < 0.05$). Lastly, as expected, the measured half-width of the collected events was reduced by pregnenolone sulfate from 419 ± 42 ms to 204 ± 27 ms ($n = 8$, $P < 0.05$). These results indicate that carbenoxolone and pregnenolone sulfate affect GABAergic epileptiform currents in a similar way, although this could still be achieved by independent sites. If the sites and mechanism(s) underlying carbenoxolone- and pregnenolone sulfate-dependent modulation of epileptiform currents were truly independent, then co-application of the two drugs should summate algebraically. In contrast, if the effect of the two molecules shared sites and/or mechanism, co-application should reveal a sublinear summation. The IC_{50} of pregnenolone sulfate for GABA_A receptors varies with the status of activation of the receptor, but typical values are in the high nanomolar (Zaman *et al.* 1992) to low micromolar range ($4.9 \mu\text{M}$, see Neelands *et al.* 1998; $7.2 \mu\text{M}$, see Park-Chung *et al.* 1999; $3\text{--}10 \mu\text{M}$,

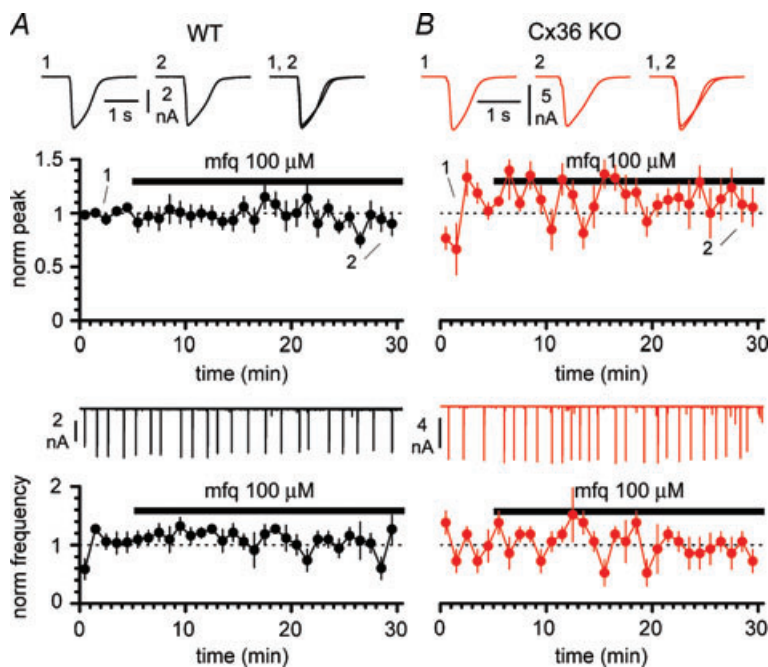


Figure 11. Mefloquine does not affect GABAergic epileptiform events in either WT (black circles and traces) or Cx36 KO animals (red circles and traces) *A*, time course of normalized peak (upper panel) and frequency (lower panel) during application of mefloquine (mfq, black bar) to slices from WT mice. Notice the lack of effect both on the amplitude and on the frequency of the events. Insets in the upper panel are averaged events in control (1), in the presence of mefloquine (2), and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. *B*, as in *A*, but experiments were performed on Cx36 KO animals. Notice that mefloquine does not affect epileptiform activity in this genotype either. Ionotropic glutamatergic synaptic transmission and GABA_B receptors blocked throughout the experiments in both *A* and *B*.

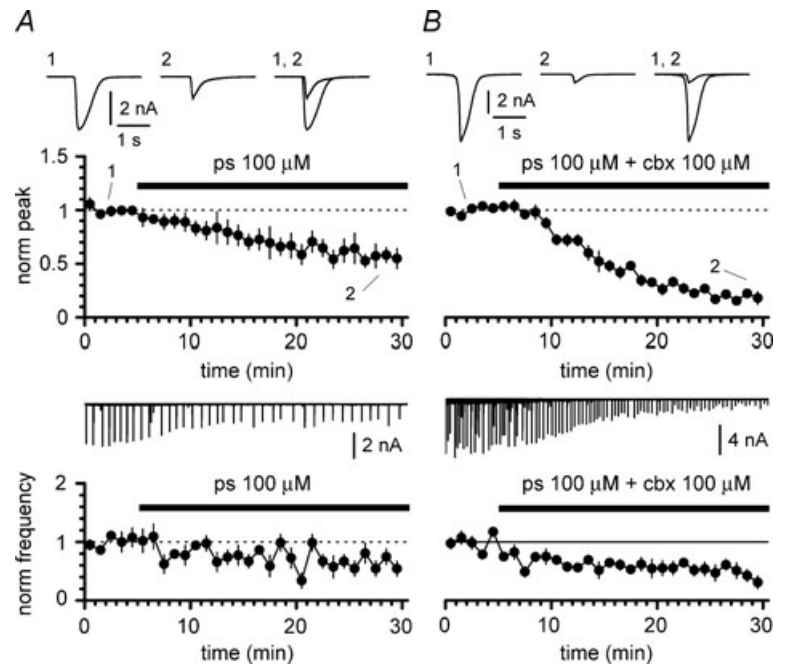


Figure 12. Effects of the application of pregnenolone sulfate on 4-AP-induced epileptiform currents and of its co-application with carbenoxolone in slices from WT animals

A, time course of normalized peak (upper panel) and frequency (lower panel) during application of pregnenolone sulfate (ps, black bar). Notice the reduction in both amplitude and frequency of the events. Insets in the upper panel are averaged events in control (1), in the presence of pregnenolone sulfate (2), and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. *B*, as in *A*, but pregnenolone sulfate and carbenoxolone (ps+cbx) were co-applied. Ionotropic glutamatergic synaptic transmission and GABA_B receptors blocked throughout the experiments both in *A* and *B*.

see Shen *et al.* 2000). Furthermore, the IC₅₀ for pregnenolone sulfate decreases in the presence of elevated GABA concentrations, and inhibition of multiquantal GABAergic responses has been shown to be especially efficient (Eisenman *et al.* 2003). Therefore, 100 μM applied to epileptiform GABAergic currents may be considered a high dose, potentially close to saturation (Zaman *et al.* 1992).

Co-application of carbenoxolone and pregnenolone sulfate (Fig. 13) powerfully decreased the peak amplitude of GABAergic epileptiform events from a control value of 3186 ± 455 pA to 610 ± 85 pA (*n* = 10, *P* < 0.05), and their frequency from 32 ± 4 mHz to 16 ± 4 mHz (*n* = 10,

P < 0.05). In addition, the half-width of the recorded epileptiform currents was also strongly reduced from 494 ± 35 ms to 236 ± 39 ms (*n* = 10, *P* < 0.05).

In order to establish whether the experimental co-application of the two drugs resulted in a linear or non-linear summation of the individual effects, we digitally added (see Methods for details) the data previously obtained with separate applications of carbenoxolone and pregnenolone sulfate (Figs 7–8 and 12–13, respectively). As shown in Fig. 14, the time course of the effect of the experimental co-application of carbenoxolone and pregnenolone sulfate on amplitude and frequency of epileptiform events was very similar to

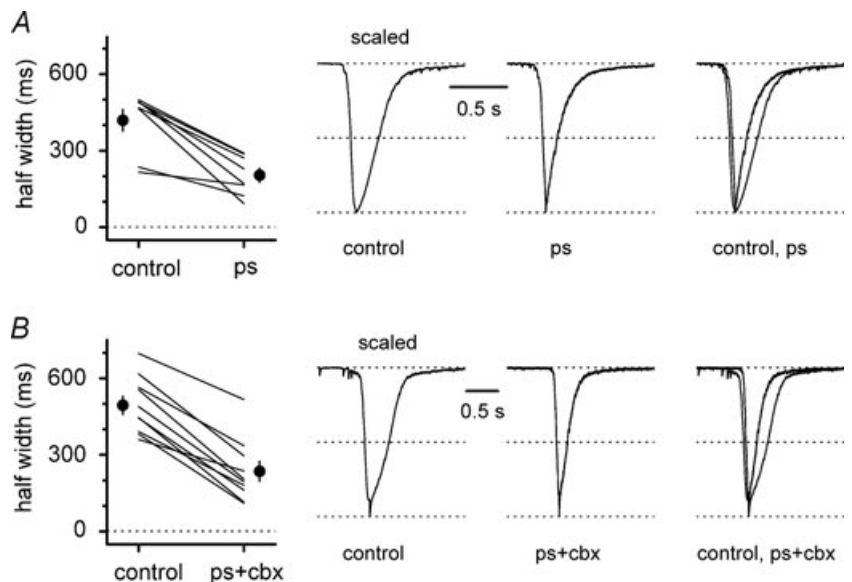


Figure 13. Kinetic changes of 4-AP-induced epileptiform currents following either application of pregnenolone sulfate or its co-application with carbenoxolone in slices from WT animals

A, left, summary graph comparing half-widths in control and in the presence of pregnenolone sulfate (ps) for individual experiments (lines) and for their overall average (black circles). Traces in control, in the presence of pregnenolone sulfate and superimposed are shown in the right panel. *B*, as in *A*, but pregnenolone sulfate and carbenoxolone were co-applied. Ionotropic glutamatergic synaptic transmission and GABA_B receptors were pharmacologically blocked in *A* and *B*.

one predicted by the digital summation of the individual effects. This would suggest that carbenoxolone and pregnenolone sulfate have independent sites of action. To further assess this possibility we calculated the confidence intervals of the difference between the experimentally measured ($n = 10$) and predicted effects (expressed as normalized values in the last 5 min of the co-application of the drugs) for amplitude, frequency and half-width of the epileptiform currents. The confidence intervals of the difference between measured and predicted effects were -0.07 ± 0.08 for the amplitude of the events, 0.02 ± 0.15 for their frequency, and 0.13 ± 0.13 for their half-width. Thus, the pharmacological profile of the combination of pregnenolone sulfate and carbenoxolone indicates that these two compounds are very unlikely to share the same site of action on GABA_A receptors.

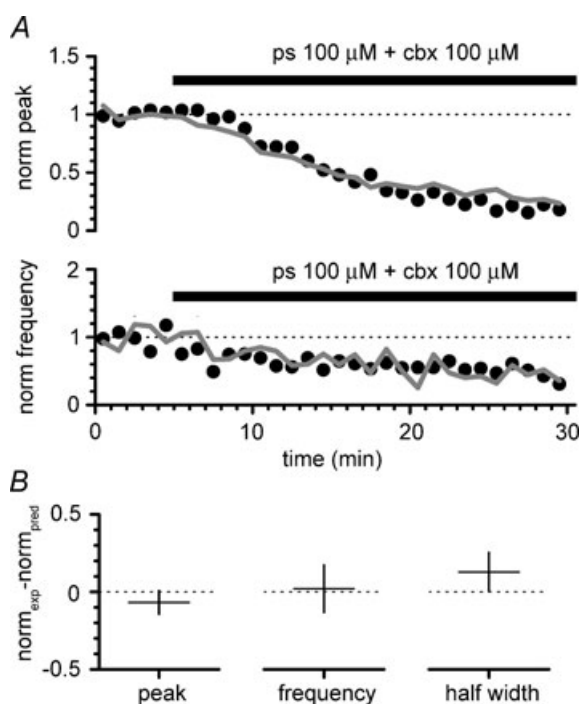


Figure 14. The effects of pregnenolone sulfate and carbenoxolone on 4-AP-induced epileptiform events add linearly

A, time course plot of the experimental effect of co-application of pregnenolone sulfate and carbenoxolone on amplitude and frequency of GABAergic currents (black circles, same data as Fig. 12B) compared to its predicted effect calculated from the linear summation of the effects of their individual applications (thick grey line). Notice the similarity of the effects. B, mean and 95% confidence intervals of the difference of the experimental effect of co-application (from left to right: on peak amplitude, event frequency and half-width) vs. the predicted effect if the two drugs act by purely independent mechanisms. Notice that all the confidence intervals of the difference ($\text{norm}_{\text{exp}} - \text{norm}_{\text{pred}}$) include a range of values indicating a very small difference between observed and predicted effects.

Therefore, we next decided to test the possibility that carbenoxolone acted as an inverse agonist at the allosteric regulatory benzodiazepine/ β -carboline binding site (Sieghart, 1995) by measuring its effect on epileptiform currents recorded in the constant presence of the specific high affinity competitive antagonist flumazenil. Experiments were performed on slices obtained from Cx36 KO mice in order to exclude effects due to blockade of gap junctions between interneurons. As shown in Figs 15 and 16, the constant presence of flumazenil did not prevent the reduction of epileptiform activity induced by carbenoxolone. GABAergic events were reduced in amplitude from 3142 ± 261 pA to 1809 ± 227 pA (first 5 min in flumazenil vs. last 5 min in flumazenil and carbenoxolone, $n = 12$, $P < 0.05$) and their frequency decreased from 26 ± 4 mHz to 14 ± 3 mHz ($n = 12$, $P < 0.05$). Also, the half-width of the epileptiform currents was shortened from 387 ± 41 ms in the presence of flumazenil alone to 292 ± 41 ms in the presence of flumazenil and carbenoxolone (Fig. 16, $n = 12$, $P < 0.05$). We directly ruled out the possibility that the lack of block of carbenoxolone-induced reduction of epileptiform activity was due to a subnanomolar affinity of carbenoxolone (as an inverse agonist) for the flumazenil binding site (affinity of flumazenil is in the low nanomolar range, see Hunkeler *et al.* 1981). If this was the case, carbenoxolone should begin to considerably affect epileptiform activity at nanomolar, or even lower concentrations. Yet, when we tested carbenoxolone at $5 \mu\text{M}$, no effect was observed. As illustrated in Fig. 15, the peak of epileptiform events was 5148 ± 1283 pA in control vs. 5844 ± 1322 pA in the presence of the drug (first 5 min in control vs. last 5 min in carbenoxolone, $n = 5$, $P > 0.05$) and their frequencies were 29 ± 6 mHz and 28 ± 6 mHz, respectively ($n = 5$, $P > 0.05$). Lastly, the half-width of the epileptiform currents did not change and was 348 ± 37 ms in control conditions and 392 ± 49 ms in the presence of $5 \mu\text{M}$ carbenoxolone ($n = 5$, $P < 0.05$).

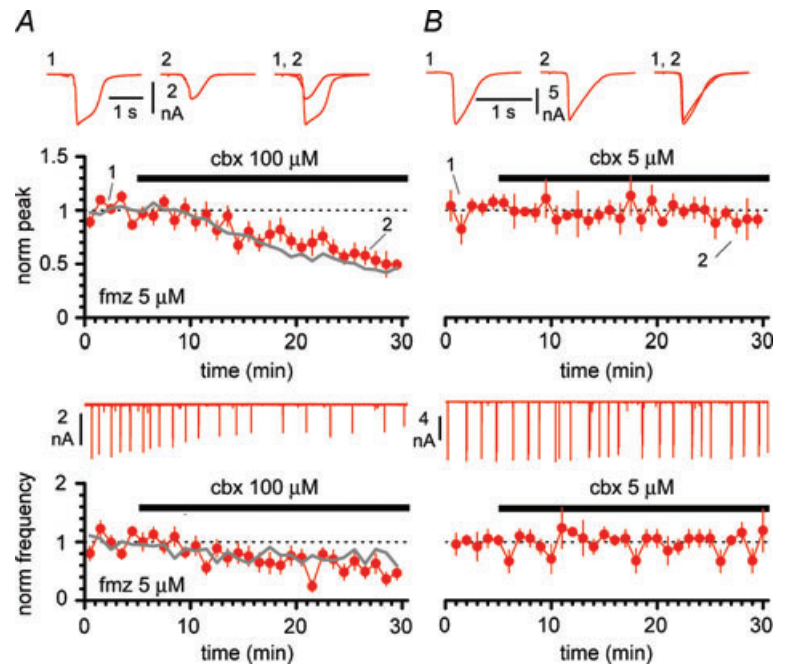
In conclusion, these results indicate that carbenoxolone does not act as an inverse agonist at the benzodiazepine allosteric regulatory site.

Discussion

This study provides direct evidence that connexin36 is not a critical requirement for 4-AP-induced epileptiform synchronization of interneurons. We also demonstrate that the antiepileptic effect of carbenoxolone on 4-AP-induced GABAergic epileptiform currents is not due to the blockade of connexin36-based gap junctions, but, most likely, to a direct action on postsynaptic GABA_A receptors. These results are important for a reinterpretation of previous studies claiming a link between gap junction blockade and antiepileptic activity

Figure 15. Carbenoxolone does not act via the allosteric regulatory site of benzodiazepines

A, time course of normalized peak (upper panel) and frequency (lower panel) during the application of carbenoxolone (cbx, black bar) in the constant presence of the competitive antagonist flumazenil: notice the similarity of these results (red circles) to those obtained in the absence of flumazenil (thick grey lines, same data as in Fig. 7B). Insets in the upper panel are averaged events in flumazenil (1), in the presence of flumazenil and carbenoxolone (2), and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. B, carbenoxolone does not change GABAergic epileptiform currents at low concentrations. Notice the lack of effects on both amplitude and frequency. Insets in the upper panel are averaged events in control (1), in the presence of low concentrations of carbenoxolone (2), and superimposed (1, 2). Inset in the lower panel shows the continuous experimental recording. Experiments shown both in A and B were performed in slices obtained from Cx36 KO mice and in the constant additional presence of NBQX (20 μ M), D-AP5 (50 μ M) and CGP55845 (5 μ M).



of carbenoxolone in the 4-AP and, possibly, other models of epilepsy (see for example Fujiwara-Tsukamoto *et al.* 2010).

Antiepileptic effect of carbenoxolone in the 4-AP model of epilepsy

At least three main different cellular populations may form electrically coupled networks in cortical circuits: glial cells (Kettenmann & Ransom, 1988; Meme *et al.* 2009), principal neurons (MacVicar & Dudek, 1981; Mercer *et al.* 2006; Wang *et al.* 2010, see review by Bennett & Pereda, 2006) and GABAergic interneurons (Galarreta & Hestrin, 1999; Gibson *et al.* 1999). Therefore, the antiepileptic effect of carbenoxolone in wild-type and Cx36 KO mice may simply suggest that other connexins expressed by glial cells or pyramidal neurons (Yamamoto *et al.* 1990; Belluardo *et al.* 2000; Nagy *et al.* 2004;

Weickert *et al.* 2005) are the real molecular targets. However, this possibility seems unlikely for the following reasons. First, the complete elimination of electrical coupling between astroglial cells by knocking out both connexin43 and connexin30 has resulted in animals with reduced spatial buffering and enhanced susceptibility to epileptiform activity *in vitro* (Wallraff *et al.* 2006). Similarly, reduced spatial buffering was observed in mice lacking connexin32 and connexin47, which is expressed by oligodendrocytes (Menichella *et al.* 2006; Odermatt *et al.* 2003). Therefore, blockade of glial gap junctions by carbenoxolone would be predicted to increase, and not to reduce, epileptiform activity. Second, our experiments were performed in the presence of ionotropic glutamate receptor antagonists. These experimental conditions should severely limit the impact of pyramidal cell activity in the network. In fact, epileptiform activity under our experimental conditions appeared fundamentally

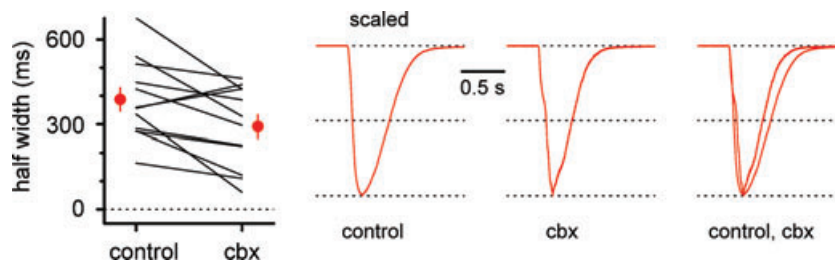


Figure 16. Kinetic changes of 4-AP-induced epileptiform currents following application of carbenoxolone in the constant presence of flumazenil

Left, summary graph comparing half-widths in flumazenil (5 μ M, control) and in the added presence of carbenoxolone (100 μ M, cbx) for individual experiments (lines) and for their overall average (red circles). Scaled traces in flumazenil, in the presence of flumazenil and carbenoxolone, and superimposed are shown in the right panel. Notice that flumazenil does not prevent carbenoxolone-induced kinetic changes.

dependent on depolarizing GABA_A receptor mediated signalling (see Fig. 4, Kantrowitz *et al.* 2005, Zsiros *et al.* 2007, and the review by Avoli *et al.* 2002). Third, epileptiform activity was not changed by a different gap junction blocker, mefloquine, which has been reported to lack non-specific effects on GABAergic synaptic transmission (Cruikshank *et al.* 2004). In conclusion, the most parsimonious explanation for our results is that the antiepileptic effect of carbenoxolone on GABAergic currents in the 4-AP model of epilepsy *in vitro* is due to direct reduction of GABA_A receptor activity, and not to the blockade of connexin36-dependent electrical coupling between interneurons. However, it needs to be acknowledged that our work was performed on slices obtained from mice. Therefore, the possibility that (when other animal species were used) species-related differences may explain some of the inconsistencies with previously published literature cannot be excluded. As mentioned earlier in the Introduction, some forms of epilepsies are thought to be sustained by depolarizing GABAergic signalling. Our conclusion that blockade of electrical coupling in interneurons does not seem to be the mechanism involved in the antiepileptic effect of carbenoxolone on GABAergic synchronous discharges is therefore important and potentially clinically relevant. We would predict that the development of a new therapeutic agent selectively targeting connexin36 would have little clinical impact in those specific forms of epilepsy.

Direct effect of carbenoxolone on GABA_A receptors

Our work indicates that the most likely site of action of carbenoxolone in suppressing GABAergic epileptiform currents is directly located on postsynaptic GABA_A receptors. Indeed, in addition to depressing GABAergic events and IPSCs, carbenoxolone was able to reduce direct responses to exogenous GABA application. The degree of block of all these events was roughly similar, suggesting that modulation of GABA release from presynaptic terminals is not a majorly contributing mechanism. These results are consistent with the observation of Tovar *et al.* (2009) in neuronal cultures, that carbenoxolone reduces the amplitude of autaptic IPSCs. The precise site mediating the effect of carbenoxolone on GABA_A receptors, however, remains to be identified. Indeed, despite the relative similarity of both the chemical structures of carbenoxolone and pregnenolone sulfate and their actions on GABAergic epileptiform activity, their effects appeared to summate algebraically, thus indicating that they are likely to act through independent sites. Neither did pharmacological blockade of the allosteric benzodiazepine regulatory site prevent the effect of carbenoxolone, thus making it unlikely that it acts as an inverse agonist at this site. Further studies in artificial

expression systems will clarify both the detailed molecular mechanism(s) of action of carbenoxolone and their potential dependence on specific GABA_A receptor subunits.

Although GABA_A receptor blockade could suppress excitatory depolarizing GABAergic signalling occurring at the epileptic focus, the lack of spatial selectivity of this approach would promote hyperexcitability and possibly epileptiform activity in healthy areas outside the focus. The advantage of our present study was to have addressed the effects of carbenoxolone in a simplified model of network activity, where the contribution of pyramidal cells was drastically reduced because of the pharmacological blockade of ionotropic glutamate receptors and epileptiform activity was essentially driven by GABA_A receptors. However, we propose that the anti-epileptic effects of carbenoxolone observed *in vivo* and *in vitro* (in more complex scenarios: i.e. with ionotropic glutamatergic transmission intact) are due to its lack of specificity and to its coordinated effects on several targets. Several studies have found that carbenoxolone reduces neuronal membrane excitability (Rekling *et al.* 2000; Rouach *et al.* 2003; Vessey *et al.* 2004; Tovar *et al.* 2009, but see also Schmitz *et al.* 2001 and Jahromi *et al.* 2002 for no effects and increased excitability, respectively). In addition carbenoxolone has been shown to reduce glutamatergic transmission by a presynaptic mechanism (Tovar *et al.* 2009) and to block postsynaptic NMDA receptors directly (Chepkova *et al.* 2008). These latter effects could compensate the potential hyperexcitability due to GABA_A receptor blockade, and maintain the balance between excitation and inhibition below the threshold for epileptiform activity.

In conclusion, the simultaneous effects on cell membrane excitability and glutamatergic and GABAergic synaptic transmission may explain the efficacy of carbenoxolone in preventing pathological activity driven by either glutamatergic or GABAergic inputs.

Epileptiform synchronization in the Cx36 KO mouse

Although several studies have highlighted the importance of connexin36 for electrical coupling between cortical interneurons, their anatomical and functional heterogeneity (Freund & Buzsaki, 1996; Maccaferri & Lacaille, 2003) prevents extending results obtained in a specific subtype to the entire population. Here, we have shown for the first time that interneurons of CA1 hippocampal stratum lacunosum-moleculare require connexin36 for the expression of a detectable electrical coupling. Similarly to what was shown by Deans *et al.* (2001) in various neocortical interneurons, the lack of connexin36 was associated to an increased membrane input resistance. This predicts a larger voltage response

to the same synaptic current, and hence might easily explain the initially unexpected larger amplitude of the epileptiform GABAergic events in Cx36 KO mice.

In addition, connexin36 does not appear critical for the propagation of GABAergic epileptiform currents across interneuronal network of different hippocampal layers, supporting the proposal by Avoli *et al.* (1996) that propagation of this type of epileptiform activity is critically mediated by transients in the external potassium concentration. This consideration also suggests the possibility that blockade of glial gap junctions by carbenoxolone might actually promote the generation and propagation of GABAergic events by impairing potassium spatial buffering. Nevertheless, this effect is overcome by the direct blocking action on GABA receptors.

Alternative mechanisms

The general conclusion that, under our experimental conditions, connexin36 does not play a significant role for the generation and propagation of GABAergic epileptiform events could imply different detailed scenarios. One possibility is simply that, during GABAergic epileptiform events, cells are already synchronized by potassium transients and their membranes undergo a significant decrease of input resistance. Both conditions would strongly attenuate the propagation of voltage signals via gap junctions, which is prominent when the membrane potentials of coupled cells are different and when membrane input resistance is high (Bennett, 1977). However, alternative mechanisms can also be envisaged. For example, a potentially alternative explanation is that bursting of interneurons during GABAergic epileptiform currents triggers some type of metabotropic signalling that negatively modulates the connexin36 gap junction channels. Connexin36 is known to be phosphorylated *in vitro* by several kinases (Urschel *et al.* 2006), which are likely to influence either the functional properties of the gap junction channels or, potentially, their trafficking at the gap junction site *in vivo* (see Flores *et al.* 2010 for connexin35, the fish orthologue of connexin36). Unfortunately, a direct experimental test of our hypothesis is challenging because, in the presence of 4-AP, interneurons receive a powerful barrage of synaptic currents that makes the direct determination of the modulation of coupling in paired cells problematic. Future studies addressing the response of connexin36-based gap junctions to various signalling pathways will be needed to clarify this issue.

Conclusions

Our work shows that connexin36 is not critically involved in shaping 4-AP-induced GABAergic epileptiform events *in vitro* and underscores the importance of a very

cautious interpretation of the antiepileptic effects of carbenoxolone. Suppression of epileptiform activity by this drug should not be taken as unequivocal evidence for the involvement of gap junctions.

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Author contributions

Conception and design of the experiments: G.M. Collection, analysis and interpretation of the data: M.B. and G.M. Drafting the article or revising it critically for important intellectual content: M.B. and G.M. Both authors approved the final version of the manuscript.

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