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Disrupted Cl^- homeostasis contributes to reductions in the inhibitory efficacy of diazepam during hyperexcited states

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Abstract

The K^+ - Cl^- cotransporter KCC2 is the major Cl^- extrusion mechanism in most adult neurons. This process in turn leads to Cl^- influx upon activation of GABA_A receptors and the canonical hyperpolarizing inhibitory postsynaptic potential. Several neurological disorders are treated with drugs that target and enhance GABA_A receptor signaling, including the commonly used benzodiazepine diazepam and the anesthetic propofol. Some of these disorders are also associated with deficits in GABA_A signaling and become less sensitive to therapeutic drugs that target GABA_A receptors. To date, it is unknown if alterations in the neuronal Cl^- gradient affect the efficacies of diazepam and propofol. We therefore used the in vitro model of glutamate-induced hyperexcitability to test if alterations in the Cl^- gradient affect the efficacy of GABA_A modulators. We exclusively utilized the gramicidin perforated-patch clamp configuration to preserve the endogenous Cl^- gradient. Brief exposure to glutamate reduced the inhibitory efficacy of diazepam within five minutes that was caused by the collapse of the Cl^- gradient, and not due to reductions in GABA_A receptor number. Unlike diazepam, propofol retained its efficacy by shunting the membrane conductance despite the glutamate-induced appearance of depolarizing GABA_A -mediated currents. Similarly, pharmacological inhibition of KCC2 by furosemide disrupted Cl^- homeostasis and reduced the efficacy of diazepam but not propofol. Collectively our results suggest pathological hyperexcitable conditions could cause the rapid accumulation of intracellular Cl^- and the appearance of depolarizing GABA_A -mediated currents that would decrease the efficacy of diazepam.

Keywords

KCC2; propofol; glutamate; rat; neurons

Introduction

The type 2 K^+ - Cl^- cotransporter (KCC2) enables most adult neurons to generate an inwardly directed Cl^- gradient that gives rise to hyperpolarizing GABA_A -mediated Cl^- currents and the canonical inhibitory postsynaptic potential (Thompson & Gahwiler, 1989b; Payne, 1997). Long term deficits in KCC2 functional expression occur in a number of neurological disorders (Blaesse *et al.*, 2009), including chronic epilepsy in humans and rats (Huberfeld *et al.*, 2007; Pathak *et al.*, 2007), ischemia (Galeffi *et al.*, 2004; Papp *et al.*, 2008; Jaenisch *et al.*, 2010), and chronic pain (Coull *et al.*, 2003; Lu *et al.*, 2008; Wu *et al.*, 2009). In some cases, these changes in KCC2 were associated with the accumulation of intracellular Cl^- . Additionally, intense GABA_A activity can overwhelm functionally

intact KCC2 resulting in the phenomenon termed Cl^- plasticity (Rivera *et al.*, 2005), which is a temporary switch from hyperpolarizing to depolarizing GABAergic currents due to Cl^- loads (Thompson & Gahwiler, 1989a; Fujiwara-Tsukamoto *et al.*, 2010; Viitanen *et al.*, 2010). Thus, at least two forms of perturbed Cl^- homeostasis can impact GABA_A-mediated currents: brief periods due to increased Cl^- channel activity, and prolonged periods due to impaired KCC2-mediated Cl^- extrusion.

GABA_A signaling is also regulated by receptor trafficking at the membrane surface, and changes in total protein and mRNA levels. Similar to the pattern observed with KCC2, changes in GABA_A receptors are associated with a number of pathophysiological states, including drug-resistant epilepsy in humans (Loup *et al.*, 2000), and after SE in rodents at the level of protein expression and trafficking (Naylor *et al.*, 2005; Goodkin *et al.*, 2008; Terunuma *et al.*, 2008) as well as at the mRNA level (Brooks-Kayal *et al.*, 1998). Further analysis utilizing combinations of biochemical and immunological techniques (Goodkin *et al.*, 2005; Saliba *et al.*, 2012) as well as single-particle tracking methods (Bannai *et al.*, 2009; Niwa *et al.*, 2012; Smith *et al.*, 2012) have revealed that GABA_A receptors are rapidly and dynamically processed within minutes under hyperexcitable conditions in cultured neurons.

GABA_A receptors are one of the most frequently targeted families of proteins for therapeutic intervention. GABA_A receptors are the principle sites of action in the brain for benzodiazepines, which are administered for multiple disorders: diazepam and lorazepam are the first-line therapy to treat status epilepticus (SE) (Goodkin & Kapur, 2009); the benzodiazepine alprazolam for anxiety, and the non-benzodiazepine GABA_A modulator zolpidem as a sleep aid (Rudolph & Knoflach, 2011). They are also the principle mediators of the general anesthetics pentobarbital and propofol (Orser *et al.*, 2002), and mediate some of the actions of neurosteroids such as THDOC (Mody & Maguire, 2011).

Considering the therapeutic pharmacology and dynamic properties of GABA_A receptors, the factors that determine receptor signaling could have clinical relevance. We therefore tested the hypothesis that intracellular Cl^- accumulation reduces the inhibitory efficacy of diazepam in dissociated rat hippocampal neurons. We exclusively utilized the gramicidin perforated-patch technique to preserve the endogenous Cl^- gradient and to demonstrate that the inhibitory efficacy of diazepam fluctuates rapidly on the same intact neurons.

Materials and methods

Animals

Eighteen timed-pregnant female Sprague Dawley rats were used (Charles River International Inc., Wilmington, MA, USA). All animal procedures were performed in accordance with NIH and institutional guidelines, and were approved by the Institutional Animal Care and Use Committee of Tufts University.

Cell culture

All experiments were performed on cultured hippocampal neurons obtained from Sprague Dawley rat E18 embryos. Briefly, neurons were plated on glass coverslips coated with poly-L-lysine in 35 mm dishes (5 coverslips per dish). Neurons were plated at a density of 250,000 per dish and maintained in Neurobasal media containing B27 serum-free supplement (2 %), glucose (0.6 %), GlutaMax supplement (1 %), penicillin (1 %, 50,000 units), and streptomycin (1 %, 50 mg). Neurons were grown in an atmosphere with 5 % CO_2 for 18–21 days prior to experimentation. We plated the neurons directly onto the coverslips in the maintenance media, we did not utilize a serum-containing solution for cell adhesion, nor did we use a glial feeding layer. All cell culture materials were purchased from Invitrogen.

Solutions and drugs

All recordings were performed using the perforated patch-clamp technique. We used gramicidin D (50 µg/mL, Sigma) to establish access resistances between 40–60 MΩ throughout the recording period. All recordings were performed at 34°C. The recording pipettes were filled with saline containing (in mM) 140 KCl and 10 HEPES, pH 7.4 KOH. Bath saline contained (in mM) 140 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 10 HEPES, 11 glucose, pH 7.4 NaOH. Stocks of bicuculline, bumetanide, DNQX, furosemide and propofol were dissolved in DMSO (0.1% final concentration), glutamate in 1M HCl (in saline, balanced with v/v 1M NaOH) and AP5 in 1M NaOH (in saline, balanced with v/v 1M HCl); all of these compounds except propofol were purchased from Tocris Bioscience. Propofol was purchased from Sigma Aldrich. Diazepam solution (5 mg/mL, purchased from Hospira Inc., Lake Forest, IL, USA) was dissolved directly into saline. We used a three-barreled (0.7 mm diameter per barrel) glass perfusion pipette (Warner Instruments) placed just above the target neuron to apply all compounds and control saline. These solutions were applied through the perfusion pipette at a rate of 0.5 mL/min and we used a computer controlled perfusion fast-step device (Warner Instruments LLC, Hamden, CT, USA) to ensure fast and complete exchange of solutions. To ensure that solutions equilibrated faster, we did not have the bath inlet pipe flowing, the only source of solution was the perfusion pipette.

Electrophysiology

We used voltage-ramp protocols to determine the reversal potential of muscimol activated currents (E_{GABA}). We first performed a voltage-ramp in the absence of muscimol to measure the background leak currents, followed 10 s later by a second voltage-ramp in the presence of muscimol. We performed off-line leak subtraction to isolate the muscimol-activated currents, which we plotted against the holding potential to obtain I-V relationships. We then obtained E_{GABA} values from linear regression fits to the leak-subtracted data at voltages near the observable reversal potential. We ramped the holding potential from –100 to –70 mV or –90 to –60 mV (20 mV/s) when muscimol was hyperpolarizing and we ramped the holding potential from –60 to –30 mV (20 mV/s) when muscimol was depolarizing. We used these two different protocols to limit perturbations in Cl[–] homeostasis that can be caused by the abnormally large driving forces on muscimol-activated currents in voltage-clamp mode. Input resistance values were obtained in current-clamp mode by using a series of 1 s current steps (–80 to +80 pA, 20 pA jumps) spaced 2 s apart; subthreshold voltage responses were then plotted against the current steps, and the data points were fit by linear regression analysis to obtain input resistance values. All data were obtained with an Axopatch 200B amplifier and pClamp 10 software (Molecular Devices LLC, Sunnyvale, CA, USA), and analyzed offline using Clampfit. All records were filtered at 2 kHz and digitized at 10 kHz.

Selection criteria of neurons

We chose only pyramidal-shaped neurons. Due to the variability of cultured hippocampal networks we used selection criteria, if neurons did not meet all of these criteria they were discarded. Neurons had to maintain membrane potential values less than –50 mV in I = 0 mode to ensure health; neurons had to reestablish membrane potential values less than –50 mV within 1 min after exposure to glutamate to ensure proper function of the Na⁺/K⁺ ATPase; neurons had to fire at least 10 APs per minute under basal conditions and after washout of furosemide or glutamate to prevent biasing the inhibitory efficacy of diazepam and propofol; and neurons had to exhibit GABAergic hyperpolarizing postsynaptic potentials and muscimol responses under basal conditions. We interleaved particular sets of experiments on the same batches of primary cultured neurons to limit the influence of different dissections: glutamate/diazepam (group 1) with glutamate/propofol (6 dissections),

furosemide/diazepam with furosemide/propofol (4 dissections), glutamate/diazepam (group 2) with glutamate + Cl^- uptake inhibitors/diazepam (3 dissections).

Sequence of electrophysiological protocols

In this section, modulator refers to diazepam or propofol. All of the data are presented as replicates of experiments performed on the same rat cultured hippocampal DIV18-21 day neuron both before and after the treatment with glutamate, furosemide, bumetanide, or carbachol. None of the data was obtained from a neuron exclusively before or after these exposures. The efficacy of the modulators was measured on all cells, but E_{GABA} and input resistance measurements were not made on every cell tested. The neurons were patched and monitored for basal activity in order to determine if they met the selection criteria as mentioned in the preceding section. After this initial period, the following sequence was performed for cells exposed to glutamate: 1) measure the input resistance in current-clamp mode, 2) measure APs for 1 min in $I=0$ mode, 3) apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 4) measure the input resistance in the continued presence of modulator in current-clamp mode, 5) washout modulator for 5 min in $I=0$ mode, 6) measure E_{GABA} in voltage-clamp mode, 7) glutamate exposure in $I=0$ mode, 8) measure E_{GABA} in voltage-clamp mode, 9) measure the input resistance in current-clamp mode, 10) measure APs for 1 min in $I=0$ mode, 11) apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 12) measure the input resistance in the continued presence of modulator in current-clamp mode, 13) washout modulator and allow the neurons to recover hyperpolarizing muscimol responses in $I=0$ mode, 14) measure E_{GABA} in voltage-clamp mode, 15) measure APs for 1 min in $I=0$ mode, 16) apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 17) washout the modulator. The following sequence was performed for cells exposed to furosemide: 1) measure APs for 1 min in $I=0$ mode, 2) apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 3) washout modulator for 5 min in $I=0$ mode, 4) measure E_{GABA} in voltage-clamp mode, 5) furosemide exposure for 4 min in $I=0$ mode, 6) measure E_{GABA} in voltage-clamp mode in the continued presence of furosemide, 7) in the continued presence of furosemide measure APs for 1 min in $I=0$ mode, 8) in the continued presence of furosemide apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 9) in the continued presence of furosemide washout modulator for 2 min, 10) washout furosemide for 3 min, 11) measure E_{GABA} in voltage-clamp mode, 12) measure APs for 1 min in $I=0$ mode, 13) apply modulator for 2 min and measure APs during the last minute in $I=0$ mode, 14) washout the modulator.

Statistical analysis

All experimental data derived from experiments described here were analyzed as previously described (Lee *et al.*, 2011). Statistical analysis was performed using GraphPad Prism 4 software (La Jolla, CA, USA). Paired t-tests (two-tailed) were used throughout except where indicated, and $P < 0.05$ was considered significant. I-V relationships were fit by linear regression analysis using GraphPad. Action potentials were counted using Minianalysis software (Synaptosoft Inc., Decatur, GA, USA). The decay rates of GABAergic PSPs were analyzed in Clampfit using first order standard exponential fits with a Chebyshev search method. All data are reported as the mean $\pm$ SEM.

Results

Glutamate caused a positive shift in E_{GABA} and reduced the inhibitory efficacy of diazepam

Glutamate is a common pathophysiological factor involved in ischemia (Ginsberg, 2008) and seizures (During & Spencer, 1993), that can also alter Cl^- homeostasis and reduce KCC2 surface expression (Kitamura *et al.*, 2008; Lee *et al.*, 2011). We therefore utilized the

glutamate model of hyperexcitability and perturbed Cl^- homeostasis to test the hypothesis that the Cl^- gradient is a significant determinant of the efficacy of diazepam. We began each experiment by demonstrating that test pulses with the selective GABA_A agonist muscimol (5 μM -500 ms, every 30 s) hyperpolarized the membrane potential (E_M), and that neurons exhibited GABA_A ergic hyperpolarizing inhibitory postsynaptic potentials (IPSPs). In every neuron tested, we observed IPSPs that appeared alone and some that immediately followed action potentials (APs) or excitatory postsynaptic potentials (EPSPs) (Fig 1A), which together formed AP-IPSP or EPSP-IPSP sequences that are typical of disynaptic inhibitory microcircuits (Pavlov *et al.*, 2011). These IPSPs that followed APs appeared as after-hyperpolarization components of the APs, but these were actually GABA_A ergic as they were sensitive to diazepam and shifts in E_{GABA} (discussed below). These observations indicated that neurons could cope with both the IPSP- and muscimol-induced Cl^- loads, which is consistent with mature neurons expressing KCC2.

We then performed a specifically ordered sequence of electrophysiological protocols (see Methods). The resting membrane potential of these neurons was -66 ± 1 mV ($n = 10$), indicating that the neurons were healthy. We then applied diazepam (3 μM) to the bath solution for 2 min and measured its ability to inhibit APs during the last minute of exposure. Diazepam reduced the number of APs/min from 40 ± 6 prior to diazepam exposure, to 7 ± 2 APs/min during exposure ($n = 10$, $t_0 = 6.72$, $P < 0.0001$), which corresponded to a percent inhibition of 84 ± 3 % (Fig 1A,E). The percent inhibition, or inhibitory efficacy, was calculated for each neuron as the arithmetic difference between the number of APs/min prior to and during diazepam exposure divided by the number of APs/min prior to diazepam exposure. We then washed out diazepam for 5 min and measured the reversal potential of muscimol-activated currents (for consistency we will refer to this value as E_{GABA}) using voltage-ramp protocols (see Methods). The average basal E_{GABA} value was -83 ± 2 mV ($n = 10$). We then calculated the driving force (DF_{GABA}) on GABA_A -mediated currents ($E_{\text{GABA}} - E_M$), which was -17 ± 2 mV and gave rise to hyperpolarizing muscimol responses under basal conditions.

We determined that three 10 s pulses of glutamate (20 μM) spaced 30 s apart caused a depolarizing muscimol response that was sustained for 5-10 min. This allowed us sufficient time to measure changes in E_{GABA} and APs in the absence and then presence of diazepam while muscimol was still depolarizing (Fig 1B). Glutamate application quickly reversed the polarity of the muscimol responses and converted the GABA_A ergic hyperpolarizing PSPs to depolarizing postsynaptic potentials (DPSPs). The AP-IPSP sequences were also transformed into AP-DPSP waveforms. Glutamate caused an average positive shift in E_{GABA} to -39 ± 4 mV ($n = 10$, $t_0 = 7.75$, $P < 0.0001$) (Fig 1E). We also observed a post-glutamate hyperpolarization to -76 ± 2 mV (Thompson & Prince, 1986), which contributed to DF_{GABA} values of $+37 \pm 5$ mV ($n = 10$). As expected, glutamate exposure significantly increased the number of APs/min from 40 ± 6 to 83 ± 12 ($n = 10$, $t_0 = 2.90$, $P = 0.0175$). The subsequent application of diazepam caused a reduction in APs/min to 36 ± 7 ($t_0 = 4.91$, $P = 0.0008$), corresponding to an inhibitory efficacy of only 57 ± 6 %, which was significantly less than diazepam's efficacy measured prior to glutamate exposure ($n = 10$, $t_0 = 4.63$, $P = 0.0012$) (Fig 1C,E). These data indicated that brief exposure to glutamate reduced the inhibitory efficacy of diazepam by 32 ± 7 % (calculated as the percentage of 1 minus the ratio of the efficacy before and after glutamate).

For several neurons we then washed out diazepam and allowed the neurons to recover hyperpolarizing muscimol responses, which indicated that the initial positive shift in E_{GABA} was temporary and immediately began to dissipate upon termination of the glutamate pulses due to sufficient amounts of KCC2. These neurons recovered E_{GABA} values of -84 ± 2 mV ($n = 5$, $t_4 = 0.52$, $P = 0.6306$ relative to the basal values of these 5 neurons only) and E_M

values of -65 ± 2 mV ($n = 5$, $t_4 = 1.32$, $P = 0.2583$ relative to the basal values of these 5 neurons only), resulting in DF_{GABA} values of -19 ± 4 mV (Fig 1D,E). We then measured the inhibitory efficacy of diazepam a third time, which recovered to near basal values and were significantly greater than values obtained immediately after glutamate exposure (95 ± 3 %, $n = 5$, $t_4 = 2.01$, $P = 0.1144$ relative to the basal values of these 5 neurons only; $t_4 = 3.25$, $P = 0.0313$ relative to the values just after glutamate exposure of these 5 neurons only) (Fig 1D,E). These data indicated that glutamate caused a loss of hyperpolarizing $GABA_A$ -mediated currents, increased excitability and decreased the ability of diazepam to suppress APs.

The most direct way to collapse the Cl^- gradient and examine its impact on diazepam's efficacy is to pharmacologically block KCC2 activity. We therefore used the KCC2 inhibitor furosemide on a separate set of neurons (see Methods). These neurons had resting membrane potential values of -65.7 ± 2.5 mV ($n = 9$). Similar to our experiments above, under basal conditions diazepam reduced the number of APs/min from 64 ± 28 to 13 ± 8 ($n = 9$, $t_8 = 2.46$, $P = 0.0393$), which corresponded to an inhibitory efficacy of 86 ± 5 %. Furosemide (500 μ M) application rapidly collapsed the muscimol response and caused a 7.1 ± 2.6 fold increase of APs/min (64 ± 28 APs/min before, 221 ± 48 APs/min during furosemide; $t_8 = 4.15$, $P = 0.0032$, $n = 9$) (Fig 2A). This indicated that KCC2 and Cl^- dependent inhibition together functioned as an endogenous break on runaway excitability in cultured neuron networks (Jarolimek *et al.*, 1996). Furosemide also temporarily depolarized the membrane potential (-58.2 ± 1.8 mV measured 1 min after furosemide onset, $n = 9$, $t_8 = 5.24$, $P = 0.0008$ compared to basal), so we waited for the membrane potential to return to basal values before proceeding (-66.2 ± 2.7 mV measured 4 min after furosemide onset, $n = 9$, $t_8 = 0.23$, $P = 0.8226$ compared to basal). We then applied diazepam in the continued presence of furosemide, which reduced the number of APs/min from 221 ± 48 to 98 ± 25 ($n = 9$, $t_8 = 4.43$, $P = 0.0022$), corresponding to an inhibitory efficacy of only 60 ± 6 %, which was significantly less than diazepam's efficacy before furosemide exposure ($n = 9$, $t_8 = 4.79$, $P = 0.0014$) (Fig 2B,D). In four of these neurons we also measured E_{GABA} values before and during furosemide exposure, and found that it shifted E_{GABA} values from -82 ± 3 mV to -39 ± 4 mV ($n = 4$, $t_3 = 6.94$, $P = 0.0061$), which corresponded to a DF_{GABA} shift from -18 ± 4 mV to $+26 \pm 5$ mV (Fig 2B,D). Furosemide's effect on diazepam was not due to direct inhibition of $GABA_A$ receptors (Korpi *et al.*, 1995; Wafford *et al.*, 1996), since the slope conductance of the muscimol-activated currents did not significantly change (slope before 10.4 ± 2.1 nS, slope during 8.1 ± 1.3 nS, $n = 9$, $t_8 = 1.72$, $P = 0.1239$; these values include 5 data points obtained from voltage-ramps performed on a separate set of neurons in the absence and presence of furosemide that are discussed below). After washout of diazepam, we then washed out furosemide, which allowed a rapid recovery of hyperpolarizing muscimol responses and suppression of neuronal activity, further indication that furosemide was the cause of the increased excitability (Fig 2C). We further examined three neurons, which reestablished E_{GABA} values of -88 ± 3 mV ($n = 3$, $t_2 = 0.57$, $P = 0.6257$ compared to the basal values of these 3 neurons only) and E_M values of -58 ± 2 mV ($n = 3$, $t_2 = 1.53$, $P = 0.2664$ compared to the basal values of these 3 neurons only), resulting in DF_{GABA} values of -30 ± 4 mV (Fig 2D). A third application of diazepam reduced the APs/min from 31 ± 4 to 5 ± 4 ($n = 3$, $t_2 = 7.21$, $P = 0.0187$). This corresponded to an inhibitory efficacy of 85 ± 10 %, which was similar to diazepam's efficacy prior to furosemide exposure on these three neurons ($t_2 = 1.44$, $P = 0.2863$), and significantly greater than diazepam's efficacy in the presence of furosemide on these neurons ($n = 3$, $t_2 = 7.98$, $P = 0.0153$) (Fig 2D). These data indicated that the effects of furosemide on diazepam's efficacy were not due to direct inhibition of certain types of $GABA_A$ receptors, and that changes in its inhibitory efficacy correlated with shifts in E_{GABA} values.

The concentration of furosemide used here is predicted to be sufficient for inhibition of the type 1 $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter (NKCC1) (Somasekharan *et al.*, 2012). We therefore examined diazepam's inhibitory efficacy in the presence of the NKCC1 inhibitor bumetanide on a separate set of neurons (Payne, 1997; Haas & Forbush, 1998). These neurons had resting membrane potential values of -64 ± 4 mV ($n = 6$), indicating that they were healthy. A 6 min application of bumetanide (10 μM), which is not predicted to significantly inhibit KCC2 function, did not shift E_{GABA} values (-94 ± 4 mV before and -93 ± 4 mV during, $n = 6$, $t_5 = 0.46$, $P = 0.6646$). Under basal conditions on this set of neurons, diazepam reduced the number of APs from 24 ± 5 to 7 ± 2 ($n = 6$, $t_5 = 4.74$, $P = 0.0052$), corresponding to an inhibitory efficacy of 72 ± 1 %. Whereas in the presence of bumetanide, diazepam reduced the number of APs from 16 ± 4 to 5 ± 1 ($n = 6$, $t_5 = 3.73$, $P = 0.0136$), corresponding to an inhibitory efficacy of 72 ± 8 %, which was not significantly different compared to the basal level ($t_5 = 0.02$, $P = 0.9831$). These data indicated that inhibition of NKCC1 did not contribute to the effects of furosemide.

Both glutamate and furosemide increased action potential frequency, so it is plausible that the reduced efficacy of diazepam was due to the increased number of APs, or conversely, that increases in action potential frequency collapse hyperpolarizing muscimol responses. We therefore used brief applications of the muscarinic receptor agonist carbachol (20 μM , 2-6 min) to increase excitability without altering E_{GABA} . We examined the effects of carbachol on a separate set of neurons, which exhibited resting membrane potential values of -62 ± 2 mV ($n = 10$). Under basal conditions, diazepam reduced the APs/min from 33 ± 8 to 6 ± 1 ($n = 10$, $t_9 = 3.72$, $P = 0.0048$), corresponding to an inhibitory efficacy of 80 ± 5 % (Fig 3A,C). After washout of diazepam for 5 min, we then applied carbachol, which did not collapse the amplitudes of the hyperpolarizing muscimol responses in any of the neurons tested (Fig 3B). In a subset of these neurons, we found that carbachol did not significantly shift E_{GABA} values (-85 ± 7 mV before, -77 ± 6 mV during, $n = 6$, $t_5 = 2.07$, $P = 0.0932$) (Fig 3C), which agrees with recent observations in hippocampal slices that brief applications of carbachol did not affect E_{GABA} (Takkala & Woodin, 2013). Carbachol did increase the number of APs/min by 4.5 ± 1.4 fold (from 33 ± 8 to 134 ± 35 , $n = 10$, $t_9 = 3.34$, $P = 0.0086$) (Fig 3C). But in the continued presence of carbachol, diazepam reduced the number of APs/min to 37 ± 11 ($n = 10$, $t_9 = 3.74$, $P = 0.0047$), corresponding to an inhibitory efficacy of 80 ± 5 %, which was not statistically different from the basal efficacy ($n = 10$, $t_9 = 0.04$, $P = 0.9723$) (Fig 3B,C). Overall, these data demonstrated that glutamate and furosemide rapidly collapsed the hyperpolarizing muscimol responses and reduced the inhibitory efficacy of diazepam, neither of which was due to increased firing rates.

Glutamate did not reduce the number of GABA_A receptors or the modulatory efficacy of diazepam

Several studies have demonstrated that glutamate can increase the lateral mobility of GABA_A receptors (Muir *et al.*, 2010; Niwa *et al.*, 2012), a process that could increase the probability of receptor internalization (Smith *et al.*, 2012). We therefore analyzed our voltage-ramp data to determine if glutamate altered the slope conductance of the muscimol-activated currents. The leak subtracted muscimol-activated currents had slope conductance values of 8.5 ± 2.1 nS before glutamate exposure and 7.2 ± 2.1 nS after glutamate exposure ($n = 10$, $t_9 = 1.55$, $P = 0.1556$). This indicated that glutamate did not significantly reduce the number of GABA_A receptors mediating the muscimol-activated currents.

In our experiments, diazepam slowed the decay rates of the GABAergic hyperpolarizing and depolarizing PSPs. We therefore analyzed if diazepam prolonged the decay of DPSPs by the same amount as it did for IPSPs. Our experiments were conducted in the perforated patch configuration, which will overestimate the actual decay rate of the GABAergic postsynaptic potential; however, this does not preclude the measurement of diazepam's prolongation of

the observed decay rates. We averaged the decay rates of 20 IPSPs in the absence and then 20 IPSPs in the presence of diazepam before glutamate exposure, and 20 DPSPs in the absence and then 20 DPSPs in the presence of diazepam after glutamate exposure on the same neurons ($n = 8$). Diazepam application slowed the decay of IPSPs from 90 ± 10 ms to 196 ± 32 ms ($n = 8$ neurons, $t_7 = 4.46$, $P = 0.0029$), corresponding to a 213 ± 16 % increase (Fig 4A,C). After the glutamate exposure, diazepam prolonged the decay of DPSPs from 163 ± 19 ms to 340 ± 39 ms ($n = 8$ neurons, $t_7 = 7.73$, $P = 0.0001$), corresponding to a 211 ± 10 % increase (Fig 4B,C). The percent increase of the decay of GABAergic PSPs in both instances were statistically similar ($t_{14} = 0.13$, $P = 0.9010$, two-tailed unpaired t-test).

The prolongation of the DPSPs by diazepam caused a biphasic waveform: a fast component that was diazepam-insensitive, and a slower component that was diazepam-sensitive (Fig 4B). We therefore examined if the fast component was actually a superimposed EPSP. Indeed, application of the ionotropic glutamate receptor antagonists DNQX ($20 \mu\text{M}$) and AP5 ($50 \mu\text{M}$) abolished the diazepam-insensitive fast component and isolated the true GABAergic DPSP (Fig 4D). We therefore ignored the fast component when fitting the DPSPs in the absence of the glutamatergic antagonists. The fits to the slow component of the diazepam-potentiated DPSP were similar in the absence and presence of the glutamatergic inhibitors, which validates ignoring the fast component (Fig 4E). These data indicated that the glutamate-induced reduction in the inhibitory efficacy of diazepam was not due to a loss of GABA_A receptors or impaired modulation of the GABAergic PSPs.

The glutamate-induced reduction of diazepam's inhibitory efficacy was due to Cl⁻ plasticity

We attempted to limit the amount of Cl⁻ loading during the glutamate pulse to demonstrate that the reduction of diazepam's efficacy was due to perturbed Cl⁻ homeostasis. We conducted these experiments on a separate group of interleaved neurons and used the same exact sequence of protocols as we did for the glutamate experiments above (*see Methods*), although here we did not measure input resistance values after glutamate exposure. We previously demonstrated that GABAergic activity was a source of the glutamate-induced positive shift of E_{GABA} (Deeb *et al.*, 2011; Lee *et al.*, 2011). However, we did not use glutamatergic antagonists and tetrodotoxin (TTX) in the current investigation, which permitted a rapid E_{GABA} shift that was temporary. Examination of the depolarizing phase of the glutamate pulse revealed a high amount of hyperpolarizing GABAergic PSPs (Fig 5A) (Deeb *et al.*, 2011). On this set of neurons, the inhibitory efficacy of diazepam was 81 ± 7 % under basal conditions (41 ± 12 APs/min before to 8 ± 3 APs/min during diazepam, $n = 5$, $t_4 = 3.03$, $P = 0.0389$). We then exposed the neurons to glutamate, which increased the APs/min from 41 ± 12 to 73 ± 16 ($n = 5$, $t_4 = 4.74$, $P = 0.0090$). We measured E_{GABA} values before and immediately after the glutamate exposure, which were shifted from -81 ± 4 mV to -44 ± 5 mV ($n = 5$, $t_4 = 5.96$, $P = 0.0040$), and resulted in depolarizing muscimol responses that were sustained throughout the post-glutamate diazepam exposure as shown above (Fig 1C). We then applied diazepam exactly 2 minutes after the last glutamate pulse. After glutamate exposure diazepam reduced the number of APs/min from 73 ± 16 to 44 ± 12 ($n = 5$, $t_4 = 3.73$, $P = 0.0203$), which corresponded to an inhibitory efficacy of 43 ± 9 % that was significantly less than the basal values ($n = 5$, $t_4 = 5.35$, $P = 0.0059$).

In a parallel set of neurons, we measured the efficacy of diazepam before and after exposure to solutions containing glutamate, the GABA_A receptor antagonist bicuculline ($50 \mu\text{M}$), and the NKCC1 inhibitor bumetanide ($10 \mu\text{M}$), which both contributed Cl⁻ loads in our earlier investigation (Lee *et al.*, 2011). This protocol allowed us to directly measure the impact of blocking the Cl⁻ load on the efficacy of diazepam despite exposure to glutamate. Under basal conditions diazepam reduced the APs/min from 19 ± 4 to 4 ± 1 ($n = 5$, $t_4 = 6.56$, $P = 0.0028$), which corresponded to a basal inhibitory efficacy of 84 ± 5 % (Fig 5C,E). After

washout of diazepam for 5 min and the measurement of the basal E_{GABA} value, we then applied the mixture of glutamate, bicuculline and bumetanide, which clearly blocked GABAergic activity during the glutamate pulse (Fig 5B). The combination of bicuculline and bumetanide limited the glutamate-induced Cl^- load, resulting in an E_{GABA} shift of only 16 ± 4 mV, from -86 ± 5 mV to -70 ± 2 mV ($n = 5$, $t_4 = 4.41$, $P = 0.0116$) (Fig 5E). This indicated that GABA_A receptors and NKCC1 were not the only mediators of the glutamate-induced E_{GABA} shift (Slemmer *et al.*, 2004). Nevertheless, all the neurons recovered hyperpolarizing muscimol responses during the post-glutamate diazepam exposure (Fig 5D). As stated above, we then applied diazepam 2 min after the last glutamate pulse. After exposure to glutamate, bicuculline and bumetanide, diazepam application reduced the number of APs/min from 48 ± 13 to 9 ± 4 ($n = 5$, $t_4 = 4.38$, $P = 0.0119$), corresponding to an inhibitory efficacy of 87 ± 6 %, which was not statistically different from the basal values ($n = 5$, $t_4 = 1.54$, $P = 0.1979$) (Fig 5E). These data demonstrated that the glutamate-induced reduction of diazepam's efficacy was due to perturbed Cl^- homeostasis and not due to other effects of glutamate on neuronal function.

Propofol retained its inhibitory efficacy after glutamate treatment by reducing input resistance

We then examined if glutamate exposure also affected the inhibitory efficacy of the intravenous anesthetic and GABA_A modulator propofol (Hales and Lambert, 1991). These experiments were conducted in parallel with our glutamate-diazepam trials shown in Figure 1 and utilized the same procedures (see Methods). The resting membrane potential of these neurons was -67 ± 2 mV ($n = 15$), indicating that these neurons were healthy. Propofol ($1.5 \mu\text{M}$) reduced the number of APs/min from 47 ± 9 to 1.4 ± 0.6 ($n = 15$, $t_{14} = 5.31$, $P = 0.0001$), corresponding to a basal inhibitory efficacy of 97 ± 1 % (Fig 6A). In contrast to diazepam, the glutamate exposure did not alter the inhibitory efficacy of propofol, it still reduced the number of APs/min from 90 ± 10 to 8 ± 5 ($n = 15$, $t_{14} = 9.34$, $P < 0.0001$), which corresponded to an inhibitory efficacy of 94 ± 3 % that was not significantly different from basal values ($n = 15$, $t_{14} = 1.05$, $P = 0.3131$) (Fig 6B,D). On a subset of these neurons, we also measured E_{GABA} values before and just after glutamate exposure, which were shifted from -82 ± 3 mV to -48 ± 5 mV ($n = 9$, $t_8 = 7.09$, $P = 0.0001$).

We also investigated the effects of furosemide on the inhibitory efficacy of propofol. These experiments were interleaved with the set of furosemide/diazepam experiments shown in Figure 2 and utilized the same protocols (see Methods). The resting membrane potential of these neurons was -64 ± 3 mV ($n = 8$). Similar to the glutamate experiments, propofol reduced the number of APs/min from 28 ± 8 to 1.4 ± 0.4 ($n = 8$, $t_7 = 3.40$, $P = 0.0115$), corresponding to an inhibitory efficacy of 94 ± 2 %. After six minutes, we then applied propofol for 2 min in the continued presence of furosemide. In sharp contrast to diazepam, furosemide did not significantly reduce the inhibitory efficacy of propofol (Fig 6C,D). It reduced the APs/min from 141 ± 20 to 19 ± 6 ($n = 8$, $t_7 = 7.62$, $P = 0.0001$), corresponding to an inhibitory efficacy of 88 ± 3 %, which was not statistically different from the basal values ($n = 8$, $t_7 = 1.47$, $P = 0.1863$). On a subset of these neurons, we waited 4 min after the onset of furosemide application before using voltage-ramp protocols to measure E_{GABA} values, which were shifted from -84 ± 1 mV to -39 ± 2 mV ($n = 5$, $t_4 = 19.63$, $P < 0.0001$). Thus, propofol's inhibitory efficacy was not reduced despite large positive shifts in E_{GABA} values and depolarizing GABA_A-mediated currents.

Propofol caused a noticeable rapid hyperpolarization of the membrane potential under basal conditions (-4.3 ± 0.4 mV, $n = 15$, $t_{14} = 10.28$, $P < 0.0001$), but we did not observe this during diazepam application ($+0.4 \pm 0.2$ mV, $n = 10$, $t_9 = 2.17$, $P = 0.0584$) (Fig 7A). We therefore tested if changes in input resistance could explain how propofol retained its inhibitory efficacy despite the glutamate-induced positive shift in E_{GABA} values. These data

were obtained from subsets of neurons presented in Figure 1 for the diazepam treatment, and Figure 6 for the propofol treatment. Prior to glutamate exposure, diazepam did not significantly affect the input resistance values, from $240 \pm 50 \text{ M}\Omega$ in the absence to $269 \pm 57 \text{ M}\Omega$ in the presence of diazepam ($n = 8$, $t_7 = 1.60$, $P = 0.1533$), which corresponded to an $11 \pm 6 \%$ shift (Fig 7B,D). Diazepam also did not significantly affect the input resistance after exposure to glutamate, from $246 \pm 57 \text{ M}\Omega$ in the absence to $244 \pm 51 \text{ M}\Omega$ in the presence of diazepam ($n = 8$, $t_7 = 0.15$, $P = 0.8874$), which corresponded to a $5 \pm 6 \%$ shift. In contrast, propofol significantly reduced the input resistance both before and after exposure to glutamate (Fig 7C,E). Propofol reduced input resistance from $355 \pm 58 \text{ M}\Omega$ to $292 \pm 42 \text{ M}\Omega$ ($n = 9$, $t_8 = 2.53$, $P = 0.0352$), which corresponded to a $15 \pm 5 \%$ reduction when muscimol was hyperpolarizing. After exposure to glutamate and when muscimol was depolarizing, propofol still reduced the input resistance from $335 \pm 46 \text{ M}\Omega$ to $266 \pm 45 \text{ M}\Omega$ ($n = 9$, $t_8 = 3.47$, $P = 0.0084$), which corresponded to a $21 \pm 6 \%$ reduction. These data demonstrated that the mechanism by which propofol retained its inhibitory efficacy was by reducing input resistance despite the loss of hyperpolarizing GABA_A-mediated currents.

Discussion

This is the first investigation to demonstrate that altered Cl⁻ homeostasis reduces the inhibitory efficacy of diazepam on intact neurons. Furosemide and the common pathophysiological factor glutamate both reduced diazepam's inhibitory efficacy. These effects were not due to reductions in GABA_A receptor number. Importantly, perturbed Cl⁻ homeostasis did not reduce the inhibitory efficacy of propofol. Our analysis revealed that propofol reduced input resistance even when muscimol was depolarizing, whereas diazepam did not significantly reduce input resistance. Our data indicate that the efficacy of diazepam is in part intrinsic to the neuronal Cl⁻ gradient and is not necessarily dependent on glia or the architecture of inhibitory circuits.

Several investigations have highlighted the anticonvulsant effects of furosemide, but the effects are likely due to inhibition of glial NKCC1 and reductions in glial swelling (Hochman, 2012), or altered kidney function (Loscher *et al.*, 2012). Our investigation supports the inhibitory role of KCC2-mediated Cl⁻ extrusion in neurons (Jarolimek *et al.*, 1996; Lewin *et al.*, 2012). Furosemide increased network excitability by abolishing hyperpolarizing GABA_A signaling and not by inhibiting GABA_A receptors. Our data supports previous experiments on cultured neurons that demonstrated furosemide did not affect GABA_A slope conductance but did significantly increase synaptic excitability (Jarolimek *et al.*, 1996). These data suggest that the anticonvulsant effects of furosemide are not caused by inhibition of KCC2. We previously demonstrated that furosemide collapsed hyperpolarizing responses mediated by GABA_A receptors without affecting the membrane potential (Lee *et al.*, 2011), however those experiments were performed in the presence of TTX, AP5 and DNQX. Thus in the current study, furosemide's effects on membrane potential were caused by increased network activity that was driven by intracellular Cl⁻ accumulation. Further support of the inhibitory role of KCC2 was provided by computational modeling of KCC2 function in neurons, which demonstrated that the partial or complete loss of KCC2 function caused a catastrophic failure of GABAergic inhibition (Doyon *et al.*, 2011).

In contrast to the inhibitory role of KCC2 that we observed, under specific experimental conditions KCC2 can drive excitation: increased KCC2-mediated K-Cl extrusion elevates extracellular K⁺, thereby transiently increasing neuronal excitability and synchrony. This effect is caused by a GABAergic Cl⁻ load and is dependent on bicarbonate ion (Viitanen *et al.*, 2010). However we performed our experiments in HEPES buffered saline, which quickly negates the influence of bicarbonate ion on GABA_A-mediated currents and on

KCC2-mediated excitation (Ruusuvuori *et al.*, 2004). Furthermore, we placed a large (0.7 mm) perfusion pipette just above the patched neuron, thereby limiting large changes in the extracellular K^+ concentration due to increased KCC2-mediated K^+ extrusion. Alternatively, KCC2 can mediate net uptake of Cl^- due to high extracellular K^+ or exceedingly low intracellular Cl^- (DeFazio *et al.*, 2000). Our data indicate that, regardless of the source, any Cl^- load should reduce the inhibitory efficacy of diazepam.

After glutamate exposure, diazepam slowed the decay rates of GABAergic DPSPs by the same amount as it modulated the IPSPs under basal conditions, indicating that the synaptic GABA_A receptors retained their sensitivity to diazepam and did not diffuse out of the synapse. In single particle tracking experiments the lateral diffusion of GABA_A receptors increases at the 10 min time-point after a 4 min exposure to glutamate (Muir *et al.*, 2010), and a 30 min treatment with NMDA declusters synaptic receptors (Niwa *et al.*, 2012). Considering that we utilized only three 10 s glutamate pulses spread over a total of 90 s, it is unlikely that enough GABA_A receptors diffused out of the synapse that could account for the reduction in diazepam's inhibitory efficacy. We also did not observe changes in the slope conductance values after glutamate exposure or in the presence of furosemide, which further indicated that there were no detectable reductions in the total number of surface GABA_A receptors in these experimental conditions.

The application of diazepam alone did not hyperpolarize the membrane potential or affect input resistance, indicating that in our experiments diazepam could only affect the membrane conductance in the presence of synaptically released GABA. Therefore, diazepam caused inhibition by two modes: by prolonging the duration of the hyperpolarizing PSP thereby reducing the probability of reaching action potential threshold, and/or by prolonging the duration of the postsynaptic GABA_A-mediated currents thereby increasing the probability of shunting the EPSPs. This interpretation is supported by data obtained in hippocampal slices from seizing and non-seizing rats: depolarizing GABAergic PSPs prevented action potential generation only if the incoming EPSP occurred when the postsynaptic GABA_A receptors were in the conducting state (Pathak *et al.*, 2007). In our experiments, glutamate caused positive shifts in E_{GABA} thereby removing the hyperpolarizing mode of diazepam-induced inhibition and reducing the inhibitory efficacy of diazepam by ~30 %. This indicated that diazepam's primary mode of inhibition was by shunting inhibition, which allowed diazepam to retain ~70 % of its inhibitory efficacy even when GABA was depolarizing. Further reductions in the inhibitory efficacy of diazepam can occur if there is a reduction in the number of GABA_A receptors leading to a decrease in GABA_A conductance (Bannai *et al.*, 2009), or by raising the E_{GABA} value beyond action potential threshold resulting in direct excitatory effects of GABA (Lee *et al.*, 2011). Indeed, high intracellular Cl^- resulted in excitatory GABA and converted the efficacy of the benzodiazepine flunitrazepam and the barbiturate pentobarbital from inhibitory to excitatory on CA1 neurons in hippocampal slices (Staley, 1992). Even though the whole-cell patch clamp technique was used throughout, to the best of our knowledge this is the only study that has examined any correlation between the inhibitory efficacy of GABA_A modulators and intracellular Cl^- concentration. Therefore, our study is the first to demonstrate that positive shifts in E_{GABA} can reduce the inhibitory efficacy of a benzodiazepine without causing it to become excitatory.

Alternatively, our experiments demonstrated that propofol directly shunted the membrane conductance even when GABA_A receptors depolarized the neurons. Benzodiazepines such as flunitrazepam and diazepam potentiate GABA-activated currents but do not directly activate a large portion of the receptors on the membrane (Farrant & Nusser, 2005). Although they do not bind to the same allosteric sites on GABA_A receptors (Bali & Akabas, 2004; Mercado & Czajkowski, 2008), the anesthetic pentobarbital shares an important

property with the anesthetic propofol: high concentrations of the anesthetics can directly activate a large portion of the receptors to generate a low amplitude Cl^- current (Thompson *et al.*, 1996; Wafford *et al.*, 1996). This is a key pharmacological effect that distinguishes the GABAergic anesthetics from the typical benzodiazepines such as diazepam. However, we applied concentrations of propofol that are well below the EC_{50} for direct activation of $\alpha 1\beta 2\gamma 2$ GABA_A receptors (Feng & Macdonald, 2004; Hales *et al.*, 2006). But our data is supported by previous experiments on cultured hippocampal neurons that demonstrated that low concentrations of propofol can activate a tonic conductance (Bai *et al.*, 2001). To date, the efficacy and potency of direct activation by propofol has not been investigated for most GABA_A receptor subtypes.

Glutamate is a known pathophysiological factor in several neurological disorders. GABA_A modulators have been tested as therapies in animal models of ischemia and humans after stroke (Ginsberg, 2008). Interestingly, the enhancement of GABA_A activity loses therapeutic efficacy at later times of treatment (Sydserff *et al.*, 1995), whereas the reduction of GABA_A activity improves outcomes at later times of treatment in a rodent model of ischemia (Clarkson *et al.*, 2010). Excessive glutamate release also has deleterious effects during seizures. Microdialysis measurements in conscious humans revealed that ambient glutamate surges into the micromolar range prior to, during and after a seizure (During & Spencer, 1993), as well as in several rat models of chemical-induced seizures (Lallement *et al.*, 1991; Liu *et al.*, 1997; Ueda *et al.*, 2002). Interestingly, rats undergoing pilocarpine-induced SE develop resistance to diazepam with increasing durations of seizures (Morrisett *et al.*, 1987; Walton & Treiman, 1988; Rice & DeLorenzo, 1999; Jones *et al.*, 2002). In this model, the efficacy of diazepam decreases by an order of magnitude within 10 min of the first stage three seizure, which is similar to the temporal development of diazepam resistance in human patients undergoing SE (Mayer *et al.* 2002; Treiman *et al.* 1998). Although not investigated directly in these particular studies, the release of glutamate correlates with the onset of diazepam-resistance during SE. It is therefore possible that surges of glutamate during the initial stages of SE cause Cl^- loading that underlies the reduction in the therapeutic efficacy of diazepam, but as our data indicates, this would not significantly reduce the efficacy of propofol, which is an often used and reliable treatment for diazepam-resistant SE (Garcia Penas *et al.*, 2007; Shorvon & Ferlisi, 2012). Thus, our data indicates that perturbed Cl^- homeostasis as a hypothetical underlying mechanism of diazepam-resistance during SE should not be ruled out simply because a different GABA_A modulator, propofol, retains its efficacy.

While it is evident in animal models of several human diseases that KCC2 functional expression is altered, it remains unclear how these changes determine the development and maintenance of pathophysiological states. It is also unknown if these deficits in KCC2 have direct impacts on the efficacy of GABA_A and glycine receptor modulators in vivo. However, our investigation has revealed a significant and rapid impact of impaired KCC2 activity and perturbed Cl^- homeostasis on the efficacy of diazepam. Our experiments also suggest that increasing KCC2 activity could maintain or restore the therapeutic effectiveness of GABA_A modulators by limiting intracellular Cl^- accumulation. Further research will undoubtedly shed more light on the impact of Cl^- homeostasis on GABAergic and glycinergic signaling as well as the therapeutic reagents that target these neurotransmitter systems.

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Abbreviations

APs	action potentials
DPSPs	depolarizing postsynaptic potentials
EC₅₀	50 % effective concentration
E_{GABA}	reversal potential of GABA _A -mediated currents
E_M	membrane potential
EPSPs	excitatory postsynaptic potentials
GABA	γ-amino butyric acid
GABA_A	γ-amino butyric acid type A receptor
IPSPs	inhibitory postsynaptic potentials
KCC2	K ⁺ -Cl ⁻ cotransporter type 2
SE	status epilepticus
NMDA	N-methyl-D-aspartate
NKCC1	Na ⁺ /K ⁺ /Cl ⁻ cotransporter type 1
PSPs	postsynaptic potentials
TTX	tetrodotoxin

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Figure 1.

Glutamate decreased the inhibitory efficacy of diazepam. **(A)** Basal activity (left) was strongly suppressed by diazepam (right). **(B)** Glutamate (arrowheads) caused a rapid depolarization of the membrane. Glutamate was applied in 10 s pulses spaced 30 s apart (only two of three pulses are shown), and muscimol test pulses (arrows) were applied in between to indicate shifts in E_{GABA} . Glutamate exposure immediately shifted the muscimol responses from hyperpolarizing to depolarizing and increased the action potential frequency. **(C)** Two minutes after the last glutamate pulse, muscimol responses were still depolarizing (left), and diazepam caused a weak suppression of neuronal activity (right). **(D)** Upon recovery of hyperpolarizing muscimol responses (left), the inhibitory efficacy of diazepam returned to basal values (right). **(E)** Summary graphs depicting the changes in the efficacy of diazepam (left) and E_{GABA} (right) before the glutamate exposure, just after the glutamate exposure, and once the neurons recovered hyperpolarizing muscimol responses. For the inhibitory efficacy plot, sticks connect data points for each cell, and the mean values are represented by horizontal lines. The n numbers are indicated within the E_{GABA} bars. * indicates statistical significance between values before and after glutamate exposure ($n = 10$, $t_9 = 4.63$, $P = 0.0012$); ** indicates statistical significance between values obtained only for the 5 neurons examined just after glutamate exposure and after full recovery ($n = 5$, $t_4 = 3.25$, $P = 0.0313$); *** indicates statistical significance between values before and after glutamate exposure ($n = 10$, $t_9 = 7.75$, $P < 0.0001$).

Figure 2.

Furosemide reduces the inhibitory efficacy of diazepam. **(A)** Furosemide (arrowhead) caused a rapid increase in action potentials and switched the muscimol responses (arrows) from hyperpolarizing to depolarizing. **(B)** Neurons exhibited stable depolarizing muscimol responses in the continued presence of furosemide (6 min) alone (left), and in the presence of diazepam (right). **(C)** Washout of furosemide (arrowhead) demonstrates the rapid recovery of hyperpolarizing muscimol responses and suppression of network activity. **(D)** Summary graphs depicting the changes in the efficacy of diazepam (left) and E_{GABA} (right) before, during and after furosemide exposure. For the inhibitory efficacy plot, sticks connect data points for each cell, and the mean values are represented by horizontal lines. The n numbers are indicated within the E_{GABA} bars. * indicates statistical significance between values before and during furosemide exposure ($n = 9$, $t_8 = 4.79$, $P = 0.0014$); ** indicates statistical significance between values obtained only on the three neurons that were examined during and after furosemide exposure ($n = 3$, $t_2 = 7.98$, $P = 0.0153$); *** indicates statistical significance between values before and during furosemide exposure ($n = 4$, $t_3 = 6.94$, $P = 0.0061$).

Figure 3.

Carbachol did not affect the efficacy of diazepam despite increased excitability. **(A)** Basal activity was suppressed by diazepam. **(B)** Neuronal excitability increased during the exposure to carbachol, which diazepam suppressed by a similar percentage. **(C)** Summary graphs of the percent inhibition by diazepam, the APs/min, and E_{GABA} values before and during carbachol exposure. For the percent inhibition graph, sticks connect the values before and during carbachol exposure for each neuron, while the horizontal line indicates the mean values. The n numbers are indicated within the APs and E_{GABA} bars. NS indicates not statistically significant ($n = 10$, $t_9 = 0.04$, $P = 0.9723$). * indicates statistical significance compared to values obtained before carbachol exposure ($n = 10$, $t_9 = 3.34$, $P = 0.0086$).

Figure 4.

Diazepam potentiated hyperpolarizing and depolarizing GABAergic PSPs. **(A)** GABAergic hyperpolarizing PSPs (black) were potentiated in the presence of diazepam (red). The exponential fits to each trace are superimposed for illustration. **(B)** Glutamate application converted the hyperpolarizing PSPs to depolarizing PSPs. Depolarizing PSPs in the absence (black) and presence of diazepam (red). Note that diazepam caused the appearance of a pronounced slow component of the PSP. The decays of both PSPs were fit with mono-exponential functions, which are superimposed for display (inset). **(C)** Summary graphs of the decay rates of the GABAergic hyperpolarizing (left) and depolarizing (right) PSPs in the absence and presence of diazepam. The *n* values representing the total number of neurons are indicated within the bars (see text). * indicates statistical significance between values in the absence and presence of diazepam before glutamate exposure (*n* = 8 neurons, $t_7 = 4.46$, $P = 0.0029$). ** indicates statistical significance between values in the absence and presence of diazepam after glutamate exposure (*n* = 8 neurons, $t_7 = 7.73$, $P = 0.0001$). **(D)** Application of the glutamatergic antagonists AP5 and DNQX revealed that the fast component of the depolarizing PSP was a diazepam-insensitive EPSP. The exponential fit to the isolated GABAergic DPSP in the presence of AP5/DNQX is superimposed on both traces (inset). **(E)** The black and red curves are the exponential fits from panel **B** and panel **D**, and are superimposed onto the combined EPSP/DPSP. Note that the exponential fits are virtually identical. The amplitudes of the events were normalized to the peaks for comparison in all panels.

Figure 5.

The glutamate-induced Cl^- load caused the reduction in diazepam's inhibitory efficacy. **(A)** Glutamate (10 s, black bar) depolarized the membrane potential (upper trace). Closer inspection of the depolarized phase revealed barrages of IPSPs (lower trace). **(B)** Application of a mixture of glutamate, bicuculline and bumetanide (10 s, black bar) also depolarized the membrane (upper trace). Closer inspection of the depolarized phase indicated that the inhibitors blocked the barrages of IPSPs (lower trace). **(C)** Before exposure to glutamate, bicuculline, bumetanide, the basal neuronal activity (upper trace) was suppressed by diazepam (lower trace). **(D)** The neuronal activity (upper trace) after exposure to glutamate, bicuculline and bumetanide was suppressed by diazepam (lower trace). Note that the muscimol response was hyperpolarizing during the diazepam application. Traces from panels **B-D** were from the same neuron. **(E)** Summary graphs of the inhibitory efficacy of diazepam (left) and E_{GABA} values (right) obtained before and after exposure to glutamate, bumetanide and bicuculline. The n numbers are indicated within the E_{GABA} bars. NS indicates not statistically significant ($n = 5$, $t_4 = 1.54$, $P = 0.1979$). * indicates statistical significance compared to values obtained before glutamate exposure ($n = 5$, $t_4 = 4.41$, $P = 0.0116$).

Figure 6.

The efficacy of propofol was not altered by glutamate or furosemide. **(A)** Basal neuronal activity (left) was strongly inhibited by propofol (right). **(B)** After exposure to glutamate (left), propofol still strongly inhibited activity even when muscimol was depolarizing (right). Data in panels **A** and **B** were from the same neuron. **(C)** After a six minute exposure to furosemide and the development of depolarizing muscimol responses (left), propofol application strongly suppressed neuronal activity (right). **(D)** Summary graphs of the inhibitory efficacy of propofol before and after glutamate exposure (left), and before and during furosemide exposure (right). The n numbers are indicated within each bar.

Figure 7.

Propofol but not diazepam reduced input resistance before and after glutamate exposure. **(A)** Application of diazepam did not affect the membrane potential (top). Application of propofol caused a rapid hyperpolarization of the membrane potential (bottom). For both traces, the arrows indicate the positions of the muscimol responses and the arrowhead indicates the onset of diazepam or propofol. The dashed lines indicate the position of the resting membrane potential to illustrate the effects of diazepam and propofol. **(B)** Membrane potential responses to the indicated current steps in the absence (red) and presence (black) of diazepam before (upper traces) and after exposure to glutamate (lower traces). **(C)** Membrane potential responses to the indicated current steps in the absence (red) and presence (black) of propofol before (upper traces) and after exposure to glutamate (lower traces). The membrane responses are superimposed to illustrate the effects of diazepam and propofol. The APs are cutoff for illustrative purposes. **(D)** Summary graphs of diazepam's effect on input resistance before (left) and after (right) exposure to glutamate. **(E)** Summary graphs of propofol's effect on input resistance before (left) and after (after) exposure to glutamate. The lines connect data points obtained for each cell. NS indicates not statistically significant before glutamate ($n = 8$, $t_7 = 1.60$, $P = 0.1533$) and after glutamate ($n = 8$, $t_7 = 0.15$, $P = 0.8874$). * indicates statistical significance between values in the absence and presence of propofol before glutamate ($n = 9$, $t_8 = 2.53$, $P = 0.0352$). ** indicates statistical significance between values in the absence and presence of propofol after glutamate ($n = 9$, $t_8 = 2.53$, $P = 0.0352$).