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Treating acute seizures with benzodiazepines: does seizure duration matter?

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ABSTRACT – Several clinical trials have shown improved seizure control and outcome by early initiation of treatment with benzodiazepines, before arrival in the emergency department and before intravenous access can be established. Here, evidence is provided and reviewed for rapid treatment of acute seizures in order to avoid the development of benzodiazepine pharmacoresistance and the emergence of self-sustaining status epilepticus. Alterations in the physiology, pharmacology, and postsynaptic level of GABA-A receptors can develop within minutes to an hour and hinder the ability of synaptic inhibition to stop seizures while also impairing the efficacy of GABAergic agents, such as benzodiazepines, to boost impaired inhibition. In addition, heightened excitatory transmission further exacerbates the inhibitory/excitatory balance and makes seizure control even more resistant to treatment. The acute increase in the surface expression of NMDA receptors during prolonged seizures also may cause excitotoxic injury, cell death, and other pathological expressions and rearrangements of receptor subunits that all contribute to long-term sequelae such as cognitive impairment and chronic epilepsy. In conclusion, a short window of opportunity exists when seizures are maximally controlled by first-line benzodiazepine treatment. After that, multiple pathological mechanisms quickly become engaged that make seizures increasingly more difficult to control with high risk for long-term harm.

Key words: GABA-A receptor trafficking, NMDA receptor trafficking, seizure, status epilepticus, epilepsy, hippocampus

Background: clinical perspective

Acute repetitive or prolonged seizures are some of the most common neurological emergencies presenting to the emergency department and can rapidly progress to status epilepticus (SE), with a mortality that approaches 23% (DeLorenzo *et al.*, 1996). Prolonged seizures themselves also may be harmful (Berg *et al.*, 1996; Dube *et al.*, 2010). Many factors contribute to the high morbidity and mortality and include aetiology (especially anoxia) and seizure duration, though it is often difficult to separate the effects of each (Maytal *et al.*, 1989; Lowenstein and Alldredge, 1993; Towne *et al.*, 1994; DeLorenzo *et al.*, 1996; Treiman *et al.*, 1998). However, several studies have shown that seizure duration

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is an independent adverse predictor of outcome (Lowenstein and Alldredge, 1993; Towne *et al*., 1994).
In addition, the longer the duration of a seizure, the more likely it is to continue (Scott *et al*., 1999; Shinnar *et al*., 2008), with an increasing likelihood of a poor outcome. In particular, 80% of seizures with a duration of less than 30 minutes respond to treatment while less than 40% seizures with a duration of greater than two hours respond well (Mayer *et al*., 2002). Also, seizures with a duration of greater than one hour are predictive of poor outcomes in all aetiological categories (Towne *et al*., 1994). After seizing for two hours, much damage may have already been done because no significant worsening of outcome is noted beyond that (Mayer *et al*., 2002).
Several long-term sequelae may result from prolonged seizure activity. These include neuronal death by 30-60 minutes of seizures (Meldrum and Horton, 1973), hippocampal injury (Ben-Ari, 1985; Cavalheiro *et al*., 1991; Fountain and Lothman, 1995; Cavalheiro *et al*., 1996), as well as a liability for chronic epileptic seizures. For example, status epilepticus increases the risk of spontaneous seizures in patients by 2.9 times (Walker, 1998), and seizure duration correlates with the development of spontaneous seizures in animal models (Dube *et al*., 2010). In addition, epilepsy occurs in 88% of cases of refractory SE but only 22% of cases of SE that respond to therapy (Mayer *et al*., 2002). Persistent disabling cognitive dysfunction also occurs in 11% of patients (Claassen *et al*., 2002), and animal studies reveal cognitive dysfunction associated with MRI hippocampal signal abnormalities in adults after prolonged febrile seizures as pups (Dube *et al*., 2009).
Seizures rapidly evolve (over minutes) through several stages as they become increasingly refractory to pharmacotherapy (Walton and Treiman, 1988; Treiman *et al*., 1998). They become self-sustaining by 20 minutes, paralleling the emergence of benzodiazepine resistance. While all seizures at early stages stop within two minutes of benzodiazepine treatment, the response rate rapidly decreases to less than 50% for more prolonged seizures. The results suggest an evolution of seizures that occurs over minutes.
Given the potential harm of prolonged seizure activity and the need for early recognition and treatment, an operational definition of status epilepticus (SE) as seizure activity greater than five minutes has been adopted by many for adult patients (Lowenstein and Alldredge, 1998; Lowenstein, 1999; Lowenstein *et al*., 1999). Activity that persists for greater than five minutes greatly exceeds (by more than two s.d.) the duration of a typical seizure and is unlikely to spontaneously arrest. Early treatment would shorten duration and help avoid adverse effects from prolonged seizure activity.
Some differences may exist for paediatric populations. First, although the majority of seizures that spontaneously abort will do so by five to ten minutes, up to 20% of both afebrile and particularly febrile seizures can spontaneously arrest after much longer durations, often exceeding 30 minutes (Hesdorffer *et al*., 2011). Lower morbidity also may be associated with these prolonged seizures compared to adults (Dunn, 1988; Maytal *et al*., 1989) and, unlike adults, rat pups that experience SE do not develop spontaneous recurrent seizures later (Zhang *et al*., 2004). In addition, SE is not associated with a rise in neuron-specific enolase (NSE) (a marker of neuronal injury) in pups while large elevations are noted in adults, and this correlates with histological evidence of damage (Sankar *et al*., 1997). Developmental differences in GABA-AR (and/or glutamatergic) subunit expression may contribute to these age-deterministic effects (Kapur and Macdonald, 1999; Zhang *et al*., 2004; Brooks-Kayal, 2005).
Despite possible age-related differences, the benefit of treating early (when treatment is most effective), in order to ensure seizure duration is short and avoid adverse outcomes, appears to outweigh potential risks such as respiratory depression, somnolence, and ataxia (Dreifuss *et al*., 1998; Kutlu *et al*., 2003). In fact, respiratory depression occurs more commonly in untreated patients who continue to seize (23%) compared to those who receive benzodiazepines in the field (10%) (Alldredge *et al*., 2001), and is not described as an adverse event in other studies (Knudsen, 1979; Dreifuss *et al*., 1998; McIntyre *et al*., 2005).
Benzodiazepines are the preferred initial therapy by neurologists and neuro-intensivists (Brophy *et al*., 2012; Riviello *et al*., 2013) for many reasons that include: rapid attainment of peak serum concentrations and onset of action/efficacy (within minutes), good CNS penetration, long duration of action (Leppik *et al*., 1983; Working Group on Status Epilepticus, 1993; Treiman *et al*., 1998), safety, and ease of use that includes the choice of multiple formulations and routes of access with high bioavailability, especially for water-soluble agents such as midazolam (Schwagmeier *et al*., 1998; Scott *et al*., 1999; Kutlu *et al*., 2003; Mahmoudian and Zadeh, 2004; McIntyre *et al*., 2005; Scott, 2005). However, clinical and animal studies show a rapid reduction in the potency of benzodiazepines with increasing seizure duration. While more than 60% of patients who present earlier with overt motor SE respond to lorazepam, less than 20% of those who present later with subtle SE achieve control of acute seizures (Treiman *et al*., 1998; Mayer *et al*., 2002). Buccal midazolam has a 100% response rate in children whose seizures are treated within 30 minutes, though only 50% respond beyond that time point (Kutlu *et al*., 2003). Also, the efficacy of rectal diazepam is significantly

attenuated after 15 minutes of seizures (Knudsen, 1979; Scott, 2005).

For several different animal models, the efficacy of benzodiazepines to stop seizures drops by 50% or more by 10-15 minutes (Walton and Treiman, 1988; Jones *et al.*, 2002), with most anticonvulsant drugs including GABAergic agents becoming completely ineffective by 35 minutes (Morrisett *et al.*, 1987). A 10-20 fold decrease in benzodiazepine potency is noted at this time (Kapur and Macdonald, 1997; Mazarati *et al.*, 1998a), and this is associated directly with alterations in the pharmacology of GABA-ARs that effectively render them unresponsive to benzodiazepines (Kapur and Macdonald, 1997).

Because the transit time to the emergency department (ED) often exceeds 15 minutes with a mean seizure duration exceeding 30 minutes upon arrival to the ED in one study (Alldredge *et al.*, 2001) and closer to 1.3 hours in another (Mayer *et al.*, 2002), several clinical trials have explored ways to avoid unnecessary delays and initiate treatment before arrival in the ED. These have included out-of-hospital delivery of benzodiazepines by paramedics or carers instructed on the use of rescue therapy in high-risk patients (Alldredge *et al.*, 1995; Lowenstein and Alldredge, 1998; Scott *et al.*, 1999; Alldredge *et al.*, 2001; Silbergleit *et al.*, 2012). Other trials attempt to reduce time-to-treat further by avoiding delays associated with obtaining intravenous access (McIntyre *et al.*, 2005; Silbergleit *et al.*, 2012).

Minimizing delays due to patient transfer or access improves seizure control and outcome. Out-of-hospital lorazepam in adults shortens seizure duration prior to treatment and terminates seizures in 59.1% before ED arrival compared to spontaneous seizure arrest in 21.1%, and decreases intensive care unit (ICU) admissions by more than 50% (an effect that was independent of aetiology) (Alldredge *et al.*, 2001). In children, pre-hospital treatment with intravenous or rectal diazepam shortens seizure duration to 32 minutes compared to 60 minutes for those initially treated in the ED and also decreases the likelihood of recurrent seizures or intubation (Alldredge *et al.*, 1995). In addition, compared to the intravenous route, the intramuscular route shortened time-to-treat by more than three minutes and stopped seizures in 73.4% *vs.* 63.4% of patients by the time of ED arrival, raising the possibility that shortening the delivery time of benzodiazepines by even a few minutes may improve outcome (Silbergleit *et al.*, 2012).

Here, basic mechanisms are considered for the rapid development of self-sustaining seizures associated with an erosion of GABAergic inhibition and the development of benzodiazepine pharmacoresistance. The argument is supported, that early intervention is the most effective treatment to prevent prolonged seizures and their harmful effects.

Seizure-induced trafficking of GABA-ARs with loss of synaptic inhibition and available sites for benzodiazepine action

Within one hour of lithium-pilocarpine-induced seizures in rats, a reduction of miniature inhibitory postsynaptic current (mIPSC) amplitude by 27% and area-under the curve (AUC) by 16% indicates a loss of synaptic inhibition mediated by postsynaptic GABA-AR in dentate gyrus (DG) granule cells (Naylor *et al.*, 2005) (*figure 1A*). Receptor kinetic models and mean-variance fluctuation analysis estimate that the number of postsynaptic GABA-ARs is decreased by 50% (Naylor *et al.*, 2005; Naylor, 2010), consistent with the correlation between mIPSC amplitude and number of synaptic receptors (Nusser *et al.*, 1997). Kinetic changes also occur that primarily involve an increase of mIPSC decay time (Naylor *et al.*, 2005; Goodkin *et al.*, 2005; Feng *et al.*, 2008) and suggest alterations of GABA-AR

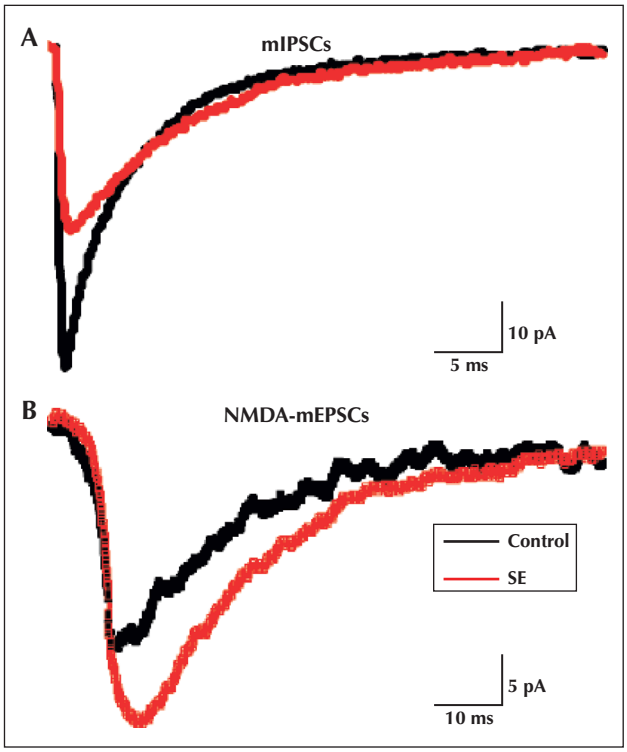

Figure 1. mIPSCs and NMDA-mEPSCs from dentate gyrus granule cells of SE and controls. (**A**) mIPSC mean traces from a typical granule cell from a control (black) and a SE animal (red), demonstrating smaller amplitude and prolonged decay in the latter. The mIPSCs were obtained by visualized whole-cell patch-clamp techniques with CsCl in the recording electrode and V_{clamp} at -70 mV. (**B**) NMDA-mEPSC mean traces from a typical granule cell from a control (black) and a SE animal (red), demonstrating larger amplitude in the latter. The NMDA-mEPSCs were obtained by visualized whole-cell patch-clamp techniques with Cs gluconate in the recording electrode and V_{clamp} at -60 mV.

functional properties, in addition to the decrease in postsynaptic receptor numbers.

Immunocytochemical labelling of the gamma 2 subunit, used to synaptically locate GABA-ARs (Nusser *et al.*, 1998) and associated with the synaptic clustering molecule gephyrin (Essrich *et al.*, 1998), confirms the decrease in the expression of synaptic receptors predicted by physiological measurements (Naylor *et al.*, 2005). The gamma 2 subunit also confers benzodiazepine sensitivity of synaptic GABA-ARs (Saxena and Macdonald, 1996); benzodiazepines bind at the pocket between alpha and gamma 2 subunits (Nusser *et al.*, 1998; Venkatachalan and Czajkowski, 2012), and gamma 2 is essential for benzodiazepine sensitivity (Pritchett *et al.*, 1989; Sigel *et al.*, 1990). Consequently, the loss of synaptic gamma2 subunit-containing GABA-ARs would be expected to decrease the number of available receptors for benzodiazepine binding and action.

The remaining synaptic GABA-ARs have a similar response compared with controls to maximal doses of diazepam, with a prolongation of mIPSC decay time and increase in AUC. But, the augmentation of synaptic inhibition by the benzodiazepine still remains insufficient to counter the initial loss by GABA-AR trafficking away from synapses during prolonged seizure activity (Naylor *et al.*, 2005). A similar study in juvenile rats shows diazepam responsiveness early and 30 minutes after acute seizures, though some blunting of the benzodiazepine response is noted at 30 minutes (Feng *et al.*, 2008). Whether or not direct alterations of GABA-AR function and pharmacology are contributory (Kapur and Macdonald, 1997; Feng *et al.*, 2008), and mIPSC kinetic changes after seizures do suggest GABA-AR functional changes, dramatic losses of synaptic gamma 2 subunit-containing GABA-ARs appear to be a major factor in the development of benzodiazepine insensitivity.

NMDAR trafficking to synapses rapidly increases excitation

Unlike synaptic GABAergic inhibition, glutamatergic excitation increases in DG granule cells by one hour of lithium-pilocarpine-induced seizures. An increase of NMDA-mEPSC amplitude and AUC to 123 and 132%, respectively, of controls (*figure 1B*) is estimated (by mean-variance fluctuation analysis) to involve a 38% increase in the number of postsynaptic NMDARs (Naylor *et al.*, 2013). NR2B subunit-containing NMDAR primarily account for the increase, and immunocytochemical labelling of NMDAR subunits confirms trafficking of receptors to synapses.

An increased contribution of non-NMDARs to mEPSCs also occurs by one hour with an increase of amplitude to 120% of controls and estimated increase of 22% in the number of non-NMDARs at synapses (unpublished results). AMPAR potentiation is noted after hypoxic seizures as well (Rakhade *et al.*, 2008; Rakhade *et al.*, 2012), and seizure-induced switches of AMPAR subunit composition to Ca++ permeant, GluA2-lacking, variants also sustains seizure activity (Rajasekaran *et al.*, 2012). This augmented excitation in the background of degraded synaptic inhibition will further upset the balance between inhibition and excitation and greatly diminish the prospect for seizure control by benzodiazepines and other anticonvulsants.

In addition, NMDARs contribute to the downregulation of GABA-ARs, either as the result of circuit hyperactivity or direct NMDAR activation (Bannai *et al.*, 2009; Muir *et al.*, 2010), *via* calcineurin phosphatase action on gamma 2 subunits (Wang *et al.*, 2003; Muir *et al.*, 2010) and lateral diffusion of GABA-ARs away from synapses and potentially towards endocytotic sites (Wang *et al.*, 2003; Bannai *et al.*, 2009). These changes affect the synaptic, gamma 2 subunit-containing, and benzodiazepine sensitive, GABA-ARs. Pretreatment with NMDAR antagonists prevents the acute loss of synaptic inhibition (Kapur and Lothman, 1990) and the loss of benzodiazepine sensitivity, even after 60 minutes of seizures (Rice and DeLorenzo, 1999). Similarly, seizure-related AMPAR activation also down-regulates synaptic inhibition *via* calcineurin activation (Sanchez *et al.*, 2005).

Activity-dependent and immediate functional losses of synaptic inhibition

Prolonged decay times of mIPSCs suggest functional alterations of postsynaptic GABA-ARs after one hour of seizures (*figure 1A*), but extracellular field recordings in the DG show that loss of evoked paired-pulse inhibition (PPI), another metric of synaptic inhibition, occurs after as little as one minute of perforant path electrical stimulation *in vivo*, and persists for greater than 20 minutes before recovery (Naylor and Wasterlain, 2005) (*figure 2A*). A similar loss of PPI for evoked postsynaptic GABA-AR responses recorded in DG granule cells occurs immediately after five minutes of stimulation *in vitro* (*figure 2B*). Because the loss of inhibition with electrical stimulation *in vivo* occurs well before the occurrence of isolated seizures and certainly before the 30 minutes of perforant path electrical stimulation necessary for self-sustaining seizures (Mazarati *et al.*, 1998b), diminished synaptic inhibition appears to precede the onset of seizures and the trafficking of GABA-AR associated with SE (Naylor *et al.*, 2005).

In addition, GABA-AR trafficking decreases of postsynaptic receptors would be expected to proportion-

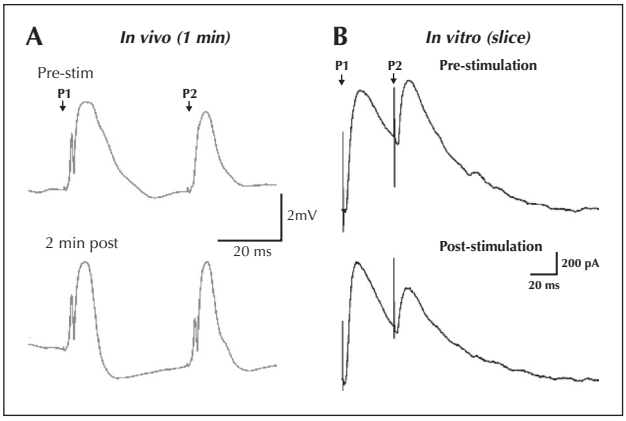

Figure 2. Loss of paired-pulse inhibition (PPI) measured in the DG after brief perforant path stimulation *in vivo* and *in vitro*. (**A)** Loss of PPI measured by field recordings *in vivo* with excitatory population-spikes on both P1 and P2 appearing within minutes after stimulation for one minute of 2-Hz continuous and 20 Hz for 10 sec/min. (**B)** Evoked IPSC responses recorded from the same DG granule cell before (top) and after (bottom) five minutes of repetitive perforant path stimulation (PPS).
Note greater inhibition with P2 before stimulation, but less after. Cs gluconate was in the recording electrode with V_{clamp} at 0 mV.

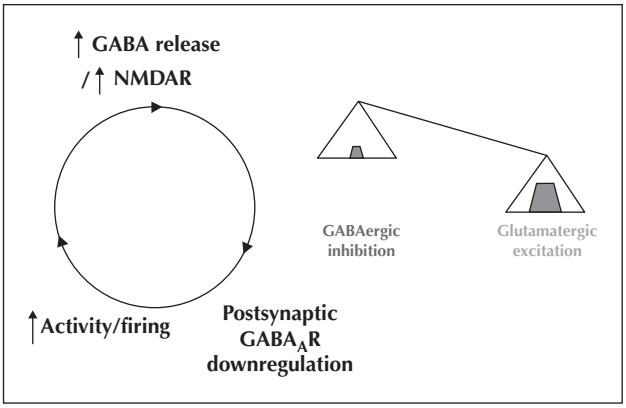

Figure 3. Schematic relating loss of inhibition to self-sustaining seizures and unbalanced glutamatergic excitation. Initial perturbation at any point in the cycle can lead to increase circuit activity with activation of NMDARs that trigger decreases of postsynaptic GABA-AR level and function. The erosion of inhibition further upsets the balance and supports persistent over-activity and seizures.

ately decrease the amplitudes of all evoked responses in proportion to the number of receptors lost and not change the ratio or interaction between pairs of evoked responses. The early loss of PPI implies a functional loss of synaptic inhibition before the absolute loss associated with postsynaptic GABA-AR decreases. This early loss of inhibition after brief convulsant-like stimulation may be sufficient to support spontaneous seizure activity (Kapur and Lothman, 1989), and spontaneous seizures maintain a loss of inhibition (Kapur *et al*., 1989), thereby perpetuating a "vicious cycle" of sustained loss of inhibition and ongoing seizures (*figure 3*).

Seizures increase extracellular GABA

Along with the reduction of mIPSC currents, an increase of tonic GABA-AR currents occurs in DG granule cells after seizing for one hour (*figure 4A*). GABA-ARs are pentomeric structures primarily involving the co-assembly of three subunits: two alphas, two betas, and a gamma or delta subunit (McKernan and Whiting, 1996). The tonic currents are mediated by extrasynaptic GABA-ARs (Nusser *et al*., 1998) that, in DG, contain subunit subtype combinations that include a delta subunit as opposed to the gamma 2 subunit of synaptic receptors.
Unlike gamma 2 subunit-containing GABA-ARs, receptors with delta subunits have much less desensitization (*figure 5C*) and lack benzodiazepine sensitivity (Saxena and Macdonald, 1996; Knoflach *et al*., 1996; Haas and Macdonald, 1999; Brown *et al*., 2002). In addition, extrasynaptic receptors are much more sensitive to low levels of ambient GABA in the extracellular space. As a result of high sensitivity and low desensitization to GABA, extrasynaptic receptors can be reliable indicators of GABA levels. The mean and variance of extrasynaptic tonic currents correlates with GABA levels and can be used to generate a dose-response curve for extracellular GABA (*figure 4B*). Based on this curve, GABA-AR tonic currents predict that GABA levels can exceed 5 μM after one hour of seizing (Naylor *et al*., 2005). In fact, tonic currents after seizures are similar to those after added GABA (*figure 4A*).
Increase in the number of extrasynaptic GABA-ARs also could explain the increased tonic currents, and increased delta subunit expression has been described with SE by some (Terunuma *et al*., 2008), but not others (Goodkin *et al*., 2008). Because the addition of 100 μM GABA occludes the difference in tonic currents between SE and control DG granule cells (Naylor *et al*., 2005), the increase with SE is attributed to an increase in extracellular GABA more than to an increase in the number of extrasynaptic receptors. Regardless of whether some change in extrasynaptic delta subunit surface expression occurs during SE, our results support micromolar increases in extracellular GABA (Naylor *et al*., 2005), and steady increases have been observed more directly with assay measurements of GABA at various time points after the onset of seizures (Walton *et al*., 1990; Wasterlain *et al*., 1993). Also, even brief stimulation may increase tonic currents. DG granule cell tonic currents increase 18.9±4.8 pA ($p<0.05$) after five minutes of hyperstimulation *in vitro* (*figure 4C*) and follow a dose-response

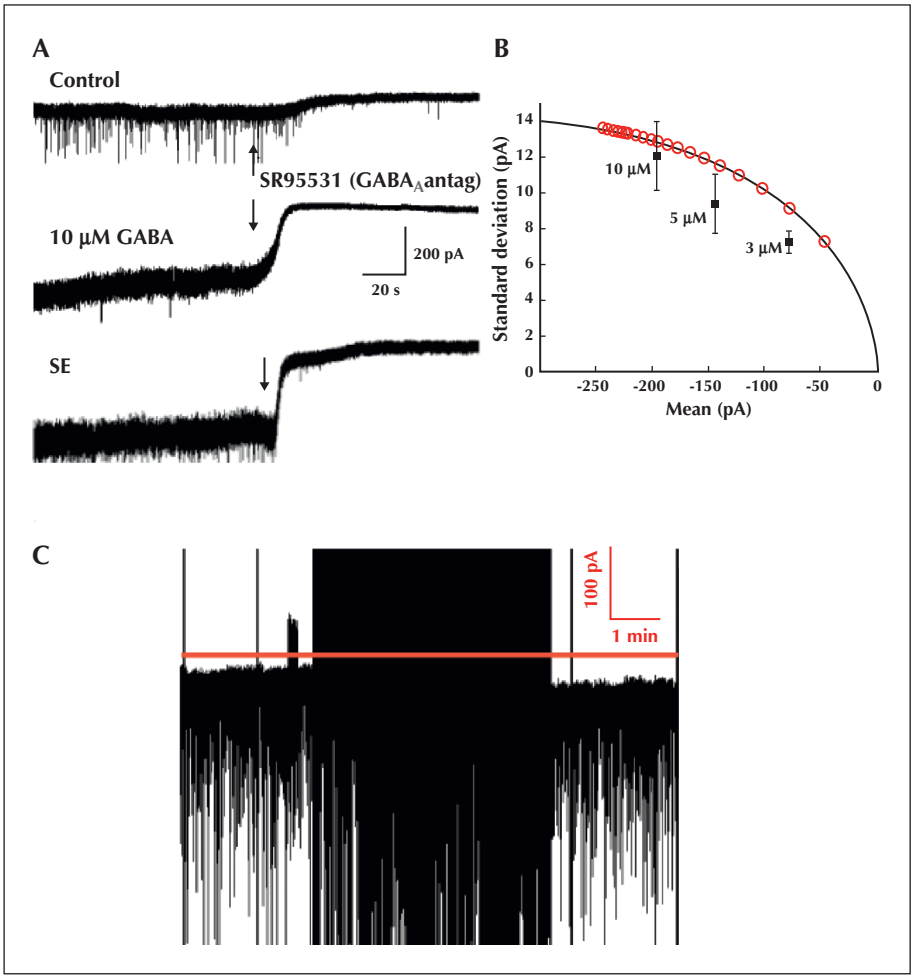

Figure 4. GABA-AR tonic currents recorded from DG granule cells. (**A)** Tonic current recordings from typical cells from control slices, slices bathed in elevated concentrations of extracellular GABA (10 µM), and slices after one hour of SE. Note the increase mean (and baseline standard deviation) of the tonic current with 10 µM GABA and SE compared to controls, as revealed by the greater baseline shift with addition of the GABA-AR antagonist SR95531. The increase in tonic currents after SE is consistent with increases in extracellular GABA. All recordings with GABA uptake inhibition (N0711; 10 µM). CsCl was in the recording electrode with V_{clamp} at -70 mV. (**B)** Dose-response curve for the mean and standard deviation of GABA-AR tonic currents calibrated for known concentrations of added GABA then used to estimate extracellular GABA after SE or perforant path stimulation (see Naylor *et al.* [2005] for methods). Round red circles represent 1-µM increases in extracellular GABA (to a total of 20 µM). Boxes with error bars: ±SEM. (**C)** Small but significant increases in GABA-AR tonic currents occurred after five minutes of perforant path stimulation *in vitro* and are best visualised using the red baseline as a reference.

curve consistent with up to a micromolar elevation in the extracellular GABA (*figure 4B*), qualitatively similar, but less than is observed after one hour of SE (*figure 4A*). Tonic extrasynaptic GABA currents in the DG appear to parallel levels of circuit activity, which occurs in the cerebellum (Brickley *et al.*, 1996).

Sources of GABA may derive from synaptic release (Glykys and Mody, 2007), but also may occur from reversals of GABA transport by glia (Wu *et al.*, 2007). Certainly, an increase in synaptic release with circuit hyperactivity is expected during prolonged seizures, and blockade of GABA uptake after SE causes an increase, not a decrease, of GABA (as indicated by the increase in tonic currents) (Naylor *et al.*, 2005).

GABA exposure (tonic or phasic) desensitizes and functionally alters synaptic GABA-ARs with early loss of paired-pulse inhibition

It is estimated that activity-dependent increases in extracellular GABA can exceed a few micromolar, especially after prolonged seizures. Moreover, adding micromolar amounts of GABA is sufficient to cause significant, rapid, and reversible desensitization of postsynaptic GABA-ARs (*figure 5A*) (Naylor, 2010), especially if uptake mechanisms are blocked and extracellular GABA can readily invade synapses and affect

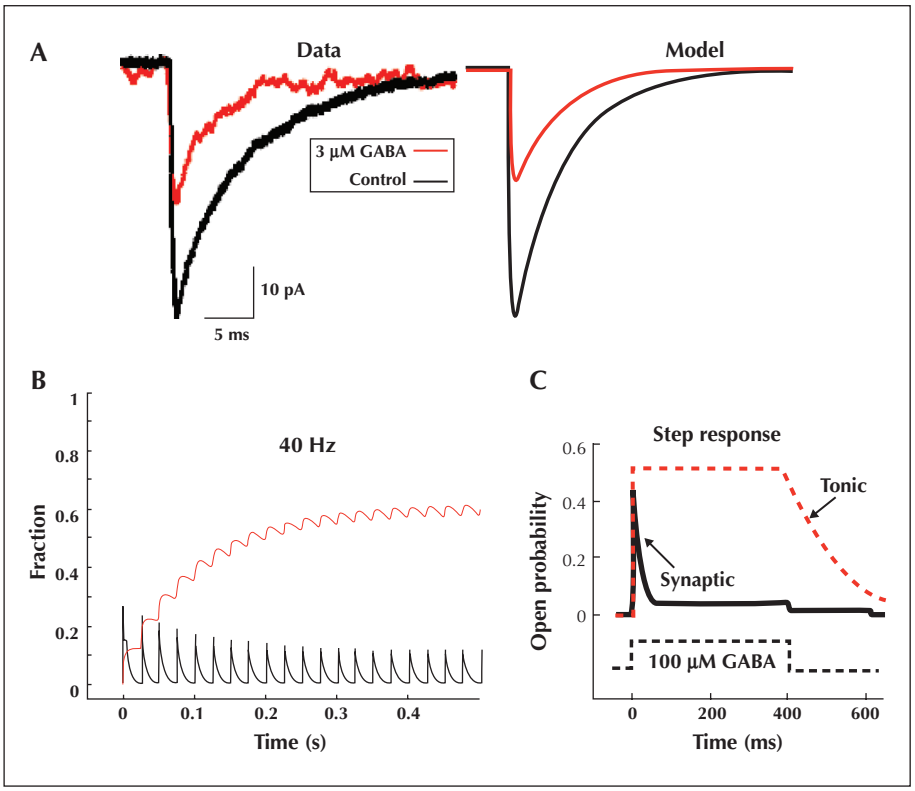

Figure 5. (A) Desensitization of synaptic GABA-ARs with addition of 3-µM GABA with a reduction of mIPSC amplitude of nearly 50%. GABA-AR receptor kinetic model prediction matches experimental results (see Naylor *et al.* [2005] for computational methods). **(B)** Model simulation of postsynaptic GABA-AR responses to high-frequency transmitter release. At 40-Hz stimulation for 200 ms, a greater than 50% loss of postsynaptic GABA-AR mIPSC is predicted (black) with nearly 50% of receptors entering desensitized states (red). (**C**) Predicted GABA-AR responses to step increases of GABA showing rapid and complete desensitization of receptors at synapses compared to extrasynaptic receptors that mediate tonic inhibition.

IPSCs. Exposure of desensitizing synaptic receptors to elevated levels of GABA may explain some reports of paradoxical worsening of seizures after treatment with tiagabine (Walton *et al.*, 1994; Shinnar *et al.*, 2001; Fitzek *et al.*, 2001), as well as instances of benzodiazepine ineffectiveness as treatment for these precipitated seizures (de Borchgrave *et al.*, 2003).

In addition, physiologically based synaptic models indicate that desensitization of postsynaptic GABA-ARs also occurs with brief high-frequency pulsatile exposure that simulates direct synaptic release under overactive conditions (using GABA-AR receptor kinetic parameters defined previously [Naylor *et al.*, 2005]). At 40 Hz, predicted desensitization of postsynaptic GABA-ARs will degrade IPSCs by more than 50% and this occurs after only 100-200 msec (*figure 5B*), raising the possibility that even very brief hyperactivity such as "fast ripples" could have an impact on GABA-AR properties.

Such rapid desensitization of gamma 2 containing receptors to high-frequency pulses of GABA has been observed with *in vitro* expression systems (Bianchi and Macdonald, 2002). In fact, exaggerated pulsatile release of GABA at synapses may desensitize postsynaptic GABA-ARs more potently than extracellular elevations of transmitter, especially when GABA uptake mechanisms are intact. GABA transporters, though numerous, have low turnover rates and regulate low (micromolar) levels of extracellular tonic (Jensen *et al.*, 2003; Hu and Quick, 2008) and spillover (Wei *et al.*, 2003) of GABA better than they can shape and control the high phasic concentrations (millimolar) of transmitter inside the synaptic cleft. Extrasynaptic delta subunit-containing receptors are more likely to be influenced by this type of transporter control, while synaptic gamma 2 subunit-containing GABA-ARs may remain vulnerable to circuit hyperactivity and desensitization. Desensitization from tonic GABA exposure or pulsatile release can contribute not only to an effective loss of available GABA-ARs (with decrease mIPSC amplitude; *figure 5A*), but also can alter postsynaptic receptor kinetic properties, including a loss of evoked PPI (*figure 6C*). Based on computational models of evoked IPSCs as a filtered sum of individual mIPSCs (with synaptic mIPSC representations and GABA-AR receptor kinetic parameters defined previously; Naylor *et al.*,

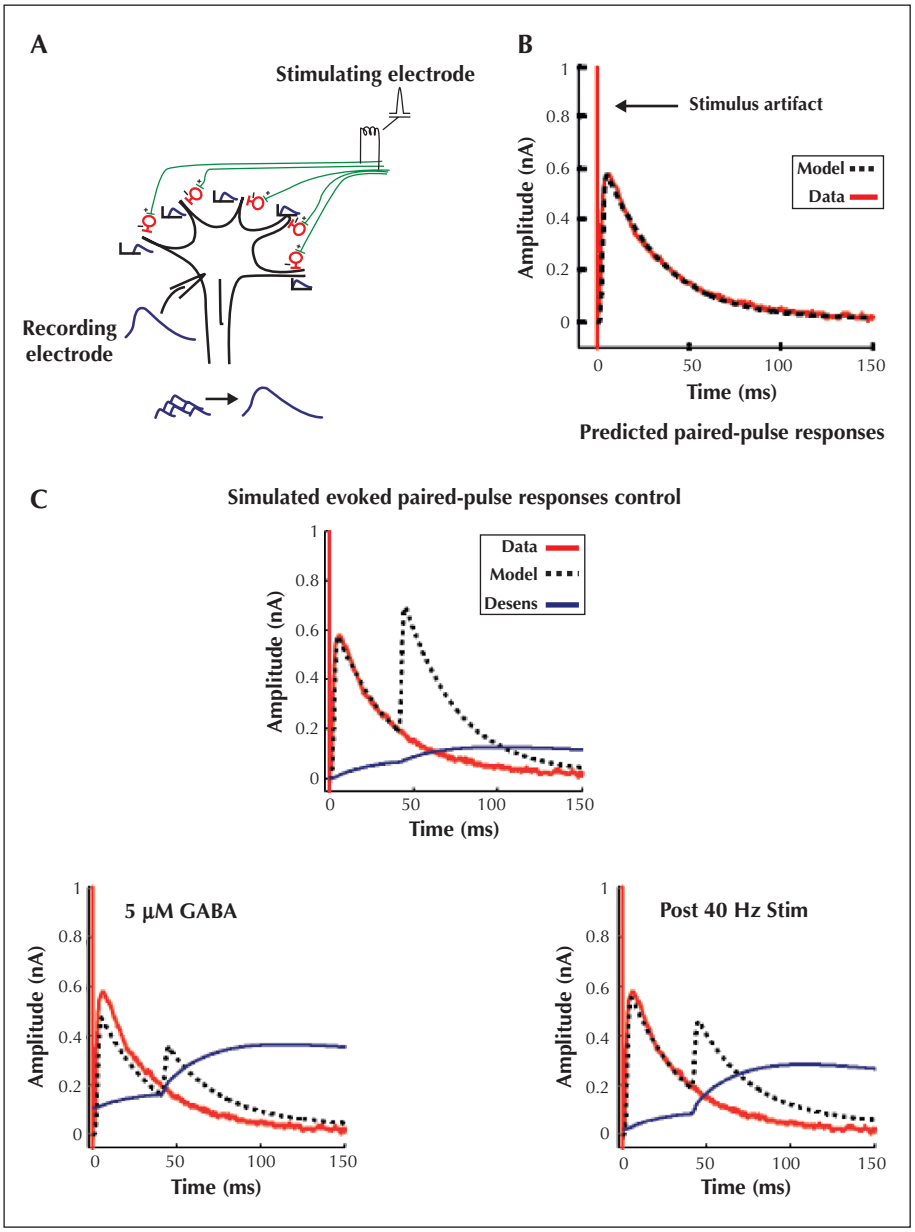

Figure 6. Perforant path evoked IPSCs from DG granule cells with simulated paired-pulse responses. (**A**) Schematic for the representation of evoked IPSCs as a filtered sum of individual synaptic events or mIPSCs. (**B**) A typical evoked IPSC recorded from a DG granule cell (red). Optimized model fit (black dot) of evoked IPSCs (previously described; Naylor *et al*., [2005]) defined parameters for paired-pulse simulations. (**C**) Simulated paired-pulse responses revealed intact PPI in controls with comparable losses of PPI with either 5-µM GABA exposure or after brief 40-Hz synaptic release. Loss of PPI is associated with hyperexcitability. Simulated paired-pulse responses correspond to postsynaptic GABA-AR contributions to loss of inhibition, not presynaptic effects on release probability. Cs gluconate was in the recording electrode with V_{clamp} at 0 mV.

[2005]), simulations of evoked paired-pulse responses that apply parameters for GABA as either 5 µM of tonic or brief 40 Hz pulsatile GABA exposure show similar losses of PPI for either condition (*figure 6C*). A similar loss of PPI is observed experimentally after perforant path stimulation *in vitro* (*figure 2B*). Because desensitization occurs rapidly, it may cause very early losses of inhibition that occur during, or even precede, seizures (*figure 2*).

In addition, gamma 2 subunit-containing GABA-ARs at synapses have properties that include not only receptor desensitization but also sensitivity to benzodiazepines (Saxena and Macdonald, 1996; Haas and Macdonald, 1999). Therefore, desensitization

affects GABA-ARs that overlap with those that are benzodiazepine sensitive and could contribute to the development of benzodiazepine insensitivity even earlier than would occur from receptor trafficking. In addition, although GABA exposure and receptor desensitization is insufficient itself to induce synaptic GABA-AR trafficking (Goodkin *et al*., 2008), it may be sufficient to trigger losses of inhibition with increases of DG circuit activity and stimulation of excitatory synaptic receptors. Activation of NMDARs (Bannai *et al*., 2009) and AMPARs (Sanchez *et al*., 2005) then can down-regulate synaptic GABA-ARs.

Discussion

Both clinical and animal studies note a rapid loss of benzodiazepine potency as seizures persist (Kapur and Macdonald, 1997; Mazarati *et al*., 1998a; Treiman *et al*., 1998; Jones *et al*., 2002), and this parallels the emergence of self-sustaining seizures with pronounced losses of synaptic inhibition. Several factors may be important. First, a decreased number of postsynaptic gamma 2 subunit-containing GABA-ARs occurs by one hour of seizures and is associated with reduced mIPSC amplitudes and selective trafficking of benzodiazepine-sensitive receptors (Naylor *et al*., 2005; Terunuma *et al*., 2008). Conversely, a rapid increase in the delivery of NMDARs containing NR2B subunits to the cell surface also occurs by one hour and contributes to increases in both synaptic and extrasynaptic excitation (Frasca *et al*., 2011; Naylor *et al*., 2013). This combination of effects causes an imbalance between inhibition and excitation that can sustain seizures and make them increasingly more difficult to treat (*figure 3*).

In addition, functional losses of synaptic inhibition in the DG occur within minutes of hyper-active perforant path stimulation and may involve rapid alterations of GABA-AR kinetic properties, such as desensitization (Naylor *et al*., 2005; Naylor and Wasterlain, 2005; Naylor, 2010). Over-exposure to excess synaptic release and/or rising tonic levels of GABA may be sufficient to desensitize the gamma 2 subunit-containing and benzodiazepine-sensitive postsynaptic GABA-ARs.

The activation of NMDAR and Ca++ entry simultaneously may trigger pathways that shift GABA-AR surface expression away from synapses, further aggravating acute losses of synaptic inhibition (Wang *et al*., 2003; Bannai *et al*., 2009), while also contributing to long-term effects that include epilepsy (Rice and DeLorenzo, 1998), cognitive dysfunction (Dube *et al*., 2009) and neuronal injury and cell death (Fujikawa, 1995; Deshpande *et al*., 2008; Frasca *et al*., 2011).

The multiple effects of seizures and circuit hyperactivity on GABA-ARs include some that change receptor functional properties (Kapur and Coulter, 1995; Kapur and Macdonald, 1997). Others, such as lateral diffusion of receptors away from synapses (Bannai *et al*., 2009; Muir *et al*., 2010) and trafficking of receptors to the cell interior (Naylor *et al*., 2005; Goodkin *et al*., 2005; Terunuma *et al*., 2008), primarily change the number of receptors available at synapses and on the cell surface, although selective trafficking of particular subtypes of GABA-ARs could skew physiological and pharmacological properties based on changes in the proportion of receptor subtypes. In addition, the lateral diffusion of GABA-ARs away from synapses and closer to endocytotic zones may herald receptor trafficking.

These effects occur by one hour and some within minutes. For example, activity-dependent lateral diffusion of GABA-ARs decreases mIPSC amplitude by five to ten minutes, with recovery over a similar time course (Bannai *et al*., 2009; Muir *et al*., 2010). Functional alteration of synaptic inhibition associated with a loss of PPI, that initially may result from postsynaptic GABA-AR desensitization among other possibilities, also occurs within minutes and may recover within minutes or can persist longer, depending on the duration of seizure activity (Kapur and Lothman, 1989; Naylor *et al*., 2002; Naylor *et al*., 2005; Naylor and Wasterlain, 2005; Holtkamp *et al*., 2005).

Many routes are available to initiate acute losses of synaptic inhibition. Heightened circuit activity, either by increases of excitation or by decreases of inhibition (Bannai *et al*., 2009), leads to stimulation of NMDARs (Bannai *et al*., 2009; Muir *et al*., 2010) or AMPARs (Sanchez *et al*., 2005; Rakhade *et al*., 2008; Rakhade *et al*., 2012). Calcineurin is a target of such activation with dephosphorylation of GABA-AR subunits and unmasking of AP2 binding sites for GABA-AR endocytosis (Bannai *et al*., 2009). Alternatively, the activity of kinases including isoforms of PKC may be decreased (or increased) with similar results (Terunuma *et al*., 2008). Changes in the phophorlylation state of GABA-AR subunits may not only alter the synaptic and cell surface numbers of receptors, but also can alter receptor physiological and pharmacological properties, including a loss of benzodiazepine sensitivity (Gao and Greenfield, 2005).

In addition, seizure-induced expression and potentiation of excitatory NMDARs (Frasca *et al*., 2011; Naylor *et al*., 2013) and AMPARs (Sanchez *et al*., 2005; Rakhade *et al*., 2008) will not only facilitate excitatory transmission and circuit hyperactivity, but also will further engage the same kinase and phosphatase transduction pathways that are responsible for GABA-AR down-regulation in the first place. The interaction

between NMDAR activation and GABA-AR regulation may explain why NMDA blockade prevents loss of benzodiazepine sensitivity as seizures progress (Kapur and Lothman, 1990; Rice and DeLorenzo, 1999).

Based on this scheme, a perturbation of inhibition or excitation could trigger circuit over-activity with movement of GABA-ARs away from synapses, loss of synaptic inhibition, and greater activity and stimulation of NMDARs (which is also increased by seizures). A "vicious cycle" of self-sustaining seizure activity could be established that preferentially drives the removal of postsynaptic gamma 2 subunit-containing GABA-ARs that are benzodiazepine sensitive.

Many anticonvulsants, including non-GABAergic drugs such as phenytoin, lose potency with prolonged seizures (Morrisett *et al*., 1987; Treiman *et al*., 1998; Jones *et al*., 2002), but benzodiazepines appear to be particularly affected (Walton and Treiman, 1988; Kapur and Macdonald, 1997). Effects on Cl- gradients that diminish GABA-AR-mediated hyperpolarisation occur during SE (Kapur and Coulter, 1995; Rivera *et al*., 2004; Lee *et al*., 2010) and certainly should diminish and possibly reverse the efficacy of benzodiazepines (Staley, 1992). However, such an effect should generalise to all drugs that act on GABA-ARs and to all GABA-ARs, including those at both synaptic and extrasynaptic sites.

However, even though the efficacy of GABAergic drugs as a class may be diminished with seizure progression, this is not uniform and differential effects have been noted between benzodiazepines, barbiturates, and propofol (Kapur and Macdonald, 1997; Treiman *et al*., 1998; Mayer *et al*., 2002; Rossetti *et al*., 2002; Shorvon, 2011). Prolonged seizures respond less well to benzodiazepines than barbiturates and propofol (Mayer *et al*., 2002; Rossetti *et al*., 2002), and GABA-ARs can become completely insensitive to benzodiazepines (Kapur and Coulter, 1995) while barbiturate sensitivity is preserved (Kapur and Macdonald, 1997). While treatment failure approaches 45% for midazolam in refractory SE, failure is indicated as 13 and 25% for barbiturates and propofol, respectively (Rossetti *et al*., 2002). A significant proportion of refractory SE responds to barbiturates after benzodiazepines have failed (Mayer *et al*., 2002).

A potential mechanism for differential loss of potency between benzodiazepines and barbiturates may relate to the preferred GABA-AR subunit binding sites for these agents and selective trafficking of GABA-ARs of particular subtypes during seizures. In particular, GABA-ARs with a gamma 2 subunit are synaptic (Nusser *et al*., 1998) and necessary for benzodiazepine sensitivity (Pritchett *et al*., 1989; Saxena and Macdonald, 1996). Benzodiazepines bind at the pocket between alpha and gamma subunits (Nusser *et al*., 1998; Venkatachalan and Czajkowski, 2012), while barbiturates and propofol bind the beta subunit that is ubiquitous for all GABA-ARs (Amin and Weiss, 1993; Serafini *et al*., 2000). Therefore, the movement of gamma 2 subunit-containing GABA-ARs away from synapses by lateral diffusion (Bannai *et al*., 2009; Muir *et al*., 2010) and/or receptor trafficking (Naylor *et al*., 2005; Terunuma *et al*., 2008) would preferentially affect the receptors with the greatest benzodiazepine sensitivity. Desensitization of the susceptible gamma 2-containing receptors (Haas and Macdonald, 1999; Bianchi and Macdonald, 2002) also may selectively restrict the availability of receptors with benzodiazepine sensitivity.

Pharmacoresistance may evolve from a combination of effects, both general and specific. The establishment, late into seizures, of self-sustaining hyperactive circuits, now characterised by alterations of both GABAergic and glutamatergic synapses, may be resistant to any intervention. However, there may be more specific effects of seizures, especially early, which focus on particular subtypes of GABA-ARs.

The subunit combinations of GABA-ARs evolve through brain development and may impact seizure characteristics, pharmacosensitivity, and long-term effects. In particular, alpha 1 and gamma 2 subunits, which combine to make up to 55% of GABA-ARs in mature synapses (Benke *et al*., 1994; McKernan and Whiting, 1996; Jacob *et al*., 2008), are at a low level at birth and increase two to three fold through adulthood (Brooks-Kayal *et al*., 1998; Brooks-Kayal, 2005). Receptors that contain this combination of alpha 1 and gamma 2 subunits are among the most benzodiazepine sensitive, with a seven fold increase in GABA efficacy (Pritchett *et al*., 1989), which explains the lack of benzodiazepine sensitivity in newborn rats (Kapur and Macdonald, 1999). In addition, the alpha 1 subunit is protective of seizures (Poulter *et al*., 1999; McIntyre *et al*., 2005; Raol *et al*., 2006), and mutations of alpha 1 and gamma 2 are associated with familial epilepsy (Bouthour *et al*., 2012). These developmental effects on GABA-AR subtypes may play a role in the longer duration of seizures in children compared to adults (Hesdorffer *et al*., 2011) as well as the high incidence of convulsive SE in children (Walker, 1998; Scott *et al*., 1999). Presumably, a lower expression level of GABA-ARs with combinations of alpha 1 and gamma 2 subunits could alter seizure responsiveness to particular pharmacological agents in an age-dependent manner as well.

Similarly, atypical GABA-AR subtypes with alpha 4 gamma 2 subunit combinations, that occur with epileptogenesis (Peng *et al*., 2004; Joshi and Kapur, 2013) and are benzodiazepine insensitive (Knoflach *et al*., 1996; Wafford *et al*., 1996; Brown *et al*., 2002), potentially could alter benzodiazepine responses in chronic epileptic patients, compared to new presentations.

When synaptic inhibition is lost and seizures become self-sustaining and benzodiazepine resistant, alternate therapies that might help restore the balance of inhibition and excitation include antagonists of NMDARs. NMDA blockade with ketamine or MK-801 is successful in several animal models of SE long after the development of benzodiazepine pharmacoresistance (Fariello *et al*., 1989; Walton and Treiman, 1991; Mazarati and Wasterlain, 1999; Borris *et al*., 2000).

Interestingly, NMDA antagonists may not be effective early or may even worsen seizures (Fariello *et al*., 1989; Bertram and Lothman, 1990), but may provide 100% control at 60 minutes (Borris *et al*., 2000). Perhaps inhibition from interneurons remains relatively intact early during seizures and NMDAR blockade not only decreases excitation of pyramidal and granule cells, but also decreases excitation of inhibitory interneurons, with disinhibition of pyramidal and granule cells. However, later after synaptic inhibition from interneurons has failed, the primary effect of NMDAR antagonism would be on excitatory cells. Combinations of NMDAR blockers and benzodiazepines may be much more effective than either agent alone (Walton and Treiman, 1991).

Clinically, success rates for treatment of SE by ketamine have been reported as high as 60-70% in epileptics (Rosati *et al*., 2012; Synowiec *et al*., 2013), but may be lower for refractory SE in patients with other aetiologies (Gaspard *et al*., 2013). An added benefit of treatment with NMDA blockers such as ketamine may not only be immediate seizure control, but also the prevention of long-term sequelae such as chronic epilepsy (Rice and DeLorenzo, 1998) and other adverse effects of excitotoxicity (Fujikawa, 1995; Deshpande *et al*., 2008; Frasca *et al*., 2011).

In conclusion, seizures rapidly become self-sustaining and pharmacoresistant secondary to multiple mechanisms that include: alterations of GABA-AR physiology and pharmacology, losses of synaptic GABA-ARs that mediate benzodiazepine action, and increases in the surface expression of excitatory NMDARs that make the task of restoring the balance between inhibition and excitation even more daunting. Very early treatment with a safe, fast, and effective drug, such as a benzodiazepine, before this intractable and deleterious sequence of events has opportunity to take hold, appears to be the best strategy. □

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