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Development of pharmacoresistance to benzodiazepines but not cannabinoids in the hippocampal neuronal culture model of status epilepticus

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Abstract

Status epilepticus (SE) is a life-threatening neurological disorder associated with a significant morbidity and mortality. Benzodiazepines are the initial drugs of choice for the treatment of SE. Despite aggressive treatment, over 40% of SE cases are refractory to the initial treatment with two or more medications. It would be a major advance in the clinical management of SE to identify novel anticonvulsant agents that do not lose their ability to treat SE with increasing seizure duration. Cannabinoids have recently been demonstrated to regulate seizure activity in brain. However, it remains to be seen whether they develop pharmacoresistance upon prolonged SE. In this study we used low-Mg²⁺ to induce SE in hippocampal neuronal cultures and in agreement with animal models and human SE confirm the development of resistance to benzodiazepine with increasing durations of SE. Thus, lorazepam (1 μ M) was effective in blocking low-Mg²⁺ induced high frequency spiking for up to 30-mins into SE. However, by 1-hr and 2-hr of SE onset it was only 10–15% effective in suppressing SE. In contrast, the cannabinoid type-1 (CB1) receptor agonist, WIN 55,212-2 (1 μ M) in a CB1 receptor dependent manner completely abolished SE at all the time points tested even out to 2 hr after SE onset, a condition where resistance developed to lorazepam. Thus, the use of cannabinoids in the treatment of SE may offer a unique approach to controlling SE without the development of pharmacoresistance observed with conventional treatments.

Keywords

Prolonged status epilepticus; benzodiazepines; cannabinoids; low-Mg²⁺ model of SE; neuronal excitability; patch clamp; hippocampal neuronal cultures

Introduction

Status epilepticus (SE) is a life-threatening neurological disorder associated with a significant morbidity and mortality (DeLorenzo, 2006, DeLorenzo, et al., 1995, Hauser and Hesdorffer, 1990). SE is defined as seizure events that last greater than 30-mins or intermittent seizures without regaining consciousness lasting for 30 minutes or longer are also classified as SE (DeLorenzo, et al., 1995, Hauser and Hesdorffer, 1990). SE accounts for as many as 55,000

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deaths per year in the United States alone (DeLorenzo, et al., 1995) and has been shown to cause significant neuronal damage especially in the limbic system in both animals and man (Drislane, 2000, Fountain and Lothman, 1995). Rapid treatment is important in controlling SE and preventing mortality (Bleck, 2005, Treiman, et al., 1998). Benzodiazepines such as diazepam (DZP), lorazepam (LZP) or midazolam are the initial drugs of choice for the treatment of SE (Chen and Wasterlain, 2006). Despite their effectiveness, a significant percent of patients with generalized convulsive SE become refractory to treatment with benzodiazepines (Bleck, 2005, Treiman, et al., 1998). Development of resistance to treatment with benzodiazepines with increasing seizure duration has also been observed in experimental models of SE (Jones, et al., 2002, Rice and DeLorenzo, 1999, Walton and Treiman, 1988). Pharmacoresistance to other antiepileptic drugs, such as phenobarbital and phenytoin used to treat SE (Chen and Wasterlain, 2006) has also been reported in animals models and humans SE (Jones, et al., 2002, Lowenstein and Alldredge, 1993, Lowenstein and Alldredge, 1998, Morrisett, et al., 1987, Walton and Treiman, 1988). Thus, loss of drug efficacy and development of pharmacoresistant seizures represents a major obstacle in the treatment of SE. It would be a major advance in the clinical management of SE to identify novel anticonvulsant agents that do not lose their ability to treat SE with increasing seizure duration.

The endocannabinoid system has been demonstrated to play an important role in regulating seizure activity in brain (Deshpande, et al., 2007, Lutz, 2004, Smith, 2005, Wallace, et al., 2003) and has been identified as a possible target for controlling SE (Blair, et al., 2006, Wallace, et al., 2003). This unique inhibitory neuroregulatory system is comprised of the cannabinoid receptors (CB1 and CB2), endogenous cannabinoid agonists (endocannabinoids: anandamide and 2-AG) and the protein machinery for their synthesis, transport and degradation (Mackie, 2006). The major psychotropic cannabinoid ⁹ THC present in marijuana (*cannabis sativa*) possess anticonvulsant properties (Karler, et al., 1974) and synthetic cannabinoids such as WIN 55,212-2 [+WIN] have been demonstrated to produce an anticonvulsant effect in a CB1 receptor dependent manner (Blair, et al., 2006, Wallace, et al., 2001). Several lines of evidence have indicated that the endocannabinoid system controls seizure threshold and seizure frequency and duration (Deshpande, et al., 2007, Wallace, et al., 2003, Wallace, et al., 2002) and prevents the development of SE in epileptic animals (Wallace, et al., 2003). The action of these agents is different than conventional anticonvulsant agents, since cannabinoids activate presynaptic CB1 receptors causing decreased neurotransmitter release with the resultant dampening of neuronal excitability (Vaughan and Christie, 2005). Using an *in vitro* model of SE, our laboratory recently demonstrated that activation of CB1 receptor via synthetic agonists produced a robust suppression of SE (Blair, et al., 2006). Thus, the use of cannabinoids in the treatment of SE may offer a unique approach to controlling SE without the development of pharmacoresistance observed with conventional treatments.

This study was initiated to evaluate the effectiveness of cannabinoids in treating SE of varying durations without the development of pharmacoresistance, employing the well-characterized hippocampal neuronal culture (HNC) model of SE (DeLorenzo, et al., 2005, Mangan and Kapur, 2004, Sombati and DeLorenzo, 1995). The HNC model is an excellent *in vitro* model to study the effects of pharmacological agents on SE, since it manifests the same neuronal spike frequency and epileptiform discharges observed with animals and human SE (DeLorenzo, et al., 2005, Mangan and Kapur, 2004, Sombati and DeLorenzo, 1995). The results of this study demonstrate that cannabinoids can control SE seizure discharges without developing pharmacoresistance under conditions where pharmacoresistance develops to the benzodiazepine, LZP and may represent a potential important advance in our ability to treat refractory SE.

Materials and Methods

Materials

LZP, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3 de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN55,212-2) [(+)-WIN] and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) was purchased from Tocris Cookson Inc (Ellisville, MO). LZP was dissolved in methanol. Stocks of (+)-WIN were made up in a vehicle stock solution of absolute ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ), and 0.9% saline at a ratio of 1:1:18 that was then diluted at a minimum of 1:500 to a final working concentration in the physiological bath recording solution (pBRS). Stock solutions of AM251 were prepared in DMSO and stored aliquoted at -20°C . The final working concentration of DMSO was 0.01%. All the drugs were bath-applied using a multi valve perfusion assembly (Warner Instrument Corp., CT, USA).

Hippocampal neuronal culture

Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications (Sombati and DeLorenzo, 1995). In brief, hippocampal cells were obtained from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a density of 2.5×10^4 cells/cm² onto a glial support layer previously plated onto poly-L-lysine coated (0.05 mg/ml) 35-mm grid cell culture dishes (Nunc, Naperville, IL). Cultures were maintained at 37°C in a 5% CO₂/95% air atmosphere and fed twice weekly with NeuroBasal-A medium supplemented with B-27 (Invitrogen Corp., San Diego, CA) containing 0.5 mM L-glutamine.

Hippocampal neuronal culture model of SE

After 2-weeks, cultures were utilized for experimentation and SE was induced as described previously (Sombati and DeLorenzo, 1995). Maintenance medium was replaced with pBRS with or without MgCl₂ containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl₂, 10 glucose, and 0.002 glycine, pH 7.3, and osmolarity adjusted to 325 ± 5 mOsm with sucrose. Continuous epileptiform high-frequency bursts (SE) were induced by exposing neuronal cultures to pBRS without added MgCl₂ (low Mg²⁺). The SE continued until pBRS containing 1 mM MgCl₂ was added back to the cultures. Unless indicated as low Mg²⁺ treatment, experimental protocols utilized pBRS containing 1 mM MgCl₂.

Whole cell current clamp recordings

Whole cell current clamp recordings were performed using previously established procedures (Sombati and DeLorenzo, 1995). Briefly, cell culture dish was mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) continuously perfused with pBRS. Patch electrodes with a resistance of 2 to 4 M Ω were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished, and filled with a solution containing (in mM): 140 K⁺ gluconate, 1 MgCl₂, and 10 Na-HEPES, pH 7.2, osmolarity adjusted to 290 ± 10 mOsm with sucrose. Whole-cell recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in current-clamp mode. Data were digitized and transferred to videotape using a PCM device (Neurocorder, New York, NY) and then played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI).

Data analysis

For assessing development of resistance to LZP or WIN, suppression of SE was determined as a percent decrease in spike frequency over increasing durations of SE. SE frequency was determined by counting individual epileptiform bursts and spikes over a recording duration of

5 min for each neuron analyzed at various durations of SE. Mean frequencies at each time-point were then represented as a percentage inhibition from control frequency (SE frequency in the absence of drug). Data were plotted using SigmaPlot analysis software 8.02 (SPSS Inc., Chicago, IL). Data are presented as mean $\pm$ SEM. Significance was determined by Student's *t*-test or one-way analysis of variance with post-hoc Dunnett's test as appropriate.

Results

Induction of SE in hippocampal neuronal cultures

The HNC model of SE is a well-established model that has been routinely used to carry out biochemical, electrophysiological and molecular investigations (DeLorenzo, et al., 2005). As shown in Fig. 1B, removal of Mg^{2+} from the recording solution resulted in the development of continuous tonic high frequency epileptiform discharges. This epileptiform activity consisted of repetitive burst discharges with each burst comprised of multiple spikes overlaying a paroxysmal depolarization shift. Spike frequency in this model of SE is greater than 3 Hz and meets the criteria of electrographic SE. Furthermore, it has been shown that SE in this model occurs synchronously throughout the interneuronal network (Sombati and DeLorenzo, 1995). Control neurons on the other hand displayed no hyperactivity and exhibited occasional action potentials (Fig. 1A).

Development of resistance to LZP with increasing duration of SE

LZP is a benzodiazepine that is the initial drug of choice for the treatment of SE (Chen and Wasterlain, 2006). LZP and DZP have been shown to be effective in controlling sustained repetitive firing of action potentials in spinal cord neurons in cultures at concentrations in the 200 nM range (McLean and Macdonald, 1988). In neurons isolated from rats undergoing SE, 3 μ M DZP was shown to elicit more enhancement of GABAR currents than 1 μ M DZP (Kapur and Macdonald, 1997). LZP at 1 μ M concentration has also been demonstrated to restore GABAergic currents in cultured hippocampal neurons undergoing ethanol withdrawal (Sanna, et al., 2003). Thus, we used a concentration of 1 μ M to maximize the effects of LZP on SE in the HNC model.

Application of LZP (1 μ M) to hippocampal neurons, 5 min after SE onset completely abolished SE (Fig. 2B). We then investigated if resistance developed to this potent anticonvulsant effect by applying LZP (1 μ M) at different time points following onset of SE. Application of LZP at 30 min post SE-onset still produced complete inhibition of SE (Fig. 2C). However, beyond this time point the blocking effects of LZP on SE started to decrease significantly. Following 45 min of SE, application of LZP produced a 60% inhibition of spike frequency (Fig. 2D). By 1 hr into SE, LZP had lost its effectiveness and produced only 15% inhibition (Fig. 2E). Two hours following SE onset, application of LZP achieved less than 10% SE inhibition and the electrographic trace resembled the no drug SE condition (Fig. 2F).

The development of resistance to LZP with increasing durations of SE is graphically shown in Fig. 3. LZP maintained a consistent level of anticonvulsant effect when applied out to 30 min following SE onset, whereas beyond the 30 min time point LZP's effect started to diminish and completely lost its effects when applied at 1–2 hr post SE-onset. All the time points beyond 30 min were significantly different from the effects of LZP at the 5 min time point.

Resistance does not develop to (+)WIN with increasing duration of SE

After demonstrating that resistance develops to LZP with increasing durations of SE in the HNC model, we then investigated if a similar phenomenon was occurring with the CB1 receptor agonist (+)WIN. In agreement with our previous studies (Blair, et al., 2006), application of (+)WIN (1 μ M) immediately following onset of SE produced a potent suppression of low- Mg^{2+}

induced high frequency spiking. We then tested the ability of (+)WIN to suppress SE activity when applied at various time points following onset of SE. Similar to the effects of LZP described above, (+)WIN (1 μ M) completely abolished the high frequency epileptiform discharges when applied up to 30 min into SE (Fig. 4B). Further, as shown in Fig. 4C and D, (+)WIN (1 μ M) maintained 100% inhibition of high frequency spiking at both 1 and 2 hr into SE, time points where LZP was found to be only 10% effective (Fig. 2E and F). This indicates that unlike LZP, resistance does not develop to (+)WIN as a result of prolonged SE.

In order to investigate if (+)WIN was blocking SE via a CB1 receptor dependent mechanism, neuronal cultures undergoing SE for two hours were pretreated with a specific CB1 receptor antagonist AM251 (1 μ M). As shown in Fig. 5C, in the presence of AM251, (+)WIN was not able abolish SE like activity. Spike frequency in the presence of AM251 and (+)WIN was similar to the frequency recorded in the presence of AM 251 alone or SE neurons alone. This suggests that (+)WIN is abolishing refractory SE via a CB1 receptor dependent mechanism. AM 251 (1 μ M) alone does not affect baseline activity in control neurons (Fig. 5A). In neurons undergoing SE it is difficult to gauge the effects of AM 251 since the neurons are already firing at peak spike frequency (Fig. 5B). DMSO vehicle had no effects on control neurons.

Discussion

Prompt treatment with anticonvulsant drugs is critical to reducing morbidity and mortality associated with SE (Bleck, 2005, Chen and Wasterlain, 2006, Treiman, et al., 1998). Despite the effectiveness of benzodiazepines and other anticonvulsant drugs in treating seizures, prolonged SE becomes refractory to treatment with currently available anticonvulsant agents (Bleck, 2005, Chen and Wasterlain, 2006, Jones, et al., 2002, Rice and DeLorenzo, 1999, Treiman, et al., 1998, Walton and Treiman, 1988). Over 40% of SE cases are refractory to the initial treatment with two or more medications (Mayer, et al., 2002, Walton and Treiman, 1988). Thus loss of drug efficacy and development of pharmacoresistant seizures is a major limiting factor in the treatment of SE (Mayer, et al., 2002, Walton and Treiman, 1988). The results of this paper demonstrate a novel discovery that the CB1 receptor agonist (+)WIN was effective in abolishing SE when applied as far out as 2 hours post SE-onset, whereas pharmacoresistance to LZP began to develop within 45 min post onset and became ineffective as an anticonvulsant by 1–2 hrs post SE-onset. This study provides the first demonstration that resistance to cannabinoid suppression of SE does not develop in the HNC model of SE and suggests that compounds in this class of drug may offer a therapeutic advance in the treatment of refractory SE. The results also suggest that further studies need to be initiated in animal models of SE and eventually in human clinical trials for the treatment of refractory SE.

SE is known to cause extensive brain damage in animals and man, resulting in further brain abnormalities (Drislane, 2000, Fountain and Lothman, 1995). Thus it is essential to terminate on-going seizure activity as rapidly as possible and prevent the development of refractory SE. Clinical trials have shown that patients treated within 20 minutes of SE had better prognoses than those who did not respond within 20 minutes (Treiman, et al., 1998). However, epidemiological studies have shown that time to seizure treatment varies broadly with only about 41% of all patients receiving their first antiepileptic drug within 30 minutes (Pellock, et al., 2004). In addition, termination of SE with benzodiazepines or phenytoin was effective in 80% of patients when administered within 30 min of seizure onset, but this effectiveness decreased to less than 40% when treatment was initiated several hours after seizure onset (Lowenstein and Alldredge, 1993, Lowenstein and Alldredge, 1998). Moreover, using animal models it has been shown that the responsiveness of SE to treatment with DZP decreases rapidly as the seizure duration increases (Jones, et al., 2002, Rice and DeLorenzo, 1999). Thus, experimental SE in rats could be stopped effectively when DZP was administered immediately after SE, but it was effective in only 17% of rats after a prolonged SE (Walton and Treiman,

1988). The results of our study utilizing the HNC model are in line with these previous findings from animal models and clinical observations with SE and demonstrates that with passage of time there is marked reduction in the ability of LZIP to terminate SE. However, (+)WIN treatment of SE in the HNC model did not develop pharmacoresistance, suggesting that the effects of cannabinoids in controlling SE seizure discharge may offer unique advantages over conventional anticonvulsant treatments.

Two main concepts have been put forward to hypothesize development of drug resistance (Remy, et al., 2003). First is the increased expression of a multidrug transporter protein (Tishler, et al., 1995, Zhang, et al., 1999) responsible for altering drug concentrations at the site of action by affecting drug uptake or increasing transport of drug cleaving enzymes. A second theory proposes that a genetic, functional or post-translational modification of the drug target itself renders the drug ineffective (Remy, et al., 2003, Spratt, 1994). Increased expression of multidrug transporter protein has been reported in human (Tishler, et al., 1995) and experimental epilepsy (Zhang, et al., 1999). However, anticonvulsant drugs such as benzodiazepines (Schinkel, et al., 1996) and carbamazepines (Owen, et al., 2001) are not transported by the multidrug transporter P-glycoprotein, thus indicating that the second theory of drug resistance could hold valid for explaining pharmacoresistance of benzodiazepines upon prolonged SE. In regards to the development of pharmacoresistance to anticonvulsant therapy with SE, several mechanisms in favor of this second theory have been proposed recently which and include changes in GABA_A receptor properties or subunit composition (Kapur and Macdonald, 1997, Macdonald and Kapur, 1999), loss of GABAergic interneurons (Houser and Esclapez, 1996, Obenaus, et al., 1993), changes in receptor-mediated regulation of GABAergic transmission (Bausch and Chavkin, 1997, Mangan and Lothman, 1996), and loss of excitatory synaptic input onto GABAergic interneurons (Bekenstein and Lothman, 1993, Doherty and Dingledine, 2001). Naylor et al. (Naylor, et al., 2005) recently demonstrated in a hippocampal slice preparation obtained from pilocarpine induced SE rats a loss of postsynaptic GABA_A receptor function which, in agreement with a previous study from our laboratory (Blair, et al., 2004), was the result of a marked increase in internalization of GABA_A receptors subunits by 1-hr following SE-onset. It will be interesting to investigate correlation between the time-course of GABA_A internalization and corresponding loss of LZIP efficacy in future studies. The *in vitro* HNC model of SE is ideally suited for such studies and can provide high enough resolution to identify these and other post-translational modifications that might underlie the development of pharmacoresistance to benzodiazepines upon prolonged SE.

Pharmacoresistance to anticonvulsant drugs is a central problem in the treatment of epilepsy and SE. Recurrent spike discharge after the removal of Mg²⁺ from extracellular medium is a commonly used model to study the mechanisms underlying seizures and seizure-induced plasticity (DeLorenzo et al., 2005; Engel et al., 2000; Goodkin et al., 2005; Sombati and DeLorenzo, 1995). Using the HNC model we recently showed that epileptogenesis caused acute and chronic increases in GABA_A receptor endocytosis that contributed to the induction and maintenance of seizures (Blair, et al., 2004). Subsequently, using the similar model, Goodkin et al., showed that prolonged SE increased intracellular accumulation of GABA_A receptors (Goodkin, et al., 2005). In these models it was also observed that prolonged SE caused a reduction of GABA-mediated synaptic inhibition (Blair, et al., 2004, Goodkin, et al., 2005). This progressive and time-dependent loss of post-synaptic GABA_A receptors is thought to underlie development of pharmacoresistance to conventional antiepileptic drugs such as phenobarbital and diazepam that are used as initial drugs of choice for the treatment of SE. Moreover, phenobarbital and phenytoin are ineffective in blocking SE in this model (Blair et al., 2006). Thus taken together, this *in vitro* model has been shown as a relevant model to study refractory SE and could prove to be a useful tool to investigate the time course and sequence of molecular events leading to the expression of benzodiazepine's pharmacoresistance upon increasing durations of SE.

Emerging evidence indicates that the endocannabinoid system plays a pivotal role in maintaining brain excitability (Lutz, 2004, Smith, 2005, Wallace, et al., 2003). The CB1 receptor is the most highly expressed $G_{i/o}$ coupled G-protein receptor in the brain (Howlett, et al., 2002). Located preferentially on presynaptic nerve terminals (Katona, et al., 1999), its activation leads to decreased c-AMP production, inhibition of N, P/Q type calcium channels, and activation of inward rectifier potassium currents, ultimately resulting in membrane stabilization and decreased neuronal excitability (Howlett, et al., 2002, Mackie, 2006). Interestingly, activation of CB1 receptors via endocannabinoids occurs in an “on-demand” fashion upon depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001) or inhibition (DSI) (Ohno-Shosaku, et al., 2001). Upon intense synaptic activity endocannabinoids are synthesized at the postsynaptic membrane. These endocannabinoids then diffuse in a retrograde fashion and activate presynaptic CB1 receptors to inhibit neurotransmitter release and modulate neuronal excitability (Vaughan and Christie, 2005). Indeed, we recently reported a significant increase in brain levels of endocannabinoid 2-AG upon pilocarpine induced SE in rats (Wallace, et al., 2003). The CB1 agonists and endocannabinoids were also reported to be anticonvulsant and blocked SE in animal and neuronal culture models (Blair, et al., 2006, Wallace, et al., 2003, Wallace, et al., 2002, Wallace, et al., 2001). More recently we also reported that the tonic CB1 receptor activation through the endocannabinoid system plays an important role in modulating seizure frequency and duration and preventing the development of SE in populations of epileptic neurons in the HNC model (Deshpande, et al., 2007). Thus disrupting the endocannabinoid tone by blocking tonic CB1 receptor activation with AM251 caused the epileptic neurons to progress to SE (Deshpande, et al., 2007). These studies along with our current observations strongly indicate that development of agents activating the CB1 endocannabinoid system could be a novel therapeutic approach for the treatment of prolonged, refractory SE and epilepsy.

Pharmacoresistance of benzodiazepines may be easily explained, as these drugs are allosteric modulators of GABAergic transmission. Thus, in the *in vitro* system, GABAergic transmission onto presumably glutamatergic neurons is required for an effect by these drugs. During SE, it appears that this connectivity is lost. For cannabinoids, the situation is completely different. Here, the most likely mechanism is that CB1 acts on cell autonomously at the presynaptic level and inhibits glutamate release. While the current study cannot pinpoint the mechanisms underlying lack of development of resistance to cannabinoids, one can speculate that the differences between the two receptor systems such as 1) number of signaling cascades activated to modulate neuronal excitability, 2) receptor kinetics, 3) cellular localization and distribution could be some of the contributing factors towards the observed differential pharmacoresistance. It will be interesting to investigate these possibilities in future studies especially using an *in vivo* model of SE.

While *in vivo* models are crucial in understanding prolonged seizure disorders and bear closer resemblance to human pathology, the *in vitro* HNC model provides an important tool in elucidation of molecular mechanisms associated with continuous high-frequency epileptiform discharges or SE. Thus, while the neuronal cultures do not have true anatomical connections and don't display clinical seizures, this HNC model of SE has several advantages over traditional *in vivo* seizure models with regard to applying various *in vitro* techniques to study molecular mechanisms involved in drug-mediated anticonvulsant effects. It is therefore well suited to further characterize molecular events underlying the development of drug resistance upon prolonged SE in a more controlled environment. This study to our knowledge represents the first demonstration of the development of pharmacoresistance to BDZs with prolonged SE in this *in vitro* model and the potential advantage of using cannabinoids to stop refractory SE. It would also be important to further investigate the effects of cannabinoids in an *in vivo* SE model in future studies.

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Figure 1.

Induction of status epilepticus in cultured hippocampal neurons. **A**, representative current clamp recording from a control neuron showing occasional spontaneous action potentials. **B**, representative current clamp recordings during low Mg^{2+} treatment showing induction of tonic high-frequency epileptiform bursts. Exposure to low Mg^{2+} induced continuous epileptiform activity (SE) with burst frequencies greater than 3 Hz. **a**, expansion of a segment from **B** showing the presence of epileptiform burst discharges. **b**, further expansion of a segment from **a**, revealing the individual epileptiform bursts characterized by paroxysmal depolarization shifts overlaid with multiple spikes.

Figure 2.

Development of resistance to lorazepam with increasing durations of SE. **A**, Removal of Mg^{2+} from the culture media induced SE in cultured hippocampal neurons. **B and C**, Treatment with LPZ (1 μM) completely stopped high frequency spiking when intervened immediately and when applied up to 30-mins into SE. **D**, Resistance starts to develop to LPZ (1 μM) by 45-mins into SE and **E**, by 1-hr LPZ (1 μM) loses its efficacy to stop low- Mg^{2+} induced SE. **F**, Two-hours into SE there is complete resistance to blocking effects of LPZ (1 μM).

Figure 3.

Graphical representation of development of resistance to lorazepam with increasing durations of SE. LPZ (1 μ M) can effectively block low-Mg²⁺ induced high frequency epileptiform burst discharges when applied up to 30-mins following SE onset. However, by 45-mins, LPZ (1 μ M) starts to lose its efficacy and by 1-hr into SE it is only 10% effective in blocking high frequency spiking. Similar results are obtained at 2-hrs into SE. Each data point represents the mean $\pm$ SEM of 3–6 experiments. * denotes significant difference from the 0-hr time point for LPZ treatment, $p \leq 0.05$.

Figure 4.

Lack of development of resistance to cannabinoids with increasing durations of SE. **A**, Cultured hippocampal neurons when exposed to a low Mg²⁺ solution exhibit high frequency epileptiform bursts analogous to SE. Treatment with the CB1 agonist WIN 55,212-2 (1 μ M) completely abolishes high frequency spiking when administered at 30-min (**B**), 60-min (**C**) and even at 120-min (**D**) following SE onset. Resistance does not develop to anti-SE effects of WIN 55,212-2 at any of the time points tested.

Figure 5.

WIN 55,212-2 is anticonvulsant via a CB1 dependent mechanism at 2-hr into SE. A, AM 251 (1 μ M) alone does not cause hyperexcitability in control neurons. B, Effects of AM 251 (1 μ M) alone on SE neurons. C, WIN 55,212-2 (1 μ M) stops low Mg^{2+} -induced high frequency spiking at 120-min following SE onset. D, Anti-SE effects of WIN 55,212-2 (1 μ M) at 120-min following SE onset were mediated via a CB1 receptor dependent mechanism since AM 251 (1 μ M) abolishes the anti-SE effects of WIN 55,212-2.