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Tetramethylenedisulfotetramine Alters Ca^{2+} Dynamics in Cultured Hippocampal Neurons: Mitigation by NMDA Receptor Blockade and $GABA_A$ Receptor-Positive Modulation

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Tetramethylenedisulfotetramine (TETS) is a potent convulsant that is considered a chemical threat agent. We characterized TETS as an activator of spontaneous Ca^{2+} oscillations and electrical burst discharges in mouse hippocampal neuronal cultures at 13–17 days *in vitro* using FLIPR Fluo-4 fluorescence measurements and extracellular microelectrode array recording. Acute exposure to TETS (≥ 2μM) reversibly altered the pattern of spontaneous neuronal discharges, producing clustered burst firing and an overall increase in discharge frequency. TETS also dramatically affected Ca^{2+} dynamics causing an immediate but transient elevation of neuronal intracellular Ca^{2+} followed by decreased frequency of Ca^{2+} oscillations but greater peak amplitude. The effect on Ca^{2+} dynamics was similar to that elicited by picrotoxin and bicuculline, supporting the view that TETS acts by inhibiting type A gamma-aminobutyric acid ($GABA_A$) receptor function. The effect of TETS on Ca^{2+} dynamics requires activation of N-methyl-D-aspartic acid (NMDA) receptors, because the changes induced by TETS were prevented by MK-801 block of NMDA receptors, but not nifedipine block of L-type Ca^{2+} channels. Pretreatment with the $GABA_A$ receptor-positive modulators diazepam and allopregnanolone partially mitigated TETS-induced changes in Ca^{2+} dynamics. Moreover, low, minimally effective concentrations of diazepam (0.1μM) and allopregnanolone (0.1μM), when administered together, were highly effective in suppressing TETS-induced alterations in Ca^{2+} dynamics, suggesting that the combination of positive modulators of synaptic and extrasynaptic $GABA_A$ receptors may have therapeutic potential. These rapid throughput *in vitro* assays may assist in the identification of single agents or combinations that have utility in the treatment of TETS intoxication.

Key Words: **Ca^{2+} oscillations; $GABA_A$ receptors; microelectrode array; NMDA receptors; rapid throughput assay; tetramethylenedisulfotetramine.**

Tetramethylenedisulfotetramine (TETS), commonly called tetramine, is a highly toxic convulsant with a parenteral LD_{50} of 0.1–0.3 mg/kg in mice or rats (Casida *et al.*, 1976; Haskell and Voss, 1957; Voss *et al.*, 1961). In adult humans, 7–10 mg is estimated as a lethal dose (Guan *et al.*, 1993). TETS was used as a rodenticide until banned worldwide in the early 1990s (Banks *et al.*, 2012; Whitlow *et al.*, 2005). It is, however, still available illegally, and is responsible for accidental and intentional poisonings, predominantly in China (Croddy, 2004; Wu and Sun, 2004; Zhang *et al.*, 2011), but also in other countries, including the United States (Barrueto *et al.*, 2003). Between 1991 and 2010 over 14,000 cases of TETS intoxication were reported in China with 932 deaths (Li *et al.*, 2012). Extreme toxicity, history of intentional mass poisonings, and the absence of a specific antidote raise concern that TETS is a potential chemical threat agent that could cause mass casualties if released accidentally or intentionally (Jett and Yeung, 2010; Whitlow *et al.*, 2005).

Mild-to-moderate poisoning with TETS leads to headache and dizziness, whereas severe intoxication produces status epilepticus and coma (Li *et al.*, 2012; Whitlow *et al.*, 2005). Animal studies demonstrate that TETS is active as a convulsant when administered orally, parenterally, and intraventricularly (Zolkowska *et al.*, 2012). Sublethal seizures are not associated with evidence of cellular injury or neurodegeneration although there is delayed transient reactive astrocytosis and microglial activation (Zolkowska *et al.*, 2012).

The primary convulsant mechanism of TETS has been thought to relate to blockade of type A gamma-aminobutyric acid ($GABA_A$) receptors and the seizures induced in animals resemble those produced by other $GABA_A$ receptor antagonists including picrotoxin and pentylenetetrazol. Limited cellular physiological studies and results from [^{35}S]*t*-butylbicyclophosphorothionate binding to brain membranes indicate that TETS inhibits $GABA_A$ receptors with an IC_{50} in the range of 1μM (Esser *et al.*, 1991; Ratra *et al.*, 2001; Squires *et al.*, 1983) and it is therefore comparable in potency to picrotoxin as an inhibitor of $GABA_A$ receptors (Cole and Casida, 1986; Ratra *et al.*, 2001; Squires *et al.*, 1983).

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Cultured hippocampal neurons display spontaneous synchronous Ca^{2+} oscillations (Tanaka *et al.*, 1996) that are driven by action potential-dependent synaptic transmission. Chemically diverse environmental toxicants have been reported to disrupt neuronal Ca^{2+} oscillations (Cao *et al.*, 2010, 2011; Choi *et al.*, 2010; Pereira *et al.*, 2010; Soria-Mercado *et al.*, 2009). Convulsant agents can also dramatically influence neuronal Ca^{2+}. For example, the organophosphate diisopropylfluorophosphate produces long-lasting Ca^{2+} elevations in hippocampal neurons (Deshpande *et al.*, 2010).

Hippocampal neurons also exhibit spontaneous electrical discharges as they form functional neuronal networks. These discharges, as detected in extracellular recordings, consist of infrequent synchronized field potentials, mixed with more frequent desynchronized random action potentials (Cao *et al.*, 2012; Frega *et al.*, 2012). Synchronous Ca^{2+} oscillations and neuronal electrical firing co-occur (Jimbo *et al.*, 1993) and are important in mediating neuronal development and activity-dependent dendritic growth (Wayman *et al.*, 2008). Genetic or environmental factors that interfere with neurotransmission influence the overall activity of neuronal networks (Frega *et al.*, 2012; Kenet *et al.*, 2007; Meyer *et al.*, 2008; Shafer *et al.*, 2008; Wayman *et al.*, 2012). For example, picrotoxin, a $GABA_A$ receptor antagonist, produces striking changes in network electrical activity (Cao *et al.*, 2012; Frega *et al.*, 2012).

In this study, we used rapid throughput assays to characterize the influence of TETS on Ca^{2+} dynamics and electrical discharges in cultured hippocampal neurons. Inasmuch as TETS induces changes in Ca^{2+} dynamics that are similar to those produced by the $GABA_A$ receptor antagonists picrotoxin and bicuculline, our results support the view that TETS acts as a $GABA_A$ receptor antagonist. Using rapid throughput Ca^{2+} measurement, we demonstrate that the $GABA_A$ receptor-positive modulators diazepam and allopregnanolone reduce or prevent TETS effects on Ca^{2+} dynamics, suggesting these agents as potential treatment strategies for TETS-induced seizures.

MATERIALS AND METHODS

Materials. Fetal bovine serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA). DNase, poly-L-lysine, cytosine arabinoside, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), hydroxypropyl-β-cyclodextran, and 3,5-dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (nifedipine) were from Sigma-Aldrich (St Louis, MO). The Ca^{2+} fluorescence dye Fluo-4, Pluronic F-127, and Neurobasal medium were purchased from Life Technology (Grand Island, NY). Tetramethylenedisulfotetramine (TETS) was synthesized as described previously (Zolkowska *et al.*, 2012). Diazepam was from Western Medical Supply (Arcadia, CA). Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) was custom synthesized and characterized as > 99% pure.

Primary cultures of hippocampal neurons. Animals were treated humanely and with regard for alleviation of suffering according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Hippocampal neuron cultures were dissociated from hippocampi dissected from C57Bl/6J mouse pups at postnatal day 0–1 and maintained in Neurobasal complete medium (Neurobasal medium supplemented with NS21, 0.5mM L-glutamine, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]) with 5% fetal bovine serum. For Ca^{2+} imaging studies using FLIPR, dissociated hippocampal cells were plated onto poly-L-lysine-coated clear-bottom, black wall, 96-well imaging plate (BD, Franklin Lakes, NJ) at a density of 0.8×10^5/well. For microelectrode array (MEA) experiments, 120 µl of cell suspension at a density of 1.5×10^6 cells/ml were added to a 12-well Maestro plate (Axion BioSystems, Atlanta, GA). After 2 h incubation, a volume of 1.0 ml of serum-free Neurobasal complete medium was added to each well. The medium was changed twice a week by replacing half the volume of culture medium in the well with serum-free Neurobasal complete medium. The neurons were maintained at 37°C with 5% CO_2 and 95% humidity.

Measurement of synchronous intracellular Ca^{2+} oscillations. Hippocampal neurons between 13 and 17 days *in vitro* (DIV) were used to investigate how TETS alters synchronous Ca^{2+} oscillations that normally occur in healthy neurons at this developmental stage. This method permits simultaneous measurements of intracellular Ca^{2+} transients in all wells of a 96-well plate as described previously (Cao *et al.*, 2010). Baseline recordings were acquired in Locke's buffer (8.6mM HEPES, 5.6mM KCl, 154mM NaCl, 5.6mM glucose, 1.0mM $MgCl_2$, 2.3mM $CaCl_2$, and 0.0001mM glycine, pH 7.4) for 10 min followed by addition of TETS and/or pharmacological agents using a programmable 96-channel pipetting robotic system, and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored for an additional 30 min. Unless otherwise indicated, pharmacological interventions were introduced 10 min prior to TETS. TETS triggered an immediate rise in $[Ca^{2+}]_i$ that was quantified by determining the area under the curve (AUC) of the Fluo-4 arbitrary fluorescence units for a duration of 5 min following TETS addition. TETS also altered the frequency and amplitude of neuronal synchronous Ca^{2+} oscillations, which were analyzed during the 10-min period after addition of TETS for 15 min.

MEA recording. All MEA recordings were conducted on 13–17 DIV hippocampal neuronal networks at 37°C in culture medium without perfusion using a 12-well Maestro system (Axion). Each well contains 64 electrodes (30 µm diameter) in an 8 × 8 grid with interelectrode spacing of 200 µm. Before recording basal electrical activity, the cultures were equilibrated in freshly prepared, prewarmed Neurobasal complete medium for 1 h. The 12-well Maestro plates were loaded onto a temperature-regulated headstage containing the recording amplifier, and raw extracellular electrical signals were acquired using Axis software (Axion BioSystems, Atlanta, GA). Signals from the amplifier were digitized at a rate of 25 kHz and filtered using Butterworth Band-pass filter (cutoff frequency of 300 Hz). The Axis software was used to detect spontaneous events that exceeded a threshold of six times the noise. Raster plot and spike rate analysis were performed by exporting the raw data to the NeuroExplorer software (version 4.0, NEX Technologies, Littleton, MA).

Data analysis. Graphing and statistical analysis were performed using GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA). EC_{50} values were determined by non-linear regression using a three-parameter logistic equation. Statistical significance between different groups was calculated using Student's *t*-test or by an ANOVA and, where appropriate, a Dunnett's multiple comparison test; p values below 0.05 were considered statistically significant.

RESULTS

Effects of TETS on Ca^{2+} Oscillations in Primary Cultured Hippocampal Neurons

Cultured hippocampal neurons (13–17 DIV) exhibit spontaneous synchronous Ca^{2+} oscillations whose frequency and amplitude can be quantitatively assessed in real time using FLIPR (Fig. 1A). Addition of vehicle (0.01% dimethyl sulfoxide [DMSO]) had no significant effect on the properties of the synchronous Ca^{2+} oscillations during the 5-min phase I period or the 10-min phase II period (Fig. 1A, top trace). By contrast,

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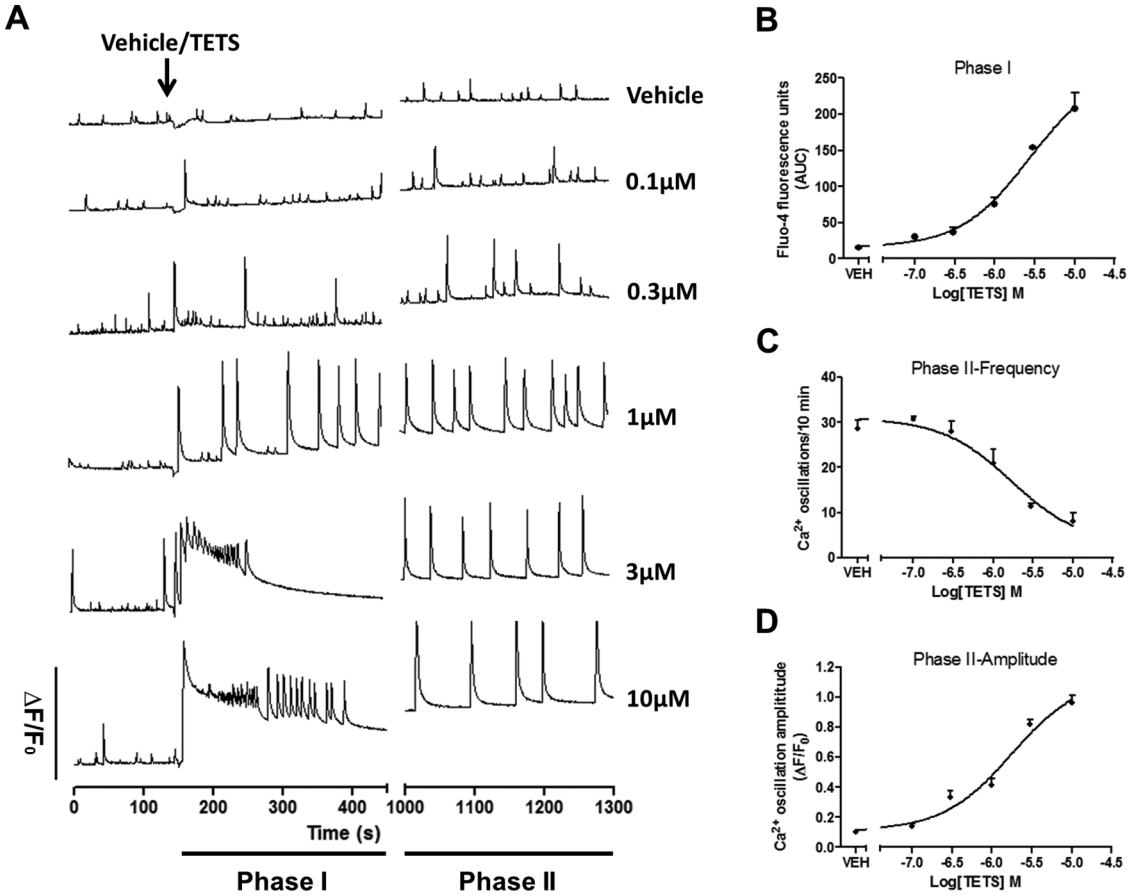

FIG. 1. TETS-induced Ca^{2+} dysregulation in hippocampal neurons. (A) Representative traces showing how acute exposure to TETS (0.1–10µM) influences Ca^{2+} fluctuations in hippocampal neurons 13–17 DIV. Note that neurons exhibit spontaneous synchronous Ca^{2+} oscillations at this developmental stage indicative of functional network connectivity. The effects of TETS were analyzed in the initial 5 min following addition (phase I) and in the subsequent 10 min (phase II). In phase I, the integrated intracellular Ca^{2+} level increased in a concentration-dependent fashion (B), and there was a plateau response at higher concentrations (3 and 10µM) that decayed slowly over the 5 min period. In phase II, there was a concentration-dependent reduction in the frequency (C) and an increase in the amplitude of the spontaneous synchronized Ca^{2+} oscillations (D). The traces shown for phase II are representative samples of the 10-min phase II period. This experiment was repeated three times with similar results.

exposure of the neurons to TETS caused an immediate increase in the amplitude of the Ca^{2+} oscillations, and at higher concentrations (3 and 10µM), a sustained plateau response that decayed slowly over the 5-min phase I period. The integrated Ca^{2+} signal (AUC) during the phase I period exhibited a concentration-dependent increase, with an EC_{50} value of 2.7µM (95% confidence interval [95% CI]: 1.4–5.2µM) (Fig. 1B). During phase II, TETS caused a concentration-dependent decrease in the frequency of the synchronous Ca^{2+} oscillations with an EC_{50} value of 1.7µM (95% CI: 0.69–4.12µM; Fig. 1C). Along with the reduction in the frequency, TETS increased the mean Ca^{2+} oscillation amplitude with an EC_{50} value of 1.8µM (95% CI: 1.12–2.80µM; Fig. 1D). TETS modestly prolonged the mean duration of individual Ca^{2+} transients compared with that measured from vehicle-exposed control neurons (data not shown). TETS-induced phase II Ca^{2+} responses (both frequency and amplitude) were reversible upon washout of TETS (Supplementary fig. 1).

For comparison, we studied the influence on Ca^{2+} dynamics in cultured hippocampal neurons of picrotoxin (PTX; 100µM), a noncompetitive blocker of $GABA_A$ receptors, and bicuculline (100µM), a competitive antagonist of $GABA_A$ receptors. Both antagonists elicited phase I and phase II responses that were similar to those induced by TETS (Fig. 2).

TETS Enhances Neuronal Electric Network Activity in Primary Cultured Hippocampal Neurons

Extracellular recordings of electrical activity from multiple sites within the neuronal cultures at a high spatial resolution provide a robust measure of network activity and connectivity (Johnstone *et al.*, 2010). After recording the basal electrical activity for 10 min, increasing concentrations of TETS were serially introduced into the wells. A 10-min recording was collected at each TETS concentration. A control well was simultaneously recorded following introduction of vehicle (0.01–0.1% DMSO). Basal recordings for up to 60 min showed that network

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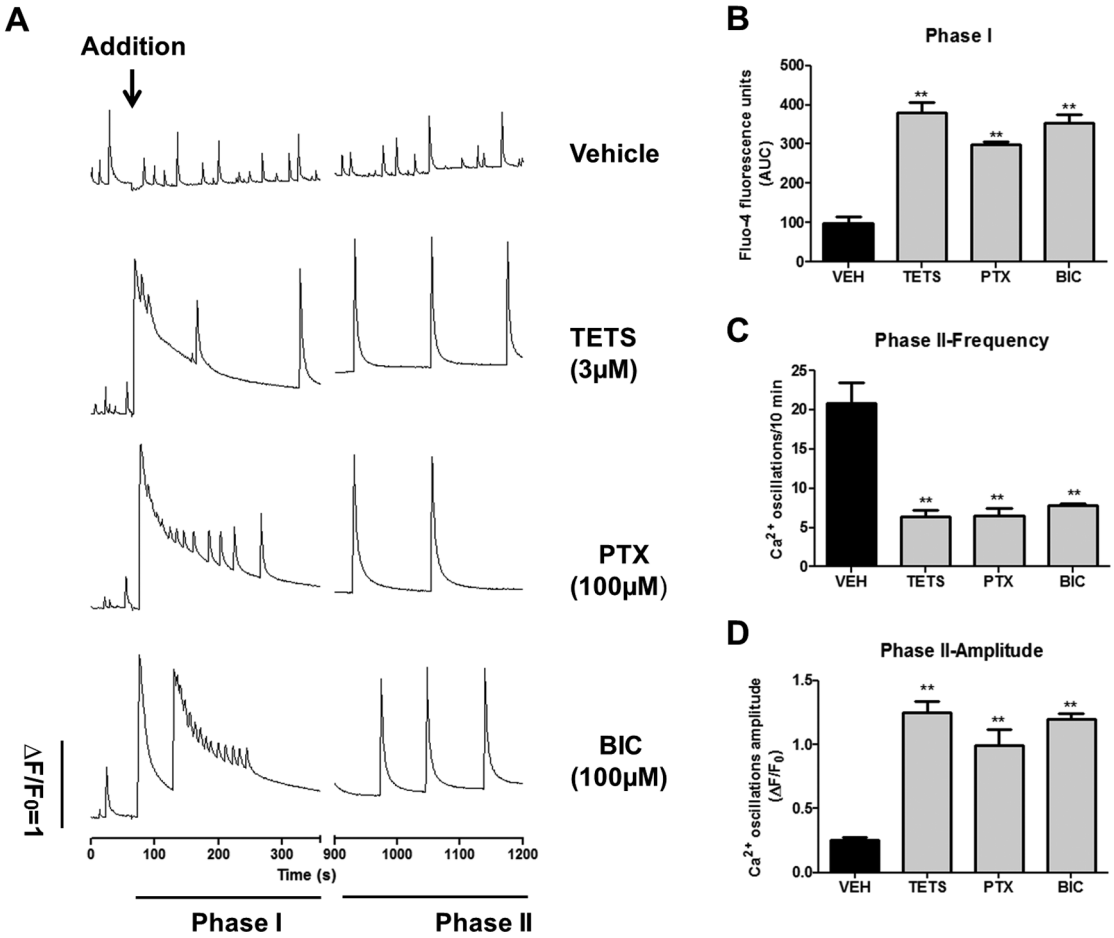

FIG. 2. TETS, picrotoxin, and bicuculline trigger similar neuronal Ca^{2+} dysregulation. (A) Representative traces from experiments comparing the effects of TETS (3μM), picrotoxin (100μM), and bicuculline (100μM) on Ca^{2+} fluctuations. The three agents produce similar acute elevation of the integrated Ca^{2+} level (B) with plateau responses in phase I, and they decreased the oscillatory frequency (C), whereas increasing the amplitude of Ca^{2+} transients in phase II (D). $^{**}p < 0.01$, inhibitors vs. control, data were pooled from three experiments performed at least in duplicate.

firing activity was stable in the absence or presence of vehicle (Fig. 3A, left panel). Exposure to TETS concentrations of 2μM and greater produced a dramatic change in discharge pattern. Events became more highly clustered (Fig. 3A, right panel and Supplementary fig. 2) and the duration of clustered bursts induced by 6μM TETS lasted up to 10 s (Fig. 3A, right panel, fourth row). There was also an overall increase in the discharge rate (Fig. 3B). After washout of TETS, the neuronal network firing recovered to basal conditions.

N-Methyl-D-Aspartic Acid Receptors, but Not L-type Ca^{2+} Channels Are Required for TETS-Induced Ca^{2+} Dysregulation

We next examined the possible involvement of N-methyl-D-aspartic acid (NMDA) receptors and L-type Ca^{2+} channels in the effects of TETS on Ca^{2+} dynamics. Preincubation of neuronal cultures for 10 min with MK-801 (1μM), an NMDA receptor blocker, attenuated both phase I and phase II effects of TETS (Figs. 4A–D). MK-801 slightly suppressed basal Ca^{2+} oscillations, which is consistent with an earlier report (Tanaka *et al.*, 1996). By contrast, nifedipine (1μM), which inhibits L-type voltage-activated Ca^{2+} channels, was without effect on TETS-induced phase I or phase II Ca^{2+} responses (Figs. 4A–D). These results indicate that NMDA receptors but not L-type Ca^{2+} channels are required for the effects of TETS on Ca^{2+} dynamics.

Diazepam and Allopregnanolone Partially Mitigate TETS-Induced Ca^{2+} Dysregulation

We next determined whether the $GABA_A$ receptor-positive modulators diazepam and allopregnanolone could protect against TETS-induced Ca^{2+} dysregulation. Figure 5A (top trace) demonstrates that the oscillatory activity of neurons exposed to vehicle remained stable over the entire recording period. Introduction of diazepam (0.1, 0.3, or 1μM) attenuated the amplitude of basal spontaneous Ca^{2+} oscillations (Fig. 5A). Pre-exposure to diazepam caused a small concentration-dependent reduction of the phase I integrated rise in $[Ca^{2+}]_i$ induced by TETS that reached statistical significance only at

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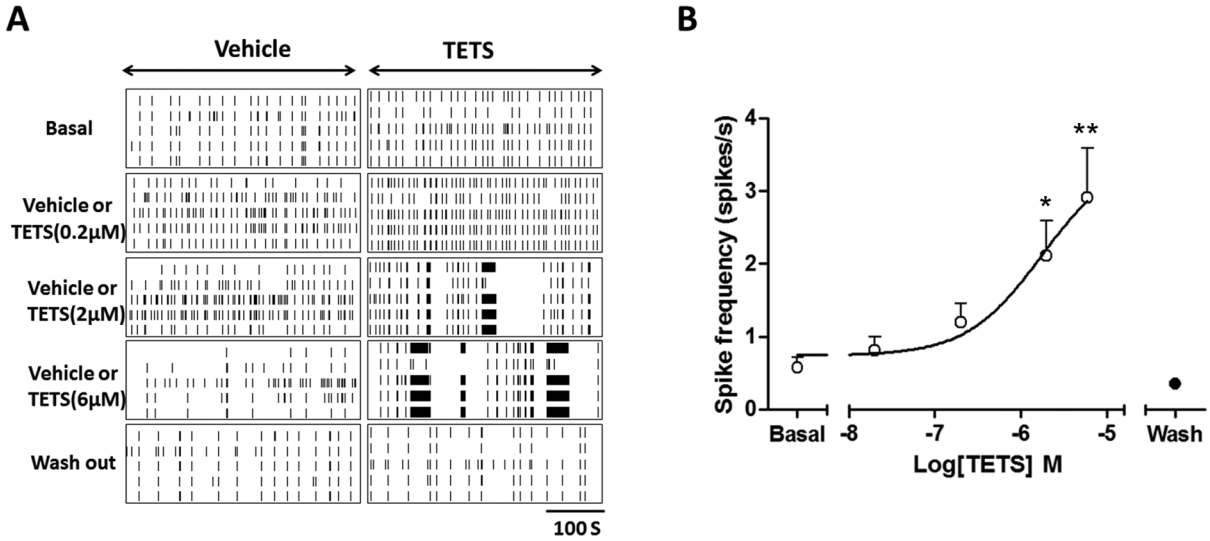

FIG. 3. TETS reversibly alters spontaneous electrical discharges in hippocampal neurons. (A) Representative raster plots of neuronal discharges before, during, and after exposure to vehicle (DMSO) (left panels) or TETS (right panels). Neuronal network activity was stable for up to 60 min in the absence or presence of vehicle. TETS solutions of increasing concentration were added serially to the wells. After recording for 10 min, the solution was removed and replaced by a higher concentration or by vehicle (wash out). TETS concentrations of 2 and 6μM caused a clustered burst discharge pattern and increased the overall discharge frequency (B). This experiment was repeated three times with each treatment performed in duplicate with similar results. $*p < 0.05$, $**p < 0.01$, TETS vs. basal.

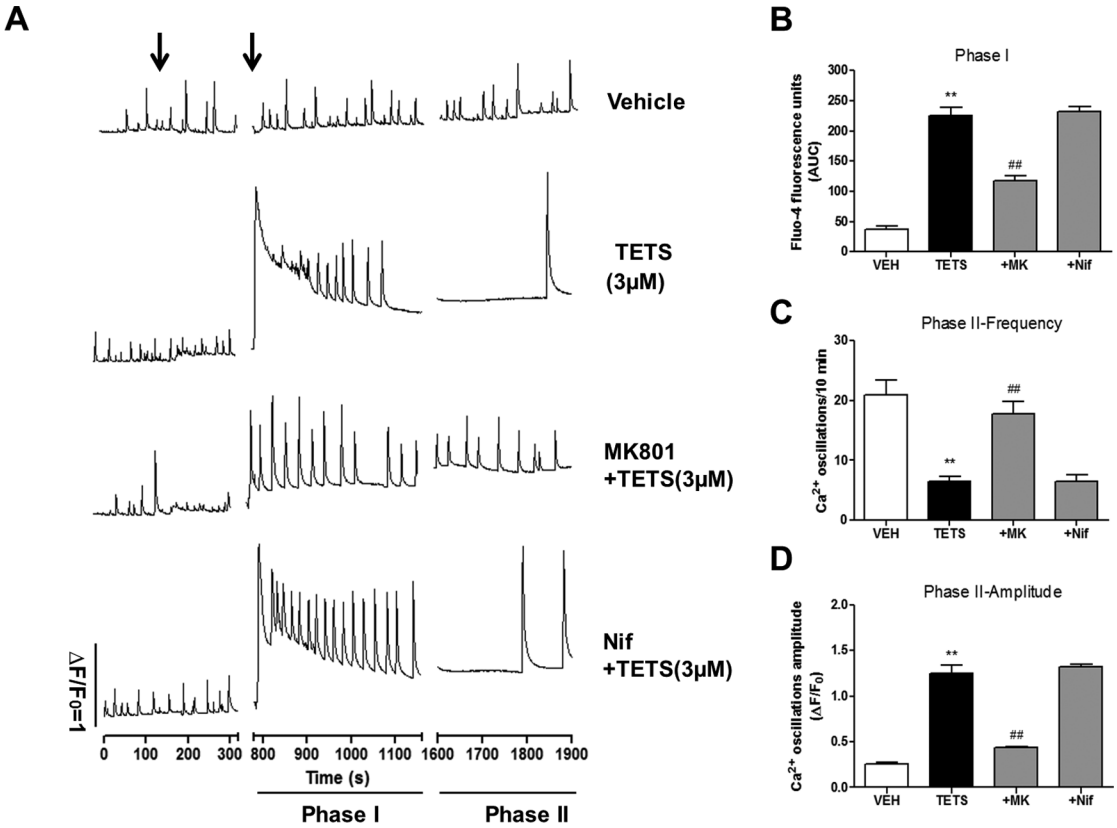

FIG. 4. MK-801, but not nifedipine, partially mitigates TETS (3μM)-induced neuronal Ca^{2+} dysregulation. (A) Representative traces illustrating effects of pre-exposure to MK-801 and nifedipine on TETS-induced Ca^{2+} dysregulation. Effects of MK-801 (MK) and nifedipine (NIF) on TETS-induced increase in integrated Ca^{2+} levels in phase I (B), the decrease in synchronous Ca^{2+} oscillation frequency in phase II (C), and the increase in Ca^{2+} transient amplitude in phase II (D). $**p < 0.01$, TETS vs. vehicle control, $##p < 0.01$, MK-801 + TETS vs. TETS, $n = 6$ pooled from two experiments.

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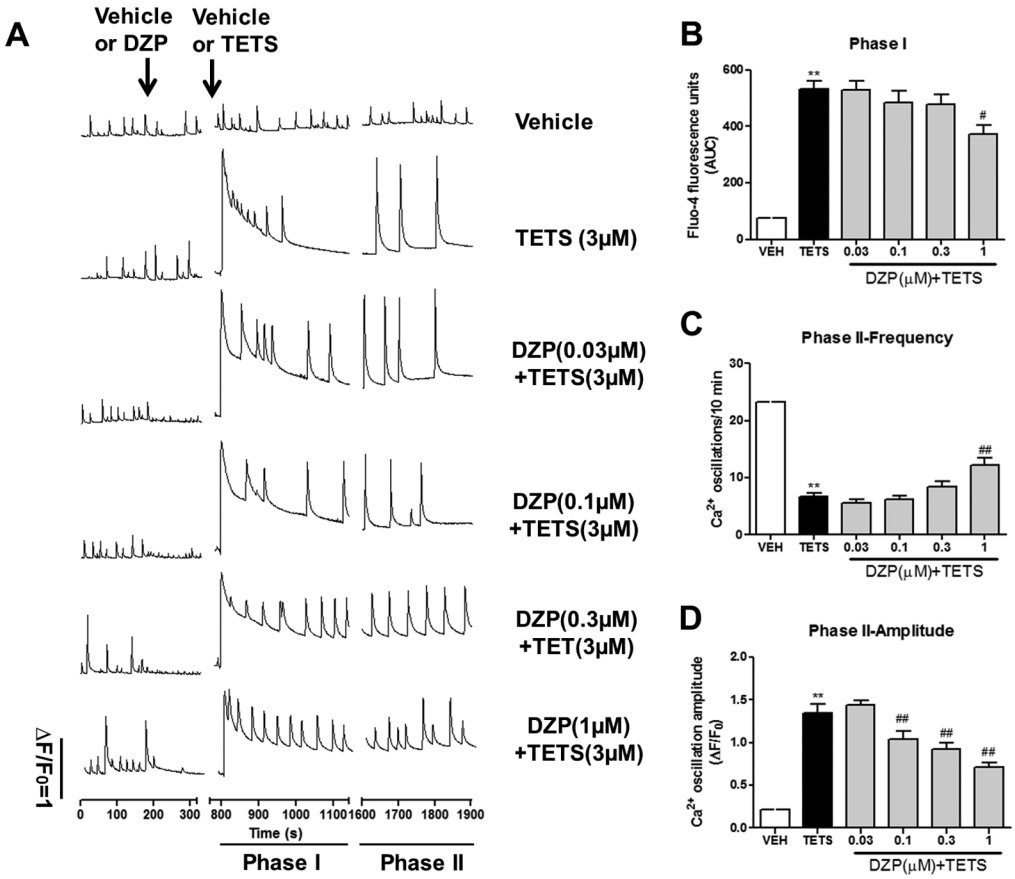

FIG. 5. Diazepam partially mitigates TETS-induced neuronal Ca^{2+} dysregulation. (A) Representative traces illustrating effects of pre-exposure to increasing concentrations of diazepam (0.03–1μM) on TETS-induced Ca^{2+} dysregulation. Effect of diazepam (DZP) on TETS-induced increase in integrated Ca^{2+} levels in phase I (B), the decrease in synchronous Ca^{2+} oscillation frequency in phase II (C), and the increase in Ca^{2+} transient amplitude in phase II (D). $^{**}p < 0.01$, TETS vs. vehicle control, $\#p < 0.05$, $\#\#p < 0.01$, diazepam + TETS vs. TETS, $n = 6$ pooled from two experiments.

1μM (Fig. 5B). Diazepam did not eliminate the phase I plateau response (Fig. 5A). Diazepam also caused a partial inhibition of the phase II frequency and amplitude effects of TETS, with the effect on amplitude reaching significance at 0.1μM (Figs. 5C and D).

As shown in Figure 6, allopregnanolone similarly attenuated the effects of TETS on Ca^{2+} dysregulation. Allopregnanolone (0.1–1μM) caused a concentration-dependent suppression of basal spontaneous Ca^{2+} fluctuations and it partially attenuated the response in phase I at 1μM without eliminating the plateau in Ca^{2+} levels (Figs. 6A and B). Allopregnanolone at 0.3 and 1μM also inhibited the phase II effect of TETS on the frequency and amplitude of Ca^{2+} oscillations, completely reversing phase II effects on transient amplitudes at 1μM (Figs. 6C and D).

Low Concentrations of Diazepam and Allopregnanolone in Combination Mitigate TETS-Induced Ca^{2+} Dysregulation

We next evaluated the effect of a combination of diazepam and allopregnanolone, each at a low concentration (0.1μM) that by itself had minimal effects on phase I or phase II Ca^{2+} dysregulation. As shown in Figure 7, the combination strongly mitigated both phase I and phase II effects. In fact, the combination treatment was able to largely eliminate the plateau response obtained with acute TETS exposure (Fig. 7A), an effect not obtained with 10-fold higher concentrations of diazepam (Fig. 5) or allopregnanolone (Fig. 6) alone.

DISCUSSION

In this study, we characterized the effects of TETS on hippocampal neurons in culture using MEA field potential recording and Fluo-4 fluorescence measurements of Ca^{2+} dynamics in the neuronal network. Over time, hippocampal neurons in culture develop a rich network of processes and form numerous functional synaptic contacts (Arnold *et al.*, 2005; Mennerick *et al.*, 1995). Cultures that have developed for 13–17 DIV as used in this study are well organized and there is robust spontaneous electrical activity mediated by excitatory and inhibitory transmission between neurons. Neurons within such cultures exhibit spontaneous action potentials and cultures of sufficient cell density may show synchronized bursting of neurons throughout the entire culture (Arnold *et al.*, 2005).

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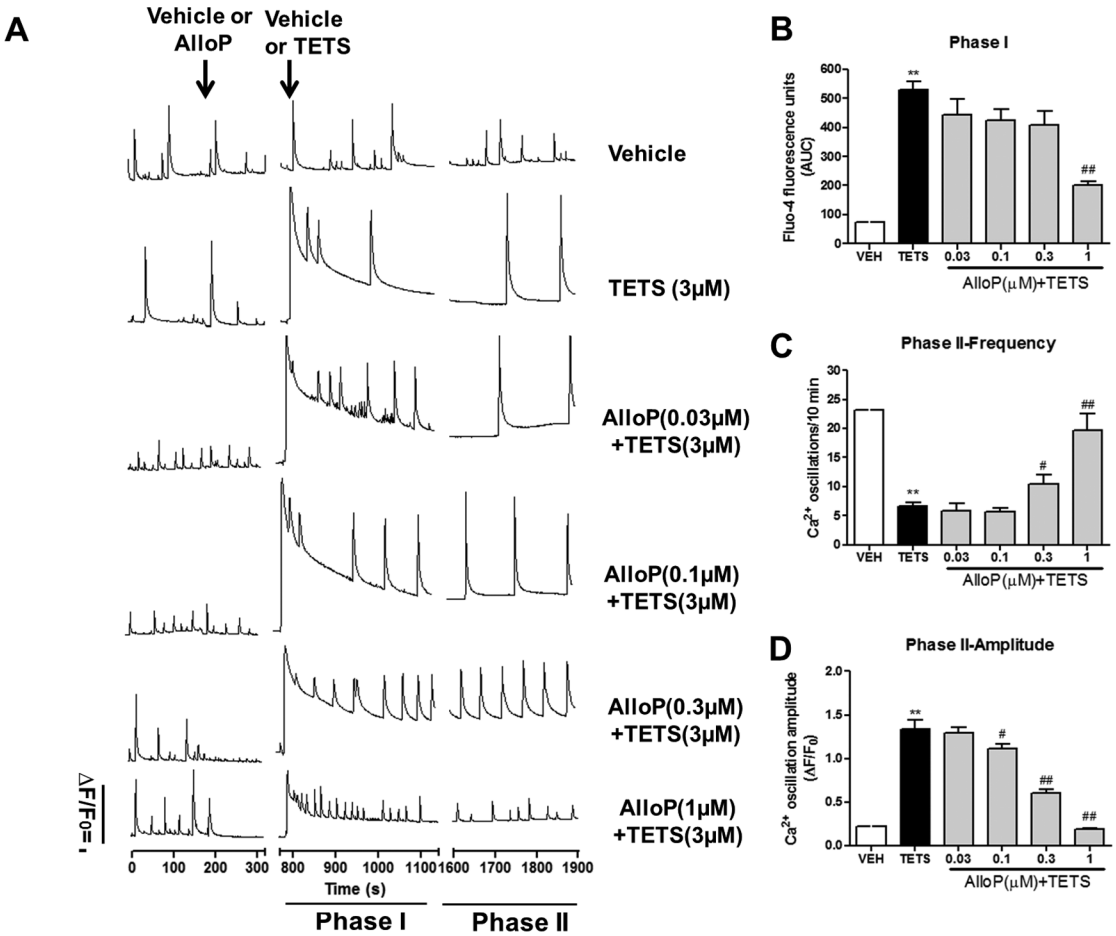

FIG. 6. Allopregnanolone partially mitigates TETS-induced neuronal Ca^{2+} signaling dysregulation. (A) Representative traces illustrating effects of pre-exposure to increasing concentrations of allopregnanolone (0.03–1μM) on TETS-induced Ca^{2+} dysregulation. Effect of allopregnanolone (AlloP) on TETS-induced increase in integrated Ca^{2+} levels in phase I (B), the decrease in synchronous Ca^{2+} oscillation frequency in phase II (C), and the increase in Ca^{2+} transient amplitude in phase II (D). $^{**}p < 0.01$, TETS vs. vehicle control, $\#p < 0.05$, $\#\#p < 0.01$, allopregnanolone + TETS vs. TETS, $n = 6$ pooled from two experiments.

Excitatory synaptic transmission is mediated by functional glutamate receptors of the NMDA and AMPA types (Abele *et al.*, 1990). Importantly, the cultures contain GABAergic neurons, which comprise ~10% of the neuronal cell population. These GABAergic neurons form robust inhibitory synaptic connections mediated by $GABA_A$ receptors that exhibit physiological properties similar to those in intact preparations (Jensen *et al.*, 1999, 2000). The GABAergic neurons impose tonic inhibition onto the network so that exposure of hippocampal cultures to $GABA_A$ receptor antagonists causes increased action potential firing, spontaneous rhythmic neuronal depolarizations, and bursting. The rhythmic depolarizations and bursting are dependent upon action potentials as they are eliminated by tetrodotoxin.

MEA recording allows the electrical activity of multiple neurons within the culture to be monitored, whereas FLIPR Fluo-4 fluorescence measurements provide a dynamic assessment of aggregate intracellular Ca^{2+} levels (Cao *et al.*, 2010, 2012). Using these assays, we found that TETS dramatically increases intracellular Ca^{2+} levels and alters Ca^{2+} dynamics, initially causing a transient increase in the $[Ca^{2+}]_i$ followed by a decrease in the frequency of synchronized Ca^{2+} oscillations but bigger transient amplitudes. MEA recordings of ongoing electric activity in the cultures showed an overall increase in discharge frequency and a change in the pattern of these discharges to a more clustered pattern interspersed by periods of electrical silence. The actions of TETS on neuronal Ca^{2+} dynamics and electrical discharge activity occur within the same concentration range, suggesting the two effects are mechanistically linked. The magnitude of the effects produced by TETS increase in a concentration-dependent manner with EC_{50} values of ~1–2μM; TETS inhibits $GABA_A$ receptor responses in other preparations at similar concentrations (Bowery *et al.*, 1975; Dray, 1975; Roberts *et al.*, 1981). Moreover, TETS-induced changes in Ca^{2+} dynamics and electrical discharges resemble those induced by the $GABA_A$ receptor antagonists bicuculline and picrotoxin (Arnold *et al.*, 2005; Cao *et al.*, 2012). Collectively, these observations support the view that the

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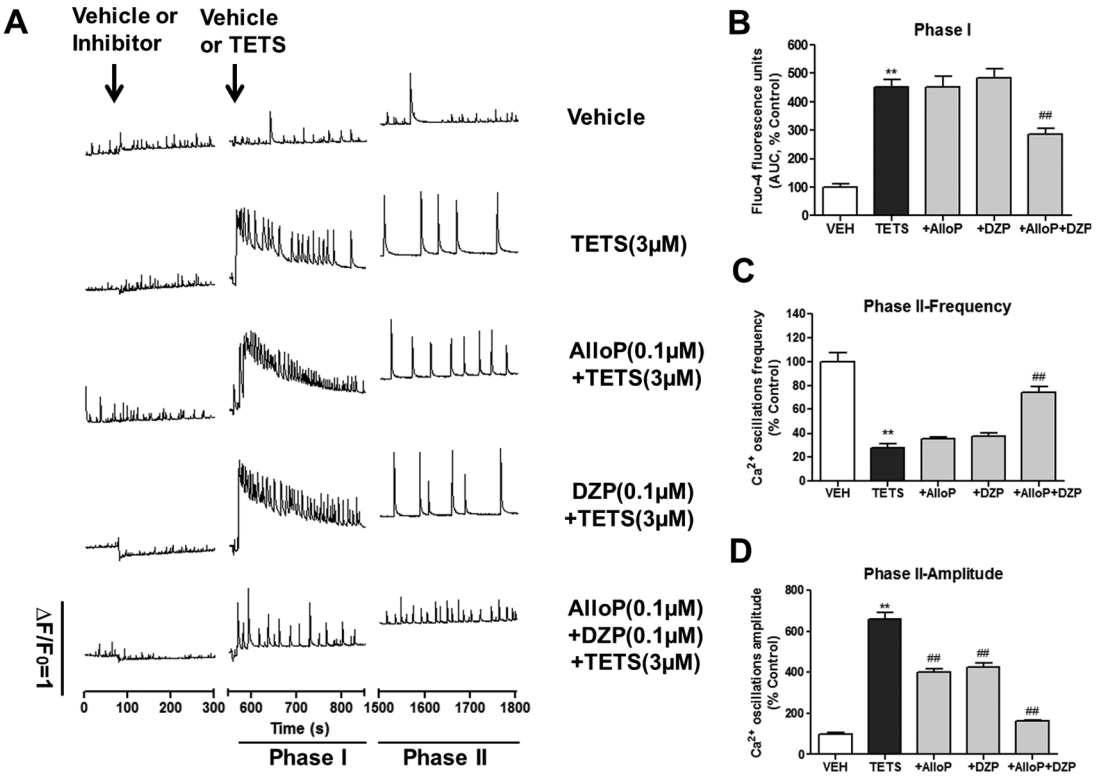

FIG. 7. Low concentrations of allopregnanolone and diazepam in combination act synergistically to mitigate TETS-induced neuronal Ca^{2+} signaling dysregulation. (A) Representative traces illustrating effect of pre-exposure to allopregnanolone (0.1μM), diazepam (0.1μM) or a combination of allopregnanolone (0.1μM) and diazepam (0.1μM) on TETS-induced Ca^{2+} dysregulation. Effects of allopregnanolone or diazepam alone or the combination on TETS-induced initial rise in $[Ca^{2+}]_i$ in phase I (B), the decrease in synchronous Ca^{2+} oscillation frequency in phase II (C), and the increase in Ca^{2+} transient amplitude in phase II (D). $^{**}p < 0.01$, TETS vs. vehicle control, $^{\#\#}p < 0.01$, allopregnanolone/diazepam + TETS vs. TETS, $n = 8$ pooled from two experiments.

$GABA_A$ receptor-blocking activity of TETS is responsible for the effects on Ca^{2+} dynamics and electrical activity. Like picrotoxin, TETS is believed to be a reversible inhibitor of $GABA_A$ receptors, which is also consistent with the rapid reversibility of its effects on burst discharges and Ca^{2+} dynamics.

TETS-triggered alterations in electrical firing and synchronous Ca^{2+} oscillations appear to rely on spontaneous action potentials because they are prevented by tetrodotoxin block of Na^+ channels (data not shown). The neuronal specificity of TETS in producing both phase I and phase II Ca^{2+} responses in hippocampal cultures is indicated by the observations that addition of TETS at concentrations of up to 3μM to the culture medium of skeletal myotubes alters neither basal Ca^{2+} levels nor electrically evoked Ca^{2+} transients (data not shown).

A key observation in this study is that the alterations in Ca^{2+} dynamics induced by TETS were largely inhibited by MK-801 demonstrating that NMDA receptors are required. The effect of TETS is unlikely to be due to direct activation of NMDA receptors inasmuch as bath application of NMDA, which directly activates NMDA receptors, fails to induce clustered burst discharges as observed with TETS and other $GABA_A$ receptor antagonists, although it does increase the overall discharge frequency (Cao *et al.*, 2012). The NMDA receptor dependence for TETS-triggered Ca^{2+} responses is consistent with earlier *in vivo* reports that the NMDA receptor antagonist MK-801 inhibits picrotoxin or bicuculline-induced convulsion in mice (Czlonkowska *et al.*, 2000; Obara, 1995). Moreover, *ex vivo* studies have demonstrated that the NMDA antagonist 2-APV suppresses picrotoxin-induced Ca^{2+} responses as well as the frequency and duration of the epileptiform discharges in the hippocampal slice preparation (Kohr and Heinemann, 1989). How NMDA receptor antagonists inhibit responses to $GABA_A$ receptor blockade remains to be determined. One possibility is that GABA antagonists cause enhanced synaptic glutamate release, leading to activation of synaptic NMDA receptors. The phase I Ca^{2+} response may therefore in part be generated by Ca^{2+} entry through these NMDA receptors. In support of this possibility, bicuculline-induced Ca^{2+} responses have been shown to involve synaptic but not extrasynaptic NMDA receptor activation (Hardingham *et al.*, 2001, 2002).

Consistent with the role of $GABA_A$ receptors in restraining bursting and altered Ca^{2+} dynamics is our observation that the $GABA_A$ receptor-positive modulators diazepam and allopregnanolone are able to protect against the effects of TETS on Ca^{2+} dynamics. Allopregnanolone was more effective in mitigating the phase I response induced by TETS than by

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diazepam. It is well recognized that benzodiazepines such as diazepam only act on synaptic $GABA_A$ receptors, whereas neurosteroids such as allopregnanolone preferentially enhance extrasynaptic $GABA_A$ receptors although they act on synaptic receptors as well (Kokate *et al.*, 1994; Lambert *et al.*, 2003; Reddy and Rogawski, 2012). Therefore, the enhanced efficacy of allopregnanolone may be due to its additional action on extrasynaptic receptors. In addition, at higher concentrations, allopregnanolone is able to directly activate $GABA_A$ receptors in the absence of GABA, whereas benzodiazepines require the presence of GABA. Therefore, there are important pharmacological differences in the action of neurosteroids and benzodiazepines at $GABA_A$ receptors that could account for the enhanced activity of allopregnanolone. However, neither diazepam nor allopregnanolone alone was fully effective, even at the highest concentrations tested (1μM). Unexpectedly, we found that the combination of diazepam and allopregnanolone, each at a threshold concentration of 0.1μM, was highly effective at protecting against the effects of TETS on Ca^{2+} dynamics, causing a nearly complete inhibition of the phase I response, including the plateau in Ca^{2+}, as well as the phase II changes. The combination of a benzodiazepine and a neurosteroid has not to our knowledge previously been studied in a simplified functional system. However, there is evidence from behavioral studies that neurosteroids can potentiate the actions of benzodiazepines (Gerak *et al.*, 2004; Molina-Hernandez *et al.*, 2003). The mechanism underlying the synergistic effect is not known. It is conceivable that the combined action on synaptic and extrasynaptic receptors accounts for the unique potency of the drug combination.

An alternative explanation of the synergism hypothesizes an interaction at the level of individual $GABA_A$ receptors. The recognition sites for neurosteroids on $GABA_A$ receptors are distinct from those that recognize benzodiazepines and barbiturates (Johnston, 1996). It is conceivable, however, that allopregnanolone and diazepam could produce a synergistic enhancement of $GABA_A$ receptors in a similar fashion as the synergism that occurs between barbiturates and benzodiazepines, where there is known to be allosteric coupling (DeLorey *et al.*, 1993). Whether the *in vitro* synergism between diazepam and allopregnanolone observed in measurements of Ca^{2+} dynamics predicts efficacy of the combination in protecting against seizures induced by TETS or caused by other factors remains to be tested.

In summary, we have developed rapid throughput methods to detect TETS-induced Ca^{2+} dysregulation and altered electrical activity in cultured hippocampal neurons. We demonstrated that two $GABA_A$ receptors allosteric modulators, allopregnanolone and diazepam, when introduced either singly or in combination prior to TETS, mitigate TETS-induced Ca^{2+} dysregulation, suggesting that the *in vitro* methods described here have translational value to identify new therapies and optimize combinatorial strategies for the prevention of TETS poisoning. The basic approaches described here are likely to be of general utility for investigating chemically diverse threat agents that elicit changes in the electrical behavior or Ca^{2+} dynamics of neuronal networks *in vitro*. The rapid throughput approaches are also expected to be useful for identifying novel targeted interventions and for optimizing therapeutic strategies involving drug combinations.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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