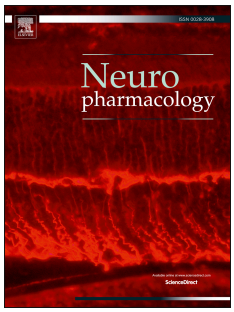
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Positive allosteric modulator of $GABA_B$ receptor alters behavioral effects but not afterdischarge progression induced by partial hippocampal kindling

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Abstract

Hippocampal seizures decreased the function of $GABA_B$ receptors, which may further increase seizure susceptibility and contribute to development of schizophrenia-like behaviors. Recent literature indicates that $GABA_B$ receptor agonist may normalize schizophrenia-like behaviors and prevent drug-induced behavioral sensitization. We hypothesized that positive modulation of $GABA_B$ receptor function during seizure induction will reduce seizure-induced schizophrenia-like behaviors. Using a partial hippocampal kindling model, afterdischarges were induced after injection of saline or dimethyl sulfoxide (vehicle-kindled rats), or a $GABA_B$ receptor positive allosteric modulator CGP7930, at 1 mg/kg i.p. (CGP1-kindled) or 5 mg/kg i.p. (CGP5-kindled). The increase in the primary afterdischarge duration during kindling was not different among the groups. However, the CGP5-kindled group showed a lower afterdischarge starting frequency as compared to vehicle-kindled or CGP1-kindled groups. Partial hippocampal kindling (21 afterdischarges) resulted in decreased prepulse inhibition and decreased gating of hippocampal auditory evoked potentials in vehicle-kindled and CGP1-kindled rats, as compared to saline-injected non-kindled rats, recorded 3-4 days after the last afterdischarge. However, CGP5-kindled rats showed normal prepulse inhibition and hippocampal auditory gating (compared to non-kindled rats), which was significantly higher than the respective measure in vehicle-kindled rats. CGP5-kindled group also showed methamphetamine–induced locomotion that was significant lower than the vehicle-kindled or CGP1-kindled group, but slightly higher than the saline-injected non-kindled rats. In conclusion, this study provides original data that a $GABA_B$ receptor positive allosteric modulator could therapeutically prevent or normalize some seizure-induced behavioral disruptions in a model of temporal lobe epilepsy.

1. Introduction

Temporal lobe epilepsy patients have a high incidence of schizophrenia-like symptoms (Slater and Beard, 1963; Stevens, 1988; Trimble et al., 2010). Positive symptoms, such as hallucinations and delusions, are commonly reported in epileptic patients (Nadkarni et al., 2007), especially in those with frequent seizures and a long history of epilepsy (Elliott et al., 2009).

The etiology of schizophrenia-like symptoms in epileptic patients is not known, but postictal, and possibly some interictal, symptoms could be induced by seizures (Logsdail and Toone, 1988; Trimble et al., 2010). In animals, changes in emotional and schizophrenia-like behaviors were observed following repeated temporal lobe seizures (Adamec, 1990; Ando et al., 2004; Kalynchuk et al., 1998; Leung et al., 2000; Ma and Leung, 2004; Pinel and Rovner, 1978). Kindling of the amygdala or hippocampus resulted in a long-lasting increase in amphetamine- or methamphetamine (METH)- induced locomotor activity (Ma and Leung, 2004; Sato, 1983), likely mediated by increased dopaminergic functions in kindled rats (Csernansky et al., 1988; Gelbard and Applegate, 1994; Strecker and Moneta, 1994). Long-term (> 1 week) prepulse inhibition (PPI) deficit was found after kindling of the dorsal hippocampus and medial prefrontal cortex (Ma and Leung, 2004, 2010). Only short-term (<30 min) PPI deficit was found after a stage 5 amygdala seizure (Koch and Ebert, 1998), and no PPI change was reported after full kindling of the perirhinal cortex or ventral hippocampus (Howland et al., 2007).

$GABA_B$ receptors ($GABA_B$Rs) have been suggested to participate in the electrographic seizures and the seizure-induced behavioral consequences. Alteration in $GABA_B$R function and expression was reported in brain tissues from temporal lobe epilepsy patients (Gambardella et al., 2003; Princivalle et al., 2003; Teichgräber et al., 2009). In animals, genetic ablation (Prosser et al., 2001; Schuler et al., 2001) or pharmacological blockade (Leung et al., 2005; Tsai et al.,

2008; Vergnes et al., 1997) of $GABA_BRs$ resulted in limbic seizures. Kindling resulted in a loss of $GABA_BR$-mediated presynaptic inhibition of GABA (Buhl et al., 1996; Wu and Leung, 1997) and glutamate receptors in the hippocampus (Poon et al., 2006) and amygdala (Asprodini et al., 1992), but an increased postsynaptic $GABA_BR$ response (Liu and Leung, 2003). Status epilepticus also induced loss of $GABA_BR$ function (Chandler et al., 2003; Haas et al., 1996; Mangan and Lothman, 1996) or expression (Sperk, 1994; Straessle et al., 2003). A loss of $GABA_BR$ function may contribute to the schizophrenia-like behaviors induced by kindling in rats. Blockade of $GABA_BRs$ in the hippocampus of normal rats resulted in decrease in both PPI and gating of auditory evoked potentials (AEPs) in the hippocampus (Ma and Leung, 2011). After prolonged early-life seizures, a decrease in hippocampal $GABA_BR$-mediated inhibition and a loss of hippocampal AEP gating was found in adult rats (Tsai et al., 2008).

In normal rats, $GABA_BR$ agonist baclofen, administered systemically, reduced the behavioral hyperactivity and PPI deficits induced by administration of MK-801 or METH (Arai et al., 2008, 2009; Bortolato et al., 2004). A class of drugs, positive allosteric modulator (PAM) of the $GABA_BR$, increases the potency and maximal efficacy of GABA at $GABA_BRs$ (Chen et al., 2006; Froestl, 2010; Urwyler et al., 2001). $GABA_BR$ PAMs have anxiolytic properties in naïve rodents, without the sedative and hyperthermic effects of baclofen (Carai et al., 2004; Cryan et al., 2004). Interestingly, administration of CGP7930 [2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethylpropyl)-phenol], a PAM of the $GABA_BR$, or baclofen, prevented the development and expression of amphetamine-induced locomotor sensitization (Cedillo and Miranda, 2012), and reduced the dependence on alcohol and other addictive drugs (Filip et al., 2015). CGP7930 was also shown to have anticonvulsant properties on pentylenetetrazol-induced seizure (Mareš, 2012) or neocortical afterdischarges (ADs) in immature rats (Mareš et al., 2013).

We are interested in preventing or treating the behavioral disturbances induced by temporal lobe seizures. Seizure-induced behavioral disturbances may involve different neural circuits and mechanisms as compared to seizure generation (Leung et al., 2000), making it possible to prevent behavioral consequences of seizures without necessarily affecting seizure generation and progression. Since loss of $GABA_BR$ function may cause seizure-induced behavioral disturbances, administration of a PAM may increase the efficacy of physiologically activated $GABA_BRs$ (Urwyler et al., 2001) and possibly reduce development of seizure-induced sensitization. Whether administration of CGP7930 during kindling could prevent the behavioral disruptions induced by hippocampal kindling is not known, which is the objective of the present study. We used partial hippocampal kindling, which was known to induce several schizophrenia-like behaviors, including a decrease in PPI and an increase in METH-induced locomotion (Ma and Leung, 2004). We also tested whether gating of auditory evoked potentials in the hippocampus (Ma et al., 2009) was affected by partial hippocampal kindling and CGP7930 administration during kindling. A decrease in PPI and decrease in gating of hippocampal auditory evoked potentials were robust measures of the schizophrenia-like behavior induced by psychoactive drugs such as ketamine, phencyclidine or MK-801 (Miller et al., 1992; Swerdlow et al., 2001; Ma and Leung, 2007; Ma et al., 2009). Our main hypothesis is that kindling with CGP7930 administration will reduce kindling-induced behavioral effects shown as alteration in PPI, gating of hippocampal auditory evoked potentials, and locomotor activity induced by METH. A secondary hypothesis is that kindling with CGP7930 administration will decrease the rate of progression of hippocampal ADs.

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2. Materials and Methods

2.1 Surgical and recording techniques

Under sodium pentobarbital (60 mg/kg i.p.) and buphrenorphine (0.03 mg/kg s.c.) anesthesia, Long-Evans rats were implanted with a pair of hippocampal electrodes bilaterally at AP -3.5 mm, ML ± 2.7 mm (Paxinos and Watson, 1998), with bregma and lambda in a horizontal plane. At each hippocampus, the pair of electrodes consisted of Teflon-coated steel wires of 127 μm inner diameter separated by ~0.7 mm vertically, and the ventral electrode was placed at 3.3 mm ventral to the skull surface. Two ground screws are placed, respectively over the frontal skull and the cerebellum.

Rats were allowed 4-7 days to recover from surgery, after which a single AD (1st AD) was given on the first recording day, without prior injection of drug or saline. The intensity for evoking a hippocampal AD was 3 times the threshold of the commissural evoked potential (Ma and Leung, 2004). A CA1 stimulating electrode was used to evoke an AD, using a 200-Hz train of 1 s duration, with single pulse duration of 0.2 ms. Five consecutive days of kindling followed, with 4 ADs delivered each day, for a total of 21 ADs. AD recordings were stored on computer and on polygraph paper. The behaviors induced by the AD were recorded. Occasional tonic clonic convulsions evoked during the AD were classified according to Racine (1972), namely stage 3 (forelimb clonus), stage 4 (forelimb clonus with rearing), and stage 5 (forelimb clonus, rearing and falling). In the first experiment, four kindling groups of rats were run: (1) **CGP1-kindled** group, which was injected with 1 mg/kg i.p. CGP7930 dissolved in dimethyl sulfoxide (DMSO) of volume (mL x body weight in kg) at 15 min before the 1st AD of the day, and again at 15 min before the 3rd AD of the day. (2) **CGP5-kindled** group, which was treated like the CGP-1 kindled group, except 5 mg/kg i.p. CGP7930 (dissolved in DMSO of volume mL x body weight in kg) was injected. (3) **DMSO-**

kindled group received DMSO injections, in the same volume (mL x body weight in kg) as CGP7930-injected rats, at 15 min before the 1st and 3rd AD of the day. (4) **Saline-kindled** group received saline injections (volume = mL x body weight in kg), at 15 min before the 1st and 3rd AD of the day. The ADs during one day were delivered at an interval of 1 h. When a rat showed a stage 3 or higher seizures, the next AD was given at 2 h instead of 1 h interval, in order to reduce the refractory effect of a generalized seizure. CGP1-kindled or CGP5-kindled rat(s) were always run together with 1-2 saline-kindled rats, to enhance the similarity of treatment between groups of rats. AD duration measurements were recorded mostly at electrodes in the contralateral CA1; a few electrodes in the contralateral dentate gyrus were included since they showed similar duration as CA1 electrodes (Leung, 1987). We found that acute injection of 1 mg/kg i.p. CGP7930 was sufficient to normalize kindling-induced deficit of PPI in a kindling-sensitive strain of Long-Evans rats (Ma and Leung, 2016).

Three groups of non-kindled rats were run. One group named **saline-nonkindled** had electrodes implanted at similar locations and received saline injections at similar times as the kindled group, but received no hippocampal kindling. The saline-nonkindled group was used as a control group for PPI and auditory gating. Two other groups of non-kindled rats, not implanted with electrodes, were run in tandem, with one group injected with CGP7930 1 mg/kg i.p. (**CGP1 only**), and another injected with saline (**saline only**), at similar times as the kindled rats. CGP1-only and saline-only groups were tested in the METH locomotion test at 5 days after the last saline/CGP7930 injection.

A subgroup of CGP1-kindled and saline-kindled rats was further tested with PPI, on the 14th and 19th day after the last AD. A rat was given an acute injection of DMSO (0.3 ml) i.p. on

the 14th day, and CGP7930 (1 mg/kg i.p.) on the 19th day; PPI measurements commenced 15 min after injection.

2.2 Prepulse inhibition, hippocampal auditory evoked potentials, and locomotor activity tests

PPI was tested three days after the last AD (21st AD) in the kindled groups, or the last injection in the saline-nonkindled group. Acoustic stimuli were presented and startle amplitude, detected by a piezoelectric accelerometer, were recorded by a microcomputer using SR-Lab (San Diego Instruments, San Diego, CA) software and interface (Ma and Leung, 2011). Bursts of acoustic noise were given by a loudspeaker above a rat placed in a semi-restraint chamber. After acclimation for 5 min with a 68 dB background noise, the rat was given a startle pulse only (120 dB 40-ms broad band burst), or a startle pulse preceded 100 ms by a prepulse of intensity of 73, 75, or 80 dB (20-ms broad band noise). For each test session, 50 trials were given in randomized order – 10 trials with startle pulse only, 10 trials with no stimulation, and 10 trials of each of the three prepulse intensities followed by a startle pulse. The intertrial interval was 15 s. PPI was measured as the difference between the response to the startle pulse alone and the response to the prepulse-startle, or PPI (in percent) = 100 * [1 - (mean startle response amplitude after a prepulse /mean amplitude of response to startle alone)].

Hippocampal auditory evoked potentials (AEPs) were recorded on the 4th day after the last AD/injection in the kindled groups, and the saline-nonkindled group, using the same semi-restraining Plexiglas chamber (Ma et al., 2009). Evoked potentials were recorded following auditory click pairs, given 15 s apart, with a 520-ms conditioning-test (C-T) interval. Twenty-five sweeps of evoked potentials (sampled at 10 kHz) were collected, and sweeps with artifacts (typically < 2 sweeps) were excluded from averaging.

On the 5-6th day after the last AD/injection, METH-induced locomotor activity was assessed in the kindled groups, CGP1-only and saline-only groups. Locomotor activity was recorded in a movement chamber with infrared beam detectors on a horizontal plane (Columbus Instruments). Counts of interruptions of infrared beams started at 15 min after injection of 1 mg/kg i.p. METH, and were accumulated in 10-min intervals, for a total duration of 120 min. Locomotion data of one saline-K rat who jumped out of the arena during testing, and one DMSO-K rat with a low weight were excluded.

2.3 Data analysis

Data were presented as mean +/- standard error of the mean (SEM). Repeated measures Analysis of Variance (ANOVA) or t-test was applied, as appropriate. A significant main or interaction effect was followed by post hoc multiple comparisons using Newman-Keuls or Fisher's Least Significant Difference (LSD) protected t test. Significance level was set as $P<0.05$. Groups that showed inhomogeneous variance were compared by a Wilcoxon signed rank test.

For the analysis of the AD starting frequency, 2.56 s (or 512 points sampled at 200 Hz) was selected from the start of the high-amplitude AD recorded at both surface and deep electrodes of the contralateral CA1, and subjected to power spectral analysis (Leung et al., 1982). The peak frequency and power were taken from the point of maximal power.

After testing and confirming a lack of statistical difference of one measure (AD duration, AD starting frequency, PPI, AEP, locomotion) between saline-kindled and DMSO-kindled groups, the two groups were combined into a single vehicle-kindled (Veh-K) group. Using a combined vehicle-kindled group gave a larger sample size for more robust statistical testing. The main results were similar if the DMSO-kindled or saline-kindled group was used instead of the vehicle-kindled group. Ensemble averages (average of all rats of each group) of the AEPs

recorded in CA1 were made for 4 groups (Veh-K, CGP1-K, CGP5-K and saline-injected non-kindled groups).

Only electrodes confirmed, after histological confirmation, to be at the apical dendritic layer of hippocampal CA1 were selected for AEP analysis. At CA1 apical dendritic layer, the AEP showed a mainly negative peak at 20-30 ms latency, with potential reversal across the CA1 cell layer, suggesting that this AEP peak was generated in CA1 (Brankack and Buzsáki, 1986; Ma et al., 2009). For each rat, the first 6 time points (1 ms per point) following the 1st conditioning (auditory) pulse were averaged and used as the baseline for the 1st response, while the 20 points before the test auditory pulse were averaged and used as the baseline for the test (2nd) response. The T/C ratio was calculated as the ratio of the peak amplitude of the AEP (above the respective baseline) in response to the 2nd test pulse (T) to that in response to the 1st conditioning pulse (C). Differences between the detailed time courses of AEPs were assessed as follows. At each digitized time point, AEP values of the two groups were compared using a unpaired Wilcoxon signed rank test, and time points that were significantly different with $P<0.05$ criterion were noted. Since multiple (100 points typically) comparisons were made, the two groups of AEPs were considered statistically different only when 5 consecutive points satisfied the $P<0.05$ criteria.

2.4 Histology

Upon completion of experiment, the rat was deeply anesthetized with pentobarbital (80 mg/kg i.p.) and transcardially perfused with 0.9% saline followed with 4% formalin. The brain of the rat was removed and cut into 40 μm coronal sections with a freezing microtome. The brain sections were mounted on glass slides and stained with thionin for identifying locations of the electrodes.

3. Results

3.1 Afterdischarge parameters and associated behaviors

Eight CGP1-kindled, 6 CGP5-kindled, 9 saline-injected (saline-kindled), and 6 DMSO-injected (DMSO-kindled) rats completed partial kindling of 21 hippocampal ADs. The first (1^{st}) and final (21^{st}) AD stimulus intensity were 133.3 ± 8.9 μA (n=29) and 169.5 ± 15.5 μA (n=29), and the AD stimulus intensity was not different among the groups [$F(3,25) = 1.4$; $P > 0.2$, one-way ANOVA]. In about one third (31%) of the rats, an increase in AD stimulus intensity during kindling was needed to reliably evoke an AD. A hippocampal kindled AD consisted of primary and secondary components (Fig. 2). The high-amplitude discharges of either the primary or the secondary AD were positive at the CA1 apical dendrites (Fig. 2A), and negative at CA1 alveus (not shown). The primary or secondary AD duration was not different between saline-kindled (n=9) and DMSO-kindled (n=6) groups [2-factor (group x kindling) ANOVA group effect $F(1,13) < 0.7$, $P > 0.4$]. A single control kindled group, named **vehicle-kindled** group (n =15), including both saline-kindled and DMSO-kindled rats, was used for statistical comparison with CGP7930-injected rats.

The primary AD duration increased with kindling (AD number) for all groups, but primary AD duration during kindling was not different among vehicle-kindled, CGP1-kindled and CGP5-kindled groups (Fig. 1A, B). This was confirmed by a two-way (group x kindling) repeated measures ANOVA, which showed a significant kindling (AD number) effect [$F(19,494) = 15.3$, $P < 0.0001$**;** first AD not included] but no significant group effect [$F(2,26) = 1.38$; $P > 0.27$]. For the ADs evoked on a single day, the AD duration tended to start relatively short for the first AD of the day (up arrow in Fig. 1B). The secondary AD, which appeared after the primary AD subsided (Fig. 1A), also increased its duration with kindling (Fig. 1C). Two-way repeated measures ANOVA of the secondary AD duration showed a significant kindling effect [$F(19,494) = 3.89$, $P < 0.0001$] but

no significant group effect [$F(2,26) = 1.7$, $P > 0.2$]. However, variability of the secondary AD duration contributed to the lack of difference among groups. Averaging the secondary AD duration for first half (2^{nd} to 10^{th} AD; early) and second half (11^{th} to 21^{st} AD; late) of kindling revealed that the average secondary AD duration was shorter for the CGP5-kindled group than the vehicle-kindled group during late kindling ($P < 0.05$, Wilcoxon; Fig. 1E1). The number of convulsive seizures (stage 3 and stage 4 seizures) induced during partial kindling was small – saline-kindled group (3 of 9 rats), DMSO-kindled (1 of 6 rats), CGP1-kindled (3 of 8 rats), and CGP5-kindled (1 of 6 rats), and the convulsive seizure frequency was not different among groups ($P > 0.7$, Chi square test).

The number of wet dog shakes (WDSs) evoked by each AD was similar among the three groups of kindled rats, but decreased in general with kindling (Fig. 1D). A two-way repeated measures ANOVA confirmed a kindling effect [$F(19,494) = 3.69$, $P < 0.0001$] but no group effect [$F(2,26) = 0.06$, $P > 0.9$]. However, when the average number of WDSs in each group was evaluated during the first and second half of kindling, a statistically significant decrease in WDS number was found for the vehicle-kindled and CGP1-kindled groups, but not for the CGP5-kindled group (Fig. 1E2).

3.2 AD starting frequency indicated CGP7930's effect on the hippocampus

In a previous study, we showed that the starting frequency of an electrically evoked hippocampal AD was increased by $GABA_B$ receptor blockade (Leung et al., 2005). Here, we evaluated the effect of $GABA_B$ positive modulator CGP7930 (1 and 5 mg/kg i.p.) and vehicle on the frequency of the first 2.56 s of the AD, for all 21 ADs evoked (Fig. 2A, B). Rats with complete digital records were evaluated from each group. The starting frequencies of saline-kindled (n=6) and

DMSO-kindled groups (n=4) were not different from each other, and they were combined into one vehicle-kindled group (n=10). The first AD of the 21 ADs delivered (called AD#1 to distinguish from primary AD) was evoked without injection, and its starting frequency did not differ among vehicle-kindled, CGP1-kindled and CGP5-kindled groups, and measured 4.31 ± 0.13 Hz (n=22; overall average). Two-factor (group x kindling from 2^{nd} to 21^{st} AD) repeated measures ANOVA of the AD starting frequency of the three groups of rats indicated a significant group effect [$F(2,19) = 6.57$, $P<0.01$], and a significant kindling effect [$F(19,361) = 1.65$, $P<0.05$]. Differences between groups were found between CGP5-kindled and vehicle-kindled groups, and between CGP5-kindled and CGP1-kindled groups, as indicated by significant post hoc comparisons at specific time points (Fig. 2B), or two-way repeated measures ANOVA comparing two groups (data not shown). The average AD starting frequency for the last 20 ADs was 3.81 ± 0.13 Hz (n=10) for the vehicle-kindled group, 4.07 ± 0.25 Hz (n=6) for the CGP1-kindled group, and 3.28 ± 0.09 Hz (n=6) for the CGP5-kindled group.

3.3 Prepulse inhibition

Prepulse inhibition (PPI) was tested 3 days after the last AD/ injection. PPI of the saline-kindled group (n=9) was not statistically different from that of the DMSO-kindled group (n=6) [non-significant group and interaction effects, $P>0.3$, two-way (prepulse intensity x group) ANOVA]. Thus, saline- and DMSO-kindled groups were combined together as a vehicle-kindled group (n=15). Among the 4 groups of rats – saline-injected non-kindled, vehicle-kindled, CGP1-kindled, and CGP5-kindled groups, two-way (group x prepulse intensity) repeated measures ANOVA showed no significant differences in the startle response amplitudes [$F(3,32) = 0.21$, $P>0.89$]. There was also no significant group effect [$F(3,32) = 2.42$, $P=0.084$], but a significant

prepulse intensity effect [F(2,64) =19.7, P<0.0001; Fig. 3A1]. Since the variance of the lowest prepulse (73 dB) response was particularly large for all kindled groups, the average PPI following 75 and 80 dB prepulses was used for group comparison. The latter average PPI value was significantly different among the 4 groups of rats [F(3,32) = 3.66, P<0.03, one-way ANOVA] with post hoc differences indicated in Fig. 3A2, i.e., the vehicle-kindled group showed a lower PPI as compared to the non-kindled or CGP5-kindled group. In summary, as compared to a non-kindled saline-injected group, partial hippocampal kindling significantly decreased PPI in response to higher prepulse intensities (75 and 80 dB), except in CGP5-kindled group.

The effect of acute CGP7930 (1 mg/kg i.p.) versus DMSO injection on PPI was tested in a subgroup of kindled rats, at 14-19 days after the last AD. Acute CGP7930 injection increased PPI as compared to acute DMSO injection in both groups of kindled animals (Fig. 3B). Two-way ANOVA showed a significant drug (acute CGP7930 versus acute DMSO) effect in 5 rats of the CGP1-kindled group [F(1,4) =8.14, P<0.05]. In 5 rats of the saline-kindled group, there was a significant drug x prepulse intensity interaction [F(2,8) = 4.94, P<0.05], without a significant drug effect [F(1,4) =1.04, P>0.3]. The interaction effect was shown as an increase in PPI by acute CGP7930 at the lowest prepulse intensity of 73 dB (P<0.01, Fisher's LSD protected t-test). Startle amplitudes were not significantly different (P>0.12, paired t-test) between acute CGP7930-injected and acute DMSO-injected groups, in either CGP1-kindled or saline-kindled rats (data not shown).

3.4 Gating of hippocampal average auditory evoked potentials (AEPs)

Hippocampal AEPs were recorded at 4 days after the last AD/ injection, using only selected electrodes at the CA1 apical dendritic layer (Fig. 4). After baseline adjustment for the AEP of each rat (Methods), the latency and magnitude of the negative peak following the

conditioning (1^{st}) and test (2^{nd}) pulse were determined. AEPs of the saline-kindled group (n=6) were not statistically different from AEPs of the DMSO-kindled group (n=4) in the T/C ratio (P>0.2, t-test) or in the detailed time course (not shown). Thus, the two groups were combined as a vehicle-kindled group (n=10). For the four groups (saline-nonkindled, vehicle-kindled, CGP1-kindled and CGP5-kindled), there was no difference in the peak latency of the 1^{st} or 2^{nd} pulse (one-way ANOVA, P> 0.18). There was also no difference in the magnitude of the conditioning-pulse peak response [$F(3, 22) = 1.93$, P> 0.15; Fig. 4A1].

The T/C ratio was significantly different among the four groups – saline-nonkindled, vehicle-kindled, CGP1-kindled, and CGP5-kindled groups [$F(3, 22) = 5.9$, P<0.005, 1-way ANOVA], with post hoc Fisher's LSD protected-t test differences illustrated in Fig. 4A2. The T/C ratio was highest in the CGP1-kindled group (0.59 ± 0.06, n=5), and lowest in the CGP-5 kindled group (0.25 ± 0.04, n=6), as compared to the vehicle-kindled group (0.46 ± 0.06, n=10), and saline-nonkindled group (0.30 ± 0.05, n=5). CGP1-kindled group, but not the vehicle-kindled group, showed a statistically higher T/C ratio than the saline-nonkindled group, while CGP5-kindled group showed a T/C ratio similar to the nonkindled group, but significantly lower than that of the vehicle-kindled group (Fig. 4A2).

The AEPs were compared between two groups at fixed time points (Methods). Similar to the peak amplitude, the conditioning-pulse (1^{st}) response was not different between group pairs (Fig. 4B1, Fig. 4D1), except being larger, before the negative peak, in the saline-nonkindled group than the CGP1-kindled group (Fig. 4C1). Robust differences between group pairs were found shortly after the negative peak evoked by the 2^{nd} (test) pulse (Fig. 4B-D, column 2). The test-pulse negative peak was larger in the CGP1-kindled group than the saline-nonkindled group (Fig. 4C2) or the CGP5-kindled group (Fig. 4D2). The test-pulse responses were different

between vehicle-kindled and saline-nonkindled groups after the negative peak (Fig. 4B2), although not in their peak T/C ratio (Fig.4A2). There were also some differences between groups in the test-pulse response before the negative peak, at ~532 ms latency or ~12 ms from the 2nd pulse (Figs. 4B2, 4C2, 4D2). The early ~12 ms AEP response may not be generated in the hippocampus (cf. Brankack and Buzsáki, 1986).

3.5 Methamphetamine-induced locomotion increased after kindling with CGP7930

When tested 5 days after kindling, METH-induced locomotion (infrared beam interruptions) was not different between saline-kindled (n=8) and DMSO-kindled (n=5) groups [two-way (group x time) ANOVA, $F > 1.1$ for both group and interaction effects, $P > 0.25$]. Thus, saline-kindled and DMSO-kindled groups were combined into one vehicle-kindled group (n=13). METH-induced locomotion was significantly different among the vehicle-kindled, CGP1-kindled, CGP5-kindled and saline non-kindled groups (Fig. 5A). Two-way (4 groups x 12 times) repeated measures ANOVA of the infrared beam interruptions after METH injection showed significant group [$F(3, 29)= 3.76$, $P < 0.03$] and time [$F(11,319) = 66.2$, $P < 0.0001$] effects, and a significant group x time interaction [$F(33,319) = 2.45$, $P < 0.0001$]. Significant post hoc comparisons, using Fisher's LSD protected t-test, are shown in Fig. 5A. The CGP1-kindled group, as compared to the vehicle-kindled group, showed higher locomotion at an early time (15 min, indicated by**), and decreased locomotion at a late time (45-55 min, indicated by*) after METH injection (Fig. 5A). METH-induced locomotion in the CGP5-kindled group was significantly lower than that of the CGP1-kindled group (indicated by $^{\#}$), and also lower than that of the vehicle-kindled group at 30-45 min post-injection (indicated by $^{\&}$ in Fig. 5A). METH-induced locomotion in the saline non-kindled group was significantly different from any of the kindled group (Fig. 5A).

METH-induced locomotion was tested in non-kindled, non-implanted rats given repeated injections (CGP1-only and saline-only groups). The saline-only and CGP1-only groups were not statistically different in their METH-induced infrared beam interruptions [2-way ANOVA, $P > 0.5$ for both group effect, and group x time interaction effect; Fig. 5B]. When kindled and non-kindled groups were compared, two-way repeated measures ANOVA showing a significant group effect for METH-induced locomotion of the vehicle-kindled [$F(1,17)= 10.8$, $P<0.001$] compared to the saline-only (non-kindled) group. Similarly, METH-induced locomotion was higher in CGP1-kindled as compared to the CGP1-only (non-kindled) group, with 2-way repeated measures ANOVA showing a significant group x time interaction [$F(11,132)= 5.2$, $P<0.0001$] and a trend for the group effect [$F(1,12)= 3.47$, $P=0.087$].

3.6 CGP7930 effects on weight gain

The non-kindled saline-injected group showed a larger weight gain than the non-kindled CGP-1 only group, on the 5th day of injection (Supplementary Fig. 1). CGP7930 or DMSO, as compared to saline, appeared to suppress weight gain. Among 4 groups of rats (saline-kindled, DMSO-kindled, CGP1-kindled, and CGP5-kindled groups), the group effect was marginally non-significant [$F(3,25) =2.45$, $P=0.087$, 1-way ANOVA]. Subsequent weight gain (from day 5 to day 10) was also not significantly different between the different kindled groups or non-kindled groups (Supplementary Fig. 1).

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4. Discussion

The present study showed that vehicle-injected hippocampal partially kindled rats, as compared to saline-injected, non-kindled rats showed a decrease in PPI and an increase in METH-induced locomotion, confirming a previous report that kindling induced schizophrenia-like behaviors (Ma and Leung, 2004). CGP1-kindled group showed a higher kindling-induced increase in METH-induced locomotion than the vehicle-kindled group, and a significant increase in hippocampus auditory T/C ratio as compared to non-kindled rats. By contrast, in the CGP5-kindled group, PPI, hippocampal auditory gating, and METH-induced locomotion were normalized as compared to the vehicle-kindled group. Kindling-induced prolongation of primary hippocampal AD duration was not affected by CGP7930, but the average secondary AD duration was decreased in the CGP5-kindled group as compared to vehicle-kindled group during late kindling.

4.1 Low doses of CGP7930 reduced weight gain

CGP7930 (1 mg/kg i.p.) as compared to saline injection alone significantly decreased weight gain during a 5-day injection period. However, CGP7930 (1 and 5 mg/kg i.p.) as compared to solvent DMSO injection during kindling had a marginally non-significant effect on decreasing weight gain in rats during the days of injection. Weight gain in response to repeated low doses of CGP7930 has apparently not been reported. However, $GABA_BR$ agonist baclofen was reported to increase short-term food intake but decrease weight gain (Patel and Ebenezer, 2010), and Ebenezer (2012) reported that a single dose of 12 mg/kg i.p. CGP7930 was required to increase short-term (< 2 h) food intake.

4.2 CGP7930 altered AD starting frequency and secondary AD but not primary AD duration

The starting frequency of an AD in the hippocampus was shown to be a novel indicator of CGP7930's action on the brain, revealing participation of $GABA_BRs$ in the hippocampal AD. The hippocampal AD starting frequency evoked by CA1 electrical stimulation was ~4 Hz, and this frequency was stable for 10-15 s, and for the 21 ADs during partial kindling. We reported previously that $GABA_BR$ blockade increased the hippocampal AD starting frequency (Leung and Shen, 2006). Here, we showed that positive allosteric modulation of the $GABA_BRs$ by 5 mg/kg i.p. CGP7930 significantly decreased the AD starting frequency (Fig. 2). The mechanism of rhythmic ADs has not been systematically studied, but we propose that the AD may arise from rhythmic bursting in a network of CA3 neurons (Miles et al., 1984; Bragin et al., 1997), in which $GABA_BRs$ modulate recurrent excitation and the level of postsynaptic hyperpolarization in CA3. Increasing postsynaptic $GABA_BR$ potency and efficacy by a PAM may reduce the depolarizing drive and rhythmic bursting frequency. Lang et al. (2014) reported that another $GABA_BR$ PAM (GS39783) reduced the occurrence of spontaneous hippocampal interictal spikes following an AD in behaving mice, which also indicated a reduction of excitability in the CA3 network.

The increase in primary and secondary hippocampal AD durations with AD number (Fig. 1B,C) is an indication of seizure progression during kindling. The kindling-related increase in primary or secondary AD duration, frequency of convulsive seizures, or decrease in number of WDSs, was not different among saline-, DMSO-, CGP1- and CGP5-kindled groups (Fig. 1). However, increase in secondary AD duration and decrease in WDSs for the late as compared to early half of the kindling period were robust in the vehicle-kindled and CGP1-kindled groups, but not statistically significant in the CGP5-kindled group (Fig. 1E). The secondary AD was also significantly shorter in the CGP5-kindled group than the vehicle-kindled group during late kindling (Fig. 1E1). Given that the secondary AD was partly generated by re-entry of temporal

(entorhinal and amygdala) paroxysmal activity to the hippocampus through the perforant path (Leung, 1987), a lack of kindling-associated increase in secondary AD duration in the CGP5-kindled group suggests reduced seizure spread from the hippocampus to the temporal cortex, and vice versa.

WDS number during hippocampal kindling was not significantly changed by CGP7930 (Fig. 1D). This is consistent with the conclusion that $GABA_BRs$ are not critically involved in the AD-associated WDSs, notwithstanding the fact that exogenous application of a $GABA_BR$ agonist (baclofen) could induce WDSs (Leung and Shen, 2006).

4.3 Effect of CGP7930 on PPI, auditory gating, and METH-induced locomotion after partial hippocampal kindling

When tested at 3 days after kindling, PPI was decreased in vehicle-kindled as compared to saline-injected, non-kindled rats. CGP7930 (5 mg/kg i.p.) injected during kindling normalized the kindling-induced decrease in PPI, such that PPI in the CGP5-kindled rats was significantly higher than that in vehicle-kindled rats, and not statistically different from that in non-kindled rats (Fig. 3A2). PPI in CGP-1 kindled rats was not significantly increased from that in vehicle-kindled rats.

We found that a single **acute** injection of CGP7930 (1 mg/kg i.p.) increased PPI in CGP1-kindled and saline-kindled rats (Fig. 3B). A $GABA_B$ PAM is expected to acutely increase PPI, since $GABA_BR$ agonist baclofen normalized PPI deficits induced by MK-801 (Bortolato et al., 2004) and methamphetamine (Arai et al., 2008), presumably by reducing of dopamine release (Bortolato et al., 2004), or normalizing neural activity in the caudal pontine reticular nucleus (Arai et al., 2008). This contrasts with an increase in PPI in heterozygous $GABA_{B1}$-deficient

mice (Prosser et al., 2001), which was not reproduced by systemic $GABA_BR$ blockade (Bortolato et al., 2004).

The T/C ratio of the auditory evoked potential in the hippocampus was higher in the CGP1-kindled group than the saline non-kindled group (Fig. 4A2). However, CGP5-kindled rats showed a mean T/C ratio that was significantly decreased from that in the vehicle-kindled or CGP1-kindled group, and not significantly different from that in saline, non-kindled group. These results suggest that 5 mg/kg i.p. CGP7930 normalized the kindling-induced auditory gating loss.

METH-induced locomotion was initially increased, and then later suppressed, in the CGP1-kindled group as compared to the vehicle-kindled group (Fig. 5). However, METH-induced locomotion in the CGP5-kindled group approached the level of saline non-kindled rats, and was significantly lower than that in the vehicle-kindled group.

4.4 Proposed mechanism of action of PAM of $GABA_B$ receptors

CGP7930 (5 mg/kg i.p) administration during kindling acted on the hippocampus, as evidenced by the decrease in starting frequency of the hippocampal AD, which gave an indicator of the CA3/CA1 output. CGP7930 (5 mg/kg i.p) also resulted in a relative lack of progression of the secondary AD, which likely depended on glutamatergic pathways from the hippocampus to the entorhinal/ perirhinal cortex. Decreased hippocampal neuronal firing and glutamatergic output may result from CGP7930's potentiation of postsynaptic $GABA_BRs$ or presynaptic heteroreceptors. CGP7930 (30 μM) in vitro was reported to potentiate autoreceptors on GABAergic terminals in the hippocampus, but did not affect heteroreceptors on glutamatergic

terminals that generated the field excitatory postsynaptic potentials in CA1 (Chen et al., 2006; Parker et al., 2008).

Hippocampal kindling resulted in a decrease in hippocampal $GABA_B$ heteroreceptor function (Poon et al., 2006), which may contribute to the kindling-induced loss in PPI and the increase in auditory T/C ratio reported here (Ma and Leung, 2011). Whether there was a kindling-induced $GABA_BR$ function decrease in the hippocampus of CGP5-kindled animals is not known.

CGP7930 may affect dopamine release of VTA neurons in the nucleus accumbens during hippocampal kindling. Glutamatergic afferents from the hippocampus to the nucleus accumbens induce dopamine release from VTA axon terminals in the nucleus accumbens (Blaha et al., 1997; Luo et al., 2013; Mogenson et al., 1993; Pennartz et al., 1994). Repeated seizures increase sensitivity of the mesolimbic dopaminergic system, and may predispose rats to schizophrenia-like behaviors (Adamec, 1990; Leung et al., 2000). The lack of kindling-induced changes in schizophrenia-like behaviors (e.g., PPI or auditory gating) in the CGP5-kindled group may result form a lack of sensitization of the mesolimbic dopaminergic system during kindling. Reducing glutamatergic inputs from hippocampal and temporal lobe to the nucleus accumbens, and potentiating postsynaptic $GABA_BRs$ on VTA dopaminergic neurons (Chen et al., 2005; Cruz et al., 2004) may reduce dopamine release during each AD and reduce sensitization of the mesolimbic dopaminergic system.

An increase in hippocampal glutamatergic output and VTA dopaminergic firing during a kindled AD may follow an injection of 1 mg/kg i.p. CGP7930, perhaps because of selective enhancement of $GABA_B$ autoreceptor function. The latter would decrease GABA release in the hippocampus and VTA, resulting in higher firing and release of dopamine/glutamate from VTA

neurons (Giorgetti et al., 2002; Laviolette and van der Kooy, 2001) during an AD. As a consequence, the CGP1-kindled as compared to the saline-kindled group may show more behavioral sensitization, manifested by decreased gating of hippocampal AEP and increased METH-induced initial locomotor activity.

4.5 Therapeutic properties of PAM of $GABA_B$ receptors in epilepsy

We suggest that $GABA_BR$ PAM may prevent postictal psychosis, and improve interictal schizophrenia-like symptoms in temporal lobe epilepsy patients. Results in the present study suggest that a PAM of $GABA_BR$ (CGP7930) at 5 mg/kg i.p. suppressed the kindling-induced behavioral changes, and ameliorated interictal schizophrenia-like symptoms in rats, without significant changes in primary seizure duration or severity. A slight concern is that a low dose of CGP7930 (~ 1 mg/kg i.p.) may increase sensitization of the mesolimbic dopaminergic system, such that a therapeutic dose has to be maintained at an appropriate level (near 5 mg/kg i.p. in the present study). Further understanding of the action of $GABA_BR$ PAMs on specific $GABA_BRs$ in the hippocampus and VTA would help to elucidate the mechanism and therapy of seizure-induced behavioral disruptions.

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Figure Legend

Figure 1. Afterdischarge (AD) duration and number of wet dog shakes (WDSs) during partial hippocampal kindling. **A.** The 19th AD of representative rats kindled after injection of saline (saline-K), CGP7930 1 mg/kg i.p. (CGP1-K) and CGP7930 5 mg/kg i.p. (CGP5-K). Duration of primary and secondary AD shown for the top trace only. Stimulation (S) was a 1-s high-frequency train; WDS episode indicated by 'x' under traces. **B.** Mean +/- SEM of primary AD duration for the 21 ADs evoked on day 0 (d0), day 1- day 5 (d1-d5). Vehicle-kindled group combined saline-kindled and DMSO-kindled groups. A dip in AD duration was observed at the start of d2-d5 in most groups (up-pointing arrow). **C**. Mean +/- SEM of secondary AD duration, and **D.** mean +/- SEM of number (#) of WDSs plotted with AD number. **E**. Secondary AD duration (**E1**) and number of WDSs (**E2**) averaged for early (2nd to 11th AD) and late kindling (12th to 21st AD) show increase in 2nd AD duration and decrease in WDSs with kindling for all groups, except for the CGP5-K group. * $P<0.05$, Wilcoxon test, different from vehicle-K group.

Figure 2. Afterdischarge (AD) starting frequencies were different following injection of vehicle, CGP7930 1 mg/kg (CGP1), and CGP7930 5 mg//kg i.p. (CGP5). **A.** Representative ADs (kindling AD#6) evoked in the saline-, CGP1-, and CGP5-kindled groups show 9.5, 11 and 8 peaks during the initial 2.5 s (calibration bar), respectively. **B.** Plot of AD starting frequency, mean +/- SEM, for the three groups during kindling. The vehicle-kindled group combined a saline-kindled (n=6) and a DMSO-kindled group (n=4). AD #1 (first of 21 ADs) of all groups were evoked without injection, while subsequent ADs (#2 to #21) were evoked following either vehicle, CGP1, or CGP5 injection. For the AD#2 to #21, AD starting frequency was significantly lower in the CGP5-kindled group (2-way ANOVA; * $P<0.05$, compared with CGP1-kindled

group; # P<0.05, compared with vehicle-kindled group; post hoc Fisher's LSD protected t-test after a significant 2-way ANOVA).

Figure 3. Prepulse inhibition (PPI) decreased in the vehicle-kindled group as compared to a non-kindled group, and was normalized in the CGP5-kindled group. **A.** Column 1: Prepulse inhibition (percent of startle-alone response) at three prepulse intensities, tested on 3 days after treatment, shown as mean +/- SEM for group of Non-kindled (Non-K) saline-injected rats, a group kindled after injection of vehicle (Vehicle-K), which included both saline and DMSO, or groups kindled after injection of CGP7930 1 mg/kg (CGP1-K) or CGP7930 5 mg/kg (CGP5-K). Column 2: The integrated (mean) PPI for the higher intensity prepulses (at 75 and 80 dB) show statistical differences among the groups (1-way ANOVA); * P<0.05, ** P<0.01, post hoc Fisher's LSD protected t-test. **B.** PPI tested following acute injection of DMSO (14 days after kindling), or acute injection of 1 mg/kg i.p. CGP7930 (19 days after kindling), in the CGP1-kindled (column 1) and the Saline-kindled (column 2) groups. B1. * indicates difference between two groups (main effect, 2-way ANOVA). B2 * indicates difference at 73 dB prepulse intensity (significant interaction effect, 2-way ANOVA).

Figure 4. Test/Conditioning (T/C) ratio of hippocampal auditory evoked potentials (AEPs) was increased by kindling and normalized by 5 mg/kg i.p. CGP7950. **A1.** Group mean + SEM of the peak conditioning-pulse amplitude (C-amp), as illustrated in an AEP trace recorded in CA1 stratum radiatum (top). There were no statistical significant differences among groups – Saline-NonK (nonK): saline-injected, non-kindled; vehicle-K (Veh-K) including both saline-kindled and DMSO- kindled groups; CGP1-K (CGP1): CGP7930 1 mg/kg i.p. injected and kindled;

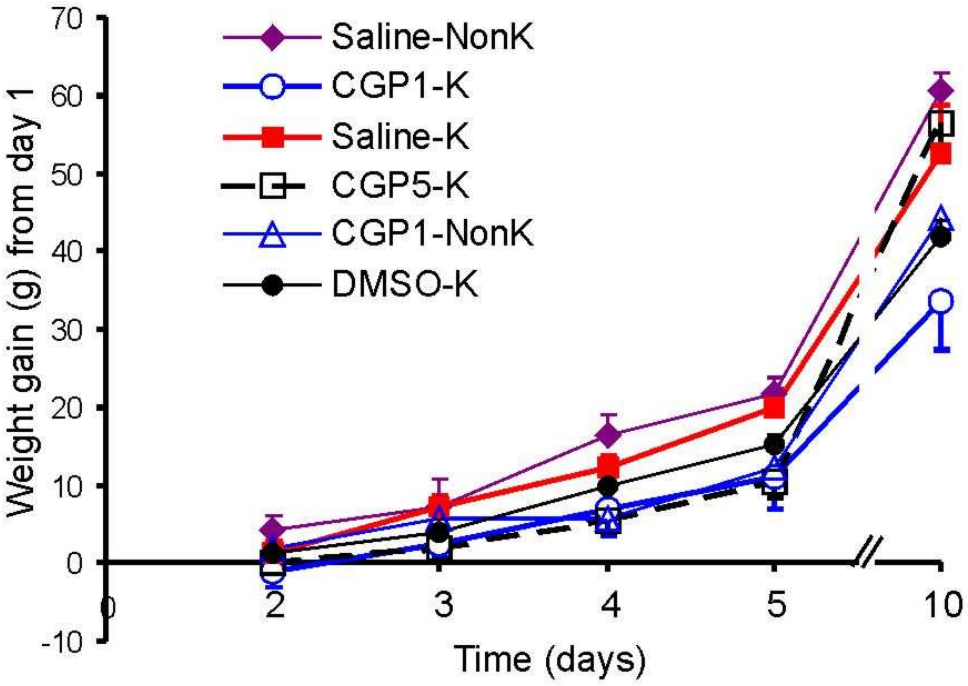
CGP5-K (CGP5): CGP7930 5 mg/kg i.p. injected and kindled. **A2.** Mean + SEM of the T/C ratio for different groups of rats. T and C, peak amplitudes of the AEP following the test and conditioning pulse, respectively. * P<0.05, ** P<0.01; Fisher's LSD protected t-test after a significant 1-way ANOVA.

B-**D**. Ensemble average of the hippocampal AEPs evoked by paired auditory stimuli (up arrows), shown as response to conditioning pulse at 0 ms (column 1) and response to test pulse (column 2); conditioning-test pulse interval was 520 ms. Statistical tests between selected group pairs: **B.** Saline-NonK (n=6 rats) versus Vehicle-K group (n=10); **C.** Saline-NonK (n=6) versus CGP1-K (n=5) groups. **D.** CGP5-K (n=6) group versus CGP1-K (n=5) group. Significant difference at a specific time bin between groups (P<0.05, Wilcoxon test, for 5 consecutive time points) is indicated by a red solid circle (labeled P).

Figure 5. Methamphetamine-induced horizontal locomotion in kindled and non-kindled groups of animals. **A**. Locomotion after injection of methamphetamine (1 mg/kg i.p.) was indicated by the number of infrared beam interruptions per 10 min in the horizontal plane (mean +/- SEM), for groups of rats kindled after injection of vehicle (vehicle kindled), CGP7930 1 mg/kg i.p. (CGP1-kindled) and CGP7930 5 mg/kg i.p. (CGP5-kindled) and a group of saline-injected, non-kindled rats (saline-only). Differences among the three kindled groups and saline-nonkindled groups were significant (2-way repeated measured ANOVA), and post hoc Fisher's LSD protected t-tests showing difference between CGP1-kindled and vehicle-kindled groups (* P<0.05,** P<0.01), CGP1-kindled and CGP5-kindled groups ($^{\#}$ P<0.05, $^{\#\#}$ P<0.01), and CGP5-kindled and vehicle-kindled groups ($^{\&}$ P<0.05); P< 0.05 difference between saline-nonkindled group and vehicle-kindled group^{0}, CGP1-kindled group^{1}, and CGP5-kindled group^{5}. **B**. Infrared

red interruptions (mean +/-SEM) after methamphetamine injection were not different between two groups of non-kindled animals, one group injected with saline only (same group as in A), and another injected with 1 mg/kg i.p. CGP7930 (CGP1 only).

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Supplementary Fig. 1

Supplementary Figure 1. Weight gain (mean +/- SEM, in grams) of rats tended to be lower in CGP7930 injected groups as compared to saline injected groups. Injection started on day 1, and weight gain was assessed at the beginning of day 2 to 5. Weight gain of CGP7930 and saline injected kindled groups (CGP1-K, CGP5-K, Saline-K, DMSO-K) were marginally not significantly different on day 5 (P>0.08, 2-way ANOVA). Injection was stopped on day 5, and weight gain at day 10 showed the weight gain in the last 5 days, which were mostly not different from each other (except for the CGP1-K group). The two groups of non-kindled rats (Saline-NonK and CGP1-NonK) were significantly different from each other.

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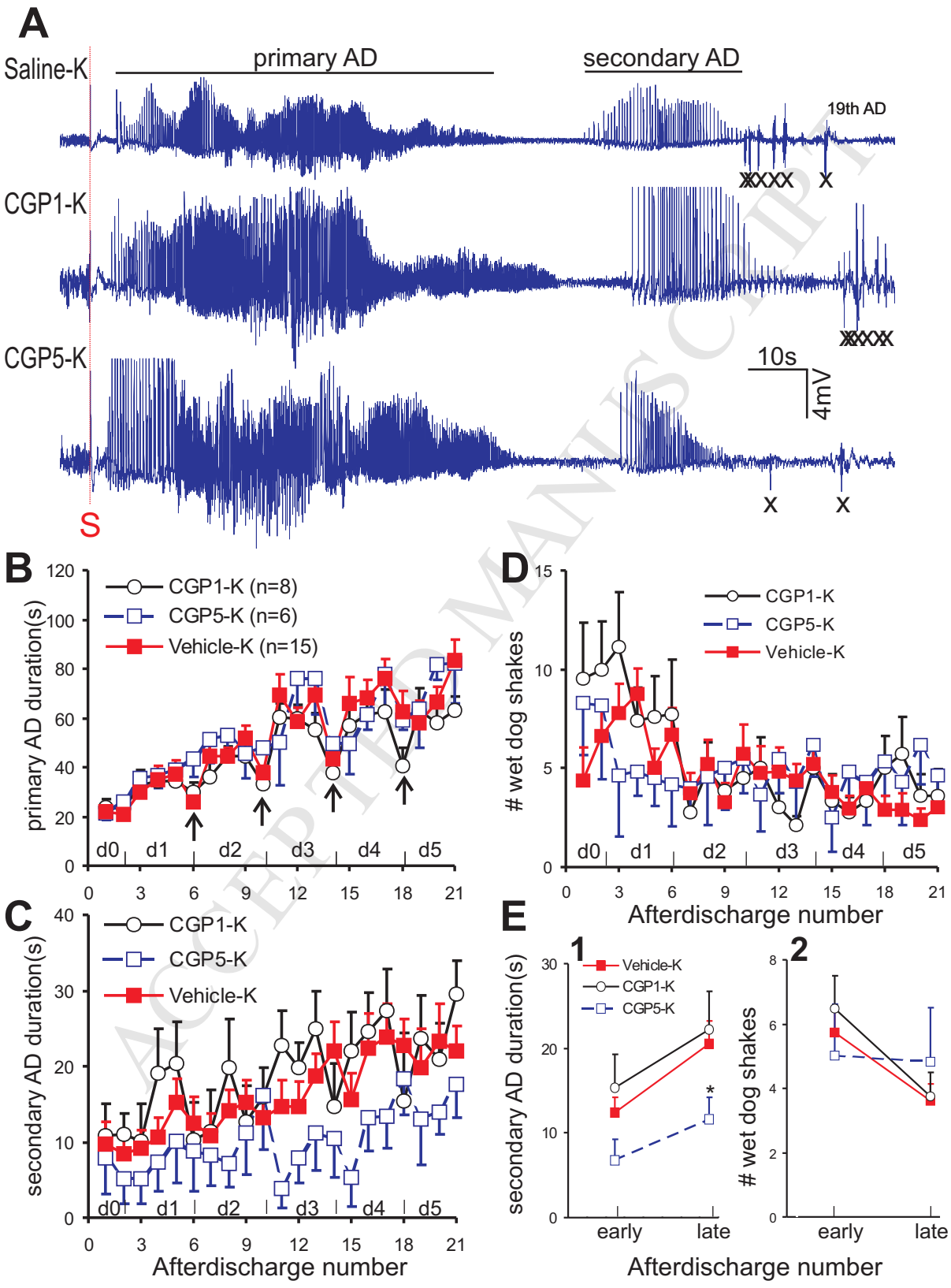

A
Saline-K
primary AD
secondary AD
19th AD
CGP1-K
CGP5-K
10s
4mV
S
B
CGP1-K (n=8)
CGP5-K (n=6)
Vehicle-K (n=15)
primary AD duration(s)
d0
d1
d2
d3
d4
d5
C
CGP1-K
CGP5-K
Vehicle-K
secondary AD duration(s)
Afterdischarge number
D
CGP1-K
CGP5-K
Vehicle-K
wet dog shakes
Afterdischarge number
E
1
2
Vehicle-K
CGP1-K
CGP5-K
secondary AD duration(s)
wet dog shakes
early
late
Afterdischarge number

Fig.1

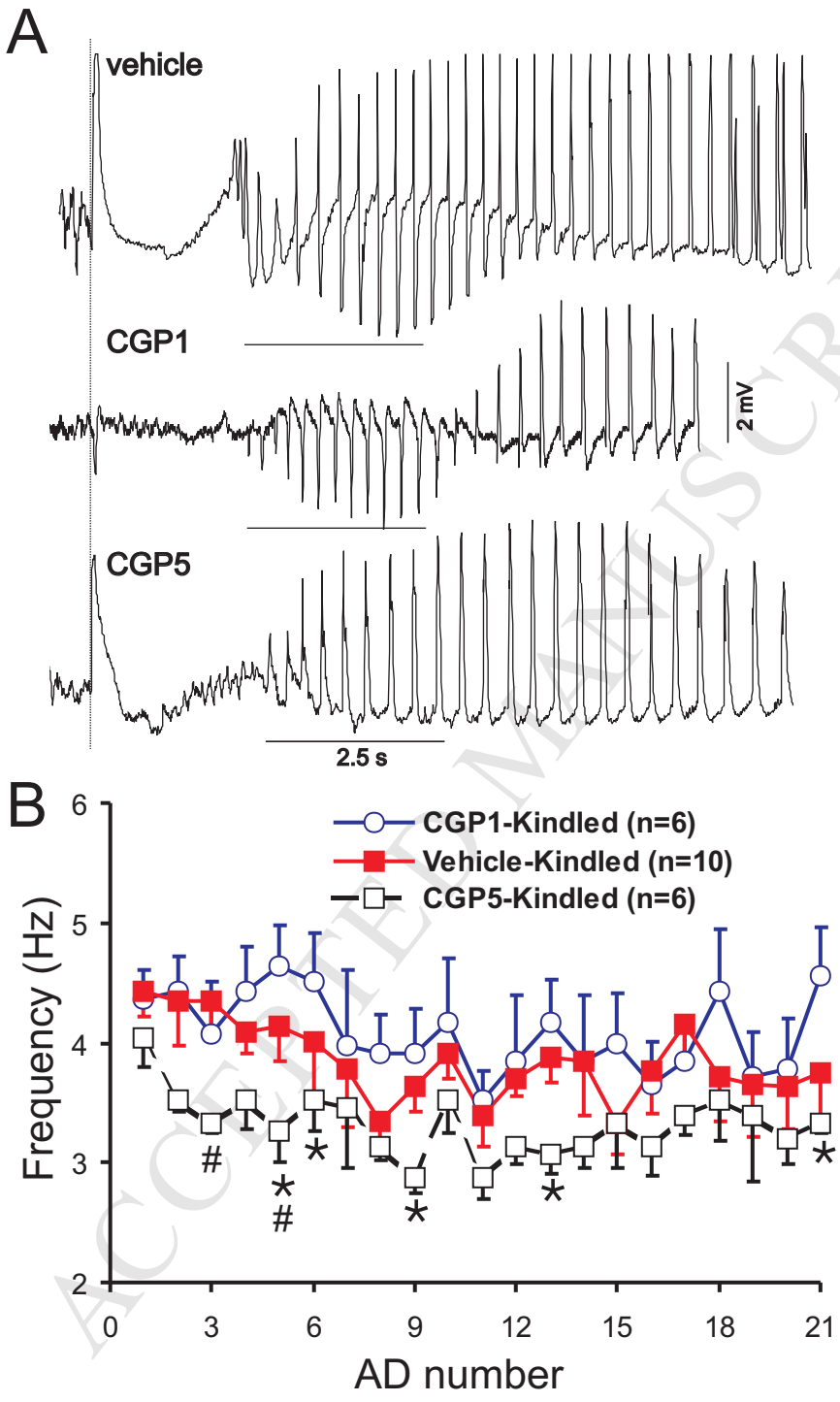


Fig. 2

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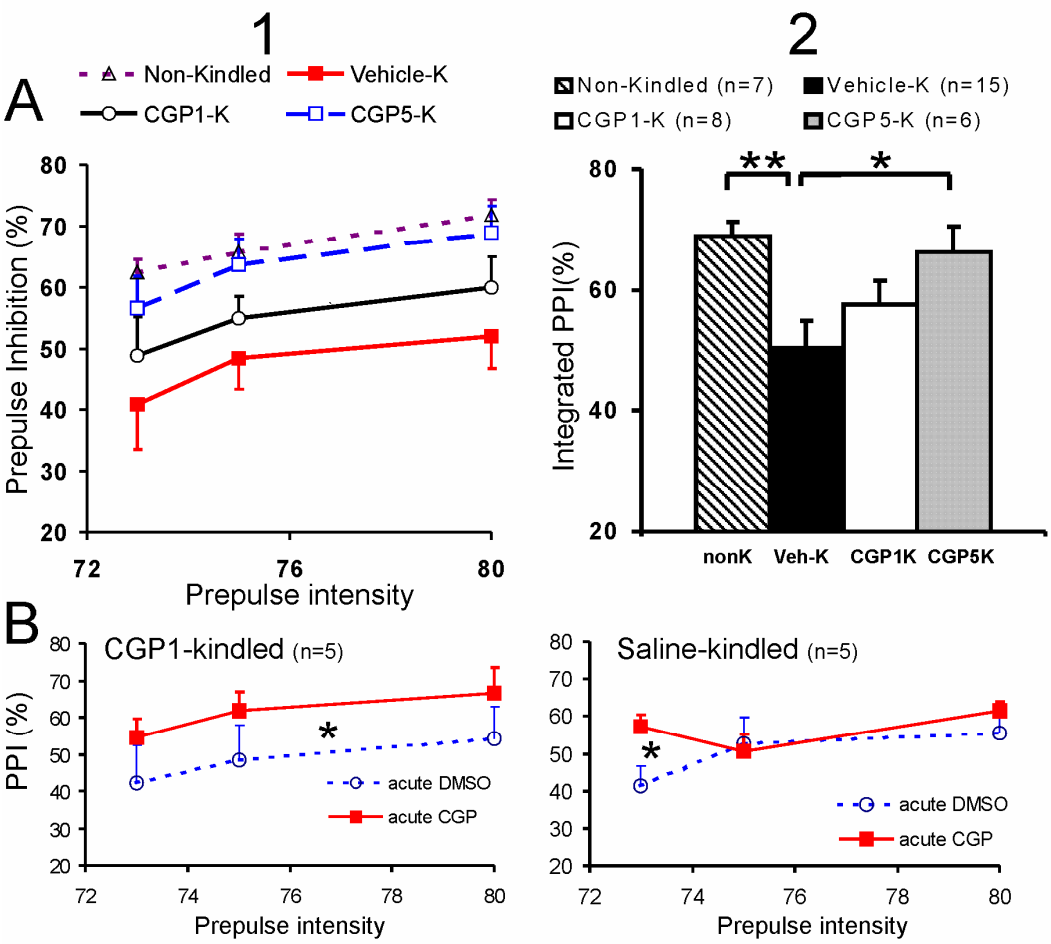

1
2
A
Non-Kindled
Vehicle-K
CGP1-K
CGP5-K
Prepulse Inhibition (%)
Prepulse intensity
Non-Kindled (n=7)
Vehicle-K (n=15)
CGP1-K (n=8)
CGP5-K (n=6)
Integrated PPI(%)
**
*
nonK
Veh-K
CGP1K
CGP5K
B
CGP1-kindled (n=5)
Saline-kindled (n=5)
PPI (%)
acute DMSO
acute CGP
Prepulse intensity

Fig.3

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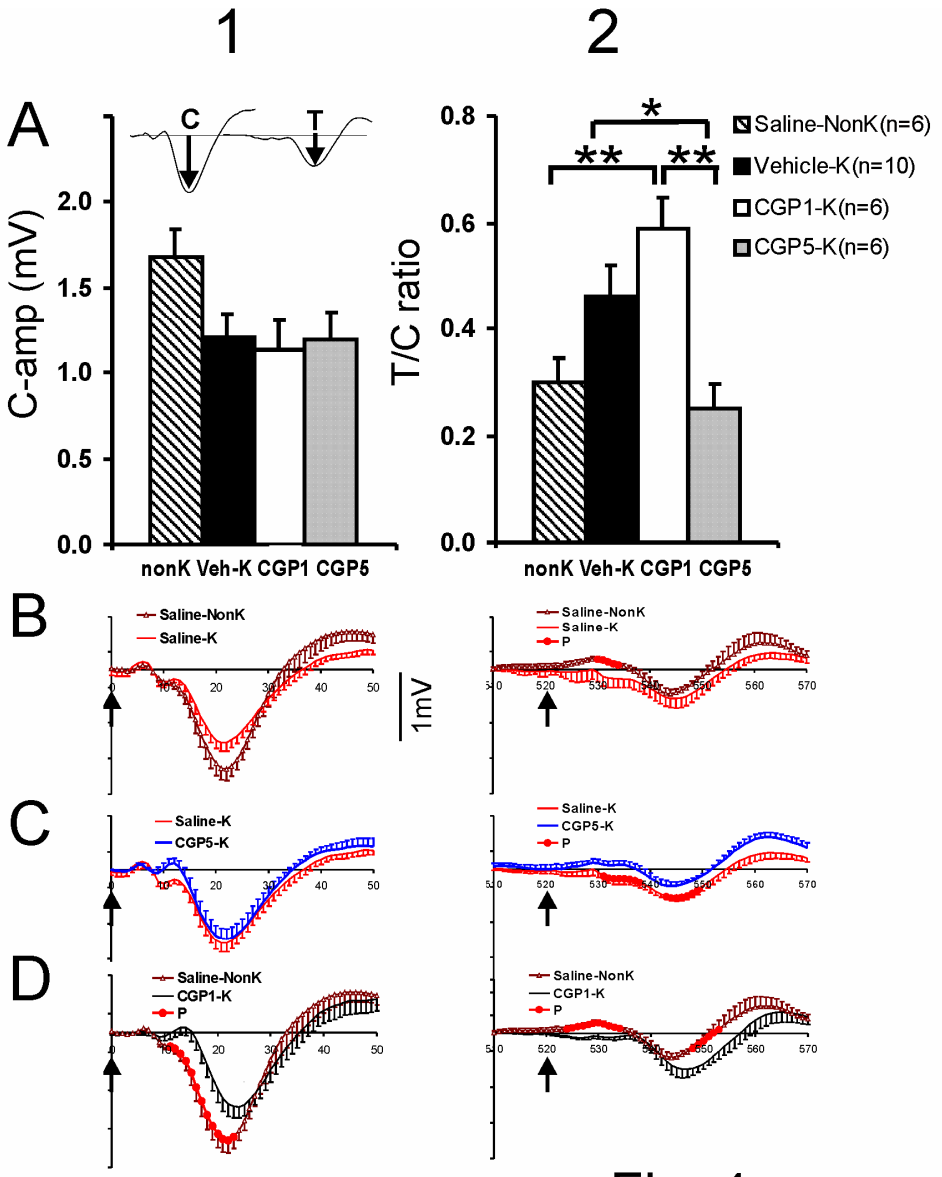

1
2
A
C
T
C-amp (mV)
2.0
1.5
1.0
0.5
0.0
nonK Veh-K CGP1 CGP5
T/C ratio
0.8
0.6
0.4
0.2
0.0
*
**
**
Saline-NonK(n=6)
Vehicle-K(n=10)
CGP1-K(n=6)
CGP5-K(n=6)
nonK Veh-K CGP1 CGP5
B
Saline-NonK
Saline-K
1mV
Saline-NonK
Saline-K
P
C
Saline-K
CGP5-K
Saline-K
CGP5-K
P
D
Saline-NonK
CGP1-K
P
Saline-NonK
CGP1-K
P

Fig. 4

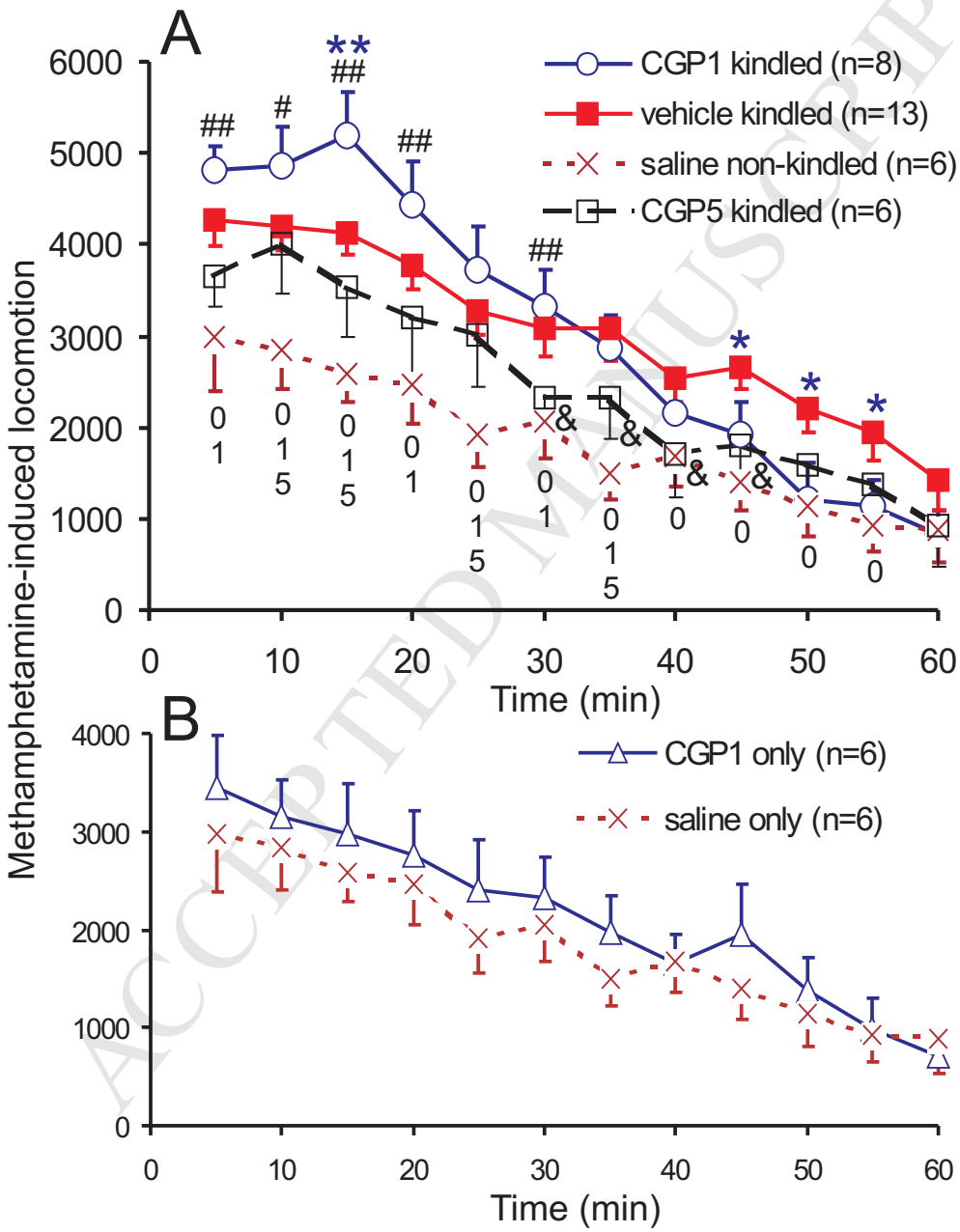


Fig.5

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Highlights

$GABA_B$ receptor modulator CGP7930 or vehicle was injected during hippocampal kindling

CGP7930 (5 mg/kg i.p.) decreased afterdischarge starting frequency but not duration

CGP7930 reduced kindling-induced loss in prepulse inhibition and auditory gating

CGP7930 reduced the hyperlocomotion induced by methamphetamine after kindling

$GABA_B$ receptor positive modulator alleviates seizure-induced psychotic symptoms

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