

Intravenous lipid emulsion modifies synaptic transmission in hippocampal CA1 pyramidal neurons after bupivacaine-induced central nervous system toxicity

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Abstract

Local anesthetics can cause severe toxicity when absorbed systemically. Rapid intravenous administration of lipid emulsion (LE) is the standard of care for severe local anesthetic systemic toxicity which can cause cardiovascular and central nervous system (CNS) injury. The biological mechanism by which LE alleviates CNS toxicity remains unknown and understudied. Previous research has suggested that local anesthetics cause an imbalance of excitatory and inhibitory transmission in the brain. Therefore, this study aimed to observe the effect of LE on glutamate- and GABA-induced currents in CA1 pyramidal neurons after bupivacaine-induced CNS toxicity. We further characterized post-synaptic modifications in these cells to try to elucidate the mechanism by which LE mediates bupivacaine-induced CNS toxicity. Sprague–Dawley rats received intravenous bupivacaine (1 mg kg⁻¹ min⁻¹) in either normal saline or LE (or LE without bupivacaine) for 5 min. An acute brain slice preparation and a combination of whole-cell patch clamp techniques and whole-cell recordings were used to characterize action potential properties, miniature excitatory, and inhibitory post-synaptic currents, and post-synaptic modifications of excitatory and inhibitory transmission in CA1 hippocampal pyramidal neurons. The expression level of GABAA receptors were assessed with western blotting, whereas H&E and TUNEL staining were used to assess cytoarchitecture and apoptosis levels respectively. Bupivacaine treatment significantly increased the number of observed action potentials, whereas significantly decreasing rheobase, the first interspike interval (ISI), and hyperpolarization-activated cation currents (I_h) in CA1 pyramidal neurons. LE treatment significantly reduced the frequency of miniature inhibitory post-synaptic currents and enhanced GABA-induced paired pulse ratio with 50 ms interval stimulation in bupivacaine-treated rats. Regulation of GABAA levels is a promising mechanism by which LE may ameliorate CNS toxicity after systemic absorption of bupivacaine.

Abbreviations used: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazol e-propionic acid receptor; BPV, bupivacaine; CNS, central Nervous System; CVS, cardio vascular System; eEPSCs, evoked excitatory post-synaptic currents; fAHPs, fast after hyperpolarizing potentials; GABA, γ-aminobutyric acid; ISI, first inter-spike interval; LAST, local anesthetic systemic toxicity; LE, lipid Emulsion; mEPSCs, miniature excitatory post-synaptic currents; mIPSCs, miniature inhibitory post-synaptic currents; NMDA, N-methyl-D-aspartic acid receptor; PPR, paired pulse ratio; RRID, research resource identifier; sAHPs, slow after hyperpolarizing potentials.

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**KEYWORDS**

bupivacaine, central nervous system toxicity, hippocampal CA1 pyramidal neurons, lipid emulsion therapy, local anesthetic systemic toxicity, synaptic transmission

1 | INTRODUCTION

Bupivacaine (BPV) is a classical long-acting amide local anesthetic used for peripheral nerve block and intraspinal anesthesia with satisfactory anesthetic effects achieved in clinical settings (Perez-Castro et al., 2009). If BPV inappropriately enters the systemic circulation, however, local anesthetic systemic toxicity (LAST) becomes a serious clinical concern (Bern, Akpa, Kuo, & Weinberg, 2011; Fettiplace & McCabe 2017; Leskiw & Weinberg, 2009; Weinberg, 2017). LAST mainly results in central nervous system (CNS) and cardiovascular system (CVS) toxicity. Relative to the CVS, the CNS is more sensitive to high plasma concentrations of local anesthetic, with toxicity occurring earlier and at lower concentrations (Oda & Ikeda, 2013; Wolfe & Butterworth, 2011). At low plasma concentrations of local anesthetic, the symptoms of CNS toxicity are paresis, dysesthesia, and dysarthria, whereas at high concentrations symptoms progress to dizziness, convulsion, circumoral paresthesia, and tremor. As the plasma concentration of local anesthetic continues to rise, uncontrollable muscular activity and generalized tonic-clonic seizures may occur (Cox, Durieux, & Marcus, 2003; Dureaun, Charbit, Nicolas, Benhamou, & Mazoit, 2016). CNS effects of BPV-LAST typically manifest as convulsion.

Lipid emulsion (LE) can be intravenously infused for parenteral nutrition or to supplement essential fatty acids. Rosenblatt, Abel, Fischer, Itzkovich, and Eisenkraft (2006) reported that the first clinical application of LE therapy successfully reversed cardiac arrest of LAST in patients in 2006. Since then, more reports have supported the early use of LE to prevent the CVS toxicity of LAST (Litz, Roessel, Heller, & Stehr, 2008; McCutchen & Gerancher, 2008). Several studies indicate that LE can effectively rescue CNS toxicity resulting from LAST (Litz et al., 2008; Oda & Ikeda, 2013; Reddy & Lahm, 2010; Spence, 2007; Wu et al., 2015), although the mechanism of LE-induced recovery has not yet been fully elucidated. Numerous successful cases of recovery after LE infusion have been reported; because rapid administration of LE is now the standard of care for severe LAST (Cave, Harrop-Griffiths, & Harbey, 2010; Neal et al., 2010), elucidating the mechanism by which LE alleviates CNS toxicity during LAST is of significant clinical interest.

Previous studies on the CNS toxicity of local anesthetic have suggested that anesthetics inhibit the growth of synaptic axons and weaken the conduction of nerve impulses, thereby weakening the ability of synapses to maintain normal physiological functions (Kanai et al., 2001). BPV-induced convulsion is closely related to an imbalance in CNS inhibition and excitatory systems, which involve ion channels, receptors, neurotransmitters, and other factors (Cousins, 1998; Miller, 2001). In this study, we used convulsive symptoms as criteria for the toxicity of CNS, which can be interpreted as a

short-term epileptic seizure. During epileptic events, high-frequency low-voltage spinous waves may arise in the cerebrum's temporal lobe, commonly beginning unilaterally in the hippocampus, that subsequently extend to the amygdala of the ipsilateral and cingulate gyrus step by step, diffuse even to the area of opposite side brain, and finally produce whole-body tonic-clonic attacks. Studying the changes of synaptic currents in nerve cells improves our understanding of how LE induces recovery after BPV-induced CNS toxicity at the level of the synapse.

Polyunsaturated fatty acids may enter the brain and alter levels of neurotransmitter release (Song, Manku, & Horrobin, 2008). The hippocampus is recognized as a key locus of BPV toxicity within the CNS (Dahmani, Rouelle, Gressens, & Mantz, 2007; Yang et al., 2003). Thus, we sought to characterize the effects of bupivacaine-induced CNS toxicity on both excitatory and inhibitory post-synaptic transmission in the hippocampus, and determine how treatment with LE may modify these processes.

2 | MATERIALS AND METHODS

2.1 | Animals & CNS toxicity model

Male Sprague-Dawley rats (Laboratory Animal Science Center of Medicine at Beijing University, China; production license number of experimental animals: SCXK[jing]2016-0010.) were housed in controlled conditions: constant temperature $22 \pm 1^\circ\text{C}$, humidity 50%–60%, 12:12 dark-light cycle. Standard food pellets and water were freely accessible. All animal procedures were performed in accordance with the guidelines of the National Institute of Health and the Medical Research Council of China and approved by the National Animal Experiment Ethics Committee of hospital of Ningxia medical university (2015–058).

Rats weighing between 300 and 340 g, aged between 9 and 10 weeks were used and were divided into BPV + LE ($n = 7$), BPV ($n = 7$), LE ($n = 7$), and Control groups ($n = 7$). No randomization was done to allocate the animals to the experimental groups, and no blinding was performed throughout the experiments. Rats in BPV + LE and BPV groups were intravenously administered with Bupivacaine ($1 \text{ mg kg}^{-1} \text{ min}^{-1}$) (Zhaohui Pharmaceuticals) using a trace syringe pump (INJECTOMAT TIVA, Fresenius Kabi) until tetanic convulsions were observed. The rats in the BPV + LE group were then immediately given LE ($3 \text{ ml kg}^{-1} \text{ min}^{-1}$) (20% lipid emulsion Kelun pharmaceuticals, China) over 5 min. A subset of animals of the BPV group was instead administered normal saline (NS) immediately for 5 min (matched to the bupivacaine + LE time point). In addition, control and LE groups of animals received either NS only ($1 \text{ ml kg}^{-1} \text{ min}^{-1}$)

or LE ($3 \text{ mL kg}^{-1} \text{ min}^{-1}$) for 5 min and served as negative sham-controls. The level of convulsive attack was rated using the Racine standard (Presley & Chyka, 2013): Level 0: no reaction; Level I: rhythmic mouth movement or facial twitch; Level II: nodding or wagging the tail; Level III: single leg jerking; Level IV: Multiple leg twitch or stiffness; Level V: comprehensive tonic-clonic seizures. For inclusion in the study, rats with convulsions were required to have a Racine level V classification. Rats were monitored for cessation of convulsions and the resumption of voluntary action (righting recovery).

The daily experiment starts at 6 am. A rat fixator was used to prevent the rats from moving during injection. The tail vein of each rat was fully expanded by soaking the tail in 38°C warm water, the puncture point was disinfected, and lidocaine hydrochloride gel was applied as an anesthetic to reduce animal pain. After injection and hemostasis, animals were transferred to a single rat cage ($320 \text{ mm} \times 220 \text{ mm} \times 160 \text{ mm}$) and recovered with heat support and free access to soft food. Posture and behavior were assessed daily after convulsions. Rats that demonstrated no convulsions within

5 min or had died were excluded from the study. Three rats were excluded based on the exclusion criteria (one rat died, two rats did not show convulsions within 5 min) during experiments. The flow diagram of the study is shown in Figure 1.

2.2 | Hippocampal slice preparations

After 6 h (at 12 am), a critical time span for convulsion effects determined *a priori*, rats were deeply anesthetized with an intraperitoneal injection of 2.5% pentobarbital (50 mg/kg) (Chun & Xiao-hui, 2008; Hájos & Mody, 1997; Soltesz & Mody, 1994) and decapitated with a hand hay cutter. The brain was rapidly removed from the skull with the least amount of damage and pressure onto the brain. The brain was then transferred into ice-cold artificial cerebrospinal fluid (aCSF, mM: 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4 , 24 NaHCO_3 , 12.5 D-Glucose, 2 CaCl_2 , and 1.5 MgSO_4 saturated with 95% O_2 and 5% CO_2 to pH: 7.3). $300\text{-}\mu\text{m}$ -thick coronal brain sections were cut into

Timeline of the experimental procedure

Sham: NS 3 mL/kg/min , 5 min

LE: LE 3 mL/kg/min , 5 min

BPV: BPV 1 mg/kg/min , tetanic convulsion,
+NS 3 mL/kg/min , 5 min

BPV+LE: 1 mg/kg/min , tetanic convulsion, +NS
 3 mL/kg/min , 5 min

(a) *In vivo*

Deeply anesthetized
and decapitated

(b) *In vitro*

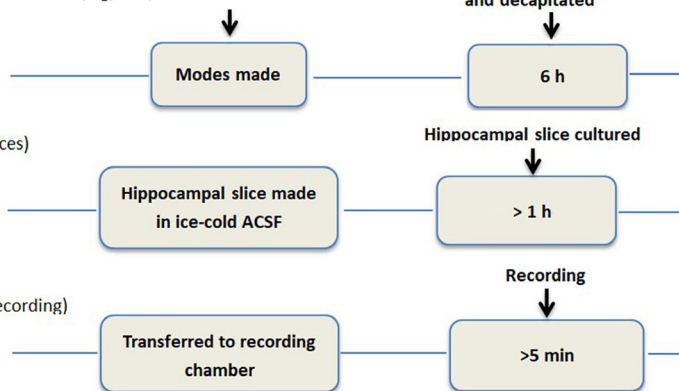
(Made and cultured slices)

Hippocampal slice cultured

(c) *In vitro*

(Electrophysiological recording)

Recording



Number of animals for each group

	Control	BPV	BPV + LE	LE
AP	4	4	4	4
mEPSCs	3	3	3	3
mIPSCs	3	4	3	3
eEPSCs	3	4	3	3
PPR of IPSCs	3	4	3	3
Died	0	1	0	0
Excluded	0	2	0	0
Included	16	19	16	16
Total	67			

FIGURE 1 Timeline of the experimental procedures for *in vivo* (a) and *in vitro* (b and c). Number of animals for each group



cold aCSF. Prepared slices were incubated in oxygenated aCSF at room temperature at least for 1 h before individual slices were transferred to a recording chamber, which was bubbled with oxygenated aCSF at $31 \pm 1^\circ\text{C}$.

2.3 | Electrophysiological studies

The temperature of aCSF in the recording chamber was $31 \pm 1^\circ\text{C}$ and the aCSF perfusion rate was set to 2 ml/min. Electrophysiological recordings were obtained using an Olympus microscope (BX50-WI, Olympus) with a 40X long-working distance objective (NA 0.8). Patch pipettes with 4–6 M Ω resistance were pulled from 110 mm long borosilicate glass capillaries (GB 150F-86–10, Sutter instrument). Ion currents were recorded using an Axopatch 700B amplifier and pClamp10.6 (Molecular Devices). Only cells that showed a high seal resistance ($>1 \text{ G}\Omega$) and a series resistance $<25 \text{ M}\Omega$ were included. The series- and input-resistances were checked before and after the recordings in each experimental sequence. Cells were excluded if the input resistance or series resistance changed more than 15% during the experiment. All current recordings were performed using whole-cell mode.

aCSF was used as the extracellular solution. The intracellular solution used was as follows: (in mM) 140 K-gluconate, 2 MgCl, 8 KCl, 10 HEPES, 0.2 NaGTP, 2 Na2ATP. The pH was adjusted to 7.3 with KOH. Five hundred ms depolarizing current pulses from -60 to 500 pA in 20 pA increments with an intertrial interval of 5 s was used to elicit action potential discharges. Afterward, a 500ms single 500pA current and three repeated 800 ms -20pA currents were delivered. The Axopatch 700B amplifier (Molecular Devices) was used for digitization and the Digidata 1440A (Molecular Devices) transducer was used to filter signals (low-pass filter 3 kHz).

During the experiment, the following data were acquired:

1. Membrane potentials (Vm);
2. Rheobase (pA).
3. The input resistance (Rin) was calculated at -20pA injecting current.
4. Peak amplitude, half width, and threshold were measured from the first spike at Rheobase.
5. ISI (the first interspike interval) was detected with a single 500 pA injecting current.
6. The fast after hyperpolarizing potentials (fAHPs) were measured in the second and third current evoked spikes. Slow after hyperpolarizing potentials (sAHPs) were measured beginning 280 ms after the end of the 800ms pulse, average potential with 50ms duration. The peak of the fAHP was calculated after subtracting threshold potential from sAHP.
7. Hyperpolarization-activated cation currents (Ih, voltage sag) were recorded by injecting a 20 pA hyperpolarizing current pulse. The voltage sag was calculated as the difference between the peak of hyperpolarization and the average voltage of 100 ms duration at 725 ms after the beginning of the injection current step.

2.4 | Miniature excitatory/inhibitory post-synaptic currents

The signal was obtained at a holding potential of -80 mV . For pharmacological isolation of α -amino-3-hydroxy-5-methyl-4-isoxazol e-propionic acid receptor (AMPA) receptor-mediated miniature excitatory post-synaptic currents (mEPSCs), 1 μM TTX and 100 μM PTX were added to the aCSF. The intracellular solution used was as follows: (in mM) 140 K-gluconate, 2 MgCl, 8 KCl, 10 HEPES, 0.2 NaGTP, 2 Na2ATP, 2QX-314. The pH was adjusted to 7.3 with KOH. For pharmacological isolation of GABA-receptor type A (GABAA)-mediated miniature inhibitory post-synaptic currents (mIPSCs), 1 μM TTX, 20 μM DNQX, and 20 μM D-AP5 were added to the aCSF. The intracellular solution used (in mM) was: 140 CsCl, 2 CaCl2, 2 MgCl2, 1.1 EGTA, 2 Na2-ATP, 10 HEPES, and 2QX-314. The pH was adjusted to 7.3 with CsOH. 5 min of recording was performed for each cell. To detect spontaneous events, the "threshold research" option was used and each event was analyzed using Clampfit software.

2.5 | AMPA/NMDA receptor currents

Electrode resistance was 4–6 M Ω after filling with intracellular solution (in mM): 135Cs-methanesulfonate, 2 MgCl, 10 NaCl, 10 HEPES, 0.2 NaGTP, 2 Na2ATP, 10 EGTA, and 2 QX-314. The pH was 7.3. The recording pipette was placed on a CA1 pyramidal neuron and a stimulating electrode was placed on the Schaffer collateral pathway 100–200 μm distal from the recorded pyramidal neuron. The stimulation duration was 0.05 ms. First, 200–300pA currents of AMPA-mediated currents were evoked by bipolar stimulation electrode at a -70 mV holding potential with 10 μM Bicuculline (GABA receptor currents antagonist) in aCSF. Next, maximal AMPA- and NMDA-mediated currents were both evoked by bipolar stimulation electrode at $+50 \text{ mV}$. Finally, subsequent application of 20 μM DNQX (AMPA receptor currents antagonist) was used to isolate NMDA currents.

2.6 | GABAA-induced paired pulse ratio

The signal was obtained at a holding potential of -70 mV . For pharmacological isolation of GABAA-receptor-mediated paired pulse ratio (PPR), 20 μM DNQX and 20 μM D-AP5 were added to the aCSF. The intracellular solution used was: (in mM) 140 CsCl, 2 CaCl2, 2 MgCl2, 1.1 EGTA, 2 Na2-ATP, 10 HEPES, and 2 QX-314. The pH was adjusted to 7.3 with CsOH. Ten traces were recorded at 20s intervals. PPR stimulation was separated by 20 ms, 50 ms, 100 ms, and 200 ms and used to distinguish between pre- and post-synaptic mechanisms.

2.7 | Histology

Fresh tissue was cut into 1–1.5 cm^2 small pieces of 3–4 mm thickness using a microtome (RM2235 Leica) and fixed in 10% neutral

formalin for 8–12 h. Tissues were washed and then dehydrated by gradient alcohol until the dimethylbenzene was transparent, and then soaked in wax. Tissues were then embedded, sliced to 3 μm thickness, and subjected to H&E staining (DP260 DAKWE auto-stain, China), neutral gum seal, and microscopic examination (BX53 OLYMPUS, Japan).

2.8 | TUNEL staining to detect apoptosis rate of hippocampal neurons

Frozen sections of rat brain tissue were prepared. The sample hippocampus tissue was fixed with 4% paraformaldehyde for 30 min before a 37°C proteinase K treatment fluid soak for 30 min, a 37°C TdT liquid enzyme reaction for 60 min in dark, and a liquid 37°C streptavidin-fluorescein reaction for 30 min in dark for link marking. Nuclei were stained with DAPI, and tissues were viewed under a fluorescence microscope. The apoptosis rate was calculated using a hippocampal neuron apoptosis ratio = (TUNEL tag number of positive cells after DAPI stain nuclei)*100%.

2.9 | Western blotting

Sample preparation: Rat hippocampal tissue was ground with liquid nitrogen and a RIPA lysate (Biyuntian, China) containing protease inhibitor (which was placed on ice for about 30 min). Oscillating vortices to aid in protein lysis. 4°C 12 000 rpm centrifugation for 15 min, the supernatant was collected as the protein sample. Protein concentration was determined by BCA method. SDS PAGE: make 10% separating gel and 5% spacer gel (distilled water, 1 M Tris-HCl, 30% acrylamide, 10% SDS, 10% ammonium persulfate, TEMED). The samples of each group were added with sample buffer and denatured at 70°C for 15 min. Gel electrophoresis: The initial voltage of electrophoresis was 80 V, and the voltage was adjusted to 120 V after the leading edge of dye enters the separation gel. Transfer: PVDF membrane was soaked in methanol to activate about 20 s, and then placed into transfer buffer together with filter paper for 10 min. Following installation of the transfer device, transfer buffer was added, and transfer was done by a 200 mA constant current for 2.5 h. Blocking buffer: 1X TBS-T with 5% non-fat dry milk, 1X TBS-T: 1X TBS (20 mM Tris, 150 mM NaCl, pH 7.6) with 1 % Tween 20. Protein extracts were subjected to western blot with primary antibodies: GABAA receptor $\alpha 1$ (Cat# AGA-001, RRID:AB_2039862, Alomone, Israel) (1:1,000) and the one protein actin (Cat# sc-47778, RRID:AB_626632, Santa Cruz, CA, USA) (1:5,000) diluted in 1% BSA and incubated overnight at 4°C and washed with TBS-T(Tris-HCl, NaCl, Tween 20) for three times, 10 min each. Subsequently, HRP-conjugated secondary antibodies were diluted with 1% BSA (IgG dilution of goat and rabbit was 1:10,000) (Cat# sc-2005, RRID:AB_631736, Cat# sc-2357, RRID:AB_628497). Incubated with PVDF membrane at room temperature for 1 h and washed with TBS-T for 3 times, 10 min each before image acquisition.

2.10 | Statistical analysis

Data were recorded, saved, and analyzed offline with pClamp10.6 for electrophysiological experiments. The graphics are generated by software GraphPad Prism7. Each set of data was tested to determine whether it followed a normal distribution. ANOVA test was used for statistical differences. Student's *t* test and Kolmogorov–Smirnov test (K-S test) were used for statistical analyses. Kolmogorov–Smirnov test (K-S test) was used for normality. Results are presented as mean \pm SEM, and differences were considered significant at $p < .05$. No sample calculation was performed. No test for outliers was performed.

3 | RESULTS

3.1 | BPV affects electrophysiological properties of CA1 pyramidal neurons in rat hippocampus

Following treatment with BPV, we observed a significant increase in the number of action potentials (Figure 2g, $n = 11$ neurons, $p < .05$, unpaired *t* test) and a significant decrease in rheobase, ISI, and Ih (Figures 2h, k and 1c, $n = 11$ neurons, $p < .05$) relative to the saline-treated control group. There were no differences in LE and BPV + LE rats compared with the saline-treated control group ($n = 11$ neurons, $p > .05$). No significant differences were observed between treated and control groups ($n = 11$ neurons, $p > .05$, unpaired *t* test) in Vm, threshold, peak amplitude, Rin, halfwidth, fAHP, or sAHP (Table 1).

4 | LE RESCUES BPV-INDUCED INHIBITION OF MIPSC FREQUENCY IN CA1 HIPPOCAMPAL NEURONS

No significant change in mEPSCs, including in amplitude or frequency of the currents, was observed (Figure 3, $n = 7$ neurons, $p > .05$). There was also no significant change in the amplitude of mIPSCs (Figure 4b, $n = 7$ neurons, $p > .05$). However, we observed a significant decrease in the frequency of mIPSCs in the BPV-treated group, which was rescued in the LE + BPV group (Figure 4c, $n = 7$ neurons, $p < .05$) (Table 2 and 3).

5 | BPV DOES NOT AFFECT AMPA/NMDA RECEPTOR CURRENTS OF CA1 PYRAMIDAL NEURONS IN RAT HIPPOCAMPUS

There were no significant differences in NMDAR or AMPAR current amplitude, AMPA/NMDA ratio, or NMDA fast tau or slow tau (Figure 5) in the hippocampal CA1 neurons of control, BPV, LE, or BPV + LE-treated rats ($n = 7$ neurons, $p > .1$) (Table 4).

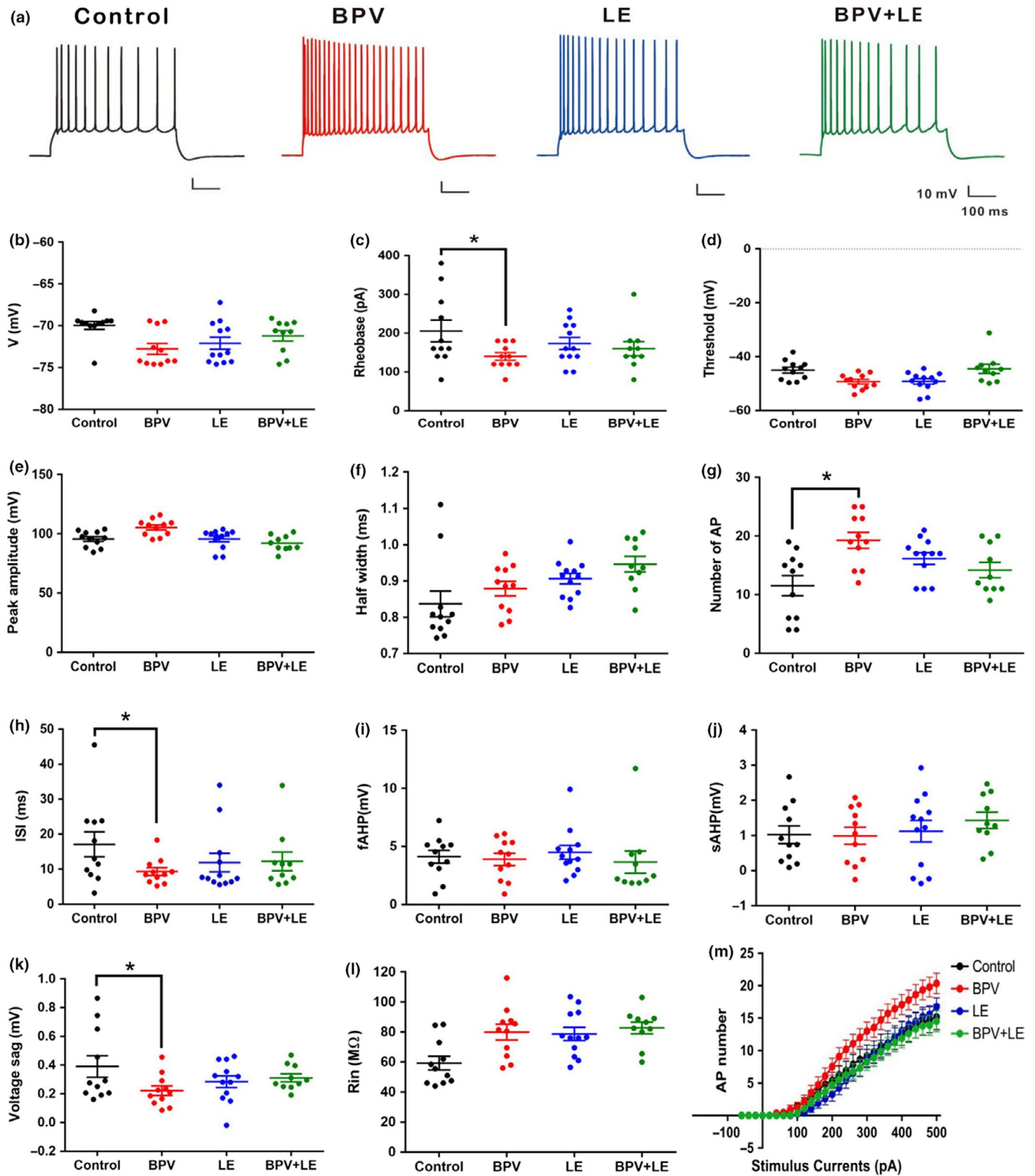


FIGURE 2 Action Potential Properties of CA1 Pyramidal Neurons in Rat Hippocampus. BPV affects electrophysiological properties of CA1 pyramidal neurons in rat hippocampus (a) Representative sample of original membrane cation current traces from saline control-treated (black), bupivacaine (BPV)-treated (red), lipid emulsion (LE)-treated (blue), and BPV + LE -treated (green) hippocampal CA1 neurons. Plots describing (b) Membrane potentials, (c) Rheobase (d) Threshold voltage, (e) Peak amplitude, (f) Half-width, (g) Number of action potential, (h) ISI (the first inter spike interval), (i) Fast after hyperpolarizing potentials (fAHPs), (j) Slow after hyperpolarizing potentials (sAHPs), (k) Hyperpolarization-activated cation currents (I_h, voltage sag), (l) Input resistance (R_{in}), and (m) action potential as a function of stimulus current in control ($n = 11$ neurons from 4 animals), BPV-treated ($n = 11$ neurons from 4 animals), LE-treated ($n = 11$ neurons from 4 animals), and BPV + LE-treated ($n = 11$ neurons from 4 animals) brains. All symbols represent means \pm SD; * $p < .05$ versus controls; unpaired t test used for statistical analysis

	Control	BPV	BPV + LE	LE
Membrane potentials (mV)	-69.96 ± 0.48	-72.76 ± 0.66	-71.2 ± 0.63	-72.09 ± 0.72
Rheobase (pA)	205.4 ± 28.04	140.0 ± 9.72 ^a	160 ± 18.14	173.33 ± 15.44
Threshold (mV)	-45.00 ± 1.10	-49.26 ± 0.85	-44.53 ± 1.69	-49.63 ± 1.01
Peak amplitude (mV)	95.68 ± 2.02	105.37 ± 2.06	92.02 ± 2.06	95.71 ± 2.35
Halfwidth (ms)	0.84 ± 0.04	0.80 ± 0.02	0.95 ± 0.22	0.91 ± 0.01
Numbers	11.55 ± 1.72	19.27 ± 1.36 ^a	14.2 ± 1.32	16.17 ± 1.01
ISI (ms)	17.07 ± 3.57	9.32 ± 1.11 ^a	12.21 ± 2.70	11.88 ± 2.66
fAHPs (mV)	4.11 ± 0.55	3.89 ± 0.53	3.65 ± 0.95	4.49 ± 0.60
sAHPs (mV)	1.02 ± 0.25	0.99 ± 0.24	1.43 ± 0.23	1.12 ± 0.31
voltage sag (mV)	0.39 ± 0.08	0.22 ± 0.03 ^a	0.31 ± 0.03	0.28 ± 0.04
Ih (MΩ)	69.96 ± 0.49	79.96 ± 5.28	82.75 ± 3.92	78.75 ± 4.43

Note: There was a significant increase in number of Action Potential ($p < .05$, Unpaired t test) and significant decrease in rheobase, ISI and Ih ($p < .05$, Unpaired t test) in the BPV rats but no differences in LE and BPV + LE rats compared with CTL group ($p > .05$, Unpaired t test). No significant differences were seen among four groups ($p > .05$, Unpaired t test) including Vm, threshold, peak amplitude, Rin, halfwidth, fAHP, and sAHP ($n = 11$, $\bar{x} \pm s$).

^a $p < .05$ versus controls.

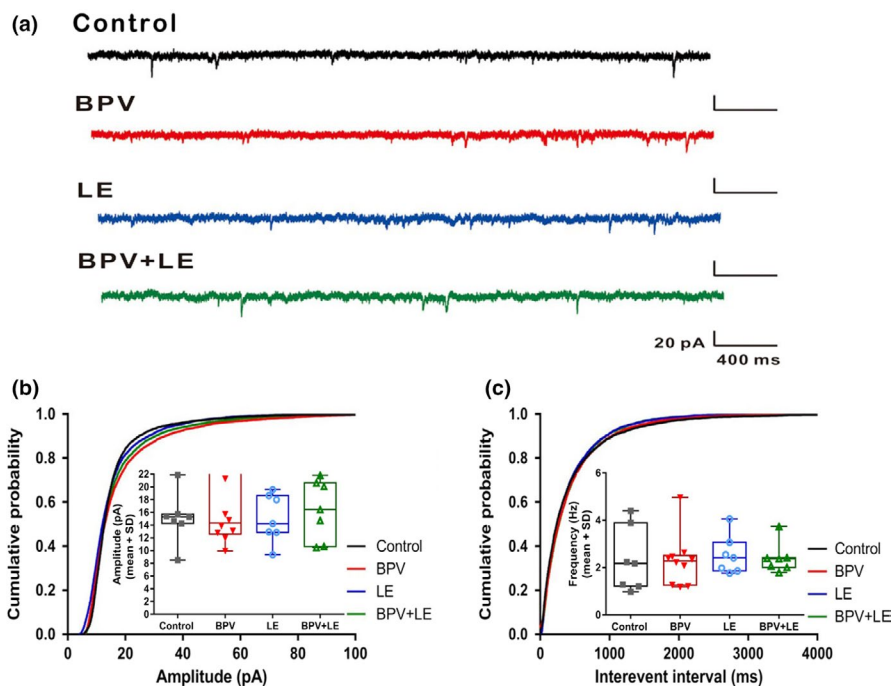


TABLE 1 Action potential properties of CA1 pyramidal neurons in rat hippocampus

FIGURE 3 Miniature excitatory post-synaptic currents (mEPSCs) of CA1 Pyramidal Neurons in Rat Hippocampus. BPV does not affect the amplitude or frequency of mEPSCs of CA1 pyramidal neurons in rat hippocampus. (a) Representative sample of original membrane cation currents traces from saline controls-treated (black), bupivacaine (BPV)-treated (red), lipid emulsion (LE)-treated (blue), and BPV + LE -treated (green) hippocampal CA1 neurons. Plots describing (b) Amplitude and (c) Frequency of mEPSCs in saline-treated controls ($n = 7$ neurons from 3 animals), BPV-treated ($n = 10$ neurons from 3 animals), LE-treated ($n = 7$ neurons from 3 animals), and BPV + LE-treated ($n = 7$ neurons) brains. All symbols represent means \pm SD; k-s test followed by unpaired t test

6 | BPV INCREASES GABA-INDUCED PAIRED PULSE RATIO OF CA1 PYRAMIDAL NEURONS IN RAT HIPPOCAMPUS

There was a significant increase in paired pulse ratio (PPR) with 50 ms interval stimulation in the hippocampal CA1 neurons of BPV-treated rats ($n = 7$ neurons, $p < .05$) relative to saline control-treated rats, but this effect was not rescued in the BPV + LE rats (Figure 6). No significant difference was seen in PPR with either 20 ms, 100 ms, or 200 ms interval stimulation among the different treatment groups (Table 5).

7 | LE REDUCES INFLAMMATORY CELL INFILTRATION IN HIPPOCAMPUS OF BPV-TREATED RATS

We performed hematoxylin and eosin staining on brains from saline-, BPV-, LE-, and BPV + LE-treated rats to determine whether treatment with LE affected tissue characteristics of hippocampi after BPV treatment. In the BPV treatment group, we observed a moderate amount of brain tissue degeneration and robust inflammatory cell infiltration (Figure 7b). In the BPV + LE group, we still

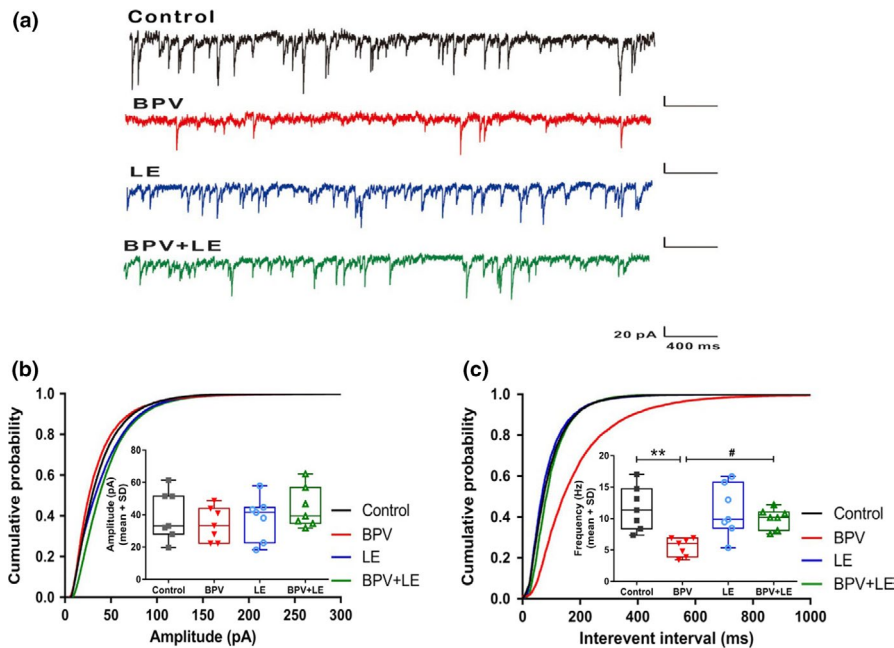


FIGURE 4 Miniature inhibitory post-synaptic currents (mIPSCs) of CA1 Pyramidal Neurons in Rat Hippocampus. LE rescues BPV-induced inhibition of mIPSC frequency in CA1 hippocampal neurons (a) Representative sample of original membrane cation currents traces from saline control-treated (black), bupivacaine (BPV)-treated (red), lipid emulsion (LE)-treated (blue), and BPV + LE-treated (green) hippocampal CA1 neurons. Plots describing (b) Amplitude and (c) Frequency of mIPSC in saline-treated controls ($n = 7$ neurons from 3 animals), BPV-treated ($n = 7$ neurons from 3 animals), LE-treated ($n = 7$ neurons from 3 animals), and BPV + LE-treated ($n = 7$ neurons from 3 animals) brains. All symbols represent means \pm SD; * $p < .05$; ** $p < .01$ versus controls; # $p < .05$; versus BPV + LE. k-s test followed by unpaired t test

TABLE 2 Miniature excitatory post-synaptic currents (mEPSCs) of CA1 Pyramidal Neurons in Rat Hippocampus

	Control	BPV	BPV + LE	LE
Amplitude (pA)	15.09 \pm 1.47	17.19 \pm 2.25	16.40 \pm 1.77	15.08 \pm 1.13
Frequency (Hz)	2.31 \pm 0.51	2.28 \pm 0.35	2.40 \pm 0.24	2.53 \pm 0.31

Note: There was no significant difference in mEPSC frequency in CTL, BPV, LE, and BPV + LE rats ($p > .05$, Unpaired t test). Similarly, no significant difference was seen in mEPSC amplitude between the four groups ($p > .05$, Unpaired t test).

TABLE 3 Miniature inhibitory post-synaptic currents (mIPSCs) of CA1 pyramidal neurons in rat hippocampus

	Control	BPV	BPV + LE	LE
Amplitude (pA)	39.62 \pm 5.73	34.25 \pm 4.00	44.42 \pm 4.75	38.06 \pm 5.13
Frequency (Hz)	11.68 \pm 1.32	5.51 \pm 0.55**	10.02 \pm 0.62 [#]	11.23 \pm 1.55

Note: There was a significant decrease in mIPSC frequency in BPV rats ($n = 7$, respectively, $p < .05$, Unpaired t test) but no differences in LE and BPV + LE rats compared with CTL group ($n = 7$, respectively, $p > .001$, Unpaired t test); There was a significant increase in mIPSC frequency in BPV+LE rats ($n = 7$, respectively, $p < .05$, Unpaired t test) compared with BPV group ($n = 7$, respectively, $p > .05$, Unpaired t test). No significant difference was seen in mIPSC amplitude among four groups ($n = 7$ respectively, $p > .05$, Unpaired t test).

** $p < .001$ versus controls; [#] $p < .05$; versus BPV + LE. k-s test followed by unpaired t test.

observed cellular degeneration in local brain tissue, but also noted a smaller degree of inflammatory cell infiltration (Figure 7d). To confirm the absence of differences in the level of cellular degeneration between BPV-treated and BPV + LE-treated groups, we performed TUNEL staining to assess the degree of apoptosis in each group relative to controls. Indeed, we found no statistically significant differences in apoptosis rates between the groups (Figure 8) (Table 6).

8 | BPV DOES NOT AFFECT EXPRESSION LEVELS OF GABAA RECEPTOR IN THE HIPPOCAMPUS

Since we found that BPV treatment decreased the GABA-induced paired pulse ratio of CA1 pyramidal neurons in rat hippocampus, we also assessed whether BPV affected expression

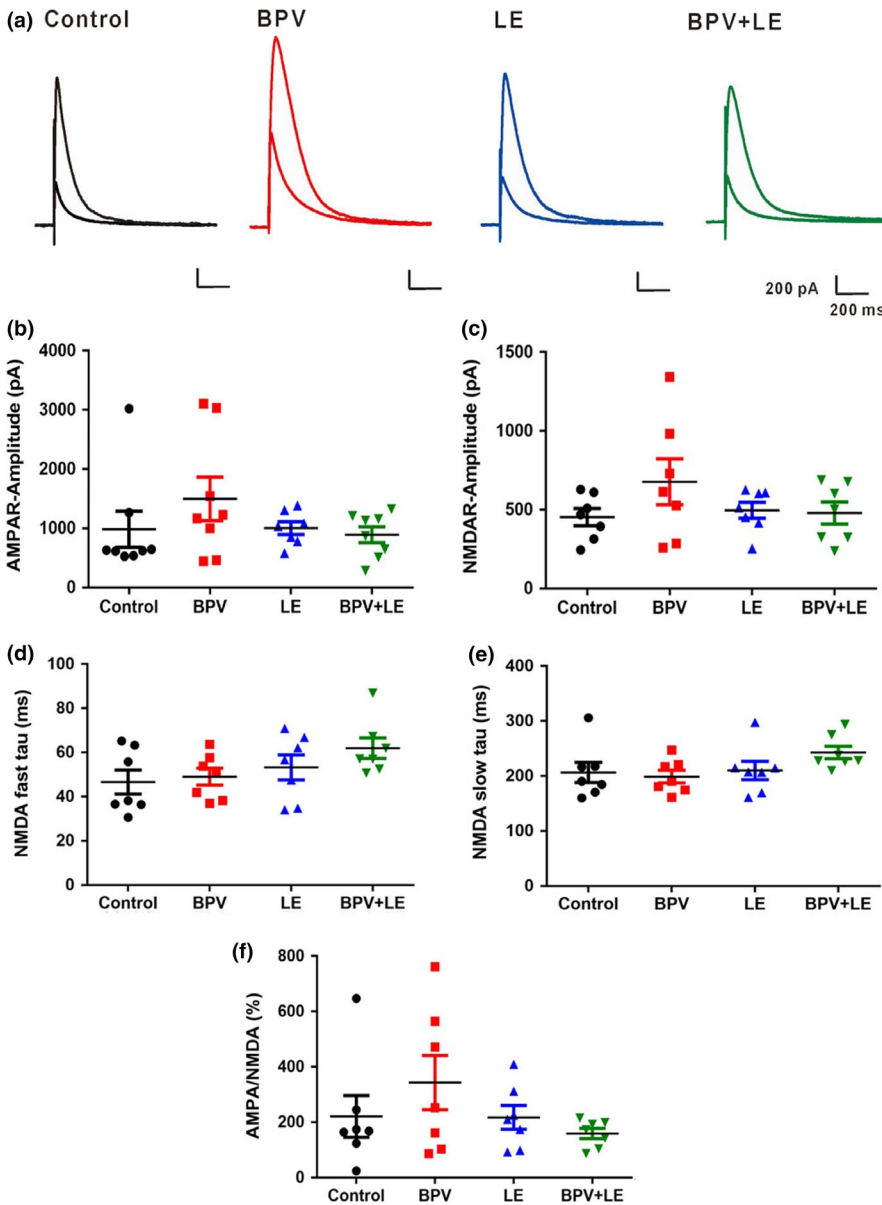


FIGURE 5 Post-synaptic Modifications of Excitatory Transmission in CA1 Pyramidal Neurons in Rat Hippocampus. BPV does not affect AMPA/NMDA receptor currents of CA1 pyramidal neurons in rat hippocampus (a) Representative sample of original membrane cation currents from saline control-treated (black), bupivacaine (BPV)-treated (red), lipid emulsion (LE)-treated (blue), and BPV + LE-treated (green) hippocampal CA1 neurons. Plots describing the (b) Amplitude of AMPA receptor currents, (c) Amplitude of the NMDA receptor currents, (d) Fast tau of the NMDA receptor, (e) Slow tau of NMDA receptor, and (f) AMPA/NMDA (100%) in saline-treated controls ($n = 7$ neurons from 3 animals), BPV-treated ($n = 7$ neurons from 3 animals), LE-treated ($n = 7$ neurons from 3 animals), and BPV + LE-treated ($n = 7$ neurons from 3 animals) brains. All symbols represent means \pm SD; Unpaired t test was performed for statistical evaluation

TABLE 4 Post-synaptic modifications of excitatory transmission in CA1 pyramidal neurons in rat hippocampus

	Control	BPV	LE	BPV + LE
NMDA amplitude (pA)	453.16 \pm 54.59	677.52 \pm 145.83	496.2 \pm 50.76	479.8 \pm 69.79
AMPA amplitude (pA)	879.98 \pm 249.93	1748.41 \pm 523.21	1,004.5 \pm 108.51	763.63 \pm 78.13
AMPA/NMDA (100%)	220.9 \pm 75.4	343.1 \pm 98.26	217.2 \pm 42.96	159.3 \pm 18.62
NMDA fast tau (ms)	46.59 \pm 5.43	49.06 \pm 3.88	53.27 \pm 5.63	61.94 \pm 4.66
NMDA slow tau (ms)	206.48 \pm 18.40	198.98 \pm 11.47	210.16 \pm 16.75	246.76 \pm 11.41

Note: There were no significant differences in CTL, BPV, LE, and BPV + LE rats including NMDA amplitude, AMPA amplitude, AMPA/NMDA, NMDA fast tau, and NMDA slow tau ($p > .05$, Unpaired t test).

levels of GABAA receptor. Our western blot analysis revealed that hippocampal levels of GABAA receptor did not vary significantly between rats treated with BPV, LE, BPV + LE, or saline (Figure 9).

9 | DISCUSSION

Central neurotransmitters are crucial in regulating the normal physiological functions of the body. In the central nervous system of



FIGURE 6 GABA-induced paired pulse ratio of CA1 pyramidal neurons in rat hippocampus. BPV increases GABA-induced PPR of CA1 pyramidal neurons in rat hippocampus (a) Representative sample of original membrane cation currents traces from saline control-treated (black), bupivacaine (BPV)-treated (red), lipid emulsion (LE)-treated (blue), and BPV + LE-treated (green) hippocampal CA1 neurons. Paired pulse ratio (PPR) stimulation separated by (b) 20 ms, (c) 50 ms, (d) 100 ms, and (e) 200 ms in saline-treated controls ($n = 7$ neurons from 3 animals), BPV-treated ($n = 7$ neurons from 3 animals), LE-treated ($n = 7$ neurons from 3 animals), and BPV + LE-treated ($n = 7$ neurons from 3 animals) brains. All symbols represent means \pm SD; * $p < .05$; unpaired t test was performed for statistical evaluation

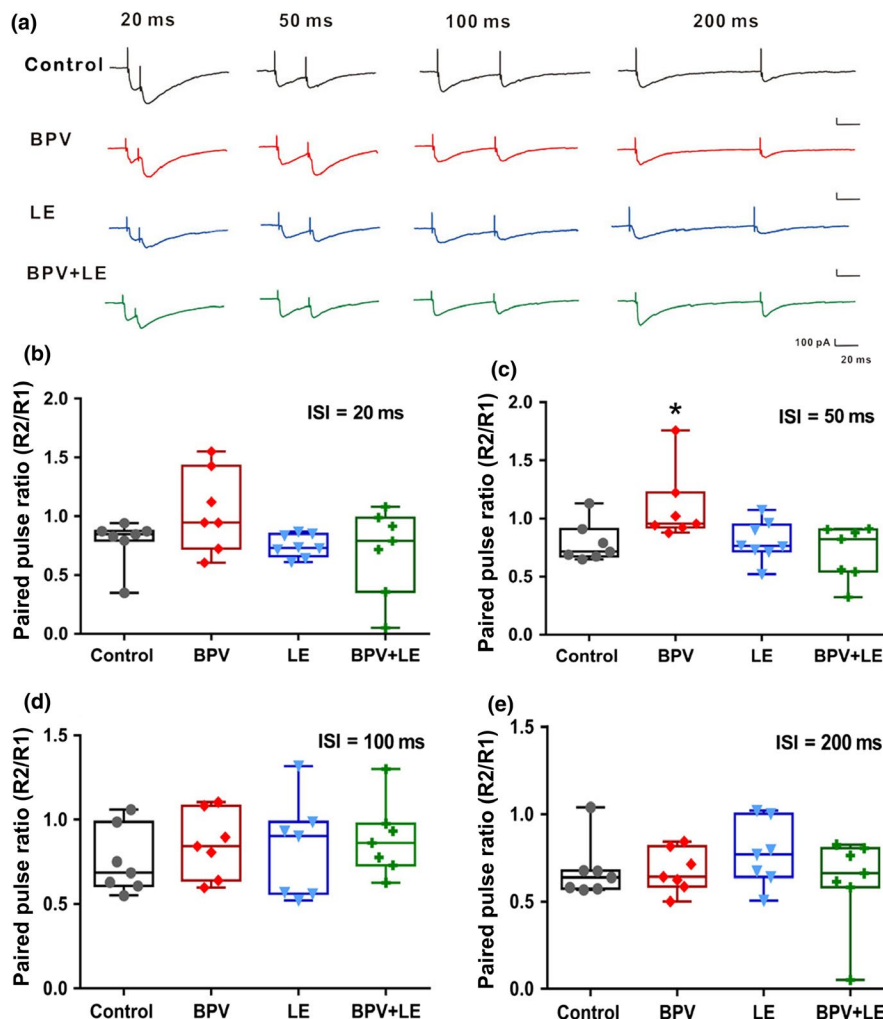


TABLE 5 GABA-induced paired pulse ratio of CA1 pyramidal neurons in rat hippocampus

	Control	BPV	LE	BPV + LE
20 ms	0.79 \pm 0.08	1.05 \pm 0.13	0.74 \pm 0.03	0.7 \pm 0.14
50 ms	0.79 \pm 0.07	1.1 \pm 0.12 ^a	0.80 \pm 0.06	0.7 \pm 0.08
100 ms	0.75 \pm 0.07	0.85 \pm 0.07	0.82 \pm 0.11	0.89 \pm 0.08
200 ms	0.68 \pm 0.06	0.68 \pm 0.05	0.77 \pm 0.07	0.62 \pm 0.10

Note: There was a significant increase in PPF with 50 ms interval stimulation in BPV rats ($p < .05$, Unpaired t test) but no differences in LE and BPV + LE rats compared with CTL group ($n = 7$, respectively, $p > .05$, Unpaired t test). No significant difference was seen in PPF with 20 ms, 100 ms, and 200 ms interval stimulation among four groups ($n = 7$, respectively, $p > .05$, Unpaired t test).

^a $p < .05$ versus controls; Unpaired t test was performed for statistical evaluation.

vertebrates, excitatory (glutamate), and inhibitory (γ -aminobutyric acid) amino acids are widespread, however, the peripheral nervous system and other organism contain very low levels. The human brain contains about four times as many excitatory amino acids as inhibitory neurotransmitters (Lener et al., 2017). The combination of glutamate and the corresponding receptors in the post-synaptic membrane induces excitation of post-synaptic neurons, and excessive activation of NMDA receptors lead to the increase in intracellular Ca^{2+} . Sudden increase in intracellular-free Ca^{2+} is an important

basis for the generation of convulsive discharge and the rapid diffusion of nerve impulses. Increased Ca^{2+} concentration in the cytoplasm can aggravate cerebral ischemia and hypoxia, forming a vicious cycle. In 1969, Olney first proposed that glutamate had a strong excitatory toxic effect on neurons, which was subsequently confirmed by a series of studies (Olney, 1969). Under pathological conditions, glutamate concentration in the extracellular space increases. Overstimulation of glutamate receptors has a significant toxic effect on the CNS, and produces excitatory central nervous

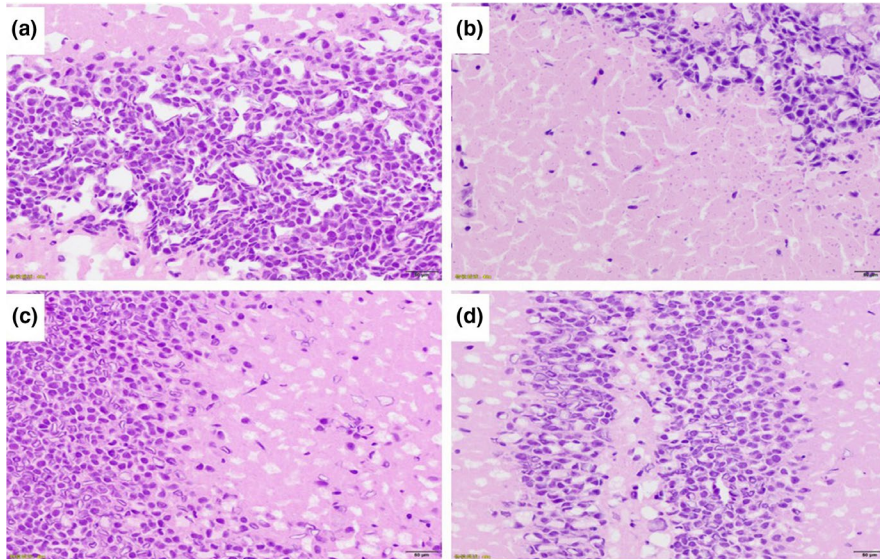


FIGURE 7 H&E staining of rat hippocampus. LE reduces inflammatory cell infiltration in hippocampus of BPV-treated rats. Hematoxylin and eosin stains (400X power). (a) Section from saline treated control group rat hippocampus showing normal nerve architecture. (b) Section from BPV-treated group rat hippocampus showing partial brain degeneration and inflammatory cell infiltration. (c) Section from LE-treated group rat hippocampus showing normal nerve architecture. (d) Section from BPV + LE-treated group rat hippocampus showing local brain degeneration and less inflammatory cell infiltration. Scale bar = 50 μm

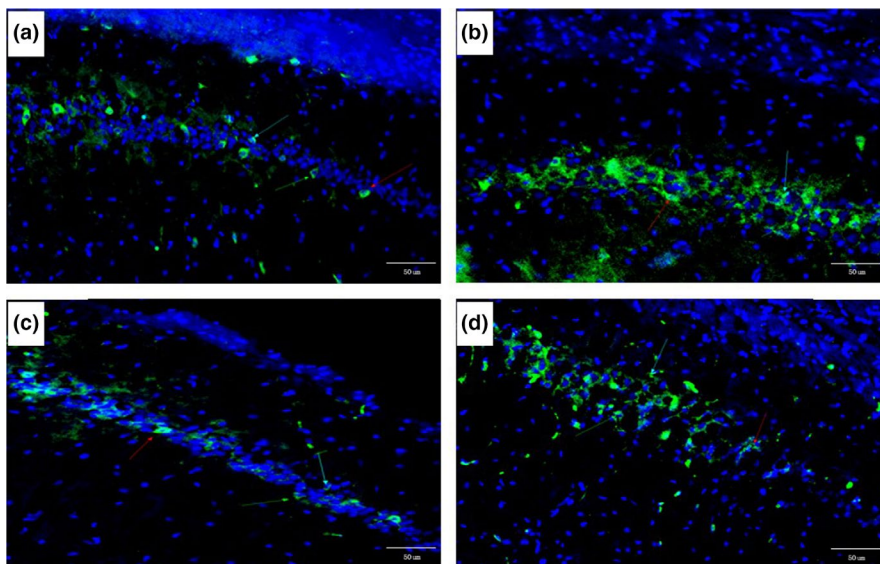


FIGURE 8 TUNEL stains of rat hippocampus. TUNEL stains in rat hippocampus from (a) saline-treated control group, (b) BPV-treated group, (c) LE-treated group, and (d) BPV + LE-treated group (400X power). Scale bar = 50 μm

	Control	BPV	BPV + LE	LE
Hippocampal neuronal apoptosis rate (%)	1.39 ± 0.89	1.46 ± 0.53	1.49 ± 0.69	1.38 ± 0.83

TABLE 6 Apoptosis rate of hippocampal cells in each group ($n = 6$, $\bar{x} \pm s$)

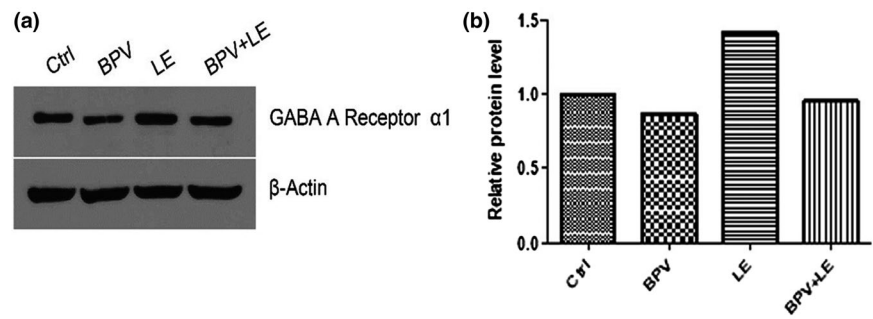
Note: There were no significant differences in hippocampal neuronal apoptosis rate in CTL, BPV, LE, and BPV + LE rats ($p > .05$, Unpaired t test).

toxicity. NMDA-Ca²⁺ not only maintains the excitability of the central nervous system, but also participates in the generation and maintenance of long-term enhancement of learning and memory, which is closely related to the establishment of hippocampal synaptic plasticity (Volianskis et al., 2015). The hippocampus is an important pathophysiological structure that is closely related to the development of convulsion. As an important part of a series of synaptic transmission loops in the hippocampus, the CA1 region must transmit effective eclampsia to other hippocampal regions through the CA1, and the CA1 is more likely to produce eclampsia such as discharge, and also

plays an important role in the persistent excitatory state of eclampsia (Milior et al., 2016).

The incidence of local anesthetic-induced CNS toxicity and convulsions is not rare and its mechanism may be related to imbalances or changes within CNS inhibition-excitation systems (Kang, Dai, Li, & Ma, 2006). Indeed, previous studies have shown that continuous infusion of BPV can inhibit glycine and glutamate-induced currents in a time-dependent manner (Yang et al., 2003). Reduced GABA neurotransmission is one of the main mechanisms underlying local anesthetic-induced convulsions (Hara, Kai,

FIGURE 9 Expression levels of GABA_A receptor in the hippocampus. BPV does not affect expression levels of GABA_A receptor in the hippocampus (a) Western blots of GABA_A receptor $\alpha 1$ in hippocampal samples from saline-treated, BPV-treated, LE-treated, and BPV + LE-treated brains. (b) Quantification of GABA_A receptor $\alpha 1$ protein levels in each group



& Ikemoto, 1995; Ikeda, Dohi, & Tsujimoto, 1982). Cocaine, tetra-*caine*, procaine, and lidocaine all have been shown to reduce synaptic GABA release without affecting its synthesis or degradation (Ikeda, Dohi, & Tsujimoto, 1983). In this study, the number of action potentials elicited by post-synaptic depolarization significantly increased after BPV, whereas following administration of LE or saline, there were no apparent effects of bupivacaine on post-synaptic excitability.

Miniature synaptic currents respond to changes in pre-synaptic transmitters by altering their frequency, and respond to changes in post-synaptic receptors by altering their amplitude. In our study, BPV did not affect either the frequency nor amplitude of miniature-excitatory post-synaptic currents (mEPSCs), implicating that BPV does not result in corresponding post-synaptic membrane changes. In theory, BPV could cause glutamate-mediated current increases. Previous studies from our laboratory have shown that excitatory glutamate levels increase within 6 h of BPV treatment (unpublished data), although this increase occurs without a functional enhancement. We observed no change in excitatory glutamate transmission in our experiments following BPV treatment. It is possible that in our model system, the amount of BPV present was insufficient to act directly on glutamate receptors, as the effect is concentration-dependent. Furthermore, although we see a change in action potential frequency, these changes will not necessarily translate into mEPSC frequency or amplitude changes, since these events are by convention considered to arise from spontaneous (action potential-independent) glutamate release. Our biochemical indicators measured the levels of both spontaneous and non-spontaneous transmitters at a certain time in the entire hippocampus. Previous data from our laboratory showed that non-spontaneous changes occur in previous experiments, including some that showed BPV has an effect on NMDA receptors (unpublished data).

LAST animals exhibit significant convulsive behavior and the main mechanism of convulsions or epilepsy is thought to be an imbalance of excitatory/inhibitory transmission. Action potential-evoked AMPA and NMDA-receptor-mediated synaptic currents were observed in two animal groups. However, we found no significant changes in either the magnitude or the kinetics of glutamate (the major excitatory transmitter) AMPA and NMDA receptors in CA1 pyramidal neurons in rat hippocampus after BPV treatment. The tau index of functional currents can be altered in some pathological

processes. While the rapid response element of tau is mostly mediated by the NR2A NMDA subunit, long-term slow elements of tau are mainly mediated by the NR2B subunit. However, because we saw no change in either tau, and no change in AMPA/NMDA properties, we hypothesized that the disturbance of excitatory/inhibitory transmission was likely because of inhibitory transmitters. As such, we next assessed the release rate of GABA by assessing paired-pulse ratio (PPR) of IPSCs.

We found that BPV treatment significantly reduced the frequency, but not the amplitude, of miniature-inhibitory post-synaptic currents. This finding indicates that BPV significantly inhibited the release of GABA. Western blot experiments showed no effect of post-synaptic GABA_A receptor expression. Central toxic seizures resulting from BPV may cause transient synaptic function inhibition or damage in the hippocampus, or may result in a decrease in the number of pre-synaptic membrane vesicles in the hippocampus. The impaired function of the GABAergic pre-synaptic membrane could reasonably result in decreased GABA synaptic vesicle release. In this case, we would expect reduced synaptic activity and a subsequent decrease in GABA synthesis and release.

10 | POSSIBLE MECHANISMS OF LIPID EMULSION PROTECTION IN THE BRAIN

After cerebral ischemia-reperfusion injury, ATP is reduced and lactic acid and free radicals begin to accumulate, resulting in serious damage to neuronal structure and function. LE not only provides the high-energy essential fatty acids necessary for brain cell metabolism, but also provides the polyunsaturated fatty acids required for the metabolism of brain biofilms and bioactive substances. Furthermore, LE oxidation is independent on insulin, meaning that the energy supplementation of impaired glucose metabolism during stress states (such as trauma and infection) can improve neurological function.

LE has also been shown to inhibit inflammatory responses in previous experiments (King et al., 2006). Omega-3 fish oil fat emulsion can reduce C-reactive protein levels in severely infected patients, inhibit the release of the inflammatory mediators TNF- α , IL-1, and IL-6, and stabilize neutrophil function. BPV-induced CNS injury activates neutrophils, releases a large number of inflammatory factors, triggers inflammatory cascades, and results in changes in microvascular beds and hemorheology. By reducing neutrophil infiltration in

brain tissue and inhibiting inflammatory responses, LE may prevent nerve cell damage, reduce brain edema, and maintain nerve function.

Finally, LE may offer protection of endothelial cells and monocyte-macrophages: Wakefield, Cohen, Craig, et al. (1989a) have suggested that LE may inhibit the procoagulant activity of monocytes and vascular endothelial cells, through a mechanism involving free fatty acids of its metabolites. Some unsaturated fatty acid derivatives in the body can also weaken the procoagulant activity of vascular endothelial cells and thus have protective effects on the vasculature (Wakefield, Cohen, Craig, et al., 1989a; Wakefield, Cohen, Rosenthal, et al., 1989b). The CNS damage caused by BPV produces a large number of inflammatory mediators, causing endothelial cell and basement membrane damage, and increased blood-brain barrier permeability. Thus, LE may exert its protective effects by inhibiting the increase in blood-brain barrier permeability and reducing CNS toxicity by acting on endothelial cells and monocytes.

This study provides an initial glance at the electrophysiological manifestations of the protective effects of LE in the CNS after BPV-induced toxicity.

11 | LIMITATIONS

Pentobarbital is a common anesthetic drug used in animal experiments for neuroelectrophysiology (Chun & Xiao-hui, 2008; Hájos & Mody, 1997; Soltesz et al., 1994) and to treat seizures by increasing the activity of the inhibitory neurotransmitter GABA. Use of this drug may be a limitation which could affect our study. We are planning further studies that will make use of different drugs that also increase the activity of the inhibitory neurotransmitter GABA. This study had several limitations. First, the rats we used were male rats weighing between 300–340 g, aged between 9–10 weeks. It is possible that, because of differences in nutritional intake, there were different basic electrophysiological states of neurons. Second, the state and electrophysiological properties of rat hippocampal neurons are closely related to age; At present, we are unable to assess the exact electrophysiological states of neurons in rats of the same weight or age, so this unavoidable factor may somehow impact outcome. Third, the acquisition of electrophysiological data is easily affected by external factors such as electromagnetic radiation. We try to minimize human error under existing conditions, but the errors generated by the equipment may still affect results. Our data were recorded, stored, and analyzed using pClamp10.6 software. Furthermore, although there are programs that automatically match the target current waveform, in this study we use manual screening; because the software does not fully recognize similar waveforms from the target current waveforms, subjective factors could impact results.

12 | CONCLUSIONS

LE can increase the GABA-mediated current and release frequency of hippocampal neurons during BPV-induced CNS toxicity in rats.

LE may have clinical utility in the treatment of convulsions related to BPV-induced CNS toxicity by balancing excitatory and inhibitory neurotransmission in the brain.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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