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[+]-Huperzine A treatment protects against *N*-methyl-D-aspartate-induced seizure/*status epilepticus* in rats

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ABSTRACT

The toxicity of organophosphorous (OP) nerve agents is attributed to their irreversible inhibition of acetylcholinesterase (AChE), which leads to excessive accumulation of acetylcholine (ACh) and is followed by the release of excitatory amino acids (EAA). EAAs sustain seizure activity and induce neuropathology due to over-stimulation of *N*-methyl-D-aspartate (NMDA) receptors. Huperzine A (Hup A), a blood–brain barrier permeable selective reversible inhibitor of AChE, has been shown to reduce EAA-induced cell death by interfering with glutamate receptor-gated ion channels in primary neuronal cultures. Although [−]-Hup A, the natural isomer, inhibits AChE approximately 38-fold more potently than [+]-Hup A, both [−]- and [+]-Hup A block the NMDA channel similarly. Here, we evaluated the protective efficacy of [+]-Hup A for NMDA-induced seizure in a rat model. Rats implanted with radiotelemetry probes to record electroencephalography (EEG), electrocardiography (ECG), body temperature, and physical activity were administered various doses of [+]-Hup A (intramuscularly) and treated with 20 μg/kg NMDA (intracerebroventricular) 20–30 min later. For post-exposure, rats were treated with [+]-Hup A (3 mg/kg, intramuscularly) 1 min after NMDA (20 μg/kg). Our data showed that pre- and post-exposure, [+]-Hup A (3 mg/kg) protects animals against NMDA-induced seizures. Also, NMDA-administered animals showed increased survival following [+]-Hup A treatment. [+]-Hup A has no visible effect on EEG, heart-rate, body temperature, or physical activity, indicating a reduced risk of side effects, toxicity, or associated pathology. Our results suggest that [+]-Hup A protects against seizure and *status epilepticus* (SE) by blocking NMDA-induced excitotoxicity *in vivo*. We propose that [+]-Hup A, or a unique combination of [+]- and [−]-Hup A, may prove to be effective for pre- and post-exposure treatment of lethal doses of OP-induced neurotoxicity.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ATCh, acetylthiocholine iodide; BChE, butyrylcholinesterase; CWNA, chemical warfare nerve agent; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EAA, excitatory amino acid; ECG, electrocardiography; EEG, electroencephalography; GD, soman; Hup A, Huperzine A; icv, intracerebroventricular; im, intramuscular; *iso*-OMPA, tetraisopropyl pyrophosphoramide; NMDA, *N*-methyl-D-aspartate; OP, organophosphate; PCP, phencyclidine; SE, *status epilepticus*.

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1. Introduction

Nerve agent and pesticide organophosphate (OP) poisoning represents an ongoing threat to public safety given their low cost and relative ease of synthesis [1–4]. OPs act by irreversible inhibition of acetylcholinesterase (AChE) leading to the build-up of acetylcholine (ACh) at the neuron–neuron and neuro-muscular junction. With the accumulation of ACh, neurons release excitatory amino acids (EAAs), such as glutamate, which bind to the *N*-methyl-D-aspartate (NMDA) receptor causing excitotoxicity and contributes to the seizure/*status epilepticus* (SE) observed with OP toxicity [5]. SE is a long seizure that produces permanent brain damage. Pharmacologic intervention of EAA-induced toxicity at the NMDA receptor is considered a promising approach to protect against seizure/SE and neuropathology [6,7].

Huperzine A (Hup A) is an alkaloid found in the Chinese club moss *Huperzia serrata*. The [−]-Hup A enantiomer is found naturally and [+]-Hup A is synthetic. [−]-Hup A has a much higher affinity for AChE than synthetic [+]-Hup A [8], and it has been demonstrated that [−]-Hup A binds to the bottom of the active site gorge of the enzyme [9]. However, both enantiomers have much higher affinities for AChE than butyrylcholinesterase (BChE) [10], which is being developed as another treatment for nerve agent toxicity [11,12]. Also, [−]-Hup A inhibits both peripheral and central AChE, unlike pyridostigmine bromide, a commonly used OP pretreatment that has been FDA-approved which only inhibits peripheral cholinesterases [13]. This is mainly because Hup A (both enantiomers), unlike pyridostigmine, does not bear a permanent positive charge.

Previous studies have shown that [−]-Hup A has a longer biological half-life than other reversible AChE inhibitors [14] and pre-treatment can prevent seizure. Our group conducted a study in primary guinea pig neuronal cell cultures and demonstrated that Hup A binds to NMDA receptors and protects against glutamate toxicity [15]. Both [+]- and [−]-Hup A were found to antagonize glutamate toxicity (Zang, 2000) although [+]-HupA has 38-fold lower AChE binding activity. [8]. A preliminary study with [+]-Hup A using a radiotelemetry rat seizure/SE model demonstrated that a [+]-Hup A pre-treatment prevents pilocarpine, a muscarinic agonist, induced seizures, but [−]-Hup A does not (data not shown). We hypothesize that [+]-Hup A-induced protection is a result of its *in vivo* NMDA receptor antagonism by blocking its ion channel without inhibiting AChE. The data using the pilocarpine model, *in vitro* Hup A receptor binding experiments [15–18] (Zhang, 2000), and previous voltage clamp experiments [19] led us to determine if the *in vivo* mechanism of action of [+]-Hup A involves NMDA antagonism, and if so, further develop [+]-Hup A as a treatment for seizure and SE induced by toxic OPs.

We used our rat radiotelemetry model to investigate the in vivo mechanism of action induced by [+]-Hup A [20]. Seizures were induced by intracerebroventricular (icv) administration of NMDA, and the animals were treated with different concentrations of [+]-Hup A pre- or post-exposure. We demonstrated that [+]-Hup A prevents NMDA-induced seizure in rats, indicating that its mechanism of action involves NMDA antagonism and blocking EAA-induced toxicity.

2. Materials and methods

2.1. Chemicals

NMDA, tetraisopropyl pyrophosphoramide (*iso*-OMPA), acetylthiocholine iodide (ATCh), and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich (St. Louis, MO). [+]-Hup A was obtained from Dr. Robert Moriarty, University of Illinois at Chicago. Isoflurane was purchased from Halocarbon Inc. (River Edge, NJ).

2.2. Animals

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition. Male Sprague–Dawley rats (200–250 g, *Rattus norvegicus*) were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed individually in microisolator cages with a 12 h light/dark cycle. Food and water were available *ad libitum*, and a 1 week stabilization period preceded surgery and experimentation.

2.3. Radiotelemetry

The Radiotelemetry system including 8 receivers and TL10M3-F50-EET bipotential radiotelemetry probes was purchased from Data Sciences International (St. Paul, MN). The probes were sterilized using 4% glutaraldehyde and handled as instructed by the manufacturer.

2.4. Surgical implantation of electroencephalography (EEG) probes

Rats were anesthetized in a chamber with isoflurane gas (2–5% isoflurane, oxygen 1 L/min flow rate). Anesthetized rats were sheared on the head and back and placed in a stereotax (David Kopf Instruments, Tujunga, CA) over a water heating jacket. The mouth and nose of the rat were placed in an adapter connected to a supply of isoflurane gas (2–3% isoflurane, oxygen 1.5 L/min flow rate). The dorsal surfaces of the rat's body and head were cleaned and two small initial incisions were made: one along the midline of the back, 7.5 cm anterior to the tail, and one along the dorsal midline of the head. Two cortical electrodes and a reference electrode were tunneled subcutaneously from the posterior (back) incision to the anterior (head) incision. The skull was cleaned with gauze, and any open veins or arteries were closed by surgical cautery. Three 1 mm burr holes were drilled, and screws were inserted: two screws 3 mm anterior to the lambdoid suture and 3 mm on each side of the sagittal suture, and one screw was inserted 3 mm to the right of the sagittal suture and 3 mm anterior to the coronal suture. The reference electrode was attached

to the forward-most screw and fixed into place. The positive electrode was placed at the right, posterior screw, and the negative electrode was placed at the left screw. VetBond (Plastics One, Roanoke, VA) was used to keep the electrodes in place. The incision was sutured using Ethicon sutures (Piscatawy, NJ).

2.5. Placement of cannula for icv NMDA administration

The cannula and dummy cannula were obtained from Plastics One (Roanake, VA). A burr hole (1 mm in diameter) was made 1 mm posterior and 1.4 mm to the right of the bregma. The cannula was inserted 5 mm below the top of the skull and immobilized using VetBond. A dummy cannula was also inserted and screwed in place until the day of NMDA administration. During NMDA administration, the animals were gently anesthetized using isoflurane and placed on the stereotaxic equipment. The dummy cannula was unscrewed and a needle connected to a Hamilton microsyringe was inserted. NMDA in a volume of 10 μL was injected, and the dummy cannula was put back into place.

2.6. Placement of electrocardiography (ECG) wires and probe

The positive ECG electrode was subcutaneously tunneled along the left side of the rat's abdomen to the xiphoid process, and the negative ECG wire was subcutaneously tunneled along the right anterior side to the right pectoral muscle. The probe was inserted subcutaneously on the left dorsal pocket of the rat. The incision was sutured, and the rat was injected with bupivacaine to alleviate the discomfort from the surgical procedure.

2.7. Development of an NMDA seizure model in rats

Rats were randomly assigned to either an experimental group ($n = 6$) or a control group ($n = 6$). The telemetry probes were activated using an external magnet, and the rat's cage was placed at the center of a telemetry receiver. Radiotelemetry data (EEG, ECG, physical activity, and body temperature) were monitored continuously beginning 30 min before any treatment. The experimental group was injected with an NMDA dose of 20 μg/kg, icv, based on a previous unpublished study by our Department (Dr. Haresh Ved, personal communication) that used mice and demonstrated "popcorn convulsions" (data not shown). The control group received icv injections of an equal volume of saline. Behavioral data, such as eating, drinking, physical activity, and seizure were noted continuously for 4–6 h after injections. After 24 h, all surviving rats were euthanized.

2.8. Treatment with [+]-Hup A against NMDA toxicity

Telemetry probe implanted Sprague–Dawley rats were randomly assigned to four experimental groups and one control group. For pre-exposure treatment, after a 30 min recording of the baseline EEG levels, the animals were administered with various doses of [+]-Hup A (1 mg/kg ($n = 3$), 2 mg/kg ($n = 3$), or 3 mg/kg ($n = 4$)) intramuscularly, im The dose of [+]-Hup A was also selected from a previous unpublished study in our Department using rats against soman exposure (Dr. Haresh Ved, personal communication). Thirty min later, NMDA (20 μg/kg) was administered icv in a volume of 10 μL. The animals were continuously monitored by radiotelemetry. For post-exposure treatment, 30 min after the baseline EEG recording, the animals were administered with NMDA (20 μg/kg) and 1 min later [+]-Hup A (3 mg/kg) was administered im ($n = 4$). The animals were monitored by radiotelemetry for 24 h.

2.9. Blood AChE assay

To determine blood AChE inhibition, about 5 mL of blood was collected by cardiac puncture from each euthanized animal. Blood was diluted 10-fold with deionized water and 20 μL of diluted sample was placed in the well of a 96-well microtiter plate for a modified microtiter assay for AChE activity [21,22]. To this, 260 μL of phosphate buffer (pH 8.0) containing 4 μM of *iso*-OMPA (BChE inhibitor) was added, along with 10 μL of DTNB followed by 10 μL of ATCh. The plate was read in a SpectraMax Plus^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA) at 412 nm. The AChE activity, measured in triplicate, was normalized to total blood protein measured by Bradford dye binding assay at 595 nm [23].

3. Results

3.1. NMDA-induced seizure/SE model

Rats administered with NMDA (20 μg/kg, icv) showed strong seizure at approximately 14–16 min post-NMDA treatment (Fig. 1A). These were "popcorn convulsion" seizures which immediately became SE. The survival of animals following NMDA administration was 50% ($n = 6$). A 10 s enlarged EEG recording at 3 h after the administration of NMDA or saline is shown in Fig. 1B. The seizure voltage increased gradually and reached an average value from +0.7 mV to −0.7 mV within 10 min (Fig. 1A and B). The seizure amplitude was higher (+0.7 mV to −1 mV) at 2.5–5 h after NMDA administration. After 5 h the seizure voltage magnitude started to drop gradually but remained higher than the baseline EEG for the remainder of the 24 h monitoring period.

3.2. Heart rate, body temperature and physical activity with the NMDA-model of seizure/SE

The heart rates of saline control animals were normal, within the range of 300–500 beats per minute (Fig. 1C). NMDA-administered animals exhibited an increase in the heart-rate beginning 15 min after NMDA administration, which lasted to 3.5 h, then slowly decreased and returned to normal levels (Fig. 1C). After 17 h, the heart-rate of NMDA-administered animals was 320 bpm which remained constant during the rest of the experiment. The body temperature decreased 6 h after NMDA administration, the lowest reading was 31 °C at 8 h following NMDA exposure. The decrease in body temperature lasted for 1 h and then returned to 34 °C and remained at that level

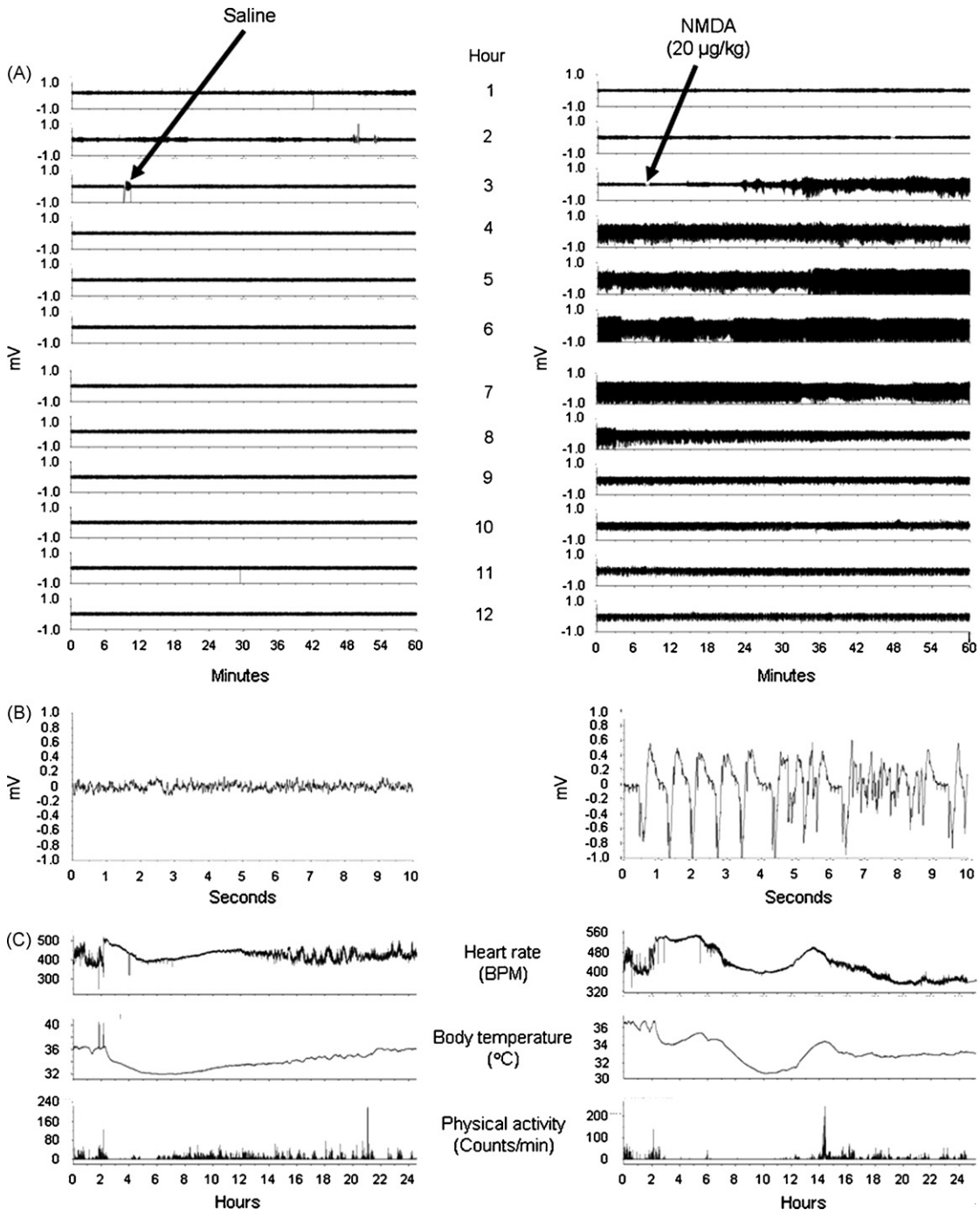

Fig. 1. NMDA-induced seizure/SE. (A) Comparison of the EEG data from a representative saline control (left) and a rat administered with NMDA by icv (right). Each line represents compressed 1 h of EEG recording and 12 h of recordings are shown. The scale on the *Y*-axis for each line is −1 mV to +1 mV. The point when either saline or NMDA (20 μg/kg, icv) was administered is noted with an arrow. (B) A 10 s segment of direct EEG recordings from radiotelemetry 3 h after NMDA or saline administration. *Y*-Axis is −1 mV to +1 mV. (C) Heart rate (bpm), body temperature (°C), and physical activity during the 24 h monitoring period.

to 24 h. Control animals showed a drop in body temperature from 36 °C to 32 °C after 4 h, which returned to baseline levels over the 24 h monitoring period. Physical activity, as calculated by the number of times the rat crossed the middle of the cage, was significantly less in NMDA-treated animals compared to controls (Fig. 1C). The quiet period was remarkable at 30 min after NMDA treatment and remained until 8 h after NMDA administration. Control animals were observed to have only a brief quiet period.

3.3. Pre-treatment with [+]-Hup A protects against NMDA toxicity

Radiotelemetry implanted animals pre-treated with 3 mg/kg of [+]-Hup A, im, and then exposed to NMDA (20 μg/kg, icv) 30 min later showed a significant decrease in seizure/SE (Fig. 2A, compared to Fig. 1A). Pre-treatment with [+]-Hup A completely abolished NMDA-induced seizure/SE. Although there were occasional marginal increases in the EEG amplitude of [+]-Hup A treated animals, the overall EEG amplitude and number of spikes were very similar to saline controls. The 24 h EEG recording of animals pre-treated with [+]-Hup A showed normal amplitudes and no seizure. A 10 s segment of the direct EEG recording of [+]-Hup A treated animal was very similar to the saline controls (Fig. 2B compared to Fig. 1B, respectfully).

3.4. The effect of a [+]-Hup A pre-treatment on heart rate, body temperature, and physical activity

Animals exposed to NMDA after pre-treatment with 3 mg/kg [+]-Hup A showed normal physical activity (Fig. 2C). Interestingly, the quiet period observed following NMDA exposure was completely eliminated in [+]-Hup A treated animals. The behavior of animals pre-treated with 3 mg/kg [+]-Hup A was very similar to normal rats by visual observation. [+]-Hup A pre-treated animals also maintained a normal heart rate of 300–500 bpm (Fig. 2C). The body temperature of rats pre-treated with [+]-Hup A, and then NMDA tended to be very unstable, but remained within the normal temperature range throughout the 24 h monitoring period (Fig. 2C).

3.5. Effect of post-NMDA exposure treatment with [+]-Hup A against NMDA toxicity

Since there is a requirement to develop a therapeutic that is effective against post-exposure to organophosphates, we determined the efficacy of post-exposure treatment with [+]-Hup A for NMDA toxicity. Animals were exposed to NMDA and treated with 3 mg/kg [+]-Hup A 1 min later and the animals were monitored by radiotelemetry as described in "Materials and Methods" (Section 2.8). One minute post-exposure treatment with [+]-Hup A also showed strong protection with complete reduction in seizure/SE (Fig. 3A compared to Fig. 1A). Seizure amplitude was close to the baseline EEG levels during the 24 h duration of the experiment. A 10 s radiotelemetry EEG segment at 3 h after NMDA treatment showed EEG amplitude between −0.2 mV and 0.2 mV which was virtually similar to saline controls (Fig. 3B).

3.6. The effect of a [+]-Hup A post-exposure treatment on heart rate, body temperature and physical activity

The physical activity of the animal was not affected by post-exposure treatment with 3 mg/kg [+]-Hup A (Fig. 3C). One min post-exposure treatment showed an occasional slight increase in the heart rate which returned to nor-

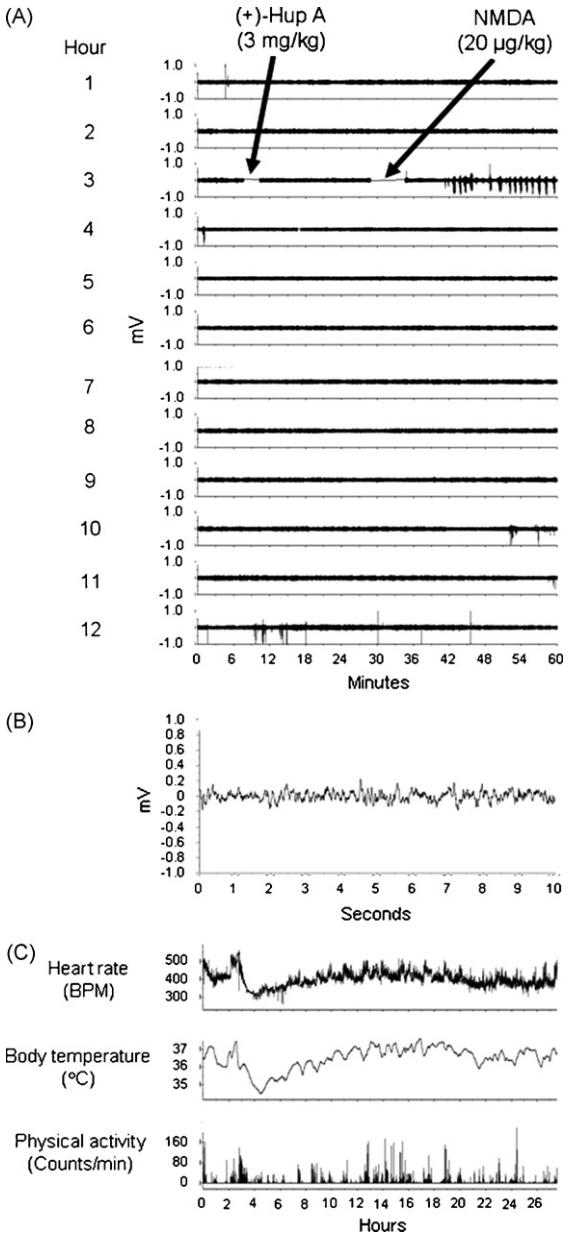

Fig. 2. Pre-treatment with [+]-Huperzine A protects against NMDA-induced seizure/SE. (A) Representative EEG recordings of [+]-Hup A (3 mg/kg), 30 min pre-treated animals showing reduction of seizure/SE induced by NMDA. Each line represents compressed 1 h of EEG recording and 12 h of recordings are shown. The scale on the *Y*-axis for each line is −1 mV to +1 mV. The times when [+]-Hup A (3 mg/kg, im) and NMDA (20 μg/kg, icv) were administered are indicated with arrows. (B) A 10 s segment of direct radiotelemetry EEG recordings of [+]-Hup A (3 mg/kg, im) pre-treated animals 3 h after NMDA administration. *Y*-Axis is −1 mV to +1 mV. (C) Heart rate (bpm), body temperature (°C), and physical activity during the 24 h monitoring period in [+]-Hup A treated animals exposed to NMDA.

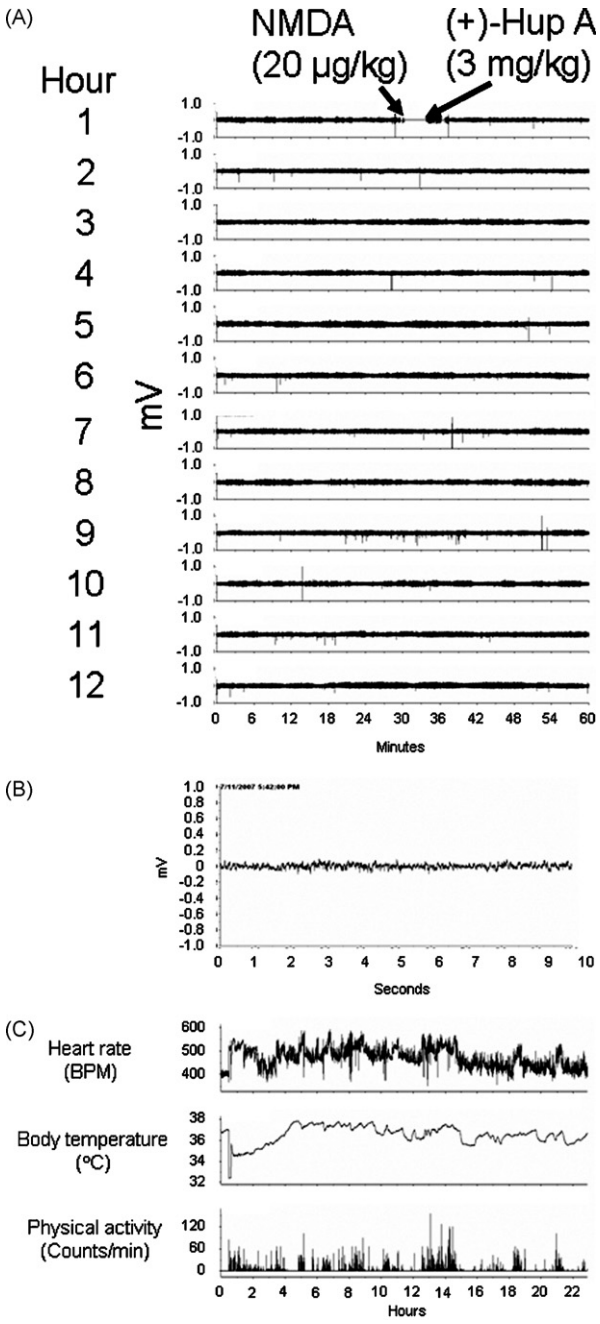

Fig. 3. Post-exposure treatment with [+]-Huperzine A protects against NMDA-induced seizure/SE. (A) Representative EEG recordings of rats treated with [+]-Hup A (3 mg/kg) 1 min after NMDA exposure showing complete reduction of seizure/SE. The times when [+]-Hup A (3 mg/kg, im, 1 min after NMDA) and NMDA (20 μg/kg, icv) were administered are indicated with arrows. Each line represents compressed 1 h of EEG recording and 12 h of recordings are shown. The scale on the *Y*-axis for each line is −1 mV to +1 mV. (B) A 10 s segment of direct radiotelemetry EEG recordings 3 h after NMDA administration in [+]-Hup A 1 min post-exposure treated rats. *Y*-Axis scale is −1 mV to +1 mV. (C) Heart rate (bpm), body temperature (°C), and physical activity during the 24 h monitoring period in [+]-Hup A post-exposure treated animal.

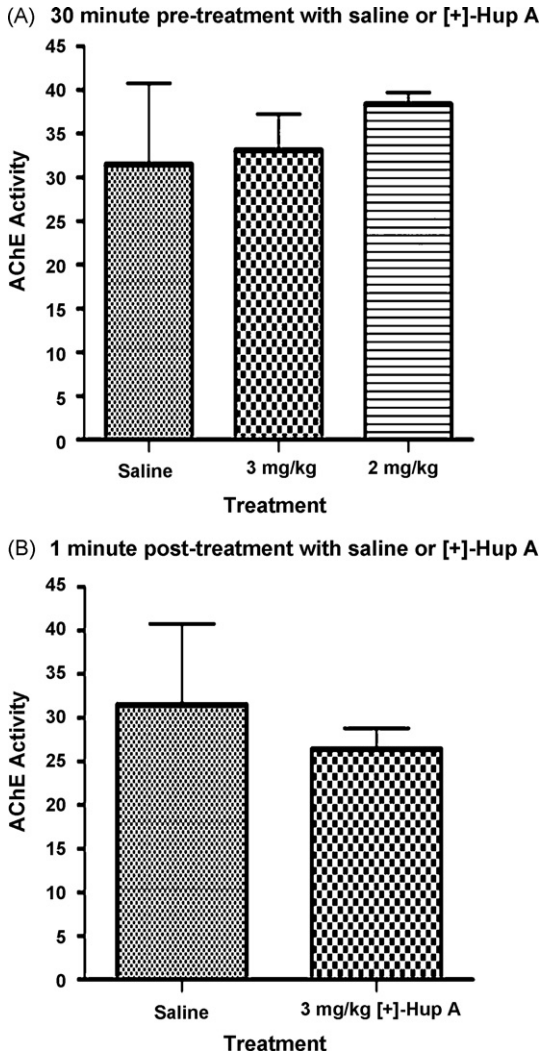

Fig. 4. Blood AChE activity in either [+]-Hup A 30 m pre-treated rats, [+]-Hup A 1 m post-treated rats, or NMDA controls. (A) A bar graph showing the similarity in AChE (units of AChE per mg of blood protein) activity between rats with a 30 min pre-treatment of [+]-Hup A (3 mg/kg or 2 mg/kg, im) and NMDA (20 μg/kg, icv) controls ($n = 4$). (B) A bar graph showing the similarity in AChE (units of AChE per mg of blood protein) activity between rats with a 1 min post-treatment of [+]-Hup A (3 mg/kg, im) and NMDA (20 μg/kg, icv) controls ($n = 4$).

mal levels 5 h after treatment (Fig. 3C). Rats treated with post-exposure [+]-Hup A showed normal baseline body temperature throughout the 24 h recording period (Fig. 3C).

3.7. Blood AChE activity

Acetylcholinesterase activity of rats pre-exposure (2 and 3 mg/kg, im) and post-exposure (3 mg/kg, im) exposure treated with [+]-Hup A, is shown in Fig. 4. The data showed no significant difference (analysis of variance or Student's *t*-test, respectfully) between the blood AChE activities in [+]-Hup A rats treated and those receiving NMDA alone. Similarly, brain AChE was also not affected by [+]-Hup A treatment.

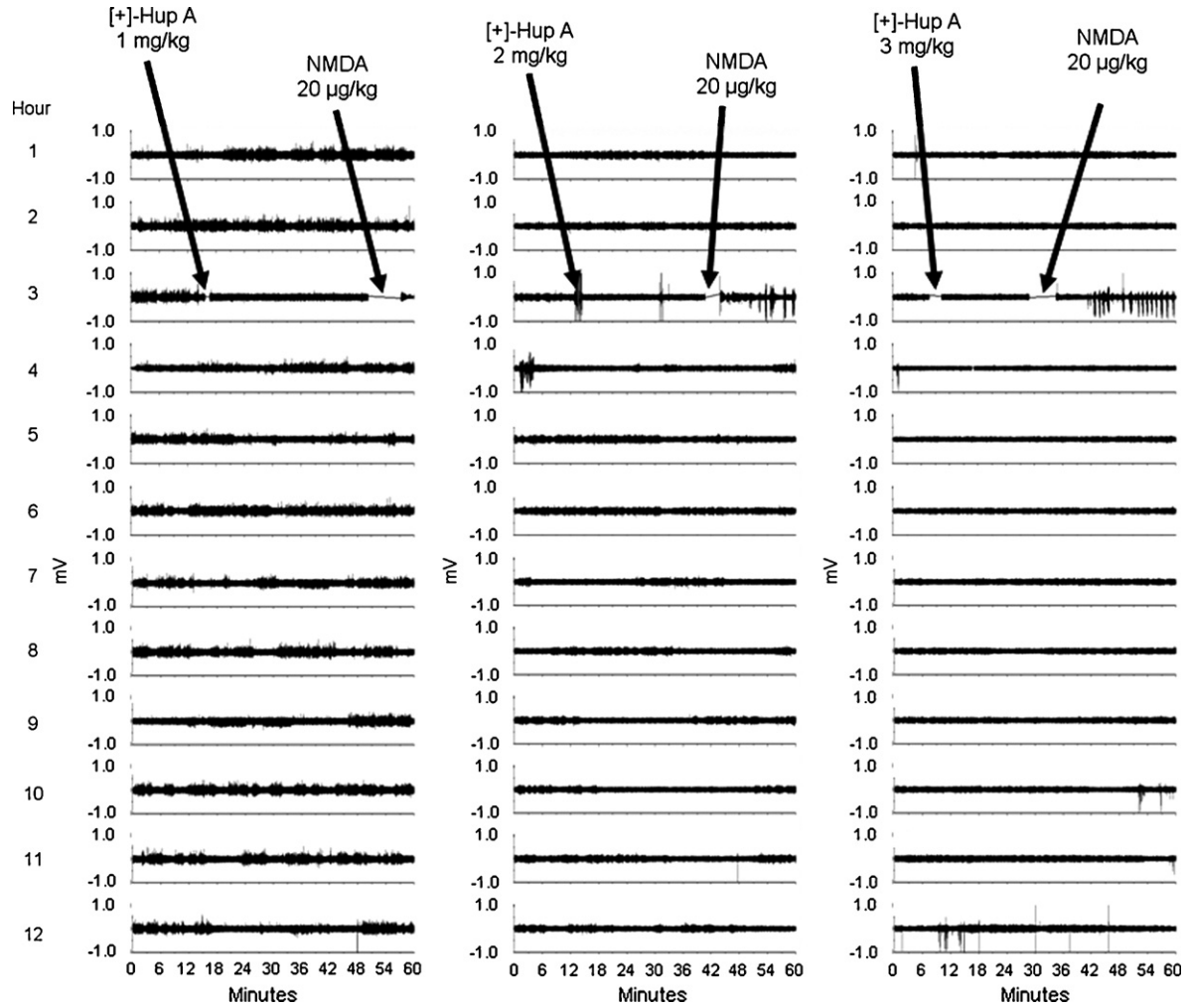

Fig. 5. Comparison of different doses of [+]-Hup A pre-treatment. The EEG recordings for animals pre-treated with 1 mg/kg, 2 mg/kg, and 3 mg/kg [+]-Hup A, im, 30 min prior to NMDA (20 μg/kg, icv) are shown. Each line represents compressed 1 h of EEG recording and 12 h of recordings are shown. The scale on the *Y*-axis for each line is −1 mV to +1 mV. All three doses of [+]-Hup A protected against NMDA-induced seizure.

3.8. Effect of lower doses of [+]-Hup A pre-treatment

To further determine the lowest concentration of [+]-Hup A for protection against seizure/SE induced by NMDA, we decreased the concentration of [+]-Hup A and assessed the protective effect. [+]-Hup A (1 mg/kg and 2 mg/kg) was administered 30 min before NMDA (20 μg/kg) exposure. The EEG data shown in Fig. 5 reveal that [+]-Hup A at these two doses also protected against NMDA-induced seizure/SE.

4. Discussion

Our major finding is that [+]-Hup A protects against NMDA-induced seizure/SE. We also demonstrated that pre-treatment and post-exposure treatment with [+]-Hup A protects against seizure/SE. Lack of blood AChE inhibition, even at the highest dose of [+]-Hup A suggests that [+]-Hup A neuroprotection is mediated by NMDA antagonism and not due to any reversible affects on AChE. Our results demonstrate that the *in vivo* mechanism of [+]-Hup A protection mainly involves NMDA antagonism. This is the first report to indicate that [+]-Hup A acts *in vivo* through NMDA antagonism. In a previous report, we showed that [−]-Hup A binds to NMDA receptors and antagonizes glutamate toxicity [15]. Here, a single dose of [+]-Hup A provided 1 min after NMDA exposure was able to protect against seizure/SE for 24 h, indicating that rapid central nervous system antagonism of the EAA pathway is very effective in mitigating seizure. Thus, [+]-Hup A shows promise for being used as a pre- and post-treatment for the EAA toxicity that accompanies many conditions including stroke, traumatic brain injury, epilepsy, and several neurodegenerative diseases, in addition to organophosphate toxicity [5,24–29].

Here, we focused on developing an EAA antagonist that can (a) easily pass through the blood–brain barrier to reach the central nervous system and (b) rapidly protect against seizure/SE for use as a post-exposure therapeutic for OP nerve agent and pesticide exposure. Despite the fact that high doses of OPs induce excitotoxicity and long-term

brain damage, there is still no approved OP treatment that focuses on NMDA receptor antagonism [1,30]. The current treatment for acute chemical warfare nerve agent poisoning symptoms is comprised of a combination of: (1) oximes to reactivate the AChE that is inhibited by the OP nerve agents; (2) atropine to antagonize the action of excess ACh at peripheral muscarinic receptor sites; (3) anti-convulsant benzodiazepines to allosterically potentiate the action of the inhibitory neurotransmitter GABA at $GABA_A$ receptors [1,4,31–33]. The effectiveness of the current combination treatment of choice is limited for several reasons. First, the therapeutic window of oximes is narrow because of the rapid "aging" of AChE coupled to CWNAs, especially with the nerve agent soman (GD) [30,34]. Second, the poor penetration of oximes across the blood brain barrier prevents reactivation of brain AChE [35,36]. Third, despite control of immediate life threatening effects of CWNAs on respiratory failure, the antimuscarinic atropine has a relatively short half-life [37]. Fourth, current treatments with atropine become refractory to control seizure only a few minutes after induction because seizure/SE is sustained by EAA neurotransmission [5]. Fifth, benzodiazepines may worsen the respiratory depression of those suffering from nerve agent toxicity [38], and sixth, benzodiazepines do not completely protect against OP-induced neuropathology [39,40]. Our preliminary experiments with diisopropylfluorophosphate, a surrogate of the nerve agents soman and sarin, showed that 1 min post-exposure treatment with [+]-Hup A (3 mg/kg, im) protected against OP-induced seizure/SE. The protection we describe was observed in the absence of atropine and 2-PAM, suggesting that [+]-Hup A offers strong protection against OP-induced seizure/SE (data not included).

NMDA receptor antagonists that block the ion-channel tend to produce phencyclidine (PCP) symptoms [41]. However, the physical activity data suggest that locomotor stimulation is not found in these animals after an effective treatment of [+]-Hup A. We also found that [+]-Hup A does not have any adverse effects on body temperature or heart rate, suggesting that [+]-Hup A has a strong affinity for NMDA receptor with little effect on other physiological variables, whereas most NMDA antagonists with strong affinities for the NMDA receptor do affect physiological processes [42]. Why NMDA antagonism by [+]-Hup A does not induce PCP-like symptoms needs to be studied further. Most likely this is an issue of low-medium affinity for the channel blocking site *in vivo* or it is also possible that [+]-Hup A might bind at a different site. Either way, an NMDA antagonist that does not produce PCP-like symptoms will be a powerful tool against excitotoxicity and major depression, among other things [5,24–29,43,44].

Although this study did not evaluate possible treatment doses, regimens, or effective post-exposure treatment duration for [+]-Hup A, it did confirm that [+]-Hup A at lower doses (1 mg/kg and 2 mg/kg) was able to protect against seizure/SE (Fig. 5). Previous studies suggest that [−]-Hup A protects against OP toxicity by the reversible inhibition of AChE [45], and this study suggests that [+]-Hup A protects against the EAA-induced seizure/SE that accompanies OP toxicity. For pre-treatment protection against OPs, a reversible inhibitor of AChE is generally desired in addition to other neuroprotectants [1,38,46–48]. We propose a combination of [−]-Hup A, a reversible AChE inhibitor, and [+]-Hup A, an NMDA antagonist, both of which pass through the blood–brain barrier. Lastly, since an im injection with [+]-Hup A protected against seizure/SE following pre- and post-exposure treatment, [+]-Hup A can be easily used with the current atropine auto injector. Consequently, we feel that this may become a great contribution to the current treatment.

Acknowledgements

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