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Huperzine A prophylaxis against pentylenetetrazole-induced seizures in rats is associated with increased cortical inhibition

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ABSTRACT

Huperzine A (HupA) is a naturally occurring compound found in the firmoss *Huperzia serrata*. While HupA is a potent acetylcholinesterase inhibitor, its full pharmacologic profile is incompletely described. Since previous works suggested a capacity for HupA to prophylax against seizures, we tested the HupA antiepileptic potential in pentylenetetrazole (PTZ) rat epilepsy model and explored its mechanism of action by spectral EEG analysis and by paired-pulse transcranial magnetic stimulation (ppTMS), a measure of GABA-mediated intracortical inhibition.

We tested whether HupA suppresses seizures in the rat PTZ acute seizure model, and quantified latency to first myoclonus and to generalized tonic–clonic seizure, and spike frequency on EEG. Additionally, we measured power in the EEG gamma frequency band which is associated with GABAergic cortical interneuron activation. Then, as a step toward further examining the HupA antiepileptic mechanism of action, we tested long-interval intracortical inhibition (LICI) using ppTMS coupled with electromyography to assess whether HupA augments GABA-mediated paired-pulse inhibition of the motor evoked potential. We also tested whether the HupA effect on paired-pulse inhibition was central or peripheral by comparison of outcomes following administration of HupA or the peripheral acetylcholinesterase inhibitor pyridostigmine. We also tested whether the HupA effect was dependent on central muscarinic or $GABA_A$ receptors by co-administration of HupA and atropine or PTZ, respectively.

In tests of antiepileptic potential, HupA suppressed seizures and epileptic spikes on EEG. Spectral EEG analysis also revealed enhanced gamma frequency band power with HupA treatment. By ppTMS we found that HupA increases intracortical inhibition and blocks PTZ-induced cortical excitation. Atropine co-administration with HupA did not alter HupA-induced intracortical inhibition suggesting independent of muscarinic acetylcholine receptors mechanism in this model. Last, pyridostigmine did not affect the ppTMS-measured cortical inhibition suggesting that HupA-induced effect is centrally-mediated.

Our data support antiepileptic HupA applications, and suggest that such activity may be via enhancement of GABAergic intracortical inhibition.

1. Introduction

Huperzine A (HupA; (1*R*,9*S*,13*E*)-1-Amino-13-ethylidene-11-methyl-6-azatricyclo[7.3.1.$0^{2,7}$]trideca-2(7),3,10-trien-5-one) is a naturally occurring sesquiterpene alkaloid compound found in the firmoss *Huperzia serrata*. Relevant to potential applications to CNS diseases, including acquired epilepsy syndromes, HupA shows anti-inflammatory (Wang et al., 2008), neuroprotective (Ma et al., 2013; Wang and Tang, 2005), anti-nociceptive properties (Bialer et al., 2015; Park et al., 2010; Yu et al., 2013), and is protective against soman-induced toxicity and seizures (Wang et al., 2011). Via the National Institute of Health Anticonvulsant Screening Program (NIH ASP; http://www.ninds.nih.gov/research/asp/index.htm), pretreatment with doses of HupA in excess of the TD_{50} protected mice and rats from brief clonic seizures triggered by subcutaneous PTZ, from seizures induced by maximal electroshock (MES) and hippocampal kindling (rats only), and at lower, well-tolerated doses, blocked seizures in the mouse 6 Hz psychomotor seizure model (S. Schachter, personal

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communication), summarized in Bialer et al. (2015). However, the range of HupA anticonvulsant capacity at a clinically-relevant dose in rats, and its mechanisms of action are not known. Given the well-described GABA-antagonistic mechanism of PTZ-induced seizures, we focused the present experiments on this model and on tests of GABA transmission that may be affected by HupA.

While the HupA effects on GABA-mediated inhibitory systems have not been studied, HupA is a demonstrated acetylcholinesterase inhibitor (Tang et al., 1999) and N-methyl-D-aspartate receptor (NMDAR) antagonist (Coleman et al., 2008). Of these two properties, The NMDAR antagonist relevance to HupA antiepileptic potential is uncertain, but atropine (a muscarinic acetylcholine receptor (mAChR) antagonist) completely blocked the HupA anticonvulsant effect in the mouse 6 Hz model in the NIH ASP screen, suggesting an important role for the cholinergic effect in this model (Bialer et al., 2015). HupA is available commercially as a dietary supplement in the US and has been tested in patients with Alzheimer's disease where it may improve cognitive function, likely via its anticholinesterase activity (Li et al., 2008; Qian and Ke, 2014). Altogether, these data suggest multipotent HupA effects that are relevant to epilepsy, and more broadly to cortical excitability.

To build upon data from the NIH ASP, we first tested the capacity of HupA to suppress clinical and electrographic seizures in the rat acute PTZ seizure model. Then, given that PTZ is a $GABA_A$ receptor antagonist (Squires et al., 1984), we conducted a series of experiments to test whether HupA increases intracortical GABAergic inhibition. Specifically, we measured intracortical inhibition by paired-pulse transcranial magnetic stimulation (ppTMS) coupled with electromyography (EMG).

In ppTMS the motor cortex is stimulated, noninvasively, by small intracranial electrical currents generated by a powerful extracranial magnetic field (Kobayashi and Pascual-Leone, 2003). It is commonly delivered unilaterally over the motor cortex such that the pair of stimuli separated by 50–300 ms interstimulus intervals produces a pair of motor evoked potentials (MEPs). When analyzed as pairs, the size of the second (test) MEP is predictably smaller than the size of the first (conditioning) MEP, most likely due to GABAergic long-interval intracortical inhibition (LICI) of the test response, triggered by the initial conditioning stimulus (Hsieh et al., 2012; Vahabzadeh-Hagh et al., 2011).

ppTMS is common protocol for measuring GABA-mediated intracortical inhibition in humans and aberrant ppTMS-derived measures of cortical inhibition are well-described in human epilepsies (Badawy et al., 2007, 2014; Brodtmann et al., 1999). Relevant to the present report, our group has adapted human ppTMS protocols to rats, where we identified a similar loss of intracortical inhibition in the PTZ acute seizure model, and enhancement of intracortical inhibition with $GABA_A$ facilitator pentobarbital (Hsieh et al., 2012; Vahabzadeh-Hagh et al., 2011). With the ppTMS protocol, we asked (1) whether HupA augments LICI, (2) whether this effect is mediated by central or peripheral cholinomimetic activity, and (3) whether the expected loss of LICI due to PTZ-induced GABA antagonism is reversed by HupA treatment.

2. Materials and methods

2.1. Animals

34 adult male Sprague-Dawley rats (260 ± 20 g) were used for the experiments. All animals were housed in a temperature controlled animal care facility with a 12-h light–dark cycle and with food and water supplied ad libitum. All procedures were approved by and in accordance with the guidelines of the Animal Care and Use Committee at Boston Children's Hospital (Boston, MA).

2.2. Pharmaceuticals

The following pharmaceuticals and stock concentrations were used in this project (per-experiment dosing is detailed below): urethane (0.2 g/ml); pentobarbital (50 mg/ml); HupA (1 mg/ml); PTZ (50 mg/ml); pyridostigmine (5 mg/ml); atropine (15 mg/ml). Saline volumes were matched to controlling drug solution volume.

2.3. Anesthesia

In the PTZ model experiment, rats were either awake or anesthetized with intraperitoneal urethane (1.2 g/kg). In the ppTMS experiment, rats were anesthetized with intraperitoneal sodium pentobarbital (65 mg/kg) or isoflurane (induction 4%, maintenance 2%). Rat body temperature was maintained throughout anesthesia on a heating pad operating at 42 °C.

2.4. Rat PTZ seizure model

We used the rat pentylenetetrazol (PTZ) seizure model as recently described (Dhamne et al., 2015). Awake rats were randomly assigned to receive intraperitoneally (i.p.) either saline or HupA (0.6 mg/kg): a maximal dose without peripheral cholinergic effects, and 10 min later all animals received PTZ (40 mg/kg; i.p.). The HupA dose was selected from preliminary experiments (data not shown) using titrating dosage (from 0 to 0.9 mg/kg in increments of 0.3 mg/kg) while monitoring for peripheral cholinergic effects such as muscle fasciculations and hypersalivation. Animals' behavior at baseline (10 min before PTZ injection) and at follow-up (10 min after PTZ injection) was video-recorded for future assessment (Fig. 1A). The latency to tonic–clonic seizures was recorded in every animal.

To assess the effect of HupA on the EEG in the PTZ model, the experiment was repeated in an additional group of urethane anesthetized animals as previously described in the kainate-induced rat epilepsy model (Hotta et al., 2010). Following anesthesia, the rats were gently restrained on a platform with two broad Velcro straps positioned over the torso behind the forelimbs and in front of the hindlimbs. The restraint allowed full range of motions of the head, limbs and tail, and enabled its view and access to the rat's head for EEG electrodes. Then rats were randomly assigned to receive either saline or HupA 15 min after urethane injection. Ten minutes after HupA, PTZ (50 mg/kg; i.p.) was administered to all rats (Fig. 1B). EEG was recorded at baseline (10 min before PTZ injection) and at follow-up (20 min after PTZ injection). The latency to first myoclonus was recorded in every animal.

2.5. Electroencephalography (EEG) acquisition

Continuous EEG in anesthetized rats was acquired using two thin silver/silver-chloride Teflon-coated EEG subdermal wire electrodes (Ives EEG Solutions, Ontario, Canada), with a reference positioned midline at the interorbital line and an active electrode unilaterally placed over the parietal region (Dhamne et al., 2015). Rats tolerated the electrodes without signs of local pain or discomfort after initial subcutaneous placement. The 1-channel cortical EEG measurements were recorded using a Cadwell EEG system (Cadwell Laboratories Inc., Kennweick, WA) at a sampling rate of 400 Hz. The signals were filtered in the bandpass cutoff range of 1–70 Hz along with a 60-Hz Notch filter. EEG traces were recorded and reviewed using Easy EEG v.2.1 (Cadwell Laboratories Inc, Wennewick, WA) and EDFbrowser v.1.22 (Teunis van Beelen, Netherland) analysis applications.