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Intrathecal Huperzine A increases thermal escape latency and decreases flinching behavior in the formalin test in rats

Paula Park[a], Steven Schachter[b], Tony Yaksh[c,*]

[a] Skaggs School of Pharmacy & Pharmaceutical Sciences, La Jolla, CA 92093, United States
[b] Osher Research Center, Harvard Medical School, Boston, MA 02138, United States
[c] Anesthesiol. Res. Lab. 0818, Department of Anesthesiology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093, United States

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ABSTRACT

Huperzine A (HupA) is an alkaloid isolated from the Chinese club moss *Huperzia serrata* and has been used for improving memory, cognitive and behavioral function in patients with Alzheimer's disease in China. It has NMDA antagonist and anticholinesterase activity and has shown anticonvulsant and antinociceptive effects in preliminary studies when administered intraperitoneally to mice. To better characterize the antinociceptive effects of HupA at the spinal level, Holtzman rats were implanted with intrathecal catheters to measure thermal escape latency using Hargreaves thermal escape testing system and flinching behavior using the formalin test. Intrathecal (IT) administration of HupA showed a dose-dependent increase in thermal escape latency with an ED50 of 0.57 μg. Atropine reversed the increase in thermal escape latency produced by 10 μg HupA, indicating an antinociceptive mechanism through muscarinic cholinergic receptors. The formalin test showed that HupA decreased flinching behavior in a dose-dependent manner. Atropine also reversed the decrease in flinching behavior caused by 10 μg HupA. A dose-dependent increase of side effects including scratching, biting, and chewing tails was observed, although antinociceptive effects were observed in doses that did not produce any adverse effects.

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Huperzine A (HupA) is an alkaloid isolated from the Chinese club moss, *Huperzia serrata*, also known in China as "*Qian Ceng Ta*." HupA has shown variable efficacy for memory, cognitive and behavioral dysfunction in patients with Alzheimer's disease when taken orally [3]. In the USA, HupA is available as a dietary supplement, and marketed in various forms, for example with vitamin E as the brand name Cerebra® [7]. It is a highly specific, reversible inhibitor of acetylcholinesterase (AchE) with potency comparable to physostigmine, galantamine, and donepezil – drugs currently approved and prescribed for Alzheimer's disease. HupA is also an *N*-methyl-D-aspartate (NMDA) receptor antagonist with additional neuroprotective mechanisms of action [11,15].

In preliminary studies, intraperitoneal HupA has shown potent anticonvulsant and antinociceptive effects in mouse models of epilepsy and pain (S. Schachter, personal communication). In previous studies, cholinesterase inhibitors administered intrathecally produce a dose-dependent antinociceptive activity in rats. The antinociceptive effects are independent of opioid and α2 adrenoceptors and are primarily due to stimulation of muscarinic cholinergic receptors [9,10,14]. To date, no prior work has been undertaken characterizing the spinal effect of HupA. In the present studies, we examine the effect of IT HupA on acute thermal escape and the formalin evoked flinching response. Importantly, we further sought to determine the degree to which the antinociceptive activity of HupA is due to stimulation of muscarinic cholinergic receptors.

The following investigations were performed under a protocol approved by the Institutional Animal Care and Use Committee. Male Holtzman rats (200–225 g; Harlan Industries, Indianapolis, IN) were housed in a cage of two rats and allowed a minimum of 2 days to acclimate after receipt prior to any surgical procedures.

Chronic intrathecal (IT) catheters were implanted at least 5 days prior to an experiment. Under isoflurane delivered in a mix of 50% oxygen and 50% air, the dorsal head of the rat is shaved and the rat is placed in a stereotaxic unit. The surgical area is cleaned with alcohol and Nolvasan solution. Skin and muscle incisions are made, and the cisternal membrane at the base of the skull is exposed as the muscle is retracted. A 22-G needle with a 75° angle is used to make a 1–2 mm incision in the cisternal membrane in order to insert the catheter. A previously prepared and sterilized catheter (PE-10 welded to PE-08; indwelling end is 8.5 cm from the joint) is flushed with saline and inserted though the cisterna 8.5 cm to the rostral edge of the lumbar enlargement, and the knot lies behind the nuchal crest. The PE-08 end of the catheter is externalized using a 19-G needle through the skin and the incisions are sutured. Rats

* Corresponding author. Tel.: +1 619 543 3597; fax: +1 619 543 6070.
E-mail address: tyaksh@ucsd.edu (T. Yaksh).

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Table 1
Incidence of side effects after intrathecal treatments by treatment groups.

Observed side effects	Intrathecal HupA dose (μg)						IT atropme + Hup 10 μg	IT vehicle + Hup 10 μg	IT atropine alone
	0	0.1	1	3	10	30			
Scratching	25%	17%	50%	50%	100%	100%	0%	50%	50%
Biting/licking PAW	25%	0%	29%	0%	80%	100%	50%	25%	0%
Chew tail	0%	0%	0%	50%	60%	100%	0%	0%	0%
Straub tail	0%	0%	0%	0%	20%	100%	100%	50%	0%
Wobble	0%	0%	0%	0%	60%	100%	100%	25%	0%
Cataleptic	0%	0%	0%	0%	40%	100%	0%	25%	0%
Facial fasciculations/chewing	0%	0%	0%	0%	0%	25%	0%	25%	25%
Hind limb rigidity	0%	0%	0%	0%	0%	0%	100%	25%	0%
Spontaneous hind paw tremors	0%	0%	0%	0%	0%	0%	50%	0%	0%
Hind paw tremor after heat	0%	0%	0%	0%	0%	0%	50%	0%	50%
Vocalization post-injection	0%	0%	0%	0%	0%	0%	75%	50%	25%

received lactated Ringers' solution with carprofen subcutaneously after the surgery, and were housed in individual cages [8].

The following drugs were used in the studies: Huperzine A, [5R-(5a,9b,11E)]-5-amino-11-ethlidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta [b] pyridin-2(1H)-one (molecular weight [MW] 242.32, Chromadex), and atropine (MW 289.4, Sigma). IT administration of drug was performed using a hand-driven syringe pump. All drugs were prepared to be injected in a 10 μl volume followed by 10 μl of vehicle to flush the catheter. HupA was dissolved in 10% cyclodextrin in 10 μg/μl concentration and then diluted to desired concentration in the following studies.

Escape latency was measured using the Hargreaves type thermal escape testing system. Rats were placed in a clear plastic cage (10 cm × 20 cm) placed on an elevated floor of clear glass 2 mm thick. A radiant heat source 4 mm in diameter projected from a movable holder beneath the glass floor. The glass floor temperature was regulated to 30 °C [4]. The rats were allowed to acclimate for at least 30 min prior to baseline testing. The movable holder was positioned under the glass in order to project the radiant heat source on the middle of the plantar surface of a hind paw. Once positioned, the light was activated with a start button and the timing circuit was initiated to measure the interval between the activation of the light beam and the withdrawal of the hind paw, which triggered a stop of light source and end of measurement. The value measured was the rat's response latency. The cutoff time in the absence of a response was 20 s.

Approximately 1 h before testing, a small metal band (0.5 g) is loosely placed around the rat's right hind paw. The rat is placed in a cylindrical Plexiglas chamber for adaptation for a minimum of 30 min. Formalin is then injected (50 μl of 5% formalin) into the dorsal surface of the right hind paw of the rat and it is placed into the chamber of the automated formalin apparatus where movement of the formalin injected paw is monitored and the number of paw flinches tallied by minute over the next 60 min. Upon completion of the test, the animal is removed and euthanized [13] (UARDG, Anesthesiology, University of California, San Diego, 92103-0818).

To determine dose and time-effect relationships, HupA was given in a range of doses to animals with spinal catheters. For thermal nociception, HupA was tested 10, 20, 30, 60, 90, and 120 min after IT administration. For formalin studies, HupA was given 10 min prior to formalin.

Side effects at different doses were noted as observed throughout the studies and recorded as a percentage of number of animals displaying the side effect out of total number of animals, regardless of the intensity of the side effect behavior (see Table 1). Control animals were tested using the vehicle, 10% cyclodextrin.

To determine whether the analgesic effects result from the anticholinesterase activity of HupA, thermal latency or flinching was tested after pretreatment with atropine, a nonspecific muscarinic antagonist. Atropine (15 μg/10 μL) was administered IT, 10 min prior to IT administration of HupA (10 μg). Any changes or prevention of previously observed side effects were recorded. The dose of atropine was determined from a previous study [14].

Data are presented as means ± SEM. The percent of the maximum possible effect (%MPE) was calculated as follows: %MPE = [(post-drug latency − baseline latency)/(cutoff time (20 s) − baseline latency)] × 100.

Dose-dependent flinching data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison *post hoc*.

A dose-dependent increase in thermal latency was observed with IT administration of HupA. Fig. 1a shows the time course of the antinociceptive effects produced by HupA at different doses along

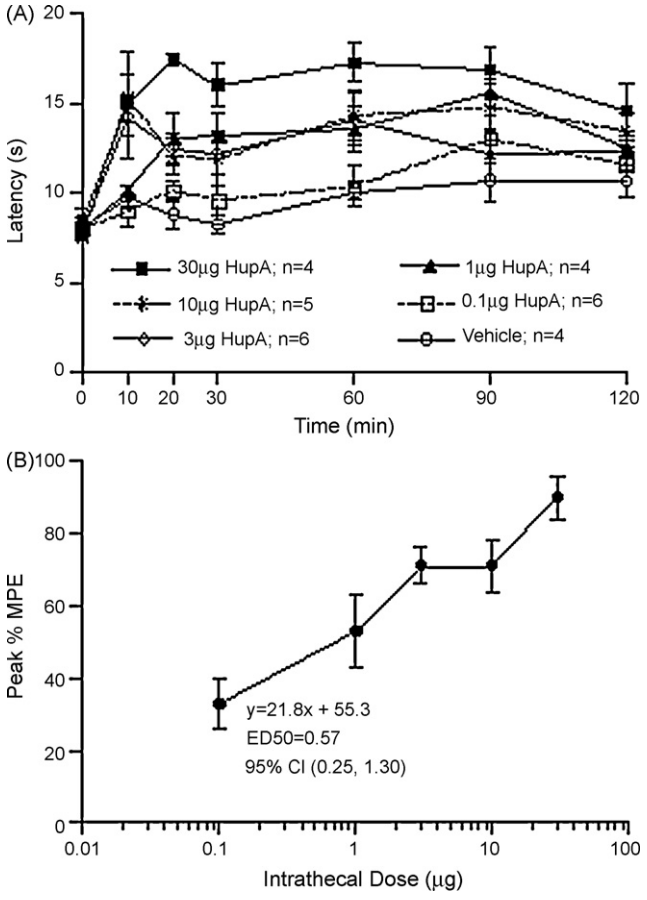

Fig. 1. (A) Thermal escape latency in s measured over time (min) after IT administration of vehicle (10% cyclodextrin), or one of five doses of HupA. (B) log dose–response curve plotting peak % MPE in the first 60 min after IT administration vs HupA doses. ED50 is 0.57 μg with a 95% CI (0.25 and 1.30).