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Intrathecal Huperzine A Increases Thermal Escape Latency and Decreases Flinching Behavior in the Formalin Test in Rats

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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.

Keywords

Cholinesterase inhibitor; spinal and analgesia

Introduction

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 (1). 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® (2). 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

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neuroprotective mechanisms of action (3,4). In preliminary studies, intraperitoneal HupA has shown potent anticonvulsant and antinociceptive effects in mouse models of epilepsy and pain (Schachter S, 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 α_2 adrenoceptors and are primarily due to stimulation of muscarinic cholinergic receptors (5,6,7). 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.

Materials and Methods

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.

Animal preparation

Chronic intrathecal (IT) catheters were implanted at least five 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 1-2mm 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 8.5cm from the joint) is flushed with saline and inserted through 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 received lactated Ringers' solution with carprofen subcutaneously after the surgery, and were housed in individual cages (8).

Drugs

The following drugs were used in the studies: Huperzine A, [5R-(5a,9b,11E)]-5-amino-11-ethylidene-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 μ l volume followed by 10 μ l of vehicle to flush the catheter. HupA was dissolved in 10% cyclodextrin in 10 μ g/ μ l concentration and then diluted to desired concentration in the following studies.

Nociceptive testing

Thermal escape latency—Escape latency was measured using the Hargreaves type thermal escape testing system. Rats were placed in a clear plastic cage (10×20cm) placed on an elevated floor of clear glass 2 mm thick. A radiant heat source 4mm indiameter projected from a movable holder beneath the glass floor. The glass floor temperature was regulated to 30° C (9). 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 20s.

Formalin testing—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 μ l 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 (10) (UARDG, Anesthesiology, University of California, San Diego, 92103-0818).

Huperzine Dose Response Study

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.

Antagonist Study

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 μ g / 10 μ L) was administered IT, 10 min prior to IT administration of HupA (10 μ g). Any changes or prevention of previously observed side effects were recorded. The dose of atropine was determined from a previous study (7).

Statistics

Data are presented as means $\pm$ SEM. The percent of the maximum possible effect (%MPE) was calculated as follows: %MPE = [(post drug latency – baseline latency) / (cutoff time (20s) – baseline latency)] $\times$ 100

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

Results

Acute thermal escape

A dose-dependent increase in thermal latency was observed with IT administration of HupA. Figure 1a shows the time course of the antinociceptive effects produced by HupA at different doses along with vehicle (10% cyclodextrin). Figure 1b shows the dose-response curve of peak % MPE on a log scale for the different HupA doses. Latency was measured for 120 min after IT administration, but the peak % MPE value was obtained from the first 60 min after IT administration. The ED 50 was 0.57 μ g with a 95% confidence interval (CI) of 0.25 and 1.30.

Atropine reversal—Atropine (15 μ g) administered IT reversed the increase in thermal latency produced by HupA at 10 μ g. Atropine alone, atropine + HupA, and vehicle + HupA were tested (Figure 2).

Formalin test

Three doses of HupA (1, 3, and 10 μ g) were tested along with vehicle as a control on the formalin test. Figure 3a shows the decrease in the rate of flinches in a dose-dependent manner compared to the control. Figure 3b shows the dose-dependent decrease in total number of flinches in phase I and phase II. Phase I measures flinching from 0-9 min after formalin injection and phase II from 10-60 min. A one-way ANOVA showed a significant decrease in total number of flinches for HupA across doses for both phase I ($p=0.0066$) and phase II ($p=0.0377$). A *post hoc* analysis indicated that the HupA 10 μ g dose was different from vehicle ($p<0.05$).

Atropine reversal—Atropine reversed the decrease in flinching behavior caused by 10 μ g HupA for phase I and phase II. Vehicle pretreatment animals showed a decrease in flinching behavior after HupA (10 μ g) as compared to atropine-pretreated rats HupA rats. (Figure 4a and 4b).

General behavior

HupA produced dose-dependent side effects throughout the time course of testing. Different behaviors were observed and major side effects were noted and listed in Table 1. Side effects observed at different doses were noted. A dose-dependent increase in behaviors such as scratching, biting, and chewing tails was observed. These tended to occur earlier in the time course. Pretreatment with atropine or vehicle produced a different set of behaviors such as extreme hind limb and tail rigidity. Atropine pretreatment seemed to cause an exaggerated hind limb rigidity and dysfunction, although one vehicle pretreated rat showed similar behaviors.

Discussion

HupA has been shown to have potent anticholinesterase effects and has blocking actions at the NMDA receptor. This profile distinguishes it from other cholinesterase inhibitors such as neostigmine. In the present work, HupA served to have a significant suppressive effect upon acute thermal escape and the formalin flinching in doses of between 3 and 10 μ g. This activity compares favorably to the activity of IT cholinesterase inhibitors such as neostigmine (5,7). Considerable data have shown that spinal cholinesterase inhibition can indeed yield potent antinociceptive effects, as measured on thermal escape and the formalin model (5,6,7). Based on pharmacological antagonism studies, these effects are thought to be mediated by a spinal muscarinic receptor, potentially of the M1 type (6,7).

Previous work has indeed shown that activation of spinal muscarinic receptors can reduce the excitability of dorsal horn projection neurons which respond to nociceptive stimuli. (11) and the role of spinal cholinergic systems in regulating nociceptive transmission (12). Spinal NMDA antagonism has also been shown to alter nociceptive processing in models of facilitated processing, e.g. the second phase of the formalin test, but has little effect upon models of acute nociception as involved in models such as that of the acute thermal escape (13).

In the present studies, IT administration of HupA produced a dose-dependent increase in the acute nociceptive threshold. On the formalin test also resulted in a dose-dependent decrease in flinching in both phase I and phase II. Importantly, in both models the observed antinociceptive effects were reversed by atropine, confirming the muscarinic activity of spinal huperzine. Although previous work has suggested an antinociceptive effect of atropine (14), at the spinal dose employed no changes in threshold when given alone were noted. The combination of the effects of IT HupA on acute nociception and the ability to reverse the observed effects by intrathecal atropine emphasizes that the HupA effects are largely dependent upon the cholinergic actions of the agent and does not support a role for the reported NMDA receptor blocking actions of the drug.

While a dose-dependent increase in the incidence of side effects was observed, it is important to emphasize that the prominent antinociceptive effects were observed at doses that did not produce impairing effects on motor function. Thus, at intrathecal doses of 10 μ g and 30 μ g, scratching and biting of hind paws and tail were noted, along with problems in balancing on hind limbs (wobble) and periods of immobility (catalepsy). Some rats showed fasciculations in the face, which may be due to a rapid chewing behavior. Interestingly, rats pre-treated with atropine developed signs of rigidity in the hind limbs and tail. Increased vocalization during and after HupA administration was noted in some animals that received atropine pretreatment. These behaviors were also noted occasionally in a vehicle-pretreated rat. Less chewing and biting of toes/tail were noted in the animals with pretreatment compared to those without any pretreatment. The exaggerated side effect profile with atropine pretreatment may indicate a possible unmasking of noncholinergic side effects that were suppressed by the acetylcholinesterase activity of HupA. It is interesting to note that the increased incidence of side effects after atropine was accompanied by a reversal of the elevated escape threshold suggesting that the side effect were not responsible for the depressed nociceptive responding.

In summary, intrathecal delivery of Hup A has an antinociceptive and pharmacological profile comparable to that identified previously for cholinesterase inhibitors. It is interesting to note that intrathecal neostigmine has been shown to be a potent analgesic in humans (15) with nausea typically being the limiting side effect. This activity suggests the potential analgesic efficacy of increasing spinal cholinergic-muscarinic activity as a future target of spinal analgesic drug development

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Figure 1.

A. Thermal escape latency in seconds 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. ED_{50} is 0.57 µg with a 95% CI (0.25, 1.30).

Figure 2.

IT Atropine (15 µg) reversed the effects of IT HupA (10 µg) on thermal latency. A two-way ANOVA showed statistically significant increase between vehicle + HupA vs atropine alone ($p=0.0049$), but not atropine + HupA vs atropine ($p < 0.05$).

Figure 3.

A. Number of flinches per minute plotted vs time after intraplantar formalin for animals receiving IT vehicle or one of three doses of IT HupA (1, 3 or 10 µg). B. Total number of flinches were recorded over phase I (0-9min) and phase II (10-60min). A dose-dependent decrease in flinching behavior is observed. (1 Way ANOVA $p < 0.05$ for Phase 1 and phase 2. * $p < 0.05$ vs vehicle *post hoc* analysis).

Figure 4.

A. Number of flinches per minute plotted vs time after intraplantar formalin for animals receiving IT vehicle or IT Atropine (Atr) (15 µg) alone, or IT HupA (10 µg) alone or IT Atropine (15 µg) followed by IT HupA (10 µg). B. Total number of flinches recorded over phase I and phase II in groups presented in top figure. Vehicle pretreatment with HupA shows a decreased total number of flinches vs Atropine alone, whereas Atropine + HupA shows flinching not different from atropine alone. * $p < 0.05$ vs atropine alone or vehicle

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

OBSERVED Side Effects	Intrathecal Hup A Dose (µg)						IT Atropine + Hup 10µg	IT Vehicle + Hup 10µg	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	25%	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%	75%	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%