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D-Leucine: Evaluation in an epilepsy model

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ABSTRACT

Background: Current medicines do not provide sufficient seizure control for nearly one-third of patients with epilepsy. New options are needed to address this treatment gap. We recently found that the atypical amino acid D-leucine protected against acutely-induced seizures in mice, but its effect in chronic seizures has not been explored. We hypothesized that D-leucine would protect against spontaneous recurrent seizures. We also investigated whether mice lacking a previously-described D-leucine receptor (Tas1R2/R3) would be protected against acutely-induced seizures.

Methods: Male FVB/NJ mice were subjected to kainic acid-induced status epilepticus and monitored by video-electroencephalography (EEG) (surgically implanted electrodes) for 4 weeks before, during, and after treatment with D-leucine. Tas1R2/R3 knockout mice and controls underwent the maximal electroshock threshold (MES-T) and 6-Hz tests.

Results: There was no difference in number of calendar days with seizures or seizure frequency with D-leucine treatment. In an exploratory analysis, mice treated with D-leucine had a lower number of dark cycles with seizures. Tas1R2/R3 knockout mice had elevated seizure thresholds in the MES-T test but not the 6-Hz test.

Conclusions: D-Leucine treatment was ineffective against chronic seizures after kainic acid-induced status epilepticus, but there was some efficacy during the dark cycle. Because D-leucine is highly concentrated in the pineal gland, these data suggest that D-leucine may be useful as a tool for studying circadian patterns in epilepsy. Deletion of the Tas1R2/R3 receptor protected against seizures in the MES-T test and, therefore, may be a novel target for treating seizures.

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1. Introduction

Novel treatments are needed for the nearly one-third of patients with epilepsy whose seizures are not controlled adequately by current medicines [1–3]. More recent data indicate that failure to achieve sustained seizure freedom for more than one year occurs in half of adults with epilepsy, and furthermore, many children and adults have repeated periods of seizure recurrence, alternating with periods of remission [4,5]. Thus, current treatments are not associated with long-term seizure control for a substantial proportion of patients. Nutrient-based therapies (including ketogenic diets, the modified Atkins diet, and the low glycemic index treatment) represent an established clinical alternative to currently used medications and lead to durable seizure control in some patients [6,7]. Although most nutrient-based epilepsy treatments have focused on fats and carbohydrates, amino acids also may control seizures [8–10]. We showed recently that L-leucine protected against seizures in mice when injected prior to the excitotoxin kainic acid, but surprisingly, only its D-enantiomer was effective in terminating ongoing seizure activity *after* the onset of seizures [11]. The importance of this finding was that the latter scenario resembles how antiseizure medicines are used in the clinic (most seizure medicines are tested in preclinical models prior to delivery of seizure-inducing stimuli). The atypical amino acid D-leucine is a component of bacterial cell walls, and although it is not incorporated de novo into mammalian proteins, it is found in trace amounts in the mammalian brain, including rat and mouse hippocampus, mouse cerebral cortex, and other regions [12,13]. D-Leucine accounts for 16–20% of total leucine in perfused mouse cortex and hippocampus (midrange among brain amino acids), but it has one of the lowest concentrations in blood [14]. No leucine isomerases have been reported, and only one enzyme (D-amino acid oxidase) accounts for nearly all its metabolism into an imino acid, ammonia, and hydrogen peroxide [15–17]. Together, these data suggest that D-leucine concentrations in brain tissue are highly regulated and may modulate neuronal function, although its exact physiological role is unclear.

D-Leucine is abundant in the pineal gland, an organ that transduces signals from light stimulation and produces melatonin, thereby partly regulating sleep–wake cycles [13,15,18]. Temporal lobe seizure activity alters the duration of different sleep stages in humans and rodents [19,20]. Therefore, the antiseizure effects of D-leucine might differ between light and dark cycles.

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Based on our prior work showing that D-leucine terminated ongoing kainic acid-induced seizures, we hypothesized that D-leucine would protect against spontaneous recurrent seizures (SRS) in a rodent model of epilepsy. We also examined a possible role in seizure protection for Tas1R2/R3, the only receptor reported to date to bind D-leucine.

2. Methods

2.1. Animals

Mice (FVB/NJ strain) FVB/NJ mice were obtained from Jackson Labs (Bar Harbor, ME) and bred locally. Tas1R2/R3 double knockout mice (on a C57BL/6 background) and wildtype C57BL/6 mice (controls) were from a colony at Henry Ford Health System (Detroit, MI) that was created at University of California, San Diego [21]. These mice were bred locally for three generations prior to seizure testing (genotype was confirmed with polymerase chain reaction (PCR) techniques). All mice were given free access to water and fed chow ad lib (Teklad Global 2018SX, Madison, WI, USA). Mice were housed 3–4 per cage, except those that had undergone surgery, who were housed singly. The light/dark cycles were 14 h and10 h in duration, respectively. All animal protocols were approved by the Johns Hopkins Animal Care and Use Committee and were in compliance with National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Electrode implantation

At six weeks of age, mice were anesthetized in an induction chamber with isoflurane (5%), then transferred quickly to a stereotactic device (Stoelting Co., Chicago, IL, USA) with anesthesia administered by nose cone (2–3% isoflurane, Baxter, Deerfield, IL, USA). Depth of anesthesia was judged by lack of response to gentle paw pinch; respirations were monitored visually while mice were anesthetized. The surgical site was shaved and then prepared with betadine and hydrogen peroxide. A midline incision was made to expose the skull. Screw holes were manually tapped with a sterile 0.025″ drill bit, located 1.5 mm lateral to the sagittal suture and 2.0 mm anterior or 4 mm posterior to bregma. At the coordinates listed, recording electrodes were therefore located over the left frontal, right frontal, and right parietal neocortex regions (confirmed by visual inspection). The ground electrode was located over the left parietal neocortex. The prefabricated headmount (Pinnacle Technology, Lawrence, KS, USA) was positioned over the screw holes after cyanoacrylate (Elmers Products, High Point, NC, USA) was applied and secured with steel screw electrodes, including one ground electrode (0.10″ and 0.12″; Pinnacle Technology, Lawrence, KS, USA). A small midline incision was made over the trapezius to insert EMG electrodes into the muscle. Silver epoxy (Pinnacle Technologies, Lawrence, KS, USA) was applied to improve contact between the screw electrodes and the headmount. Dental acrylic coating (Lang Dental Manufacturing Co., Wheeling, IL) was applied to insulate and protect the EEG leads. Chromic gut 4-0 sutures (Med-Vet International, Mettawa, IL, USA) were used to close the skin layer around the implant and antibiotic ointment (bacitracin/neomycin/polymixin B, Johnson & Johnson, New Brunswick, NJ, USA) was applied. Ketoprofen (5 mg/kg, s.c., Zoetis, Parsippany, NJ, US) was injected after surgery. Postoperatively, the mouse was moved to a cage that was prewarmed with a heating pad and monitored until ambulating to food and water sources. Mice were treated with either topical lidocaine 5% (Amneal, Bridgewater, NJ) or ketoprofen in the first 18 h following surgery.

2.3. EEG monitoring

Continuous video-EEG monitoring was performed 5–7 days per week for 8–24 h per day (typically, 12–18 h, 6 days per week). Mice were housed in a clear cylinder with ad lib access to food and water during monitoring (Pinnacle Technology, Inc., Lawrence, KS, USA). Electrodes were connected to the data collection system via an amplifier and low-torque swivel; dark cycle illumination was provided by an infrared camera (Pinnacle Technology, Inc., Lawrence, KS, USA). Raw data were collected and analyzed using Sirenia Acquisition and Seizure Basic software (version 1.7.5, Pinnacle Technology, Inc., Lawrence, KS, USA). The raw EEG tracing was inspected manually for evidence of seizures and then rescreened using a power level of 100 μV^2. When seizures were noted on EEG, the video was used to clinically stage the seizure [22]. Because this was our initial cohort, assessments were unblinded. Video of each mouse was reviewed (with corresponding inspection of raw EEG data) to determine the amount of sleep (quantified in minutes) over 15 min in three hourly epochs on four different days during the pretreatment, treatment, and posttreatment periods (i.e., 12 epochs per treatment period). Sleep was defined as immobility, lying in a curled-up posture with eyes closed and a consistent change in EEG activity from the awake state. Specific days and epochs of time were selected using a random number generator. None of the epochs included seizures.

2.4. Induction of status epilepticus

One week after surgery, kainic acid (5.3 mg/ml PBS; Cayman Chemical, Ann Arbor, MI, USA) was injected intraperitoneally at a dose of 25–100 mg kainic acid/kg mouse body weight (in increments of 10–25 mg/kg) until the mouse went into status epilepticus, defined as persistent epileptiform activity on the EEG accompanied by recurrent clinical seizures for at least 12 h (the minimum time we determined in pilot experiments to induce epilepsy). Some mice received diazepam i.p. (5 mg/kg) (Hospira, Lake Forest, IL, USA) if clinically evident convulsions were persistent and severe. Mice were injected with PBS (i.p.) 4–8 h after induction of status epilepticus to maintain adequate hydration.

2.5. D-Leucine treatment

D-Leucine (Oakwood Chemicals, Estill, SC, USA) was administered ad lib in drinking water (1.5% w/v, using sterile water as a diluent) from a standard water bottle (Pinnacle Technology, Inc., Lawrence, KS, USA) continuously for 28 days during the treatment period.

2.6. Maximal electroshock threshold (MES-T) test

The MES-T was performed as previously described [23]. Tas1R2/R3 knockout mice (4 males, 3 females) or C57BL/6 controls (5 males, 1 female), all 10 weeks of age at the beginning of testing, were used. Briefly, tetracaine was applied (as in the 6-Hz test) before corneal stimulation using a Rodent Shocker 221 (Harvard Apparatus, Holliston, MA, USA). Settings included shock duration 0.2 s, 500 v, 150 mA; current settings were adjusted in a staircase-like manner, adjusted based on responses in serial testing (i.e., if no seizure occurred, then current was increased to the next level). Scoring was based on the presence or absence of tonic hindlimb extension (i.e., extension of the hindlimbs to 180° in the rostral-caudal plane). The person performing testing was blinded to genotype, and mice were selected for testing in random order by an assistant.

2.7. 6-Hz test

The 6-Hz electroshock test was performed as described [23]. Tas1R2/R3 knockout mice (4 males, 3 females) or C57BL/6 controls (5 males, 1 female), all 6 weeks of age at the beginning of testing, were used (note that this test was performed in this cohort prior to the MES-T test). Briefly, tetracaine 0.5% ophthalmic solution (Bausch & Lomb, Tampa, FL, USA.) was administered to the corneas before current was delivered (ECT Unit #57800, Ugo Basile North America, Collegeville, PA, USA) at a frequency of 6 Hz, pulse width of 0.2 ms, and shock