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Harmine, a natural beta-carboline alkaloid, upregulates astroglial glutamate transporter expression

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ABSTRACT

Glutamate is the predominant excitatory amino acid neurotransmitter in the mammalian central nervous system (CNS). Glutamate transporter EAAT2/GLT-1 is the physiologically dominant astroglial protein that inactivates synaptic glutamate. Previous studies have shown that EAAT2 dysfunction leads to excessive extracellular glutamate and may contribute to various neurological disorders including amyotrophic lateral sclerosis (ALS). The recent discovery of the neuroprotective properties of ceftriaxone, a beta lactam antibiotic, suggested that increasing EAAT2/GLT-1 gene expression might be beneficial in ALS and other neurological/psychiatric disorders by augmenting astrocytic glutamate uptake. Here we report our efforts to develop a new screening assay for identifying compounds that activate EAAT2 gene expression. We generated fetal derived-human immortalized astroglial cells that are stably expressing a firefly luciferase reporter under the control of the human EAAT2 promoter. When screening a library of 1040 FDA approved compounds and natural products, we identified harmine, a naturally occurring beta-carboline alkaloid, as one of the top hits for activating the EAAT2 promoter. We further tested harmine in our *in vitro* cell culture systems and confirmed its ability to increase EAAT2/GLT1 gene expression and functional glutamate uptake activity. We next tested its efficacy in both wild type animals and in an ALS animal model of disease and demonstrated that harmine effectively increased GLT-1 protein and glutamate transporter activity *in vivo*. Our studies provide potential novel neurotherapeutics by modulating the activity of glutamate transporters via gene activation.

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1. Introduction

Glutamate is the predominant excitatory amino acid (EAA) neurotransmitter in the mammalian CNS. It activates ligand-gated ion channels that are named after their agonists *N*-methyl-D-aspartate (NMDA), amino-3-hydroxy-5- methyl-4-isoxazolepropionate (AMPA), kainate and G-protein-coupled metabotropic receptors. Extracellular accumulation of EAAs and excessive activation of EAA receptors contribute to neuronal cell death observed both in acute insults to the CNS and in chronic neurodegenerative diseases, including ALS, Huntington's disease, Alzheimer disease and behavioral disorders such as depression (Gegelashvili et al., 2001).

Low extracellular levels of glutamate are maintained by transport into neurons and astrocytes. Five distinct glutamate transporters have been cloned that allow for sodium-dependent high-affinity glutamate transport, designated GLT1/EAAT2, EAAC1/EAAT3, GLAST/EAAT1, EAAT4, and EAAT5 (Danbolt, 2001). The EAAT (excitatory amino acid transporter) nomenclature refers to the human transporter species while GLT refers to the rodent transporter homologue of EAAT. Expression of EAAT1 and EAAT2 is generally restricted to astroglia; other EAATs are neuronal. The astroglial transporter EAAT2 is the dominant transporter in brain and spinal cord, accounting for up to 95% of all activity (Danbolt, 2001).

Excess levels of glutamate in the cerebral spinal fluid (CSF) (10 fold increase) are found in up to 40% of ALS patients (Rothstein et al., 1990). Reduced functional transport of glutamate has been observed in postmortem tissue from patients with ALS (Rothstein et al., 1992). This is due to a selective loss of EAAT2, whose immunoreactivity is dramatically reduced in ALS (Rothstein et al., 1995). At least 40%

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(and as much as 75%) of all sporadic ALS patients appear to have defects in glutamate transport and excessive extracellular glutamate levels (Spreux-Varoquaux et al., 2002). SOD1^{G93A} transgenic ALS rats also show reduced synaptosomal glutamate uptake activity as well as increased extracellular levels of glutamate and reduced clearance of glutamate (Dunlop et al., 2003; Howland et al., 2002). All SOD1 mutant rodent animal models of ALS show reduced levels of GLT1 (Dunlop et al., 2003; Howland et al., 2002). Alterations of glutamate transport protein expression have also been reported in other neurodegenerative and demyelinating diseases including Huntington's disease, Alzheimer's disease, and multiple sclerosis.

Genetic overexpression of the EAAT2/GLT1 protein is protective *in vitro* and delays disease *in vivo* (Guo et al., 2003). A recent unbiased small molecule screen employing low throughput spinal cord tissue slices revealed that beta lactam antibiotics, including ceftriaxone, increase GLT1/EAAT2 expression, protect against neural injury and delay disease in ALS mice (Rothstein et al., 2005).

Here we report our effort to employ a new drug discovery approach by developing a cell-based luciferase reporter screening system for identifying EAAT2 promoter activators. By screening a library of up to 1040 FDA approved compounds and natural products, we identified harmine, a naturally occurring beta-carboline alkaloid, as one of the top hits for turning on the EAAT2 promoter. We demonstrated that harmine effectively increased GLT-1 expression both *in vitro* and *in vivo*. Our studies provide a potential new neurotherapeutic strategy by modulating the activity of glutamate transporters *via* gene activation.

2. Material and methods

2.1. Generation of CC4 line and luminescence based screening assay

We obtained a human immortalized astroglial-fetal derived cell (HIA) line from Dr. Ahmet Hoke at Johns Hopkins University (Hoke et al., manuscript in preparation). To be able to use this line in our screening assay, we stably transfected HIA cells with a plasmid encoding firefly luciferase reporter under the control of a short fragment of the human EAAT2 promoter (2.5 kb) as described previously (Rothstein et al., 2005). The obtained cell line (CC4 line) was then validated to see if it is suitable for our screening assay, through testing its reproducibility and calculating its S/N ratio and Z′ factor. CC4 cells were seeded on 96-well plates and treated with different compounds. After 3-days incubation, cells were lysed in plates and luciferase activity was measured with Fluostar OPTIMA plate reader (BMG Labtech, NC), following manuals from Promega.

2.2. Primary neuron-astrocyte co-culture

Primary astroglial cells were cultured from postnatal 2–3days mouse pups. Cortices were dissected out and dissociated with papain and subsequently cultured on collagen-coated T75 flask in DMEM containing 10% fetal bovine serum (FBS). At DIV14 astrogial cells were seeded into collagen-coated 6-well plates at a concentration of 7×10^5 cells/well. Primary cortical neurons were isolated from cortices of E16 embryonic mice. After dissociated with papain, 1 million neurons were seeded per well on the top of the confluent astrocytes. Co-cultures were first maintained in Neurobasal medium supplemented with 5% FBS and 2% B-27. After 4 days half of the medium was changed into serum free Neurobasal medium supplemented with 2% B27. Cells were treated with various compounds at DIV5 for 3–6 days accordingly.

2.3. Differentiation of human stem cell derived astrocyte

We obtained a human neural stem cell line (HSC) from Dr. Clive Svendsen at Wisconsin Medicine (Svendsen et al., 1998). HSC spheres were maintained in maintenance medium (DMEM/F-12 supplemented with 1% N2, 0.1% LIF and 20 ng/ml EGF). To differentiate the stem cells into astrocytes, HSC spheres were dissociated with acutase (Chemicon) and seeded on Poly-L-Ornithine/Laminin coated 12-well plates. After being cultured in DMEM/F-12 supplemented with 10% fetal bovine serum for at least 7 days, cells were treated with various compounds.

2.4. RT-qPCR

Total RNA from cultured cells or tissues were isolated by using RNA EASY kit (Qiagen) which was then reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems). Real-time PCR was performed using iCycler iQ Real-Time Detection System from BioRad.

2.5. Western Blot

Cultured cells were directly lysed with ice-cold lysis buffer (20 mM Tris–HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X and 0.1% SDS). Tissues harvested from mice were first homogenized in ice-cold lysis buffer with glass homogenizers, then sonicated, followed by centrifugation at 13,000 × g for 15 min at 4 °C. The protein concentrations of the supernatants were quantified with DC protein assay kit (BioRad, CA). 5–10 μg of total proteins per lane were loaded on precasting 10% polyacrylamide gel (BioRad) for SDS-PAGE. After being transfered onto PVDF membrane, immunoblots were probed with primary antibodies: anti-GLT-1 (1:2000), anti-GLAST (1:200), anti-GFAP (1:5000, Chemcon), and anti-Actin (1:5000, Sigma); and subsequently incubated with HRP conjugated secondary antibody (1:10,000, GE Healthcare). Immunoblots were detected with SuperSignal West Pico chemiluminescent substrates (Thermo Scientific) and visualized by VersaDoc system (BioRad). Intensities of bands were determined by ImageJ software.

2.6. Glutamate uptake assay with cultured cells or crude synaptosomes prepared from mice cortical tissues

0.5 μM L-glutamate (cold:radioactive = 99:1) and 0.3 μCi L-[^{3}H]glutamate per sample (PerkinElmer) was used for measuring glutamate uptake with cultured cells; while 0.5 μM L-glutamate (cold:radioactive = 99:1) and 0.075 μCi L-[^{3}H]glutamate per sample was used for measuring glutamate uptake with crude synaptosomes prepared from mice cortical tissues. For glutamate uptake assay with cultured cells, cells were first washed and pre-incubated at RT for 10 min in Na^+ buffer (5 mM Tris–HCl, pH 7.2, 10 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, and 10 mM D-glucose) in the presence or absence of various glutamate transporter inhibitors. Glutamate uptake reaction was started by incubating cells for 5 min at 37 °C in Na^+ buffer containing 0.5 μM L-glutamate and 0.3 μCi L-[^{3}H]glutamate per sample, followed by rapid washing twice with ice-cold Na^+-free assay buffer (5 mM Tris–HCl, pH 7.2, 10 mM HEPES, 140 mM Choline-Cl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, and 10 mM D-glucose). Cells were then lysed with 0.1N NaOH solution and radioactivity was measured using a scintillation counter. For glutamate uptake assay with crude synaptosomes, mice cortical tissues were first homogenized in ice-cold 0.32M sucrose and then centrifuged at 800 × *g* for 10 min. Supernatants were subsequently centrifuged at 20,000 × g for 20 min at 4 °C. Pellets which contain crude synaptosomes were then washed in 0.32M sucrose, centrifuged and re-suspended in ice-cold 0.32M sucrose. Glutamate uptake reactions were started by adding crude synaptosomes in Na^+ buffer containing 0.5 μM L-glutamate and 0.075 μCi L-[^{3}H]glutamate per sample and incubated for 3 min at 37 °C, and stopped by rapidly adding four volumes of ice-cold Na^+-free assay buffer. Radioactive crude synaptosomes were then collected on filter paper using a Brandel Harvester and washed with Na^+-free assay buffer. Each piece of filter paper containing radioactive crude synaptosomes was transferred into scintillation vials and radioactivity was measured using a scintillation counter. Protein concentrations were measured by Bradford method.

2.7. Animals and pharmocokinetics studies

Six to eight week old SOD^{G93A} mice were ordered from Jackson Lab. Wild-type C57/B6 mice (8 weeks) were ordered from Charles River. The care and treatment of animals in all procedures strictly followed the NIH guide for the Care and Use of Laboratory Animals and Guidelines for the use of animals in neuroscience research and the Johns Hopkins University IACUC. To perform pharmacokinetics studies, 8 week old wild-type C57/B6 mice were injected intraperitoneal with 30 mg/kg harmine at time zero. At designated time points three animals were sacrificed and plasma, cortical and spinal cord tissues were collected. Tissues were homogenized in 2 mM SDS solution. Plasma and homogenate were sent out to a commercial research organization and analyzed using LC/MS/MS detection method.

3. Results

3.1. Astroglial-based EAAT2 promoter reporter screening assay reveals Harmine as potent activator of gene expression

A reporter cell line (CC4) was generated by stably expressing a firefly luciferase reporter under the control of a 2.5 kb human EAAT2 (GLT-1) promoter fragment in human immortalized astroglial-fetal derived cells (Rothstein et al., 2005; Yang et al., 2009). Compared to other reporter systems, luminescence based assays are most sensitive therefore suitable to develop a screening assay that can be converted into a high throughput screening (HTS) assay to screen large chemical libraries (100,000s of compounds). We reasoned that after long-term (i.e., days) exposure of CC4 to various compounds, we could assess the compounds' efficacy on increasing