Published in final edited form as:
Neuropharmacology. 2011 June ; 60(7-8): 1168–1175. doi:10.1016/j.neuropharm.2010.10.016.

Harmine, A Natural Beta-Carboline Alkaloid, Upregulates Astroglial Glutamate Transporter Expression

Yun Li[1], **Rita Sattler**[1,2], **Eun Ju Yang**[1], **Alice Nunes**[1], **Yoko Ayukawa**[1], **Sadia Akhtar**[1], **Grace Ji**[1], **Ping-Wu Zhang**[1], and **Jeffrey D. Rothstein**[1,2,*]

[1]Department of Neurology, Johns Hopkins University, School of Medicine, Baltimore, MD 21205

[2]BSI NeuroTranslational Program, Johns Hopkins University, School of Medicine, Baltimore, MD 21205

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.

Keywords

harmine; GLT-1; EAAT2; glutamate transporter; astroglia; ALS

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

*Corresponding Author: Jeffrey D. Rothstein MD, PhD, Professor of Neurology and Neuroscience; Director, Robert Packard Center for ALS Research; Co-Director, Brain Science Institute (BSI); Johns Hopkins University, School of Medicine; The John G. Rangos Sr. Building, 855 N. Wolfe St., Rm. 270, Baltimore, MD 21205 410-614-3846; FAX: 410-502-5459, jrothstein@jhmi.edu.

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 EAAT2. 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% (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.

Material and Methods

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.

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.

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 20ng/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.

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.

Western Blot

Cultured cells were directly lysed with ice-cold lysis buffer (20mM This-HCl, pH 7.4, 140mM NaCl, 1mM 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 15min 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.

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 0.5μM 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 (5mM Tris-HCl, pH 7.2, 10mM HEPES, 140mM NaCl, 2.5mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM K_2HPO_4, and 10mM D-glucose) in the presence or absence of various glutamate transporter inhibitors. Glutamate uptake reaction was started by incubating cells for 5 minutes 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 (5mM Tris-HCl, pH 7.2, 10mM HEPES, 140mM Choline-Cl, 2.5mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM K_2HPO_4, and 10mM 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 10min. Supernatants were subsequently centrifuged at 20,000 × g for 20min 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 minutes 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.

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 30mg/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 2mM SDS solution. Plasma and homogenate were sent out to a commercial research organization and analyzed using LC/MS/MS detection method.

Results

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 2.5kb 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 EAAT2 promoter activity by measuring luciferase activity. We validated the suitability of the CC4 cell line for our drug screening assay by

testing its reproducibility as well as by assessing drug screening assay parameters such as signal-to-noise ratio (S/N) and Z' factor (Figure 1A). We concluded that the CC4 cell-based luminescent assay is a robust assay with high reproducibility, a reasonable S/N (close to 11) and Z' factor (close to 0.6). We therefore started to screen a library of 1040 drugs and nutritionals that are FDA approved in the USA (US drug collection; MicroSource Discovery Systems, Inc.). Each drug was tested at a 10μM concentration and was examined in triplicate. A representation of a 96-well plate from the screening is illustrated in Figure 1B. From this screen, we identified a series of compounds that were capable of increasing luciferase activity, presumably due to the enhancement of EAAT2 promoter activity (Table 1). A naturally occurring beta-carboline alkaloid, harmine, was identified as one of the most effective compounds. It was subjected to a dose response curve analysis (0.001-30μM) resulting in an EC_{50} value of 3.2μM (Figure 1C). High concentrations of harmine (≥ 30μM) were toxic to our cultured cells (see single data point in dose response curve). We therefore calculated the EC_{50} value using the 10μM response as our maximal value.

Luminescence based screening assays may lead to false positive hits that non-specifically interfere with the luciferase activity rather than increasing luciferase expression *via* promoter activation (Auld et al., 2008). In order to test the specificity of harmine, we performed the assay in cells expressing luciferase driven by a gene promoter that is not related to glutamate transporters (Monocarboxylate Transporter 4). We found that harmine specifically increased the EAAT2 promoter driven luciferase but not the unrelated control (Figure 2A). Parallel experiments demonstrated that actinomycin, a blocker for transcriptional activation, completely abolished the effect of harmine (Figure 2B). This indicated that harmine increased luciferase activity of CC4 cells through transcriptional regulation. Last, we performed reverse transcription-polymerase (RT)- quantitative PCR and found that harmine increased luciferase mRNA after 24-hour treatment in CC4 cells (Figure 2C). These data supported our hypothesis that harmine acts as a transcriptional activator of EAAT2.

Harmine increases endogenous GLT-1 mRNA in primary cultures of mouse and human astrocytes

We next validated harmine in our primary cortical neuron-astrocyte co-culture system to determine whether harmine is capable of increasing endogenous GLT-1 mRNA. Harmine efficiently increased GLT-1 mRNA after 48-hour treatment (Figure 3A). Unexpectedly, harmine also significantly increased the expression of GLAST, another astrocytic glutamate transporter (Figure 3B). When other genes were examined, including Glial fibrilary acidic protein (GFAP), Neuron-specific class III beta-tubulin (Tuj1) and neuronal glutamate transporter (EAAC1), an increase in their gene expression (data not shown) was not seen. The specific induction of harmine on both GLT-1 and GLAST suggests that there might be a common transcriptional regulation of the key proteins in glial glutamate uptake.

Mouse GLT-1 and GLAST promoter sequences are not 100% homologous to the human gene and the primary screen with CC4 cells only tested a partial fragment of the EAAT2 promoter. It was unclear if harmine could show activity on the human full-length EAAT2 and/or EAAT1 promoters. We then tested its effect in human stem cell derived astrocytes, employing a human neural stem cell. This is not an immortalized cell line, but a human neural stem cell line maintained as a neurosphere that has the full potential to either differentiate into neurons or astrocytes (Svendsen et al., 1998). We dissociated the neurospheres and cultured the cells with a specific medium to differentiate them into astrocytes. We then treated those astrocytes with harmine and showed a significant induction of both human EAAT1 (GLAST) and EAAT2 (GLT-1) expression after 48 hour treatment (Figure 3C & D).

Harmine increases GLT-1 and GLAST protein levels and glutamate uptake in primary cell cultures

We next asked if harmine is capable of increasing endogenous GLT-1 and GLAST proteins in our primary mouse cortex co-culture system. Indeed, we demonstrated that harmine significantly increased both endogenous GLT-1 and GLAST protein after a 6-day treatment (Figure 4A) while other astroglial proteins like GFAP or neuronal protein Tuj1 were unaffected by drug treatment.

To verify that the harmine-induced newly formed protein is functional, we performed a glutamate uptake assay in our cortical co-culture system. As shown in Figure 4B, harmine dose dependently increased Na^+ dependent glutamate transporter activity. To determine which of the glutamate transporter subtypes contribute to the measured glutamate uptake we performed the uptake assays in the presence of subtype specific glutamate transporter blockers. TBO-A, a non-specific glutamate transporter blocker, inhibits all Na+ dependent glutamate uptake, while DHK specifically blocks GLT-1-dependent glutamate uptake. Subtraction of specific from non-specific uptake revealed that harmine increased both GLT-1 and GLAST dependent uptake (Figure 4C).

Specificity of harmine's activity

Harmine is a harmala alkaloid (Figure 5A) and generally these alkaloids have a wide spectrum of pharmacological actions, including MAO inhibition, binding to benzodiazepine receptors, convulsive or anticonvulsive actions and tremorogenesis (Aricioglu et al., 2003; Glennon et al., 2000; Guan et al., 2001; Husbands et al., 2001; Kim et al., 1997; Lutes et al., 1988; Song et al., 2004). To gain some insight into the specificity of harmine's ability to up-regulate glutamate transporter gene expression, we tested the activities of anumber of harmine analogs. Harmol and harmane showed ~ 50% and 20% activity respectively compared to harmine, while other derivatives showed no activity (Figure 5B). Figure 5C shows a full dose response curve of harmol using our primary screening assay. We calculated its EC_{50} value (3.8 μM) using the 10μM response as our maximal value, since higher concentrations of harmol (≥ 30μM) were toxic to our cultured cells. Then we tested harmol (3μM) in our neuron-astrocyte co-culture system found that there was a positive trend towards a GLT-1 upregulation and a significant increase in GLAST expression (Figure 5D).

Harmine readily crosses the blood brain barrier (BBB)

To confirm BBB penetration, we performed a simple pharmacokinetics study on WT mice. Animals were dosed i.p. with 30mg/kg harmine at time zero and at designated time points three animals were sacrificed. Plasma and brain homogenate were analyzed by LC/MS/MS detection method. Figure 6A shows the time course of harmine concentrations in plasma, spinal cord and cortex. As described previously, harmine penetrates the brain very rapidly, most likely due to its highly lipophilic structure (Zetler et al., 1974). Harmine is eliminated from the brain very quickly, reaching 10% of its maximum levels at about 90min after dosing. Pharmacokinetic parameters differed slightly between cortex and spinal cord with C_{max}*cortex* = 24377 ng/g and C_{max}*spinal cord* = 18623 ng/g. As reported before, plasma levels of harmine were lower than tissue levels (about 1/3 at time zero) and rapidly declined over the time course of our measurements (Zetler et al., 1974). An estimation of the molarity of harmine in the brain led to a value of 3μM at the 2 hr time point, which parallels the concentration needed *in vitro* to achieve mRNA and protein upregulation of glutamate transporters.

Harmine increases GLT-1 protein levels and glutamate uptake *in vivo*

To test whether harmine increases protein levels of GLT-1 *in vivo* we treated $SOD1^{G93A}$ mice. These mice show ALS-like symptoms at around 90 days (disease onset), and they reach disease end-stage typically around 130 days. $SOD1^{G93A}$ mice (10-11 mice per group) were treated from D90 to D100 with either harmine (10mg/kg and 30mg/kg; i.p.) or saline twice a day. At the end, animals were sacrificed and tissues (cortex, spinal cord) were harvested and analyzed by western blot. After normalization to actin, we observed that 10mg/kg of harmine significantly increased GLT-1 protein expression ($p< 0.05$, t test) in cortex, and lead to a positive trend of protein upregulation in the spinal cord (Figure 6B).

Next, we asked if harmine-induced increase in GLT-1 protein would increase glutamate transporter activity *in vivo*. We injected wild type mice (10-11 mice per group) twice a day with harmine (10mg/kg, i.p.) or saline for 10 days. At the end, animals were sacrificed and glutamate uptake was measured from cortical tissue homogenates (Figure 6C & D). We demonstrated that harmine significantly increased both Na^+ dependent glutamate uptake (which represents both, GLT-1 and GLAST-dependent uptake) and GLT-1 specific glutamate uptake ($p<0.05$, t test).

Discussion

The discovery and development of new drugs that could modulate glutamate transporters could be a novel approach to multiple neurological and psychiatric disorders. Dysfunctional glutamate transmission and consequent accumulation of extracellular glutamate has been a target for therapy in ALS, Huntington's disease, epilepsy, multiple sclerosis as well as depression and schizophrenia. In this paper, we have outlined a new cell-based screening assay for the identification of small molecule compounds that activate gene expression of glutamate transporters, proteins that are known to become dysfunctional and downregulated during ALS disease progression, as well as in other neurological disorders (Gegelashvili et al., 2001) (Lepore et al., 2007; Rothstein, 2009; Rothstein et al., 2005).

This assay was designed to be readily translated into a high-throughput screening (HTS) platform to screen large chemical libraries (e.g. 10^5-10^6 compounds). A pilot screen was successfully performed with an external HTS facility screening a library of about 30,000 compounds (R. Sattler and J. Rothstein, unpublished observation). In addition, this cell-based assay was used to perform a combinatorial screen of the Microsource library used in this publication (Li *et al*., manuscript in preparation).

Our laboratory had performed a recent screen for glutamate transporter activators using primary organotypic spinal cord slice cultures (Rothstein et al., 2005). In this screen, we identified numerous beta lactam antibiotics, such as ceftriaxone, as effective small-molecule compounds that upregulated GLT-1 gene expression and observed that it protected against motor neuron cell death. Since primary cultures cannot be used for high throughput screening, we developed the current screening assay and validated it by testing the newly updated Microsource library. A recent report shows the development of a HTS for translational activators of glutamate transporter EAAT2 (Colton et al., 2010). Using a cell-based enzyme-linked immunosorbent assay in a stably transfected astrocyte cell line, Colton and colleagues developed a screening assay to specifically screen small molecule compounds that activate EAAT2 translation, although the active compounds in that assay were not shown to actually increase endogenous protein *in vivo*. That assay differs from our assay in that we screen for compounds acting on a transcriptional level, which means we screen for the formation of new mRNA followed by new protein synthesis. Studies have shown that mRNA levels of GLT-1 are downregulated even before disease onset (Yang et

al., 2009), hence, it will be crucial to boost new mRNA synthesis to overcome the loss of GLT-1/EAAT2 protein during disease.

The use of luciferase-based reporter gene assays has to be taken with care, as some compound structures can lead to false positive hits by stabilizing the luciferase protein instead of modulating gene transcription (Auld et al., 2008). In our study, we focused on only a few of the positive hits we obtained from our screening assay. While most compounds showed specific activity towards the EAAT2 promoter, some compounds with increased luciferase activity failed to be blocked with actinomycin treatment and consequently failed to increase mRNA levels of GLT-1 *in vitro*. None of these compounds were further tested for transporter activity.

With the newly developed assay, we identified harmine, a naturally occurring small molecule, as an effective compound to induce transcriptional activation of astrocytic glutamate transporters in cultured astroglia- but more significantly, in vivo following repeated administration. Harmine is a beta-carboline alkaloid that was first isolated in 1847 from seeds of *Peganum harmala* (Syrian rue) and *Banisteriopsis caapi*, both of which have traditionally been used for ritual and medicinal preparations in the Middle East, Central Asia, and South America (Sourkes, 1999). It is also present in common plant-derived foods and in human tissues (Guan et al., 2001). Known pharmacologic effects of harmine include hallucinogenesis (Sourkes, 1999), convulsive or anticonvulsive actions (Aricioglu et al., 2003), and tremor (Guan et al., 2001; Lutes et al., 1988). Several potential molecular targets for these central pharmacologic effects of harmine have been identified. These include MAO-A (Kim et al., 1997), 5-HT2A (Glennon et al., 2000), imidazoline receptors (I1 and I2 sites) (Husbands et al., 2001), and cyclin-dependent kinases (CDK1, 2, and 5) (Song et al., 2004). To our knowledge, activation of any of these targets does not lead to increased glutamate transporter expression.

Few compounds have been shown to activate increased glutamate transporter-EAAT2/GLT1 protein expression and function *in vivo*. Our new cellular screen has provided insight into a class of compounds that could serve as a starting point for new GLT1 activators. Given harmine's CNS side effects and its multi-target activities, harmine itself is not a suitable candidate for immediate clinical applications. However, it may eventually be possible to separate its effects on GLT-1 upregulation and on CNS pharmacology through optimization of its chemical structure. Medicinal chemistry efforts to do so are ongoing, which will hopefully lead to a more potent and selective drug candidate that can be moved through the drug development process reaching from preclinical studies to clinical trials. These trials may include clinical applications such as ALS, but also other neurodegenerative disorders as well as psychiatric diseases.

Acknowledgments

We thank Ahmet Hoke for HIA cells and Clive Svendsen for HSC line. This work was supported by the Cinque Foundation and the Maryland Stem Cell Research Foundation. The findings from this study are dedicated to the memory of Steven Cinque.

References

Aricioglu F, Yillar O, Korcegez E, Berkman K. Effect of harmane on the convulsive threshold in epilepsy models in mice. Ann N Y Acad Sci. 2003; 1009:190–195. [PubMed: 15028587]

Auld DS, Thorne N, Nguyen DT, Inglese J. A specific mechanism for nonspecific activation in reporter-gene assays. ACS Chem Biol. 2008; 3:463–470. [PubMed: 18590332]

Colton CK, Kong Q, Lai L, Zhu MX, Seyb KI, Cuny GD, Xian J, Glicksman MA, Lin CL. Identification of translational activators of glial glutamate transporter EAAT2 through cell-based

high-throughput screening: an approach to prevent excitotoxicity. J Biomol Screen. 2010; 15:653–662. [PubMed: 20508255]

Danbolt NC. Glutamate uptake. Prog Neurobiol. 2001; 65:1–105. [PubMed: 11369436]

Dunlop J, Beal McIlvain H, She Y, Howland DS. Impaired spinal cord glutamate transport capacity and reduced sensitivity to riluzole in a transgenic superoxide dismutase mutant rat model of amyotrophic lateral sclerosis. J Neurosci. 2003; 23:1688–1696. [PubMed: 12629173]

Gegelashvili G, Robinson MB, Trotti D, Rauen T. Regulation of glutamate transporters in health and disease. Prog Brain Res. 2001; 132:267–286. [PubMed: 11544995]

Glennon RA, Dukat M, Grella B, Hong S, Costantino L, Teitler M, Smith C, Egan C, Davis K, Mattson MV. Binding of beta-carbolines and related agents at serotonin (5-HT(2) and 5-HT(1A)), dopamine (D(2)) and benzodiazepine receptors. Drug Alcohol Depend. 2000; 60:121–132. [PubMed: 10940539]

Guan Y, Louis ED, Zheng W. Toxicokinetics of tremorogenic natural products, harmane and harmine, in male Sprague-Dawley rats. J Toxicol Environ Health A. 2001; 64:645–660. [PubMed: 11766171]

Guo H, Lai L, Butchbach ME, Stockinger MP, Shan X, Bishop GA, Lin CL. Increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset but not the outcome of ALS in mice. Hum Mol Genet. 2003; 12:2519–2532. [PubMed: 12915461]

Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). Proc Natl Acad Sci U S A. 2002; 99:1604–1609. [PubMed: 11818550]

Husbands SM, Glennon RA, Gorgerat S, Gough R, Tyacke R, Crosby J, Nutt DJ, Lewis JW, Hudson AL. beta-carboline binding to imidazoline receptors. Drug Alcohol Depend. 2001; 64:203–208. [PubMed: 11543990]

Kim H, Sablin SO, Ramsay RR. Inhibition of monoamine oxidase A by beta-carboline derivatives. Arch Biochem Biophys. 1997; 337:137–142. [PubMed: 8990278]

Lepore AC, Haenggeli C, Gasmi M, Bishop KM, Bartus RT, Maragakis NJ, Rothstein JD. Intraparenchymal spinal cord delivery of adeno-associated virus IGF-1 is protective in the SOD1G93A model of ALS. Brain Res. 2007; 1185:256–265. [PubMed: 17963733]

Lutes J, Lorden JF, Beales M, Oltmans GA. Tolerance to the tremorogenic effects of harmaline: evidence for altered olivo-cerebellar function. Neuropharmacology. 1988; 27:849–855. [PubMed: 2851115]

Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol. 2009; 65(Suppl 1):S3–9. [PubMed: 19191304]

Rothstein JD, Martin LJ, Kuncl RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N Engl J Med. 1992; 326:1464–1468. [PubMed: 1349424]

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature. 2005; 433:73–77. [PubMed: 15635412]

Rothstein JD, Tsai G, Kuncl RW, Clawson L, Cornblath DR, Drachman DB, Pestronk A, Stauch BL, Coyle JT. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol. 1990; 28:18–25. [PubMed: 2375630]

Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol. 1995; 38:73–84. [PubMed: 7611729]

Song Y, Kesuma D, Wang J, Deng Y, Duan J, Wang JH, Qi RZ. Specific inhibition of cyclin-dependent kinases and cell proliferation by harmine. Biochem Biophys Res Commun. 2004; 317:128–132. [PubMed: 15047157]

Sourkes TL. “Rational hope” in the early treatment of Parkinson’s disease. Can J Physiol Pharmacol. 1999; 77:375–382. [PubMed: 10537223]

Spreux-Varoquaux O, Bensimon G, Lacomblez L, Salachas F, Pradat PF, Le Forestier N, Marouan A, Dib M, Meininger V. Glutamate levels in cerebrospinal fluid in amyotrophic lateral sclerosis: a

reappraisal using a new HPLC method with coulometric detection in a large cohort of patients. J Neurol Sci. 2002; 193:73–78. [PubMed: 11790386]

Svendsen CN, ter Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostenfeld T, Caldwell MA. A new method for the rapid and long term growth of human neural precursor cells. J Neurosci Methods. 1998; 85:141–152. [PubMed: 9874150]

Yang Y, Gozen O, Watkins A, Lorenzini I, Lepore A, Gao Y, Vidensky S, Brennan J, Poulsen D, Won Park J, Li Jeon N, Robinson MB, Rothstein JD. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron. 2009; 61:880–894. [PubMed: 19323997]

Zetler G, Back G, Iven H. PHARMACOKINETICS In the rat of the hallucinogenic alkaloids harmine and harmaline. Naunyn Schmiedebergs Arch Pharmacol. 1974; 285:273–292. [PubMed: 4282118]

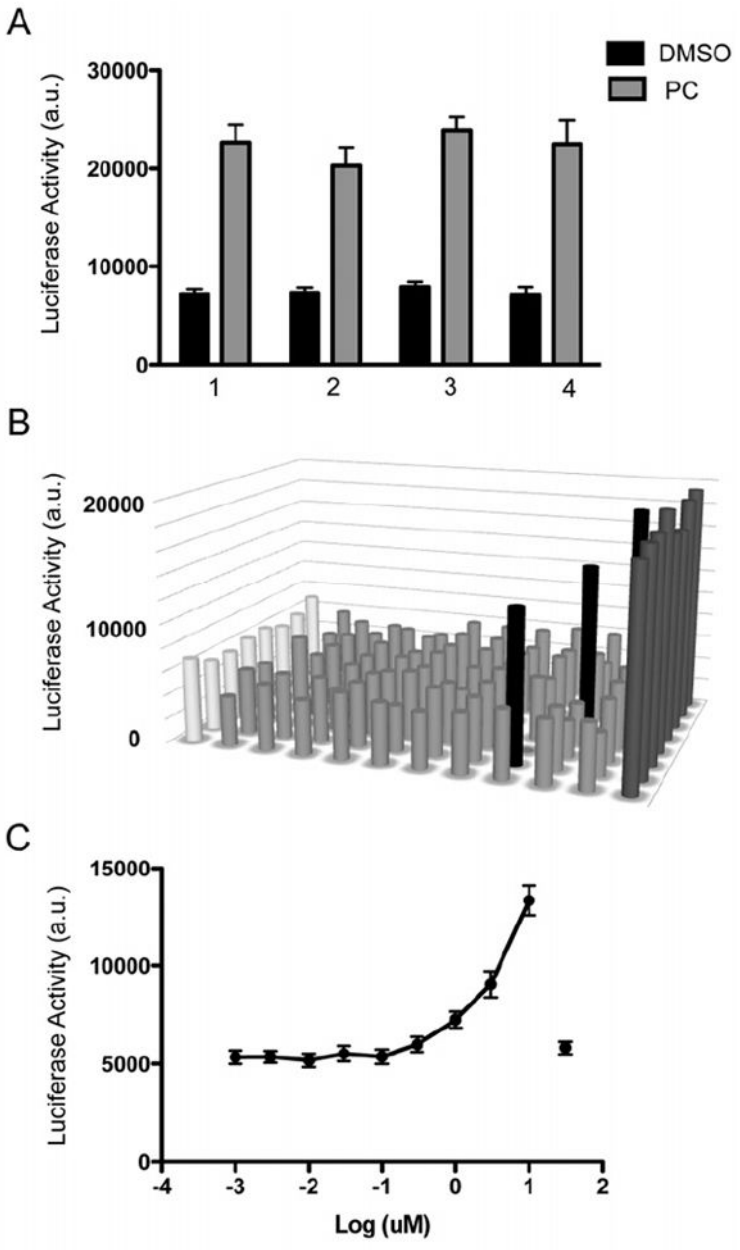

Figure 1.

Luminescence based screening assay for transcriptional activation of EAAT2. Human immortalized astrocytes were stably transfected with a luciferase reporter under the control of an EAAT2 promoter fragment. (**A**) Cells were treated with a known activator (positive control, PC) or vehicle for 3 days. Luciferase activity was measured on day 4. The positive control compound gave reproducible luciferase activity in 4 independent experiments. Error bars represent standard deviations. **B**: Representative individual 96-well plate from the screening assay. Positive controls were positioned on the right side column (dark gray) and vehicle controls were on the left (light gray). Three hits (black) were identified in this plate. Z' factor is 0.6. **C**: Harmine, one of the hits from the screen, was tested with a full dose response curve. Concentrations of harmine > 30μM were toxic to the cells. Estimated EC_{50} for harmine is 3.2μM.

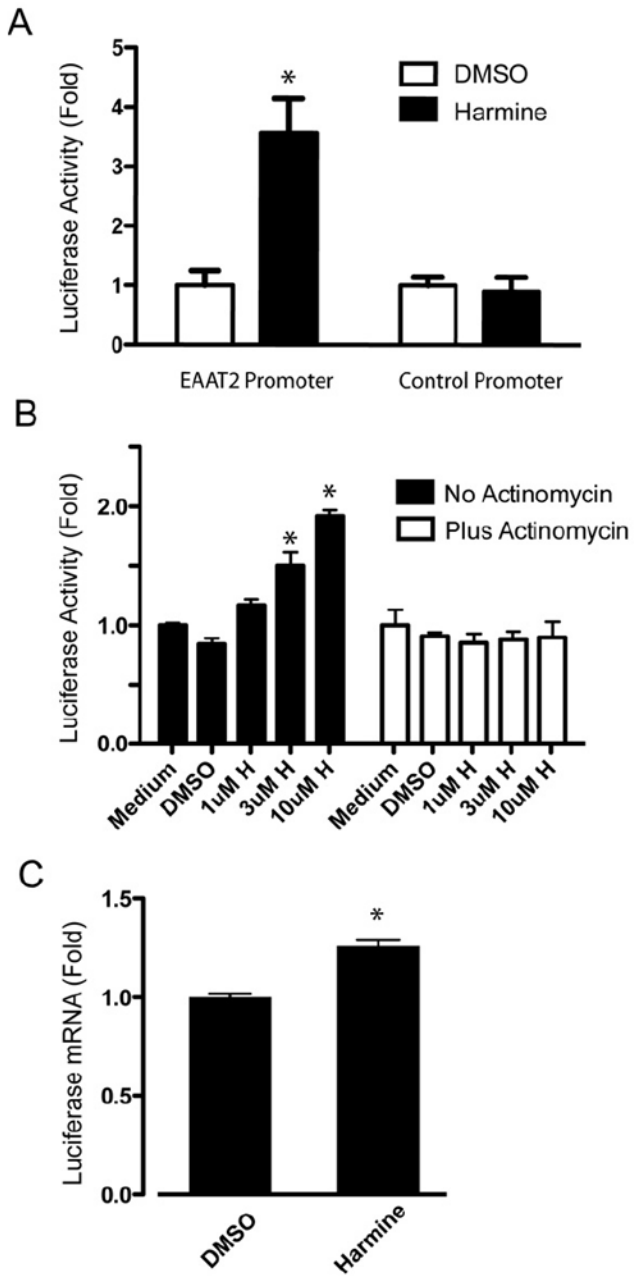

Figure 2.

Harmine increased EAAT2 promoter activity in CC4 cells. **A**: Harmine (10μM) specifically increased EAAT2 promoter activity but did not activate a control promoter, illustrated by luminescent activity assay. **B**: Actinomycin, a transcriptional inhibitor, completely blocked the effect of harmine in the luciferase assay with CC4 cells. **C**: RT-qPCR analysis demonstrated that harmine (10μM) increased luciferase mRNA in CC4 cells. * dedicated $p < 0.05$, t-test vs DMSO control.

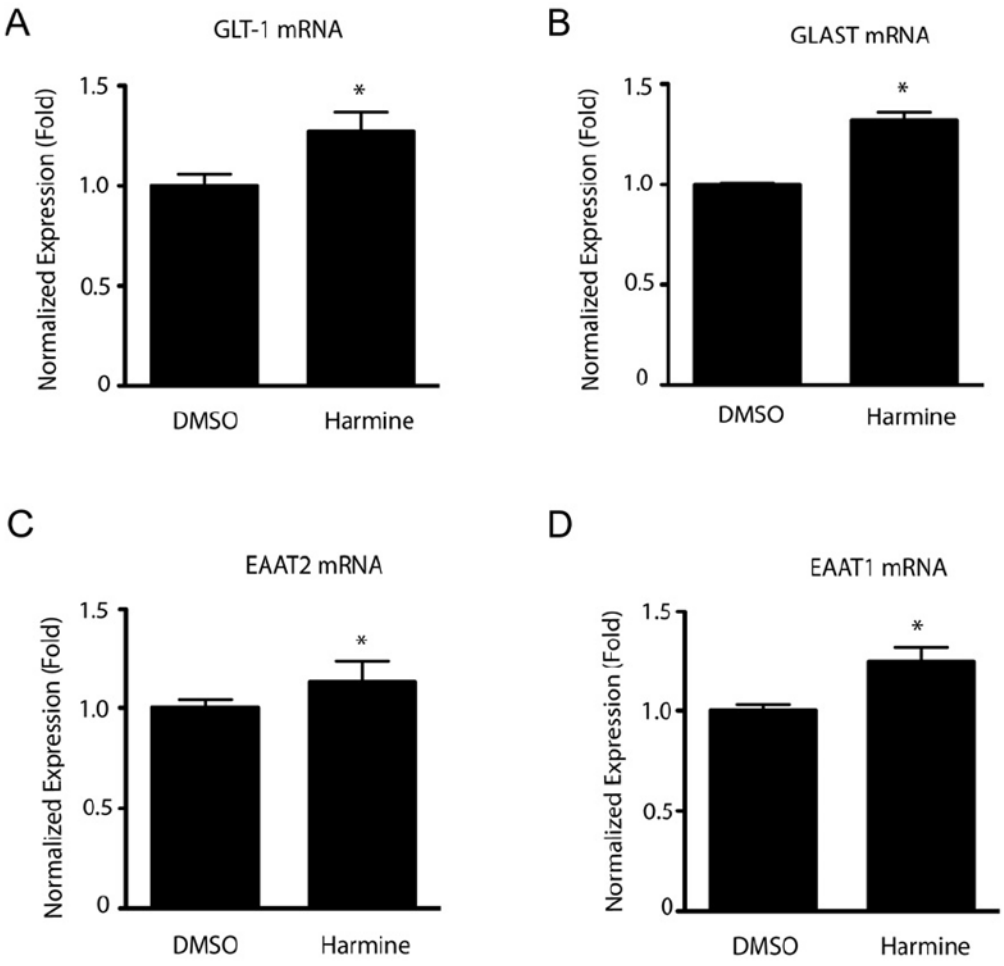

Figure 3.

Harmine increases mRNA levels of endogenous glutamate transporters in mouse and human astrocytes: Harmine (3μM) increases mRNA levels of GLT-1 (**A**) and GLAST (**B**) in mouse cortical co-culture. **C**/**D**: Harmine (3μM) increases mRNA levels of EAAT2 (human version of GLT-1; **C**) and EAAT1 (human version of GLAST; **D**) in human stem cell derived astrocyte culture. * dedicated $p < 0.05$, t-test vs. DMSO control.

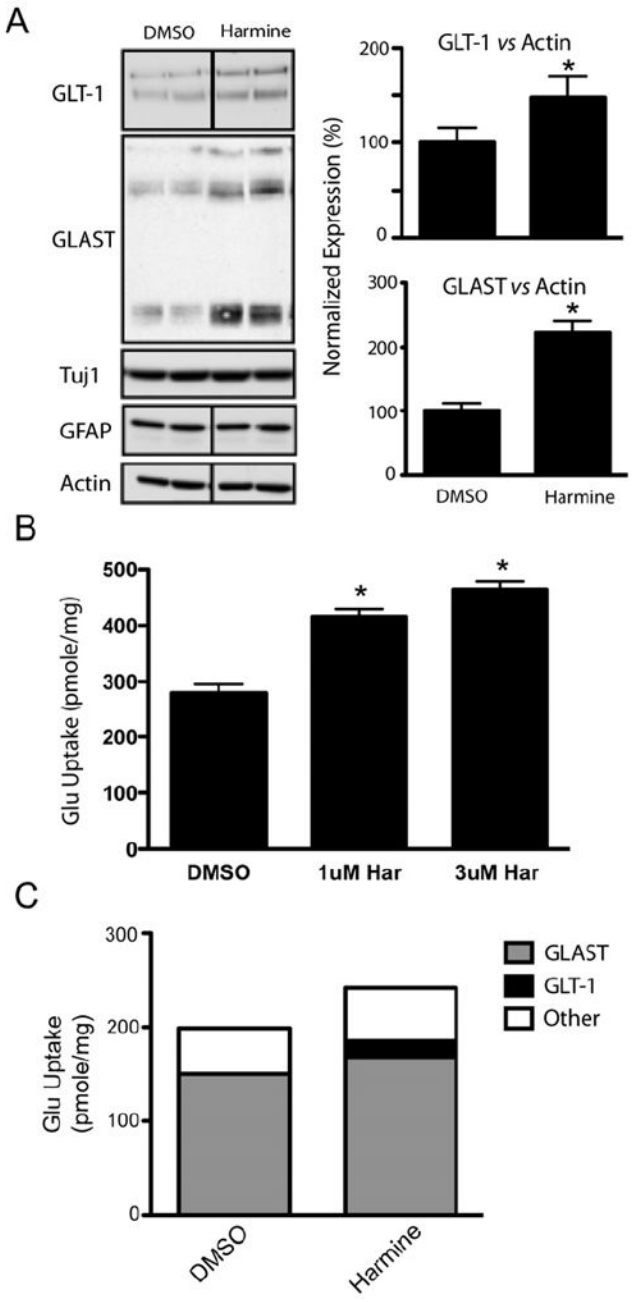

Figure 4.

Harmine increases protein levels of glutamate transporters in mouse cortical neuron-astrocyte co-culture. **A**: Harmine treatment (3μM) increases GLT-1 and GLAST protein compared to DMSO treated control. Left panels show western blots, right panels show quantification of WB. **B**: Harmine dose dependently increases Na^+ dependent glutamate uptake in mouse cortical co-culture. **C**: Harmine-induced increase in glutamate uptake is due to an up-regulation of both, GLAST and GLT-1.

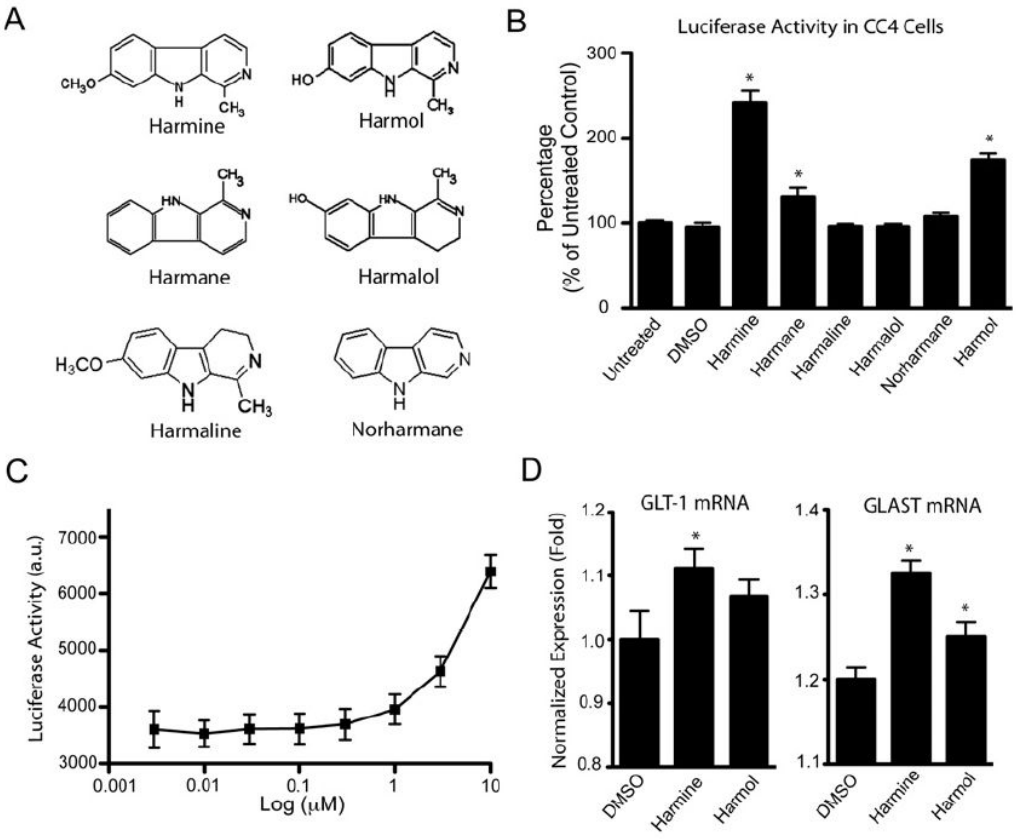

Figure 5.

Harmol, a derivative of harmine, increases EAAT2 promoter activity. **A**: Chemical structures of harmine derivatives. **B**: Effect of harmine's derivatives in luminescent activity assay with CC4 cells (all at a dose of 10μM). **C**: Dose response curve of harmol in luminescent activity assay with CC4 cells. Estimated EC_{50} for harmol is 3.8μM. **D**: RT-qPCR demonstrated that harmol (3μM) increased GLT-1 and GLAST mRNA levels. * dedicated $p < 0.05$, t-test vs DMSO control.

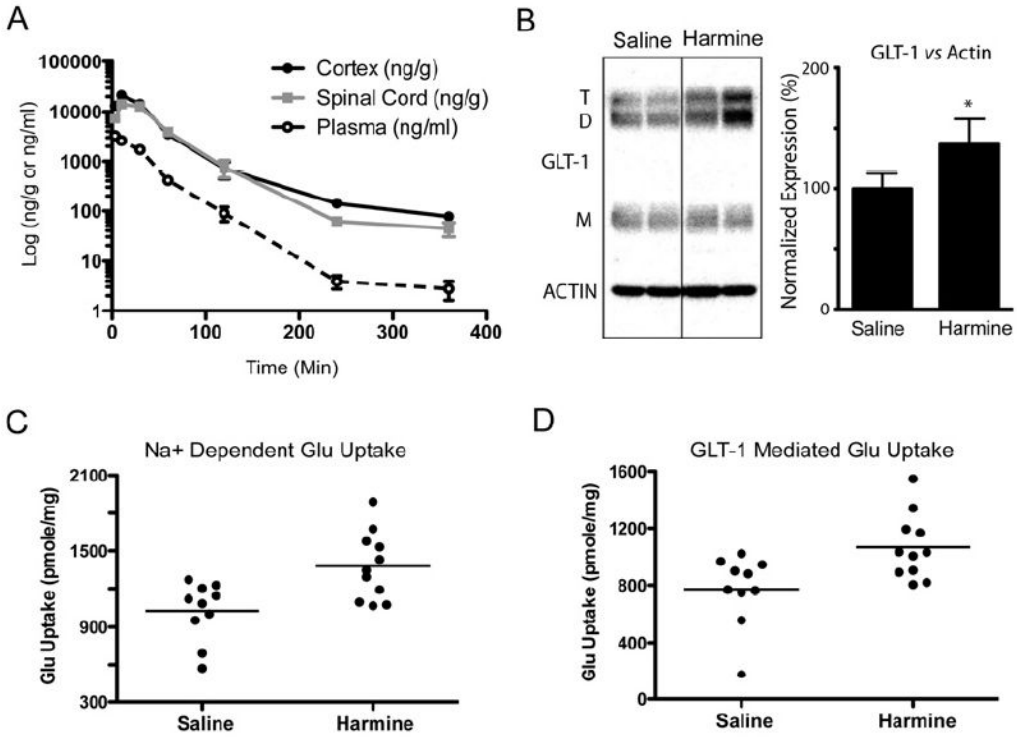

Figure 6.

Harmine increases GLT-1 expression *in vivo*. **A**. Time course of harmine concentrations in plasma and brain tissue. WT mice were dosed i.p. with 30mg/kg harmine and at each time point 3 animals were sacrificed and analyzed for drug levels. **B**. GLT-1 western blot analysis of cortical tissues collected from harmine (10mg/kg; twice a day) and saline injected $SOD1^{G93A}$ mice. Right panel shows quantification of the western blot. **C**. WT mice were treated with harmine (10mg/kg; twice a day) for 10 days. Na^+ dependent total glutamate uptake was measured from freshly collected cortical tissue and shows increased uptake in the harmine treated animals. **D**. GLT-1 mediated glutamate uptake of cortical tissue isolated from harmine and saline injected wild type mice.

Table 1

Top 10 hits from screening of 1,040 MicroSource NINDS compound collection.

Name	Formula	Increase in E2 Promoter Activity	EC50
HARMINE	C13H12N2O	**2.5 fold**	**3.2μM**
JH02	C15H16O2	**2.7**	**1.2μM**
JH03	C16H12O3	**2.3 fold**	**0.7μM**
JH04	C10H7N3S	**2.7 fold**	**1.5μM**
JH05	C17H19NO3	**2.6 fold**	**1.9μM**
JH06	C10H18ClN3O2	**2.3 fold**	**0.8μM**
JH07	C14H8O4	**2.2 fold**	**3.3μM**
JH08	C17H15NO3	**2.3 fold**	**0.025μM**
JH09	C11H12ClN5	**2.2 fold**	**0.8μM**
JH10	C14H12O3	**2.2 fold**	**7.2μM**