

Regular Article

Human nasal olfactory epithelium as a dynamic marker for CNS therapy development

Rita Sattler^{a,c}, Yoko Ayukawa^a, Luke Coddington^a, Akira Sawa^{b,d}, David Block^e,
Richard Chipkin^e, Jeffrey D. Rothstein^{a,b,c,*}

^a Department of Neurology, Johns Hopkins University, Baltimore, MD 21205, USA

^b Department of Neuroscience, Johns Hopkins University, Baltimore, MD 21205, USA

^c Brain Science Institute, Johns Hopkins University, Baltimore, MD 21205, USA

^d Department of Psychiatry, Johns Hopkins University, Baltimore, MD 21205, USA

^e Psyadon Pharmaceuticals Inc. (formerly Ruxton Pharmaceuticals), Germantown, MD 20876, USA

ARTICLE INFO

Article history:

Received 17 January 2011

Revised 12 July 2011

Accepted 4 September 2011

Available online 16 September 2011

Keywords:

Nasal biopsy

Olfactory epithelial tissue

Glutamate transporter

ALS

Astroglia

Astrocyte

Surrogate marker

ABSTRACT

Discovery of new central nervous system (CNS) acting therapeutics has been slowed down by the lack of useful applicable biomarkers of disease or drug action often due to inaccessibility of relevant human CNS tissue and cell types. In recent years, non-neuronal cells, such as astrocytes, have been reported to play a highly significant role in neurodegenerative diseases, CNS trauma, as well as psychiatric disease and have become a target for small molecule and biologic therapies. We report the development of a method for measuring pharmacodynamic changes induced by potential CNS therapeutics using nasal olfactory neural tissue biopsy. We validated this approach using a potential astrocyte-targeted therapeutic, thiamphenicol, in a pre-clinical rodent study as well as a phase 1 human trial. In both settings, analysis of the olfactory epithelial tissue revealed biological activity of thiamphenicol at the drug target, the excitatory amino acid transporter 2 (EAAT2). Therefore, this biomarker approach may provide a reliable evaluation of CNS glial-directed therapies and hopefully improve throughput for nervous system drug discovery.

© 2011 Elsevier Inc. All rights reserved.

Introduction

A biomarker is a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention. The discovery of new CNS acting therapeutics has been slowed down by the lack of useful relevant biomarkers and/or pharmacodynamic measures of disease or drug action. The difficulty for biomarkers in the vast majority of neurologic and psychiatric diseases lies in part in the lack of availability of appropriate tissue samples. Therefore, finding peripheral markers or easily accessible central nervous system (CNS) markers that accurately reflect biology in the CNS is a major goal and critical endeavor in clinical pharmacology of CNS drug development (Collins, 2011). Recently, non-neuronal cells, such as astrocytes, have been reported to play a highly significant role in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia, Huntington's disease, as well as psychiatric disease, such as depression (Lobsiger and Cleveland, 2007; Yamanaka et al., 2008). For example in ALS, Huntington's disease, and multiple sclerosis astrocytes are characterized by a loss of excitatory amino acid transporter 2 (EAAT2; rodent nomenclature: glutamate transporter 1

(GLT-1)) protein and mRNA expression (Edwards, 2009; Ronnett et al., 2003; Rothstein et al., 1995; Werner et al., 2001; Yang et al., 2009). The loss of this transporter results in inefficient clearance of extracellular glutamate, which in the case of ALS leads to glutamate-mediated motor neuron cell death (excitotoxicity), one pathway implicated in ALS pathogenesis (Rothstein et al., 1996; Rothstein et al., 1995). At the same time, manipulation of synaptic glutamate by altering transporter efficacy has been proposed as a novel approach to behavioral disorders such as depression (Banasr et al., 2010; Mineur et al., 2007).

While drugs are increasingly considered for targeting various glial cell types, astroglia are restricted to the central nervous system and thus not readily accessible to assess phenotypic or functional changes in response to targeted drug therapy. Cultured embryonic human or rodent astroglia show some of the characteristics of *in vivo* astroglia cells (e.g. expression of minor subtypes of glutamate transporter proteins) but are not reliable predictors of functional responses similar to those observed *in vivo* and in adult tissues. Therefore it is crucial to develop a method that allows us to follow the biological activity of a drug *in vivo* using an effective and cell specific but minimally invasive approach.

Here we report the development of a pharmacodynamic marker employing nasal olfactory epithelial (OE) tissue as a drug validation tool for expression of astroglial proteins, including EAAT2/GLT-1 and glial fibrillary acidic protein (GFAP). EAAT2/GLT-1 is an astrocytic

* Corresponding author: Brain Science Institute, Johns Hopkins University, 855 N. Wolfe St., Rangos 2-Rm 278, Baltimore, MD 21205, USA. Fax: +1 410 502 5459.

E-mail address: jrothstein@jhmi.edu (J.D. Rothstein).

protein that is downregulated in diseased states, plays a significant role in triggering excitotoxic neuronal cell death during disease progression, and is a potential target for drug development for both neurological and psychiatric disease. GFAP is an astroglial specific intermediated filament protein.

It was recently shown that rodent olfactory epithelial mucosa does express specific markers of CNS cell types including astrocytic marker GFAP, as well as neuronal markers such as glutamate receptor subtypes, nerve growth factor receptors and neurotransmitters GABA and glutamate (Au and Roskams, 2002; Au and Roskams, 2003; Priest and Puche, 2004; Thukral et al., 1997). Therefore, nasal olfactory tissue has been used to study CNS disease mechanisms either by performing histological pathology analyses or by culturing OE cells *in vitro* (Hahn et al., 2005; Seal and Edwards, 2006; Voglmaier and Edwards, 2007; Voglmaier et al., 2006). However, to our knowledge, no studies have used OE tissue samples as a surrogate pharmacodynamic marker for astroglial drug targets.

Based on our interest in glutamate transporter function and dysfunction, we chose to test drugs that upregulate EAAT2/GLT-1 protein and mRNA. Upregulation of astroglial glutamate transporter gene expression has been suggested to protect against glutamate-mediated neuronal cell death by removing excess extracellular glutamate (Rothstein et al., 1996). The neuroprotective properties of glutamate transporter upregulating compounds has since been shown in a number of animal models of neurodegenerative diseases including ALS, HD, ischemia and psychiatric disease such as depression (Hota et al., 2008; Lipski et al., 2007; Miller et al., 2008; Rothstein et al., 2005; Thone-Reineke et al., 2008).

The drugs used in our study, ceftriaxone and thiamphenicol, were discovered from a screen of FDA approved compounds (Rothstein et al., 2005) and were subsequently tested for CNS penetration. Therefore, they were ideal candidates to use for the validation of an assay which monitors EAAT2/GLT-1 levels in peripheral easily accessible neural tissue which we discovered serves as an accurate pharmacodynamic reporter of drug action in the CNS.

To access olfactory tissue mice and human volunteers underwent a biopsy of neural olfactory epithelial tissue. Treatment of mice with EAAT2/GLT-1-upregulating compound thiamphenicol (TAP) showed increased EAAT2/GLT-1 gene expression in nasal neuro-epithelial rodent tissue samples that was similar to the increase in rodent brain tissue. These observations were then validated in human tissue with a human clinical trial measuring EAAT2/GLT-1 levels in OE tissue samples before and after drug treatment with the same EAAT2/GLT-1 upregulating compound in healthy volunteers. The results suggest that the nasal olfactory biopsy, a simple, repeatable and safe procedure, is a valuable approach to evaluate the biological activity of drugs targeted for astrocytes or neurons in patients during the course of clinical drug development.

Materials and methods

Ethics

This study was conducted according to the principles expressed in the Declaration of Helsinki. This study was approved by the Institutional Review Board of the Johns Hopkins University, USA (Protocol Approval NA_00016223) and SGS Life Science Services, Belgium (EC Approval #2987), which conducted the nasal biopsies in drug-treated healthy volunteers. All patients provided written informed consent for the collection of samples and subsequent analysis. For animal experiments, the full study was reviewed and approved by the Johns Hopkins University Animal Care and Use committee following IACUC guidelines (Protocol Approval MO06M209). All animals were handled in strict accordance with good animal practice as defined by the Johns Hopkins IACUC animal welfare committee.

Primary neuron-astrocyte co-culture

Primary astroglial cells were cultured from postnatal 2–3 days 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 astroglial 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.

Promoter reporter assay

A human immortalized astroglial-fetal derived cell line (HIA) was stably transfected 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; Su et al., 2003). The cells were seeded on 96-well plates and treated with TAP for 3 days. Cells were lysed in plates and luciferase activity was measured using a luciferase activity kit (Promega) and the Fluostar OPTIMA plate reader (BMG Labtech, NC).

Drug treatment of WT mice

WT mice (N = 10 per group) were treated with EAAT2/GLT-1 activating agent thiamphenicol (TAP) or vehicle for 10 days 1× a day by intraperitoneal injections (ip) at a dose of 25 mg/kg. TAP was dissolved in saline and 0.3 mL was injected. On day 11, animals were sacrificed and brain tissue, spinal cord and nasal epithelial tissue were isolated.

Isolation of nasal olfactory epithelial protein and mRNA in mice

Mouse nasal turbinates were dissected on dry ice and stored at -80°C . For protein isolation, nasal tissue was homogenized in Tris buffer and solubilized in 0.1% SDS followed by sonication. Tissue lysate was separated on a SDS gel and immunoblotted (see detailed methods below). For mRNA isolation nasal tissue was homogenized using a Polytron tissue grinder and RNA was isolated using the RNA easy kit (Qiagen) by following the instruction of the manufacturer's protocol. RNA was stored at -80°C until RT-qPCR analysis.

Nasal biopsies in human volunteers

Volunteers were selected to participate in the study of the approved protocol after written informed consent was obtained. The technique for a safe biopsy in the living was developed in 1982 (Lovell et al., 1982) and has since then been used continuously without affecting olfactory function (Jafek et al., 2002; Lanza et al., 1994). An increasing number of laboratories have performed this technique using local anesthesia to study olfactory physiology as well as dysfunction in both patients as well as an even larger number of healthy controls (Feron et al., 1999; Gomez et al., 2000; Hahn et al., 2005; Holbrook et al., 2005; KleinJan et al., 2006; McCurdy et al., 2006; Rawson et al., 1997; Ronnett et al., 2003).

Otolaryngologists with sinus surgery experience performed the nasal biopsy. The 30 min procedure was performed under local nasal anesthesia (lidocaine liquid 4% (Xylocaine®), oxymetazoline HCl 0.05% (Afrin®), and following biopsy subjects were observed for an additional 15 to 30 min. If needed, 1% lidocaine with 1/100,000 epinephrine was injected to provide both additional anesthesia and vasoconstriction. The endoscopic procedure was performed using either a small curette or biting forceps for tissue removal. To avoid trauma to the cribriform plate, the biopsies were usually taken from the

upper nasal septum. Occasionally, a small piece of superior turbinate (which is usually lined with neural olfactory tissue) was removed from the lateral nasal wall. The tissue was removed from either the front or the back of the olfactory cleft, and sometimes both. Up to two blocks of tissue (approximately 2 mm each) were collected from each side of the nose.

The side effects of nasal biopsy were uncommon, but included mild nasal- or head-ache for a few hours, and a small amount of bloody nasal discharge. Subjects were instructed not to blow their nose and not to perform any strenuous physical activity for 24 h after the procedure.

Study design for drug treatment of healthy volunteers (inpatient)

Healthy volunteers underwent a nasal biopsy on day – 1 on one side of the nose only. TAP was administered in 3 groups (placebo (N = 6), 750 mg/day (N = 12), 1500 mg/day (N = 12)) starting day 1 to day 14. Blood samples were taken throughout the trial period to obtain basic pharmacokinetic analyses as well as to monitor for safety and tolerability. Every volunteer underwent a spinal tap for cerebral spinal fluid (CSF) sampling on day 13. On day 14, a repeat nasal biopsy was done on the contralateral side of the nose. These two samples were used to determine the fold difference of EAAT2/GLT-1 before and after drug treatment. After the final biopsy, subjects were observed for another 2–3 h before being released.

Quantitative RT-PCR

Total RNA was reversed transcribed using Applied Biosystems' high capacity cDNA archive kit. The quantitative real time RT-PCR assay was performed using the iCycler iQ Real-Time Detection System from BioRad. TaqMan Probes, which allow for specific mRNA amplification, were purchased from Applied Biosystems and included: human (Hs) EAAT2 (# Hs 00188189_m1), Hs GFAP (# Hs 00157674_m1), Hs OMP (# Hs 00255144_s1) and Hs 18S rRNA (# Hs 99999901_s1). All probes were kept in the dark with PCR reactions prepared on ice at 4 °C. Each sample was run in triplicate in a reaction volume of 50 µL in iCycler iQ PCR plates, 96 well (Bio-Rad, # 2239441) containing: 25 µL of 2× TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, # 4324018), 2.5 µL of 20× TaqMan Probe, 5 µL of sample cDNA for EAAT2/GPAP/EAAT1/ OMP or 2 µL of sample cDNA (diluted cDNA in 1:1000 with RNase-free water) for 18 s. Distilled water was added for a total reaction volume of 50 µL. All four genes (EAAT2, GPAP, EAAT1, OMP, and 18S rRNA as control gene) were amplified on the same 96 well plate to minimize assay variability. The reaction in a thermal cycler used the following settings: 95 °C for 10 min (hold), 95 °C for 15 s (40 cycles), 60 °C for 1 min. The amount of the target gene was calculated on the basis of the threshold cycle (C_T) value, and is standardized by the amount of housekeeping gene (18S rRNA) by means of the $2^{-\Delta\Delta C_T}$ method: $\Delta\Delta C_T = (C_{T, \text{target}} - C_{T, 18S \text{ rRNA}}) - (C_{T, \text{target}} - C_{T, 18S \text{ rRNA}})_{\text{reference}}$ ("target", means the gene of interest, and "reference" means the untreated control. PCR results are expressed as fold increases over control values, which are the values obtained from tissue samples on day – 1. Therefore, each data point represents the fold increase in gene expression of one individual subject.

Immunoblotting

EAAT2/GLT-1 protein was detected by standard immunoblotting techniques with anti-GLT-1 antibody. Human EAAT2 is recognized by an anti-GLT-1 antibody (Rothstein et al., 1994). Tissue was lysed in SDS-containing Tris buffer and semi-quantitatively analyzed by SDS-PAGE Western Blot analysis. Immunopositive bands were normalized to the levels of actin within the same sample (anti-Actin, 1:5000, Sigma).

Immunohistochemistry

Fresh nasal tissue was fixed with 4% paraformaldehyde in PBS for 1 h at RT. After rinsing with PBS, tissue was cryoprotected in 30% sucrose in PBS until tissue sunk and embedded in tissue freezing medium (Triangle Biomedical Sciences, Inc.) for cryostat sectioning. 12 mm sections were prepared for immunofluorescence or DAB staining. Sections were post fixed with 4% paraformaldehyde in PBS and incubated in blocking buffer (10% normal goat serum, 0.2% Triton X-100 in PBS) for 60 min at RT, and then incubated with primary antibodies against GLT-1 (rabbit, 1:200), p75 NGFR (mouse, 1:500, Sigma) or GFAP (rabbit, 1:100, DAKO) diluted in 3% normal goat serum, 0.2% Triton X-100 in PBS overnight at 4 °C. For immunofluorescence, Alexa 594/647 goat anti-mouse or anti-rabbit IgG (1:1000, Invitrogen) was applied as a secondary antibody for 2 h at RT, followed by nuclear counter staining with Hoechst 33342 (2 mg/mL, Invitrogen). For DAB staining, biotinylated goat anti-mouse or anti-rabbit IgG (1:200, Vector Laboratories) was applied to the tissue sections for 60 min at RT, followed by avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories), according to the manufacturer's protocol. Immunoreactivity was visualized using DAB (Vector Laboratories) as a chromogen. Sections were then counterstained with Gill's hematoxylin. All double staining was performed sequentially. Confocal images of stained tissues were taken using a Zeiss LSM510 or 710 Meta.

Data analysis

All statistical calculations and/or graphic analyses were performed using SAS® version 8.2 (SAS Institute, Cary, NC, USA) or Graph Pad Prism version. Statistical analyses on safety were performed by SGS Life Science Services. Statistical analysis on mRNA data from human samples was done using the Kruskal Wallis nonparametric test to compare the equality of the medians and the nonparametric test for trend. Statistical analysis on protein data from the preclinical rodent study was done using an independent two sample t-test. Johns Hopkins Biostatistics Department provided fee-for-service statistical oversight.

Results

Expression of EAAT2/GLT-1 in olfactory epithelial tissue

To study the expression of astroglial EAAT2/GLT-1 in the olfactory epithelium, we first analyzed mouse and human nasal tissue for EAAT2/GLT-1 protein levels using standard immunoblotting techniques. Human olfactory mucosa was obtained via outpatient nasal biopsy, a low-risk procedure that is well established with no discernible adverse effects including on olfactory function (Lanza et al., 1994; Lovell et al., 1982). Lysates of mouse and human nasal tissue samples were separated on SDS gels and compared with mouse spinal cord tissue lysates for their glutamate transporter expression levels (Fig. 1A). While expression levels in the nasal tissue were much lower compared to levels in spinal cord tissue, both mouse and human OE showed similar immunopositive bands staining positive for monomeric as well as multimeric EAAT2/GLT-1 transporter complexes (Fig. 1A) (Danbolt, 2001). Pooled human tissue samples were required for the initial protein analysis (Fig. 1), as single human biopsies contained insufficient tissue and protein for Western blot detection of EAAT2. In addition, we performed quantitative mRNA measurements on mouse tissue obtained via quantitative real time reverse transcriptase PCR (qRT-PCR) (Fig. 1B), confirming the expression of EAAT2/GLT-1 mRNA in the nasal biopsy sample compared to cortex, spinal cord and other peripheral tissues. mRNA levels of EAAT2/GLT-1 were about 3–4 fold lower in the OE compared to brain tissue, but higher than any other tested organs, except for

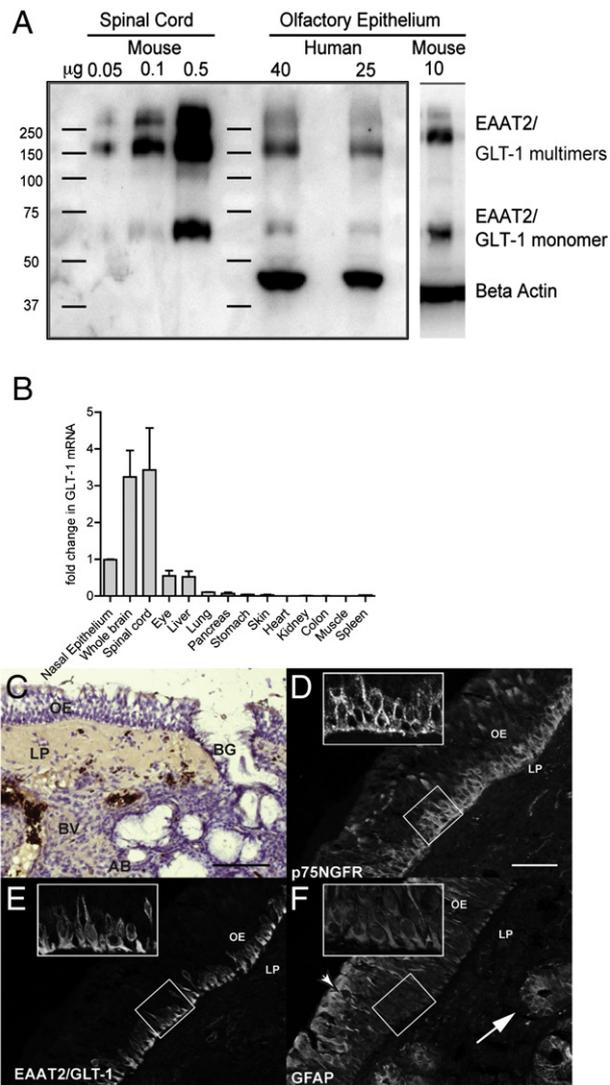


Fig. 1. Astroglial EAAT2/GLT-1 mRNA and protein is expressed in nasal tissue samples from rodents and humans. **A.** Western blot analysis of human and mouse nasal olfactory mucosa tissue showed expression of EAAT2/GLT-1 protein, (monomeric and multimer complexes). The protein expression level in the nasal tissue is significantly lower than it is in spinal cord tissue. **B.** Quantitative mRNA analysis confirmed a lower expression of EAAT2/GLT-1 in the olfactory epithelium (OE) compared to cortex or spinal cord, and very low expression of GLT-1 in other peripheral tissues. **C.** EAAT2/GLT-1 is expressed in the basal cell layer of human olfactory epithelium. H&E staining in human nasal biopsy samples confirms the anatomy of the olfactory mucosa: olfactory epithelium (OE), lamina propria (LP), Bowman's gland (BG), blood vessels (BV), axon bundles (AB) in the LP. **D–F.** Nasal biopsy samples were immunostained for basal cell marker p75NGFR (**D**), glutamate transporter EAAT2/GLT-1 (**E**) and astrocyte marker GFAP (**F**). GLT-1 co-localizes with basal cell marker p75NGFR, while GFAP is highly expressed in sustentacular cells of the OE (**F**, arrowhead) as well as ensheathing cells surrounding axon bundles in the LP (**F**, Arrow). ** $P < 0.01$ vs. OE control, Students t-test.

eyes and pancreas, which showed RNA levels equal to OE. Similarly, astroglial-specific GFAP mRNA was also detected in the rodent and human nasal specimens, thereby confirming the presence of two well-known unique astroglial proteins in nasal olfactory tissue (data not shown).

EAAT2/GLT-1 is expressed in global basal cells of the OE

To determine which cells of the OE express the glutamate transporter, we fixed and immunostained human nasal biopsy samples. The olfactory mucosa is composed of the olfactory epithelium, which contains olfactory receptor neurons (ORN), supporting cells and basal cells, and the lamina propria (LP) (Fig. 1C) (Breipohl et al.,

1974; Graziadei, 1973; Morrison and Costanzo, 1992; Okano and Takagi, 1974). The supporting cells, also known as sustentacular cells, are believed to share similar functions with central nervous system glial cells such as insulating the olfactory receptor cells and providing guidance for the receptor cell axons. The basal cells consist of two subtypes of cells, horizontal and global basal cells, which were identified as the neuroblast stem cells of the olfactory epithelium (Moulton, 1974). The lamina propria contains the axons of the olfactory receptor neurons that project into the olfactory bulb, thereby linking olfactory mucosa to the CNS compartment. These axons are usually found in axon bundles surrounded by olfactory ensheathing cells and supported by connective tissue composed of fibroblasts, Bowman glands as well as blood vessels (Fig. 1C).

Double labeling with antibodies to EAAT2/GLT-1 and the basal cell marker p75 nerve growth factor receptor (p75NGFR) indicated a colocalization of these two proteins in the basal cell layer of the OE (Figs. 1D and E) suggesting that the transporter is expressed in progenitor cell types. This finding parallels the expression pattern of EAAT1 in the CNS where during development, EAAT1 is commonly expressed in neural progenitors (Berger and Hediger, 2006).

The astrocytic marker GFAP was found in sustentacular cells of the OE as well as olfactory ensheathing cells of the lamina propria surrounding axon bundles, similar to what has been reported in mouse OE (Fig. 1F) (Au and Roskams, 2003). EAAT2/GLT-1 was not detected in these cells, suggesting that in peripheral tissue GFAP protein expression does not overlap with EAAT2/GLT-1 protein expression.

Thiamphenicol increases EAAT2/GLT-1 gene expression and function in vitro and in vivo

In a recent study a number of compounds that upregulate the gene expression of EAAT2/GLT-1 were reported (Rothstein et al., 2005) (complete data set: <http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi>; Bioassay AID: 1587). Follow up studies with two of the lead compounds, ceftriaxone and thiamphenicol, showed that the compounds increase transcriptional activation, protein expression and transporter function in rodent astroglia *in vitro* and *in vivo* (Rothstein et al., 2005) (Figs. 2A–G). Specifically, TAP dose-dependently activated the EAAT2/GLT-1 promoter as tested in an overexpressed cell system using a luciferase reporter construct driven by the EAAT2/GLT-1 promoter (Su et al., 2003) (Fig. 2A). When applied to primary astrocyte/neuron co-cultures, TAP increased EAAT2/GLT-1 endogenous protein levels as well as functional glutamate uptake (Figs. 2B and C). The increase in protein did not correlate linearly with increased transporter function, most likely because newly formed transporters may not all be trafficked to the plasma membrane to provide functional glutamate uptake, as has been seen previously *in vitro* (Danbolt, 2001). The TAP-mediated increase in functional glutamate uptake effectively protected against oxygen glucose deprivation, an excitotoxic *in vitro* model (Fig. 2D). These results confirmed the neuroprotective potential of astrocytic transporter upregulation during an ischemic *in vitro* insult shown by others (Weller et al., 2008). In addition, similar to ceftriaxone, TAP significantly increased rodent EAAT2/GLT-1 gene expression and functional activity in WT mice after chronic oral treatment ($N = 10$). The actions of TAP on functional transport were due to gene/protein induction, as the drug had no acute direct effects on transporter function (Rothstein et al., 2005; Figs. 2E, F and G).

While the increase in gene expression for both lead compounds, ceftriaxone and thiamphenicol, was equally moderately significant, TAP was studied further as a “proof of principle” compound, predominantly because TAP is orally active while ceftriaxone has to be administered intravenously for chronic human use. Thiamphenicol has good CNS penetration (Azzollini et al., 1970; Ferrari, 1984; Pfenninger et al., 1977), but due to known modest marrow-toxicity it is not considered an ideal drug candidate for chronic treatments in patients. Nevertheless, given the significant efficacy in the *in vitro* experiments,

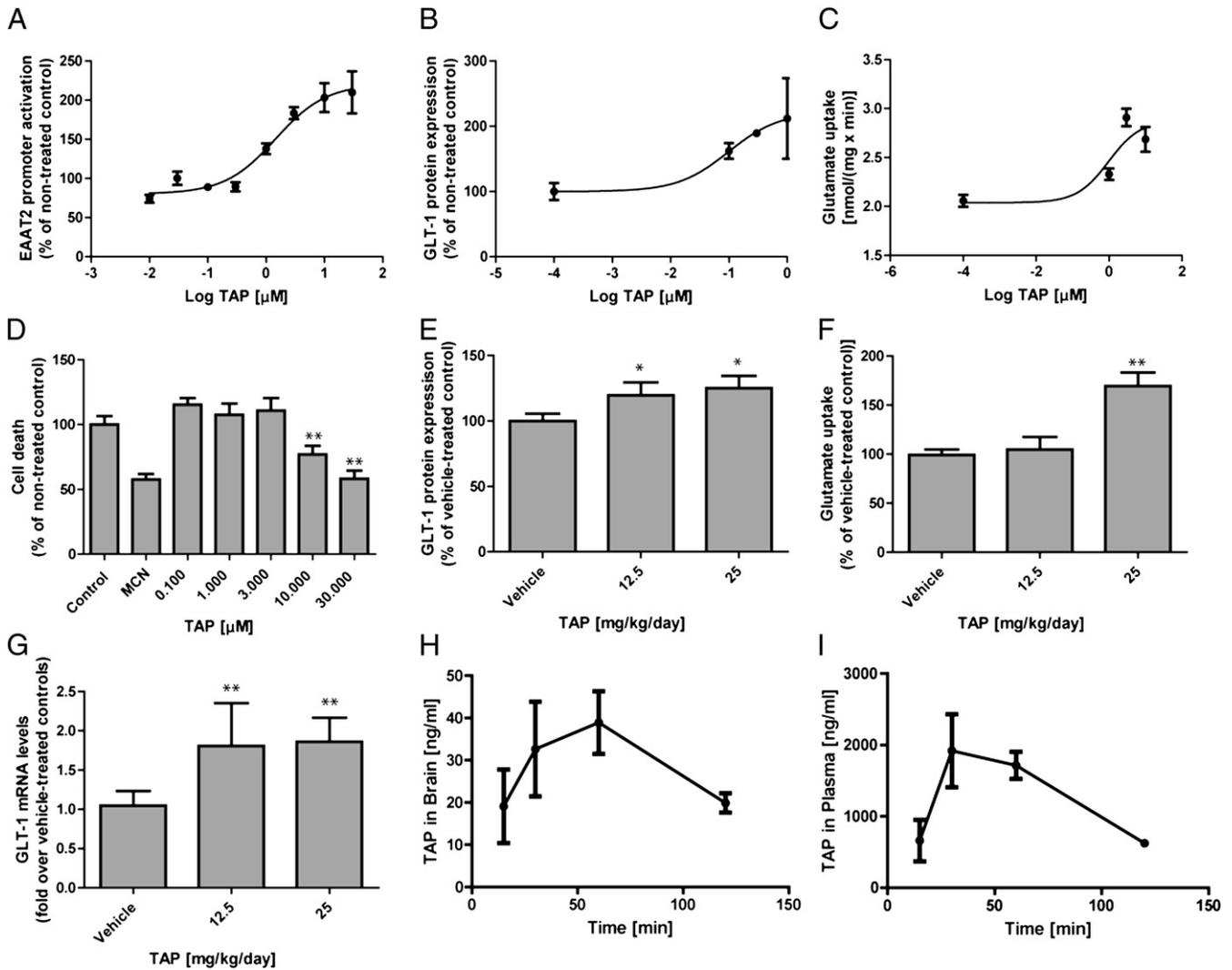


Fig. 2. Thiamphenicol (TAP) therapy increases EAAT2/GLT-1 gene expression and function in cultured mouse astrocytes and in mouse brain. A. TAP activates the EAAT2/GLT-1 promoter. Promoter activation was examined by measuring luciferase activity using a reporter gene construct driven by a 2.5 kB promoter fragment of EAAT2 (Li et al., 2011; Rothstein et al., 2005). Human immortalized astrocytes stably transfected with the reporter gene construct were treated for 72 h with various concentrations of TAP. B. TAP increases EAAT2/GLT-1 protein expression in primary mixed cortical neurons. Primary mouse co-cultures of neurons and astrocytes were treated with TAP for 72 h. A dose-dependent increase of EAAT2/GLT-1 protein was revealed by western blot analyses. C. TAP increases functional glutamate transport. Glutamate transport was measured in primary mouse co-cultures of neurons and astrocytes following 72 h of TAP treatment. TAP dose-dependently increased glutamate uptake in these cultures. D. Primary mouse co-cultures of neurons and astrocytes were treated with TAP for 72 h. Cells were then exposed to an oxygen-glucose-free environment for 1.5 h (Goldberg et al., 1988). After the oxygen-glucose deprivation, cells were washed back into normal ACSF solution and maintained for another 23 h at 37 °C, 5% CO₂. Neuronal cell death was determined by measuring LDH release. Cells treated with MK-801, CNQX and nimodipine (MCN) were used as a positive control for neuroprotective activity. TAP-dependent upregulation of EAAT2/GLT-1 resulted in dose-dependent neuroprotection in the OGD assay using the present *in vitro* culture model. E/F/G. TAP increases GLT-1 mRNA, protein and glutamate uptake *in vivo*. WT mice were treated for 10 days with 12.5 or 25 mg/kg/day TAP via oral delivery. TAP dose-dependently increased EAAT2/GLT-1 protein (as measured in brain cortex) **P<0.01 vs. control, ANOVA (E) and mRNA (F). Synaptosomal preparations obtained from brain cortex showed a TAP-dependent increase in functional glutamate transport (G). *P<0.05, **P<0.01 vs. vehicle control, Student t-test. H/I. To test for TAP brain penetration, we injected WT mice with 25 mg/kg TAP ip and collected plasma and brain tissue at different time points. Samples were analyzed for TAP using LC/MS/MS analysis.

TAP served as the best proof of principle compound for evaluating neural OE as a potential pharmacodynamic marker assay.

Thiamphenicol increases EAAT2/GLT-1 gene expression in rodent olfactory epithelial tissue

To determine if nasal OE tissue could serve as a pharmacodynamic marker for glutamate transporter EAAT2/GLT-1 upregulation as seen in CNS tissue, we treated WT mice (N=10) with TAP (Glycinate form of TAP) for 14 days at 25 mg/kg ip daily. These rodent experiments allowed us to directly test our hypothesis that peripheral tissue will respond similarly to CNS tissue with treatment of a BBB-penetrable, CNS-targeted drug. Therefore, lysates of cortex, spinal cord and OE of TAP-treated mice were analyzed for EAAT2/GLT-1 protein in comparison with saline-treated animals (N=10). Western

blot analysis confirmed that chronic TAP therapy in mice led to an increase in glutamate transporter protein EAAT2/GLT-1 throughout the CNS, including the cortex (Fig. 3B) and spinal cord (data not shown) and similar increases were found in nasal OE samples (Fig. 3A). In this representative blot we loaded for the nasal tissue both, olfactory neural epithelial tissue (labeled OE) as well as adjacent non-neural-epithelial mucosa tissue (labeled M), which should express little to no EAAT2/GLT-1. The quantification of all samples showed that peripheral OE tissue showed a 3 fold increase in EAAT2/GLT-1 expression over saline-treated animals, while cortex showed about 50% protein upregulation (Fig. 3C). Pharmacokinetic studies performed in mice and measuring TAP using LC/MS/MS analysis showed a peak brain level of TAP 1 h after ip injection at 39 ng/mL, with a corresponding plasma level of 1714 ng/mL (Figs. 2H and I). This may explain the higher drug efficacy in the peripheral tissue compared to

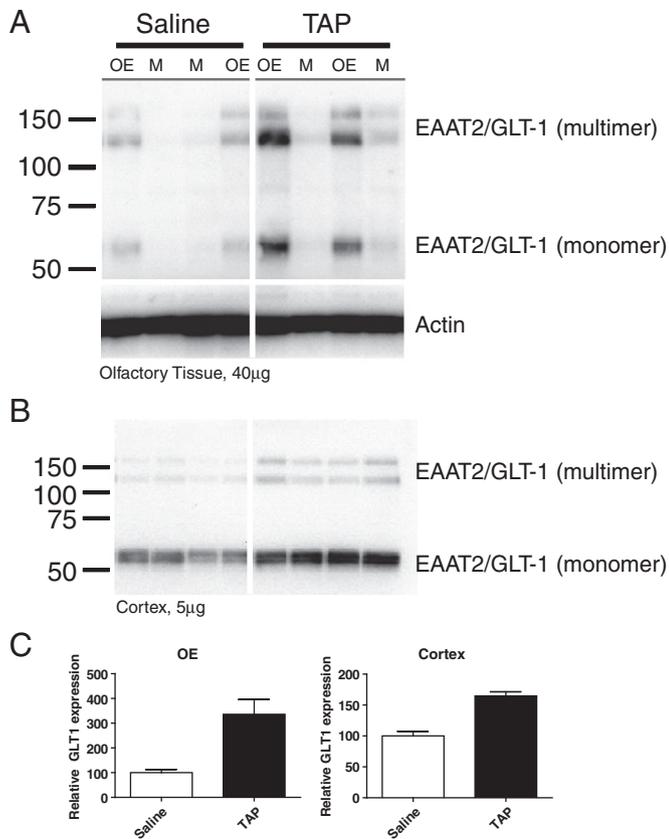


Fig. 3. TAP increases EAAT2/GLT-1 protein in mouse nasal olfactory epithelium and brain. WT mice were treated with TAP (N=10) or saline (N=10) for 10 days (25 mg/kg/day, ip). Cortex and olfactory epithelial tissue was isolated and probed for EAAT2/GLT-1 protein expression using standard Western Blot analysis. A. TAP increases EAAT2/GLT-1 protein levels in comparison to saline treated animals in olfactory epithelial tissue (OE) tissue, but not non-neural epithelial mucosa (M). B. This upregulation was paralleled with an upregulation of EAAT2/GLT-1 in tissue samples of cortex. Protein loading was normalized to levels of beta actin in each sample. C. Quantitative analysis of all samples showed a significant upregulation of EAAT2/GLT-1 in both, olfactory epithelial tissue and cortex samples (N=10 for each group, t-test p value ≤ 0.05).

the brain tissue. The brain tissue levels of TAP sufficient to achieve transporter upregulation are nevertheless much lower than what is needed to achieve similar upregulation in cultured astrocytes (compare to Figs. 2B and C), which suggests that *in vivo* CNS drug efficacy of TAP is much higher than *in vitro*.

In summary, these results demonstrate that olfactory epithelial tissue analysis of glutamate transporter levels could serve as a peripheral biomarker for astrocytic CNS protein expression. Based on this rodent assay, it may be possible to predict a human response to TAP therapy by comparing effective drug concentrations between mouse brain and human CSF samples.

Thiamphenicol increases EAAT2/GLT-1 gene expression in human olfactory epithelial tissue

To determine if a similar upregulation of EAAT2/GLT-1 does indeed occur in human nasal olfactory tissue, we analyzed nasal biopsy tissue samples from healthy volunteers, before and after a 14 day oral TAP treatment in a double blinded placebo controlled inpatient trial. We knew from the rodent studies and from historical data on TAP that the drug easily penetrates through the blood brain barrier and enters the brain (Azzollini et al., 1970; Ferrari, 1984; Pfenninger et al., 1977). The human study was therefore designed to obtain updated pharmacokinetic and safety data on TAP and to collect drug-induced pharmacodynamic data in the form of EAAT2/GLT-1

mRNA levels from nasal OE. (Note: the clinical trial data are summarized in the online supplemental documents). Because the material from human nasal biopsy is not sufficient for actual EAAT2/GLT-1 protein quantification we quantified EAAT2/GLT-1 mRNA levels from the biopsies. A potential limitation of this approach would be a lack of close correlation between EAAT2/GLT-1 protein and mRNA levels. However, prior studies have shown relatively close correlation between EAAT2/GLT-1 mRNA and protein (Danbolt, 2001; Rothstein et al., 2005).

Thirty normal volunteers were divided into three groups: placebo (N=6), TAP 750 mg once a day po (N=12) and TAP 1500 mg once a day po (N=12). Nasal biopsies were taken on day -1 (before drug treatment) and day 14. Plasma pharmacokinetics were monitored on day 1 and 13. Cerebral spinal cord fluid (CSF) samples were taken on day 13 to measure levels of TAP which allowed for an estimate on how much drug had entered the brain. In addition to EAAT2/GLT-1, mRNA levels of the following genes: GFAP (astrocytic expression in CNS), EAAT1 (astrocytic expression in CNS, expressed in neuronal precursor cells during early development in CNS, expressed in precursor neuronal cells in OE (Regan et al., 2007), OMP (olfactory marker protein; neuronal expression in OE and CNS) were measured. Validation of the measurements of mRNA levels in human OE samples obtained from normal volunteers in earlier studies revealed a low inter- and intra-patient variability (Supplement Fig. 1). The intra-patient variability was obtained by comparing mRNA levels obtained from the left and right side of the nose of each volunteer (Supplement Fig. 1). This was important since the drug study was designed to obtain a biopsy from one side of the nose before drug treatment and from the opposite side of the nose after drug treatment. This experimental design allowed each person to serve as their own control and to follow changes of mRNA levels within each person individually.

TAP was well tolerated at every dose and no serious adverse side effects were reported. The pharmacokinetic profile revealed dose-related plasma concentrations, no drug accumulation, and an average half-life of 4–6 h (see Supplementary Table 1). Furthermore, TAP dose-dependently penetrated into the brain at day 13 with CSF levels of 101.8 ± 23.1 ng/mL at 750 mg/day and 220.2 ± 123.5 ng/mL at 1500 mg/day (see also Fig. 4). These levels were about 8 fold higher than the *in vivo* concentrations found in TAP-treated WT mice which lead to a 20% increase in functional EAAT2/GLT-1 protein levels (Figs. 2E, F, H and I). In addition, there was a significant correlation between CSF drug levels and levels measured in plasma suggesting

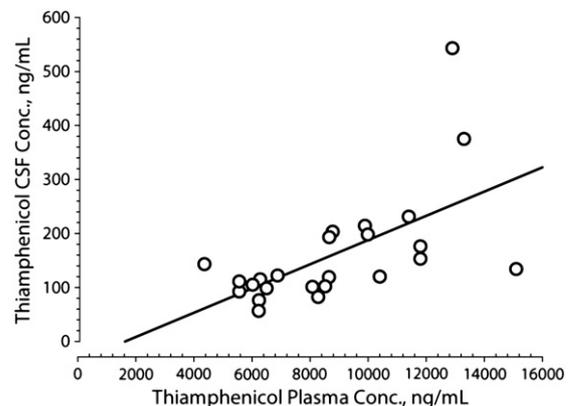


Fig. 4. Significant correlation between plasma and CSF TAP concentrations. Plasma and CSF levels of TAP in human volunteers were determined by LC/MS/MS at the end of the trial. Concentrations of TAP were measurable in all CSF samples collected 1.5 to 3 h post-dose on day 13. Mean \pm SD CSF concentrations ranged from 101.8 ± 23.1 ng/mL to 220.2 ± 123.5 ng/mL for the 750 and 1500 mg dosages, respectively. The CSF concentration measured 1.5 to 3 h post-dose was significantly (p value ≤ 0.05) correlated with the C_{max} in plasma with a coefficient of determination (r^2) of 0.811. The CSF/ C_{max} ratios were similar for both dosages: 1.6 ± 0.6 (750 mg) and $2.0 \pm 0.9\%$ (1500 mg).

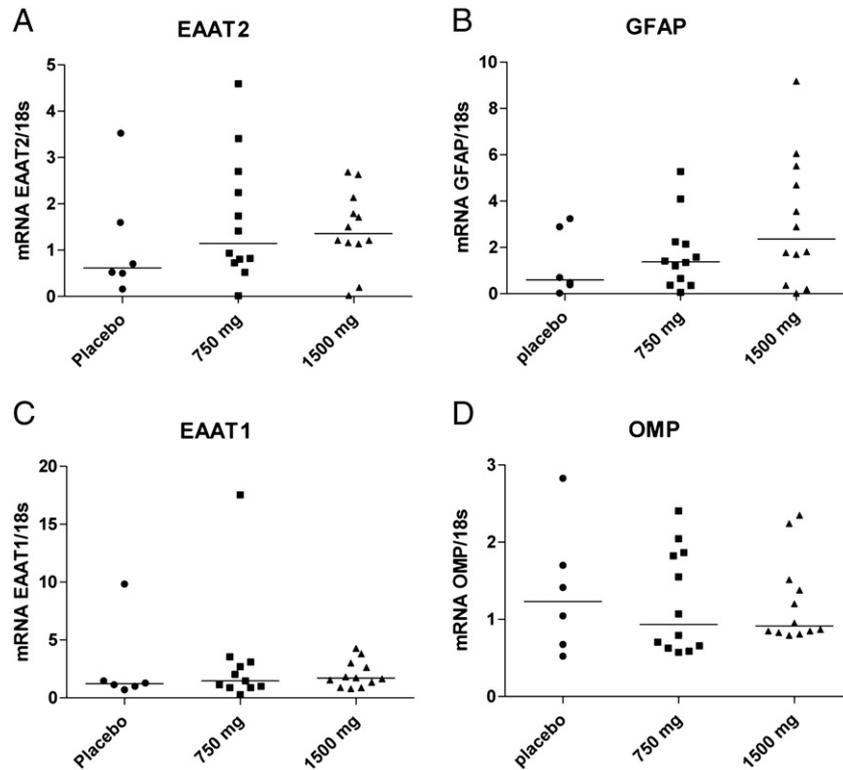


Fig. 5. TAP increases EAAT2/GLT-1 mRNA levels in human nasal olfactory epithelium after chronic oral treatment. Healthy volunteers were treated with TAP for 14 days (0 (N = 6); 750 mg (N = 12); 1500 mg (N = 12) once a day, oral delivery). Nasal biopsies were performed the day before the beginning of drug treatment (day – 1) and on day 14. mRNA levels of several genes were quantified by qPCR analysis. Data are presented as fold increases in mRNA levels after drug treatment compared to before drug treatment for each individual subject. A and B. Astrocytic mRNA EAAT2 and GFAP confirmed a positive trend towards increased median gene expression levels (red lines) while other CNS/olfactory genes EAAT1 (C) and OMP (D) showed no changes in expression after drug treatment. Statistical analysis using Kruskal Wallis nonparametric test and nonparametric test for trend showed no statistical significance for either gene.

that CNS tissue received proportional levels of drug in comparison to peripheral tissue (Fig. 4).

The tissue samples collected from the subjects before and after the drug treatment were analyzed in a blinded fashion and only after the final data analysis were the samples unblinded and blotted according to their individual groups. The data shown in Fig. 4 suggests a dose-dependent positive trend towards an increase in mRNA levels of both astrocytic genes, EAAT2/GLT-1 and GFAP (Figs. 5A and B). Specifically, median values for EAAT2/GLT-1: placebo = 0.62, 750 mg/kg = 1.14, 1500 mg/kg = 1.27; for GFAP: placebo = 0.71, 750 mg/kg = 1.38, 1500 mg/kg = 2.36. Non-astrocytic or neuronal genes OMP and EAAT1 showed no trend towards an increase in mRNA levels after drug treatment (Figs. 5C and D), suggesting a specificity of drug-induced increases in astrocytic gene expression. Median values for OMP: placebo = 1.23, 750 mg/kg = 0.93, 1500 mg/kg = 0.91; EAAT1: placebo = 1.22, 750 mg/kg = 2.05, 1500 mg/kg = 1.66. Analysis on these data using the Kruskal Wallis (KW) nonparametric test and the nonparametric test for trend (nptrend) did not show any statistical significance for either gene (EAAT2: p-value = 0.7086 (KW), 0.515 (nptrend); GFAP: p-value = 0.3039 (KW), 0.138 (nptrend); OMP: p-value = 0.846 (KW), 0.835 (nptrend); EAAT1: p-value = 0.69 (KW), 0.397 (nptrend)). However, the positive trend towards an increase in mRNA levels in EAAT2/GLT-1 confirmed the data obtained in our animal study and hence supports the use of nasal biopsy samples as a biomarker for astrocyte biology in drug discovery.

Discussion

These studies provide both pre-clinical and human validation of a new relatively simple approach to measuring biological activity of

CNS targeted pharmaceuticals in peripheral tissue samples. The development of CNS relevant pharmacodynamic and biomarker tissue tools is a serious unmet need in the development of CNS acting pharmaceuticals. Biomarkers provide the opportunity to relate long-term clinical outcome measures to immediate validated biochemical changes caused by drug therapy. A validated biomarker can help make early decisions on whether or not a new drug candidate shows biological activity instead of waiting for drug efficacy outcome measures that are substantially delayed, such as in human survival trials. These disease-specific markers can often also be used to monitor disease progression in addition to testing drug efficacy in clinical trials (Au and Roskams, 2002; Au and Roskams, 2003; Doke et al., 1999; Priest and Puche, 2004; Seal and Edwards, 2006; Thukral et al., 1997).

Here we report that human and rodent olfactory epithelial tissue contain astroglial-like EAAT2/GLT-1 expressing cells and that these cells accurately recapitulate CNS responses to an astrocyte-modifying drug in brain. We therefore chose to use these peripheral cells as a biomarker for pharmacodynamic measurements of drug efficacy of EAAT2/GLT-1 upregulating compounds. The rodent study nicely illustrated that drug effects seen in the CNS were also seen in nasal olfactory tissue samples, confirming our hypothesis that nasal OE can serve as a peripheral pharmacodynamic marker for CNS drug activity.

Based on the data obtained from the rodent pre-clinical study, we performed a human phase 1 study validating the use of the peripheral nasal OE tissue as a pharmacodynamic marker for drug activity of EAAT2/GLT-1 activating agents. We should emphasize that detecting biological activity in the human OE does not ultimately confirm an effect in the human CNS, especially considering that BBB penetration in rodents may not parallel exactly the penetration characteristics in humans. In other words, since OE can be reached by drugs without

having to cross the BBB it can show higher efficacy than what is seen in CNS tissue. Since we are not able to collect human CNS tissue, we cannot confirm whether the observed trend parallels the actual upregulation of EAAT2/GLT-1 in human brain tissue. However, drug levels measured in the CSF confirmed BBB penetration of thiamphenicol suggesting biological activity of thiamphenicol in CNS tissue, similar to what our pre-clinical studies of TAP in rodents demonstrated. More importantly, the nasal assay allows use of peripheral human tissue to evaluate actions of a drug towards a potential important therapeutic astroglial target—EAAT2/GLT-1.

Previous work comparing rodent to human astroglial EAAT2/GLT-1 revealed that the gene promoter is regulated very similarly between the two species (Lee et al., 2008; Rothstein et al., 2005; Su et al., 2003). But only brain imaging approaches such as the use of glutamate transporter PET ligands will allow us to ultimately confirm in the CNS the biological activity of transporter upregulating compounds seen in human nasal biopsy samples.

The present studies also provide new data on the expression pattern of astroglial glutamate transporter protein EAAT2/GLT-1 in nasal epithelial mucosa. EAAT2/GLT-1 was not expressed in olfactory ensheathing cells located in the lamina propria of the olfactory mucosa. Based on rodent studies, these cells had been suggested to be closely related to olfactory bulb ensheathing cells (Au and Roskams, 2003) and are often referred to as olfactory Schwann cells due to similarities in molecular and cellular properties (Barber and Lindsay, 1982). While we confirmed the presence of astrocytic protein GFAP in the ensheathing cells, we were able to detect EAAT2/GLT-1 only in the p75-positive basal cells located in the region near the lamina propria. Based on animal studies, basal cells are considered the neuroblast stem cells of the olfactory neuroepithelium (Graziadei and Monti Graziadei, 1985; Moulton, 1974). Recent studies suggest that basal cells are multipotent progenitor cells that could give rise to both, neuronal and non-neuronal cells (Beites et al., 2005; Iwai et al., 2008). It is therefore possible that these progenitor cells express EAAT2/GLT-1 as an early stage astrocyte marker protein.

Astroglial dysfunction was first described as a contributing event to ALS pathogenesis and neurodegenerative diseases with the discovery of excitatory neurotransmitter transporter defects (Howland et al., 2002; Rothstein et al., 1993; Rothstein et al., 1994; Rothstein et al., 1992). More recently, multiple laboratories have described a role for astroglia and EAAT2/GLT-1 in neurodegenerative disease, including ALS, HD and spinocerebellar ataxia (Custer et al., 2006; Shin et al., 2005; Yamanaka et al., 2008). Both *in vitro* and *in vivo* model systems suggest that either loss of function (e.g. EAAT2/GLT-1-mediated glutamate transport) and the gain of a toxic event (e.g. secretion of toxins) act as propagators of neural degeneration (Di Giorgio et al., 2007). Furthermore, astroglia have recently been the target of psychiatric disease therapies, EAAT2/GLT-1 in depression and D-amino acid oxidase in schizophrenia (Sanacora et al., 2008). The development of a biomarker for astroglia therapeutic targets may thus be an important tool for more efficient drug discovery in many neurological and/or psychiatric disorders.

In summary, our study confirms the use of nasal epithelial biopsy tissue samples as a simple and safe pharmacodynamic marker for monitoring biological activity of astroglial EAAT2/GLT-1 targeted drug therapy. Equally important, this tissue sampling approach could also be useful for monitoring biological activity of drugs targeting CNS neurons.

Supplementary materials related to this article can be found online at doi:10.1016/j.expneurol.2011.09.002.

Conflict of interest

J.D.R. and R.C. have an equity interest in Psyadon Pharmaceuticals; J.D.R. and R.C. are on the Psyadon Board of Directors. D. B. has equity interest and serves on the Board of Directors of Gliken Pharma. R.S.

and J.D.R. are on a pending patent for the use of thiamphenicol in various neurological and psychiatric disorders. No other authors have competing interests to declare.

Author contributions

A.Y. and L.C. performed animal and human qPCR; A.Y. performed all immunohistochemistry; L.C. performed immunoblotting assays; R.S., D.B., R.C., J.D.R. designed rodent and human experiments; A.S. developed biopsy method; A.S., D.B. and R.C. edited the manuscript; R.S. and J.D.R. wrote the manuscript.

Acknowledgment

The authors thank Drs. Jeffrey Wolf and Rodney Taylor, University of Maryland and Dr. Sandra Lin, Johns Hopkins University for performing the surgical biopsies; Dr. Yongjie Yang, Johns Hopkins University for assistance in quantitative PCR data analysis; Rebecca Michaud and Jackie Gutenkunst for technical assistance, Drs. Randy Reed, Johns Hopkins University and Frank Margolis, University of Maryland with isolation and analysis of rodent nasal tissue. This work was supported by NIH NS33958, Ruxton Pharmaceuticals, Robert Packard Center for ALS Research at Johns Hopkins, and the Brain Science Institute at Johns Hopkins.

References

- Au, E., Roskams, A.J., 2002. Culturing olfactory ensheathing glia from the mouse olfactory epithelium. *Methods Mol. Biol.* 198, 49–54.
- Au, E., Roskams, A.J., 2003. Olfactory ensheathing cells of the lamina propria *in vivo* and *in vitro*. *Glia* 41, 224–236.
- Azzollini, F., Gazzaniga, A., Lodola, E., 1970. Thiamphenicol excretion in subjects with renal insufficiency. *Int. Z. Klin. Pharmakol. Ther. Toxikol.* 4, 303–308.
- Banasr, M., Chowdhury, G.M., Terwilliger, R., Newton, S.S., Duman, R.S., Behar, K.L., Sanacora, G., 2010. Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. *Mol. Psychiatry* 15 (5), 501–511.
- Barber, P.C., Lindsay, R.M., 1982. Schwann cells of the olfactory nerves contain glial fibrillary acidic protein and resemble astrocytes. *Neuroscience* 7, 3077–3090.
- Beites, C.L., Kawauchi, S., Crocker, C.E., Calof, A.L., 2005. Identification and molecular regulation of neural stem cells in the olfactory epithelium. *Exp. Cell Res.* 306, 309–316.
- Berger, U.V., Hediger, M.A., 2006. Distribution of the glutamate transporters GLT-1 (SLC1A2) and GLAST (SLC1A3) in peripheral organs. *Anat. Embryol. (Berl.)* 211, 595–606.
- Breipohl, W., Laugwitz, H.J., Bornfeld, N., 1974. Topological relations between the dendrites of olfactory sensory cells and sustentacular cells in different vertebrates. An ultrastructural study. *J. Anat.* 117, 89–94.
- Collins, F.S., 2011. Reengineering translational science: the time is right. *Sci. Transl. Med.* 3 90cm17.
- Custer, S.K., Garden, G.A., Gill, N., Rueb, U., Libby, R.T., Schultz, C., Guyenet, S.J., Deller, T., Westrum, L.E., Sopher, B.L., La Spada, A.R., 2006. Bergmann glia expression of polyglutamine-expanded ataxin-7 produces neurodegeneration by impairing glutamate transport. *Nat. Neurosci.* 9, 1302–1311.
- Dambolt, N.C., 2001. Glutamate uptake. *Prog. Neurobiol.* 65, 1–105.
- Di Giorgio, F.P., Carrasco, M.A., Siao, M.C., Maniatis, T., Eggen, K., 2007. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat. Neurosci.* 10, 608–614.
- Doke, T., Hayashi, T., Kikuchi, J., Nagaoka, S., Nakano, T., Sakaguchi, T., Terasawa, K., Badhwar, G.D., 1999. Application of real-time radiation dosimetry using a new silicon LET sensor. *Mutat. Res.* 430, 191–202.
- Edwards, R., 2009. What the neuron tells glia. *Neuron* 61, 811–812.
- Feron, F., Perry, C., Hirning, M.H., McGrath, J., Kay-Sim, A., 1999. Altered adhesion, proliferation and death in neural cultures from adults with schizophrenia. *Schizophr. Res.* 40, 211–218.
- Ferrari, V., 1984. Salient features of thiamphenicol: review of clinical pharmacokinetics and toxicity. *Sex. Transm. Dis.* 11, 336–339.
- Goldberg, M.P., Monyer, H., Weiss, J.H., Choi, D.W., 1988. Adenosine reduces cortical neuronal injury induced by oxygen or glucose deprivation *in vitro*. *Neurosci. Lett.* 89, 323–327.
- Gomez, G., Rawson, N.E., Hahn, C.G., Michaels, R., Restrepo, D., 2000. Characteristics of odorant elicited calcium changes in cultured human olfactory neurons. *J. Neurosci. Res.* 62, 737–749.
- Graziadei, P.P., 1973. Cell dynamics in the olfactory mucosa. *Tissue Cell* 5, 113–131.
- Graziadei, P.P., Monti Graziadei, G.A., 1985. Neurogenesis and plasticity of the olfactory sensory neurons. *Ann. N. Y. Acad. Sci.* 457, 127–142.
- Hahn, C.G., Gomez, G., Restrepo, D., Friedman, E., Josiassen, R., Pribitkin, E.A., Lowry, L.D., Gallop, R.J., Rawson, N.E., 2005. Aberrant intracellular calcium signaling in olfactory neurons from patients with bipolar disorder. *Am. J. Psychiatry* 162, 616–618.

- Holbrook, E.H., Leopold, D.A., Schwob, J.E., 2005. Abnormalities of axon growth in human olfactory mucosa. *Laryngoscope* 115, 2144–2154.
- Hota, S.K., Barhwal, K., Ray, K., Singh, S.B., Ilavazhagan, G., 2008. Ceftriaxone rescues hippocampal neurons from excitotoxicity and enhances memory retrieval in chronic hypobaric hypoxia. *Neurobiol. Learn. Mem.* 89, 522–532.
- Howland, D.S., Liu, J., She, Y., Goad, B., Maragakis, N.J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., DeGennaro, L.J., Cleveland, D.W., Rothstein, J.D., 2002. 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.* 99, 1604–1609.
- Iwai, N., Zhou, Z., Roop, D.R., Behringer, R.R., 2008. Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. *Stem Cells* 26, 1298–1306.
- Jafek, B.W., Murrow, B., Michaels, R., Restrepo, D., Linschoten, M., 2002. Biopsies of human olfactory epithelium. *Chem. Senses* 27, 623–628.
- Kleinjan, A., Willart, M., van Rijt, L.S., Braunstahl, G.J., Leman, K., Jung, S., Hoogsteden, H.C., Lambrecht, B.N., 2006. An essential role for dendritic cells in human and experimental allergic rhinitis. *J. Allergy Clin. Immunol.* 118, 1117–1125.
- Lanza, D.C., Deems, D.A., Doty, R.L., Moran, D., Crawford, D., Rowley III, J.C., Sajjadian, A., Kennedy, D.W., 1994. The effect of human olfactory biopsy on olfaction: a preliminary report. *Laryngoscope* 104, 837–840.
- Lee, S.G., Su, Z.Z., Emdad, L., Gupta, P., Sarkar, D., Borjabad, A., Volsky, D.J., Fisher, P.B., 2008. Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. *J. Biol. Chem.* 283, 13116–13123.
- Li, Y., Sattler, R., Yang, E.J., Nunes, A., Ayukawa, Y., Akhtar, S., Ji, G., Zhang, P.W., Rothstein, J.D., 2011. Harmine, a natural beta-carboline alkaloid, upregulates astroglial glutamate transporter expression. *Neuropharmacology* 60 (7–8), 1168–1175.
- Lipski, J., Wan, C.K., Bai, J.Z., Pi, R., Li, D., Donnelly, D., 2007. Neuroprotective potential of ceftriaxone in *in vitro* models of stroke. *Neuroscience* 146, 617–629.
- Lobsiger, C.S., Cleveland, D.W., 2007. Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. *Nat. Neurosci.* 10, 1355–1360.
- Lovell, M.A., Jafek, B.W., Moran, D.T., Rowley III, J.C., 1982. Biopsy of human olfactory mucosa. An instrument and a technique. *Arch. Otolaryngol.* 108, 247–249.
- McCurdy, R.D., Feron, F., Perry, C., Chant, D.C., McLean, D., Matigian, N., Hayward, N.K., McGrath, J.J., Kay-Sim, A., 2006. Cell cycle alterations in biopsied olfactory neuroepithelium in schizophrenia and bipolar I disorder using cell culture and gene expression analyses. *Schizophr. Res.* 82, 163–173.
- Miller, B.R., Dorner, J.L., Shou, M., Sari, Y., Barton, S.J., Sengelaub, D.R., Kennedy, R.T., Rebec, G.V., 2008. Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. *Neuroscience* 153, 329–337.
- Mineur, Y.S., Picciotto, M.R., Sanacora, G., 2007. Antidepressant-like effects of ceftriaxone in male C57BL/6J mice. *Biol. Psychiatry* 61, 250–252.
- Morrison, E.E., Costanzo, R.M., 1992. Morphology of olfactory epithelium in humans and other vertebrates. *Microsc. Res. Tech.* 23, 49–61.
- Moulton, D.G., 1974. Dynamics of cell populations in the olfactory epithelium. *Ann. N. Y. Acad. Sci.* 237, 52–61.
- Okano, M., Takagi, S.F., 1974. Secretion and electrogenesis of the supporting cell in the olfactory epithelium. *J. Physiol.* 242, 353–370.
- Pfenninger, J., Furrer, H., Furst, M., Vogt, J., Widmer, H., 1977. Thiamphenicol in treatment of *Haemophilus influenzae* meningitis. *Helv. Paediatr. Acta* 32, 207–216.
- Priest, C.A., Puche, A.C., 2004. GABAB receptor expression and function in olfactory receptor neuron axon growth. *J. Neurobiol.* 60, 154–165.
- Rawson, N.E., Gomez, G., Cowart, B., Brand, J.G., Lowry, L.D., Pribitkin, E.A., Restrepo, D., 1997. Selectivity and response characteristics of human olfactory neurons. *J. Neurophysiol.* 77, 1606–1613.
- Regan, M.R., Huang, Y.H., Kim, Y.S., Dykes-Hoberg, M.I., Jin, L., Watkins, A.M., Bergles, D.E., Rothstein, J.D., 2007. Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. *J. Neurosci.* 27, 6607–6619.
- Ronnett, G.V., Leopold, D., Cai, X., Hoffbuhr, K.C., Moses, L., Hoffman, E.P., Naidu, S., 2003. Olfactory biopsies demonstrate a defect in neuronal development in Rett's syndrome. *Ann. Neurol.* 54, 206–218.
- Rothstein, J.D., Martin, L.J., Kuncl, R.W., 1992. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. *N. Engl. J. Med.* 326, 1464–1468.
- Rothstein, J.D., Jin, L., Dykes-Hoberg, M., Kuncl, R.W., 1993. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6591–6595.
- Rothstein, J.D., Martin, L., Levey, A.I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., Kuncl, R.W., 1994. Localization of neuronal and glial glutamate transporters. *Neuron* 13, 713–725.
- Rothstein, J.D., Van Kammen, M., Levey, A.I., Martin, L.J., Kuncl, R.W., 1995. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann. Neurol.* 38, 73–84.
- Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16, 675–686.
- Rothstein, J.D., Patel, S., Regan, M.R., Haenggeli, C., Huang, Y.H., Bergles, D.E., Jin, L., Dykes-Hoberg, M., Vidensky, S., Chung, D.S., Toan, S.V., Bruijn, L.I., Su, Z.Z., Gupta, P., Fisher, P.B., 2005. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* 433, 73–77.
- Sanacora, G., Zarate, C.A., Krystal, J.H., Manji, H.K., 2008. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nat. Rev. Drug Discov.* 7, 426–437.
- Seal, R.P., Edwards, R.H., 2006. The diverse roles of vesicular glutamate transporter 3. *Handb. Exp. Pharmacol.* 137–150.
- Shin, J.Y., Fang, Z.H., Yu, Z.X., Wang, C.E., Li, S.H., Li, X.J., 2005. Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. *J. Cell Biol.* 171, 1001–1012.
- Su, Z.Z., Leszczyniecka, M., Kang, D.C., Sarkar, D., Chao, W., Volsky, D.J., Fisher, P.B., 2003. Insights into glutamate transport regulation in human astrocytes: cloning of the promoter for excitatory amino acid transporter 2 (EAAT2). *Proc. Natl. Acad. Sci. U. S. A.* 100, 1955–1960.
- Thone-Reineke, C., Neumann, C., Namsolleck, P., Schmerbach, K., Krikov, M., Schefe, J.H., Lucht, K., Hortnagl, H., Godes, M., Muller, S., Rumschüssel, K., Funke-Kaiser, H., Villringer, A., Steckelings, U.M., Unger, T., 2008. The beta-lactam antibiotic, ceftriaxone, dramatically improves survival, increases glutamate uptake and induces neurotrophins in stroke. *J. Hypertens.* 26, 2426–2435.
- Thukral, V., Chikaraishi, D., Hunter, D.D., Wang, J.K., 1997. Expression of non-N-methyl-D-aspartate glutamate receptor subunits in the olfactory epithelium. *Neuroscience* 79, 411–424.
- Voglmaier, S.M., Edwards, R.H., 2007. Do different endocytic pathways make different synaptic vesicles? *Curr. Opin. Neurobiol.* 17, 374–380.
- Voglmaier, S.M., Kam, K., Yang, H., Fortin, D.L., Hua, Z., Nicoll, R.A., Edwards, R.H., 2006. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. *Neuron* 51, 71–84.
- Weller, M.L., Stone, I.M., Goss, A., Rau, T., Rova, C., Poulsen, D.J., 2008. Selective overexpression of excitatory amino acid transporter 2 (EAAT2) in astrocytes enhances neuroprotection from moderate but not severe hypoxia-ischemia. *Neuroscience* 155, 1204–1211.
- Werner, P., Pitt, D., Raine, C.S., 2001. Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann. Neurol.* 50, 169–180.
- Yamanaka, K., Chun, S.J., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D.H., Takahashi, R., Misawa, H., Cleveland, D.W., 2008. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat. Neurosci.* 11, 251–253.
- 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, M.B., Rothstein, J.D., 2009. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. *Neuron* 61, 880–894.