Shannon Mythen, TAAR1-dependent astrocyte dysregulation during HAND and METH exposure**,** Master of Science (Biomedical Sciences), September, 2018, 63 pp., 2 tables, 8 illustrations, bibliography, 67 titles.

Abstract:

Excitatory amino-acid transporter (EAAT)-2 is predominantly expressed in astrocytes and clears glutamate from tripartite synapses preventing excitotoxicity. EAAT-2 dysregulation occurs during human immunodeficiency virus (HIV)-associated neuroinflammation and methamphetamine (METH) abuse, leading to neurotoxic outcomes. Trace amine associated receptor (TAAR) 1, a METH receptor in astrocytes, triggers EAAT-2 dysfunction. Protein kinase C (PKC) signaling promotes ubiquitination of EAAT-2 C-terminal lysine residues, resulting in EAAT-2 internalization. As a G protein coupled receptor, TAAR1's signaling is implicated in PKC activation. In this work, we investigated the role of TAAR1 in PKC-mediated EAAT-2 ubiquitination during HIV-associated neurocognitive disorders (HAND) and METH comorbidities. We evaluated a TAAR1 overexpression model in primary astrocytes to elucidate TAAR1-mediated functional changes. We found that TAAR1-selective inhibitor, EPPTB, reduced EAAT-2 ubiquitination, and a PKC activator decreased glutamate clearance in METH-pretreated human astrocytes. Therapies targeting astrocyte dysfunction may improve outcomes during HAND, METH abuse and other neuroinflammatory disorders.

TAAR1-DEPENDENT ASTROCYTE DYSREGULATION DURING HAND AND METH EXPOSURE

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TAAR1-DEPENDENT ASTROCYTE DYSREGULATION DURING HAND AND METH EXPOSURE

THESIS

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Abbreviations

ART	Antiretroviral therapy
ASM	Astrocyte synthetic medium?
BIS II	Bisindolylmaleimide II, PKC inhibitor
BBB	Blood-brain barrier
β-PEA	Beta phenylethylamine
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CPE	CNS penetration effectiveness
DAG	Diacylglycerol
DAT	Dopamine transporter
DAPI	4', 6-diamidino-2-phenylindole
EAAT-2	Excitatory amino acid transporter 2
EPPTB	N-(3-Ethoxyphenyl)-4-(1-pyrrolidinyl)-3-(trifluoromethyl)benzamide, Selective antagonist for TAAR1
FBS	Fetal bovine serum
$G\alpha_s$	G-protein α stimulating- GPCR subunit
$G\alpha_q$	G-protein α q-GPCR subunit
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Go	Go6976, PKC inhibitor

GPCR	Guanine protein coupled receptor
HA	Hemagglutinin
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
ICC	Immunocytochemistry
IL	Interleukin
IP	Immunoprecipitation
METH	Methamphetamine
MPER	Mammalian protein extraction reagent
mRNA	Messenger ribonucleic acid
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NEDD4-2	Neural precursor cell expressed developmentally down-regulated protein 4-2
PBS	Phosphate buffered saline
PK	Protein kinase
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
RT-PCR	Real time-polymerase chain reaction
σ-1R	Sigma receptor 1
TAAR1	Trace amine associated receptor 1
UTR	Untranslated region
WES	Simple western

WB	Western blot

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CHAPTER I

INTRODUCTION

Human immunodeficiency virus (HIV) and methamphetamine (METH) comorbidities altered excitatory amino acid transporter-2 (EAAT-2) expression and function. Trace amine associated receptor 1 (TAAR1), a novel astrocyte receptor for METH down regulates EAAT-2 transcription and alters funciton.[1-3] Protein kinase C (PKC) is also known to post-translationally modify EAAT-2 through ubiquitination, thus altering receptor function.[4-7] As a G protein coupled receptor (GPCR), TAAR1 may induce calcium (Ca^{2+}) and diacylglycerol (DAG) signaling, thus, activating PKC.[8,9] We propose that TAAR1 induction of PKC activation may also regulate post-translational modifications of EAAT-2 in primary human astrocytes during METH and HIV central nervous system (CNS) disease.

1.1 HIV/HAND:

According to the Joint United Nations Programme on HIV/AIDS, an estimated 36.7 million people are currently living with HIV globally, with 1.1 million residing in the U.S. In 2016, about 1.8 million new HIV infections were reported worldwide.[10] Approximately 59% of adults and 52% of children living with HIV have access to antiretroviral therapy (ART).[11] With ART, the prevalence of the most severe neurocognitive disorder, HIV-associated dementia (HAD), has decreased but not been eliminated. Less aggressive forms of impairment are on the rise, which can afflict up to 70% of the HIV population.[12,13] Suppression of HIV in the brain has proven difficult due to poor CNS penetration of ART. Latent reservoirs are a barrier for HIV cure and their presence can explain very low levels of viremia in patients undergoing ART.[14] Lack of ART medication in the CNS has also been linked to the presence of HIV RNA in cerebrospinal fluid.[15] Considering the difficulty of viral eradication in the CNS and the large number of people affected by HIV-associated

neurocognitive disorders (HAND), expanding therapeutic treatments to reduce or prevent HAND development must continue.

1.2 METH:

METH is a highly addictive substance that causes the release of dopamine into the synaptic cleft by reversing the dopamine transporter (DAT) on neurons.[16,17] In 2012, 1.2 million people reported using METH in the previous year, with 133,000 new users age 12 or older.[12] METH can be smoked, snorted, injected or eaten, and long term use of impure, poor-quality METH can lead to weight loss, tooth loss and decay and skin sores.[16,18] As a water and lipid soluble substance, METH easily crosses the blood-brain barrier (BBB) within minutes of administration, causing powerful euphoric effects.[18,19] A study conducted by Volkow et al. (2001) measured METH brain to serum ratios in rats injected with 1 mg/kg dose, a concentration comparable to that observed following human consumption. METH levels peaked at a 13:1 ratio (6 μM concentration) within 20 minutes, and remained elevated at an 8:1 ratio between 2–6 hours.[20] Rapid METH administration (i.e., intravenous or smoking) along with METH tolerance and dosage needed to achieve euphoric effects can influence METH stimulation. Binge consumption and repeated use can also impact METHs lasting presence in the CNS.[21] Long-term METH use has been linked to decreased DAT and increased METH tolerance, ultimately leading to CNS abnormalities including deficits in memory, executive function, anxiety and depression.[18-20]

1.3 HIV & METH:

The National Institute on Drug Abuse reported that 25% of HIV positive individuals seek treatment for drug and alcohol abuse in 2012.[12] METH use is associated with risky

sexual behavior and lowered inhibitions that increase the likelihood for acquiring HIV infection.[22,23] METH abuse can influence HIV-1 pathogenesis by accelerating the emergence of HAND.[22] Previous reports have also linked METH abuse to decreased ART adherence and immune dysregulation.[22-24] Together, METH and HIV can increase BBB permeability and neuroinflammation.[22] Ultimately, METH abuse increases oxidative stress and excitoxicity in astrocytes, promoting a neurotoxic environment.[2,3,25]

1.4 Astrogliosis:

Astrocytes, the prominent glial cells in the CNS, perform a variety of functions to maintain a homeostatic environment. They play a large role in neuroprotection by providing metabolic support to neurons, regulating the integrity of the BBB, and aiding in transmitter uptake and release. They also play a role in wound healing with glial scar formation.[26] Astrocyte secretion of factors such as tissue inhibitor of metalloproteinases, anti-oxidants and neurotrophins promote neuronal protection.[25,27] During chronic neuroinflammation, astrocytes can adopt a reactive phenotype termed “astrogliosis” and forego their neuroprotective role. Astrogliosis comprises reactive changes in glial cells in response to a broad range of insults including tissue damage, disease or infection. This can be characterized by upregulation in astrocyte specific glial fibrillary acidic protein (GFAP) expression, morphological and proliferative changes, and increased secretion of inflammatory mediators.[2,28] Prolonged environmental insults can lead to decreased metabolic support, increased excitotoxicity and oxidative stress.[2,29,30] HIV can invade the CNS early during infection through infiltrating monocytes that cross the BBB and infect resident microglia. In astrocytes, expression of and exposure to virus, viral proteins and HIV-1-relevant cytokines, induce functional changes that ultimately influence neuronal

survival and function.[14,28,31] Chronic CNS HIV-1 infection perpetuates neuroinflammation in HAND. Reactive astrogliosis is common to both HIV-1 infection and METH abuse.[22,24] METH exacerbates HIV dysregulation of astrocyte glutamate clearance and induces oxidative stress, thus contributing to neurotoxicity.[25] HAND-relative stimuli, HIV and interleukin-1β (IL-1β), coupled with METH exposure alter astrocyte morphology.[1-3] As part of the tripartite synapse (pre-synaptic and post-synaptic neurons and astrocytes), dysregulation of astrocyte function is detrimental to neuronal health and function.[28]

1.5 TAAR1:

TAAR1 is a GPCR functionally expressed in astrocytes,[1,2] with G protein α-stimulating ($G\alpha_s$) and G protein α-q ($G\alpha_q$) subunit activity.[8] TAAR1 is an intron-less gene with seven transmembrane domains. TAAR1 contains minimal N-terminal glycosylation sites that typically facilitate plasma membrane expression in GPCRs.[32] TAAR1 binds biological trace amines, such as β-phenylethylamine.[1] METH is a TAAR1 agonist, and has a similar structure to most trace amines.[33] During METH exposure and TAAR1 activation, dopamine levels in the synapse are transiently increased. METH also directly reverses neuronal DAT activity from reuptake of dopamine to secretion of dopamine, yielding euphoric effects.[34,35]

To investigate astrocyte activation and dysregulation, our previous studies have utilized two different treatment paradigms for METH and HIV-relevant stimuli. The acute, high dose paradigm consisted of METH (500 μM), HIV_{ADA} (p24, 10 ng/ml), and IL-1β (20 ng/ml) treatment for 24 hours.[1,3] The chronic exposure consisted of 7-day METH pretreatment (50 nM), followed by low level HIV-associated activation, HIV_{ADA} (1000 reverse transcriptase activity/ml) and IL-1β (0.2 ng/ml) for 24 hours, mimicking low-dose

longer term effects.[2,25] Utilizing both METH treatment paradigms, TAAR1 expression increased with METH and HAND-relevant stimuli. METH-induced activation of TAAR1 signaling increased cAMP levels, but decreased EAAT-2 expression and glutamate clearance abilities.[1,2] Selective inhibition of METH-induced TAAR1 activation using the reverse agonist EPPTB, N-(3-Ethoxyphenyl) 4-(1-pyrrolidinyl)-3-(trifluoromethyl)benzamide), significantly reduced cyclic adenosine monophosphate (cAMP) levels and restored glutamate clearance abilities to that of control cells.[1,30] TAAR1 may also induce Ca^{2+} flux, by activating phospholipase C (PLC).[2,8] Knockdown of TAAR1 using specific siRNA improved glutamate clearance abilities in control and METH treated astrocytes. TAAR1 knockdown also reduced METH-mediated cAMP signaling, indicating TAAR1 serves as a receptor for METH in primary human astrocytes.[1] Taken together, astrocyte TAAR1 may serve as a potential therapeutic target to improve astrocyte function during HIV-associated neuroinflammation and METH exposure.

1.6 EAAT-2 regulation:

Excitatory amino acid transporter-2 (EAAT-2) is a membrane-bound protein responsible for clearing glutamate from the extracellular environment in the brain. Of the EAAT family, EAAT-2 is the most abundantly expressed in the CNS, and is primarily expressed by astrocytes. EAAT-2 is responsible for over 90% of glutamate reuptake in the CNS.[36,37] Clearance of excess glutamate is essential to prevent neuronal excitoxicity and damage. EAAT-2 expression and function is highly regulated, and downregulation of this protein in animals caused susceptibility to excitotoxic insults and lethal seizures.[38] Dysregulation of EAAT-2 occurs with multiple neuroinflammatory diseases such as

Alzheimer's,[39] amyotrophic lateral sclerosis,[40] HIV,[1,2,30] stroke,[41] and traumatic brain injury.[42]

During HIV infection, neuroinflammation and METH exposure, astrocyte EAAT-2 regulation is multifaceted. Both high and low dose METH exposure decreased EAAT-2 mRNA and protein levels, producing functional outcomes like reduced glutamate clearance.[2,3] METH coupled with HAND-relevant stimuli also dysregulated EAAT-2 in astrocytes. Chronic METH combined with HIV and IL-1β treatment decreased EAAT-2 mRNA expression and impaired glutamate clearance abilities in astrocytes. Blocking TAAR1 restored glutamate clearance abilities in METH pretreated cells.[2] As glutamate clearance is a critical function for overall CNS and neuronal health, therapies targeting EAAT-2 regulation could be used to combat neurocognitive decline during HIV infection, neuroinflammation and METH abuse.

1.7 PKC:

The PKC family consists of 10 isozymes involved in altering the function of target proteins through phosphorylation of hydroxyl groups of serine and threonine amino acid residues.[43] PKC family members can be divided into three groups based on activation. Classical PKC enzymes (PKCα, PKCβ, and PKCγ) are activated by Ca^{2+} and DAG binding. Novel PKC enzymes (PKCδ, PKCε, PKCη, and PKCθ) also require DAG binding but do not require Ca^{2+} for activation. Atypical enzymes (PKCζ and PKCι/λ) require no second messenger binding for enzyme activation; they are instead allosterically activated by partitioning defective 6-CDC42 complex binding to the Phox/Bem1 domain. [43,44] Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), have activity similar to DAG and cause long lasting activation of PKC. Bisindolylmaleimide II (BIS II) acts as a

general PKC inhibitor for all subtypes, while Go6976 (Go) inhibits the classical, PKC isoforms α and β1.[4,6,44] Previous studies in C6 glioma and fibroblast-like COS-7 cell lines, implicate PKC signaling in EAAT-2 post-translational modification by promoting ubiquitination of C-terminal lysine residues. This results in the internalization and degradation of the glutamate transporter; however, EAAT-2 degradation mechanisms are a source of debate.[4-7] The role of PKC in the regulation of astrocyte EAAT-2 is not well understood during HIV-infection, neuroinflammation and METH comorbidities. As a GPCR, TAAR1 can signal through $G\alpha_q$ subunit, to activate PLC and DAG, resulting in increased cytosolic Ca^{2+}.[8,9,45] Thus, we propose astrocyte TAAR1 may regulate PKC-dependent EAAT-2 post-translational modifications and function. Through GPCR activity, METH induction of TAAR1 may activate PKC signaling, leading to the ubiquitination and subsequent internalization and degradation of EAAT-2 from the plasma membrane of astrocytes **(Fig. 1.1)**. Thus, contributing to decreased astrocyte glutamate clearance and excitoxicity of neurons.

Fig. 1.1

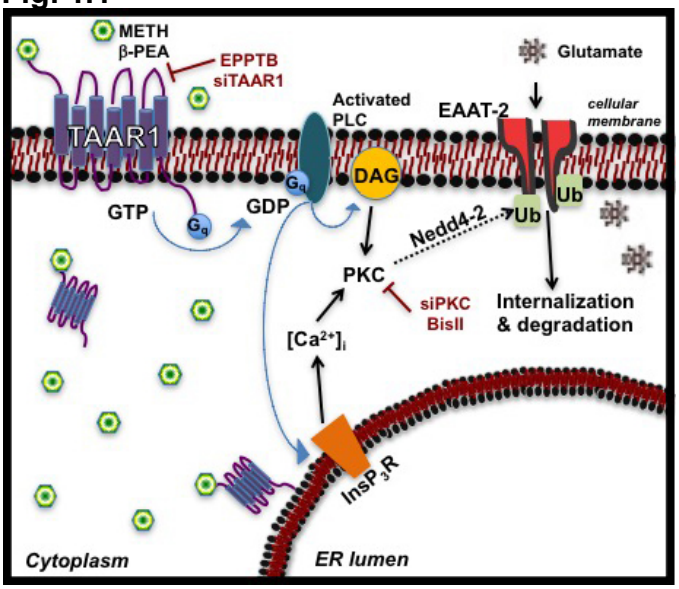


Fig. 1.1- TAAR1-mediated PKC-induced EAAT-2 ubiquitination: Depicted here is the proposed mechanism of TAAR1 involvement in PKC-mediated post-translational modification of EAAT-2 on the plasma membrane of primary human astrocytes.

1.8 Hypothesis & Objectives:

Fig. 1.2

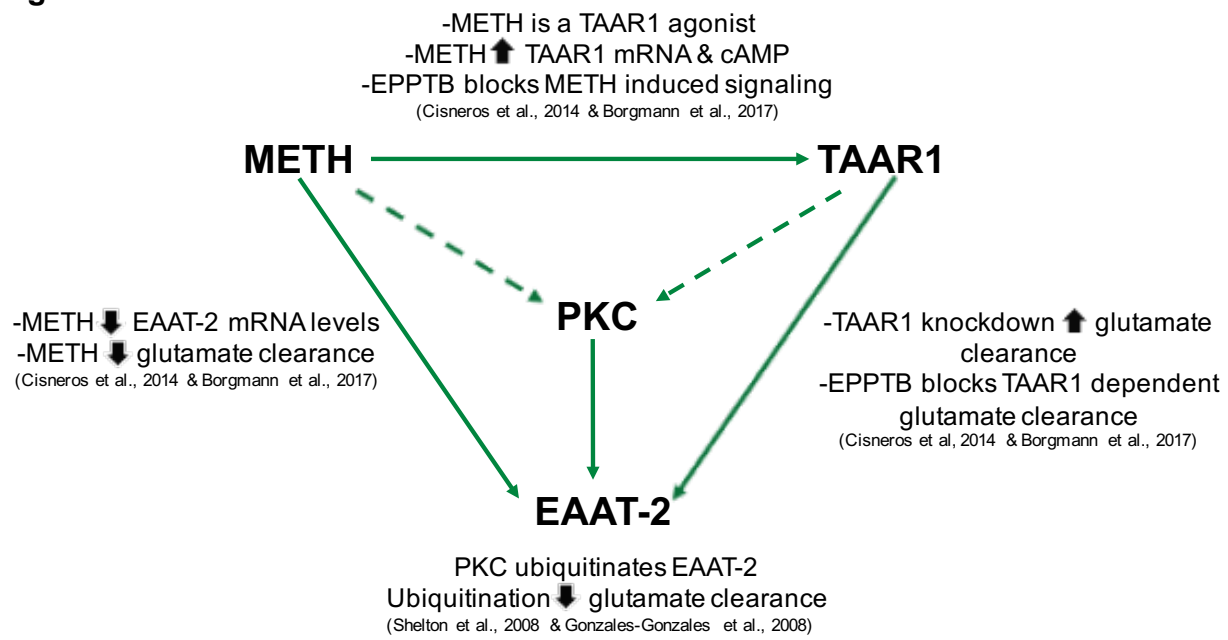


Fig. 1.2- Known and proposed links between METH, TAAR1, EAAT-2 and PKC: METH and TAAR1 have been cited to both regulate EAAT-2 in various ways **(solid lines)**. PKC is implicated in EAAT-2 post-translational modification **(solid lines)**. Based on literature, we hypothesize that METH and TAAR1 may play a role in PKC-induced EAAT-2 regulation **(dashed lines)**.

Whether TAAR1 directly influences PKC-dependent signaling to alter EAAT-2 function has yet to be identified. In the present study, we explored TAAR1-mediated PKC activation in EAAT-2 post-translational regulation during HAND and METH comorbidities **(Fig. 1.2)**. **Our overarching hypothesis is TAAR1 dysregulates astrocyte intracellular signaling during METH abuse in HAND, thus contributing to disease pathogenesis.**

Our study aims to delineate therapeutically targetable mechanisms that regulate astrocytes during neuroinflammation in HAND and METH comorbidity, and propose astrocyte TAAR1 as a potential target to combat neurocognitive decline, particularly in the context of EAAT-2 dysfunction. To mimic upregulation of TAAR1 during HIV CNS disease, we used a combination of tagged/untagged TAAR1 overexpression constructs in primary human astrocytes. We then used a physiologically relevant model of extended METH exposure and low level HIV-associated activation to mimic levels of human disease.[2,25]

We explored the role of TAAR1, neuroinflammation and PKC activation to study astrocyte regulation by assessing glutamate clearance and astrocyte activation. Thereafter, we visualized TAAR1 overexpression by live cell imaging and immunocytochemistry (ICC). Next, TAAR1 mRNA levels were quantified by real-time polymerase chain reaction (RT-PCR) and protein expression measured by western blot (WB). Then, EAAT-2 post-translational modification ubiquitination was evaluated by immunoprecipitation (IP). To assess the role of PKC, inhibitors, BIS II and Go, along with the PKC activator PMA, were used to measure EAAT-2 ubiquitination and glutamate clearance. Lastly, to observe TAAR1 mediated PKC activation, we used EPPTB to block TAAR1 signaling and measure EAAT-2 ubiquitination.

1.9 Significance and impact:

In the current HIV era, ART medication effectively controls viremia in the periphery; however, due to low CPE of ART, the brain serves as a viral reservoir, with difficulties eradicating virus.[14] Chronic HIV CNS infection exacerbates neuroinflammation, promoting the onset of HAND.[13] Reactive gliosis is a common feature found during HIV

infection and METH abuse, further inducing neuroinflammation.[29] Alone and in combination, exposure to METH and HAND-relevant stimuli reduced astrocyte EAAT-2 expression and glutamate clearance abilities.[2,3] Recently, PKC was identified as a post-translational regulator of EAAT-2 though ubiquitination, decreasing glutamate clearance.[4,6] As a METH receptor, astrocyte TAAR1 can also regulate EAAT-2 expression and function. EPPTB-mediated inhibition of TAAR1 signaling recovered glutamate clearance of astrocytes.[2,3] As a GPCR, TAAR1 dysregulation of EAAT-2 may be mediated by PKC activation in astrocytes.[8,9,45] Potential therapies targeting astrocyte TAAR1 may restore EAAT-2 function and improve CNS function during METH abuse, HIV infection and neuroinflammation. We are investigating a direct neurotoxic mechanism common to both HIV and drug abuse. In astrocytes, understanding how this process is regulated is a step in the right direction toward developing a therapy to combat it, which is likely to improve the lives of the HIV+ population and drug abusers worldwide.

CHAPTER II

METHODS AND MATERIALS

2.1 Isolation, cultivation and activation of primary human astrocytes:

Human astrocytes were isolated from first and early second trimester elective fetal brain tissues as previously described.[21,46] Tissues were procured in full compliance with the ethical guidelines of the National Institutes of Health, Universities of Washington and North Texas Health Science Center. Cell suspensions were mechanically dissociated by filtering through a Nitex mesh and trituration. When a single cell suspension was achieved, cells were washed with several low speed centrifugation/trituration steps and cultured in astrocyte medium (ASM, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 with 10% fetal bovine serum, 1% penicillin/streptomycin/neomycin and 1% amphotericin B) initially at a density of $50x10^6$ cells/150 cm^2 at 37°C and 5% CO_2. Every 7 – 10 days, adherent astrocytes were passaged with trypsin-ethylenediaminetetraacetic acid and cultured at $20x10^6$ cells/150 cm^2.

2.2 METH exposure and dosing:

By back calculating the concentration of METH in rodent brain lysates, the peak (6 μM – 2 mM) and prolonged ranges (60 – 600 nM) was determined for *in vitro* investigations.[21,47] In this context, astrocytes were pretreated with METH (50 nM, Sigma-Aldrich Inc., St. Louis, MO) for 7 days, with passaging every 7-8 days. Astrocytes were plated for experimental assays with METH for 24 hours followed by additional treatments of IL-1β (20 ng/ml, R&D Systems, Minneapolis, MN), EPPTB (5 μM, Tocris, Minneapolis, MN), PMA (100 nM, Tocris), BIS II (2 μM, AdipoGen, San Diego, CA) and Go6976 (100 nM, Tocris). Acute treatments in immunoprecipitation studies ranged from 0.5-2 hours. All other assays utilized 8–24 hour treatment time points.

2.3 Glutamate clearance assay:

Primary human astrocytes were plated in triplicates in 48-well tissue culture plates at a density of 0.15×10^6 cells/well with METH for 24 hours followed by additional treatments of IL-1β (20 ng/ml, R&D Systems, Minneapolis, MN), EPPTB (5 μM, Tocris, Minneapolis, MN), PMA (100 nM, Tocris), BIS II (2 μM, AdipoGen) and Go6976 (100 nM, Tocris). Glutamate (400 μM) with and without continued METH treatment in phenol-free ASM was added into each well, and clearance was assayed 20 hour post-glutamate addition. The assay was performed and analyzed according to manufacturer's guidelines (Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit, Thermo Fisher Scientific). Glutamate clearance was normalized to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) levels in each donor. MTT is a colorimetric assay for measurement of metabolic activity.[48] Briefly, five percent MTT reagent in ASM was added to astrocytes and incubated for 30 minutes at 37°C. The MTT solution was removed, and crystals were dissolved in dimethyl sulfoxide (DMSO) for 15 minutes with gentle agitation. Absorbance was assayed at 490 nm in a Spectromax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Following MTT normalization, variation across donors was accounted for by calculating glutamate clearance as a percent of control samples.

2.4 Immunocytochemistry:

Adherent cultures were fixed with acetone:methanol (1:1) or 4% paraformaldehyde, rehydrated & blocked with 2% bovine serum albumin (BSA), 0.1% Triton X-100 in phosphate buffered saline (PBS) for 30 min. Cultures were labeled overnight at 4°C with antibodies specific to hemagglutinin (HA) (rabbit, 1:1000, R&D Systems), TAAR1 (rabbit 1:200, Osenses, Australia), EAAT-2 (mouse, 1:500, Santa Cruz

Biotechnologies, Dallas, TX), GFAP (chicken, 1:1000, Biolegend, San Diego, CA) and subsequently conjugated with AlexaFluor® secondary antibodies (1:400, goat, Life Technologies). Finally, nuclei were labeled with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) 1:1000, Life Technologies). Micrographs were taken at 200X/400X original magnification on an Eclipse Ti-300 (Nikon, Melville, NY) and then pseudocolored with NIS-Elements software (Nikon).

2.5 Nucleofection:

HA-TAAR1 and TAAR1-mcherry expression plasmids (Genecopoeia, Rockville, MD) along with TAAR1-glial fibrillary acid protein (GFP) and untagged-TAAR1 expression plasmids (Origene) were transfected in 1.6 million astrocytes with 0.25 µg plasmid DNA, performed with the shuttle Nucleofector using program CL133 (Lonza, Switzerland). Cells recovered in media 48 hour post-transfection before analysis.

2.6 Real-time gene expression analysis:

Astrocyte RNA was isolated by the Trizol method (Thermo Fisher Scientific, Waltham, MA) followed by DNA digestion and precipitation. A Nanodrop fluorospectrometer (Thermo Fisher Scientific) was used to assess RNA purity and to quantify total RNA levels. Following total RNA isolation, the Dynabeads mRNA Direct Kit (Life Technologies, Carlsbad, CA) was used to enrich mRNA. The kit relies on base pairing between the poly A tail of mRNA and the oligo dT residues covalently linked to the surface of the dynabeads. Following mRNA enrichment, transcripts were made into cDNA with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) at 10 ng/µl.

Expression levels were measured by RT-PCR using Taqman® gene expression assays and the StepOnePlus detection system (Thermo Fisher Scientific). Assay identifiers used in human cells were compiled in **Table 2.1**. The 20 µl reactions with 10 ng cDNA were carried out at 50°C for 2 minutes, 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds in 96-well optical, RT-PCR plates. Transcript levels were normalized to GAPDH or mRNA samples quantified in a duplex PCR reaction. Astrocyte expression levels are represented as fold changes to respective control as calculated by the comparative ΔΔCT method.[49] To assess residual DNA in our mRNA samples, mRNA and cDNA were assayed in parallel on RT-PCR.

Table 2.1: Gene expression assay target:	**Assay number (Thermo Fisher)**	**Dye**
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	4310884E	(VIC/TAMRA)
Trace amine associated receptor 1 (TAAR1)	Hs00373229_s1	(FAM/MGB)

2.7 Live cell imaging:

Adherent cultures were plated and recovered in media 24 hours before imaging. Media was then removed, and PBS added to all wells. Micrographs were taken at 200X/400X original magnification on an Eclipse Ti-300 (Nikon) and then pseudocolored with NIS-Elements software (Nikon).

2.8 Protein isolation, identification and analysis:

Total cellular proteins were isolated by lysing cells directly with mammalian protein extraction reagent (MPER, Thermo Fisher Scientific). Protein levels were determined by Precision Red advanced protein reagent (Cytoskeleton, Inc., Denver, CO) or the BCA

protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Whole cell lysate protein levels were determined by western blot using the Bolt electrophoresis system, the iblot transfer system (Thermo Fisher Scientific) and imaged in a Fluorochem HD2 (ProteinSimple, San Jose, CA). Between 20 – 40 µg of protein was loaded in Bolt gels (Invitrogen). Blots were probed with antibodies for TAAR1 (rabbit, 1:200, Osenses), GFP (mouse, 1:2000, Origene, Rockville, MD) or/and GAPDH (mouse, 1:1000, Santa Cruz Biotechnologies). Secondary detection was achieved using goat anti-rabbit/mouse-HRP (Cell Signaling, Danvers, MA). Alexa Fluor plus antibodies (Thermo Fisher Scientific)

2.9 Primer design, PCR & gel electrophoresis:

Custom primers for were designed using Sigma-Aldrich Easy Oligo design tool. Primer length, molecular weight, secondary structures, primer dimer, melting temperature and GC rich content were controlled for and optimized. Product length varied by primer set: TAAR1-1= 207 base pair (BP), TAAR1-2= 185 BP, GFP-3= 172 BP, GFP-4= 238 BP, UTR-5= 279 BP and TAAR1-GFP-6= 659 BP. PCR amplification was performed using manual Taq DNA Polymerase Kit (Fisher Scientific) according to manufacture's instructions. For amplification of products less than 300 bp, 20 µl reactions with 10 ng mRNA or cDNA were carried out at initial denaturation at 95°C for 5 minutes followed by 34 cycles of annealing and extension at 95°C for 20 seconds, 64°C for 20 seconds and 68°C for 30 seconds and final extension at 68°C for 5 minutes. For amplification of products above 600 bp, 20 µl reactions with 10 ng mRNA or cDNA were carried out at initial denaturation at 95°C for 5 minutes followed by 36 cycles of annealing and extension at 95°C for 20 seconds, 64°C for 20 seconds and 68°C for 1 minute and final extension

at 68°C for 5 minutes. Samples were loaded onto 1.2% agarose gels containing (1.0 µg/ml) ethidium bromide (Fisher). Gels were imaged in a Fluorochem HD2 (ProteinSimple).

Table 2.2- TAAR1-GFP custom primers:

Primer name	Primer sequence	Primer length
TAAR1-1 Forward	TGGGGTGTCTGGTCATGCCT	20
TAAR1-1 Reverse	TGGAGTGTCCCTGCTGTTTT	20
TAAR1-2 Forward	ACTGCAGAGGAGGTTGCTCTGTCT	24
TAAR1-2 Reverse	AGGAAAGCTGTGAAGACATTGGGGA	25
GFP-3 Forward	GAGAGCGACGAGAGCGGCCT	20
GFP-3 Reverse	GGCTTCTACCACTTCGGCAC	20
GFP-4 Forward	GATCGGCGACTTCAAGGTGAT	21
GFP-4 Reverse	GTGGAGGAGGATCACAGCAA	20
UTR-5 Forward	TCTATTGGGAACCAAGCTGGA	21
UTR-5 Reverse	AGCAGGAGGACGTCCAGACA	20
TAAR1-GFP-6 Forward	ACTGCAGAGGAGGTTGCTCTGTCT	24
TAAR1-GFP-6 Reverse	GGCTTCTACCACTTCGGCAC	20

2.10 Immunoprecipitation:

Protein isolation and quantification was measured as previously described in Section 2.9. Immunoprecipitation was performed using Pierce AG magnetic beads, according to manufacturer's instructions. Protein isolation was performed using an antibody for EAAT-2 (Rabbit- 5 µg, Proteintech, Rosemont, IL). Immunoprecipitation samples were determined by western blot using the Bolt electrophoresis system, the iblot transfer system (Thermo Fisher Scientific) and imaged in a Fluorochem HD2 (ProteinSimple). Blots were probed with antibodies for EAAT-2 (mouse 1:500, Millipore, Burlington, MA) and Ubiquitin (mouse, 1:1000, Santa Cruz Biotechnologies). Secondary detection was achieved with goat anti-rabbit/mouse-HRP antibodies (Cell Signaling).

2.11 Simple Western:

Protein isolation and quantification was measured as previously described in section 2.9. Whole cell lysate protein levels were determined by simple western (WES)

capillary protein detection system (ProteinSimple) according to manufacturer's directions. Columns were probed with antibodies for EAAT-2 (rabbit, 1:50, Abcam, Cambridge, MA), PKC (mouse, 1:50, Santa Cruz Biotechnologies) and Vinculin (mouse, 1:10000, Cell Signaling). Secondary detection was achieved with anti-rabbit/mouse-HRP antibodies.

CHAPTER III

RESULTS

3.1 Induction of astrogliosis with METH, IL-1β and PKC activation:

A major function of astrocytes in the CNS is their ability to clear excess glutamate from the environment. Accumulation of extracellular glutamate can lead to overstimulation of glutamatergic receptors and induce neuronal excitotoxicity.[36,37] As a popular psychostimulant in HIV infected individuals, a physiologically relevant, chronic low level METH exposure (7-day pretreatment, 50 nM) was used to mimic CNS levels following METH binges.[25] Reactive astrogliosis and function was evaluated using IL-1β (20 ng/ml) as a classical activator of astrocytes.[50] PKC is likely involved in the mechanism behind EAAT-2 regulation in astrocytes, as recent literature cites PKC mediation of EAAT-2 post-translational modification in various cell lines.[4-7,51] As such, astrocytes were exposed to PKC activator, PMA (100 nM). Cells were then treated with glutamate (400 μM) for 20 hours and assayed for glutamate clearance **(Fig. 3.1 A).** Positive control IL-1β reduced glutamate clearance in astrocytes ($P<0.001$) as compared to control. Interestingly, PMA significantly ($P<0.001$) decreased glutamate clearance abilities. Chronic low level METH exposure has a variable effect on human donors. In these studies, METH alone decreased glutamate clearance in one donor, which drove overall significance ($P<0.001$), but had little effect in two others. Our research has previously reported METH-mediated effects in multiple studies with human astrocytes.[2,3,30] We also visualized induction of a reactive phenotype in astrocytes using 7-day METH pretreatment and 24 hour IL-1β stimuli. To observe the role of METH-induced TAAR1 signaling on astrocyte activation, cells were also treated with TAAR1 inhibitor, EPPTB (5 μM) for 24 hours. Cells were fluorescently labeled for GFAP (red) and EAAT-2 (green) with a merged image showing DAPI labeled nuclei (blue) **(Fig. 3.1 B)**. Astrocytes exhibited classical morphology in

control cells, displaying long extended processes with wide, rounded cell bodies. Classical activation of astrocytes was seen using inflammatory IL-1β stimuli as a positive control. METH stimulated cells also displayed activated phenotypes with shrunken cell bodies and thin processes. Addition of TAAR1 inhibitor, EPPTB, to METH pretreated cells showed morphology comparable to control cells.

3.2 TAAR1 overexpression plasmids and mRNA detection:

To amplify TAAR1-mediated effects on astrogliosis, several TAAR1-tagged plasmids along with an untagged plasmid were used to overexpress the TAAR1 protein in primary human astrocytes **(Fig. 3.2 A)**. Using multiple-tagged plasmids allowed assessment of TAAR1 by live-cell fluorescence microscopy (GFP, mCherry) and detection of changes in TAAR1 localization or function by comparing tagged and untagged plasmids. To first confirm overexpression in our cells, TAAR1 and GAPDH levels were measured by RT-PCR. Because TAAR1 is an intronless gene, any primer-probe assays (like RT-PCR) will detect cDNA, residual-overexpression plasmid DNA and genomic DNA. This is evident in **(Fig. 3.2 B)**, as TAAR1 expression is significantly ($P<0.001$) increased as compared to untransfected control in all plasmid pairs. To account for remaining DNA, paired mRNA and cDNA samples were assayed in parallel. The difference in cDNA and mRNA CTs were calculated and plotted as a fold change **(Fig. 3.2 C)**. Of all the overexpression vectors, only TAAR1-GFP samples showed a significant ($P<0.05$) increase in TAAR1 expression as compared to untransfected control. This indicates that TAAR1-GFP samples show the greatest increase in TAAR1 mRNA expression.

3.3 TAAR1 overexpression imaging and protein levels:

After correcting for residual DNA to measure TAAR1 mRNA overexpression, TAAR1 expression was evaluated at the protein level **(Fig. 3.3)**. Astrocytes were transfected with plasmids encoding Con-GFP, TAAR1-GFP, Con-mCherry and TAAR1-mCherry; live cell imaging for was performed **(Fig. 3.3 A-B)**. Astrocytes were transfected with plasmids encoding HA-Con (HA-GFP), HA-TAAR1, Con-Untagged and TAAR1-Untagged; cells were fixed and stained for HA **(Fig. 3.3 C)** and TAAR1 **(Fig. 3.3 D)**. In control transfection conditions, all tagged samples displayed fluorescence of their specific tag. While the Con-Untagged cells displayed endogenous TAAR1 staining. In all TAAR1 overexpression conditions, fluorescence was comparable to Mock, resulting in TAAR1 expression being indistinguishable from background fluorescence. As a more quantitative measure of protein expression, western blots were probed for TAAR1 and GFP **(Fig. 3.3 E-F)** and TAAR1 alone **(Fig. 3.3 G)**, with GAPDH as a loading control for all blots. Although endogenous TAAR1 protein was visualized in all conditions, exogenous expression of TAAR1 was not detected. Control vectors tagged with GFP, Con-GFP **(Fig. 3.3 E)** and HA-Con (HA-GFP) **(Fig. 3.3 F)** displayed GFP protein. We have tested short and long time points for TAAR1-GFP expression using both lipofectamine and nucleofection transfection methods (Data not shown) and have not been able to visualize protein expression. Through extensive trials with multiple TAAR1 overexpression vectors, we conclude there is something impeding TAAR1 overexpression in human astrocytes.

3.4 Detection of TAAR1-GFP full length transcript:

To determine if an expression blockade exists between transcription and translation, we examined if the full length TAAR1-GFP transcript was being transcribed; Various regions throughout the TAAR1-GFP sequence were amplified **(Table 2.2),** including the beginning and end of TAAR1 and GFP, as well as TAAR1-GFP fusion and the 3'UTR **(Fig. 3.4 A)**. All parts of the TAAR1-GFP transcript were present in our cDNA samples, with little to no amplification in our mRNA samples indicating no residual amounts of genomic or plasmid DNA remaining **(Fig. 3.4 B)**. All together, these data indicate full length TAAR1-GFP mRNA expression and have confirmed that the full-length TAAR1-GFP transcript is synthesized in primary human astrocytes; however, TAAR1-GFP protein is undetectable by live cell imaging, immunocytochemistry and western blot. The purpose of the TAAR1 overexpression model was to isolate TAAR1-dependent astrocyte dysregulation. Thus, we proceeded with TAAR1 inhibition studies using EPPTB to understand the regulation of EAAT-2 by PKC-mediated pathways.

3.5 Post-translational regulation of EAAT-2 by TAAR1 and PKC

As previously mentioned, PKC regulated EAAT-2, by mediating EAAT-2 ubiquitination in C6 glioma and Cos 7 cells.[4-7,51,52] Here, the role of METH-induced PKC on EAAT-2 post-translational modification and altered function in primary human astrocytes was explored. Our lab previously identified TAAR1 as a functional METH receptor on astrocytes, and linked TAAR1 activation to decreased EAAT-2 expression and function.[1-3] We hypothesize that TAAR1, as a GPCR, can induce PKC activation, leading to EAAT-2 ubiquitination. METH pretreated cells along with control culture were stimulated by PKC activator PMA, with and without PKC inhibitors BIS II and Go and

TAAR1 antagonist EPPTB. To confirm EAAT-2 immunoprecipitation, immunoblotting for EAAT-2 demonstrated specific pulldown of target protein. EAAT-2 aggregates were identified at (150 kD) in EAAT-2 IP samples and this band was not visualized in IgG control samples **(Supplementary Fig. 3.1)**. While the levels of PMA-mediated PKC activity differed from donor to donor due to human variability, PMA induced robust EAAT-2 ubiquitination in control cells. Further, broad PKC inhibitor BIS II, effectively blocked PKC-induced EAAT-2 ubiquitination in control and METH pretreated cultures. While chronic low-level METH treatment alone did not significantly increase EAAT-2 ubiquitination, blocking METH-induced TAAR1 signaling with EPPTB decreased EAAT-2 ubiquitination **(Fig. 3.5 A)** This suggests that TAAR1 may be playing a role in EAAT-2 ubiquitination in METH exposed cells.

To narrow down the classes of PKC isoforms involved,[53] astrocytes were treated with commonly used PKC activator PMA, broad PKC inhibitor BIS II and classical PKC isoform inhibitor Go. Go effectively inhibited PMA-induced EAAT-2 ubiquitination **(Fig. 3.5 B)**, at the same level as the broad PKC inhibitor BIS II, in the presence of PMA. This indicated, with PMA activation, blocking all isoforms of PKC reduced EAAT-2 ubiquitination. Ubiquitination directly impacts EAAT-2 function due to internalization and degradation of the plasma membrane associated receptor. METH pretreated and control cultures were stimulated with PMA, BIS II, Go and EPPTB. Cells were then treated with glutamate (400 μM) for 20 hours and assayed for glutamate clearance **(Fig. 3.5 C)**. Low-level METH had a variable response in human donors, as also discussed in **(Fig. 3.1 A)**, and showed a significant ($P<0.005$) decrease in clearance as compared to control **(Fig. 3.5 C)**. Glutamate clearance significantly decreased in control and METH exposed cells

treated with PMA ($P<0.001$), PMA+BIS II ($P<0.001$) and PMA+Go6976 ($P<0.001$); however, in control and METH cultures, PMA and BIS II significantly increased clearance abilities compared to PMA alone ($P<0.001$). The general inhibitor BIS II blocked PMA-induced decrease in glutamate clearance better than Go, although neither inhibitor fully restored EAAT-2 function.

Taken together with results in **(Fig. 3.5 A-B)**, PKC-mediated EAAT-2 ubiquitination was reduced by inhibiting all PKC isoforms. However, recovery of EAAT-2 function was only ameliorated by inhibition of non-classical PKC isoforms, implicating novel and atypical PKC isoform in the regulation of EAAT-2 glutamate clearance. Surprisingly, METH+EPPTB showed no significant improvement in glutamate clearance compared to METH alone, despite METH+EBBTB decrease in EAAT-2 ubiquitination **(Fig. 3.5 A)**. Further studies are needed to determine if TAAR1 plays a direct or indirect role in PKC-mediated EAAT-2 ubiquitination with METH exposure, and if blocking TAAR1 can improve PKC-mediated EAAT-2 dysregulation.

Fig. 3.1- Induction of astrogliosis by IL-1β, PMA and METH and recovery with EPPTB: Astrocytes were kept in culture for one week +/- METH (50 nM) passaged and treated with IL-1β (20 ng/ml), PMA (100 nM), METH for 8-24 hours. Astrocytes were assayed for glutamate clearance, at 20 hours post-glutamate (400 μM) addition **(A)**. METH cultures also received EPPTB treatment (5 μM) for 24 hours **(E)**. Cells were fluorescently imaged for GFAP and EAAT-2 with nuclear label DAPI **(B-E)**. Statistical significance was determined in cumulative data from three astrocyte cultures (n=3) by two-way ANOVA followed by Dunnett's multiple comparison test (*** $P<0.001$) as compared to control. For ICC, one representative donor is shown of three independent experiments.

Fig 3.1

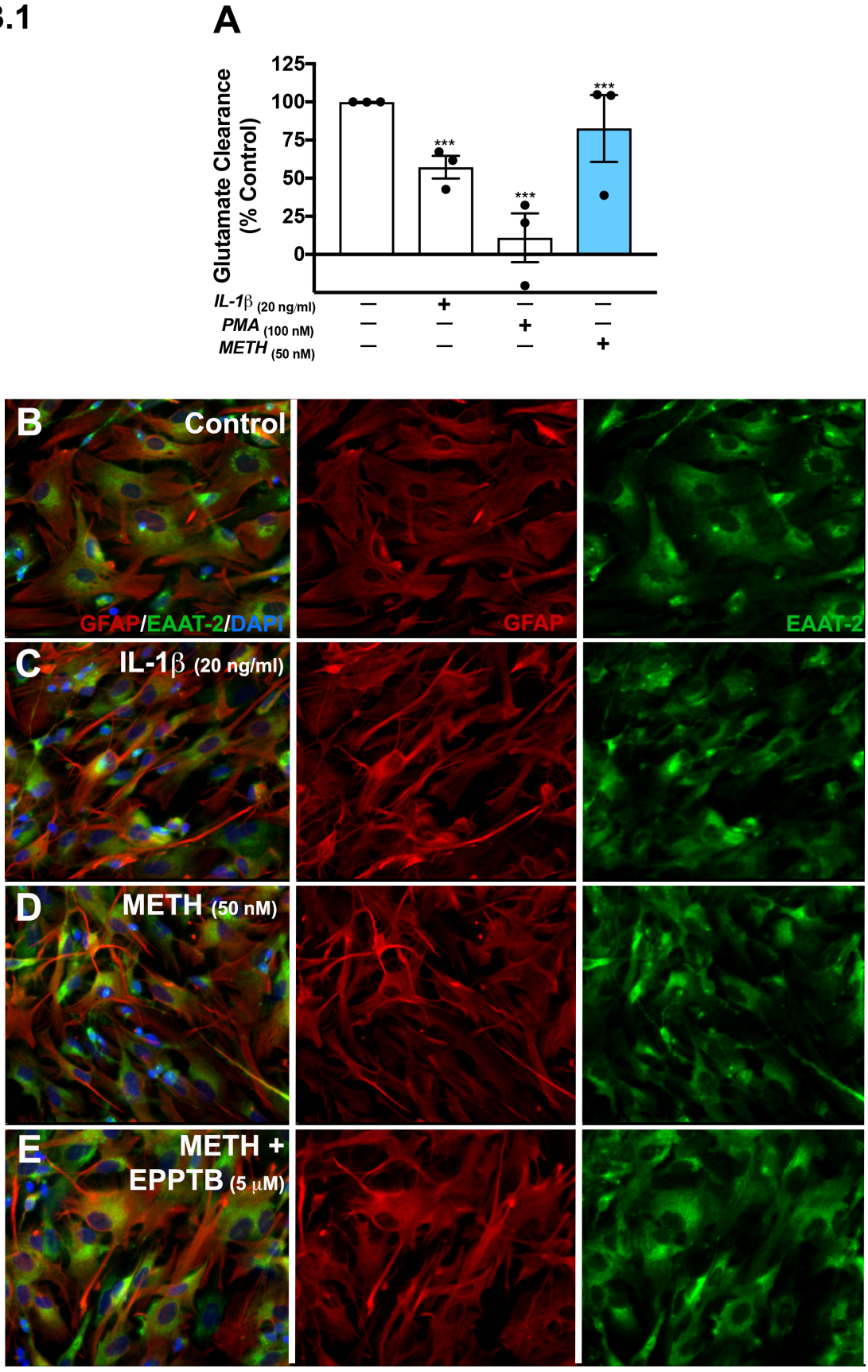

A
Glutamate Clearance (% Control)
125
100
75
50
25
0
***
***
***
IL-1β (20 ng/ml)
PMA (100 nM)
METH (50 nM)
B Control
GFAP/EAAT-2/DAPI
GFAP
EAAT-2
C IL-1β (20 ng/ml)
D METH (50 nM)
E METH + EPPTB (5 μM)

Fig. 3.2- Overexpression of TAAR1-GFP mRNA levels and testing of multiple constructs: TAAR1 fused with C-terminal GFP, C-terminal mCherry, N-terminal HA and untagged-TAAR1 overexpression constructs along with vector backbone controls were transfected into primary human astrocytes **(A)**. All constructs were driven by a CMV promoter and contained the TAAR1 open reading frame. Transfected cells were allowed to recover 48 hours before analysis. Total RNA was isolated, followed by mRNA enrichment. TAAR1 mRNA expression was measured by RT-PCR in transfected astrocytes **(B-C)**. TAAR1 to GAPDH ΔCT values were calculated and compared to untransfected control **(B)**. The fold change of ΔCT cDNA – $\Delta\Delta$CT mRNA was calculated and compared to untransfected control **(C)**. Statistical significance was determined in cumulative data from three astrocyte cultures (n=3) by one-way ANOVA followed by Uncorrected Fisher's LSD test (* $P<0.05$, *** $P<0.001$) comparisons ae made to untransfected control. One representative donor is shown.

Fig. 3.2

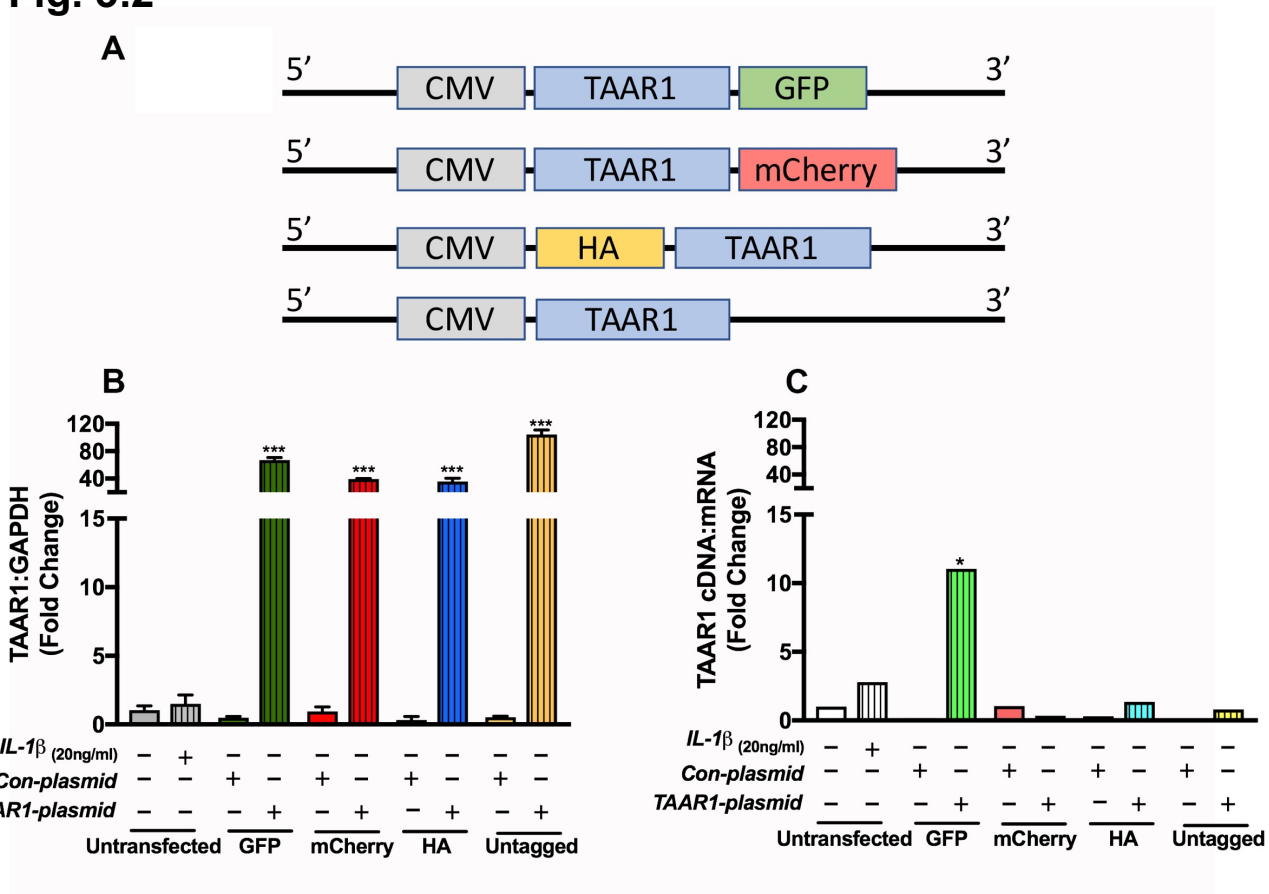

A
5′
CMV
TAAR1
GFP
3′
5′
CMV
TAAR1
mCherry
3′
5′
CMV
HA
TAAR1
3′
5′
CMV
TAAR1
3′
B
TAAR1:GAPDH
(Fold Change)
120
80
40
15
10
5
0
***
***
***
***
IL-1β (20ng/ml)
Con-plasmid
TAAR1-plasmid
Untransfected
GFP
mCherry
HA
Untagged
C
TAAR1 cDNA:mRNA
(Fold Change)
120
80
40
15
10
5
0
*
IL-1β (20ng/ml)
Con-plasmid
TAAR1-plasmid
Untransfected
GFP
mCherry
HA
Untagged

Fig. 3.3- Undetectable TAAR1 protein overexpression: Mock, control backbone and TAAR1 overexpression constructs were transfected into primary human astrocytes. Live cell images were taken for GFP **(A)** and mCherry **(B)**. Cells were PFA fixed and immunostained for HA **(C)** and TAAR1 **(D)** with nuclear marker, DAPI. Total protein lysates were harvested for GFP constructs **(E)**, HA constructs **(F)** and untagged constructs **(G)**. Blots were immunoprobed for TAAR1, GFP and loading control GAPDH. One representative donor is shown for each panel.

Fig. 3.3

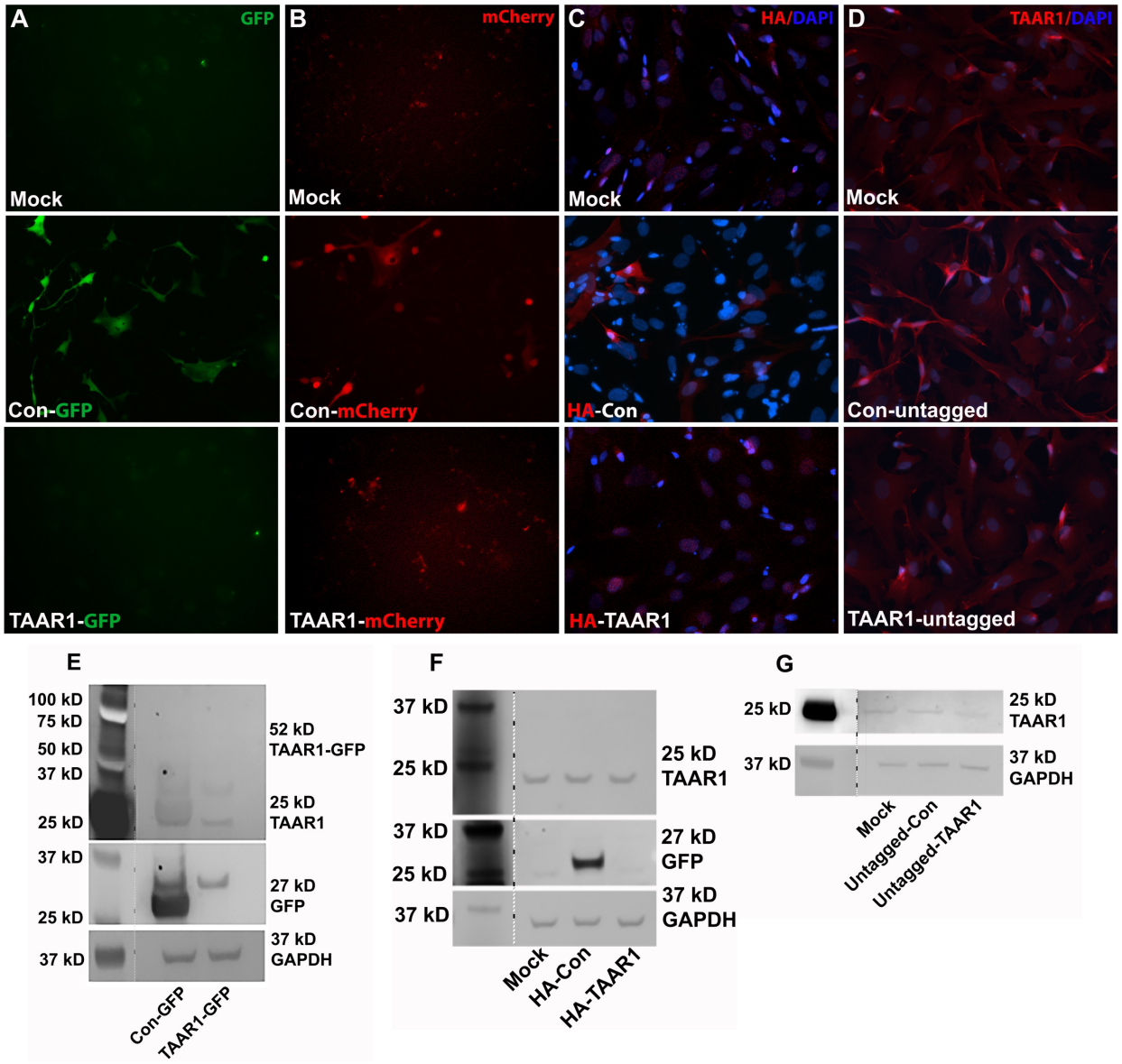

A
GFP
Mock
B
mCherry
Mock
C
HA/DAPI
Mock
D
TAAR1/DAPI
Mock
Con-GFP
Con-mCherry
HA-Con
Con-untagged
TAAR1-GFP
TAAR1-mCherry
HA-TAAR1
TAAR1-untagged
E
100 kD
75 kD
50 kD
37 kD
25 kD
37 kD
25 kD
37 kD
52 kD
TAAR1-GFP
25 kD
TAAR1
27 kD
GFP
37 kD
GAPDH
Con-GFP
TAAR1-GFP
F
37 kD
25 kD
37 kD
25 kD
37 kD
25 kD
TAAR1
27 kD
GFP
37 kD
GAPDH
Mock
HA-Con
HA-TAAR1
G
25 kD
37 kD
25 kD
TAAR1
37 kD
GAPDH
Mock
Untagged-Con
Untagged-TAAR1

Fig. 3.4

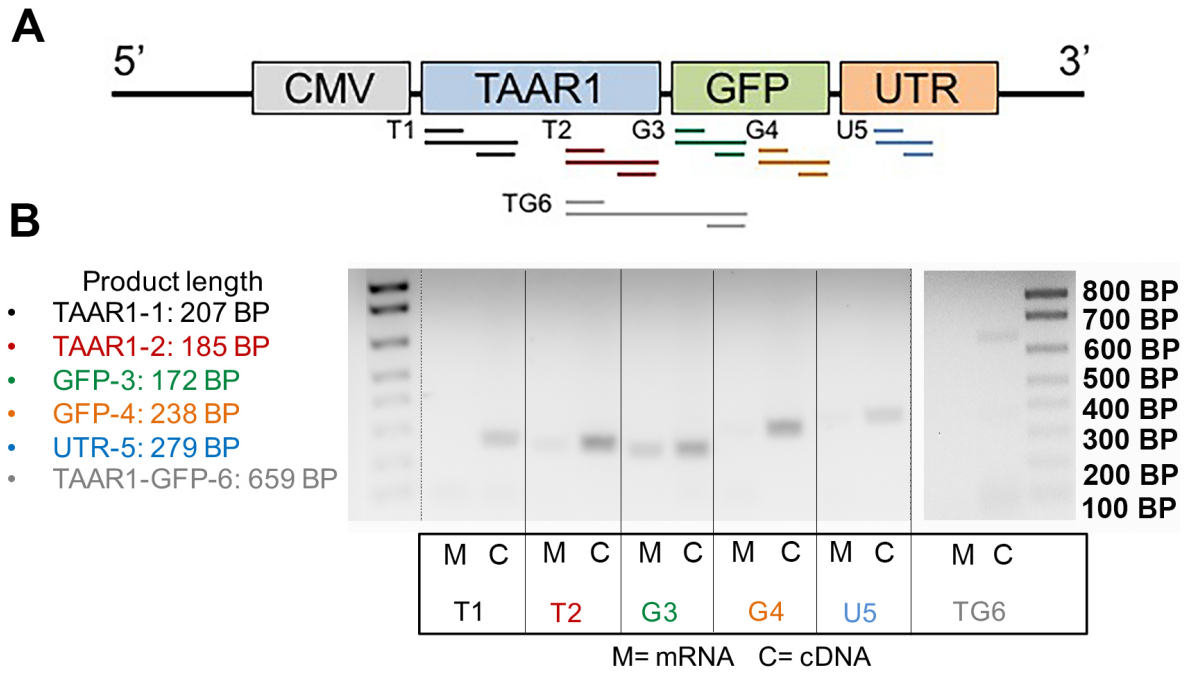
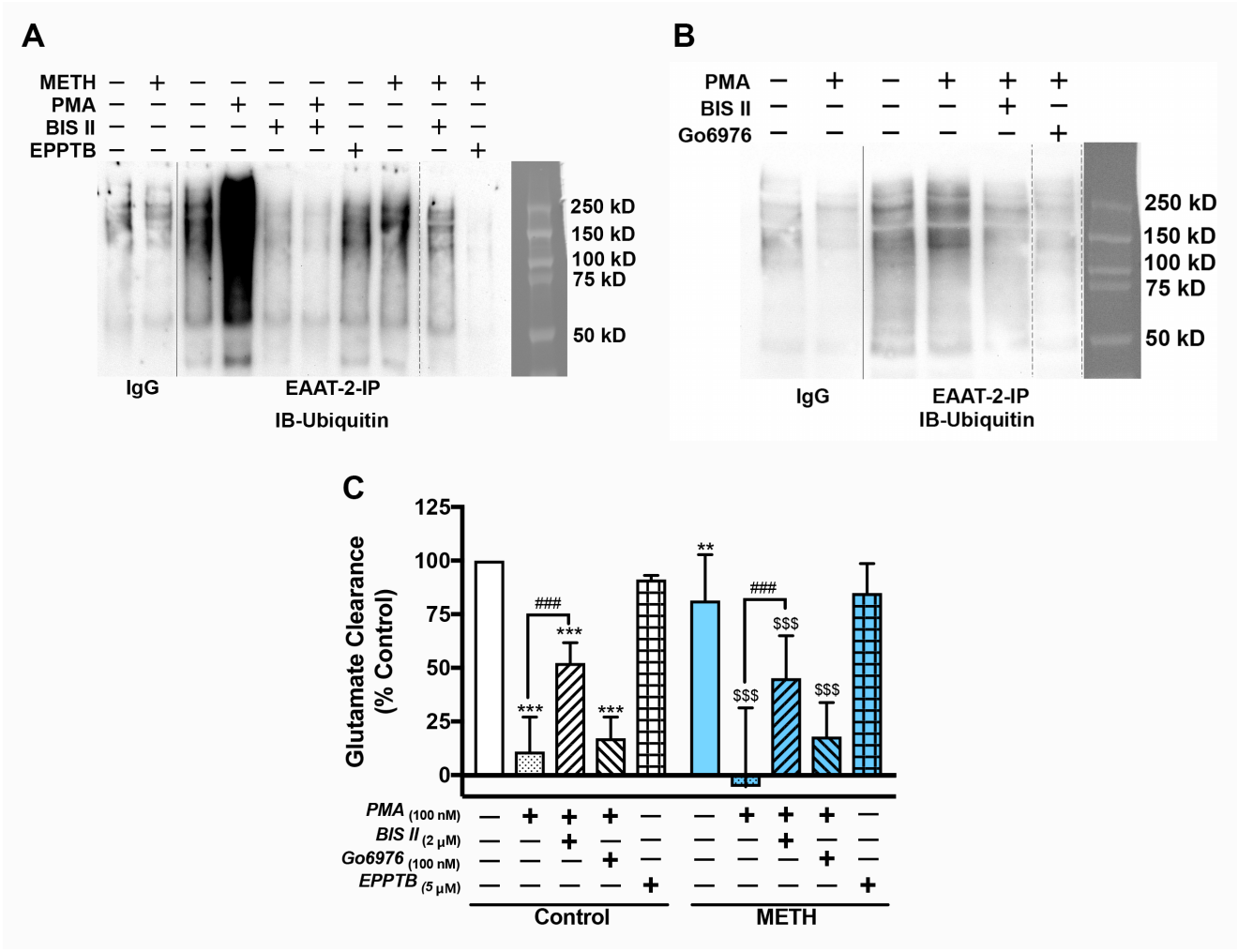


Fig. 3.4- Full length TAAR1-GFP transcript is synthesized in astrocytes: Custom primers were designed to detect the full length TAAR1-GFP in transfected astrocytes **(A)**, **(Table 2.2)**. TAAR1-GFP plasmid was transfected into primary human astrocytes. Total RNA was harvested followed by mRNA (M) enrichment and cDNA (C) synthesis. Both mRNA (M) and cDNA (C) samples were amplified by PCR using our custom primers **(B)**. To visualize presence or absence of the appropriate sized band for each primer set, agarose gels were imaged and product length visualized. One representative donor is shown.

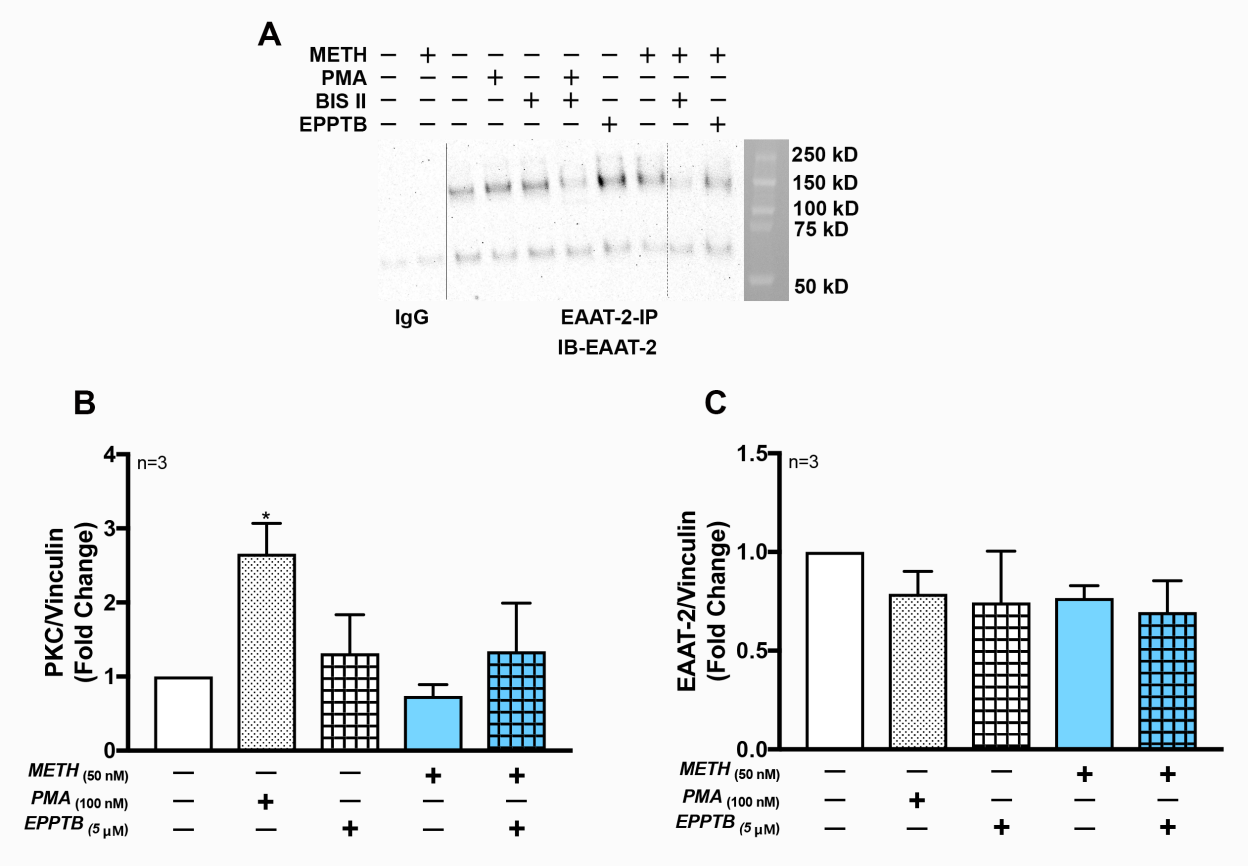
Fig. 3.5- EAAT-2 ubiquitination and functional changes by PKC and TAAR1: Astrocytes were kept in culture for one week +/- METH (50 nM) and stimulates with METH, PMA (100 nM), BIS II (2 μM), Go6976 (100 nM) and EPPTB (5 μM) for 0.5-24 hours. Total protein was harvested and immunoprecipitated to pulldown EAAT-2 protein, IgG antibody was used as a control. Immunoblots were probed for ubiquitin **(A-B)**. For immunoprecipitation, one representative donor is shown. Functional changes in EAAT-2 were assessed by glutamate clearance assay, at 20 hours post-glutamate addition **(C)**. Statistical significance was determined in cumulative data from three astrocyte cultures (n=3) by two-way ANOVA followed by Dunnett's multiple comparison test (*** $P<0.001$). Statistical comparisons in **C** are as follows: (***) compared to control, ($$$) compared to METH and (###) compared to PMA+BIS II.

Fig. 3.5


A
METH
PMA
BIS II
EPPTB
250 kD
150 kD
100 kD
75 kD
50 kD
IgG
EAAT-2-IP
IB-Ubiquitin
B
PMA
BIS II
Go6976
250 kD
150 kD
100 kD
75 kD
50 kD
IgG
EAAT-2-IP
IB-Ubiquitin
C
Glutamate Clearance (% Control)
125
100
75
50
25
0
###
***
**
$$$
PMA (100 nM)
BIS II (2 μM)
Go6976 (100 nM)
EPPTB (5 μM)
Control
METH

Supplementary Fig. 3.1- Total EAAT-2 and PKC protein levels in control and METH pretreated cells: Astrocytes were kept in culture for one week +/- METH (50 nM) passaged and treated with METH, PMA (100 nM), BIS II (2 μM), Go (100 nM) and EPPTB (5 μM) for 0.5-24 hours. Total protein was harvested and immunoprecipitation was performed to enrich EAAT-2 protein, IgG antibody was used as a control for immunoprecipitation **(A)**. One representative donor is shown out of three independent experiments. Total protein levels for PKC, EAAT-2 and vinculin were also measured using the WES capillary protein detection system (Protein Simple) **(B-C)**. Statistical significance was determined in cumulative data from three astrocyte cultures (n=3) by one-way ANOVA followed by uncorrected Fisher's LSD test (* $P<0.05$).

Supplementary Fig. 3.1



CHAPTER IV

DISCUSSION

The goal of the present study was to investigate the links between METH induced TAAR1 signaling, PKC activation and EAAT-2 post-translational modification and function in primary human astrocytes. Our studies show that exogenous TAAR1 expression is blocked at the transcriptional level in primary human astrocytes. We conclude that PKC is a potent regulator of human astrocyte EAAT-2 ubiquitination and function. TAAR1 may play a role in EAAT-2 ubiquitination as selective inhibition using EPPTB reduces basal METH-induced EAAT-2 levels. Further investigation is needed to determine the outcomes of extended METH exposure and TAAR1 signaling, PKC activation and EAAT-2 dysfunction in astrocytes.

We utilized a combination of tagged and untagged TAAR1 overexpression vectors as an impactful approach to observe TAAR1-dependent astrocyte regulation. The mechanism behind the lack of TAAR1 overexpression in primary human astrocytes poses an intriguing yet unanswered question. Shi et al. (2016) successfully overexpressed TAAR1-GFP plasmid (Origene) in Chinese hamster ovary cells.[54] Other groups have also reported difficulty with transient transfection in cell lines.[32,33,55,56] Visualization of TAAR1 protein overexpression is rarely published in TAAR1 studies and endogenous TAAR1 is expressed at very low levels, making detection difficult.[1,32,33,57] As TAAR1 is a GPCR, a common determinant of active and functional TAAR1 expression is the assessment of second messenger signaling induced by a TAAR1 selective agonist.[35] Classical GPCR signaling through the $G_{\alpha s}$ subunit activates cAMP; however, TAAR1 can also signal though $G_{\alpha q}$ subunit and activate PLC to translocate to the endoplasmic reticulum and release Ca^{2+} into the cytosol.[8,9,45] In primary human astrocytes, we demonstrated increased mRNA levels using TAAR1-GFP transfection yet unsuccessful protein

expression, suggesting post-transcriptional or early translational regulation leading to rapid degradation of the protein. TAAR1 localization has been largely associated with intracellular membranes;[54-56,58] however, expression on plasma membranes and the ER has been detected in primary human astrocytes.[2,3] Classically, GPCRs are regulated through beta-arrestin internalization and degradation or recycling of the receptor. This mechanism is internally initiated to prevent prolonged GPCR signaling *via* agonist desensitization due to excess ligand binding.[59] GPCR regulation can also be influenced by microRNA activity. This mechanism is externally initiated as GPCR-ligand binding can activate or inhibit microRNA transcription in the nucleus. MicroRNAs can attenuate target proteins involved in GPCR internalization and trafficking, thus influencing GPCR expression and signaling.[59] Further studies investigating microRNA regulation of TAAR1 may identify post-transcriptional regulation blocking TAAR1 protein synthesis during TAAR1-overexpression. Inhibition of specific protein degradation mechanisms, such as classical GPCR lysosomal degradation or ubiquitin-mediated proteasomal degradation, may shed light of the type of regulation occurring and if successful transient expression of TAAR1 is a possibility in primary human astrocytes.

Alternative approaches studying astrocyte dysregulation uncovered a link between TAAR1, PKC activation and EAAT-2 ubiquitination. The mechanism behind PKC regulation of EAAT-2 is not fully understood. Several groups have identified PKC-mediated ubiquitination, not phosphorylation of EAAT-2 at C-terminal lysine residues as the factor leading to internalization and degradation of the receptor.[4,6] Decreased EAAT-2 function can greatly impact neuronal health and signaling, leading to neuronal excitoxicity and CNS damage.[2,3,60] Recently, E3 ubiquitin ligase NEDD4-2 has been

identified as a possible mediator of PKC-induced EAAT-2 ubiquitination. Active PKC phosphorylates NEDD4-2 on several domains, which subsequently ubiquitinates EAAT-2.[5] Expression of NEDD4-2 in primary cortical astrocytes of C57BL/6 mice has been reported,[7] and NEDD4-2 is likely involved in PKC regulation of EAAT-2 in primary human astrocytes.

In this study, presence of PKC activator PMA and broad PKC inhibitor BIS II not only blocked EAAT-2 ubiquitination, but also significantly recovered EAAT-2 function compared to PKC activation alone in METH-pretreated astrocytes. Classical PKC inhibitor, Go, was also shown to downregulate EAAT-2 ubiquitination. Interestingly, Go did not recover EAAT-2 glutamate clearance abilities in control and METH cultures. This suggests that various PKC isoforms may regulate EAAT-2 ubiquitination, as seen using both PKC inhibitors; however, inhibition of the non-classical PKC isoforms recovered EAAT-2 function during PMA activation and METH exposure. This indicates that multiple mechanisms (including PKC signaling) are involved in regulation of EAAT-2 function. Although multiple studies demonstrate PKC-mediated ubiquitination of exogenously overexpressed EAAT-2 in various cell lines,[4-7] the connection to a specific PKC isoform has yet to be determined. Isolating PKC isoform(s) involved in astrocyte EAAT-2 dysregulation, needs further investigation.

METH is a known regulator of EAAT-2 at the transcriptional level in primary human astrocytes. METH induced TAAR1-activation of second messenger cAMP signals through PKA. Active PKA induces translocation of cAMP responsive element binding (CREB) protein into the nucleus, thus altering EAAT-2 transcription.[3] METH modulation of astrocyte EAAT-2 function has also been reported.[2,30] We hypothesize that METH may

mediate PKC-induced EAAT-2 regulation in a TAAR1-dependent manner. Here, METH had variable responses across donors in both EAAT-2 ubiquitination and effects on glutamate clearance.

Variation in METH signaling among human donors could be attributed to multiple sources. Two receptors have been proposed to mediate METH response in astrocytes, TAAR1 and the Sigma receptor 1 (σ-1R). Both TAAR1 and σ-1R are known to induce Ca^{2+} flux in response to METH,[1,2,61] which may lead to PKC activation in astrocytes. Recently, σ-1R has been implicated in astrocyte activation in response to METH. A study by Zhang et al., (2015) demonstrated that inhibition of σ-1R, using σ-1R antagonist SN79, blocked METH-induced astrocyte activation and inflammatory cytokine expression.[62] The σ-1R has been identified as a therapeutic target in the context of drug abuse and neurodegeneration. The receptor is known to bind several agonists and is involved in the regulation of a number of signaling mechanisms, as reviewed by Maurice et al., (2009).[63] TAAR1 responds to fewer agonists, consisting of trace amines and amphetamine derivatives, suggesting TAAR1 as a target involved in less signaling pathways, reviewed by Rutigliano et al., (2017).[35] Overall, σ-1R may be involved in astrocyte activation, but this project focused on the regulation of EAAT-2 expression and function in primary human astrocytes. Based on our previous work implicating TAAR1 in EAAT-2 regulation,[1-3] our current study investigated METH-induced TAAR1 signaling on PKC activation in astrocytes.

At present, astrocyte activation by METH may be altered by TAAR1 genetic variability. Currently, there are 50 synonymous and 50 non-synonymous single nucleotide polymorphisms (SNPs) detected in human TAAR1 (hTAAR1).[64] Functional effects of

SNPs in hTAAR1 have not been fully characterized, and presence of SNPs may alter hTAAR1 receptor binding and signaling abilities. One study reports identification of sub-functional and non-functional hTAAR1 receptors was mediated by SNPs.[54] TAAR1 SNPs in primary human astrocytes could very well affect METH receptor binding, signal transduction and cellular localization. As TAAR1 mRNA increased in response to METH and HAND relevant stimuli,[1,2] the presence of SNPs may alter cellular response to viral infection, inflammatory mediators and drugs of abuse. Recent studies have shown that TAAR1 induction of dopamine reuptake in neurons is dependent on PKA and PKC activation,[8,9,34] and the effects on DAT can be blocked by using PKA and PKC inhibitors.[9,52,56] Further investigation is needed to determine whether TAAR1 initiates PKC activation during METH exposure and HIV-associated neuroinflammation.

Our work demonstrated that METH may mediate PKC-induced EAAT-2 regulation in a TAAR1-dependent manner in astrocytes. Blocking TAAR1 signaling with EPPTB, decreased EAAT-2 ubiquitination in METH pretreated cells, implicating METH and TAAR1 in EAAT-2 post-translational modification and thus, function. The effect of EPPTB as a “selective” TAAR1 inhibitor is attributed to a lack of off-target binding. TAAR1 has been shown to induce activation an inwardly rectifying potassium current, which decreases the firing frequency of dopaminergic neurons in a murine model. Addition of EPPTB blocked the current, thus increasing dopaminergic neuron firing. In TAAR1 knockout mice, the effect of EPPTB on dopaminergic neuronal firing was not detected, indicating no off-target effects.[65] Unpublished observations from our group indicates side-effects of EPPTB exposure, such as alterations in cell metabolic activity, proliferation, and organelle function, particularly in mitochondria (data not shown).[2] Although, as the only

identified TAAR1 specific inhibitor, EPPTB may serve as a potential therapy to block METH-induced TAAR1-mediated astrocyte dysregulation of EAAT-2.

Conclusions and future directions:

The ultimate goal is to highlight potential therapeutic targets that regulate astrocytes, with recovery of astrocyte function as an outcome. In the present study, we specifically examined the post-translational regulation of EAAT-2 function downstream of METH and TAAR1. However, previous data demonstrate that HIV-associated neuroinflammation is a potent regulator of EAAT-2 function. Future studies will investigate the role of PKC-mediated EAAT-2 ubiquitination in the context of HIV-CNS disease. Such research will be essential as PMA is used to reactivate HIV as part of "shock and kill" therapy.[66] While this may help identify and eradicate viral reservoirs in the CNS, our findings suggest it will also exacerbate excitoxicity by promoting EAAT-2 ubiquitination, internalization and degradation in astrocytes, infected or not. Our data show that inhibition of TAAR1 decreases EAAT-2 ubiquitination, and blocking TAAR1 using EPPTB may help preserve EAAT-2 glutamate clearance abilities during HIV shock and kill therapy. As TAAR1 levels are shown to increase with inflammatory insults, and TAAR1 knockdown improves EAAT-2 levels and function, future studies could include neurological disorders in which excitotoxicity contributes to disease pathology.[39-42,67] Further, METH exposure in a physiological model of binging and escalating METH abuse may highlight TAAR1 mediated effects not evident in the chronic low level in vitro model used in this study.

Thus, we propose that targeting astrocyte EAAT-2 dysregulation during METH abuse and HIV-associated neuroinflammation may restore astrocyte function, decrease

neurotoxicity and improve CNS health. Targeting direct mechanisms regulating the vital function that is glutamate clearance, may be an innovative way to restore CNS function, applicable to multiple neurodegenerative diseases.

CHAPTER V

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