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Glutamate buffering capacity and blood-brain barrier protection of opioid receptor agonists biphalin and nociceptin

Saeideh Nozohouri*[a], Yong Zhang*[a], Thamer H. Albekairi[a,b], Bhuvaneshwar Vaidya[a], Thomas J. Abbruscato[a]

[a]Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX 79106, USA.

[b]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia.

*The authors contributed equally to the study.

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Running title: Opioid receptor agonists protect the BBB

Address correspondence and reprint requests to:

Abbruscato, T.J., Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, 1300 S Coulter St, Amarillo, TX-79106, Email: Thomas.abbruscato@ttuhsc.edu, Phone: 806-414-9234; Fax: 806356-4034

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Abbreviations:

BBB: Blood-brain barrier; Bi: Biphalin; CNS: Central Nervous System; EAAT: Excitatory Amino Acid Transporter; GLAST: Glutamate-aspartate Transporter; NTX: Naltrexone; NMDA: N-methyl- D -aspartate receptor; NOP: Nociceptin Opioid receptor; NOC-I: Nociceptin Inhibitor; OGD: Oxygen, glucose deprivation; OR: Opioid Receptor; ROS: Reactive Oxygen Species; TJ: Tight junction; t-PA: Tissue Plasminogen Activator.

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Abstract:

Opioids play crucial roles in the regulation of many important brain functions including pain, memory, and neurogenesis. Activation of opioid receptors is reported to have neuroprotective effects following ischemic reperfusion injury. The objective of this study was to understand the role of biphalin and nociceptin, opioid receptor agonists, on blood-brain barrier (BBB) integrity during ischemic stroke. In this study, we aimed to measure the effect of biphalin and nociceptin on astrocytic glutamate uptake and on expression of excitatory amino acid transporter (EAAT) to study the indirect role of astrocytes on opioid receptor-mediated BBB protection during in vitro stroke conditions. We used mouse brain endothelial cells (bEnd.3) and primary astrocytes as in-vitro BBB model. Restrictive BBB properties were evaluated by measuring [^{14}C] sucrose paracellular permeability and the redistribution of the tight junction proteins. The protective effect of biphalin and nociceptin on BBB integrity was assessed after exposing cells to oxygen-glucose deprivation (OGD) and glutamate. It was observed that combined stress (2mM glutamate and 2hours OGD) significantly reduced glutamate uptake by astrocytes however, biphalin and nociceptin treatment increased glutamate uptake in primary astrocytes. This suggests a role of increased astrocytic buffering capacity in opioid meditated protection of the BBB during ischemic stroke. It was also found that the combined stress significantly increased [^{14}C] sucrose paracellular permeability in in-vitro BBB model. Biphalin and nociceptin treatment attenuated the effect of the combined stress, which was reversed by the opioid receptor antagonists, suggesting the role of opioid receptors in biphalin and nociception's BBB modulatory activity.

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Significant statement:

There is an unmet need for discovering new efficacious therapeutic agents to offset the deleterious effects of ischemic stroke. Given the confirmed roles of opioid receptors in the regulation of CNS functions, opioid receptor agonists have been studied as potential neuroprotective options in ischemic conditions. This study will greatly add to the knowledge about the cerebrovascular protective effects of opioid receptor agonists and provide insight about the mechanism of action of these agents.

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1. Introduction:

Ischemic stroke is one of the major causes of disability and death worldwide, which comprises a serial of complex pathogenic processes such as reactive oxygen species production, excitotoxicity, inflammation, blood-brain barrier (BBB) disruption and eventually neuron death. Amelioration of these deleterious processes will improve post-ischemic outcomes in patients and ultimately neuronal recovery. However, only one FDA-approved therapeutic drug exists for ischemic stroke patients, tissue plasminogen activator (t-PA), which has limitations regarding side effects and stroke patient eligibility. Therefore, there is an unmet need for discovering new efficacious therapeutic agents to offset the deleterious effects of ischemic stroke (Nozohouri *et al.* 2020b).

Following ischemic injury, the major excitatory neurotransmitter, glutamate, is increased through excess release from activated presynaptic neurons and malfunctioned clearance by the astrocytic glutamate transporters. Excess extracellular glutamate is excitotoxic to neurons. It has also been reported to increase BBB permeability in cultured brain endothelial cells through activation of NMDA receptors (Xhima *et al.* 2016). The BBB functions as a highly specialized interface which comprises cellular and vascular components. The selective nature of the BBB allows to maintain a unique extracellular milieu within the brain that is essential for normal CNS function. Astrocytes play an important role in BBB integrity through regulating cerebral endothelial cell function, buffering extracellular neurotransmitter concentrations, and modulating CNS ionic balances and fluid accumulation. Due to its toxicity at higher concentrations, extracellular glutamate is required to be rapidly removed by a set of excitatory amino acid transporters (EAATs) primarily expressing in astrocytes including glutamate-aspartate transporter (GLAST, also known as EAAT1) and glutamate transporter-1 (GLT-1, also known as

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EAAT2). Importantly, increased glial EAAT1 (but not EAAT2) has been observed in patients with traumatic brain injury which reflects a neuroprotective potential of EAAT1 (Choi *et al.* 2015). The neuroprotective role of EAAT1 has been also reported in post-ischemic brain damage in EAAT1-deficient animals and in stroke patients (Yamashita *et al.* 2006; Chen *et al.* 2015). These studies suggest a superior role of astrocytic EAAT1 over EAAT2 in maintaining the excitatory neurotransmitters hemostasis after brain injuries. Therefore, developing novel agents to regulate astrocytic EAAT1 and glutamate buffering capacity could be a promising neurovascular protective strategy in the management of ischemic stroke. Targeting glutamate clearance may provide an advantage over glutamate receptor antagonists, which have been found to decrease neurodegeneration in some nondamaged areas which produced failed results in clinical trials (Ikonomidou & Turski 2002).

Opioids play crucial roles in the regulation of CNS functions and hemodynamics through activation of the opioid receptors (ORs) distributing widely throughout the brain. ORs comprise four subtypes, i.e., the classical mu-OR, delta-OR, kappa-OR and the newer nociceptin opioid peptide receptor (NOP). The classical ORs have gained extensive interest of researchers for their role as neuroprotective targets and have been reported to demonstrate potential neuroprotective activities in ischemic stroke by several overlapping mechanisms (Vaidya *et al.* 2018). There are numerous studies regarding neuroprotective effects of both opioid agonists and antagonists. Liao et al. have shown that naloxone decreases brain infarction, chemokine expression and neutrophil accumulation through blocking mu opioid receptors following ischemic injury (Peyravian *et al.* 2019). Although earlier preclinical reports have shown neuroprotective activity of OR antagonists, clinical trials failed to verify those findings due to the adverse effects of high doses of antagonists on neuronal functions (Vaidya *et al.* 2018). Our lab has published several studies

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reporting the neuroprotective effects of a non-selective OR agonist biphalin, which is associated with reduced ROS production, brain edema, infarct size, and neuron death, tested in ischemic stroke models (M Cowell & Sun Lee 2016; Yang *et al.* 2015; Rashedul Islam *et al.* 2016). Structure of biphalin is the combination of two tetrapeptides derived from enkephalin pharmacophore which have high affinity to delta-OR and mu-OR low affinity to kappa-OR, but no affinity to NOP (Yang *et al.* 2015). Nociceptin is a natural ligand of NOP with no affinity to the classical ORS. Furthermore, a recent study reports that activation of NOP serves as a regulator of EAAT1 and glutamate uptake in developing astrocytes (Meyer *et al.* 2017). This study raises the hypotheses that 1) if a potent OR activator, such as biphalin, has similar regulating capacity of astrocytic EAAT1 and glutamate uptake, and 2) if the glutamate buffering activity of OR agonists ameliorate ischemia/glutamate induced BBB damage.

In the present study, we examine glutamate buffering activity of biphalin and nociceptin under ischemic conditions with primary astrocytes and their protective potential against ischemia/glutamate-induced paracellular permeability with an endothelial cells/astrocytes coculture BBB model.

2. Materials and methods

2.1 Cell culture and bEnd3/Astrocyte coculture:

bEnd3 cells (Passage 27-29) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented by fetal bovine serum (FBS) (Atlanta biologicals, Minneapolis, MN) and non-essential amino acid and penicillin/streptomycin (PS) (Sigma, St. Louis, MO). Cells were maintained in a humidified incubator at 37 °C and with 5% CO2. Mouse primary astrocytes were obtained from the cerebral cortices of one day old CD-1 mouse pups (CD-1 mouse, Charles Rivers Laboratory) according to the method explained

previously by (Du *et al.* 2010). After isolating the brain, cortices were removed and placed in Hank's balanced salt solution (HBSS) supplemented with gentamycin (10 µg/mL). Then cortices were digested with 0.25% trypsin for 10-15 minutes at 37 °C followed by neutralizing with FBS containing DMEM. The cells were then seeded into a cell culture flask and the medium was refreshed every 3 days until reaching confluency.

For bEnd3 and astrocyte co-culture, the Transwell inserts (0.4-lm pore size, 12-well; Corning, Lowell, MA) were inverted and astrocytes with a density of 150,000 cells/filter were seeded onto the basolateral side of the filter membrane and allowed to adhere. After 4 hours, the Transwells were inverted back, and astrocytes were allowed to grow for 2 more days in astrocyte medium. Then, bEnd3 cells with a density of 50,000 cells/filter were added onto the apical side of the Transwell filter. The co-culture of primary astrocytes and bEnd3 cells were grown for 8 days. Media were changed every other day (Li *et al.* 2010). Barrier integrity was assessed by measuring trans endothelial electrical resistance (TEER) by EVOM resistance meter (World Precision Instruments, Sarasota, FL, USA) using the STX-2 electrodes.

2.2 Oxygen glucose deprivation:

In order to simulate the stroke condition in-vitro, cells were exposed to oxygen glucose deprivation (OGD) condition following an established protocol (Yang *et al.* 2015). For this purpose, cell culture medium was removed and cells were rinsed with Dulbecco's Phosphate Buffered Saline Solution (DPBS) and glucose-free Earle's balanced salt solution (EBSS) (in mM 140 NaCl, 0.83 MgSO4, 5.36 KCl, 1.02 NaH2PO4, 1.18 CaCl, 26.19 NaHCO3) bubbled with 95% N2/5% CO2 was added to the cells to create aglycemic condition. Then cells were transferred to a custom-made hypoxic chamber (Coy Laboraties, Grasslake, MI) with 95%

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N2 and 5% CO2 at 37°C to induce hypoxia (1% oxygen). Cells were exposed to OGD condition for 2 hours.

2.3 Glutamate +/- OGD +/- biphalin and nociceptin treatment on the in-vitro BBB models:

The permeability experiments were performed on day 8-10 following mono- or co-culture establishment. On the day of experiment, the in-vitro BBB model was incubated with 1 µM naltrexone in HEPES buffer (in mM 120 NaCl, 1 CaCl2, 25 HEPES, 1 KH2PO4, 2 KCl, 1 MgSO4, 10 d-Glucose) in both compartments for 30 minutes, then the media was changed to 1 µM naltrexone and 0.1 µM biphalin in HEPES. After 30 minutes, naltrexone, biphalin and 2mM glutamate was added to the cells in EBSS which simulates if glutamate was released from neurons. Then the cells were transferred to OGD chamber. The same treatment strategy was used to evaluate the effect of 1µM nociceptin and its inhibitor. For the co-culture of bEnd3 cells and astrocytes, the treatment was only added to the basolateral side of the Transwell insert to study the role of astrocytes in the BBB protection.

2.4 Permeability measurement:

The paracellular permeability was assessed by measuring the diffusion profile of [^{14}C] sucrose (Nozohouri *et al.* 2020a). Briefly, after 2 hours of OGD, the media was removed from both compartments and 1.5 mL HEPES was added to the basolateral compartment and 0.5 mL HEPES containing 0.1 µCi/mL of [14C] sucrose was added to apical compartment for evaluation of BBB permeability. 100 µL samples were removed from the basolateral chamber at (5, 15, 30, 60 and 120 minutes) and replaced with fresh HEPES. The radioactivity of collected samples were evaluated using a liquid scintillation counter (Beckman Coulter, USA). Then the permeability coefficient was calculated using the following formula:

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$$PC = \frac{dQ}{dt} \times \frac{1}{\mathrm{C0} \times A}$$

Where dQ/dt is the diffusion rate of [^{14}C] sucrose across the membrane, A is the area of Transwell insert and C_0 is the initial concentration of paracellular marker added in donor compartment.

2.5 Western blot analysis:

Astrocytes and bEnd3 cultures were lysed using RIPA buffer supplemented with proteinase and phosphatase inhibitor cocktails and then collected. Protein concentration in each sample was measured using BCA protein assay. After solubilizing samples using Laemmli buffer, equal quantities of proteins (20 µg/Lane) were loaded and separated by SDS-polyacrylamide gel electrophoresis (SDSPAGE) in 10% acrylamide and then transferred to a PVDF membrane for 2 hours at 100V. Then the membrane was incubated in blocking solution containing 5% nonfat dry milk to block non-specific binding. Then the membranes were immunoblotted with the appropriate primary antibodies overnight at 4°C with the following dilutions: anti EAAT1/GLAST (1:1000) rabbit mAb (Cell signaling technology, Danvers, MA), anti-claudin-5 (1:500) mouse mAb (Invitrogen, Waltham, MA), anti-actin (1:10000) mouse mAb (Sigma, St. Louis, MO). After rinsing, the membrane was incubated with the appropriate anti-rabbit or anti mouse secondary antibody (Cell signaling technology, Danvers, MA), for 2 hours and the bands were visualized with enhanced chemiluminescence. Beta-actin immunoreactivity was used as the loading control in each sample and was used to normalize the corresponding data. Data were analyzed using N.I.H. ImageJ software and is reported as the ratio of control group.

2.6 [^{3}H] glutamate uptake:

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For this sets of experiments, nociceptin (1 μM), biphalin and selective opioid receptor agonists (0.1 μM) with or without the non-selective opioid receptor antagonist naltrexone (1 μM) and nociceptin receptor antagonist BAN ORL 24 (0.1 μM) were added to the primary astrocyte culture grown on 6-well plates. After overnight incubation, the selected groups were transferred to OGD chamber following the above-mentioned method and after 2 hours of OGD, [^{3}H] glutamate uptake assay was performed on all groups. In one set of experiments, the astrocytes were incubated with 10 μM of EAAT1 inhibitor, UCPH 101 (Abcam, Cambridge, UK), for 15 minutes after the OGD and before performing the glutamate uptake assay. First, a 20mM stock solution of UCPH 101 was prepared in dimethyl sulfoxide and the final concentration of 10μM was prepared in HEPES and then was added to cells (Liang *et al.* 2014). The uptake was measured by incubating the cells with [^{3}H] glutamate (0.1 μCi/mL) for 7 minutes. An incubation time of 7 minutes was selected based on the results of the time-dependent uptake study performed in our lab (data not shown). Uptake study was conducted by incubating the cells grown on 6-well plates with HEPES that contains 25 μM glutamate, which mimics the physiological concentration of glutamate. The uptake was terminated by removing radioactive HEPES buffer and rinsing the cells with ice-cold HEPES. Cells were then lysed using 1% Triton X-100 (ARCOS). Radioactivity was then measured using a liquid scintillation machine (Beckman Coulter). Protein estimation was performed on lysates using a BCA protein estimation kit (Pierce BCA Protein Assay Kit) following manufacturer protocol. The data was normalized by protein estimation and presented as the percentage change compared to the control group.

2.7 Immunocytochemistry:

The bEnd3 cells were seeded into 4-well chamber slides (Lab-Tek II Chamber slides). After reaching confluency, the cells were exposed to OGD and glutamate with or without

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treatment with biphalin and nociceptin. Afterwards, the cells were fixed with 4% paraformaldehyde, washed and permeabilized with 0.1% Triton X-100 for 5 minutes. Cells were incubated with blocking solution containing 1% bovine serum albumin (BSA) and 2% goat serum for 1 hour at room temperature followed by incubation with primary antibody (1:100) in the same blocking solution overnight at 4°C. Next day, the cells were washed with 0.1% BSA solution in PBS and were stained with fluorescence-tagged corresponding secondary antibody at room temperature for 2 hours followed by 3 times wash with PBS. Slides were mounted with DAPI containing Prolong Gold antifade mounting media (Invitrogen). Fluorescent Images were obtained using a multiphoton microscope (A1R; Nikon, NY, USA) in the confocal mode, using a 60x objective.

2.8 Statistical analysis:

All the data are presented as the mean ± standard deviation (SD) of at least three independent replicates. Unpaired student's t-test was used to compare two groups whereas one-way ANOVA followed by Tukey's post hoc multiple comparison test was used to compare more than two groups (Prism, version 8.0; GraphPad Software Inc., San Diego, CA, USA). P values < 0.05 were considered statistically significant.

3. Results:

3.1 Biphalin and nociceptin increase glutamate uptake and upregulate glutamate transporter EAAT1 expression in primary astrocytes in a concentration and time-dependent manner.

To investigate the effect of biphalin and nociceptin on the glutamate buffering capacity of astrocytes, increasing concentrations of biphalin and nociceptin were added to mouse primary cortical astrocytes and [^{3}H] glutamate uptake was measured in these cells. As shown in figures

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1A and 1B, biphalin and nociceptin increase the uptake of glutamate by astrocyte in a concentration-dependent manner in normoxic condition. We suggest that biphalin and nociceptin mediate glutamate uptake through upregulation of glutamate transporter, EAAT1/GLAST, in astrocytes. As depicted in figure 1C, increasing concentrations of biphalin and nociceptin upregulate the expression of EAAT1 in primary astrocytes hence increase the glutamate buffering capacity of these cells. With these experiments, one concentration of biphalin (0.1 µM) and nociceptin (1µM) was selected, and the cells were incubated with that concentration for various time points. It was observed that biphalin and nociceptin increase the expression of EAAT1 in a time-dependent manner, as well (Figure 1D). To further evaluate the mechanism involved in this upregulation, we pre-incubated the cells with antagonists of opioid receptors, naltrexone (NTX) for biphalin and BAN ORL 24 (NOC-I) for nociceptin. The results indicated that, the overexpression of EAAT1 is inhibited by the antagonists of NOP and classical opioid receptors suggesting that the role of nociceptin and biphalin in increasing the expression of glutamate transporter is mediated through classical and nociceptin opioid receptors (Fig 1E). Based on above-mentioned findings, we selected 12 hours of treatment with 0.1 µM biphalin and 1 µM nociceptin for future experiments of this study.

3.2 Biphalin and nociceptin pretreatment increases the [^{3}H] Glutamate uptake in primary cortical astrocytes challenged with 2 hours of oxygen-glucose deprivation (OGD) by increasing the expression of EAAT1.

After confirming the effect of biphalin and nociceptin in increasing the expression of EAAT1 and glutamate uptake by astrocytes in normoxic condition, we studied their effect on OGD exposed cells as well to mimic ischemic stroke condition. As depicted in figure 2A and 2C, 2 hours of OGD increases the capacity of astrocytes in clearing the excess extracellular

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glutamate by upregulating the level of EAAT1 in these cells however, pretreatment with 0.1 μM biphalin or 1 μM nociceptin, further increases glutamate uptake by primary astrocytes which is mediated by significant upregulation of glutamate transporter, EAAT1 (Figure 2B, 2D). Also, like normoxic condition, this protective effect is reversed by corresponding antagonists of opioid or NOP receptors during OGD condition.

3.3 Biphalin and nociceptin improve glutamate uptake in astrocytes exposed to OGD and glutamate combined stress condition.

2 hours of OGD alone, did not compromise the capacity of astrocytes in glutamate uptake and in fact increased the uptake however, exposure of astrocytes to 2mM of glutamate along with 2 hours of OGD, significantly decreased the capacity of astrocytes in uptake of glutamate by 45% compared to control group (P <0.0001) (Figure 3A). Interestingly, pretreatment of cortical astrocytes with 0.1 μM of biphalin or 1 μM of nociceptin significantly increased the uptake of [^{3}H] glutamate in damaged astrocytes exposed to 2 hours OGD and 2mM glutamate compared to untreated groups (Figure 3B). Moreover, compatible with the uptake study, in these groups of astrocytes the expression of EAAT1 has been improved significantly by biphalin and nociceptin pretreatment compared to untreated group (3C). In another set of experiments, primary astrocytes were preincubated with 10 μM EAAT1 inhibitor, UCPH 101, prior to glutamate uptake to evaluate the role of EAAT1 in the clearance of extracellular glutamate. As depicted in figure 3D, 15 minutes exposure of astrocytes to UCPH 101 significantly decreased [^{3}H] glutamate uptake in the biphalin and nociceptin-treated cells by blocking the transport of glutamate via EAAT1.

3.4 Non-selective OR agonist, biphalin, shows superior glutamate buffering activity compared to subtype-selective OR agonists.

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To further investigate the potency of biphalin, we compared the effect of it with different selective opioid receptor agonists. As shown in figure 4, with the same concentration (0.1 μM), biphalin increases the uptake of [^{3}H] glutamate in astrocytes 44%, 52% and 45% more than selective kappa (U-50488), delta (DPDPE) and mu (DAMGO) opioid receptor agonists respectively confirming a better excitatory amino acid protective effect of biphalin compared to selective agonists. Similarly, biphalin increases the expression of EAAT1 more than selective opioid receptor agonists.

3.5 Biphalin and nociceptin stabilize BBB integrity in an *in vitro* co-culture model exposed to OGD and glutamate combined stress condition.

To investigate the possible role of astrocytes in increasing the barrier properties of the BBB and buffering the ions and transmitters, we established and used in-vitro coculture model of primary astrocytes and bEnd3 cells. We exposed this model with the combined stressor of 2mM glutamate and 2 hours OGD, which was intended to compromise the BBB and increase the paracellular permeability. We found that the percentage increase in the paracellular permeability in the monoculture of bEnd3 cells was 54% (0.00059 cm/min to 0.00091 cm/min), whereas in the coculture model this change was 32% (0.0005 cm/min to 0.00066 cm/min) (Figure 5A). These data suggest that astrocytes have an important role in influencing the BBB integrity during in vitro stroke conditions and with elevated levels of glutamate. To potentiate the protective role of astrocytes in maintaining the BBB integrity, the co-culture of bEnd3 cells and astrocytes were pre-treated with 0.1 μM biphalin or 1 μM nociceptin in the basolateral side of the model, where astrocytes reside. It was found that biphalin pretreatment significantly decreased the paracellular permeability compared to 2 hours OGD and 2mM glutamate exposed group (0.00058 cm/min vs 0.00041 cm/min; P <0.0001). To confirm that this effect is mediated through opioid receptors we

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further pretreated the coculture with 1 μM naltrexone prior to the experiment. Interestingly, we found that naltrexone significantly negates the effect of biphalin suggesting the role of opioid receptors in the mechanism of biphalin protection (0.00041 cm/min vs 0.00053 cm/min; *P* <0.01) (Figure 5B). Similar to biphalin, nociceptin treatment decreased the permeability of paracellular marker and improved the BBB integrity compared to combined stress exposed group (0.00058 cm/min vs 0.00036 cm/min; *P* <0.0001) and this effect was partially blocked by the nociceptin receptor's inhibitor, BAN ORL 24.

3.6 Biphalin and nociceptin attenuate the increased BBB permeability and improve the expression of tight junction protein in bEnd3 monolayers following combined stress condition (2 hours OGD + 2mM glutamate)

To evaluate the protective effect of biphalin and nociceptin directly on bEnd3 cells, monolayers of bEnd3 cells were exposed to either 2mM glutamate at normoxic condition or 2mM of glutamate and 2 hours of OGD. As depicted in figure 6A, 2 hours of OGD alone did not significantly increase the paracellular permeability of [^{14}C] sucrose compared to normoxic group however, addition of 2mM glutamate increased the BBB permeability significantly (0.00074 cm/min vs. 0.00051 cm/min; *P*<0.01). The most significant level compared to normoxia was obtained when both 2 hours of OGD and 2mM glutamate were combined (0.00091 cm/min vs 0.00051 cm/min; *P* <0.001). Pre-treatment of bEnd3 monolayers with 0.1 μM biphalin or 1 μM nociceptin significantly attenuated glutamate-mediated increase in the BBB permeability in OGD condition (0.00077 cm/min vs 0.00052 cm/min; *P* <0.05) (Figure 6B).

The most prominent structural and molecular components of the BBB are tight junctional proteins between the brain microvascular endothelial cells. To provide further mechanistic insight on the protective effect of biphalin and nociceptin on barrier function, in another set of

experiments we evaluated the expression of tight junction protein claudin-5 in bEnd3 cells after exposing the cells to 2 hours of OGD and 2mM of glutamate using western blotting and immunofluorescence staining. It was observed that, pretreatment of bEnd3 cells with biphalin or nociceptin significantly improved the expression of claudin-5 compared to untreated group following exposure to combined stress condition. The representative images of claudin-5 are depicted in figure 6C. Significant disruptions were seen in the distribution and expression of claudin-5 following combined stress which is attenuated with biphalin or nociceptin pretreatment since they resulted in more intense staining of claudin-5 at cell-to-cell contact points.

4. Discussion:

The main challenge in developing new therapeutics for the management of ischemic stroke is the complex pathophysiology of this disorder along with the incomplete understanding of contributing cellular and molecular mechanisms (Nozohouri *et al.* 2020b). It is well-known that paracellular permeability of compounds across BBB increases significantly following ischemic injury which is due to the opening of the tight junction proteins. Endothelial cells of the cerebral microvasculature function to protect neurons and glial cells from harmful insults. Among tight junction proteins, claudins, a family of transmembrane proteins, play a crucial role in sealing the gaps between cells and limiting paracellular passage of ions. Among various forms of claudin, claudin-5 is mainly expressed in cerebral microvascular endothelial cells.

Since ischemic stroke initiates multiple pathways resulting in neuronal cell death, successful neuroprotection needs stimulation of several pathways. Therefore, activation of opioid receptor subtypes to simultaneously target various neuroprotective pathways is a rational therapeutic strategy. The neuroprotective role of opioid receptor agonists in ischemic stroke has been widely reported by different research groups. Opioid receptor activation attenuates several

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pathophysiological factors such as excitotoxicity, inflammation and anoxic depolarization. Moreover, these receptors prevent BBB disruption, regulate astrocytic alterations, and offset potential adverse effects of tPA. It has also been reported that non-selective opioid receptor agonists improve stroke outcomes better than selective agonists (Popiolek-Barczyk *et al.* 2017; Yang *et al.* 2015).

However, opioid agonists have also contributed to ischemic stroke pathogenesis. Along with common side effects of abuse and addiction related to opioids, long-term pain management using prescribed opioids present neurovascular complications that contribute to ischemic stroke (Voon *et al.* 2018; Fallon *et al.* 2018). This class of drugs such as morphine, act primarily by activating μ-opioid receptors and result in mitochondrial dysfunction, oxidative stress thus BBB impairment (Feng *et al.* 2012; Woller *et al.* 2012; Moqaddam *et al.* 2009). Therefore, opioid antagonists could also provide an appealing therapeutic strategy for the management of ischemic stroke (Anttila *et al.* 2018; Chen *et al.* 2001; Liao *et al.* 2003).

Biphalin, is an example of non-selective, potent opioid receptor agonist that has high affinity to mu and delta opioid receptors. Nociceptin on the other hand, is an endogenous peptide that binds to G-protein coupled receptor, opioid receptor like-1 (ORL1), which is another member of opioid receptor family. Despite structural similarity of nociceptin to dynorphin A, it does not have binding affinity to the classical opioid receptors. In this study, we evaluated the cerebrovascular protective properties of biphalin and nociceptin.

This study focused on understanding how BMECs respond to in-vitro ischemic conditions after treatment with opioid receptor agonists. To accomplish this goal, we utilized in-vitro mono- and co-culture models which are composed of bEnd3 cells cultured with astrocytes positioned opposed on a polyester Transwell inserts. To simulate ischemia in-vitro, we optimized

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the timing of BBB disruption which was exposure of cells to 2 hours of hypoxic condition with the addition of 2 mM glutamate, as glutamate level was reported to increase up to 10 mM during ischemic injury (András *et al.* 2007). BMEC monolayers and co-culture of bEnd3 cells and astrocytes exposed to OGD and glutamate showed a significant increase in [^{14}C] sucrose permeability compared to control groups. These data are consistent with other published findings claiming increased permeability in BMECs treated with hypoxia for various durations (Mark & Davis 2002).

One important finding was that biphalin and nociceptin pretreatment prevented the increase in BBB opening through opioid receptors since the protective effect was negated by their corresponding inhibitors. The protective effect of biphalin and nociceptin on the BBB integrity is proposed to be mediated through glutamate. Therefore, it is crucial to understand how the increased level of glutamate during ischemia- reoxygenation disrupts the BBB mechanistically. The effect of glutamate-induced alterations of the tight junctional proteins in cultured brain microvascular endothelial cells occurred after exposure to glutamate and resulted in cellular redistribution of these proteins followed by a decrease in the expression level of them. Interestingly, biphalin and nociceptin treatments were effective in improving the expression of claudin-5 hence attenuating the paracellular permeability of [^{14}C] sucrose. However, pretreatment with biphalin and nociceptin did not improve the expression level and distribution of ZO-1 and occludin in damaged cells (data not shown).

Since stroke is both a vascular and neuronal disorder, it is advantageous to activate opioid receptors in different sites of neurovascular unit, mainly neurons and astrocytes, to exhibit the maximum beneficial effects. Astrocytes have been reported to express opioid receptors and the expression level of these receptors can be modulated by the exposure to different stimuli such as

glutamate (Thorlin *et al.* 1997). Astrocytes provide various housekeeping functions including formation of the BBB, modulation of cellular communications and protection against oxidative stress. Moreover, astrocytes play crucial roles in the removal of extracellular glutamate and potassium during the synaptic plasticity (Yao *et al.* 2014). It is well-known that in the ischemic brain, the most common neurotoxic transmitter is glutamate and excess amount of it results in excitotoxicity and neuronal damage. Various approaches such as NMDA receptor antagonists have been studied in clinical settings for the prevention of glutamate-induced excitotoxicity. However, application of NMDA antagonists for cerebral ischemia has failed in these trials since these antagonists hinder neuronal survival by blocking the synaptic transmission (Ikonomidou & Turski 2002; Wang & Shuaib 2005). Another therapeutic approach to inhibit glutamate mediated excitotoxicity is to decrease extracellular glutamate concentration through increasing the uptake of glutamate by EAATs (Simantov *et al.* 1999; Williams *et al.* 2005). The glutamate-aspartate transporter named EAAT1 in humans, is one of the main transporters during brain development and plays important role in protecting brain against glutamate-induced cytotoxicity. These transporters are predominantly expressed on astrocytes and their expression can be regulated pharmacologically. The up regulation of EAAT on astrocytes has been shown to improve neurological score and decrease infarct volume in experimental stroke animal models (Verma *et al.* 2010; Beschorner *et al.* 2007). In our study we also focused on evaluating the protective effect of biphalin and nociceptin on the glutamate clearance following glutamate-induced injury in primary cortical astrocytes. To perform these experiments, we measured the uptake of [^{3}H] glutamate in primary cortical astrocytes. First, we validated the published method reported in primary rat cortical astrocytes in mice cortices using nociceptin treatment as a positive control since nociceptin regulates the levels of the glutamate/aspartate transporters GLAST/EAAT1 in

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both human and rodent brain astrocytes (Meyer *et al.* 2017). GLAST/EAAT1 upregulation by nociceptin is mediated by nociceptin opioid receptors and the downstream participation of a complex signaling cascade that involves the interaction of several kinase systems.

Interestingly, biphalin acted similarly as nociceptin and increased the glutamate uptake in primary cortical astrocytes. In addition, biphalin effect on glutamate uptake was observed at 0.1 μM concentration which is the same effective concentration that stabilized the BBB permeability compromised by OGD and glutamate. Exposure of primary cortical astrocytes to 2 hours of OGD increased the glutamate uptake however, combined stress decreased the capacity of astrocytes in [^{3}H] glutamate uptake significantly. Pretreatment with 0.1 μM biphalin and 1 μM nociceptin increased the protective effect of astrocytes and their effect was negated by opioid receptor and nociceptin receptor antagonists. Interestingly, biphalin was more effective than nociceptin and selective kappa (U-50488), delta (DPDPE) and mu (DAMGO) opioid receptor agonists in increasing the capacity of astrocytes for glutamate uptake and nociceptin showed similar effect to the selective OR agonists. The mechanism behind this effect was evaluated by western blotting to investigate the expression level of glutamate transporters.

As expected, pretreatment with biphalin and nociceptin increased the expression of astrocytic EAAT1. Additionally, the effect of upregulated EAAT1 was significantly blocked by the selective inhibitor of this transporter which further confirms that the effects of Bi and NOC in glutamate uptake is mediated through EAAT1 overexpression and that EAAT1 is the major contributor to glutamate clearance in astrocytes. Overall, the BBB can be considered as one of the main targets of biphalin and nociceptin when treating ischemic stroke. These opioid receptor agonists have protective effects on both the function and structure of the BBB under stress conditions. Biphalin and nociceptin maintain the integrity of tight junctions by improving the

expression of claudin-5. Moreover, they enhance the protective function of astrocytes in clearing the extracellular glutamate by increasing the expression of glutamate transporter, EAAT1. These results have important clinical implications about possible prevention of vasogenic edema, which can be a life-threatening result of ischemic stroke.

Future studies are warranted to investigate the effect of nociceptin and biphalin on neurons in ischemic conditions. It is also crucial to consider the signaling mechanisms behind the increased expression of EAAT1 during stress condition. Since BBB comprises various cell types, studies using a triple culture model of the neurovascular unit cells would be remarkable to evaluate the impact of other cells on the expression of EAAT1 and the role of opioid receptors. The results of the current study provide an opportunity to design a preclinical study to investigate the BBB protection potential of opioid receptor agonists, as putative stroke treatments alone or in combination with thrombectomy or tPA administration.

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Figure legends:

Figure 1: Concentration and time-dependent effect of biphalin and nociceptin in glutamate uptake and EAAT1 expression in primary cortical astrocytes. Increasing concentrations of biphalin (A) and nociceptin (B), increase [^{3}H] glutamate uptake in primary cortical astrocytes in normoxic condition. (C) Which is due to the increased expression of glutamate transporter, EAAT1 in these cells. (D) Biphalin and nociceptin increase the expression of EAAT1 in a time-dependent manner and different exposure time results in overexpression of EAAT1 accordingly. (E) The overexpression of EAAT1 is blocked by the antagonists of NOP and classical opioid receptors which suggests that the role of these compounds in increasing the expression of glutamate transporter is mediated through opioid receptors. Data represented as Mean ± SD of 3 to 4 independent replicates. ** P <0.01, **** P <0.0001.

Figure 2. **Effect of biphalin and nociceptin pretreatment in [^{3}H] Glutamate uptake and EAAT1 expression in primary cortical astrocytes following exposure to 2 hours of OGD.** 2 hours of OGD increase the capacity of astrocytes in glutamate uptake and pretreatment with biphalin (A) or nociceptin (C) further increase this capacity which is inhibited by the opioid receptor antagonists. This effect is mediated by significant up-regulation of EAAT1 in primary

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cortical astrocytes (B, D). Data represented as Mean ± SD of 3 to 4 independent replicates. * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001.

Figure 3. Effect of biphalin and nociceptin on glutamate uptake in astrocytes exposed to OGD and glutamate combined stress condition. (A) Exposure of astrocytes to 2mM of glutamate along with 2 hours of OGD, significantly decreased the capacity of astrocytes in uptake of glutamate. (B) Pretreatment of cortical astrocytes with 0.1 µM of biphalin or 1 µM of nociceptin significantly increased the uptake of [^{3}H] glutamate in damaged astrocytes exposed to 2 hours OGD and 2mM glutamate compared to untreated groups. (C) This effect is mediated through upregulation of EAAT1 in primary cortical astrocytes. (D) The effect of EAAT1 in the transport of extracellular glutamate in biphalin and nociceptin-treated astrocytes was significantly blocked by the selective inhibitor of EAAT1, UCPH 101 (10 µM), reflecting the major contribution of this transporter in the clearance of glutamate. Data represented as Mean ± SD of 3 to 4 independent replicates. ** *P* <0.01, **** *P* <0.0001.

Figure 4. Comparison between the effect of biphalin and selective opioid receptor agonists in inducing the glutamate uptake by primary astrocytes. (A) with the same concentration (0.1 µM), biphalin increases the uptake of [^{3}H] glutamate in astrocytes more than selective kappa (U-50488), delta (DPDPE) and mu (DAMGO) opioid receptor agonists confirming better protective effect of biphalin compared to selective agonists. (B) Also, biphalin increases the expression of EAAT1 more than selective opioid receptor agonists. Data represented as Mean ± SD of 3 to 4 independent replicates. ** *P* <0.01, *** *P* <0.001.

Figure 5. (A) The % increase in the paracellular permeability in response to glutamate and OGD. It was found that the monoculture of bEnd3 cells was almost 20% more permeable than the coculture model which is much resistant to combined stress condition

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indicating the role of astrocytes in the BBB integrity and protection. **(B) Role of opioid receptors in the leakiness of the BBB in coculture model.** Biphalin significantly stabilized the BBB and attenuated the increase in paracellular permeability due to the combined stress condition. Naltrexone pretreatment negated this effect indicating that the action is mediated through opioid receptors. Nociceptin treatment also, showed similar effect on the BBB permeability confirming the involvement of opioid receptors in the integrity of BBB. Data represented as Mean ± SD of 3 to 4 independent replicates. ** P <0.01, *** P <0.001**.**

Figure 6. Effect of biphalin and nociceptin on the BBB permeability and the expression of tight junction proteins in bEnd3 monolayers following combined stress condition (2 hours OGD + 2mM glutamate). (A) 2 hours of OGD did not cause significant alteration in [^{14}C] sucrose paracellular permeability but addition of 2 mM glutamate increased the permeability of paracellular marker in normoxic condition. Combination of 2mM glutamate and 2 hours OGD significantly increased the permeability of radio-labeled marker. (B) Biphalin or nociceptin pretreatment significantly attenuated the effect of 2mM glutamate and 2 hours OGD by decreasing the paracellular permeability of [^{14}C] sucrose compared to untreated group, suggesting the involvement of glutamate in the protective mechanism of action of these opioid agonists. (C) Representative immunofluorescence images showing protein expression and localization of claudin-5 in bEnd3 cells after exposure to combined stress condition and treatment with biphalin or nociceptin. (D) Western blot studies also confirmed that Biphalin and nociceptin treatment increase the expression of claudin-5 in cells exposed to combined stress conditions. Data represented as Mean ± SD of 3 to 4 independent replicates. * P <0.05, ** P <0.01, *** P <0.001.

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Figure 1.

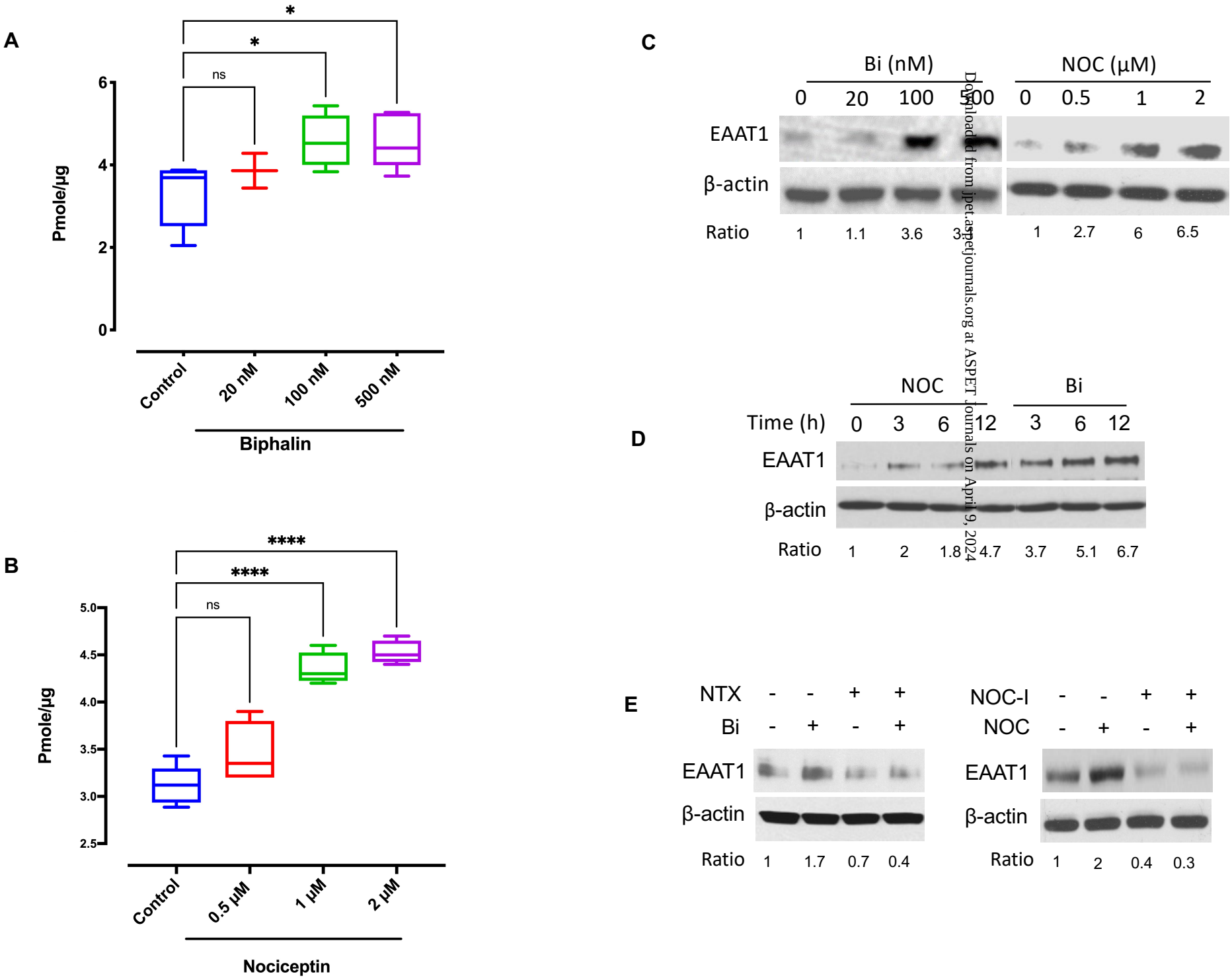
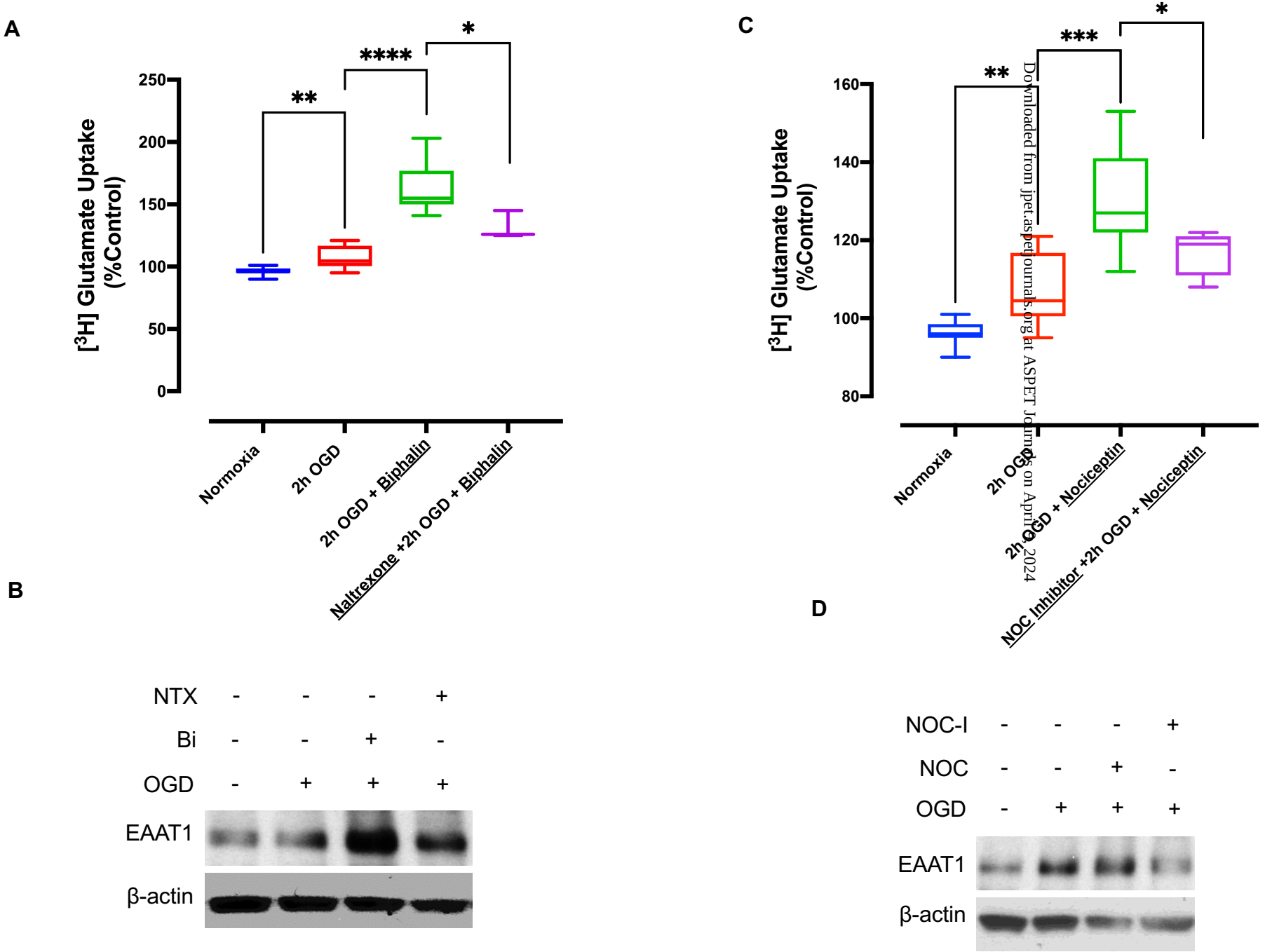


Figure 2.



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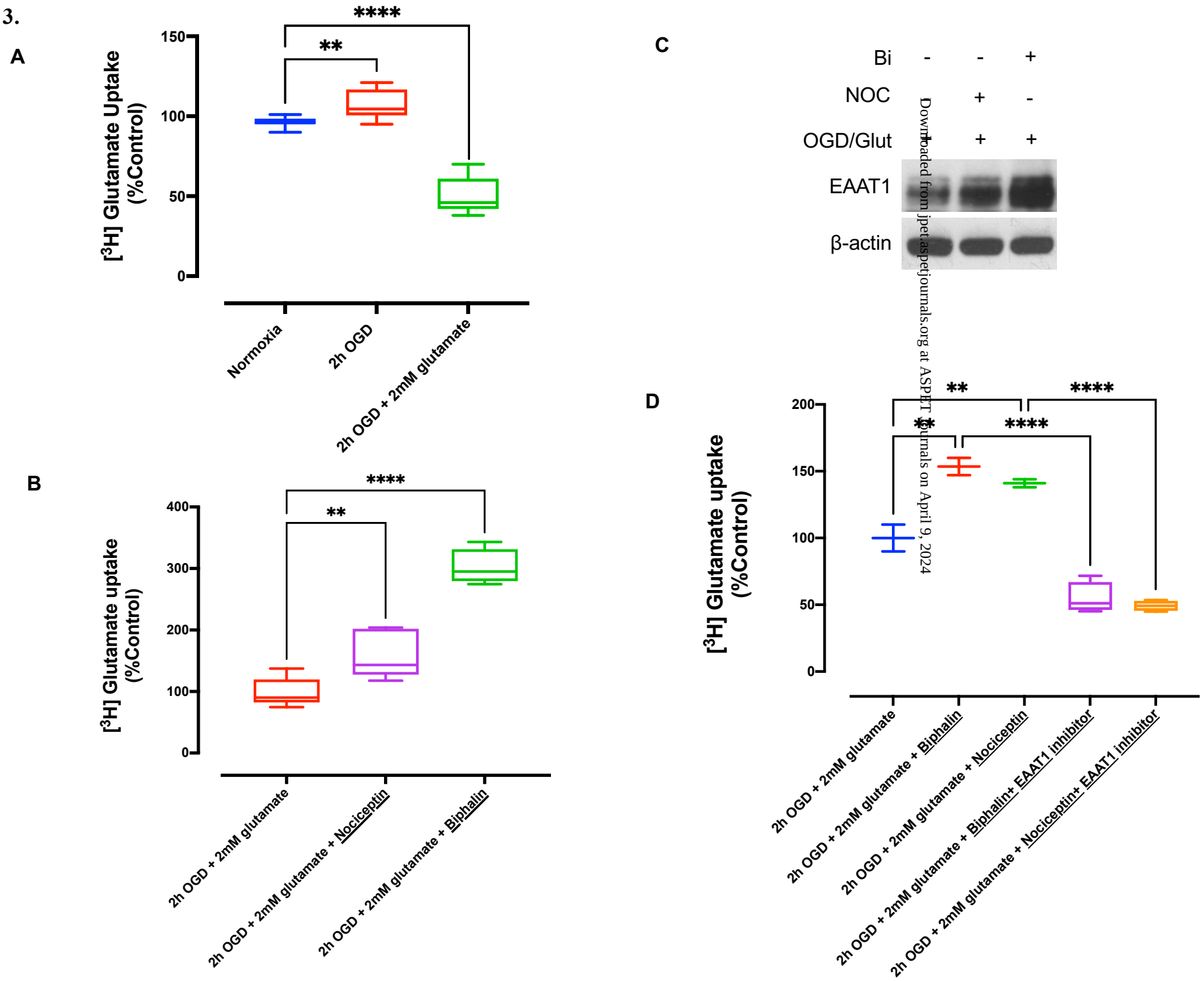

Figure 4.

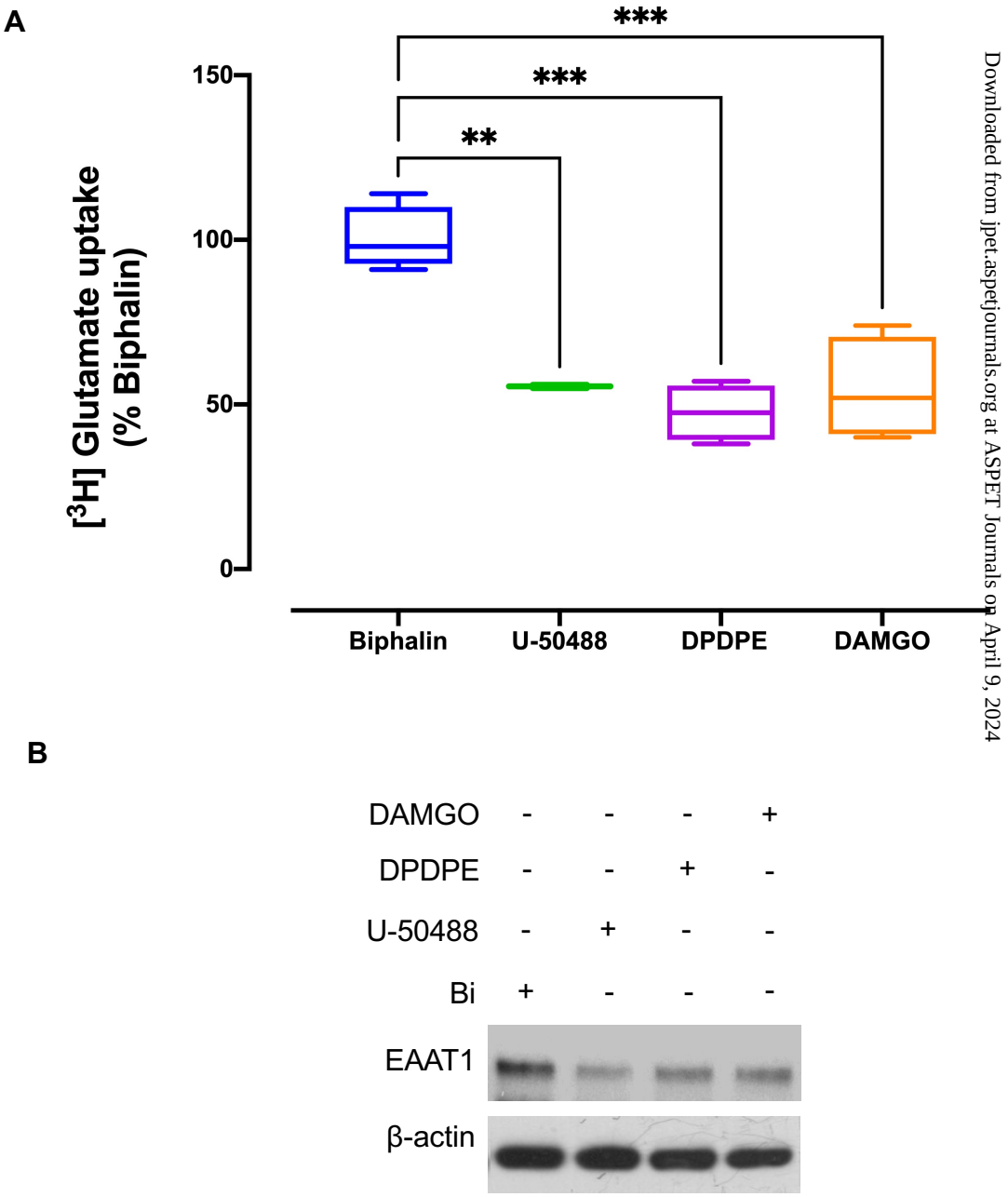

Figure 5.

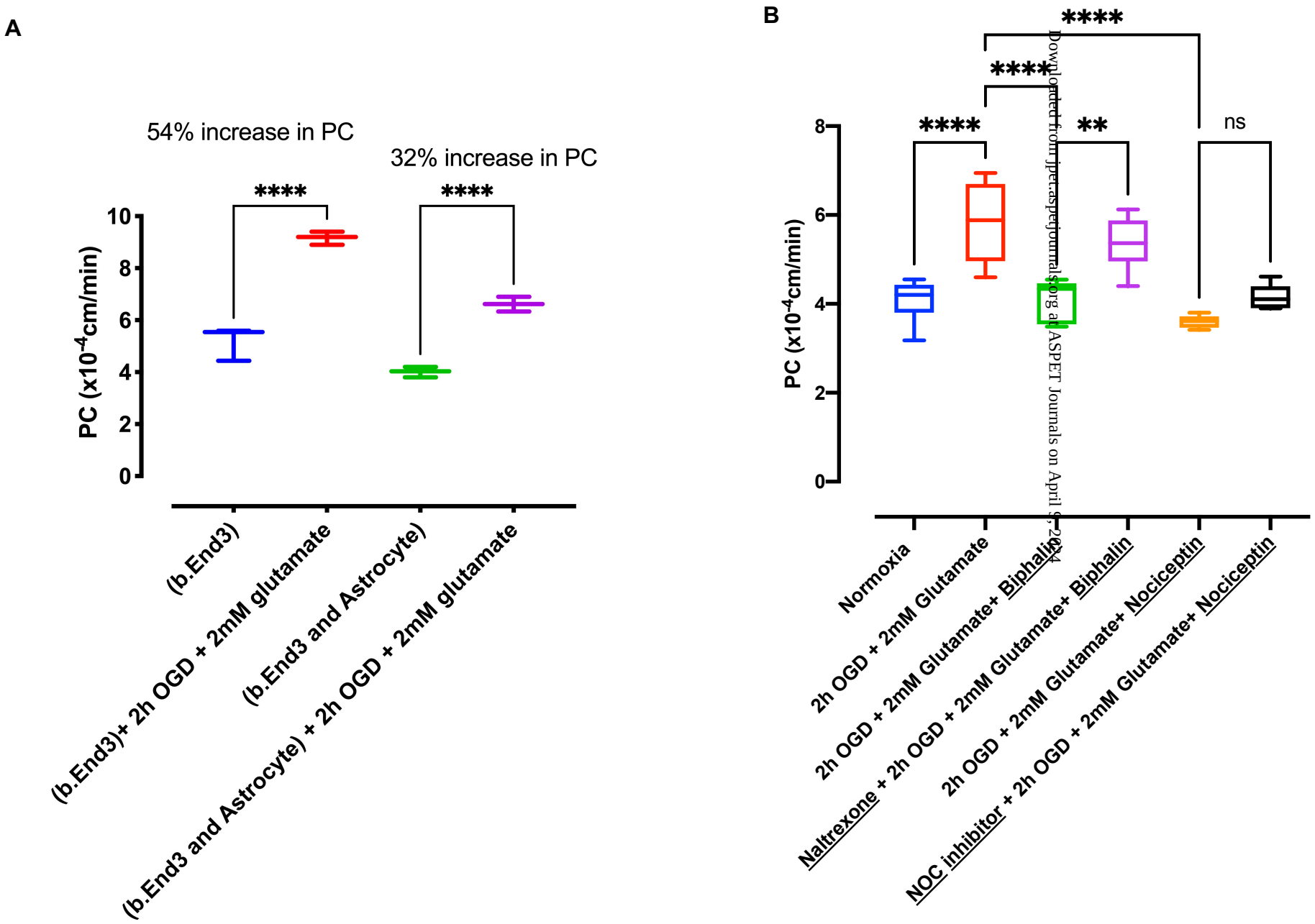

Figure 6.