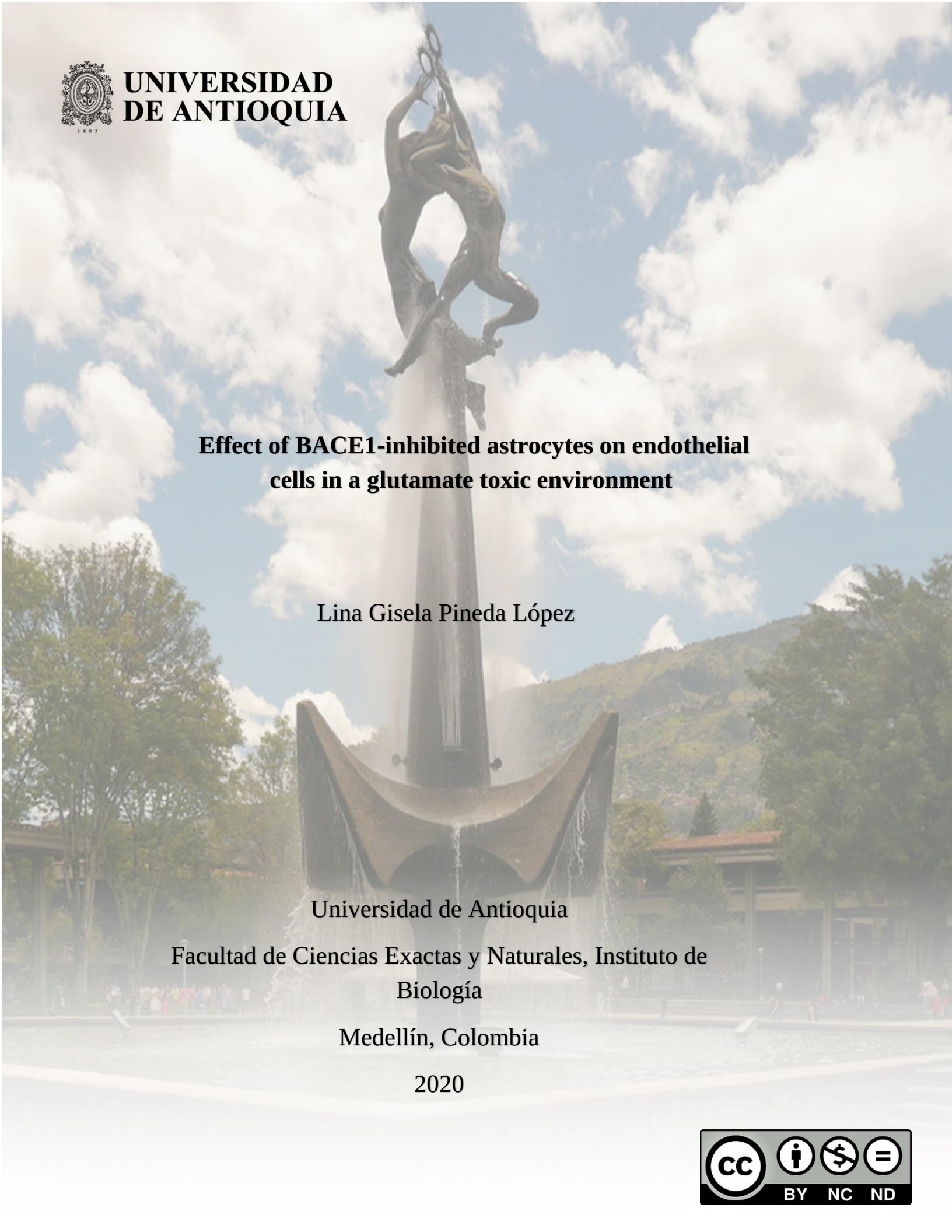

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Effect of BACE1-inhibited astrocytes on endothelial cells in a glutamate toxic environment

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Abstract

After cerebral ischemia or during several central nervous system (CNS) pathologies, exacerbated high glutamate concentration are released into the cerebral parenchyma producing a toxic environment that damages the components of the neurovascular unit (NVU) including the endothelial cells; which affects the function and structure of the blood-brain barrier. Astrocytes, glial cells that are closely related to the endothelium, change their gene expression profile in the face of toxicity and, depending of such profile, can be either protective or damage mediators. The enzyme BACE-1, involved in amyloidosis and vascular deterioration, increases its expression after an ischemic event even in astrocytes. Given the above, our aim was to determine the effect of BACE-1-inhibited astrocytes on endothelial cells integrity in a glutamate toxic environment by observing the effect of BACE-1 inhibition in endothelial cells and astrocytes cultures and endothelium-astrocyte co-cultures, through cytotoxicity and immunofluorescence assays. The results obtained suggest BACE1 inhibition protects endothelial cells integrity by reversing structural and inflammatory damage, also reverses astrocytic reactivity causing structural changes in cytoskeleton and cell inflammation. Furthermore, BACE1-inhibited astrocytes protect endothelial cells integrity regulating the Zonula occludens-1 distribution and decreasing inflammatory processes caused by glutamate toxicity. In summary, these results suggest inhibition of BACE1 under a glutamate toxicity has a protective effect on endothelium and astrocytes and their interactions.

Key words: BACE1 inhibition, astrocytes, endothelium, glutamate, protection.

Significance Statement:

The present research shows that inhibition of BACE1 in endothelial cells has a protective effect against glutamate toxicity, by regulating the distribution of tight junction proteins. Furthermore, in astrocytes, the BACE1 inhibition causes reactivity changes and cytoskeleton remodeling. Finally, BACE1 inhibition in astrocytes protect functional and structural

endothelial cells integrity decreasing the pro-inflammatory environment. These results lead to new questions about the role of BACE1 in the neurovascular unit and the mechanisms underlying it.

INTRODUCTION

The blood brain barrier (BBB) is a structure that acts as an interface between the CNS and the peripheral circulatory system. The BBB selectively filters through the endothelium allowing the influx of oxygen and nutrients, in addition to the delivery of cellular debris, but restricting the entry of cells or molecules that can alter brain parenchyma integrity [1]. The properties of BBB are regulated by the function of the neurovascular unit (NVU), which is composed of endothelial cells (EC), in contact with the basal lamina, pericytes, astrocytes and neurons, which are immersed in an interstice with the extracellular matrix [2,3]. Astrocytes are closely related to the EC that structure the BBB and support its properties through trophic factors release that maintain the integrity of tight junctions such as Claudin-5 (CLDN5) and Zonula occludens-1 (ZO-1) [4–7]. Those astroglial cells maintain the brain parenchymal homeostasis by regulating energy and metabolism, blood flow and synapse function. The NVU components are susceptible to damage by high glutamate concentration; specifically in EC, glutamate trigger apoptosis mediated by oxidative stress [8,9]. Although, glutamate is the main excitatory neurotransmitter in the brain, in high extracellular concentrations can cause massive damage by excitotoxicity, due to a prolonged or excessive activation of excitatory receptors that trigger apoptosis and/or necrosis signaling pathways [10,11]. This process is common in ischemic stroke and neurodegenerative diseases, in which occur the morphological and functional alterations of EC affecting BBB integrity [12–15].

Complementarily, astrocytes can undergo cellular, molecular and functional changes in response to tissue disturbances, by excitotoxicity, producing astrogliosis [16]. Reactive astrocytes may be involved in both neurodegeneration and neuroprotection; in terms of neurodegeneration, they mediate and propagate pro-inflammatory signals, in addition to activating catabolic processes and triggering apoptosis; but they also intervene in the uptake and synthesis of neurotransmitters, induce angiogenesis and mediate antioxidation,

contributing to neuroprotection. The role of reactive astrocytes depends on the profile of genes that are expressing differentially and the post-injury time [17].

Among some proteins of interest in this context is the enzyme BACE1 (beta-site amyloid precursor protein cleaving enzyme 1). BACE1 is a protease located in lipid rafts, localized in endosomes and the trans Golgi network [18]. It has been characterized by its participation in amyloidosis and vascular deterioration. Although its expression had been initially described in neurons, it has already been reported to be expressed in astrocytes and EC, and also that its levels increase on stressful events such as an ischemic stroke where a pro-inflammatory microenvironment is generated [19–23]. The inhibition of BACE1 has been studied as a possible therapeutic target for the Alzheimer´s disease treatment; however it has been done mainly in neurons, both *in vitro* and *in vivo*, and several beneficial results have been obtained, among them: blocking toxic peptides Aβ production, reduction pro-inflammatory response and recovery of cognitive function in 3xTgAD mice [18,24,25].

According, since the BACE1 enzyme is involved in vascular deterioration given by stressful conditions such as toxicity induced by high glutamate concentrations, leading to disruption of NVU function and therefore of BBB, in which there is a close relationship between the astrocyte and endothelium, our aim was to determine the effect of BACE1-inhibited astrocytes on endothelial cells in a toxic environment by glutamate, to understand the role of BACE1 at NVU components, which has not completely understood in neurological diseases yet.

MATERIALS AND METHODS

bEnd.3 cell line cultures

The bEnd.3 (ATCC CRL-2299) murine cell line was used as an endothelial cell model as we described previously [26]. The bEnd.3 cells were thawed in DMEM culture medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum (FBS, Eurobio) and 1% of penicillin–streptomycin mixture (GIBCO). After 24 hours, the medium was replaced to the maintenance medium (DMEM supplemented with 10% fetal bovine serum (FBS, Eurobio) and 1% of penicillin–streptomycin mixture (GIBCO)). The cells were incubated at 37 ° C and 5% CO2.

To perform the subcultures, the cells were trypsinized using 0.25% trypsin/EDTA mixture (GIBCO) during 5 min and subcultured in 12-well plates at a density of 2.5×10^5.

Astrocytes primary cultures

The astrocytes were obtained from cortical primary cultures of brain extracted from Wistar rats on post-natal day 1 or 2 as we described previously [26,27]. The cortex was enzymatically dissociated with trypsin, cultured in T75 flasks (surface area $75cm^2$) and maintained in DMEM (Sigma Aldrich) supplemented with 10% FBS (Eurobio) and 1% of penicillin–streptomycin mixture (GIBCO). The cells were incubated at 37 °C and 5% CO2. The culture medium was changed every 2 days. From day in vitro (*DIV*) 8 until *DIV* 10, the flasks were shaken at 350 rpm in a sequence of 6 h, 18 h, and 24 h to minimize the amount of microglia and oligodendrocytes. Then, the cells were trypsinized using 0.25% trypsin/EDTA mixture (GIBCO) during 5 min and subcultured in 12-well plates at a density of 7.5×10^4.

bEnd.3 and astrocytes co-culture

Astrocytes were subcultured on the *DIV* 10 on coverslips in 12-well plates. The bEnd.3 cells were thawed and subculture on gelatinized coverslips with 4 paraffin dots at the ends until *DIV* 15 when the co-culture was performed. On *DIV* 21, the co-culture was disassembled to inhibit BACE1 on astrocytes, 24 hours later the co-culture was reassembled and treated with glutamate. In *DIV* 23, the culture medium was collected to measure cytotoxicity and both cell types were fixed to perform immunofluorescences.

Glutamate-induced toxicity assay and inhibitor treatment

i. **bEnd.3:** The bEnd.3 cells cultures were pre-treated on the *DIV* 8 with the β-secretase Inhibitor IV (CAS 797035-11-1, Merck) at 1 μM, 24 hours later were treated with glutamate at 125 μM for 20 minutes and treated again with the inhibitor. On the *DIV* 10 the culture medium was collected to measure cytotoxicity and the cells were fixed to perform immunofluorescences.

ii. **Primary astrocytes:** Astrocytes were pre-treated on the *DIV* 21 with the β-secretase Inhibitor IV at 1 μM, 24 hours later were treated with glutamate at 125 μM for 24 hours and treated again with the inhibitor. On the *DIV* 23 the culture

medium was collected to measure cytotoxicity and the cells were fixed to perform immunofluorescences.

iii. **Co-culture:** In the co-culture, BACE1 was inhibited in the astrocytes at the previously described concentration, however both cell types were exposed to glutamate treatment for 20 minutes. On the *DIV* 23, the medium was collected to measure cytotoxicity and the co-culture was disassembled to fix both cell types.

LDH assay

The cytotoxicity was measured through the percentage of LDH release with the Roche LDH Cytotoxicity Detection Kit. The assay was performed by mixing the media with the mixture of the two kit solutions, which was incubated for 30 minutes in the dark and finally the absorbances of the samples were measured at 490 nm in a microplate reader. The percentages of LDH release were calculated using the following formula: % LDH release = [(A- Low control) / (High control- Low control)]*100, where A was the absorbance indicating the LDH activity level, the low control was the LDH activity of basal release from untreated cells and the high control was the measure of maximum LDH release from cells (Cells treated with Triton X-100 at 1%).

Immunofluorescence

The cell cultures were fixed with 4% paraformaldehyde prepared in cytoskeleton buffer (CBS). Autofluorescence was eliminated using 50 mM ammonium chloride (NH_4Cl) for 10 min. The cells were permeabilized with 0.2% Triton X-100 prepared in CBS and then blocked with blocking solution (2.5% FBS in CBS). The cultures were incubated overnight at 4°C with mouse primary antibodies against mouse CLDN5 (1:750, Invitrogen), rabbit ZO-1 (1:500, Thermo Fisher Scientific), mouse GFAP (1:750, Sigma), rabbit BACE1 (1:500, Abcam) and mouse IL-1β (1:500, Abcam). Subsequently, incubation for 1 hour with secondary antibodies was performed with Alexa 594 o 488 (1:500, Molecular Probes), the nuclei and cytoskeleton were stained with Hoechst 33258 (1:5000, Invitrogen) and phalloidin conjugated with Alexa 594 or Alexa 488 (1:500, Molecular Probes. Then, serial washings with PBS (Phosphate-buffered saline) were performed and the coverslips with the immunolabelled cells were fixed to slides with Fluorosave (Calbiochem). The cells were

observed under an Olympus IX 81 epifluorescence microscope and the images were captured with an oil immersion objective (60X, NA 1.42) and then processed.

Morphological analysis

i. Condensed nuclei

The average diameter of each nucleus was quantified by automatic measurements using the "Count and measure objects" tool of Image Pro-Plus software. Nuclei with diameter between 3.0 and 6.0 μm were defined as condensed. The percentage of condensed nuclei were calculated using the following formula: Percentage of condensed nuclei = [condensed nuclei / (condensed nuclei + normal nuclei)] $*$ 100.

ii. Number and area of gaps

In the Adobe Photoshop software, a binary image of each analyzed field was generated where the gaps were plotted using the "Magic Wand" tool. In the images obtained, the number and area of gaps were measured using the "Count and measure objects" tool of Image Pro-Plus software.

iii. Fluorescence intensity quantification

Fluorescence intensity measurements of each protein were obtained from colors channels through the calculation of the intensity, using the "Measure" tool, which was relativized with the cell area. These analyzes were performed using Image J (NIH software).

iv. Fluorescence profiles

To determine the Claudin-5 distribution, a fluorescence profile was established by drawing a line (50 μm) throughout the cells using the "Line profile" tool of Image Pro-Plus software.

Statistical analysis

The parametric data were compared using one-way ANOVA followed by the Tukey-Kramer post-hoc test to determine the means that was significantly different from each other. The non-parametric data were compared using the Kruskal-Wallis test and Dunn's test with Bonferroni correction. The results were considered significant when $p<0.05$ (* indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$). Data analysis were performed using the software R version 3.4.4 (2018, The R Foundation for Statistical Computing).

RESULTS

BACE1 inhibition protects EC integrity under glutamate toxicity by reversing structural and inflammatory damage

The BACE1 inhibitor (β-secretase inhibitor IV) was used at 1, 5, 7.5 and 10 μM on EC to determine the concentration for experiments through cytotoxicity measurement (data not shown). The concentration at 1 μM had the least toxic effect, therefore, it was used for the treatments. To characterize the effect of glutamate and BACE1 inhibitor (BACE1i) on the endothelial cell line bEnd.3, cytotoxicity was measured through the percentage of LDH release and immunolabelling were performed to determine the cells state. In terms of percentage of LDH, there were no significant differences between the treatments but a tendency to increase by glutamate and an apparent reversal of this effect by BACE1i were observed (Fig. 1A). However, glutamate caused a significant increase in the percentage of condensed nuclei (Fig. 1B) and in the number and area of gaps between cells (Fig. 1C and D). These effects, except the number of gaps, were significantly reduced by the BACE1i. Despite glutamate treatment produced depolymerization of actin cytoskeleton which remained on BACE1 inhibition, tight junction protein CLDN5 immunoreactivity was partially recovered by BACE1i; distributing it at the membrane from cytoplasm under glutamate condition (Fig. 1E). This finding was confirmed by the CLDN5 profile fluorescence intensity, which BACE1 inhibition prevents the increase immunoreactivity at the cytoplasm caused by glutamate, and it was partially was redistributed at the cell membrane. (Fig. 2A and B).

Complementarily, BACE1 inhibition decreases the BACE1 immunoreactivity, which had been increased by the glutamate treatment, and reversed the glutamate-induced inflammatory damage, through the reduction of IL-1β immunoreactivity (Fig. 3B and C). BACE1 distribution in controls is apparently perinuclear while IL-1β is throughout the entire cell in a mainly diffuse pattern with few brighter spots that may be vesicles. Glutamate treatment extended BACE1 distribution to the entire cell although the highest concentration remains in the perinuclear zone where it apparently collocates with IL-1β. BACE1 inhibition upon stimulation with glutamate prevents IL-1β perinuclear concentration, but not in the case of

BACE1 which is maintained mainly in such zone but with a diffuse pattern throughout the cells (Fig. 3A).

Together, these results suggest that BACE1 inhibition maintains the endothelial cell integrity preventing cell death and inflammation and recovering cellular junctions disruption under a glutamate toxicity event.

BACE1 inhibition reverses astrocytic reactivity causing cytoskeleton remodeling and cell inflammation

To assess the astrocytes morphology with reduction in BACE1 expression under a glutamate toxicity environment, immunolabeling was performed to determine astrocytic reactivity using the GFAP (Glial Fibrillary Acidic Protein) marker and furthermore, cytotoxicity was measured through percentage of LDH release. Glutamate treatment increased the percentage of LDH with respect to controls and BACE1 inhibition did not significantly reduce it, although there was a downward trend (Fig. 4A). On the other hand, GFAP fluorescence intensity increased with glutamate treatment and BACE1 inhibition reduced this effect to baseline levels observed in controls (Fig. 4B). Morphological characterization shows the above as well as changes in the microfilaments and intermediate filaments of the cytoskeleton (Fig. 4C). BACE1 inhibition induces filopodia-like process of actin and it also depolymerizes the GFAP and throw out to the extracellular space, these effects were found even when the astrocytes had been treated with glutamate. In addition, BACE1 inhibition reversed glutamate-induced inflammatory damage (Fig. 5A). IL-1β immunoreactivity had a clear trend to decrease, although there was not significant, while BACE1 protein levels decreased significantly to baseline levels of controls (Fig. 5C and B). The distribution of BACE1 immunolabeling extends throughout whole cell, with stronger bright spot at the perinucleus, where looks overlapping with IL-1β, whose subcellular organelle must be confirmed (Fig. 5A). In summary, these findings suggest that BACE1 is involved in the astrocytes activation and that its inhibition under toxic events could reverse astrocytic hyperreactivity and inflammation.

BACE1-inhibited astrocytes protect EC integrity regulating ZO-1 distribution and decreasing inflammation caused by glutamate

To validate the effect of BACE1-inhibited astrocytes on endothelium inflammation and cell disruption, astrocyte-endothelium coculture under glutamate toxicity were performed. Immunolabeling of ZO-1 and IL-1β and the percentage of LDH release was performed to determine the cellular effect. Glutamate treatment significantly increased the percentage of LDH with respect to DMSO control, effect did not reverse by BACE1-inhibited astrocytes. However, the cytotoxicity values that were presented were low since the increase in cells treated with glutamate was approximately 2.2% compared to the controls (Fig. 6A). Accordingly, glutamate treatment increased the percentage of condensed nuclei by 3% compared to DMSO control and BACE1-inhibited astrocytes did not significantly reduce it, although there was a downward trend (Fig. 6B). The percentage of condensed nuclei, caused by glutamate treatment, in endothelial cells not cocultured with astrocytes was 38% (Fig. 1B) while in coculture this value was 5% (Fig. 6B), indicating the protective effect of astrocytes on the endothelium in the face of stressful stimuli. In terms of structural damage, both the number and area of the gaps were increased by treatment with glutamate, and BACE1-inhibited astrocytes only significantly reduced the number (Fig. 6C), although there was an evident tendency to decrease the area (Fig. 6D). Regarding inflammation, glutamate treatment caused a significant increase in IL-1β immunoreactivity with respect to the inhibitor alone, but this increase was not statistically different from the DMSO control. BACE1-inhibited astrocytes reduced a non-significant 3.91% of such an increase, which is a clear downward trend (Fig. 6E). Furthermore, the basal levels of this cytokine were lower in this case compared to those of cells not co-cultured with astrocytes (Fig. 3C).

The observed phenomenon of cytoplasmic distribution and immunoreactivity change of CLDN5 by glutamate treatment also occurs with the tight junction protein ZO-1 (Fig. 7). Tight junction protein ZO-1 immunoreactivity was partially recovered by BACE1-inhibited astrocytes; distributing it at the membrane from cytoplasm under glutamate condition (Fig. 6F). This finding was confirmed by the ZO-1 profile fluorescence intensity, which BACE1-inhibited astrocytes prevents the increase immunoreactivity at the cytoplasm caused by glutamate, and it was partially was redistributed at the cell membrane (Fig. 7A and B)

Together, these results suggest that BACE1-inhibited astrocytes have a beneficial effect on endothelial cells against tight junction damage and inflammation under glutamate toxicity.

Discussion

Our findings suggest that BACE1 inhibition protects cellular integrity from glutamate-induced damage in both endothelium and astrocytes. In the case of EC, the percentage of LDH released after treatments were very low, probably because of exposure time to glutamate, but the observed trend may indicate the glutamate harmful effect and its reversal due to BACE1 inhibition, which is clearly evident in condensed nuclei quantification due to the fact that chromatin condensation occurs in early stages of excitotoxicity and is prior to plasma membrane disruption and, therefore, to LDH release that occurs later in the cell death process [28,29].

Cellular damage in EC was apparently reversed by BACE1 inhibition through reducing inflammation, as suggested by IL-1β immunolabeling results, and CLDN5 partial redistribution to the cell periphery. Pro-inflammatory cytokines upregulation in the NVU or peripheral circulation has been associated with changes in BBB permeability due to tight junctions disruption between EC and phenomena such as monocyte adhesion or leukocyte trafficking, in addition to cytoskeleton conformation changes [30]. The relationship between excitotoxicity and the inflammatory phenomenon, focusing on IL-1β, has been extensively studied [31] but the relationship between this cytokine and BACE1 has not. However, the tumor necrosis factor-α (TNF- α) has been linked to the disruption of tight and adherent junctions between EC by BACE1 levels increased. Among BACE1 multiple substrates is ST6Gal-l, which is a protein involved in sialylation: a fundamental post-translational modification involved in endothelial function. BACE1 levels increased leads to cleavage and subsequent degradation of St6Gal-I, decreasing its activity and therefore the proteins stability that maintains endothelial monolayer integrity [32]. The above partially explains why BACE1 inhibition in the context of glutamate-induced toxicity, which is related to inflammation, decreased the area of gaps between EC, thus protecting the cellular monolayer.

Regarding the CLDN5 redistribution phenomenon, it has been previously reported that in presence of inflammatory or starvation stimuli, there is an increase in the cytosolic fraction of the protein and a consequent decrease in the membrane [33–35]. CLDN5 distribution dynamics seems to be regulated by autophagy, a mechanism that acts as a protector, in that

context, against BBB damage [35]. Because, it has been reported that BACE1 inhibition recovers lysosomal and autophagic defects in neurons [36]; therefore, a future study is needed to determine whether such inhibition enhances autophagy in EC and through that mechanism the CLDN5 normal distribution is recovered.

In the case of astrocytes, the levels of LDH released after treatments were also very low although the exposure time to glutamate was longer. Astrocytes can perform the uptake process of such neurotransmitter and subsequently metabolize it so their cell viability is not significantly affected by that stimulus [37]. Interestingly, BACE1 inhibition in astrocytes with or without the glutamate treatment caused cytoskeleton changes, both in actin and intermediate filaments. Interestingly, BACE1 overexpression has been reported to decrease the amount of F-actin rich filopodia-like protrusions in neurons through regulation of B4, an auxiliary subunit of a voltage-gated sodium channel [38]. Additionally, BACE1 has also been related to growth cone collapse regulation [39]. In both cases, our protein of interest is related to dynamic changes in actin, however, this regulation occurs in specific molecules or processes of neurons so additional studies are needed to clarify the mechanisms through which BACE1 is changing the actin dynamics in astrocytes, and also of GFAP for which there are not reports yet.

The observed morphological changes in astrocytes, in addition to inflammation condition, could be related to BACE1 overactivation on astrogliosis, where BACE1 inhibition recover astrocytes function. To date there is only evidence that focal activation of astrocytes coincides with BACE1 levels increased [40], but the mediating mechanisms in this process should be elucidated. The increase in IL-1β protein expression has been observed in astrocytes after excitotoxicity induction [31], which is consistent with the observed increase in immunoreactivity when stimulated with glutamate. It has been reported that such increase in glial cells can cause the activation of the inflammatory mediator COX-2 in neurons, which in turn upregulates BACE1 through PGE2 and cAMP in a reciprocal interaction of both cell types [41]. Since astrocytes interact with the different components of the NVU where there are conserved signaling pathways whose molecules have an action on each cell type, it is probable that IL-1β increase in astrocytes also promotes cyclooxygenases activity in EC

which would have as a consequence not only the increase of prostaglandins action as vasodilators but also the BACE1 upregulation [41,42].

Specific interactions between the brain endothelium and astrocytes at NVU, like the hypothetical one above, could influence BBB phenotype and its function under physiological and pathological conditions [43]. In this sense, our findings suggest that BACE1-inhibited astrocytes maintain EC integrity by preventing cell adhesion disruption, contributing to correct distribution of the tight junction protein ZO-1, and therefore it could improve the barrier impermeability probably through decreasing pro-inflammatory cytokine IL-1β immunoreactivity under toxicity by glutamate, which is involved in multiple harmful effects. Regarding ZO-1, it has been described that factors released by astrocytes, for example Sonic hedgehog (SHH) and Glial-derived neurotrophic factor (GDNF), have a protective effect on EC by regulating ZO-1 levels [44], however there are no studies that widely cover the mechanisms and processes of the protein distribution. As with the CLDN5 protein, we conclude that research is needed to describe the relationship between the BACE1 enzyme and tight junction proteins. About the inflammation, several authors have proposed a feedback mechanism between pro-inflammatory processes, such as those involving IL-1β, and neurodegenerative processes, where BACE1 plays an active role [21,40,45]. Products of BACE1 enzymatic action can induce inflammation and this in turn increases the enzyme levels . It is likely that by inhibiting BACE1 overexpression in an excitotoxic environment, a compensatory balance will occur where inflammation is reduced, and therefore, in the case of astrocytes, hyperreactivity is reduced so their regulatory profile becomes protective instead of mediator of damage, which explains the maintenance of EC integrity.

Endothelial damage in BBB has been discussed as a possible cause or consequence of multiple neurodegenerative diseases [46]. The study of modulation and induction of EC phenotype by astrocytes and pericytes has gained much importance in order to determine possible therapeutic targets. In conclusion, this research determined that under conditions of glutamate toxicity, while BACE1 inhibition directly in the endothelium has a beneficial effect, BACE1-inhibited astrocytes further protect the EC integrity in co-culture by decreasing the inflammatory phenomenon and regulating protein ZO-1 dynamics. The above

provides relevant information for the prosecution of BACE1 enzyme study in NVU and its role in the interaction of its different components.

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Figure legends

Figure 1. BACE1 inhibition protects endothelial cells from damaging effects of glutamate. (A) Percentage of LDH release by endothelial cells (bEnd.3) after 24 hours of treatments. Data are presented as the mean ± SEM of n=7. Kruskal-Wallis test. (B) Percentage of condensed nuclei for each treatment. These were quantified from 20 fields per treatment for each n (n=4). Data are presented as the mean ± SEM. (C) Number of gaps per field from threshold images, gaps= spaces between cells indicating disruption of the monolayer, presented as the mean ± SEM of n=4. Kruskal- Wallis test followed by Dunn test with Bonferroni correction. (D) Area of gaps between cells expressed in μm^2, it was quantified as the mean of black portion area in threshold images from 20 fields per treatment for each n (n=4). ANOVA followed by Tukey's test. (E) Morphological characterization showing nuclei stained with Hoechst (blue), F-actin cytoskeleton visualized with Alexa Fluor

488 phalloidin (green) and the tight junction protein Claudin-5 visualized with Alexa Fluor 594 (red). Magnification X 60, scale bar 20 μm. In the zoomed insets are observed the condition of the nuclei, the state of actin cytoskeleton and the Claudin-5 distribution. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment. All experiments were performed in duplicate. Significance codes: * P<0.05, ** P <0.01, *** P <0.001.

Figure 2. BACE1 inhibition reverses Claudin-5 cytoplasm redistribution caused by glutamate on endothelial cells. (A) Fluorescence intensity of Claudin-5 per unit area, it was quantified from 20 cells per treatment for each n (n=4 performed in duplicate) and divided per unit area, the data are presented as the mean ± SEM, ANOVA followed by Tukey's test, significance code: * P<0.05. (B) Fluorescence profiles, in the right column, showing the cellular distribution of Claudin-5 (red line) in relation to the nucleus (blue line) and cytoskeleton (green line) based on representative images of the left column. The black arrows indicate the tight junction peaks showing the changes in its distribution by the treatment. White line drawn on each cell: 50 μm. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment.

Figure 3. BACE1 inhibitor decreases the increase of BACE1 immunoreactivity and the IL-1β inflammation marker on endothelial cells caused by glutamate. (A) Representative images showing the BACE1 (red, visualized with Alexa Fluor 594) and IL-1β (green, visualized with Alexa Fluor 488) expression on endothelial cells (bEnd.3) under different treatments. Magnification X 60, scale bar 20 μm. (B) and (C) Fluorescence intensity of BACE1 and IL-1β, respectively, per unit area, these were quantified from 20 cells per treatment for each n (n=4 performed in duplicate) and divided per unit area. Data are presented as the mean ± SEM, ANOVAs followed by Tukey's tests. significance code: *** P <0.001.

Figure 4. BACE1 inhibition reduces astrocytic reactivity and induces filopodia-like processes. (A) Astrocytic cytotoxicity expressed as percentage of LDH release against 24 hours of treatments. Data are presented as the mean ± SEM of n=8. Kruskal-Wallis test followed by Dunn test with Bonferroni correction. (B) Fluorescence intensity of GFAP (Glial Fibrillary Acidic Protein) per unit area, it was quantified from 20 cells per treatment for each

n (n=4) and divided per unit area, the data are presented as the mean ± SEM. ANOVA followed by Tukey's test. (C) Morphological characterization showing nuclei stained with Hoechst (blue), F-actin cytoskeleton visualized with Alexa Fluor 594 phalloidin (red) and GFAP visualized with Alexa Fluor 488 phalloidin (green). Magnification X 60, scale bar 20 μm. In the zoomed insets, the white arrows indicate projections of actin cytoskeleton and the yellow arrows indicate extracellular GFAP IR dots. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment. All experiments were performed in duplicate. Significance codes: * P<0.05, ** P <0.01, *** P <0.001.

Figure 5. BACE1 and IL-1 β are decrease by BACE1 inhibition on glutamate-stressed primary astrocytes. (A) Representative images showing the BACE1 (red, visualized with Alexa Fluor 594) and IL-1β (green, visualized with Alexa Fluor 488) expression on primary astrocytes under treatments. The zoomed insets show the distribution of both proteins and an apparent perinuclear colocalization in vesicles (yellow). Magnification X 60, scale bar 20 μm. (B) Fluorescence intensity of BACE1 per unit area, it was quantified from 20 cells per treatment for each n (n=4) and divided per unit area. ANOVA followed by Tukey's test (C) Fluorescence intensity of IL-1β per unit area, it was quantified from 20 cells per treatment for each n (n=4) and divided per unit area. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment. Kruskal-Wallis test followed by Dunn test with Bonferroni correction. Data are presented as the mean ± SEM, the experiments were performed in duplicate. Significance codes: * P<0.05, ** P <0.01, *** P <0.001.

Figure 6. BACE1-inhibited astrocytes protect the integrity of co-cultured endothelial cells from damage caused by glutamate. (A) Percentage of LDH released by astrocyte-endothelium co-culture after treatments. (B) Percentage of condensed nuclei for each treatment. These were quantified from 20 fields per treatment for each n. (C) The number of gaps per field from threshold images, gaps= spaces between cells indicating disruption of the monolayer. (D) The area of gaps between cells expressed in μm^2, it was quantified as the mean of black portion area in threshold images from 20 fields per treatment for each n. (E) Fluorescence intensity of IL-1β per unit area, it was quantified from 20 cells per treatment for each n and divided per unit area. All data just described are presented as the mean ± SEM of n=4. (F) Morphological characterization of endothelial cells that were co-cultured with

primary astrocytes showing nuclei stained with Hoechst (blue), IL-1β visualized with Alexa Fluor 488 (green) and the tight junction protein Zonula occludens-1 (ZO-1) visualized with Alexa Fluor 594 (red). Magnification X 60, scale bar 20 μm. In the zoomed insets are observed the condition of the nuclei and the ZO-1 and IL-1β distribution. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment. All experiments were performed in duplicate. (A-D) ANOVA followed by Tukey's test and (E) Kruskal-Wallis test followed by Dunn test with Bonferroni correction. Significance codes: * $P<0.05$, ** $P<0.01$.

Figure 7. BACE1-inhibited astrocytes reverse the increased immunoreactivity and cytoplasmic distribution of ZO-1 protein caused by glutamate in endothelial cells. (A) Fluorescence intensity of ZO-1 per unit area, it was quantified from 20 cells per treatment for each n (n=4 performed in duplicate) and divided per unit area, the data are presented as the mean ± SEM. ANOVA followed by Tukey's test. Significance code: * $P<0.05$. (B) Fluorescence profiles, in the right column, showing the cellular distribution of ZO-1 (red line) in relation to the nucleus (blue line) and IL-1β (green line) based on representative images of the left column. The black arrows indicate the tight junction peaks showing the changes in its distribution by the treatment. White line drawn on each cell: 50 μm. DMSO= vehicle, Inh= β-secretase Inhibitor IV, - or Glu refers to glutamate treatment.