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"Study of the role of System X_c^- in neuroinflammation and neurodegenerative diseases"

"Studio del ruolo del System X_c^- nella neuroinfiammazione e nelle malattie neurodegenerative"

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ABSTRACT

Free radical production and oxidative stress play an important role in the pathogenesis of various neurodegenerative diseases, including HIV-associated neurocognitive disorders (HANDs) and Alzheimer's disease (AD). Oxidative stress is defined as an imbalance between the production of pro-oxidant molecules, for example reactive oxygen species (ROS), and antioxidant system, such as intracellular glutathione (GSH) production, in favor of pro-oxidant molecules. In several cell types including astrocytes, ROS can trigger a protective antioxidant cell response through the transcriptional induction of phase II detoxifying and antioxidant genes (i.e. ARE genes). Several transcription factors are involved in these cellular defence processes, including nuclear factor erythroid 2 related factor 2 (Nrf2), that has a fundamental role against oxidative stress. Among other ARE genes, System X_c^- plays a critical role in the manteinance of GSH homeostasis, because it transports simultaneously a glutamate out of the cell and a cystine into the cell, where it is reduced in cysteine, and it is used for the synthesis of GSH.

We hypothesize a double role of System X_c^- transporter in CNS: on the one hand, System X_c^- has a protective role towards astrocytes due to the production of GSH; on the other hand, System X_c^- could be dangerous towards bystanding neurons, because of its ability to export glutamate in extracellular space that could induce excitotoxicity and neuronal death. Accordingly, in a lot of neurodegenerative diseases, for example HAND and AD, System X_c^- antiporter could have a pivotal role.

Our previous findings indicate that the HIV protein Tat, endogenously produced in astroglial cells, can induce an effective antioxidant response mediated by Nrf2 and ARE-driven gene expression. In this study, we demonstrated for the first time that Tat-induced neurotoxicity is mediated by the increase of glutamate release due to astrocytic System X_c^- up-regulation.

Free radical production and oxidative stress play pivotal role also in Alzheimer's disease. First, we analyzed the effect of amyloid-β (Aβ) in astrocytes. We found that Aβ is able to induce Nrf2 nuclear translocation and, consequently, to increase the transcription of ARE genes. In particular, Aβ is able to induce up-regulation of System X_c^-. We found that Aβ-induced antioxidant response was not able to protect astrocytes from death, indeed

we demonstrated a significant reduction of astrocytes viability, regardless of the System X_c^- activity.
Once the response in astrocytes was characterized, we evaluated the effects of Aβ directly on neuronal cells cultured alone. First, we found that neurons were not able to activate Nrf2/ARE pathway, however, despite the lack of Nrf2 activation, System X_c^- protein and mRNA were up-regulated in differentiated SH-SY5Y neuronal cells treated with Aβ (50 μM) for 24 hours. Second, we observed that treatment with Aβ did not interfere with neuronal cell viability.
Successively, we explored the role of astrocytic response to Aβ in neuronal viability. Although the induction of Nrf2-driven gene expression has been widely indicated as a protective mechanism to counteract the effects of oxidative stress, in many cell types such as astrocytes, the up-regulation of xCT elicited by Nrf2 could be a potential source for excitotoxicity due to excessive glutamate release. We investigated the effect of Aβ-induced astrocytic antioxidant response on neuronal cell viability, focusing in particular on the role of System X_c^-. In order to study the effect of astrocytic System X_c^- activation on surrounding neurons, we performed co-cultures with U373 and differentiated SH-SY5Y cells. The co-culture system represents a better tool for understanding the role played by astrocytes in mediating neuronal protection or toxicity. The up-regulation of System X_c^- can increase extracellular glutamate release and potentially cause excitotoxicity. Thus, we wondered whether astroglial cells treated with Aβ (50 μM) were able to release higher levels of glutamate in the extracellular space in comparison to control cells. As expected, Aβ treated cells released 50% more glutamate than untreated cells. We found that SSZ treatment prevented Aβ-induced glutamate release, reducing its levels in extracellular space. These findings clearly indicate that astroglial cells treated with Aβ induce an increased glutamate release by eliciting System X_c^- up-regulation. Moreover, we demonstrated that at 24 hours post-treatment there is a significant 50% less viability of differentiated SH-SY5Y cells and a significant 35% less viability of U373 cells than the respective control cells. To verify whether the reduced neuronal viability, induced by Aβ, was effectively due to the increased export of glutamate through System X_c^- transporter, we performed an MTT assay on co-cultures treated for 24 hours with Aβ alone or in the presence of SSZ. SSZ was able to prevent neuronal toxicity, restoring cell viability at the control level. These findings demonstrate for the first time that Aβ-induced neurotoxicity is mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells. Moreover, our findings demonstrate also that Aβ-induced

neurotoxicity is mediated by the activation of NMDA receptor in neuronal cells, as indicated by the effect of NMDA receptor inhibitor that was able to restore neuronal viability.

Given the involvement of astrocytes in CNS pathology, it is not surprising that the ability to exacerbate neurodegeneration through the conversion of oxidative stress to excitotoxicity via system X_c^- has been linked to a variety of disorders, including Alzheimer's disease. The present study shed light on Nrf2/system X_c^- pathway and may help to better understand the role of astrocytes as the cell population responsible for the bulk of the neuronal death in Alzheimer's disease.

SINTESI

Lo stress ossidativo e la produzione di radicali liberi giocano un ruolo importante nella patogenesi di varie malattie neurodegenerative, come la neurodegenerazione associata all'HIV (HAND) e il morbo di Alzheimer (AD). Lo stress ossidativo è definito come uno squilibrio tra la produzione di molecole pro-ossidanti, ad esempio le specie reattive dell'ossigeno (ROS), e il sistema antiossidante, come la produzione di glutatione intracellulare (GSH), a favore delle molecole pro-ossidanti. In diversi tipi cellulari, inclusi gli astrociti, le ROS possono innescare una risposta antiossidante attraverso la trascrizione di geni detossificanti e antiossidanti di fase II (i geni ARE). Tra i fattori coinvolti nella trascrizione di tali geni troviamo nuclear factor erythroid 2 related factor 2 (Nrf2), principale regolatore della risposta contro lo stress ossidativo. Tra i geni ARE, il trasportatore System X_c^- svolge un ruolo critico nel mantenimento dell'omeostasi del GSH, in quanto trasporta contemporaneamente una molecola di glutammato fuori dalla cellula e una di cistina all'interno della cellula, dove viene ridotta in cisteina ed utilizzata per la sintesi di GSH.
In questo lavoro di tesi è stato studiato il ruolo del trasportatore System X_c^- nel contesto della neuroinfiammazione e della neurodegenerazione.
In particolare, è stato ipotizzato un duplice ruolo del System X_c^- nel sistema nervoso centrale: da una parte, un ruolo protettivo nei confronti degli astrociti, visto il suo coinvolgimento nella produzione di GSH; dall'altra, un effetto dannoso per i neuroni circostanti, a causa della sua capacità di esportare glutammato il quale, accumulandosi nell'ambiente extracellulare, potrebbe causare eccitotossicità e morte neuronale. Di conseguenza, in molte malattie neurodegenerative, come HAND e AD, il System X_c^- potrebbe avere un ruolo cruciale.
Nel nostro laboratorio, in precedenza, era stato dimostrato che la proteina Tat dell'HIV, prodotta negli astrociti, può indurre in tali cellule l'attivazione di una efficiente risposta antiossidante, ossia del pathway Nrf2/geni ARE responsabile dell'aumento di espressione del trasportatore System X_c^-.
Successivamente, nel lavoro svolto per la presente tesi è stato dimostrato per la prima volta che negli astrociti che producono Tat (U373-Tat) aumenta il rilascio di glutammato nell'ambiente extracellulare. Il trattamento con sulfasalazina (SSZ), uno specifico inibitore del System X_c^-, riporta il rilascio di glutammato ai livelli del controllo (U373-mock), dimostrando che l'effetto è causato dall'up-regolazione del System X_c^-.

Dato che il glutammato extracellulare può causare eccitotossicità, abbiamo valutato la vitalità neuronale in condizioni di co-coltura, e il risultato ha mostrato che i neuroni (cellule SH-SY5Y) in co-coltura con le U373-Tat presentano una vitalità ridotta rispetto al controllo, rappresentato da cellule SH-SY5Y coltivate insieme a U373 che non producono la proteina Tat (SH-SY5Y/U373-mock). Come atteso il trattamento con SSZ ha ripristinato la vitalità ai livelli del controllo.
Questi risultati dimostrano per la prima volta che la neurotossicità indotta da Tat è mediata da un aumento del rilascio di glutammato a causa della up-regolazione del System X_c^- nelle cellule astrogliali.
La produzione di radicali liberi e lo stress ossidativo svolgono un ruolo chiave anche nel morbo di Alzheimer. Importante nello sviluppo della patologia è la proteina β-amiloide (Aβ).
Come prima cosa abbiamo studiato l'effetto del Aβ negli astrociti (cellule U373) e abbiamo dimostrato che è in grado di indurre un incremento della traslocazione nucleare di Nrf2 e, di conseguenza, un aumento della trascrizione dei geni ARE, tra cui: GCL, HO-1, SOD1, SOD2, GPX, CAT e System X_c^-. In particolare, Aβ causa negli astrociti un'aumentata espressione del System X_c^- sia a livello trascrizionale che proteico; al contrario, l'espressione di SOD1 e CAT a livello proteico risulta ridotta.
Tuttavia la risposta antiossidante indotta da Aβ non è in grado di proteggere gli astrociti dalla morte. Abbiamo osservato infatti una significativa riduzione della vitalità, che non viene ripristinata ai livelli del controllo in seguito a trattamento con la SSZ, indicando che gli astrociti muoiono in seguito a trattamento con Aβ indipendentemente dall'attività del System X_c^-.
Successivamente, abbiamo studiato l'effetto del Aβ nei neuroni (cellule SH-SY5Y differenziate) e abbiamo dimostrato che, in seguito a trattamento con Aβ, i neuroni non sono in grado di attivare il pathway Nrf2/geni ARE. Osserviamo infatti una ridotta traslocazione di Nrf2 nel nucleo, che tuttavia non impedisce l'attivazione di una risposta antiossidante, dimostrata da un aumento di espressione degli mRNA di alcuni geni antiossidanti. Tra questi troviamo il System X_c^-, per il quale è stato osservato anche un aumento a livello proteico.
È stato dimostrato che Aβ non influisce sulla vitalità neuronale, mentre la SSZ è in grado di ridurla, dimostrando che il System X_c^- ha un ruolo fondamentale nella protezione dei neuroni dalla morte.
Per ultimo, abbiamo studiato l'effetto della risposta antiossidante indotta dal Aβ negli astrociti sulla vitalità neuronale, concentrandoci in particolare sul ruolo del System X_c^-. Per valutare l'effetto dell'attivazione del System X_c^-

negli astrociti sui neuroni circostanti, abbiamo eseguito i successivi esperimenti in condizioni di co-coltura astrociti/neuroni.
Il Aβ causa un aumento del rilascio di glutammato da parte degli astrociti in co-coltura in seguito all'up-regolazione del System X_c^- in tali cellule, infatti la SSZ è in grado di riportare i livelli di glutammato pari al controllo.
Inoltre, abbiamo dimostrato che Aβ causa morte neuronale in co-coltura, e che co-trattamenti con SSZ ed MK801, un'antagonista del recettore NMDA, sono in grado di prevenire la neurotossicità ripristinando la vitalità neuronale a livello del controllo.
Questi risultati dimostrano per la prima volta che la neurotossicità indotta dal Aβ è mediata da un aumento del rilascio di glutammato in seguito all'up-regolazione del System X_c^- negli astrociti e dall'attivazione, da parte del glutammato rilasciato nell'ambiente extracellulare, del recettore NMDA delle cellule neuronali.
Concludendo, questo lavoro di tesi sottolinea l'importanza della via di segnale Nrf2/System X_c^- e può aiutare a comprendere meglio il ruolo degli astrociti come popolazione cellulare responsabile della maggior parte della morte neuronale nelle malattie neurodegenerative, tra cui HAND e AD.

INTRODUCTION

Oxidative stress in neurodegenerative diseases.

Free radical production and oxidative stress play an important role in the pathogenesis of various neurodegenerative diseases, including HIV-associated neurocognitive disorders (HANDs) and Alzheimer's disease (AD).

Oxidative stress is defined as an imbalance between the production of pro-oxidant molecules, for example reactive oxygen species (ROS), and antioxidant system, such as intracellular glutathione (GSH) production, in favor of pro-oxidant molecules (Fig. 1).

Molecular entities or molecular fragments which contain one or more unpaired electrons in an atomic orbital or molecular orbital are defined as free radical *(Halliwell, 1999).*

ROS together with reactive nitrogen species (RNS) are the major pro-oxidant molecules in the cells. The most common ROS/RNS are the hydroxyl radical ($OH^{\cdot}$), the superoxide radical anion ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), and peroxyl-radicals ($ROO^{\cdot}$), as well as non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($O_2^{\cdot}$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{-}$) *(Lushchak V.I., 2014).*

ROS are mainly present in the brain and they are produced in glial cells and neurons, that are particularly sensitive to oxidative damage.

In the cell, ROS are extremely reactive and they are responsible of a lot of processes, such us oxidation of lipids, proteins and DNA with the production of toxic molecules *(Uttura et al., 2009).*

It has been reported that ROS are responsible of neuronal loss in several neurodegenerative diseases. In the 1990s, the scientific community focused their studies on the genetic and biochemical characterization of superoxide dismutase (SOD1), the enzyme that catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide, demonstrating that SOD1 plays an important role in diseases as heart failure, cancer, diabetes, Down's syndrome, and amyotrophic lateral sclerosis *(Milani et al., 2011).*

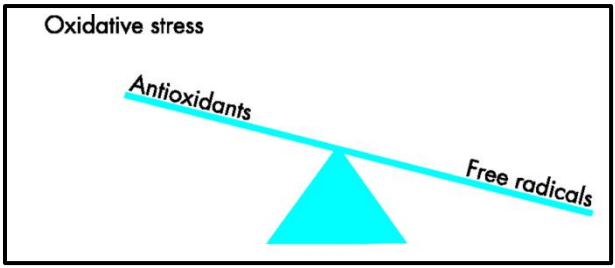

Figure 1. Graphic representation of oxidative stress *(oem.bmj.com/content/60/8/612)*.

HIV-associated neurocognitive disorders (HAND).

HIV-related diseases continue to be a severe public health problem worldwide, with around 40 million people being infected with HIV. Despite highly active antiretroviral therapy, in the late phase of HIV infection, a subset of patients develops neurological complications collectively termed as HIV-associated neurocognitive disorders (HANDs). Several mechanisms have been proposed to explain why and how low level of viral replication may lead to neurocognitive disorders in the absence of direct infection of neurons. One likely possibility is that glial cell activation and neurotoxicity may arise because of the effects mediated by viral proteins as gp120 and Tat. Note that antiretroviral drugs do not affect Tat availability in infected patients and Tat it is found in the cerebrospinal fluid (CSF) and infiltrating brain macrophages, in treated HIV patients with very low blood viral load. Tat can directly interact with neurons after being released by infected macrophages or glia into the extracellular space in the brain (Fig. 2). As we previously demonstrated in neuronal SH-SY5Y cells, Tat can lead to reduced cell viability eliciting H_2O_2 production by a mechanism involving both polyamine metabolism and N-methyl-D-aspartate (NMDA) receptor activation. However, Tat also induces neuronal loss indirectly at a distant site and in the meantime, supports the survival of infected astrocytes. Among other HIV-1 proteins, Tat elicits oxidative stress conditions leading to intracellular glutathione decrease in brain endothelial cells and different cell types, including neuronal cells. During oxidative stress conditions, various cell types can up-regulate the activity of nuclear factor erythroid 2-related factor 2 (Nrf2), the main orchestrator of the antioxidant response, thus becoming able to counteract intracellular reactive oxygen species (ROS) accumulation and glutathione (GSH) depletion. Astrocytes are more

resistant to oxidative insults than isolated neurons, indeed, unlike neurons they are able to strongly up-regulate Nrf2-mediated gene expression. Upon modifications in cellular redox state, Nrf2 migrates to the nucleus and sequentially binds to promoter regions, known as antioxidant response element (ARE), of many phase II detoxifying and antioxidant genes such as system X_c^- subunit xCT, γ-glutamyl-cysteine ligase (GCL), superoxide dismutase (SOD), glutathione peroxidase (GPX), heme-oxygenase-1 (HO-1). System X_c^- is an amino acid antiporter that mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the plasma membrane. While the import of L-cystine through this transporter is critical to glutathione production and protection from oxidative stress in astrocytes, the export of glutamate represents a further route of release through which this neurotransmitter may cause excitotoxicity. Thus, system X_c^- has currently been linked to both physiological and pathological processes in the CNS. Although the induction of Nrf2-driven gene expression has been widely indicated as a protective mechanism to counteract the effects of oxidative stress, in many cell types such as astrocytes, the up-regulation of xCT elicited by Nrf2 could be a potential source for excitotoxicity due to excessive glutamate release. Despite a lot of experimental and clinical findings indicate astrocytes as the cell population responsible for the bulk of the neuronal death in many neurodegenerative diseases, the cellular pathways leading to such a damage, particularly in HANDs, are not yet clearly defined *(Mastrantonio et al., 2018).*

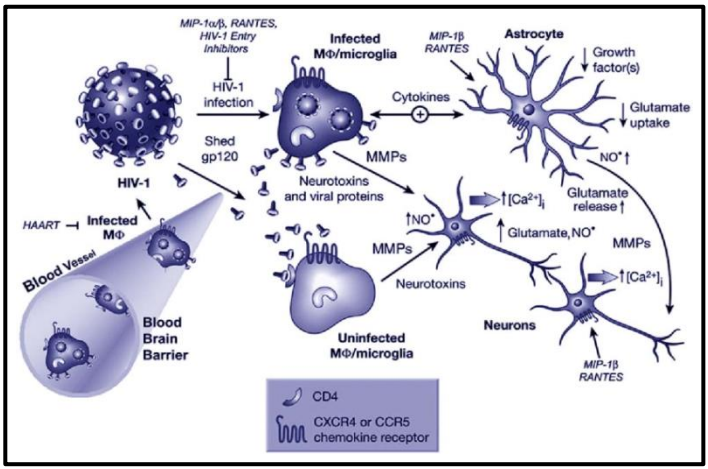

Figure 2. Current model of HIV-1 neuropathology: direct and indirect effects of virus infection in the CNS. *(https://www.bioscience.org/2008/v13/af/2860/figures.htm).*

Alzheimer's Disease.

Alzheimer's disease (AD) is a global public health priority, recognized by World Health Organization. AD is the leading cause of dementia, responsible of 50-75% of all world cases and results in the deterioration of selective cognitive performance, including memory and mental processing.
AD is characterized by deposition of amyloid-β peptide (Aβ) in senile plaques, intracellular neurofibrillary tangles comprising hyper-phosphorylated tau, synaptic dysfunction and neuronal death. Some or all of which are believed to cause the cognitive and behavioral deficits that typify this disease *(Garwood et al., 2011).*
Aβ was first isolated as the principal component of amyloid deposit in the brain and cerebrovasculature of AD patients and it derives from the larger peptide APP (amyloid-β precursor protein), a transmembrane glycoprotein of 695-770 aminoacids which is trafficked through both secretory and endocytic pathways.
APP is metabolized by the action of secretases: the 90% of APP is processed by α-secretases that produce a fragment of 83 aminoacids called sAPPα, that remains embedded in the plasma membrane, and the remaining 10% of total APP is processed by β-secretases that produce a fragment of 99 aminoacids called sAPPβ, released in extracellular space; sAPPα and sAPPβ are both processed by γ-secretases, composed by two catalytic subunits presenilin-1 (PS1) and presenilin-2 (PS2), to obtain P3 fragment by sAPPα and Aβ by sAPPβ (Fig. 3) *(Wilkins and Swerdlow, 2017).*
The Aβ degradation occurs by the action of Aβ degrading proteases (AβDPs), a large set of proteases including neprilysin (NEP), matrix-metalloproteinases (MMPs), insulin-degrading enzyme (IDE), that are vulnerable to a range of insults, for example pharmacological inhibition, environmental insults and oxidative damage.
Aβ is continuously produced and removed, and these opposing forces defined the absolute concentration of Aβ in the brain *(Saido and Leissring, 2012).* In physiological conditions, Aβ monomers are innocuous and they have a rapid turnover; conversely, under pathological conditions, the monomers turnover decreases about 30% and they start self-association. Indeed, on the one hand mutations in APP or in PS1/PS2 are associated with familial AD forms and lead to accumulation of a greater amounts of total Aβ. On the other hand, β-secretases activities and expression levels are increased in AD patients and the cleavage by γ-secretases is sometimes imprecise, resulting in a C-terminal heterogeneity of the Aβ peptide population. $A\beta_{1-42}$ is about 5-10% of total Aβ peptides and it is the most

represented in patients with Alzheimer's disease *(Wilkins and Swerdlow, 2017)*. The increase of $A\beta_{1-42}$ levels promotes oligomerization of monomers and the small soluble oligomers, composed by 12 units, are the most toxic form of Aβ *(Ferreira and Klein, 2011)*.

Although the molecular mechanisms leading to neuronal damage in AD have not been completely understood, it is well established that increased production of Aβ, in soluble and/or aggregate form, is a key causative event for Alzheimer's disease.

It is known that AD brain is under intense oxidative stress, manifested by increased protein oxidation, lipid peroxidation, free radical formation, DNA/RNA oxidation, nitro-tyrosine levels, and advanced glycation end products *(Butterfield, 2002)*.

In our lab, it has been demonstrated that exogenous administration of the biologically active fragments $A\beta_{25-35}$ and $A\beta_{1-42}$ to cultured endothelial cells results in the production of ROS.

Most of the known genetic, medical, environmental, and lifestyle-related risk factors for AD are associated with increased oxidative stress. The early involvement of oxidative stress in AD is demonstrated by recent studies on cell culture models, transgenic animal models, postmortem brains, and biologic fluids from subjects with AD, mild cognitive impairment (MCI), and Down syndrome. For example, up-regulation of HO-1, a sensitive marker of oxidative stress, is observed in astroglial cells in postmortem brains of AD. Moreover, increased lipid peroxidation, protein oxidation and decreased copper/zinc SOD activity were observed in transgenic mouse and *C. elegans* models of AD amyloidosis *(Nunomura et al., 2006)*.

Aβ-containing astrocytes have been described in brain of AD patients and they seem to be altered in function. Indeed, in physiological conditions, astrocytes are able to take up Aβ and to carry-out Aβ clearance through NEP, IDE and MMP-9 action, on the contrary, in pathological conditions Aβ accumulation in astrocytes suggests a failure in the clearance of Aβ. This data demonstrate that astrocytes have a pivotal role in the pathogenesis of Alzheimer's disease *(Thal, 2012)*.

Moreover, Aβ can form pore in astrocytes membranes and allow the influx of calcium from the extracellular space. This modulation of calcium levels can induce the activation of NADPH oxidase and the subsequent ROS production, thus leading to Aβ-induced oxidative stress in astrocytes.

Our hypothesis is that oxidative stress induced in astrocytes by Aβ, may elicit the pathway mediated by Nrf2 transcription factor and ARE genes as we described above for HAND. Thus, given the important role played by

astrocytes in maintenance of neuronal functions, their antioxidant response could be involved in neuronal death in AD *(Abramov et al., 2004).*

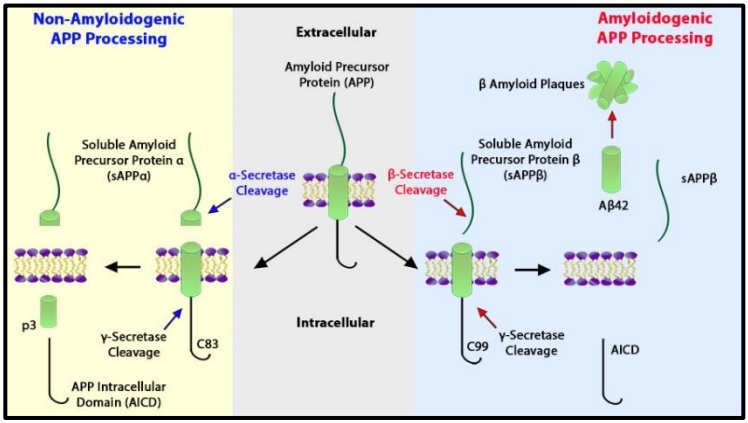

Figure 3. Aβ production process: the 90% of APP is processed by α-secretases that produce a fragment of 83 aminoacids called sAPPα, and the remaining 10% of total APP is processed by β-secretases that produce a fragment of 99 aminoacids called sAPPβ; sAPPα and sAPPβ are both processed by γ-secretases, to obtain P3 fragment by sAPPα and Aβ by sAPPβ *(www.biolegend.com/en-us/amyloid-precursor-protein).*

The antioxidant cell response.

Cells are evolved an efficient system that allows to counteract oxidative stress: they continuously measure intracellular ROS levels and, if they are too high, can activate an antioxidant response.
In several cell types including astrocytes, ROS can trigger a protective antioxidant cell response through the transcriptional induction of phase II detoxifying and antioxidant genes (i.e., ARE genes).
Several transcription factors are involved in this cellular defense process, including nuclear factor erythroid 2 related factor 2 (Nrf2) that has a fundamental role against oxidative stress by acting as sensor.
Nrf2 is a basic leucine zipper (bZIP) protein that is a member of cap'n'collar (CNC) family of transcription factors, it contains six functional domains, that are called Nrf2-ECH homologies (Neh 1-6).
Kelch-like ECH associated protein 1 (KEAP1) is a homodimer with three major domains: two DRG domains, one for each C-terminal part of KEAP1

dimers, that binds to Nrf2, and one at N-terminal part, that binds to E3 ubiquitin ligase complex (Rbx-1), through an adaptor, cullin-3.
Under physiological conditions, Nrf2 binds KEAP1 into the cytoplasm and KEAP1 directs Nrf2 to continuous ubiquitination and degradation by proteasome.
Under oxidative stress conditions, phosphorylation of binding sites allows the release of Nrf2 by KEAP1 into the cytoplasm and the consequent translocation of Nrf2 into the nucleus. In the nucleus Nrf2 associates with small MAF proteins (small musculoaponeurotic fibrosarcoma), and the heterodimers bind specific DNA sites, ARE elements, in the promoter of antioxidant genes, ARE genes, that codify protein that able to restore the redox state of the cell (Fig. 4) *(Sandberg et al., 2014).*
A lot of enzymes associated with GSH production, inactivation of ROS and phase II detoxification enzymes contain ARE sequence in their promoters.
Therefore, the increase of Nrf2 translocation into the nucleus causes the increase of transcription of typical ARE genes, including:

- GCL (glutamate-cysteine ligase) is the limiting enzyme in the biosynthesis of GSH. It is composed by two subunits, a catalytic subunit (GCLC) and a regulatory subunit (GCLM). The increased expression of GCL causes an increase of GSH production levels in the cell and its augmented secretion. Astrocytes transport GSH in the extracellular space through Mrp1 (multidrug resistance-associated protein 1) protein and the secreted GSH acts as antioxidant in neurons protecting them by oxidative stress, NO production and apoptosis *(Vargas et al., 2008).*
- HO-1 (heme oxygenase 1) is an enzyme that catalyzes heme's degradation. It is induced by heme, but also by heavy metals, endotoxins, thermal shocks, inflammatory cytokines, and prostaglandins. HO-1 has a fundamental role in the maintaining of cellular homeostasis, because it is induced by many oxidizing agents *(Choi et al., 1996).*
- NQO1 (NADPH:quinone oxidoreductase 1) is a cytosolic flavoprotein that is able to catalyze the reduction of two electron of compounds usually present in the cells as Coenzyme Q. It is involved in reduction of reactive intermediates *(Bruge et al.,2008).*
- SOD (superoxide dismutase) is an oxidoreductase enzyme that catalyzes the dismutation of superoxide anion free radical (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2). The high efficiency of SOD is essential for the maintenance of cellular redox state, because SOD isoforms are able to eliminate superoxide ions

(in physiological state they are continuously produced in small quantities, but in pathological state they are produced in higher quantities) that, in the presence of transition metals, can be transformed in oxygen radical species with high toxicity. SOD1 is the mitochondrial superoxide dismutase, while SOD2, is the cytoplasmic form *(Younus, 2018)*.

- CAT (catalase) is an enzyme with two different enzymatic activities. In presence of high concentration of H_2O_2, CAT catalyzes the transformation of H_2O_2 in H_2O and O_2; conversely, in presence of low concentration of H_2O_2, and in presence of hydrogen donator, CAT acts as a peroxidase oxidizing the substrate *(Scibior et al., 2006)*.
- GPX (glutathione peroxidase) is an enzyme that acts mainly as peroxidase, but it also has an antioxidant function and it can take diverse metabolic roles *(Brigelius-Flohè et al., 1999)*.
- SYSTEM X_c^-, subunit xCT: is the catalytic subunit of a cystine-glutamate antiporter, essential for the production of GSH *(Dun et al., 2006)*.

Among other genes, Nrf2 regulates the transcription of KEAP1, cullin-3, Rbx-1, and at the same time the complex KEAP1/cullin-3/Rbx-1 degrades Nrf2. Also the KEAP1 complex can be transported into the nucleus where it is able to take back Nrf2 into the cytoplasm for its degradation by proteasome *(Sandberg et al., 2014)*.

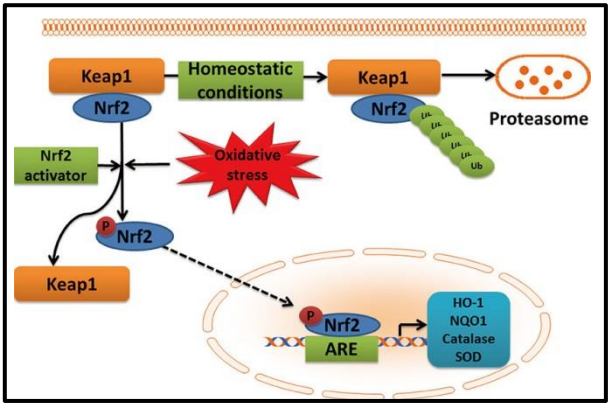

Figure 4. Nrf2/ARE pathway. Under physiological conditions, Nrf2 binds Keap1 into the cytoplasm and it is continuously degraded by proteasome; under oxidative stress conditions Nrf2 dissociates from Keap1 and goes into the nucleus where it increases the transcription of ARE genes *(https://e-dmj.org/ViewImage.php?Type=F&aid=588690&id=F2&afn=2004_DMJ_38_5_337&fn=dmj-38-337-g002_2004DMJ).*

System X_c^- antiporter.

Aminoacids deprivation, xenobiotic exposure and oxidative stress can regulate System X_c^- expression and function.
System X_c^- is a member of heteromeric amino acid transporters (HAT) family, sodium-independent, chloride-dependent, electroneutral.
The transporter is composed of two subunits, covalently linked through a disulphide bond:

- 4F2hc (also known as CD98), the "heavy chain", that is a cell surface glycoprotein that is responsible of the localization of the transporter on plasma membrane.
- xCT, the "light chain", that is a 502 aminoacids protein with twelve transmembrane domains (TMDs) and intracellular N- and C- terminals ; it is responsible of the transporter activity *(Bridges et al., 2012).*

System X_c^- is a cystine-glutamate transporter that transports simultaneously a glutamate out of the cell and a cystine into the cell, where it is reduced in cysteine. Inside the cell, cysteine is used for the synthesis of GSH, the major antioxidant of the cell, that is a tripeptide consisting of glutamate, cysteine and glycine (Fig. 5) *(Dun et al., 2006).*
System X_c^- has been characterized in a lot of cell type, both inside and outside the CNS, for example it has been characterized in astrocytes, microglia, immature cortical neurons and glioma cell lines. Both astrocytes and neurons express xCT subunit, but several studies reveal that the subunit is not functional in mature neurons, that are not able to transport cystine into the cell through System X_c^- activity *(Bridges et al., 2012).*
System X_c^- plays a critical role in the manteinance of GSH homeostasis, because the intracellular levels of cysteine are the limiting factor for GSH synthesis.
GSH, and thus the System X_c^- function, has a pivotal role for the cell protection against oxidative stress-mediated damage and for the execution of an efficient antioxidant response *(Dun et al., 2006).*
This function is important in the CNS, where there are high level of oxygen consumption and a lot of number of enzymes and metabolites that are able to generate ROS. Accordingly, in the CNS, where there is very high request of GSH, the cysteine/cystine ratio in the extracellular space widely favors cystine and the transporters are organized to allow the transport of cystine into astrocytes and the transport of cysteine into neurons.
This process is called "cystine/cysteine cycle" and starts with System X_c^- activity that allows the transport of cystine into astrocytes, where it is

reduced in cysteine and successively used for the synthesis of GSH. Astrocytic GSH is released in the extracellular space and metabolized by γ-glutamil-transpeptidase and aminopeptidase-N. In this way, a tank of extracellular cysteine is generated and cysteine is transported into the surrounding neurons and used for the synthesis of neuronal GSH. Therefore, given that GSH is essential for the protection of the cell against oxidative damage, System X_c^- becomes fundamental as a transporter associated with the antioxidant machinery of the cell *(Dun et al., 2006)*.

However, it has also been reported that the up-regulation of System X_c^- can increase extracellular glutamate release and potentially cause excitotoxicity *(Bridges R. et al., 2012) (Bridges R.J. et al, 2012)*. Thus, we hypothesize a double role of System X_c^- transporter in the CNS.

On the one hand, System X_c^- has a protective role towards astrocytes due to the production of GSH inside these cells. On the other hand, it could be dangerous towards bystanding neurons, because of its ability to export glutamate in extracellular space thus promoting excitotoxicity and neuronal death.

Accordingly, in a lot of neurodegenerative diseases, as HAND and AD, a pivotal role of System X_c^- antiporter could be hypothesized *(Dos-Santos-Pereira et al., 2018*).

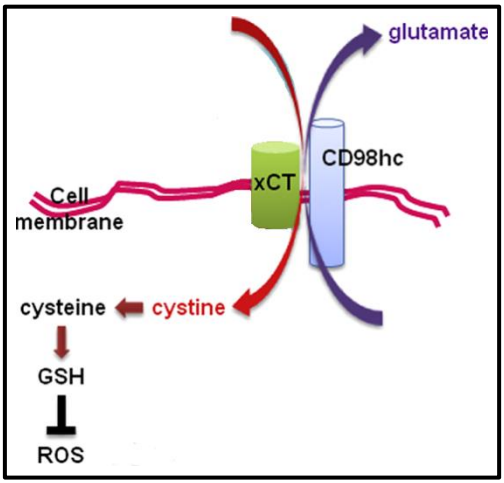

Figure 5. System X_c^- antiporter: it transports a glutamate out of the cells and a cystine into the cell, where it is reduced in cysteine and used for the synthesis of GSH *(www.cell.com/cancer-cell/fulltext/S1535-6108(11)00050-X).*

MATERIALS AND METHODS

Materials

Amyloid-β Protein fragment 25-35 ($A\beta_{25-35}$), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% Trypsin–EDTA solution, gentamicin solution 50 mg/ml, sulfasalazine (SSZ), a specific System X_c^- inhibitor, MK-801 hydrogen maleate (MK-801), and MTT assay kit were obtained from Sigma–Aldrich (Milan, Italy). Bradford reagent was obtained from Bio-Rad Italia (Milan, Italy).

All chemicals were of analytical or reagent grade and were used without further purification.

ARP6017 HIV-1 Tat-B recombinant protein (101 aa), anti-Tat antibody (EVA 3069.1 Mab to HIV-1 Tat Fit Biotech) and pC63.4.1 plasmid were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, the NGIN Consortia, and the Bill and Melinda Gates GHRC-CAVD Project. ARP697 was donated by FIT Biotech, Estonia, Dr. J. Karn.

The kit Go Taq 2-Step RT-qPCR System is obtained from Promega.

For Western Blot analysis and immunofluorescence, the following primary antibodies were used: anti-actin 1:1000 (Sigma), anti-Nrf2 1:1000 (Abcam), anti-Lamin A 1:1000 (Abcam), anti-Lamin B1 1:1000 (Abcam), anti-System X_c^- 1:5000 (OriGene), anti-SOD1 1:2000 (Abcam), anti-CAT 1:20000 (Rockland). For Western Blot, secondary peroxidase-labeled anti-rabbit IgG antibodies were from Bio-Rad Italia (Milan, Italy). For immunofluorescence secondary anti-Rabbit IgG Alexa Fluor 488 was obtained from Invitrogen.

Cell cultures, treatments and transfection

U373-MG human glioblastoma astrocytoma cells and SH-SY5Y human neuroblastoma cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 40 μg/ml gentamicin at 37 °C in a humidified 5% CO_2 incubator. Confluent monolayers of U373 cells were subcultured by conventional trypsinization. For the experiments, 2.5×10^5 or 4×10^5 cells were seeded in 35- or 60-mm tissue culture dishes respectively, and grown up to 80% confluence for 18–24 h before treatments.

Working solutions of Aβ were prepared in culture medium from stock solutions stored at −20 °C.

Working solutions of Tat were freshly prepared in culture medium from stock solutions stored at -80°C. Where indicated, SH-SY5Y cells were treated with 200 ng/ml HIV-1 Tat recombinant protein in serum free DMEM.
For transfection, 4 x 10^5 U373 cells were seeded in 60 mm dishes and transfected with Lipofectamine 3000 (Invitrogen) and with 1 μg of pcDNA3.1 or pcDNA3.1-Tat, thus obtaining two stable-transfected cell lines, termed U373-mock and U373-Tat, respectively. After 48 h, the transfected cells were selected adding to the DMEM medium 400 μg/ml of G418 (Geneticin, Sigma). For the maintenance of transfected cells in culture, 200 μg/ml of G418 was used.

SH-SY5Y differentiation
SH-SY5Y cells were seeded in a confluent monolayer in culture dishes necessary for the experiments. To be differentiated, cells were coltured in Neurobasal medium (Gibco) with 2 mM L-glutamine, 10 μM Retinoic Acid (Sigma) and 1X B-27 supplement (Gibco). The medium was changed every two days for a week.

MTT assay
For the experiments to test neuronal viability and astrocytes viability, U373 and SH-SY5Y cells were co-cultured in poli L-lysined cover glass (20x20 mm). For each sample, 10^5 neuronal cells were seeded in cover glass and 2.5×10^5 astroglial cells were plated in the lower compartment of six-well plate and allowed to grow for 24 hours.
MTT assay was performed as indicated by manufacturer's instructions on SH-SY5Y cells and U373 cells at the end of each incubation period. Briefly, MTT solution (stock solution of 0.5 mg/ml) was added to the cell culture at the final concentration of 10%. After incubation at 37 °C for 4 hours, formazan crystals were dissolved in lysis buffer (4 mM HCl, 0.1% NP40 (v/v) in isopropanol) and the optical density (O.D.) of each sample was measured using a microplate reader at 570 nm (BioTek ELx800 Absorbance Microplate Reader, Winooski, VT, USA).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
Total RNA was purified by using TRIzol Reagent (Life technologies Italia-Invitrogen, Monza, Italy) and reverse transcribed into cDNA with GoTaq 2-step RT-qPCR system (Promega Italia Srl, Milan, Italy). cDNA was amplified for the following genes: catalase (CAT), superoxide dismutase

(SOD1 and SOD2), glutathione peroxidase (GPX), System X_c^- (xCT subunit), and glutamate-cysteine ligase (GCLC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was examined as the reference cellular transcript. The sequences of primers were reported below. PCR product quantification was calculated by applying the SYBR-Green method. Reactions were performed in a Agilent Aria Mx machine (Agilent technologies) using the following program: 45 cycles of 95°C for 15 s, 60°C for 60 s, 72°C for 20 s. GAPDH mRNA amplification products were present at equivalent levels in all cell lysates. The data are calculated relative to the internal housekeeping gene according to the second derivative test (delta–delta Ct ($2^{-\Delta\Delta CT}$) method).

The primers sequences are:

CAT	Forward 5′- TCA GGT TTC TTT CTT GTT CAG -3′
	Reverse 5′- CTG GTC AGT CTT ATA ATG GAA TT -3′
SOD1	Forward 5′- AGT AAT GGA CGA GTG AAG G -3′
	Reverse 5′- GGATAG AGG ATT AAA GTG AGG A -3′
SOD2	Forward 5′- AAT GGT GGT GGT CAT ATC A -3′
	Reverse 5′- CCC GTT CCT TAT TGA AAC C -3′
HO-1	Forward 5′- CGG GCC AGC AAC AAA GTG -3′
	Reverse 5′- AGT GTA AGG ACC CAT CGG AGA A -3′
GPX3	Forward 5′- CAT TCG GTC TGG TCA TTC TG -3′
	Reverse 5′- CCT GGT CGG ACA TAC TTG A -3′
System X_c^-	Forward 5′- GGT GGT GTG TTT GCT GTC -3′
	Reverse 5′- GCT GGT AGA GGA GTG TGC -3′
GCLC	Forward 5′- TTG CAA AGG TGG CAA TGC -3′
	Reverse 5′- GAA ACA CAC CTT CCT TCC -3′
GAPDH	Forward 5′- TTG TTG CCA TCA ATG ACC C -3′
	Reverse 5′- CTT CCC GTT CTC AGC CTT G -3′

Preparation of Nuclear and Total Extracts

After treatments at the indicated time points, the cells were mechanically detached with a scraper in cold PBS. Nuclear extracts were prepared by adding buffer A (10 mM Hepes, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.1% NP40, protease inhibitor cocktail) to the cell pellets to separate nuclei from cytosol. After incubation for 10 min on ice and subsequent centrifugation at 12,000 rpm for 10 min at 4 °C, pellets containing nuclear fractions were resuspended in buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 0.05% NP40, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) and incubated on ice for 30 min. A final centrifugation at 14,000

rpm was carried out, and the supernatants were collected, quickly frozen in liquid nitrogen, and stored at − 80 °C. The total protein content of nuclear extracts was determined according to Bradford method. To show the quality of the nuclear separation, both nuclear and cytosolic fractions were blotted for the nuclear marker lamin A or B1 and cytoplasmic marker actin, respectively.

For total extracts, the cells were mechanically detached with a scraper in cold PBS. Total extracts were prepared by adding buffer TEEN (10 Mm Tris HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail) to the cell pellets. After incubation for 20 min on ice and subsequent centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatants were collected, quickly frozen in liquid nitrogen, and stored at − 80 °C. The total protein content was determined according to Bradford method.

Analysis of Nrf2 Activation and System X_c^-, SOD1 and CAT Expression by Western Blotting

To evaluate Nrf2 activation, equal amounts (20 μg proteins/sample) of nuclear extracts were subjected to electrophoresis in an 8% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 1 hours and incubated overnight at 4 °C with a polyclonal anti-Nrf2 antibody (1:1000; Abcam Italy, Prodotti Gianni S.p.A., Milan, Italy) or with a polyclonal anti-lamin A (1:1000, Abcam, Italy) or with polyclonal anti-lamin B1 (1:1000, Abcam, Italy).

To evaluate System X_c^- and CAT expression, equal amounts (15 μg proteins/sample) of total extracts were subjected to electrophoresis in an 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 1 hours and incubated overnight at 4 °C with polyclonal anti-System X_c^- antibody (1:5000; OriGene), anti-CAT antibody (1:20000, Rockland) or polyclonal anti-actin (1:1000, Sigma, Italy).

To evaluate SOD1 expression, equal amounts (15 μg proteins/sample) of total extracts were subjected to electrophoresis in an 14% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 1 hours and incubated overnight at 4 °C with polyclonal anti-SOD1 antibody (1:2000, Abcam) or polyclonal anti-actin (1:1000, Sigma, Italy).

Lamin A/B1 and actin were used as the reference protein amounts for nuclear and total extracts, respectively. Secondary peroxidase-labeled anti-rabbit IgG antibody (1:10000) was from Bio-Rad Italia (Milan, Italy).

Immunoreactive bands were detected using ECL Western blotting detection reagents (GE Healthcare, Milan, Italy) and captured by Chemi Doc TM XRS 2015 (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis was performed using Image Lab software (Version 5.2.1; © Bio-Rad Laboratories). The optical density of each band was measured and normalized with respect to the relative loading control band (lamin A/B1 for Nrf2 and actin for System X_c^-).

Measurements of Glutamate Concentration in Cell Supernatants

To evaluate glutamate release in supernatants of co-cultures, Glutamate Assay (BioVision) was performed as indicated by manufacturer's instructions. Briefly, 20 µl of each sample supernatant was collected in a 96-well plate and assay buffer was added up to 50 µl final volume. Then 100 µl of the reaction mix was added to each well, the plate was incubated for 30 min at 37 °C, protected from light, and optical density (OD) was measured at 450 nm in a microplate reader. Glutamate concentration of each sample was calculated using glutamate standard curve (0, 1.3, 6.5, 13, 26, 40, 53, 67 µM).

Immunofluorescence analysis

7×10^4 SH-SY5Y cells were seeded on poli L-lysined cover glass and 1.5×10^5 U373 cells were seeded on six-well plate with poli L-lysined cover glass.

After suitable treatments, cells were washed in PBS and fixed in PFA 4%. The permeabilization was performed in methanol for 10 minutes at -20 °C. Cells were washed and incubated for 1 hour at room temperature with a blocking solution (5% FBS, 1% BSA in PBS). After cells were incubated overnight at 4 °C with polyclonal anti-Nrf2 diluted 1:100 in blocking solution or anti-System X_c^- diluted 1:100 in blocking solution. After being washed , cells were incubated for 1 hours at 37 °C with anti-Rabbit IgG Alexa Fluor 488 conjugated diluted 1:500. Cells were then washed and closed with a preserving fluorescence medium (Vectashield) and observed under a fluorescence microscope.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) of n observations. Statistical analysis was performed by one-way ANOVA and subsequently by Bonferroni post-test. Differences are considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of Tat-induced System X_c^- activation in astroglial cells on HIV-associated neurotoxicity.

We previously found that, in astroglial cells, endogenously produced Tat can induce Nrf2 activation, ARE-driven gene expression and System X_c^- up-regulation. These findings indicate an effective antioxidant response and suggest how infected astrocytes can counteract oxidative stress induced by HIV infection *(Mastrantonio et al., 2018).*

However, the up-regulation of System X_c^- can increase extracellular glutamate release and potentially cause excitotoxicity *(Bridges R. et al., 2012) (Bridges R.J. et al, 2012)*. Thus, we wondered whether Tat-producing astroglial cells release higher levels of glutamate in the extracellular space in comparison to control cells. To this aim, we measured the glutamate levels in the supernatant of U373-Tat co-cultured with SH-SY5Y neuronal cells in comparison with U373-mock/SH-SY5Y co-cultures. As expected, Tat-producing cells released twice the amount of glutamate respect to control cells. The higher levels of glutamate were reached after 48 hours of co-culture and were kept unchanged up to 72 hours of co-culture (the last time point analyzed), as shown in Fig. 6A.

To demonstrate that glutamate was produced by astroglial cells, we measured the glutamate levels in the supernatant of transfected U373 mono-cultured for 48 hours. As shown in the insert of Fig. 6A, Tat-producing cells (U373-Tat) released about twice the amount of glutamate respect to control cells (U373-mock). It is noteworthy that our findings are consistent with previous data indicating increased glutamate levels in CSF of patients with HIV dementia *(Ferrarese et al., 2001)*, thus providing an explanation about the involved mechanisms at molecular and cellular level. In our opinion, this represents an important point and confers a physiological relevance to our study.

Finally, to verify whether Tat-elicited glutamate release occurred through System X_c^- activation, we analyzed the levels of the amino acid in the supernatants of co-cultures treated without or with sulfasalazine (SSZ), a specific System X_c^- inhibitor. As shown in Fig. 6B, we found that SSZ treatment prevented Tat-induced glutamate release, maintaining its levels comparable to the control at all the time points analyzed.

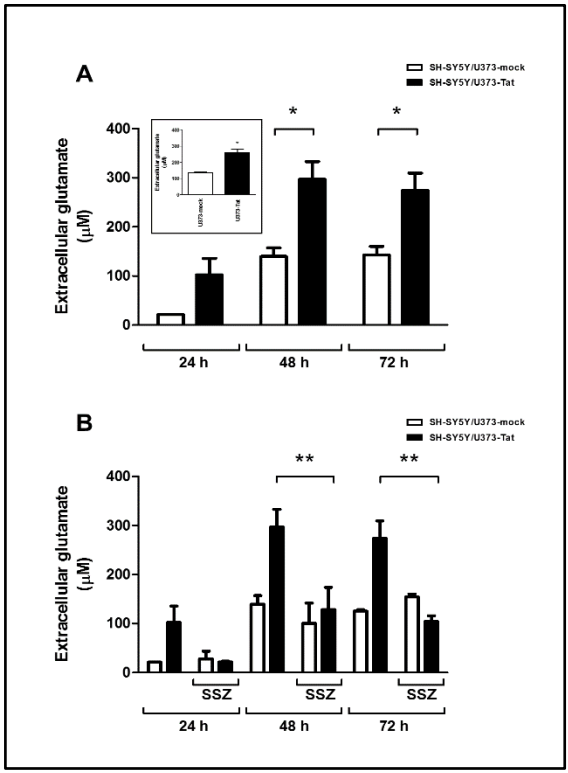

Figure 6. Effects of endogenously produced Tat and system X_c^- activity on extracellular glutamate release in SH-SY5Y/U373-Tat co-cultures. (A) 10^5 SH-SY5Y cells were co-cultured with 2.5×10^5 U373 cells stably transfected with pcDNA3.1 (U373-mock) or with pcDNA3.1-Tat (U373-Tat) for 24, 48 and 72 hours. The Glutamate Assay was performed as specified in the Materials and methods section. The histogram shows the extracellular glutamate release (μM). Values are calculated relative to a glutamate standard curve (μM). Results are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * p≤0.05 vs SH-SY5Y/U373-Mock (white). The inset shows glutamate release in stably transfected mono-cultures, U373-mock or U373-Tat, for 48 hours. * p≤0.05 vs U373-mock. (B) Co-cultures were treated for 24, 48 and 72 hours alone or with SSZ and Glutamate Assay was performed. The histogram shows the extracellular glutamate release (μM). Values are calculated relative to a glutamate standard curve (μM). Results are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0.01 between SH-SY5Y/U373-Tat and SH-SY5Y/U373-Tat +SSZ.

These findings clearly indicate that Tat released by astroglial cells induces an increased glutamate release by eliciting System X_c^- up-regulation.

Afterwards, the viability of neuronal cells under these experimental conditions has been investigated. As shown in Fig. 7A, the viability of neuronal cells co-cultured with U373-Tat for 48 hours was significantly decreased with respect to neuronal cells co-cultured with U373-mock. In particular, we observed 20% less viability in SH-SY5Y cells co-cultured with astrocyte-producing Tat. To verify whether the reduced neuronal viability, induced by Tat, was effectively due to the increased export of glutamate through System X_c^-, we performed an MTT assay in co-cultures treated for 48 hours alone or in the presence of SSZ. As shown in Fig. 7B, SSZ was able to prevent neuronal toxicity in U373-Tat/SH-SY5Y co-cultures, restoring neuronal viability at the control level.

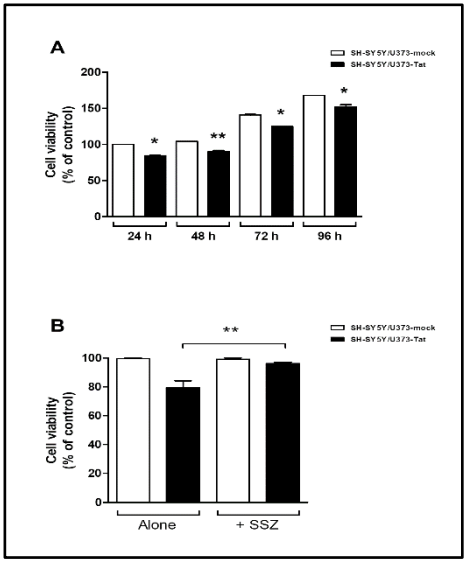

Figure 7. Effects of endogenously produced Tat and system xc- activity on SH-SY5Y viability co-cultured with U373-Tat cells. (A) SH-SY5Y cells were co-cultured with U373 cells stably transfected with pcDNA3.1 (U373-mock) or with pcDNA3.1-Tat (U373-Tat) for 24, 48, 72, and 96 hours. (B) Co-cultures were treated for 48 hours alone or in the presence of SSZ. MTT cell viability assay was performed as specified in the Materials and methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of neuronal cells co-cultured with U373-mock cells and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. (A) * $p \leq 0.05$ and ** $p \leq 0.01$ vs SH-SY5Y/U373-mock. (B) ** $p \leq 0.01$ between SH-SY5Y/U373-Tat alone and SH-SY5Y/U373-Tat+SSZ.

These findings demonstrate for the first time that Tat-induced neurotoxicity is mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells. Moreover, our study suggests that latently infected astrocytes, as occur in the CNS of HIV-infected patients, may mediate neurodegeneration through this pathway.

Note that glutamate release via System X_c^- from both astrocytes and microglia has been shown to enhance excitotoxicity of cortical neurons *(Fogal et al., 2007)*. Very recently, it has been found that system X_c^- contributes to increase glutamate excitotoxicity in the neocortex of a mouse model (Dach-SMOX) displaying a constant and chronic oxidative stress *(Pietropaoli et al., 2018)*. Several data reported that during neuroinflammation activated microglia and astrocytes release and maintain high level of extracellular glutamate *(Takeuchi et al., 2006)*. Moreover, it has been recently observed that System X_c^- could mediate methamphetamine-induced neurotoxicity by eliciting oxidative stress, microgliosis, and glutamate-related toxicity *(Dang et al., 2017).*

Early evidence reporting that System X_c^- could be a source of excitotoxic glutamate derives from a study on microglia that, because of a sustained need for oxidative protection, expresses high levels of the transporter *(Piani and Fontana, 1994).*

More recently, Gupta et al. reported that Tat elicits microglial glutamate release via System X_c^- induction, thus suggesting that Tat-induced glutamate release might contribute in part to neurologic dysfunctions associated with HIV infection *(Gupta et al., 2010*). In astrocytes, it has been reported that the up-regulation of System X_c^- by interleukin-1β (IL-1β) was able to enhance hypoxic neuronal injury. In fact, neurons co-cultured with astrocytes were found to be more susceptible than neurons alone to hypoxic cell death after treatment of cultures with IL-1β, an effect that was mediated by increased efflux of glutamate through system X_c^- *(Fogal et al., 2007).* Previously, we reported that the expression of IL-1β as well as of nitric oxide synthase was elicited in astrocytes by HIV gp120 *(Persichini et al., 2014).*

These findings indicate how inflammatory pathways and nitrosative stress may converge to an intersection point, represented by System X_c^- activation, and suggest a likely explanation for the mechanism involved in excitotoxicity induced by HIV infection *(Mastrantonio et al., 2016)*.

Effect of Amyloid-β on antioxidant response of astroglial cells.

Free radical generation and oxidative stress play pivotal roles in many neurodegenerative diseases, including Alzheimer's disease *(Abramov et al., 2004).*

In several cell types, including astrocytes, ROS can trigger a protective antioxidant cell response through the transcriptional induction of phase II detoxifying and antioxidant genes (i.e., ARE genes).

Here, we analyzed the effects of Aβ ($A\beta_{25-35}$ 50 μM) on gene expression of several antioxidant enzymes in human U373 astroglial cells treated for 4, 8 and 16 hours. By Real time PCR analysis, we demonstrated that mRNA levels of CAT, SOD1, SOD2, HO-1 and GPX were increased in Aβ-treated cells with respect to the control. As shown in Fig. 8, the maximum was reached between 4 and 8 h post-treatment for all the genes.

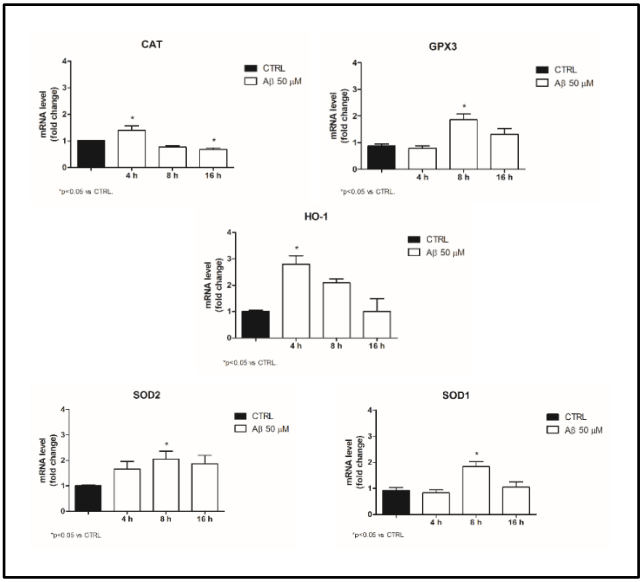

Figure 8. Effects of Aβ on ARE-driven gene expression in U373 cells. U373 were treated with Aβ (50 μM) for 4, 8 and 16 h. After incubation at 37°C, the cells were homogenized and total RNA has been purified to assess mRNA levels of several genes (CAT, SOD1, SOD2, GPX and HO-1) by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * p≤0.05 vs CTRL.

Among ARE genes, there are genes involved in GSH homeostasis, the main antioxidant of the cell. Therefore, we focused our attention particularly on GCL and System X_c^-, both involved in the maintenance of the redox state. In the same experimental conditions described above, we observed by Real Time PCR analysis that amyloid-β treatment of U373 cells leads to a significant increase of GCL and System X_c^- mRNA levels in comparison with control cells. As shown in Fig. 9, the results indicate a significant 1,5- or 2,5-fold increase of mRNA levels at 8 hours post-treatment.

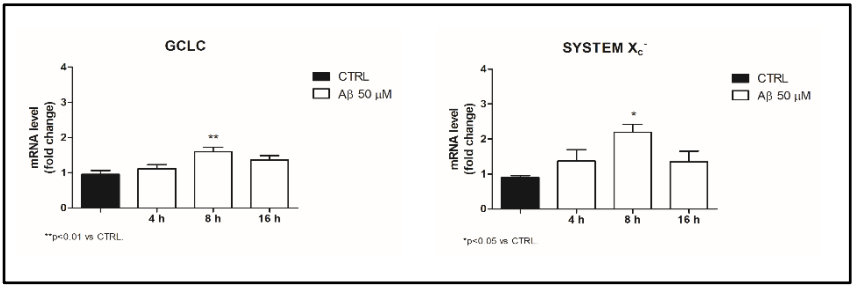

Figure 9. Effects of Aβ on GCLC and System X_c^- gene expression in U373 cells. U373 were treated with Aβ (50 μM) for 4, 8 and 16 h. After incubation at 37°C, the cells were homogenized and total RNA has been purified to assess mRNA levels of GCLC and System X_c^- by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *p≤0.05 vs CTRL; **p≤0.01 vs CTRL.

Given that ARE genes are mainly regulated by Nrf2, we investigated whether Aβ could activate this transcription factor in astroglial cells. To this aim, U373 cells were treated with Aβ (50 μM) for 2, 4 and 24 hours and the levels of Nrf2 was measured in nuclear extracts by Western blot analysis. The results shown in Fig. 10 indicate that Aβ induced a 1.8-fold increase of the nuclear Nrf2 levels already at 2 hours post-treatment. While, at 24 hours post-treatment we observed a significant decrease of Nrf2 levels with respect to the control. However, this time frame was compatible with the transcriptional induction of antioxidant ARE genes.

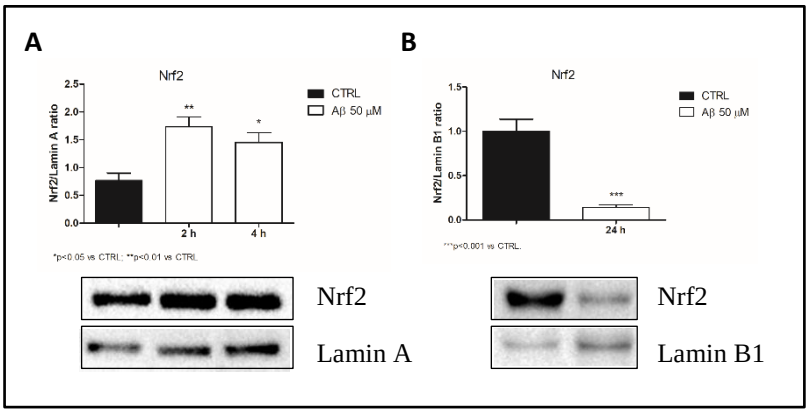

Figure 10. Effects of Aβ on Nrf2 nuclear translocation in U373 cells. A) Cells were treated with Aβ (50 μM) for 2 and 4 hours. B) Cells were treated with Aβ (50 μM) for 24 hours.
The histograms show the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear lamin A or nuclear lamin B1 content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL; ** p≤0.01 vs CTRL; *p≤0.05 vs CTRL.

In order to confirm the nuclear translocation of Nrf2 in U373 cells treated with Aβ (50 μM), the transcription factor localization was evaluated also by confocal analysis at 4 hours post-treatment. As expected, the data show an increase of nuclear localization of Nrf2 in treated cells compared to control cells (Fig. 11).

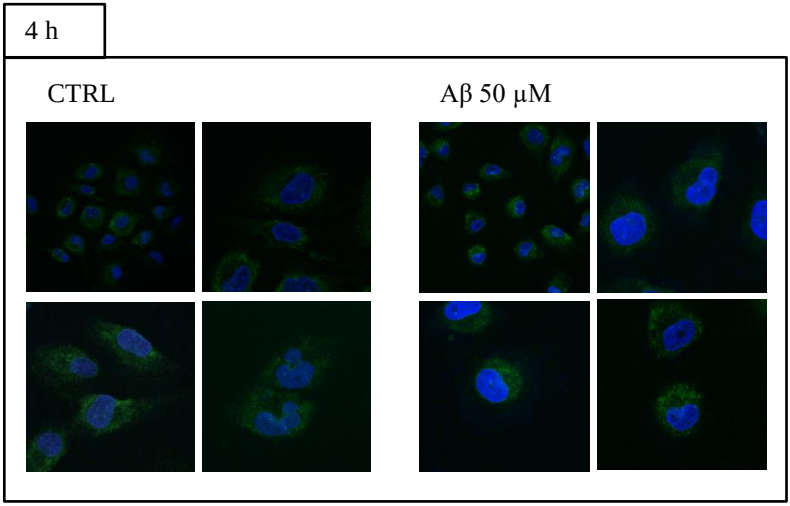

Figure 11. Nrf2 localization in Aβ-treated U373. Cells were treated with Aβ (50 μM) for 4 hours and subjected to immunofluorescence staining using anti-Nrf2 1:100 (Abcam, green) antibody as specified in Methods and analyzed by immunofluorescence microscopy. The magnification are obtained with a 3x zoom. Nuclei (blue) are stained with DAPI.

Since activation of the astrocytic Nrf2 pathway represents a principal regulator of the large array of Nrf2-dependent antioxidant genes, these findings would be in harmony with the idea that astrocytes play a key role in providing antioxidant support to nearby neurons. Indeed, it is thought that post-mitotic neurons survive and are functional for many decades despite their relatively low intrinsic antioxidant defenses because of the strong antioxidant support they receive from surrounding glial cells, particularly astrocytes *(Baxter et al., 2016).*

To test this idea, and given that astrocytes are able to activate Nrf2-ARE pathway, we investigated if Aβ was able to up-regulate SOD1, CAT and System X_c^- also at protein expression level. We evaluated SOD1 protein expression in U373 cells treated with Aβ (50 μM) for 24 and 48 hours by western blotting whole extracts. Despite the up-regulation of SOD1 mRNA expression, the data presented in Fig. 12 show a significant decrease of the protein levels in treated cells compared to controls at both 24 and 48 hours.
Noteworthy, the increase of mRNA levels not always leads to an actual increase of protein production. Post-transcriptional regulation of mRNAs as well as post-translational modifications and the rate of protein degradation contribute to determine the global levels of specific proteins. In this respect, it should be noted that SOD1 mRNA levels, which peaked at 8 h post-treatment, decreased near to control levels already at 16 h, thus indicating that the up-regulation of gene expression was not maintained for longer time. The high efficiency of SOD is essential for the mateinance of cellular redox state, because its ability to eliminate superoxide ions. In previous studies, it has been demonstrated that decreased SOD activity contributes to increase oxidative damage in APP transgenic mouse used as AD animal models *(Schuessel et al., 2005; Milani et al., 2011).*

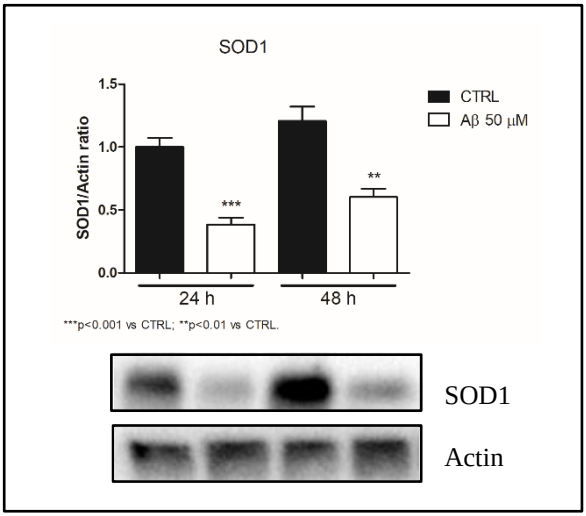

Figure 12. Effects of Aβ on SOD1 protein expression in U373 cells. Cells were treated with Aβ (50 μM) for the indicated time points. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to actin content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL; ** p≤0.01 vs CTRL.

We evaluated also CAT protein expression since this protein plays an essential role in cell defense against oxidative stress because of its ability to break down hydrogen peroxide into water and molecular oxygen.
As well as what observed for SOD1, the results shown in Fig. 13 indicate that also CAT was not modulated by Aβ treatment. Rather, we observed a significant decrease of CAT expression at 48 hours post-treatment.
Altogether, these results suggest that astrocytes may be susceptible to oxidative stress damage. Indeed, in this specific context, they seem to be unable to sustain an effective antioxidant response, in spite of an early activation of the Nrf2/ARE pathway (see Fig. 8-11).

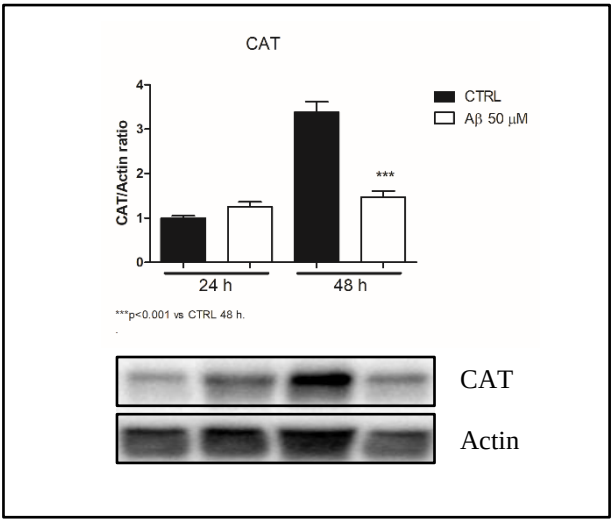

Figure 13. Effects of Aβ on CAT protein expression in U373 cells. Cells were treated with Aβ (50 μM) for the indicated time points. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to actin content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL.

Finally, we studied System X_c^- protein expression given its role in providing the cell with cystine for the biosynthesis of GSH and consequently for redox homeostasis. Indeed, the augmented expression and activity of the transporter can increase intracellular levels of cysteine, that is the rate-limiting substrate for the synthesis of glutathione.
We found that Aβ (50 μM) augmented System X_c^- protein levels in U373 cells at 24 hours post-treatment in whole extract analyzed by Western blot. The data show a significant 2-fold increase of System X_c^- expression in treated cells compared to controls (Fig. 14).

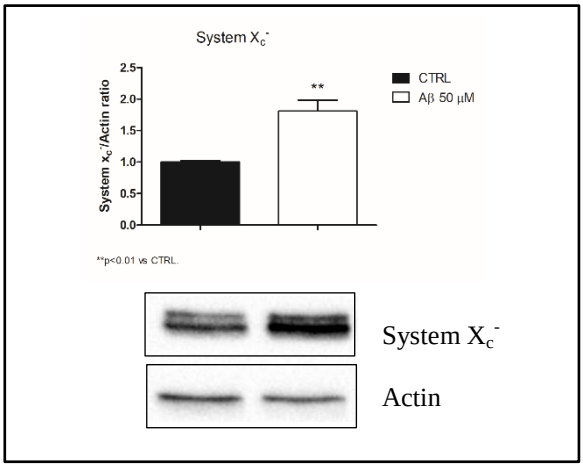

Figure 14. Effects of Aβ on System X_c^- protein expression in U373 cells. Cells were treated with Aβ (50 μM) for 24 hours. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to actin content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0.01 vs CTRL.

We have evaluated also by confocal microscopy the expression of System X_c^- in astroglial cells treated with Aβ (50 μM) for 24 and 48 hours. The data shown in Fig. 15 confirm the increase of System X_c^- expression in treated cells compared to controls at both 24 and 48 hours post-treatment.

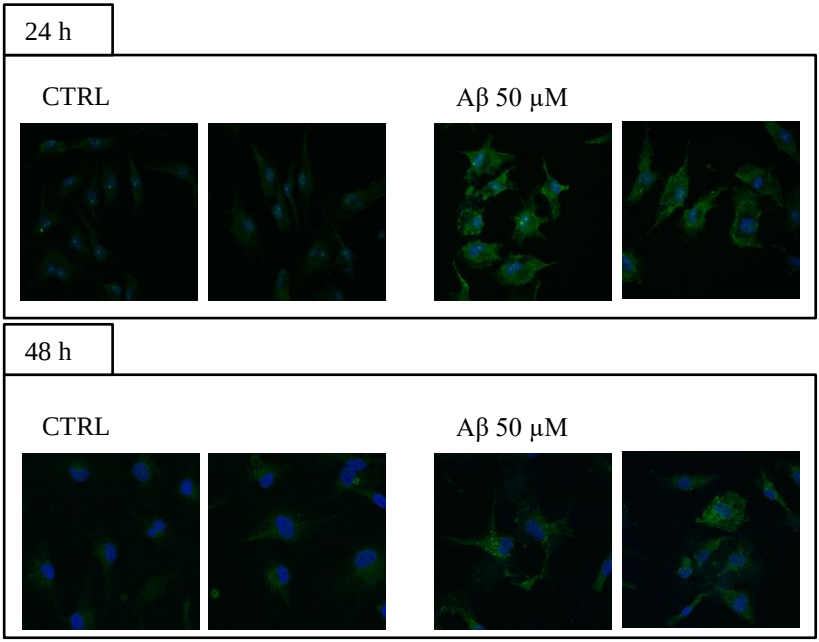

Figure 15. System X_c^- expression in Aβ-treated U373. Cells were treated with Aβ (50 μM) for 24 and 48 hours and subjected to immunofluorescence staining using anti-System X_c^- 1:100 (OriGene, green) antibodies as specified in Methods and analyzed by immunofluorescence microscopy. Nuclei (blue) are stained with DAPI.

These data indicate that astrocytes, upon stimulation with Aβ, can activate an antioxidant response through System X_c^- up-regulation, that allows the cell to increase its GSH content. However, this response is only partially effective because of the lack of prolonged expression over the time of SOD1 and CAT antioxidant enzymes.

In order to evaluate whether Aβ-induced antioxidant response can protect astrocytes from death, we analyzed with MTT assay the viability of U373 cells treated with Aβ (50 μM) for 24 hours. The results show a significant decrease of astrocytes viability of about 35% at 24 hours post-treatment.
To study the role of System X_c^- on the viability of U373 cells treated with Aβ, we performed MTT assay on astroglial cells cultured with or without sulfasalazine (SSZ, 300 μM), a specific System X_c^- inhibitor. As expected, the results shown in Fig. 16 indicate that Aβ-induced astrocyte death is not dependent by System X_c^- activity.

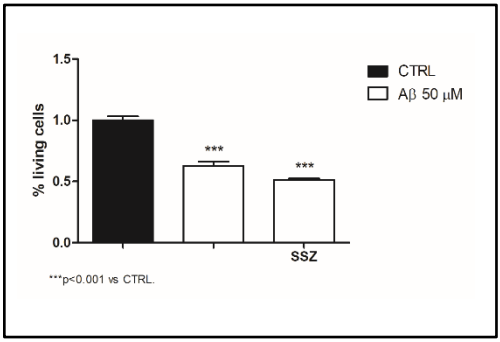

Figure 16. Effects of Aβ and System X_c^- activity on U373 cells viability. U373 cells were treated for 24 hours with Aβ alone or in the presence of SSZ. MTT cell viability assay was performed as specified in the Materials and methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of untreated U373 cells and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ***p≤0.001 vs CTRL.

In conclusion, astrocytes try to counteract Aβ-dependent oxidative damage activating an early antioxidant response, but this antioxidant response is not sufficient to counteract Aβ effects that lead astrocytes to death.

Effect of Amyloid-β on antioxidant response of neuronal cells.

It is known that antioxidant response in neurons is less efficient than in astrocytes *(Baxter et al., 2016)*, indeed previous findings from a study in neuronal and glial cells indicate that the Nrf2/ARE pathway was particularly weak in neurons *(Ahlgren-Beckendorf et al., 1999).*
In order to investigate whether amyloid-β can induce an antioxidant response in neuronal cells as in astrocytes, we first differentiated SH-SY5Y cells by culturing them in neurobasal medium supplemented with retinoic acid (RA) 10 μM and B-27 growth factor. Differentiated SH-SY5Y cells showed a complete decrease in cellular proliferation rate, block of cell cycle and induction of extensive neurites outgrowth (Fig. 17) *(Lopes et al., 2010).*

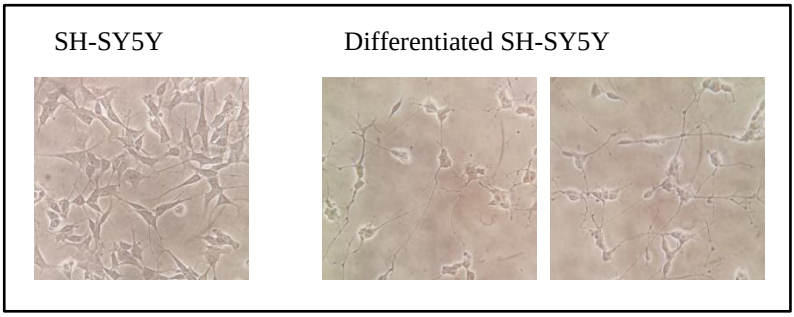

Figure 17. Changes in morphological features of SH-SH5Y before and after differentiation with Retinoic Acid 10 μM. Neuronal differentiation was performed as specified in the Materials and methods section.

In order to evaluate if Aβ treatment was able to activate Nrf2/ARE pathway in neuronal cells, we prepared nuclear extract from differentiated SH-SY5Y cells treated with Aβ (50 μM) for 2 and 4 hours. As shown in Fig. 18, western blot analysis demonstrated a significant decrease of Nrf2 nuclear levels at both times of treatment.

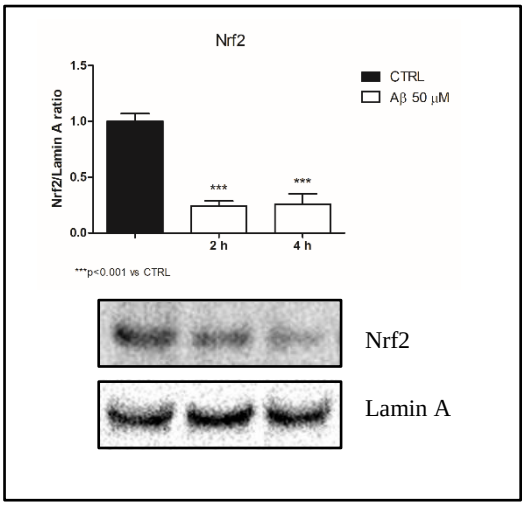

Figure 18. Effects of Aβ on Nrf2 nuclear translocation in differentiated SH-SY5Y cells. Cells were treated with Aβ (50 μM) for the indicated time points. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear lamin A content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL.

Then, we evaluated the mRNA and protein levels of SOD1 and System X_c^-. By Real time PCR analysis we found that in differentiated SH-SY5Y cells the treatment with Aβ (50 μM) induced a 2-fold increase of SOD1 mRNA expression at 16 hours post-treatment (Fig. 19A).

A possible explanation of these opposing results would be that some antioxidant genes can be regulated by transcription factors other than Nrf2, as reported in the literature. Baxter and colleagues reported that neuronal activity regulates ARE-driven gene expression independently on Nrf2, thus protecting cells from oxidative stress. Moreover, NF-κB was one of the first transcription factors shown to be redox regulated, and Rojo and colleagues

demonstrated that its activation leads to SOD1 transcriptional induction *(Baxter et al., 2016; Rojo et al., 2004)*.
We evaluated also SOD1 protein expression in differentiated SH-SY5Y cells treated with Aβ (50 μM) for 24 hours. Western blot analysis of whole cell extracts demonstrated that Aβ was able to decrease SOD1 protein expression in neuronal cells (Fig. 19B). Also in this case, we obtained opposing results between mRNA and protein expression that may suggest how neuronal cells are not fully equipped to counteract Aβ-induced oxidative stress.

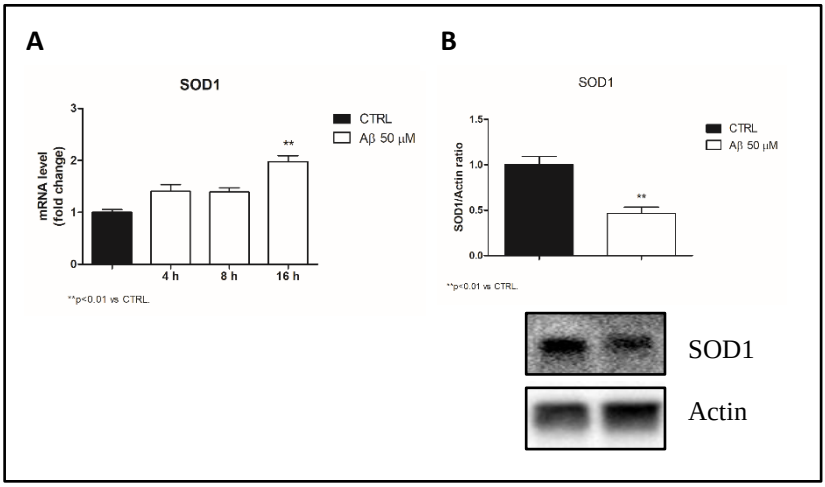

Figure 19. Effects of Aβ on SOD1 gene (A) and protein (B) expression in differentiated SH-SY5Y cells. (A) Differentiated SH-SY5Y cells were treated with Aβ (50 μM) for 4, 8 and 16h. After incubation at 37°C, the cells were homogenized and total RNA has been purified to assess mRNA levels of SOD1 gene by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0.01 vs CTRL. (B) Differentiated SH-SY5Y cells were treated with Aβ (50 μM) for 24h. Data are calculated relative to the internal housekeeping protein actin and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0. 01 vs CTRL.

With respect to System X_c^- activation, it should be noted that, besides Nrf2, activating transcription factor 4 (ATF4) can mediate the transcription of xCT subunit of System X_c^- under oxidative stress condition *(Ye et al., 2014)(Baxter et al., 2016).* Therefore, we have investigated xCT mRNA expression by Real Time PCR analysis on differentiated SH-SY5Y cells treated with Aβ (50 μM) for 4, 8 and 16 hours. The results show that Aβ was able to increase xCT mRNA levels with a peak at 4 hours post-treatment (Fig. 20A).
We analyzed System X_c^- protein expression in differentiated SH-SY5Y cells treated with Aβ (50 μM) for 24 hours by western blotting whole cell extracts. The data show a significant 1.5-fold increase of System X_c^- in treated cells compared to controls (Fig. 20B).

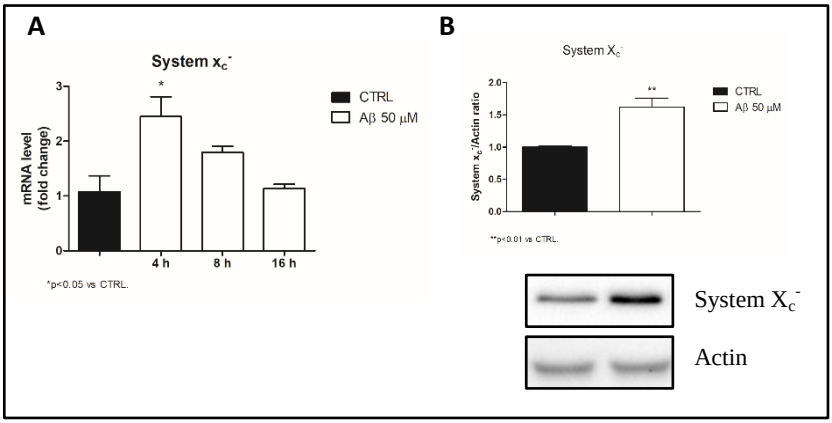

Figure 20. Effects of Aβ on System X_c^- gene (A) and protein (B) expression in differentiated SH-SY5Y cells. (A) Differentiated SH-SY5Y cells were treated with Aβ (50 μM) for 4, 8 and 16h. After incubation at 37°C, the cells were homogenized and total RNA has been purified to assess mRNA levels of System X_c^- gene by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * p≤0.05 vs CTRL. (B) Differentiated SH-SY5Y cells were treated with Aβ (50 μM) for 24h. Data are calculated relative to the internal housekeeping protein actin and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0. 01 vs CTRL.

Successively, we analyzed the effect of Aβ on neuronal cells viability.
To this aim, we performed MTT assay on SH-SY5Y cells treated with Aβ (50 μM) for 24 hours. The data show that treatment with Aβ did not interfere with neuronal cell viability. However, the pre-treatment with System X_c^- inhibitor, SSZ 300 μM, reduced of about 30% cells viability (Fig. 21).

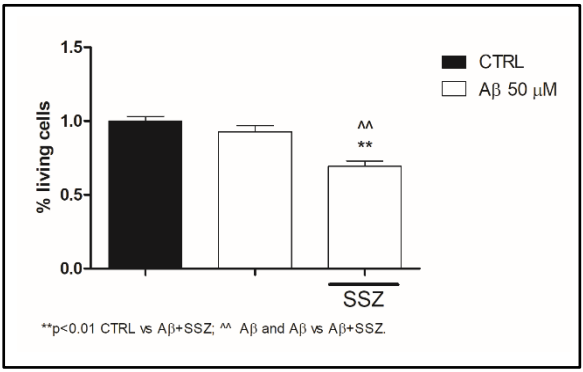

Figure 21. Effects of Aβ and System X_c^- activity on differentiated SH-SY5Y cells viability. Differentiated SH-SY5Y cells were treated for 24 hours with Aβ alone or in the presence of SSZ. MTT cell viability assay was performed as specified in the Materials and methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of untreated differentiated SH-SY5Y cells and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. **p≤0.01 vs CTRL; ^^p p≤0.01 vs Aβ.

This data indicate a fundamental role of System X_c^- in maintaining neuronal viability.

Effects of astrocytic antioxidant response on neuronal cell viability.

Although the induction of Nrf2-driven gene expression has been widely indicated as a protective mechanism to counteract the effects of oxidative stress, in many cell types such as astrocytes, the up-regulation of xCT elicited by Nrf2 could be a potential source for excitotoxicity due to excessive glutamate release *(Bridges R.J. et al., 2012)*.
Despite a lot of experimental and clinical findings indicate astrocytes as the cell population responsible for the bulk of the neuronal death in many neurodegenerative diseases, the cellular pathways leading to such a damage, in Alzheimer's disease, are not yet clearly defined.
Here, we investigated the effect of Aβ-induced astrocytic antioxidant response on neuronal cell viability, focusing in particular on the role of System X_c^-.
In order to study the effect of astrocytic System X_c^- activation on surrounding neurons, we performed co-cultures with U373 and differentiated SH-SY5Y cells. The co-culture system represents a better tool for understanding the role played by astrocytes in mediating neuronal protection or toxicity.

As shown in Fig. 22, System X_c^- protein was up-regulated in astrocytes co-cultured with neuronal cells.
We have evaluated System X_c^- protein expression in U373 cells co-cultured with differentiated SH-SY5Y cells treated with Aβ (50 μM) for 24 hours by western blot analysis of whole cell extracts. The results indicate a significant 1.5-fold increase of System X_c^- expression in astrocytes co-cultured in the presence of Aβ (50 μM) for 24 hours, compared to controls.

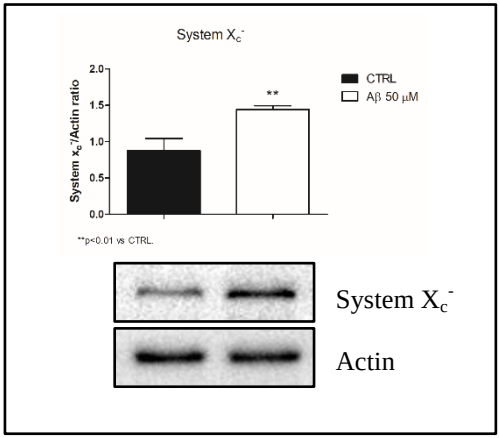

Figure 22. Effects of Aβ on System X_c^- protein expression in U373 cells co-cultured with differentiated SH-SY5Y cells. Co-cultures were treated with Aβ (50 μM) for 24 hours. The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to actin content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0.01 vs CTRL.

The up-regulation of System X_c^- can increase extracellular glutamate release and potentially cause excitotoxicity. Thus, we wondered whether astroglial cells treated with Aβ (50 μM) were able to release higher levels of glutamate in the extracellular space in comparison to control cells.
To this aim, we measured the glutamate levels in the supernatant of differentiated SH-SY5Y/U373 cell co-cultures untreated or treated with Aβ (50 μM) for 24 hours. As expected, Aβ treated cells released 50% more glutamate than untreated cells (Fig. 23).
Therefore, to verify whether Aβ-elicited glutamate release occurred through System X_c^- activation, we analyzed the levels of the amino acid in the supernatants of co-cultures treated without or with SSZ (300 μM).
We found that SSZ treatment prevented Aβ-induced glutamate release, reducing its levels in extracellular space. These findings clearly indicate that astroglial cells treated with Aβ induce an increased glutamate release by eliciting System X_c^- up-regulation (Fig. 23).

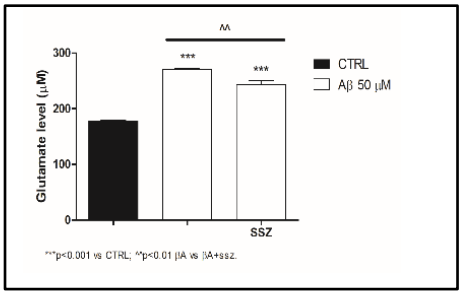

Figure 23. Effects of Aβ and System X_c^- activity on extracellular glutamate release in differentiated SH-SY5Y/U373 co-cultures. Co-cultures were treated for 24 hours. The Glutamate Assay was performed as specified in the Materials and methods section. The histogram shows the extracellular glutamate release (μM). Values are calculated relative to a glutamate standard curve (μM). Results are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL; ^^p≤0.01 between Aβ an Aβ+SSZ.

Afterwards, the viability of neuronal cells under these experimental conditions has been investigated.
The viability of differentiated SH-SY5Y cells and U373 cells grown in co-cultures was evaluated at 24 hours after treatment with Aβ (50 μM).
The results demonstrate that at 24 hours post-treatment there is a significant 50% less viability of differentiated SH-SY5Y cells and a significant 35% less viability of U373 cells than the respective control cells (Fig. 24).
To verify whether the reduced neuronal viability, induced by Aβ, was effectively due to the increased export of glutamate through System X_c^- transporter, we performed an MTT assay on co-cultures treated for 24 hours with Aβ alone or in the presence of SSZ. SSZ was able to prevent neuronal toxicity, restoring neuronal viability at the control level. These findings demonstrate for the first time that Aβ-induced neurotoxicity is mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells.
Also, excitotoxicity may play a pivotal role in neurodegeneration.
NMDA receptors (NMDARs) are important therapeutic targets for many CNS disorders including Alzheimer's disease. NMDARs have a relatively high affinity for glutamate, and can lead to excitotoxicity and neuronal apoptosis, as a result of perturbed cellular calcium homeostasis and mitochondrial alterations *(Newcomer et al., 2000).*
To verify whether the reduced neuronal viability, induced by extracellular glutamate release through System X_c^-, was effectively due to the activation of NMDA receptor, we performed MTT assay of co-cultures treated for 24 hours with Aβ alone or in the presence of NMDA receptor antagonist, MK801 (10 μM). As shown in Fig. 24, MK801 was able to prevent neuronal toxicity restoring the percentage of neuronal living cells at the control level.
These findings clearly demonstrate that Aβ-induced neurotoxicity is mediated by the activation of NMDA receptor in neuronal cells.

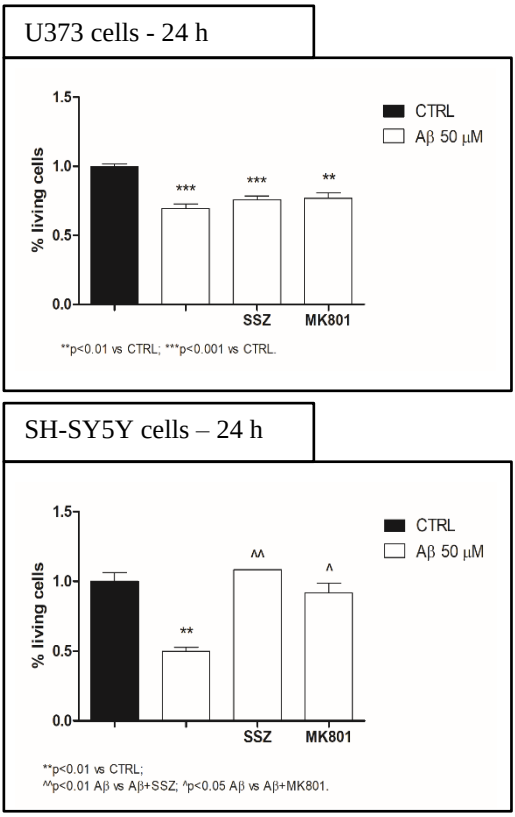

Figure 24. Effects of Aβ, System X_c^- activity and NMDA receptor activation on differentiated SH-SY5Y cells viability and U373 cells viability in co-cultures. Differentiated SH-SY5Y cells were co-cultured with U373 cells for 24 hours. Co-cultures were treated for 24 hours with Aβ alone or in the presence of SSZ, and with Aβ alone or in presence of MK801. MTT cell viability assay was performed as specified in the Materials and methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of control co-cultures and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** p≤0.01 vs CTRL; ^ p≤0.05 between Aβ and Aβ +MK801; ^^ p≤0.01 between Aβ and Aβ+SSZ.

In order to verify a short-term effect of Aβ, the viability of differentiated SH-SY5Y cells co-cultured with U373 was assessed after treatment with Aβ (50 μM) for 5 minutes.
At 5 minutes post-treatment we observed a significant 60% less viability of differentiated SH-SY5Y cells and a significant 50% less viability of U373 cells compared to the respective control cells.
To verify whether the reduced neuronal viability, evoked by Aβ, was effectively due to the increased export of glutamate through System X_c^-, we performed an MTT assay in co-cultures treated for 5 minutes with Aβ alone or in the presence of SSZ. As shown in Fig. 25, SSZ was able to prevent neuronal toxicity, partially restoring the percentage of neuronal living cells at the control level. These findings demonstrate for the first time that Aβ-induced neurotoxicity is a short-term effect mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells. The neurotoxic effect is maintained over time, at least up to 24 hours post-treatment as demonstrated in Fig. 24.

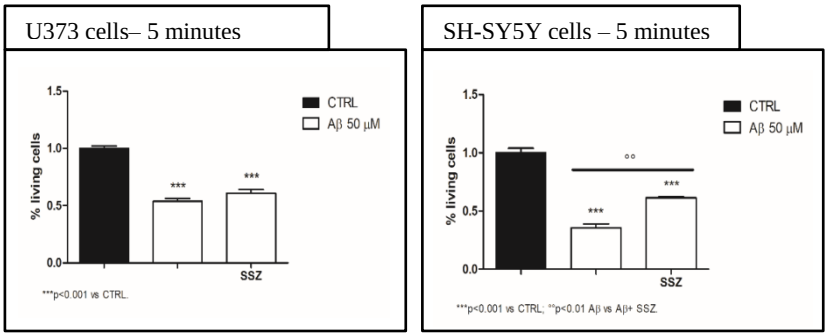

Figure 25. Effects of Aβ and System X_c^- activity on differentiated SH-SY5Y cells viability and U373 cells viability in co-cultures. Differentiated SH-SY5Y cells were co-cultured with U373 cells for 5 minutes. Co-cultures were treated for 5 minutes with Aβ (50 μM) alone or in the presence of SSZ. MTT cell viability assay was performed as specified in the Materials and methods section. The histogram shows the percentage of control. Values are calculated relative to the viability of control co-cultures and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. *** p≤0.001 vs CTRL; °° p≤0.01 between Aβ and Aβ+SSZ.

As a whole, Aβ can induce in astrocytes both Nrf2 activation and up-regulation of ARE-driven genes, including System X_c^-. The latter being responsible of reduced cell viability of neuronal cells co-cultured with astrocytes, because of sustained glutamate release and neuronal NMDA receptor activation.
It should also be noted that upon stimulation with Aβ, human neuronal cells were not able to activate Nrf2-mediated antioxidant response but instead, they were able to up-regulate phase II detoxifying and antioxidant genes through the action of other transcription factor, such us NF-κB and ATF4.

CONCLUSIONS

In this work we focused our attention on the role of System X_c^- transporter in the contest of neuroinflammation and neurodegeneration.
We hypothesized a double role of System X_c^- in the CNS: on the one hand, System X_c^- has a protective role towards astrocytes due to the production of GSH inside these cells; on the other hand, it could be dangerous towards bystanding neurons, because of its ability to export glutamate in extracellular space thus promoting excitotoxicity and neuronal death. Accordingly, in a lot of neurodegenerative diseases, for example HAND and AD, System X_c^- could have a pivotal role.
Our previous findings indicate that, in astroglial cells, endogenously produced Tat can induce an effective antioxidant response through the activation of Nrf2/ARE pathway and suggest how infected astrocytes can counteract oxidative stress induced by HIV infection.
In this work we demonstrated that Tat-producing astrocytes (U373-Tat) released twice the amount of glutamate respect to control cells (U373-mock) and we found that treatment with SSZ, a System X_c^- inhibitor, prevented Tat-induced glutamate release, maintaining its levels comparable to the control.
Therefore, we evaluated the effect of glutamate release on neuronal cell viability (SH-SY5Y cells) in co-cultures conditions. We observed 20% less viability of SH-SY5Y cells co-cultured with U373-Tat and we demonstrated that SSZ was able to prevent neuronal toxicity restoring neuronal viability at the control level.
These findings demonstrate for the first time that Tat-induced neurotoxicity is mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells. Moreover, our study suggests that latently infected astrocytes, as occur in the CNS of HIV-infected patients, may mediate neurodegeneration through this pathway.
Free radical generation and oxidative stress play pivotal roles in many neurodegenerative diseases, including Alzheimer's disease.
Firstly, we evaluated the effect of the aggregated form of Aβ, a key marker of AD, on astrocytes (U373 cells).
We demonstrated that Aβ is able to induce the transcription of ARE genes in astrocytes. In particular, we demonstrated a significant increase of CAT, SOD1, SOD2, HO-1, GPX, GCL and System X_c^- genes transcription with a peak between 4- and 8-hours post-treatment for all the genes analyzed.

Given that ARE genes are mainly regulated by Nrf2, we investigated whether Aβ could activate this transcription factor in U373 cells. We found that Aβ induced a 1.8-fold increase of the nuclear Nrf2 levels already at 2 hours post-treatment. Accordingly with these findings, we investigated whether Aβ was able to induce also the increase at protein expression level of SOD1, CAT and System X_c^-. We found that Aβ induced a significant decrease of SOD1 and CAT proteins expression in treated cells compared to controls. Post-transcriptional regulation of mRNAs as well as post-translational modifications and the rate of protein degradation contribute to determine the global levels of specific proteins. In this respect, it should be noted that SOD1 mRNA levels, which peaked at 8 h post-treatment, decreased near to control levels already at 16 h, thus indicating that the up-regulation of gene expression was not maintained for longer time. On the contrary, we found that Aβ induced a significant 2-fold increase of System X_c^- protein expression in treated cells compared to controls.

All together these data indicate that astrocytes try to counteract Aβ-induced oxidative stress through System X_c^- activity, that allows the cell to increase its GSH content. However, System X_c^- activity is not supported by the action of phase II detoxifying and antioxidant enzymes such as SOD1 and CAT. Indeed, the evaluation of cell viability shows a significant 35% less viability of astrocytes treated with Aβ compared to control; moreover, the treatment with SSZ was not able to restore to control levels the U373 viability of Aβ+SSZ co-treated cells, indicating that astrocytes death was independent on System X_c^- activity.

Secondly, we evaluated the effect of Aβ on neuronal cells (differentiated SH-SY5Y cells).

It is known that antioxidant response in neurons is less efficient than in astrocytes, indeed we demonstrated that Aβ can induce a significant decrease of Nrf2 nuclear translocation at 2- and 4-hours post-treatment. Nevertheless, we observed the increase of System X_c^- levels and no reduction of viability upon treatment with Aβ. Moreover, our findings show that the System X_c^- inhibitor, SSZ, decreased neuronal viability, thus indicating that the activation System X_c^- in neuronal cells cultured in the absence of astrocytes, play a protective role being able to counteract the effects of Aβ.

Finally, we investigated the effect of Aβ-induced astrocytic antioxidant response on neuronal cell viability, focusing in particular on the role of System X_c^-. We observed that Aβ treatment in neuronal/astrocyte co-culture system leads to a significant 50% less viability of differentiated neuronal cells and to a significant 35% less viability of astroglial cells than the

respective control cells. SSZ was able to prevent neuronal toxicity, restoring neuronal viability at the control level. Our findings demonstrate for the first time that Aβ-induced neurotoxicity is mediated by an increase of glutamate release due to System X_c^- up-regulation in astroglial cells. It should be pointed out also that the activation of System X_c^- in different cell populations may lead, as we demonstrated here, to opposing effects and great attention should be paid in designing new therapeutic targets.

Given the involvement of astrocytes in CNS pathology, it is not surprising that the ability to exacerbate neurodegeneration through the conversion of oxidative stress to excitotoxicity via system X_c^- has been linked to a variety of disorders (Fig. 26). It should be noted that all the experiments of this work have been performed with cancer cell lines and this is surely a limiting factor, but it represents a first step to understand the process responsible of neuronal death in HAND and AD. Anyway, the present study shed light on Nrf2/system X_c^- pathway and may help to better understand the role of astrocytes as the cell population responsible for the bulk of the neuronal death in HAND and AD.

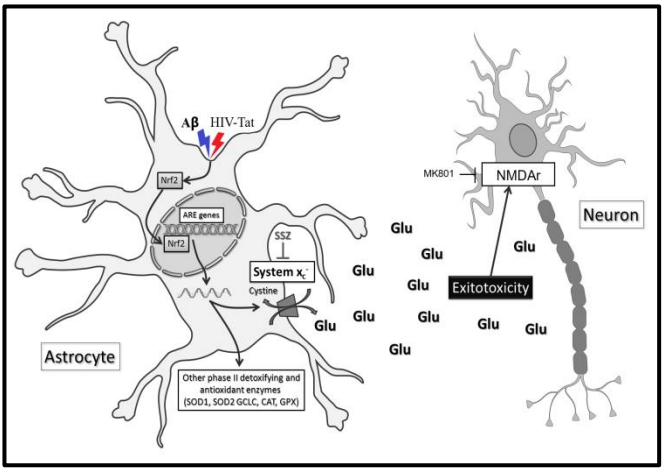

Figure 26. Proposed model for HIV-Tat and Aβ effects on Nrf2 translocation, ARE genes up-regulation, System X_c^- activation and glutamate release during respectively HAND and AD. In astrocytes, Tat and Aβ trigger an antioxidant response through the transcriptional induction of Nrf2-dependent ARE genes (e.g., SOD1, SOD2 GCLC, CAT, GPX, HO-1 and System X_c^-). While the import of L-cystine through the transporter System X_c^- is critical to protection from oxidative stress (i.e., glutathione production), the export of glutamate represents a route of release through which this neurotransmitter may cause neuronal death. We propose that astrocytes can exacerbate neurodegeneration through the conversion of oxidative stress to excitotoxicity via System X_c^-. Moreover in AD, we observed that released extracellular glutamate induces NMDA receptor activity on neuronal cells; its activation mediates neurotoxicity. To analyze the process, the experiments on glutamate release and neuronal viability have been performed using co-cultures of neuronal cells with astrocytes. For more details see text.

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FIGURE LIST

ABBREVIATION LIST

AD: Alzheimer's disease
APP: β-amyloid precursor protein
ARE: antioxidant response element
ATF4: Activating transcription factor 4
Aβ: amyloid-β peptide
AβDPs: Aβ degrading proteases
bZIP: basic leucine zipper
CAT: catalase
CNC: cap'n'collar
CNS: central nervous system
CSF: cerebrospinal fluid
GCL: glutamate-cysteine ligase
GPX: glutathione peroxidase
GSH: glutathione
H2O2: hydrogen peroxide
HAND: HIV-associated neurocognitive disorders
HAT: amino acid transporters
HO-1: heme oxygenase 1
HOCl: hypochlorous acid
IDE: insulin-degrading enzyme
IL-1β: interleukin 1 beta
KEAP1: Kelch-like ECH associated protein 1
MMPs: matrix-metalloproteinases
MAF: small musculoaponeurotic fibrosarcoma
Neh: Nrf2-ECH homologies
NEP: Neprilysin
NF-κB: nuclear factor kappa B
NMDA: N-methyl-D-aspartate
NMDAR: N-methyl-D-aspartate receptor
NO-: nitric oxide
NQO1; NADPH:quinone oxidoreductase 1
Nrf2: nuclear factor erythroid 2 related factor 2
O2-: superoxide radical anion
O2•: singlet oxygen
OH-: hydroxyl radical
ONOO-: peroxynitrite

PS1: presenilin-1
PS2: presenilina-2
RA: retinoic acid
Rbx-1: E3 ubiquitin ligase complex
RNS: reactive nitrogen species
ROO-: peroxyl-radicals
ROS: reactive oxygen species
SOD: superoxide dismutase
SSZ: sulfasalazine