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"INVESTIGATING THE OXIDATIVE/NITROSATIVE STRESS RESPONSE INDUCED BY HIV-TAT PROTEIN IN GLIAL AND NEURONAL CELLS"

"STUDIO DELLA RISPOSTA ALLO STRESS OSSIDATIVO/NITROSATIVO INDOTTO DALLA PROTEINA TAT DEL VIRUS HIV IN CELLULE GLIALI E NEURONALI"

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ABSTRACT

In the last years, evidence has been accumulated suggesting that oxidative stress plays a major role in the HIV associated neuropathogenesis. Oxidative stress is defined as an imbalance between the pro-oxidant and the anti-oxidant systems, with the shift towards the pro-oxidant system. To balance ROS levels and counteract their toxic effects, cells employ several enzymatic and non-enzymatic antioxidant systems. The transcription factor Nrf2, is an important regulator of cell survival and adaptive mechanisms, in conditions of elevated oxidative stress, translocates into the nucleus, binds to the promoter regions of many phase II detoxifying and antioxidant genes, the antioxidant-response elements.

As previously demonstrated in our lab, the neurotoxic effects of the HIV protein Tat are associated with the stimulation of the NMDA receptors that in turn induce an increased spermine oxidase activity and a consequent ROS accumulation. Since in many cell types ROS are also able to induce an antioxidant response, we analyzed the effect of Tat-induced spermine oxidase activation on the Nrf2/ARE pathway in SH-SY5Y human neuroblastoma cells. We found out that Tat was able to induce Nrf2 activation, and this effect was reverted to control levels by chlorexidine, a strong competitive inhibitor of spermine oxidase. Next we evaluated, by RT-qPCR analysis, the expression of some ARE genes in Tat-treated cells. The results indicate a significant up-regulation of all the genes analyzed (HO-1, SOD 1, SOD 2, NQO1 and CAT) at 4h post-treatment. This effect was reverted to control levels by chlorexidine pre-treatment. Since Tat-induced spermine oxidase activation is mediated by the stimulation of NMDAR, we analyzed Nrf2 activation and ARE genes expression in Tat-stimulated cells pretreated with MK801, a specific NMDAR antagonist. We found out that MK801 completely prevented Tat-induced Nrf2 activation and ARE genes expression thus indicating the involvement of NMDAR in this pathway. The results strongly suggest a role for this receptor and for spermine oxidase in Tat-induced antioxidant response in human neuronal cells.

Since in neurons the activation of NMDAR leads to enhancement of NO production by the calcium-dependent neuronal NOS, and NO is an endogenous inducer of Nrf2-dependent phase-2 enzymes both *in vitro* and *in vivo*, we also analyzed the role of NO in Nrf2-ARE pathway activation in Tat-stimulated neuronal cells. The results showed that L-NAME, a specific NOS inhibitor, was able to significantly reduce the nuclear translocation of Nrf2 induced by Tat. Next, we studied the involvement of NO in Tat-induced SMO activation. Our data indicate that the pre-treatment of SH-SY5Y cells with L-NAME significantly reduced Tat-induced spermine oxidase activation. Therefore, we wondered whether Tat could directly affect the

enzyme activity by *S*-nitrosylation. To this aim we carried out *biotin-switch assay* on protein extracts of SH-SY5Y cells treated with Tat and found out that, although the treatment with NO donors induced SMO *S*-nitrosylation, the treatment with Tat was not able to induce this protein modification. Thus, further studies are needed to deeply understand the effects of NO in Tat-elicited SMO activation that could be due to modifications of other targets. Moreover, since oxidative stress leads to protein misfolding and aggregation, we evaluated the induction of p62-mediated selective autophagy in SH-SY5Y cells treated with Tat. We found out that Tat was able to induce a 2/3-fold increase of p62 expression at 8h, 16, 24h post-treatment. Conversely we didn't observe any induction of LC3 in the same experimental conditions. Next, we evaluated the presence of Tat in both cytosol and nucleus compartments at different time points and we found out that Tat was present in the cytosol at 4h and 8h post-treatment. Tat levels strongly decreased at 16h and 24h when p62 protein increased, thus suggesting a role for p62 in Tat degradation. Based on this consideration, we evaluated Tat/p62 interaction by co-immunoprecipitation experiments and we found that p62 partially co-precipitated with Tat.

Besides the study on neuronal cells we analyzed also the effect of HIV proteins on astroglial cells. Astrocytes play a critical role in mediating neuronal toxicity or neuronal rescue. In neurodegeneration associated with HIV-1, chronic inflammation and oxidative stress play a crucial role and these conditions are often related. As an example, excessive amount of NO, as produced by inducible NO synthase upon the exposure of activated astrocytes to cytokines and/or viral proteins, is assumed to contribute to neuronal dysfunction associated to HIV infection. We observed that gp120 and Tat are able to induce a cPLA2-dependent arachidonic acid production, this response being critical for allowing activation of the transcriptional factor NF-kB and subsequent iNOS and interleukin-1β transcription in astroglial cells. Tat and gp120 effects were evaluated in the absence and presence of a cPLA2 inhibitor and/or arachidonic acid. The results demonstrate that treatment of cells with these two HIV proteins was able to activate NF-κB, this activation being inhibited by pre-treatment with cPLA2 inhibitor and restored by pre-treatment with arachidonic acid. Since NF-kB is involved in the transcription of a variety of pro-inflammatory genes, including iNOS and IL1β, we have analyzed the role of the cPLA2-AA pathway in the regulation of iNOS and IL1β transcription. Tat and gp120 induced in a dose-dependent manner both iNOS and IL1β mRNA levels. The pre-treatment with the cPLA2 inhibitor restored mRNA levels of iNOS and IL1β to control levels. Altogether, these results suggest that HIV proteins induce an early arachidonic acid production that seems to act as an upstream proinflammatory effector.

Moreover, we analyzed the effect of Tat on the antioxidant response of astroglial cells. In particular, we demonstrated that Tat was able to induce Nrf2 and Nrf2-driven gene expression in U373 cells. The activation of Nrf2 was also evaluated in U373 cells transfected with Tat. Also in this model system, endogenously produced Tat was able to induce an antioxidant response as indicated by Nrf2 nuclear translocation and ARE gene expression (GCLC, GPX, SOD1, SOD2, CAT, NQO1). In particular, we found out increased levels of SystemXc, an amino acid transporter that transports cystine into the cell in exchange for glutamate. SystemXc plays a crucial role in the regulation of extracellular glutamate and the maintenance of glutathione levels therefore it is involved in both excitotoxicity and antioxidant response. Based on the above considerations, we performed co-culture experiments to evaluate neuronal viability and Nrf2/ARE pathway activation in the presence of stably transfected astrocytes expressing Tat. Here, we reported a 20% reduction of viability of SY5Y cells co-cultured with Tat-expressing astrocytes. In addition, Tat also led to Golgi dispersal in neuronal cells. Besides this detrimental actions, we also demonstrated the induction of an antioxidant response in neuronal cells as elicited by astrocyte-released Tat, this effect being due to Nrf2 activation. It should be reminded that Tat-induced ROS/RNS generation may play crucial role in the canonical pathway of Nrf2 activation since they can directly modify the stress sensor protein Keap1.

In summary, our findings provide evidence of an antioxidant response activation and may help our understanding of the mechanism by which Nrf2 can mediate protection against neurodegenerative diseases associated with HIV infection.

SINTESI

Negli ultimi anni, è stato evidenziato come lo stress ossidativo svolga un ruolo centrale nella patogenesi dei disturbi neurocognitivi associati all'infezione da HIV. Lo stress ossidativo è definito come uno sbilanciamento tra i sistemi pro-ossidanti e antiossidanti, con uno spostamento verso il sistema pro-ossidante. Per bilanciare i livelli di ROS e neutralizzare i loro effetti tossici, le cellule impiegano diversi sistemi antiossidanti, enzimatici e non-enzimatici. Il fattore di trascrizione Nrf2 è un importante regolatore della sopravvivenza cellulare e dei meccanismi adattativi messi in atto dalle cellule. In condizioni di elevato stress ossidativo, Nrf2 trasloca nel nucleo e si lega agli elementi ARE presenti nel promotore dei geni detossificanti e antiossidanti.

Come dimostrato precedentemente nel nostro laboratorio, l'effetto neurotossico di Tat è associato alla stimolazione dei recettori NMDA, che a sua volta induce un incremento dell'attività della spermina ossidasi e il conseguente accumulo di ROS. Poiché in alcuni tipi cellulari la produzione di ROS è in grado di indurre una risposta antiossidante, abbiamo analizzato l'effetto dell'attivazione della spermina ossidasi indotta da Tat nella via Nrf2/ARE in cellule di neuroblastoma umano SH-SY5Y. I risultati di questo lavoro mostrano che Tat è in grado di indurre l'attivazione di Nrf2 e che questo effetto può essere riportato ai livelli del controllo, pre-trattando le cellule con clorexidina, un potente inibitore competitivo della spermina ossidasi. Inoltre, l'attivazione di Nrf2 indotta da Tat provoca l'aumento di espressione dei geni HO-1, SOD1, SOD2, NQO1, CAT come indicato dagli esperimenti di RT-qPCR. In particolare abbiamo osservato un incremento significativo di tutti i geni analizzati dopo 4h di trattamento, tale aumento viene ricondotto ai livelli del controllo pretrattando le cellule con clorexidina. Poiché l'attivazione della spermina ossidasi indotta da Tat è mediata dalla stimolazione del recettore NMDA, abbiamo studiato il coinvolgimento di questo recettore nell'attivazione di Nrf2 in cellule SH-SY5Y trattate con Tat. Come riportato nella tesi, il trattamento con MK801, l'antagonista specifico del recettore NMDA, previene completamente sia l'attivazione di Nrf2 sia l'espressione di alcuni geni ARE nelle cellule trattate con Tat. Nell'insieme questi risultati indicano il coinvolgimento del recettore NMDA nella via di attivazione Nrf2/ARE, e suggeriscono un ruolo per questo recettore nella risposta antiossidante indotta da Tat in cellule neuronali umane.

Dal momento che nei neuroni l'attivazione del recettore NMDA porta all'aumento della produzione dell'NO da parte della NOS neuronale e che l'NO è un induttore endogeno degli enzimi di fase II dipendenti da Nrf2 sia *in vitro* che *in vivo*, abbiamo analizzato il ruolo dell'NO nell' attivazione della via NRF2/ARE nelle stesse cellule neuronali stimolate con Tat. I risultati

mostrano che l'inibitore specifico delle NOS, L-NAME, è in grado di ridurre significativamente la traslocazione nucleare di Nrf2 indotta da Tat. Successivamente abbiamo osservato che il pretrattamento delle cellule SH-SY5Y con L-NAME riduce significativamente l'attivazione della spermina ossidasi indotta da Tat indicando il coinvolgimento dell'NO nell'attivazione dell'enzima. Inoltre, è stata analizzata l'eventuale *S*-nitrosilazione della spermina ossidasi tramite la metodica di *biotin-switch.* Benché i risultati mostrino come i donatori di NO siano in grado di indurre questa modificazione nell'enzima, il trattamento con Tat non è in grado di svolgere lo stesso effetto. Questo dato suggerisce che gli effetti dell'NO nell'attivazione della spermina ossidasi indotta da Tat potrebbero essere dovuti alla modificazione di altri target.
Lo stress ossidativo è correlato a disfunzioni nel ripiegamento delle proteine e ad aggregazione proteica. La cellula è in grado di contrastare queste condizioni attraverso meccanismi di degradazione selettiva tramite la proteina p62. L'espressione di tale proteina è regolata da Nrf2. Pertanto abbiamo analizzato i livelli di p62 nelle cellule SH-SY5Y trattate con Tat. I risultati mostrano che Tat è in grado di indurre un incremento dell'espressione di p62 a 8, 16 e 24 h; al contrario, la proteina LC3, coinvolta nell'autofagia, non viene modulata nelle stesse condizioni sperimentali. Inoltre, abbiamo valutato la presenza di Tat in entrambi i compartimenti, citosolico e nucleare, a differenti tempi ed abbiamo osservato che i livelli di Tat diminuiscono fortemente a 16 e 24 h in corrispondenza dell'aumento di p62. Nell'ipotesi che p62 potesse avere un ruolo nella degradazione di Tat, abbiamo valutato l'interazione tra Tat e p62 attraverso esperimenti di co-immunoprecipitazione e abbiamo dimostrato che le due proteine interagiscono parzialmente.
Accanto allo studio delle cellule neuronali, abbiamo valutato l'effetto delle proteine del virus HIV su cellule astrogliali. Infatti, gli astrociti svolgono un ruolo critico nel mediare sia la protezione sia la tossicità neuronale. Nella neurodegenerazione associata ad HIV-1 l'infiammazione cronica e lo stress ossidativo giocano un ruolo cruciale e queste condizioni sono spesso correlate. Ad esempio, un eccesso di NO prodotto dalla NOS inducibile da parte di astrociti attivati esposti a citochine o proteine virali (come Tat e gp120 di HIV) può contribuire alla disfunzione neuronale. In questo lavoro è stato dimostrato come Tat e gp120 siano in grado di indurre la produzione di acido arachidonico da parte della cPLA2. Questa risposta permette l'attivazione del fattore di trascrizione NF-kB e l'espressione dei geni da esso attivati in cellule astrogliali, come iNOS e interleuchina 1β. I risultati ottenuti suggeriscono che le proteine dell'HIV inducono una produzione precoce di acido arachidonico che sembra agire precocemente come effettore pro-infiammatorio.

In seguito, abbiamo analizzato l'effetto della proteina Tat sulla risposta antiossidante in cellule astrogliali. In particolare abbiamo dimostrato che Tat è in grado di indurre l'espressione di Nrf2 e dei geni da esso regolati in cellule U373. La traslocazione di Nrf2 è stata valutata anche in cellule U373 trasfettate con Tat; anche in questo modello, Tat è in grado di indurre una risposta antiossidante come indicato dall'induzione della traslocazione di Nrf2 e dell'espressione dei geni ARE. In particolare, abbiamo focalizzato la nostra attenzione sulla sovra-regolazione del SystemXc, un trasportatore amminoacidico che trasporta cistina nelle cellule in cambio di glutammato. Il SystemXc gioca un ruolo cruciale nella regolazione del glutammato extracellulare e nel mantenimento dei livelli di glutatione, essendo così coinvolto sia nella eccitotossicità che nella risposta antiossidante. Basandoci su queste considerazioni, abbiamo effettuato esperimenti in co-cultura per valutare la vitalità delle cellule neuronali e l'attivazione della risposta antiossidante in presenza di astrociti stabilmente trasfettati esprimenti Tat. I risultati indicano una riduzione significativa della vitalità delle cellule neuronali in co-cultura con U373-Tat, nonostante una leggera attivazione della risposta antiossidante in cellule neuronali. Inoltre abbiamo dimostrato che sia il trattamento con Tat che Tat secreta dagli astrociti sono in grado di indurre la dispersione del Golgi in cellule neuronali. Complessivamente i risultati di questo lavoro mostrano come Tat sia in grado, da una parte, di mediare degli effetti neurotossici e dall'altra parte, di indurre una risposta antiossidante in cellule neuronali umane. Infine viene messo in evidenza il ruolo delle cellule astrogliali nella modulazione degli effetti protettivi e/o dannosi anche in base alle funzioni peculiari di questa popolazione cellulare.

1) INTRODUCTION

Oxidative/nitrosative stress and neurodegenerative disorders

Oxidative/nitrosative stress can be defined as an imbalance between the production of free radicals and the antioxidant cell systems, with the shift towards free radicals generation. Living organisms possess finely regulated systems to maintain very low ROS/RNS levels, i.e. their production and elimination are well balanced resulting in a steady-state ROS/RNS level. However, under certain circumstances this balance can be disturbed. There are several reasons for that: increased level of endogenous and exogenous compounds, depletion of reserves of antioxidants, inactivation of antioxidant enzymes, decrease in production of antioxidant enzymes and, finally, combinations of two or more of the listed above factors.

Molecular entities or molecular fragments which contain one or more unpaired electrons in an atomic orbital or molecular orbital is referred as free radical (Halliwell, 1999). These unpaired electron(s) usually give a considerable degree of reactivity to the free radical. The most important class of radical species generated in living systems are radicals derived from oxygen species. Molecular oxygen has a unique electronic configuration and is itself a radical. The addition of one electron to O_2 forms the superoxide anion radical (O_2^-) (Miller and Buettner, 1990). Superoxide anion arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the primary ROS, and can further interact with other molecules to generate secondary ROS, either directly or prevalently through enzyme or metal-catalyzed processes (Valko and Morris, 2005). RNS are another class of free radicals including nitric oxide (NO) and its derivates. NO is generated in biological tissues by specific nitric oxide synthases (NOS) (Ghafourifar, 2005). NO is a reactive radical that acts as an important biological signaling molecule in a large variety of diverse physiological processes like neurotransmission, defence mechanisms, smooth muscle relaxation, blood pressure regulation, and immune regulation (Bergendi, 1999). Overproduction of RNS is called nitrosative stress (Ridnour et al., 2004) and it occurs when in a system the generation of RNS exceeds the system's ability to eliminate and neutralize them. Nitrosative stress leads to post-translational protein modifications that can inhibit the normal function of many proteins.

Several pathologies such as cancer (Leinone et al., 2014) diabetes mellitus (Yan 2014), cardiovascular (Mei et al., 2014) and neurodegenerative diseases (Ahmad et al., 2014) are linked to chronic oxidative stress. However, the cause-effect relationship is still not completely understood. In many cases, the occurrence of such events depends on the possibility of living organisms to adapt their defense systems to enhanced ROS/RNS generation. These

systems usually operate at the level of expression of specific genes encoding antioxidant and associated enzymes, or enzymes responsible for production of antioxidants (Volodymyr, 2014).

In neurodegenerative diseases, oxidative stress leads to protein misfolding and upon polyubiquitination the misfolded proteins accumulate in cytoplasmic and intracellular inclusions forming protein aggregates (Damme et al., 2015). Neurons are long-lived, terminally-differentiated cells that do not undergo renewal. Due to their extreme polarization, size and post-mitotic nature, they are uniquely sensitive to the accumulation of misfolded proteins, dysfunctional organelles and protein aggregates, because they cannot rely on the dilution of cellular waste occurring during cell division. An example of neurodegenerative disease associated with the accumulation of aggregates is the Parkinson disease (PD), where α-synuclein and parkin represent the major protein components of the inclusion bodies found in the brain (Goedert 2001). Other examples of protein aggregates include Amyloid plaques and neurofibrillary tangles in Alzheimer disease (AD), Lewy bodies in PD, Mallory bodies (MBs) in steatohepatitis, and intracytoplasmic hyaline bodies in hepatocellular carcinoma (HCC) (Kuusisto et al. 2001). Cellular homeostasis requires a constant balance between biosynthetic and catabolic processes. In particular, protein turnover is essential, both for maintaining the pool of amino acids required for continued protein synthesis and for removing defective proteins that are translated or folded incorrectly. Furthermore, many essential cellular functions, such as cell division, transcription and signal transduction, are regulated by the modulation of protein levels accomplished by altering the balance of protein synthesis and degradation (Nedelsky et al., 2008).

The two major intracellular protein degradation pathways are the ubiquitin proteasome system (UPS) and autophagy. The term "autophagy" refers to a range of processes, including chaperone-mediated autophagy, microautophagy and macroautophagy, the latter being the major and best-characterized subtype of autophagy (Frake et al., 2015).

Oxidative/nitrosative stress in HAND

The world has observed the development of HIV-related diseases across countries as a severe global health problem, with the number of people living with HIV infection reaching an estimated 34.0 million and an estimated 2.5 million new HIV infections occurring in 2011 (UNAIDS 2012). It is generally assumed that in HIV-1-infected patients, the virus not only destroy the immune system and leads to acquired immunodeficiency syndrome (AIDS), but also penetrates the central nervous system (CNS) soon after it infects target peripheral immune cells, presumably via infiltration of HIV-1-infected macrophages and lymphocytes, leading to several neurological

disorders, collectively known as HAND (HIV-1 associated neurological disorders). The American Academy of Neurology (AAN) modified the research diagnostic criteria of HAND in 2007 by recognizing three major categories: asymptomatic neurocognitive impairment (ANI), HIV-associated mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) as the most severe form of neurocognitive impairment (Jianxun et al., 2013). The development of HAD is one of the most devastating consequences of HIV-1 infection in CNS and it involves a variety of neuropathological complications directly triggered by HIV-1, including peripheral neuropathies, vacuolar myelopathy, and a syndrome of cognitive and motor dysfunction (Kaul et al., 2001 and 2005). In fact, HAD is characterized by neurocognitive impairment (forgetfulness, slowing of thought and poor concentration), emotional disturbance (apathy and social withdrawal), and motor abnormalities (weakness, ataxia, clumsy gait, and tremor) (Yadav and Collman, 2009; del Palacio et al., 2012). The introduction of highly active antiretroviral therapy (HAART) has successfully increased life expectancies by reducing morbidity and mortality of patients infected with HIV-1 and has dramatically decreased incidence of HAD to as low as 10.5%. In addition, improved control of peripheral viral load and the treatment of opportunistic infections continue to prolong survival time. Nonetheless, HAART is insufficient to provide protection from HAD, or to reverse the disease in most cases, because it is unable to prevent the entry of HIV-1 into the CNS (Kaul et al., 2006; Xia et al., 2011). Consequently, as the incidence of dementia (estimated in the early 1990s as high as 20-30%) has declined to a current 10% in individuals with low CD4 T cell counts and advanced HIV disease (McArthur et al., 1993), as many as 40% of HIV-positive patients still suffer from HAND (Lindl et al., 2010). Indeed, with the longer lifespan of patients with HIV-1 infection and AIDS in recent years, the prevalence of HAND is on the rise even in patients with well-controlled symptoms (McArthur et al., 2003).

The CNS is susceptible to infection by retroviruses of various species and by members of the lentivirus family, in particular. The specific requirements for entry to the brain and the many cell types in the CNS increase the complexity of virus-cell interactions in the brain. In theory, five main cell types (astrocytes, oligodendrocytes, neurons, perivascular macrophages, and microglia) are susceptible to retroviral infection, but of these five, the latter two are the most commonly infected by HIV-1 (Kramer-Hammerle et al., 2005). HIV-1 enters the brain early in the course of infection, presumably via infected macrophages and lymphocytes, and then persists primarily in perivascular macrophages and microglia (Koenig et al., 1986). Three pathways have been proposed for viral entry into the brain: (i) carriage of HIV-1 by infected leukocytes (“Trojan horse” hypothesis); (ii) passage of

cell-free virus into the brain; and (iii) release of virus into the brain by infected endothelial cells (Kramer-Hammerle et al., 2005) (Figure 1).

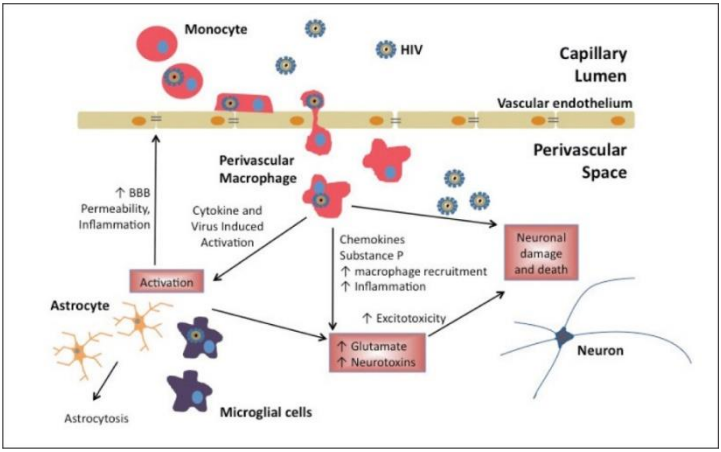

Fig 1: HIV-1 neuroinvasion. According to the " Trojan Horse hypothesis" entry of HIV-1 into the brain takes place by the migration of infected monocytes, which differentiate into perivascular macrophage. These cells can release cytokine and chemokines, HIV virus and viral particles that lead to astrocytes activation. All of these pathways increase the glutamate and neurotoxins rates, leading to neuronal damage and death.

This can occur within 1–2 weeks after the virus enters into the systemic circulation (Gray et al., 1993). An important player may be the blood brain barrier (BBB), which separates the CNS from the periphery and supposedly controls the traffic of low-molecular-weight nutrients, peptides, proteins, and cells in and out of the brain (Banks et al., 2006). Thus, the condition of the BBB may potentially determine continuing or repeated neuroinvasion during the course of HIV disease. Following extravasation, infected monocytes/perivascular macrophages begin to actively produce and release HIV, as well as a variety of proinflammatory mediators that impacts the function of surrounding cells and enhances further monocyte recruitment (Librizzi et al., 2006). Among others, neuronal and glial cells are susceptible to the virus presence in the CNS; most studies have found a lack of infection of neurons, there are some reports of HIV-1 DNA and protein expression in these cells and it has previously shown that neuroblastoma cells are susceptible to HIV-1 infection (AlvarezLosada et al., 2002). The possibility of neuronal infection certainly would be an important factor in neuropathology, because infected neurons, like it happens in astrocytes, may provide a reservoir of virus with the capacity for reactivation. Because approximately 70% of the cells in brain are astrocytes, they are a significant

reservoir of latent HIV-1 DNA, being a significant factor in HIV-1-mediated neuropathogenesis (del Palacio et al., 2011). Although the low number of infected cells in the brain cannot explain the extent of damage observed in HIV-1 encephalopathy, it is widely accepted that viral proteins shed by infected cells, as well as a variety of toxic products secreted by activated cells (infected or uninfected), are the major factors involved in the underlying neuropathology (Gendelman et a., 1994). Excessive production of inflammatory biomolecules or mediators of inflammation produced by different cell types of CNS may induce neurotoxicity. Monocytes, lymphocytes, and activated macrophages after entering into CNS release various pro-inflammatory, inflammatory cytokines, reactive oxygen, and other biomolecules with high neurotoxic potential. These mediators individually, additively, or synergistically disrupt normal functioning of cells of CNS by inducing neurotoxicity (Munoz-Fernandez and Fresno, 1998). Among these NO is produced by microvascular endothelial cells, macrophages, and neurons which may result in N-methyl- D-aspartate (NMDA) type glutamate-associated neurotoxicity. Elevated levels of NOS have been reported in the brain of HAD patients, whereas a 40-fold increase in expression of NOS has been described in neurons of drug addict HIV-1 adults (Minagar et al., 2002). (Figure 2).

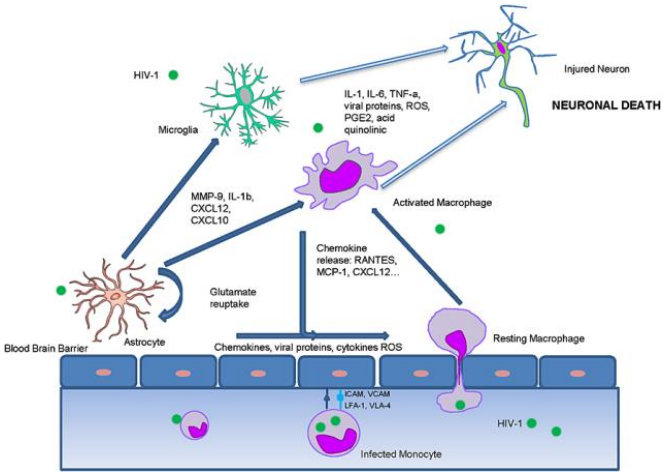

Fig 2: Mechanism of neuropathogenesis. The two main components of this mechanism are the direct effect of the HIV-1 infection, including HIV-1 proteins, and the indirect consequence of infection comprising the secretion of cytokines and neurotoxins. The infected macrophages and microglia participate actively in the neurodegeneration shedding viral proteins and releasing significant amount of cytokines and neurotoxins into the CNS. The alteration of astrocytes function results in an increase in the level of neurotoxicity in the brain. Neurotoxins released from several sources lead to neuronal injury

Glutamate excitotoxicity is thought to be one of several mechanisms by which HIV exerts neurotoxicity that culminates in HAND. The amino acid glutamate is the principal excitatory neurotransmitter in mammalian CNS where it is synthesized and stored in the neuronal cytosol in synaptic vesicles in millimolar concentrations (Nedergaard et al. 2002). Extracellular concentrations of glutamate in the synaptic cleft are kept low (nanomolar ranges) by excitatory amino acid transporters (EAATs). These are glutamate transporters which are located mainly on astrocytes and function in removing excess glutamate from the synaptic cleft after the completion of a signaling event, returning it to homeostatic levels.

The presence of excess glutamate in the synaptic clefts activates glutamate gated ion channels and results in high levels of ion influx into neuronal cells allowing the over activation of downstream calcium ion-dependent effectors and signaling pathways, culminating in neuronal damage. Neuronal damage then causes further release of intracellular glutamate into the extracellular space affecting nearby neurons. Most acute and chronic neuronal diseases, including HAD, have implicated this type of bystander pathology of excitotoxicity (Potter et al., 2013).

In the CNS, cystine–glutamate exchange is critical for the maintenance of extracellular glutamate concentrations as well as supplying cystine to astrocytes for glutathione production (Moran et al., 2005). The systemXC, an abundant amino acid transporter, transports intracellular cystine in exchange for extracellular glutamate (Ye and Sontheimer, 1999). SystemXC is a heterodimeric protein complex consisting of a catalytic light chain (xCT) and a regulatory heavy chain (4F2hc) (Sato et al., 1999), which is essential for membrane localization of the transporter (Bassi et al., 2001). It is highly expressed in glioma cells in which it exists in two splice variants, hxCTa and hxCTb, both of which are upregulated after oxidative stress (Kim et al., 2001). SystemXC-mediated glutamate release has been implicated in a number of conditions in which excitotoxic cell injury occurs. These include inflammation (Barger and Basile, 2001), virally induced encephalopathy (Espey et al., 1998), and periventricular leukomalacia (Oka et al., 1993). Specifically, cystine is an essential precursor for the biosynthesis of cellular glutathione (GSH), which is a key regulator of the redox status of the cells (Jefferies et al., 2003); therefore, glutamate release from glioma cells is an obligatory by-product of cystine uptake by glioma cells, which is necessary to maintain the high synthetic rates of glutathione biosynthesis in condition of oxidative and inflammatory stress. (Chung et al., 2005).

i. Neurotoxicity induced by HIV-1 Tat

HIV-1 genome contains three structural genes (gag, pol and env), four accessory (vif, vpr, vpu and nef) and two regulatory (tat and rev) genes, the products of which are responsible for establishing sophisticated interactions between the virus and human host (da Silva et al., 2006; Zuo et al., 2006). HIV-1 Tat is well-known as a transactivator protein that contributes to transactivation of viral and cellular genes (Ju et al., 2009; Mahlknecht et al., 2008; Nekhai et al., 2007). Tat is an early regulatory protein that has a variable length of 86–104 aa, encoded by two exons.

The first exon encodes the first 72 aa (Schwarze et al., 1999). Due to the variable length of Tat, its weight varies from 14 to 16 kDa. Incomplete forms of this viral protein (from 58 to 72 aa) may also be able to induce the biological effects of the full-length protein. A double splicing mechanism occurs after the transcription of Tat mRNA. This is a post-transcriptional modification that consists of cutting of the Tat mRNA and removal of unnecessary sequences. The process is followed by the joining together of nucleic acid sequences (Fanales-Belasio et al., 2009). The extracellular form of Tat, which is released from productively infected cells, is also able to enter target cells and induce its effects (Zheng et al., 2005). Studies of Tat-derived peptides have demonstrated that residues 48–60 from the basic domain (the protein transduction domain or PTD) account for the functional internalization into cells (Futaki et al., 2001) and that cellular heparan sulfate proteoglycans act as low-affinity cellular receptors for extracellular Tat (Tyagi et al., 2001). Mutational analysis of HIV-1 Tat has identified two important functional domains: an activation domain that mediates its interactions with cellular machinery and an arginine-rich region that is required for binding to the transactivation responsive element (TAR) RNA (Hwang et al., 2003). Functions proposed for HIV-1 Tat include chromatin remodelling, phosphorylation of RNA polymerase II that is involved in the transcription of the full-length viral mRNAs, transactivation of viral genes and binding to a specific structure of HIV-1 mRNAs (Richman et al., 2009). The thorough study of Tat suggests all of these functions are sequentially triggered as a cascade for a single purpose, namely, HIV-1 gene expression. In fact, HIV expression is limited by cellular barriers which inhibit effective mRNA transcription. The HIV-1 provirus overcomes these barriers through the action of its own activator, Tat (Pumfery et al., 2003). Tat recruits cellular proteins to relieve the repression of the viral long-terminal repeat (LTR), and thereby the viral promoter can induce the expression of viral genes (Richman et al., 2009).

In the absence of any stimulation, the integrated HIV-1 provirus remains silent in latently infected cells such as T cells, monocytes and macrophages. Expression of the viral genome is regulated by the enhancer and promoter

elements contained within the HIV LTR located at the 5′end of the integrated HIV provirus (Rohr et al., 2003). HIV-1 Tat interacts with several chromatin modifying complexes and histone modifying enzymes to relieve the virus LTR.

The results of several studies suggest that Tat is a potential contributor to HIV-1 dementia (Wallace et al., 2005). Tat increases intracellular Ca2+ in neurons, followed by mitochondrial calcium uptake which results in the generation of ROS, activation of caspases and eventually apoptosis of neurons. Tat can be transported efficiently across the intact BBB. The mRNA levels for Tat have been reported to be elevated in the brain extracts from HAD patients. Tat plays an important role during neuropathogenesis, both as an intracellular and extracellular mediator of neurotoxicity (Fig 3) (Wong et al., 2005).

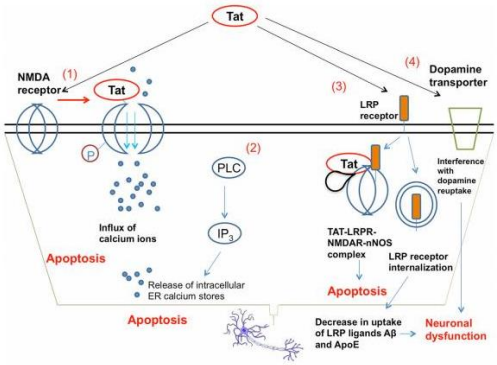

Fig 3. Mechanisms of Tat neurotoxicity. (1) Tat binds to the NMDA receptor and drives the phosphorylation of an intracellular NMDAR subunit, causing excess opening of cation channels and toxic accumulation of calcium. (2) When applied to neurons, Tat is able to induce the activation of PLC and drive the IP3-mediated release of intracellular calcium from ER stores, further contributing to calcium toxicity and apoptosis. (3) Tat can bind to LRP receptors, and be taken up as part of a macromolecular complex including NMDAR and neuronal nitric oxide synthase (nNOS) that induces cellular apoptosis [65]. Tat can also drive the internalization of the LRP receptor, reducing the uptake of LRP receptor ligands amyloid-β peptide and Apolipoprotein E, which may contribute to systemic neuronal dysfunction. (4) Tat interferes with the activity of dopamine transporter, diminishing the reuptake of dopamine by pre-synaptic neurons and interfering with signal transmission.

Tat also binds the low density lipoprotein receptor related protein (LRP) in neurons, causing LRP internalization and a decrease in uptake of natural LRP ligands such as amyloid-β peptide and Apolipoprotein E (Liu et al., 2000) (Fig 3). The interaction of Tat with LRP can lead to the formation of an apoptosis-promoting complex including postsynaptic density protein-95 (PSD- 95), NMDA receptors and neuronal nitric oxide synthase (nNOS) (Eugenin et al., 2007). Tat has been found to interfere with the expression of

miRNAs in neurons, increasing the levels of CREB-targeting miR-34a and leading to neuronal dysfunction (Chang et al., 2011). Tat can also interfere with the ability of dopamine transporter to reuptake dopamine (Zhu et al., 2011). This likely contributes to the particularly severe damage rendered to dopaminergic-rich regions in the brains of patients with severe HAND (Chang et al., 2008).

It is noteworthy that, Tat is able to activate the NMDAR, leading to excitotoxicity and neuronal apoptosis, as a result of perturbed cellular calcium homeostasis and mitochondrial alterations (Self et al., 2004).

It has been demonstrated that Tat causes apoptotic neuronal death, which can be antagonized by NMDAR blockers, indicating that Tat-induced neuronal injury is due to persistent activation of NMDAR (Li et al., 2009). Evidence suggests that Tat is an important neurotoxic effector, being able to interact with and activate NMDAR, both directly and indirectly (Eugenin e al., 2007).

In 2013 Capone et al. provided clear evidence that the origin of ROS generation is related to Spm catabolism and Tat is able to induce SMO enzyme activity in neuroblastoma cells through the stimulation of NMDAR. The pivotal role of SMO as the primary source of the cytotoxic H_2O_2 has already been demonstrated in a human breast cancer model, using a stable, short hairpin RNA knockdown strategy (Pledgie et al., 2005). Regarding the involvement of NMDAR in this pathway, it has been demostrated that the inhibition of the polyamine metabolism by the SMO-specific inhibitor CHL completely prevented NMDA-induced cell death as well as ROS production. In agreement with the stimulatory effect of Tat on SMO activity, it was observed a significant decrease in the intracellular content of Spm, that may have a detrimental effect on cell viability for two main reasons. On one hand, Spm is responsible for intrinsic gating of ion channels by direct plugging of the channel pore. In particular, intracellular Spm can block the channel pore of NMDAR (Igarashi and Kashiwagi, 2010). Therefore, the reduction of Spm concentration induced by Tat treatment may cause hyperactivity of NMDAR thus leading to excitotoxicity. On the other hand, the depletion of Spm also makes cells more sensitive to ROS damage, because Spm has been demonstrated to act as a free radical scavenger that can protect DNA from H^2O^2-induced damage (Casero and Pegg, 2009). In this respect, it has been also observed a marked depletion of GSH content in neuroblastoma cells treated with Tat, which may have dramatic consequences on cell viability. In the literature, a reduction in GSH levels has been associated with physiological processes such as aging (Finkel and Holbrook, 2000) and neurological disorders such as Alzheimer and Parkinson diseases (Gu et al., 1998); in fact the GSH content in the brain of such patients has been stated to decrease by 40–50%, compared to controls (Sofic et al., 1992).

ii. Neurotoxicity induced by HIV-gp120

The process of HIV-1 infection begins by HIV-1 binding to CD4 receptor on the target cell surface, through the viral envelope protein gp120 (Matthews et al., 1987). Both monomeric and oligomeric gp120 have neurotoxic capabilities (Bardi et al., 2006), and transgenic mice expressing gp120 have a spectrum of neuronal and glial changes resembling abnormalities in brains of HIV-1-infected humans (Toggas et al., 1994). HIV-1 gp120 directly binds NMDAR on human embryonic neurons and can cause a lethal influx of calcium ions (Lannuzel et al., 2005). HIV-1 gp120 can bind to either CCR5 or CXCR4 and induce death in neuroblastoma cells (Catani et al., 2000) (Figure 4). This apoptosis apparently takes place through a p38-MAPK-mediated signaling cascade (Kaul et al., 1999). Cognitive testing of gp120 transgenic mice showed age-dependent deficits in open field activity and spatial reference memory tests (D'Hooge et al., 1999). The natural ligands of both CCR5 (eg. CCL5, CCL3) and CXCR4 (CXCL12) were found to be neuroprotective against gp120 neurotoxicity (Khan et al., 2003). However, CXCL12 displays neurotoxicity after the N-terminal cleavage of a tetrapeptide in CXCL12 by MMP-2 (Zhang et al., 2003). Another factor up-regulated by the interaction of gp120 with CXCR4 is the neuronal nicotinic receptor α7, which increases cellular permeability to [Ca2+] influx and contributes to cell death (Ballester et al., 2010).

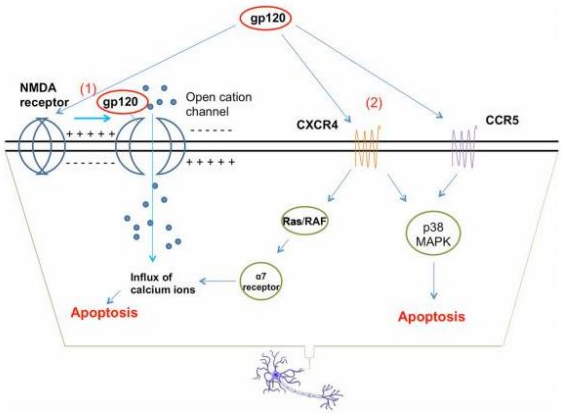

Fig 4. Mechanisms of gp120 neurotoxicity. (1) gp120 can bind to the NMDA receptor and lead to excessive opening of NMDAR-gated cation channels, allowing the influx of calcium ions to toxic levels. (2) gp120 can directly bind to either CCR5 or CXCR4, activating an
p38-MAPK mediated signaling cascade that leads to neuronal apoptosis. The gp120-CXCR4 binding also up-regulates the expression of the nicotinic receptor α7, which increases cellular permeability to [Ca2+] influx and contributes to cell death.

Several studies indicate that HIV proteins (e.g.,gp120), which are assumed to contribute to neuronal abnormalities in HAND (Alfahad and Nat, 2013; Schouten et al., 2011), can induce NF-κB and relative target genes (Genis et al., 1992), as well as arise in intracellular calcium levels (Medina et al., 1999). In this contest, the cytosolic phospholipase A2 (cPLA2), a calcium-dependent enzyme, hydrolyzes membrane phospholipids to release arachidonic acid (AA) (Burke et al., 2009). Its metabolites play a number of functions in the organism, among them the regulation of the activity of several kinases, including protein-tyrosine kinases (Joubert et al., 2001), these being able to elicit constitutive NOS phosphorylation and inactivation (Colasanti et al., 1999). NO is synthesized by at least three distinct isoforms of NOS: NOS-I or neuronal NOS (nNOS), NOS-II or inducible NOS (iNOS), and NOS-III or endothelial NOS. NOS-I and NOS-III are both calcium/calmodulin-dependent, are constitutively expressed (so that they are both called cNOS), and release physiological concentrations of NO. In contrast, iNOS can produce a greater amount of NO after induction by bacterial lipopolysaccharide (LPS), cytokines, and/or viral proteins (Colasanti and Suzuki, 2000). The existence of a crosstalk between cNOS and iNOS has been widely reported; in fact under physiological conditions, low levels of NO produced by cNOS suppress the transcription factor NF-κB, which is involved in the transcription of several pro inflammatory genes, including iNOS (Colasanti and Persichini, 2000). After a pathological stimulus (e.g.,LPS and/or cytokines), cNOS is phosphorylated early on a tyrosine residue, thus leading to enzymatic inhibition and subsequent reduction of physiological NO levels (Colasanti et al., 1999). Under these conditions, NF-κB is freed from the inhibitory action of NO; it enters the nucleus and participates in the transcription of genes, including iNOS and interleukin-1β (IL-1β) (Colasanti and Suzuki, 2000). Note that NF-κB, in turn, is able to induce cPLA2 mRNA expression with a subsequent AA release, thus establishing a positive feedback (Hernandez et al., 1999). The expression of these genes promotes a wide production of inflammatory effectors (e.g.,NO,AA,cytokines), which could contribute to HAND pathogenesis.

The antioxidant cell response: the p62/keap1/Nrf2/ARE pathway

In response to intrinsic and extrinsic stimuli, cells activate various adaptive mechanisms to promote ROS/RNS detoxification. The cap'n'collar (CNC) family proteins are transcription factors, which contain basic leucine zipper (bZIP) and CNC domains. By regulating various antioxidant genes and phase II detoxifying enzymes, which are required for metabolic detoxification of xenobiotics, they play a pivotal role in the cellular response to oxidative or electrophilic stresses (Motohashi and Yamamoto, 2007). The CNC family

consists of nuclear factor erythroid-derived 2 (NF-E2), NF-E2-related-1 [NRF1 or NF-E2-like 1 (NFE2L1)], NRF2 (NFE2L2), NRF3 (NFE2L3), and distantly related broad complex–tramtrack–bric-a-brac (BTB) and CNC homology 1 (BACH1) and BACH2. Functionally, the CNC transcription factors form heterodimers with small MAF proteins (Kannan et al., 2012). These heterodimers regulate genes containing the antioxidant response element (ARE) or the MAF recognition element (MARE) such as heme oxygenase 1 (*HO-1*), NADP(H):quinone oxidoreductase 1 (*NQO-1*), glutamylcysteine ligase (*GCL*), peroxiredoxin (*PRDX*), superoxide dismutase (*SOD*), catalase (CAT), glutathione peroxidase (GPx), sulfiredoxin (Srx), thioredoxin reductase (Txnrd) and glutathione S-transferase (GST), which are involved in detoxification and drug metabolism (Blank, 2008; Eychene et al., 2008). ARE core sequence, 5′-RGTGA(C/G)NNNGC-3′, acts as a *cis*-acting enhancer and shows significant homology to the MARE enhancer, 5′-TGCTGAG(C)TCAGCA-3′ (Nerland, 2007). Interestingly, many studies demonstrate that among CNC members, NRF2 is heavily involved in the regulation of antioxidant genes (Zhang et al., 2008).

NRF2 is a soluble protein primarily localized to the cytoplasm. It is highly conserved across species and contains seven functional NRF2-ECH homology (Neh) domains (Figure 5) (Itoh et al., 1999), responsible for heterodimerization and ARE binding, interacting with the transcriptional coactivators (Nioi et al., 2005) and for stabilization by recruiting an ubiquitin ligase complex (McMahon et al., 2004). Moreover, there is a binding site for KEAP1 (Kelch-like erythroid cell-derived protein with CNC homology ECH-associated protein 1) to regulate the ubiquitination (Wang et al., 2013).

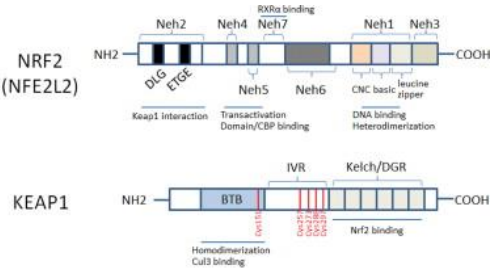

Fig 5. Structural domains of NRF2 and KEAP1. NRF2 includes the CNC-bZIP domain and functional Neh domains. The CNC-bZIP domain including Neh1 is required for DNA binding and interaction with ubiquitin conjugating enzymes. The transactivation domain contains Neh4 and Neh5. Neh2 is involved in Keap1 binding. KEAP1 contains the BTB domain for KEAP1 homodimerization and interaction with Cul3. Six kelch repeats in the Kelch/DGR domain interact with the Neh2 domain of NRF2 for binding. The IVR domain links the BTB and Kelch/DGR domains and has several critical cysteine residues for KEAP1 activation and NRF2 repression.

Under basal conditions, NRF2 is localized in the cytoplasm through a direct interaction with the KEAP1 protein that leads to ubiquitination and proteasomal degradation (Itoh et al., 1999). KEAP1 contains three functional domains, a BTB domain, an intervening region (IVR), and a double glycine repeat (DGR) or a Kelch motif, which contains six copies of the conserved kelch repeat that form a β-propeller structure. The BTB domain is required for KEAP1 homodimerization and the interaction with Cul3, which regulates KEAP1 through ubiquitination and proteasomal degradation. Twenty-seven cysteines within the IVR domain residues act as active stress sensors in human KEAP1 (Dinkova-Kostova et al., 2002). Among these, Cys151, Cys257, Cys273, Cys288, and Cys297 are known to be highly reactive toward oxidative and electrophilic stresses (Sekhar et a., 2010). In response to stress, these cysteine residues become oxidized and form disulfide bonds or covalent adducts. The cysteine modifications cause a conformational change in KEAP1 to prevent NRF2 ubiquitination (Zhang and Hannink , 2003). Free NRF2 then translocates into the nucleus, heterodimerizes with small MAFs, and binds to ARE sequences in promoter regions of antioxidant genes (Moon and Giaccia, 2015).

In 2010, it was discovered that the antioxidant transcription factor Nrf2 can be activated by p62/SQSTM1 (sequestosome 1) by a 'non-canonical' pathway. The underlying mechanism is completely redox independent, and involves the recruitment of Keap1 that functions as an adapter protein of the Cul3-ubiquitin E3 ligase complex responsible for degrading Nrf2 (Komatsu et al., 2010). In agreement with this model, p62 binds to aggregates of ubiquitylated proteins and increases its affinity for Keap1 when phosphorylated at Ser351 (Ichimura et al., 2013) This event induces Keap1 degradation via autophagy (Taguchi et al., 2012) and leaves Nrf2 free to accumulate and translocate in the nucleus (Filomeni et al., 2014).

The p62 protein was originally identified because it bound to the tyrosine kinase Lck (Joung et al., 1996). Subsequently, p62 was found to bind atypical protein kinase C (Sanchez et al., 1998) and shown to act as a scaffold or adaptor protein in NFkB signaling pathways following activation of tumor necrosis factor-α (TNF- α) (Sanz et al., 1999), interleukin-1 (Sanz et al., 2000), and nerve growth factor (Wooten et al., 2000) receptors. In addition, p62 is involved in activation of caspase-8 upon stimulation of cell death receptors (Jin et al., 2009). p62 is required for Ras-induced tumorigenesis *in vitro* and *in vivo* and is up-regulated in different human tumors (Duran et al., 2008). Because p62 binds to ubiquitin and to LC3, it is both a selective autophagy substrate and a cargo receptor for autophagic degradation of ubiquitinated targets (Ichimura et al., 2008; Komatsu et al., 2007; Lamark et al., 2009). During the evolutionary conserved process of autophagy, p62 forms cytosolic inclusion bodies, which contain ubiquitinated protein

aggregates that subsequently can be degraded by autophagy (Bjørkøy et al., 2005). Isolation membranes embrace and envelop part of the cytoplasm, sequestering the content into a double-membrane vesicle called an autophagosome, that in turn, fuse with lysosomes, and their contents are degraded by lysosomal hydrolases (Mizushima et al., 2008). The expression of the p62 gene is induced by NRF2 upon exposure to electrophiles, ROS and NO (Kosaka et al, 2010). Furthermore, p62 protein has been reported to stimulate expression of genes containing ARE sequence in their promoter regions (Liu et al., 2007). A positive inter-relationship of some sort exists between NRF2 and p62, which influences the ARE-gene battery. In 2010, Jain et al. showed that NRF2 can induce p62 expression by binding directly to a conserved ARE in its promoter/enhancer. Noteworthy p62 protein is able to establish a positive feedback loop responsible for maintaining its high expression levels. Indeed, p62 is able to stimulate NRF2 activity by binding to KEAP1, sequestering it, and directing its degradation by autophagy. This association between p62 and KEAP1 leads to stabilization of NRF2, enabling the transcription factor to induce ARE-driven gene expression (Figure 6). The p62 ARE is conserved in mammals, and it contains the 5' flanking region TGC-, and the core sequence -TGAGTCA- classifying it as a MAF-like recognition element (MARE). The NRF2 responsive p62 MARE contains a GC to CG substitution in the 3' flanking region compared with the consensus MARE (Motohashi et al., 2002). It has been reported that MARE-like sequences with a G to C substitution in the 3' flanking region preferentially bind to small MAF:NRF2 heterodimers (Jain et al., 2010).

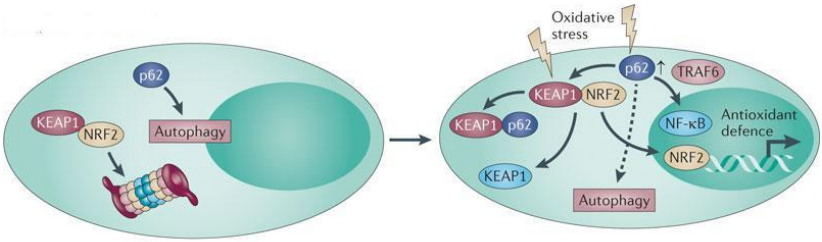

Fig 6. Under normal conditions, NRF2 is bound to KEAP1 and is inactivated as a transcription factor for antioxidant-defence genes by proteasome-mediated degradation. p62 is degraded through autophagy under normal conditions. In the presence of oxidative stress, KEAP1 is either modified so that it can no longer bind NRF2 or it is sequestered by p62, the expression of which is increased in response to oxidative stress. This displaces KEAP1 from NRF2 so that NRF2 can activate antioxidant-defence genes and promote survival. Oxidative stress also activates nuclear factor-κB (NF-κB) as a result of p62 upregulation and tumour necrosis factor receptor-associated factor 6 (TRAF6) complex formation, or by other mechanisms, to turn on antioxidant-defence gene expression. From "Deconvoluting the context-dependent role for autophagy in cancer" Eileen White Nature Reviews Cancer 12, 401-410 (June 2012)

2) AIMS OF THE WORK

HIV-1 infection is associated with neurological complications, recognized as disorders of various degree, known as HAND. Resident cells in the CNS, including microglia and astrocytes, can be infected and/or activated by HIV and subsequently release a variety of neurotoxins, including viral particles, such as Tat and gp120 in the brain microenvironment. Tat and gp120, more than other HIV proteins, play a pivotal role in HAND neuropathogenesis. The major factors involved in HAND are inflammation and oxidative/nitrosative stress. Excessive production of inflammatory biomolecules or mediators of oxidative stress produced by different cell types of CNS may induce neurotoxicity.

This PhD project deals with three topics, all linked to a major context. It is already known that Tat-induced neuronal injury is due to persistent NMDAR activation. Recently, our group provided clear evidence that the origin of ROS generation was related to Spm catabolism and that Tat was able to induce SMO enzyme activity in neuroblastoma cells through the stimulation of NMDAR.

As ROS also function as signaling molecules able to trigger many pathways in the cell, the first aim of this PhD work was to evaluate Tat-mediated induction of antioxidant response in neuronal cells. Here, we wanted to assess the activation of Nrf2/ARE pathway and the role of NO in SH-SY5Y neuroblastoma cells taken as cellular model. Moreover, since protein aggregation is a common marker of many neurodegenerative diseases, the involvement of p62 protein in Nrf2/ARE pathway and its role in the degradation of Tat will be analyzed.

Besides the study on neuronal cells we will also analyze the effect of HIV proteins on astroglial cells. Astrocytes are indeed the brain cells that profoundly affect neuronal functions and, in a variety of neurological disorders, are thought to play a critical role in mediating neuronal toxicity or neuronal rescue. In neurodegeneration associated with HIV-1, both chronic inflammation and oxidative stress play a crucial role and these conditions are often related. Thus, the second aim was to evaluate the role of cPLA2-AA pathway in the pro-inflammatory response induced by Tat and gp120, and then we wondered whether Tat was able to activate the antioxidant response in astroglial cells, evaluating Nrf2/ARE pathway induction and the involvement of NO. In particular, we planned to focus our attention on the pivotal role of SystemXc, a cystine/glutamate antiporter that allows astrocytes to maintain the high synthetic rates of glutathione to counteract ROS accumulation. Finally, we wanted to analyze the neurotoxic effect of Tat, performing neuronal-astroglial cell co-culture experiments. Since is noteworthy that during oxidative stress, the steady-state structure and proper

physiological function of the Golgi apparatus were often affected, we analyzed GA structure change in neurons treated with Tat and co-cultured with Tat-secreting astrocytes. Another consequence of the neurotoxicity induced by Tat could be the reduced viability of neuronal cells, or the simultaneous activation of the antioxidant response.

3) RESULTS AND DISCUSSION

HIV-Tat Induces the Nrf2/ARE Pathway through NMDA Receptor-Elicited Spermine Oxidase Activation in Human Neuroblastoma Cells.

The HIV-Tat protein is known as a significant promoter of neurotoxicity (Kaul et al., 2001; Jin et al., 2012). Several mechanisms underline these toxic effect; Tat can freely penetrate neuronal cell membranes and induce lipid peroxidation (Haughey et al., 2004), leading to ROS generation such as superoxide and hydrogen peroxide. In addition, Tat can trigger iNOS expression thus increasing the production of NO, which binds superoxide anion yielding the highly reactive molecule peroxynitrite (ONOO-) (Nicotera et al., 1995) which can have deleterious CNS effects (Nakhostin-Roohi et al., 2013).

Moreover, it is noteworthy that HIV proteins, such as Tat, are able to activate NMDAR, leading to excitotoxicity and neuronal apoptosis, as a result of perturbed cellular calcium homeostasis and mitochondrial alterations (Self et al., 2004).

Here, we found out that the treatment of human SH-SY5Y neuroblastoma cells with 200 ng/ml recombinant Tat for 4, 8, and 24 h was able to increase the mRNA expression of antioxidant enzymes, such as NQO1, CAT, SOD1, SOD2 and HO-1. Tat concentration was chosen based on previous experiments (data not shown). As shown in figure 7, we have observed an increase of mRNA expression of all the genes analyzed reaching the maximum at 4 h (8 h for HO-1) after Tat treatment. At 24h post-treatment, gene expression was reverted to nearly control levels.

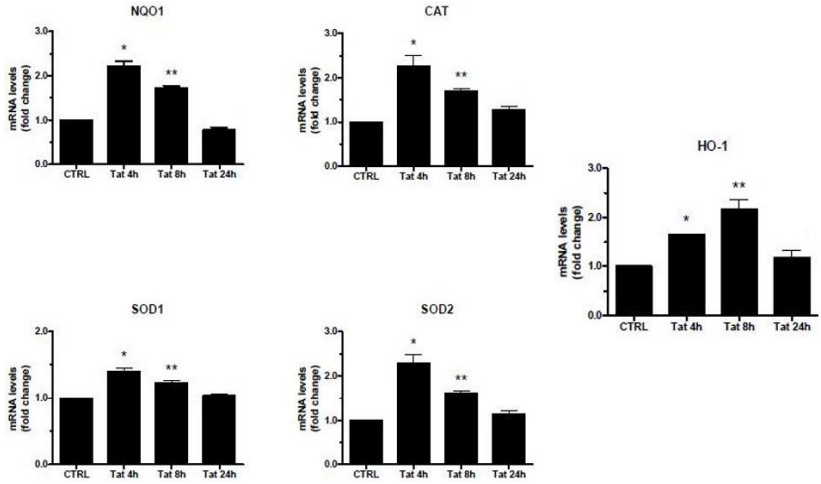

Fig 7: Effects of Tat on ARE-driven gene expression in SH-SY5Y cells. SH-SY5Y were treated with Tat (200 ng/ml) for 4, 8, and 24 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of several genes (NQO1, CAT, SOD1, SOD2, HO-1) by RT-qPCR. Data 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.
NQO1: * p≤0.01 vs CTRL, ** p≤0.01 vs CTRL; CAT: * p≤0.01 vs CTRL, ** p≤0.05 vs CTRL; SOD1: * p≤0.01 vs CTRL, ** p≤0.05 vs CTRL; SOD2: * p≤0.01 vs CTRL, ** p≤0.05 vs CTRL; HO-1: * p≤0.05 vs CTRL, ** p≤0.01 vs CTRL.

Recently, another HIV-1 protein, reverse transcriptase (RT), was shown to induce an antioxidant response (Isaguliants et al., 2013). In particular, RT increased the transcription of the phase II detoxifying enzymes NQO1 and HO-1 in human embryonic kidney cells, revealing a direct link between the propensity of the viral proteins to induce oxidative stress and their immunogenicity (Isaguliants et al., 2013). Furthermore, it has been reported that gp120 significantly upregulates HO-1 and NQO1 in human astrocytes, suggesting a possible role of the antioxidant defense mechanism in promoting cell survival (Reddy et al., 2012). Notably, a progressive increase in serum catalase activity has been detected in advancing HIV infection, reflecting and/or compensating for systemic glutathione and other antioxidant deficiencies in HIV-infected individuals (Leff et al., 1992).
Normally, the expression of antioxidant enzymes is induced in response to oxidative stimuli, including ROS production. As reported elsewhere, the treatment of SH-SY5Y cells with Tat for 1 and 4 h was able to induce ROS generation by the upregulation of the SMO enzyme activity (Capone et al., 2013). Here, we hypothesize that Tat can elicit an antioxidant response in neuronal cells through a SMO-dependent activity. To address this question,

we first assessed the ability of Tat to upregulate the activity of SMO at an early time point (i.e., 15 min). Therefore, a chemiluminescence analysis was performed to measure H_2O_2 production in extracts from cells treated with Tat (200 ng/ml) for 15 and 60 min. As shown in figure 8, the SMO activity was already increased at 15 min post-Tat treatment; this effect was maintained for up to 60 min.

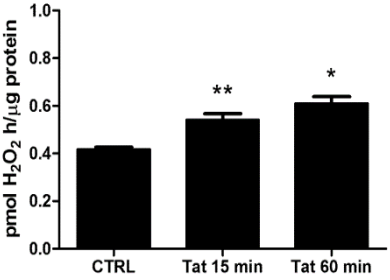

Fig 8: Effects of Tat on spermine oxidase activity. SH-SY5Y cells were treated with Tat (200 ng/ml) for 15 and 60 min. At the end of incubation cell extracts were analyzed for SMO activity. The data shown are the means of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** $p \leq 0.05$ vs CTRL, * $p \leq 0.01$ vs CTRL.

Next, we wanted to determine whether the expression of detoxifying and antioxidant genes in response to Tat treatment was elicited by the SMO-induced activity. Therefore, we treated SH-SY5Y cells with 10 nM chlorhexidine digluconate (CHL), a strong competitive inhibitor of SMO (Cervelli et al., 2013), for 16 h before treatment with Tat (200 ng/ml). As expected, CHL completely prevented Tat-induced up-regulation of all the genes analyzed (Fig 9), thus suggesting that Tat may elicit an antioxidant response in neuronal cells through the activation of SMO.

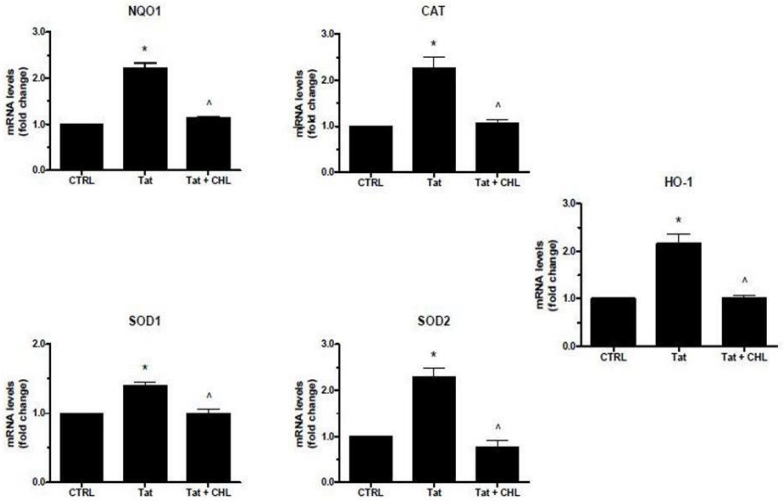

Fig 9: Effects of SMO inhibition on Tat-induced gene expression in SH-SY5Y cells. Cells were overnight pretreated with CHL (10 nM) or medium alone before the addition of Tat (200 ng/ml) for 4 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of NQO1, CAT, SOD1, SOD2, HO-1 genes by RT-qPCR. Data are calculated relative to 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. NQO1: * p≤0.01 vs CTRL, ^ p≤0.01 vs Tat; CAT: * p≤0.01 vs CTRL, ^ p≤0.01 vs Tat; SOD1: * p≤0.01 vs CTRL, ^ p≤0.01 vs Tat; SOD2: * p≤0.01 vs CTRL, ^ p≤0.01 vs Tat; HO-1: * p≤0.01 vs CTRL, ^ p≤0.01 vs Tat;

Considering that ARE genes are mainly regulated by Nrf2, we investigated whether Tat was able to activate this transcription factor in human neuroblastoma cells. To this aim, we examined the Nrf2 translocation into the nucleus, after treatment with Tat (200 ng/ml) for 15 minutes, 2 hours and 16 hours. Nrf2 levels were measured in nuclear extracts by using Western blot analysis. As shown in figure 10, Tat induced a 2.68-fold increase of the nuclear Nrf2 levels already at 15 min post-treatment, which was maintained up to 16h.

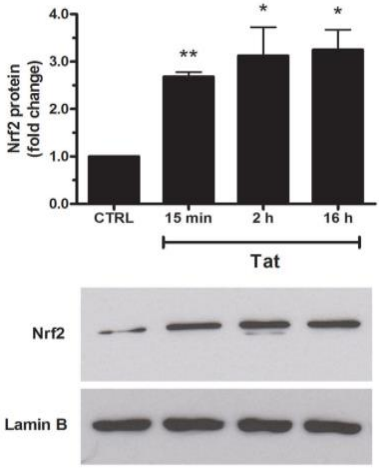

Fig 10: Effects of Tat on Nrf2 nuclear translocation in SH-SY5Y cells. Cells were treated with Tat (200 ng/ml) for the indicated time points. After incubation at 37 °C, cells were mechanically harvested, and the nuclear extracts were prepared as specified in the Materials and Methods section to assess Nrf2 levels by western blot analysis with anti-Nrf2 1:1000 (Abcam) and anti-laminB 1:4000 (Abcam). The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear Lamin B 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. ** $p \leq 0.05$ vs CTRL, * $p \leq 0.01$ vs CTRL.

The effect of Tat on Nrf2 activation was also confirmed by a TransAm kit based on ELISA method (see below in figure 12).

We found, for the first time, that Tat activates Nrf2 in neuronal cells, and our results are consistent with data reporting that Tat enhances the cellular expression of Nrf2 at the transcriptional and protein levels in MAGI cells (Zhang HS et al., 2009). Moreover, it has been reported that Nrf2 is also up regulated in response to gp120 in primary astrocytes, thereby suggesting a possible protective role of gp120-induced Nrf2 in regulating the levels of pro-oxidative and pro-inflammatory molecules in HANDs (Reddy et al., 2012).

As described above (see figure 2), Tat is able to upregulated the activity of SMO at an early time point (i.e., 15 min). To investigate on the role of SMO in Tat-elicited Nrf2 activation, SH-SY5Y cells were exposed overnight to 10 nM CHL before treatment with Tat (200 ng/ml) for 15 min. Then, a western blot analysis was performed on nuclear extracts using an anti-Nrf2 specific antibody. As shown in figure 11, the pretreatment of SH-SY5Y cells with CHL completely prevented Tat-induced Nrf2 nuclear translocation, thereby suggesting an involvement of SMO in this mechanism.

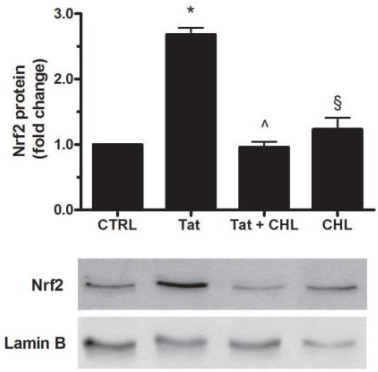

Fig 11: Effects of SMO inhibition on Tat-induced Nrf2 activation in SH-SY5Y cells. Cells were overnight pretreated with CHL (10 nM) or medium alone before the addition of Tat (200 ng/ml) for 15 min. After incubation at 37 °C, cells were mechanically harvested, and the nuclear extracts were prepared as specified in Methods to assess Nrf2 levels by western blot analysis with anti-Nrf2 1:1000 (Abcam) and anti-laminB 1:4000 (Abcam). The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the nuclear Lamin B content and are the means ± SEM from three separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used tom determine significant differences. * $p \leq 0.01$ vs CTRL, ^ $p \leq 0.01$ vs Tat, § Not significant vs Tat + CHL.

Previously, it has been reported that Tat was able to induce ROS production through the stimulation of SMO activity in neuroblastoma cells (Capone et al., 2013). Here, we evaluated the role of SMO-dependent ROS generation in Tat-induced Nrf2 activation by pretreating SH-SY5Y cells with the antioxidant NAC, which is able to prevent Tat-induced ROS generation (Capone et al., 2013). As shown in Fig 12A, the pre-treatment of cells with NAC (2 mM) for 1 h before treatment with Tat (200 ng/ml) for 15 min inhibited Nrf2 activation.

As we observed elsewhere, Tat induces SMO activity and ROS production through the stimulation of NMDAR in neuroblastoma cells (Capone et al., 2013). To investigate whether NMDAR was involved in Tat-induced Nrf2 activation, we pretreated SH-SY5Y cells for 2 h with the NMDAR antagonist MK-801 (10 μM) and then treated with Tat (200 ng/ml) for 15 min. Figure 12A shows the strong inhibitory effect of MK-801 on the activation of Tat-induced Nrf2. Consistently, NMDA induced Nrf2 activation, clearly indicating that the stimulation of NMDAR can be responsible for the observed Nrf2 activation in neuroblastoma SH-SY5Y cells. As expected, Tat-induced mRNA expression of the two ARE genes, NQO1 and CAT was prevented by MK-801 pretreatment (Fig 12B). Altogether, these results indicate that Tat induces the Nrf2 pathway through NMDAR-elicited SMO activation in human neuroblastoma cells.

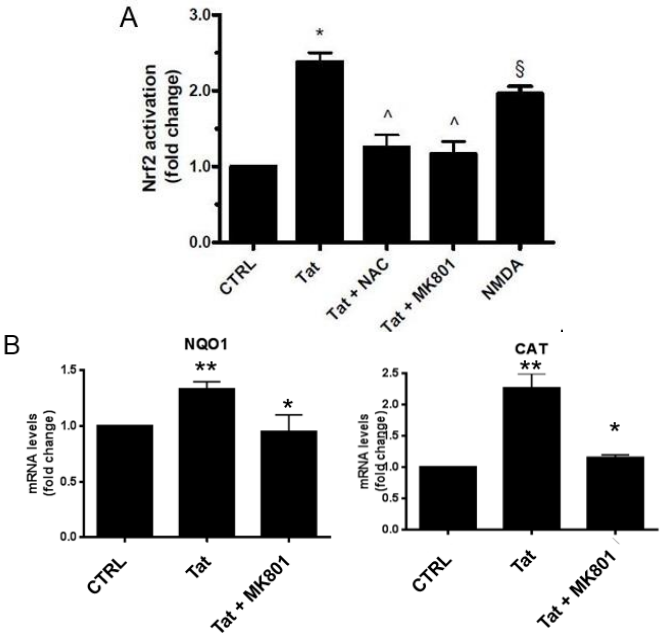

Fig 12: Role of NMDA receptor in Tat-induced Nrf2 activation. (A) SH-SY5Y cells were pretreated for 2 h with MK-801 (10 μM), NAC (2 mM), or medium alone before the addition of Tat (200 ng/ml) for 15 min. After incubation at 37 °C, the cells were homogenized, and Nrf2 activation was quantified by TransAM assay as detailed in the Materials and Methods section. Data points are the means ± S.E.M. from 3 separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * $p \leq 0.01$ vs CTRL, ^ $p \leq 0.01$ vs Tat, § $p \leq 0.01$ vs CTRL. (B) Cells were pretreated for 2 h with MK-801 (10 μM) or medium alone before the addition of Tat (200 ng/ml) for 4 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of NQO1 and CAT genes by RT-qPCR. Data are calculated relative to GAPDH and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of two independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. NQO1: * $p \leq 0.05$ vs Tat, ** $p \leq 0.01$ vs CTRL; CAT: * $p \leq 0.05$ vs Tat, ** $p \leq 0.01$ vs CTRL;

The question now is whether the modulation of NMDAR/SMO/ROS/Nrf2/ARE pathways is sufficient for protection against Tat-induced oxidative stress (see figure 13 for a schematic overview). As we described elsewhere, NMDAR/SMO/ROS activation leads to a weak (approximately 30%) neurotoxicity in Tat-treated SH-SY5Y cells (Capone et al., 2013), so, cell death occurs (although weakly) despite the activation of Nrf2/ARE pathway. A similar paradox has been reported by Akay et al. (2014) in a study on the effects of ARV drugs in the central nervous system. In particular, the neuronal damage and death that occur following exposure to ARV drugs, despite the endogenous antioxidant response, suggest that this response may be insufficient or too delayed to protect cells from Tat toxicity.

Accordingly, Zhang et al. (2009) report that Nrf2 activation induced by Tat in MAGI cells is not sufficient for protection. It is important to take into account that the activation of an antioxidant response is not only regulated by the induction of Nrf2 but also by post-induction responses that tightly control Nrf2 activation and repression back to the basal state, finally 'switching off' Nrf2-activated gene expression (Niture et al., 2014). Notably, HIV-1 induces accelerated aging, and the redox imbalance may actively promote senescence (Davinelli et al., 2014). Compared to age-matched controls, HIV-1 transgenic rats have been shown to have a significant reduction in the protein levels of Nrf2 and HO-1, suggesting a weakening in the protection exerted by the Nrf2/HO-1 system (Davinelli et al., 2014).

A goal of future research may be to spatially and temporally modulate the molecular pathways involved in the potentiation of the antioxidant responses versus oxidative stress. It should be noted that high levels of SMO can be neurotoxic in the brain, not only generating ROS (i.e., H2O2) but also producing spermidine and reactive aldehydes such as 3-AP (Cervelli et al., 2004). In this respect, increased brain polyamine catabolism, with concomitant generation of toxic metabolites (e.g., 3-AP), has been observed after traumatic brain injury, silent brain infarction and stroke (Park and Igarashi, 2013). Interestingly, it has been hypothesized that agents that can chemically neutralize reactive aldehydes should demonstrate neuroprotective synergic actions with the antioxidant response (Wood et al., 2006). To this end, hydroxylamines have been proposed as aldehyde-trapping agents both in an *in vitro* model of neurodegeneration induced by the reactive aldehyde 3-AP and in an *in vivo* rat model of hippocampal neurodegeneration (Wood et al., 2006). However, in murine keratinocyte cells, spermidine and spermine were able to trigger an antioxidant response, increasing the expression of phase II genes through the activation of the Nrf2-ARE pathway by acrolein, a 3-AP-derived aldehyde (Kwak et al., 20 3).

Thus, a treatment strategy may be on the one hand to act at the NMDAR level, by using specific inhibitors, and/or at the SMO level (either upstream using CHL or downstream by inhibiting 3-AP and/or ROS), and on the other hand to potentiate the antioxidant responses using Nrf2-activating compounds. Currently, the Nrf2-ARE pathway is a high-value therapeutic target for several neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis), and numerous cell-based and in silico high-throughput screens have identified novel Nrf2-activating compounds (Johnsonet l., 2015). In this respect, dimethyl fumarate (DMF) has been found recently to attenuate neurotoxicity in SH-SY5Y cells and in an animal model of Parkinson's disease by enhancing Nrf2 activity (Jing et al., 2015). Furthermore, DMF has been proposed to be beneficial for the treatment of neurodegenerative

diseases, such as HANDs (Gill et al., 2013). In particular, monomethyl fumarate (MMF), an active DMF metabolite *in vivo*, blocked ARV-induced ROS generation and neuronal damage/death, enhancing the endogenous antioxidant responses in vitro (Akay et al., 2014). As recently suggested, an interesting new twist to Nrf2-dependent therapeutic approaches is that not only the pharmacological target but also the cell type targeted may be relevant (Johnson et al., 2015). Finally, with respect to Nrf2 overexpression, neurological disorders appear as promising targets for gene therapy (Kanninen et al., 2015).

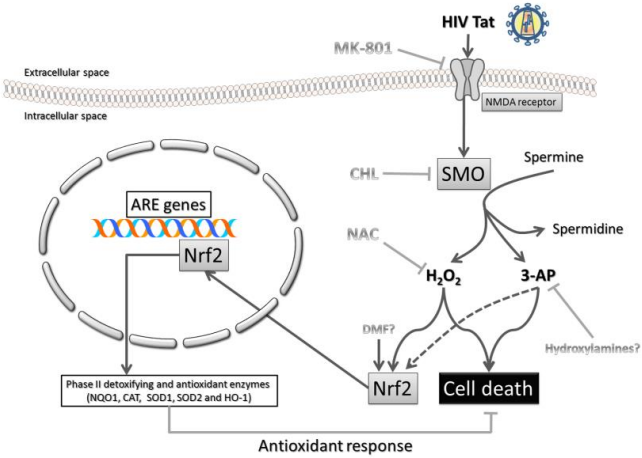

Fig. 13 Proposed model of the role of NMDAR-elicited SMO activation in the Tat-induced Nrf2 pathway. On the one hand, HIV-1 Tat induces neuronal cell death through the production of H_2O_2 and 3-AP by a mechanism involving NMDAR-induced SMO activation. On the other hand, the same pathways are able to trigger an antioxidant response through the transcriptional induction of Nrf2-dependent ARE genes. For more details, see text.

The role of nitric oxide in SMO-mediated activation of Nrf2 by HIV-Tat in human neuronal cells

Nitrosative stress plays a critical role in HAND pathogenesis (Ridnour et al., 2004). During nitrosative stress, post-translational modifications such as *S*-nitrosylation may occur which can inhibit the normal functioning of several proteins.

Since in neurons the activation of NMDAR leads to the enhancement of nitric oxide production by the calcium-dependent nNOS, and NO is an endogenous inducer of Nrf2-dependent phase-II enzymes, both *in vitro* and *in vivo*, we analyzed the role of NO in the activation of the Nrf2-ARE pathway in Tat-stimulated neuronal cells.

To this aim, we analyzed SMO activity in SH-SY5Y cells treated with Tat (200 ng/ml) in the absence or presence of the specific NOS inhibitor L-NAME (1 mM). As shown in figure 14 a 30 min pre-treatment with L-NAME significantly reduced Tat-induced SMO activation.

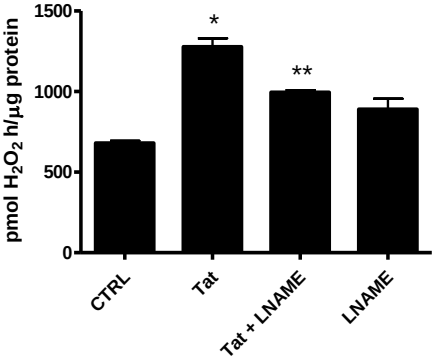

Fig 14: Effects of Tat on spermine oxidase activity: the role of NO. SH-SY5Y cells were pretreated with L-NAME (1 mM) and with Tat (200 ng/ml) for 4 h. At the end of incubation cell extracts were analyzed for SMO activity. The data shown are the means of three independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** $p \leq 0.01$ vs Tat, * $p \leq 0.001$ vs CTRL.

As previously described in the same cell culture model, Tat induced SMO activity and ROS production through the stimulation of NMDA receptor leading to Nrf2 activation. To investigate whether NO was involved in Tat-induced Nrf2 activation, we pretreated SH-SY5Y cells for 30 min with L-NAME and prepared nuclear extracts. We evaluated Nrf2 translocation to the nucleus by a TransAm kit based on ELISA method. As shown in figure 15, Tat induced a 2.3-fold increase of Nrf2 activation that was restored to nearly control levels by L-NAME pre-pretreatment.

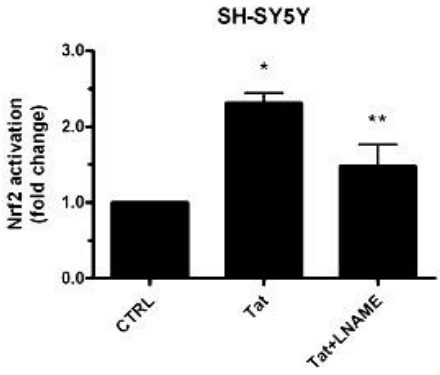

Fig 15: Role of NO in Tat-induced Nrf2 activation. SH-SY5Y cells were pretreated for 30 min with L-NAME (1 mM), or medium alone before the addition of Tat (200 ng/ml) for 15 min. After incubation at 37°C, the cells were homogenized, and Nrf2 activation was quantified by TransAM assay as detailed in the Materials and Methods section. Data points are the means ± S.E.M. from 3 separate experiments, each performed in duplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * $p \leq 0.001$ vs CTRL, ** $p \leq 0.05$ vs Tat.

Next, we wanted to better understand if Nrf2 activation was able to induce an effective antioxidant response in SH-SY5Y cells. As shown in figure 16, Tat-induced HO-1 mRNA up-regulation was completely abolished by L-NAME pretreatment.

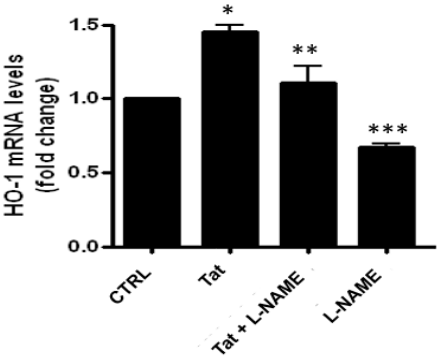

Fig 16: Role of NO in Tat-induced HO-1 mRNA expression. Cells were pretreated for 30 min with L-NAME (1 mM) or medium alone before the addition of Tat (200 ng/ml) for 4 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of HO-1 gene by RT-qPCR. Data are calculated relative to GAPDH and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of two independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * $p \leq 0.05$ vs Tat, ** $p \leq 0.05$ vs Tat,*** $p \leq 0.001$ vs Tat;

These results indicate the involvement of endogenously produced NO in this pathway and suggest a possible role for NO in modulating SMO activity. Therefore, we wondered whether Tat could affect SMO activity by inducing *S*-nitrosylation. To this aim we carried out biotin-switch assay on protein extracts of SH-SY5Y cells treated with Tat. In figure 17, we observed that although the treatment with NO donors, such as DPTA NONOate induced SMO S-nitrosylation, the treatment with Tat was not able to induce the same protein modification. Thus, the effects of NO in Tat-elicited SMO activation could be due to modifications of other targets. Further studies are needed to deeply understand this mechanism.

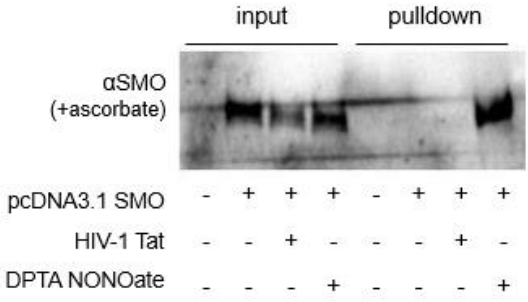

Fig 17: Role of NO in Tat-induced SMO *S*-nitrosylation. SH-SY5Y cells were transfected with pcDNA3.1 SMO vector; after 24 h cells were treated with Tat (200 ng/ml) for 1 h. After incubation at 37 °C, the cells were homogenized, and *S*-nitrosylation was evaluated by the biotin-switch assay as detailed in the Materials and Methods section. Anti-SMO 1:1000 (Proteintech)

Analysis of autophagy markers in neuronal cells: the role of p62.

Oxidative stress leads to protein misfolding and aggregation, and these two conditions are often linked to many neurodegenerative diseases. P62 protein plays a crucial role in the clearance of misfolded and aggregated proteins by mediating their degradation through two major intracellular pathways, the ubiquitin proteasome system (UPS) and selective autophagy. We evaluated the induction of p62-mediated selective autophagy in SH-SY5Y cells treated with Tat. To this aim we analyzed by western blot the expression of p62 and of both forms of LC3 (LC3-I) and (LC3-II), the cytosolic and the conjugated to phosphatidylethanolamine, respectively. As shown in figure 18, we found out that Tat was able to induce a 2-3-fold increase of p62 expression at 8h, 16, 24h post-treatment. Conversely we didn't observe any induction of LC3 in the same experimental conditions as shown in figure 19.

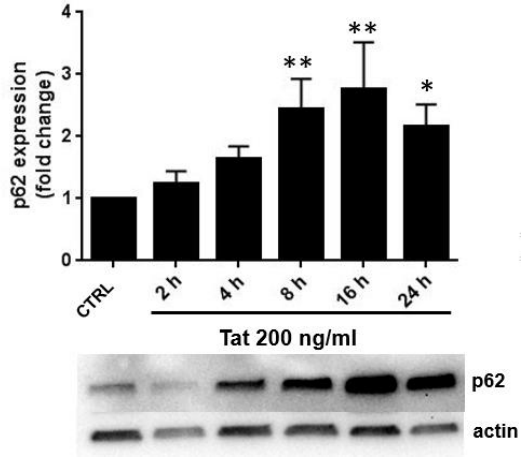

Fig 18: p62 expression in Tat-induced SH-SY5Y cells. Cells were treated with Tat (200 ng/ml) for 2h, 4h, 8h, 16h and 24h. After incubation at 37 °C, cells were mechanically harvested, and total extracts were prepared as specified in Methods to assess p62 levels by western blot analysis with anti-p62 1:4000 (MLB) and anti-actin 1:2000 (Sigma). The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the actin content and are the means ± SEM from three separate experiments, each performed in triplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. * $p \leq 0.05$ vs CTRL, ** $p \leq 0.001$ vs CTRL.

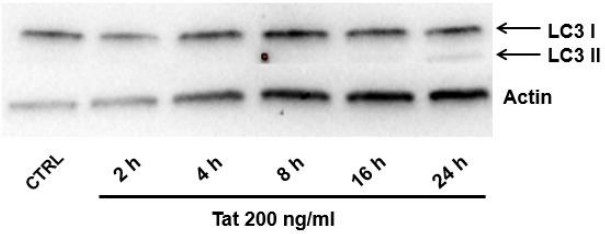

Fig 19: LC3 I-II expression in Tat-induced SH-SY5Y cells. Cells were treated with Tat (200 ng/ml) for 2h, 4h, 8h, 16h and 24h. After incubation at 37 °C, cells were mechanically harvested, and total extracts were prepared as specified in Methods to assess LC3 levels by western blot analysis with anti-LC3 1:4000 (Cell signaling) and anti-actin 1:2000 (Sigma).

P62 is a scaffold protein that binds, among others, to ubiquitin and, in turn, is involved in UPS pathway. As demonstrated in figure 20, after 16 h of Tat treatment, both p62 and ubiquitin levels were induced; the fig 20 also show that after 16h of Tat treatment is enhanced the merge from p62 and ubiquitin. With regard to this result, we could hypothesize that, in neuronal cells treated with Tat, p62 could be involved in UPS pathway, but further studies are needed to confirm this hypothesis.

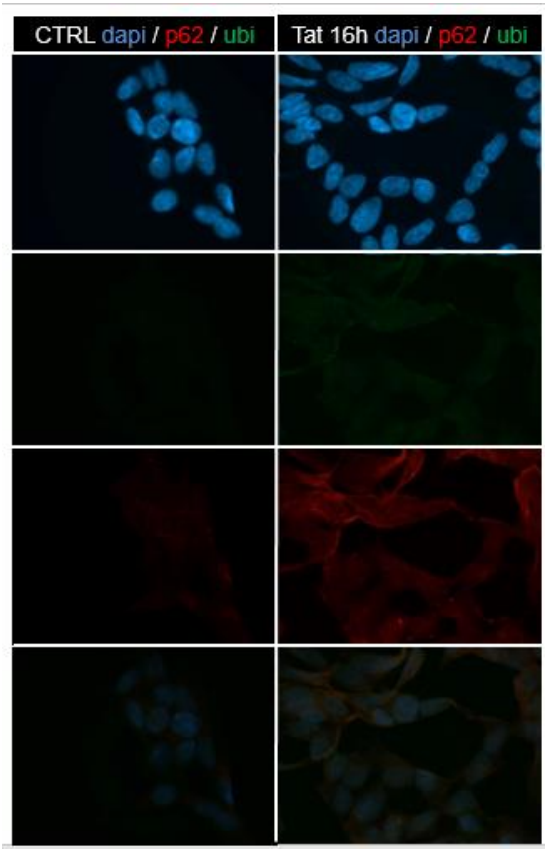

Fig 20: p62 and ubiquitin localization in Tat-treated SH-SY5Y. Cells were treated with Tat (200 ng/ml) for 16h and subjected to immunofluorescence staining using anti-p62 1:200 (MLB) or anti-ubiquitin 1:500 (Millipore) antibodies as specified in Methods and analyzed by immunofluorescence microscopy.

We tested the expression of p62 also in ETNA cells (Embryonic Telencephalic Naı¨ve Apaf). The results in figure 21 indicate that p62 was induced by Tat treatment at 16 h. These results confirm that Tat is able to induce p62 protein expression in two neuronal cell lines.

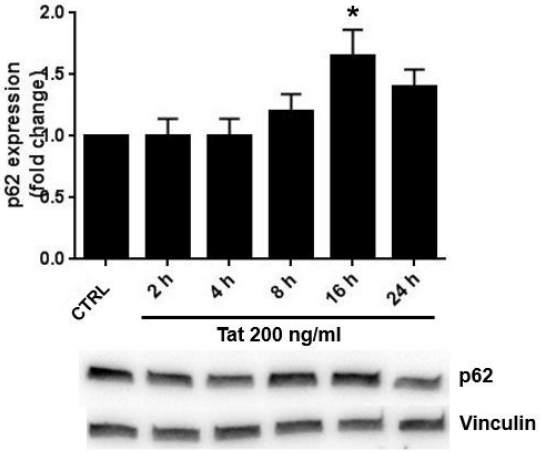

Fig 21: p62 expression in Tat-induced ETNA cells. Cells were treated Tat (200 ng/ml) for 2h, 4h, 8h, 16h and 24h. After incubation at 37 °C, cells were mechanically harvested, and total extracts were prepared as specified in Methods to assess p62 levels by western blot analysis with anti-p62 1:4000 (MLB) and anti-vinculin 1:4000 (Sigma). The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the actin content and are the means ± SEM from three separate experiments, each performed in triplicate. One-way ANOVA, followed by Bonferroni's test, was used tom determine significant differences. * $p \leq 0.05$ vs CTRL.

In recent years, it was discovered that Nrf2 was activated by p62 by a 'non-canonical' pathway, in fact p62 binds to the Nrf2 inhibitor, Keap1, responsible for degrading Nrf2. Since Nrf2 can induce p62 expression by binding directly to a conserved ARE in its promoter/enhancer, p62 protein is able to increase its own expression via this element. Therefore, we analyzed in our system p62 mRNA expression, by RT-qPCR on total RNA from SH-SY5Y cells treated with Tat at different time points. As shown in figure 22 Tat was able to significantly increase p62 mRNA levels at 16 h post-treatment.

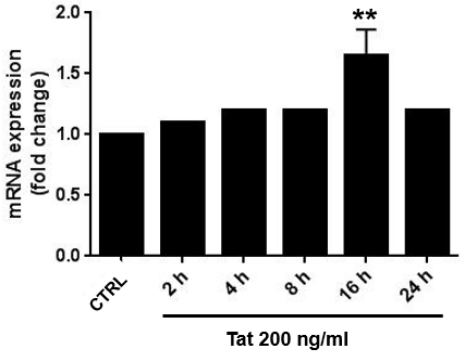

Fig 22: p62 mRNA expression in Tat-induced SH-SY5Y. Cells were treated with Tat (200 ng/ml) for 2h, 4h, 8h, 16h and 24h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of p62 gene by RT-qPCR. Data are calculated relative to actin and are expressed as the mean fold change compared with control. Each value represents the mean ± SEM of two independent experiments. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. ** $p \leq 0.001$ vs CTRL.

To evaluate the role of Nrf2 in Tat-induced p62 expression, we transfected SH-SY5Y cells with a dominant-negative mutant of Nrf2. As shown in figure 23 Tat-induced p62 and NQO1 up-regulation was prevented by the expression of Nrf2 dominant-negative mutant. These results clearly indicate the involvement of Nrf2 in Tat-induced p62 expression.

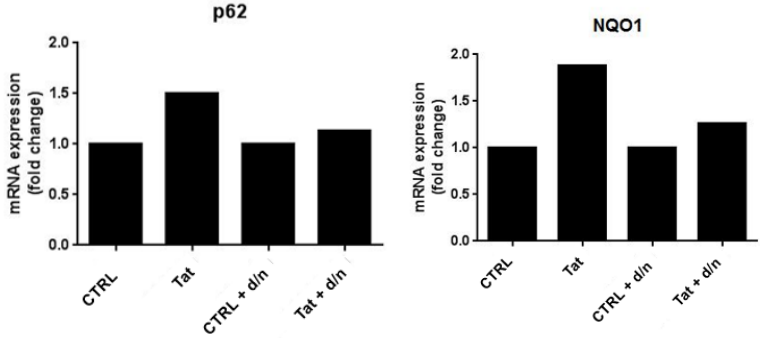

Fig 23: Nrf2-induced p62 mRNA expression in Tat-induced SH-SY5Y. Cells were transfected with a dominant negative mutant of Nrf2 for 48 h and next treated with Tat (200 ng/ml) for 24 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of p62 and NQO1 genes by RT-qPCR. d/n: dominant negative mutant of Nrf2.

Next, we evaluated the presence of Tat in both cytosol and nucleus compartments at different time points. As shown in figure 24, Tat was present

in the cytosol with a peak at 4h and 8h post-treatment and was detected also in the nucleus at the same time points.

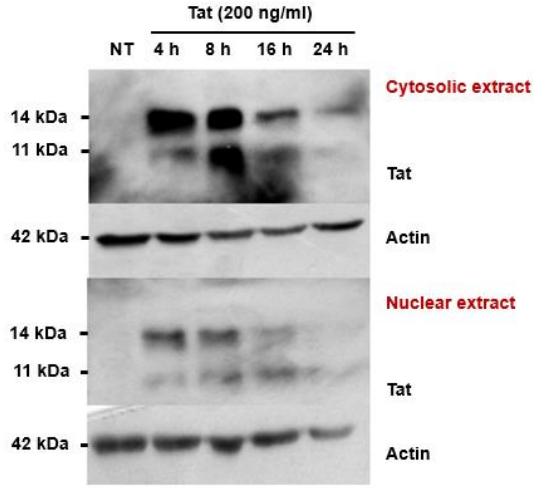

Fig 24: Tat levels in nuclear and cytosolic compartment of Tat-treated SH-SY5Y cells. Cells were treated with Tat (200 ng/ml) for 4h, 8h, 16h, 24 h. After incubation at 37 °C, cells were mechanically harvested, and the nuclear extracts were prepared as specified in Methods to assess Tat levels by western blot analysis with anti-Tat 1:1000 (Fit Biotech) and anti-actin 1:2000 (Sigma).

Tat levels strongly decreased at 16h and 24h when p62 protein increased, thus suggesting a role for p62 in Tat degradation. Based on this consideration, we evaluated Tat/p62 interaction by co-immunoprecipitation experiments. The figure 25 shows that p62 co-precipitated with Tat, as demonstrated by immunoprecipitation experiments performed with anti-Tat and anti-p62 antibodies. However, the two proteins only partially interacted, as indicated by the presence of high levels of Tat in the unbound extract.

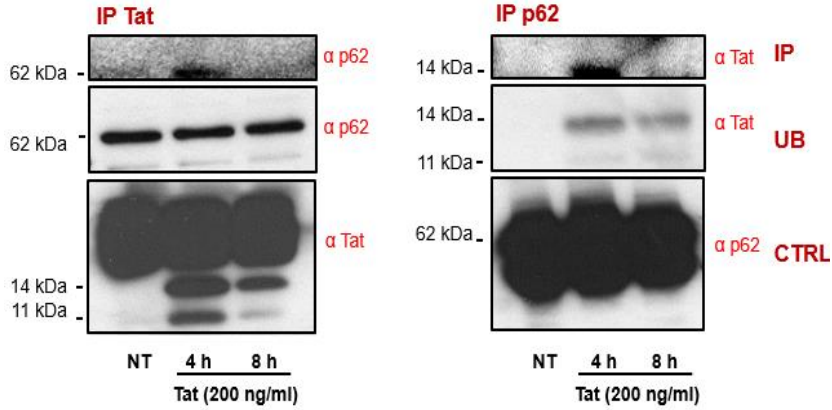

Fig 25: Co-immunoprecipitation of Tat and p62 in Tat-treated SH-SY5Y cells. Cells were treated with Tat (200 ng/ml) for 4h and 8h. After incubation at 37 °C, cells were mechanically harvested, the total extracts were prepared and immunoprecipitation were performed with anti-Tat and anti-p62, as specified in Methods; Western blot analysis was performed with with anti-p62 1:4000 (MLB) and anti-Tat 1:1000 (Fit Biotech). IP immunoprecipitated, UB unbound, CTRL immunoprecipitation control.

The effects of Tat and gp120 HIV proteins on astroglial cells: induction of proinflammatory and antioxidant response

Over the past 10 years, a significant amount of research has gone into understanding the extensive role played by astrocytes in the CNS, overturning their previous status as merely space holders, structural scaffolding, and scavengers. Their newly described roles include uptake of neurotransmitters, modulation of synaptic transmission, BBB maintenance, vaso-modulation, and long-term potentiation. The new concept of neuronal-glial intercommunication, where astrocytes play a dynamic role by integrating neuronal inputs and modulating synaptic activity, has helped us better comprehend astrocyte dysfunction in the context of HAND (Perez-Alvarez et al., 2013; Navarrete et al., 2013).

It has been recently shown that astrocytes can be infected by HIV-1 *in vitro*, and additionally that infected astrocytes can impair BBB function (Eugenin et al., 2011), but the infected astrocytes are rarely seen in patient brain autopsies (although one recent study has shown 20% of the astrocytes are infected in patients with HAD (Churchill et al., 2009)). Furthermore, recombinant Tat protein has been shown to be responsible for the induction of chemokines (Conant et al., 1998), cytokines (Nath et al., 1999) and nitric oxide synthetase (Liu et al., 2002) in cultured primary human astrocytes. Release of toxic cytokines, inability to take up excess glutamate and damage to the BBB make astrocytes a central offender in the pathogenesis of HAND. Besides the study on neuronal cells, in this part of work, we also analyzed the effect of HIV proteins on astroglial cells. Astrocytes are indeed the brain cells

that profoundly affect neuronal functions and, in a variety of neurological desorders, are thought to play a critical role in mediating neuronal toxicity or neuronal rescue. In neurodegeneration associated with HIV, chronic inflammation and oxidative stress play a critical role and these conditions are often related.

Proinflammatory response in astrocytes: the two HIV proteins, gp120 and Tat, induce NFkB activation via cPLA2/AA pathway.

Excessive amounts of NO, as produced by iNOS upon exposure of activated microglia and astrocytes to cytokines and/or viral proteins (e.g.,HIV tat and gp120), are assumed to contribute to neuronal abnormalities in HAND. Evidence exists supporting the notion that iNOS induction takes place after a nearly decline in physiological NO levels (i.e., NO released by constitutive NOS). In this part of work, we demonstrated that HIV-1 gp120 and Tat are able to inhibit nNOS through cPLA2-dependent AA production, this response being critical for allowing activation of the transcriptional factor NF-κB and subsequent iNOS and interleukin-1β transcription in astroglial cells.

The experiments are performed on normal human astrocytes (NHA). We analyzed the effects of gp120 and Tat on NF-κB activation. In particular, these viral particles effects were evaluated in the absence and presence of AACOCF3, the cPLA2 inhibitor, and/or AA. Our results demonstrate that treatment of cells with gp120 (30nM) for 30' and with Tat (100ng/ml) for 15' was able to activate NF-κB, this activation being inhibited by pretreatment with AACOCF3 (75 μM) and restored by pretreatment with 300nM AA (Fig. 26). Thus, it is feasible to assume that HIV proteins activate NF-κB through a mechanism involving cPLA2 activation and consequently AA production.

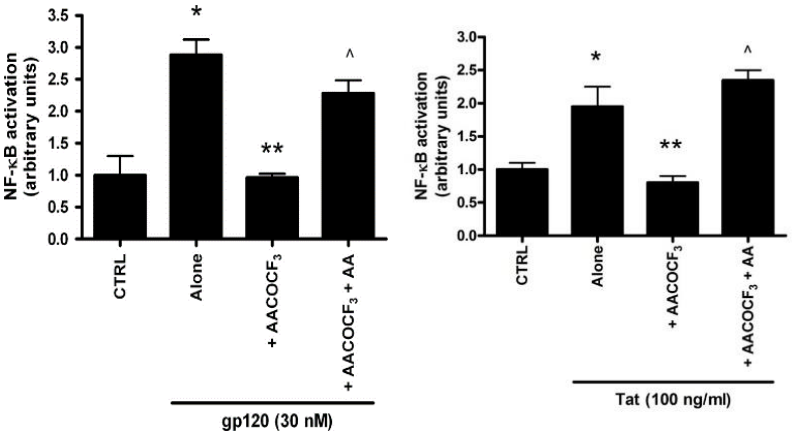

Fig 26: A critical role for AA in the gp120-induced NF-κB activation. Cells were incubated for 5 min in the absence or presence of AACOCF3 (75 mM) alone or associated with AA (300nM) and then treated with gp120 (30nM) and Tat (100 ng/ml). Cells were analyzed after 0.5h for NF-κB activation, as detailed under Materials and methods. Data points are the means ± SEM from three separate experiments, each performed in duplicate.gp120: *$p \leq 0.001$ vs CTRL;**$p \leq 0.001$ vs gp120 alone (30 nM); ^ $p \leq 0.01$vs gp120 + AACOCF3. Tat: *$p \leq 0.001$ vs CTRL;**$p \leq 0.001$ vs Tat alone (100 ng/ml); ^ $p \leq 0.01$vs Tat + AACOCF3.

It is well known that NF-κB is involved in the transcription of a great number of inflammatory genes, including iNOS and IL-1β. To verify whether AA-dependent nNOS inhibition is associated with induction of NF-κB-dependent genes by gp120, we investigated the role of the cPLA2–AA pathway in iNOS mRNA expression induced by gp120.

As shown in figure 27 A, treatment of NHA cells with gp120 (1–100nM) for 4h induced iNOS transcriptional expression in a dose-dependent manner, with a peak of stimulation at 30nM. The effect mediated by 30nM gp120 was inhibited by a pretreatment with 75 μM AACOCF3 (Fig. 27 B). Under similar conditions, with a further 4-h incubation, gp120 was also found to induce iNOS protein expression via a mechanism sensitive to AACOCF3.

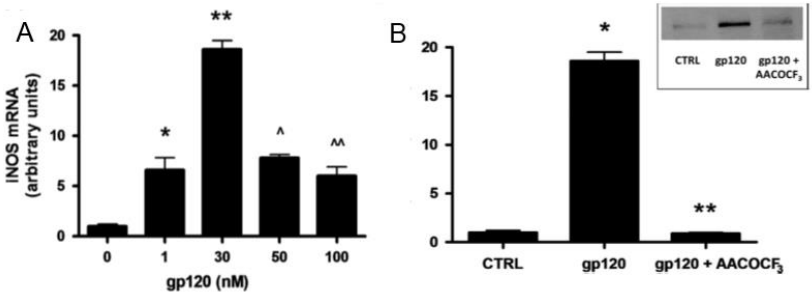

Fig 27: Role for the cPLA2 pathway in the gp120-induced iNOS mRNA accumulation. (A) Cells were treated for 4h with increasing concentrations (0–100nM) of gp120. (B) Cells were incubated for 5 min in the absence or presence of AACOCF3 (75 mM) and then treated for 4h with gp120 (30nM). The PCR product for the GAPDH gene was taken as the reference cellular transcript. Estimates of the relative iNOS mRNA amounts were obtained by dividing the peak densitometry of the iNOS band by the peak densitometry of the GAPDH band. Setting the value of iNOS mRNA from untreated cells equal to 1 unit, values for the other samples were calculated relative to it. Data points are the means ± SEM from three separate experiments,each performed in duplicate. The insert shows the Western blot for iNOS protein.(A)*p ≤ 0.01,**p ≤ 0.001, ^ p ≤ 0.001, and ^^ p ≤ 0.01 vs untreated cells; (B)*p ≤ 0.001 vs CTRL; **p ≤ 0.001vs gp120.

The same results were obtained with a Tat treatment. The figures 28 A shows that iNOS mRNA increase in dose-dependent manner with pick at 100 and 200 ng/ml; the effect mediated by 100 ng/ml of Tat was inhibited by a pretreatment with 75 μM AACOCF3 (Fig 28 B).

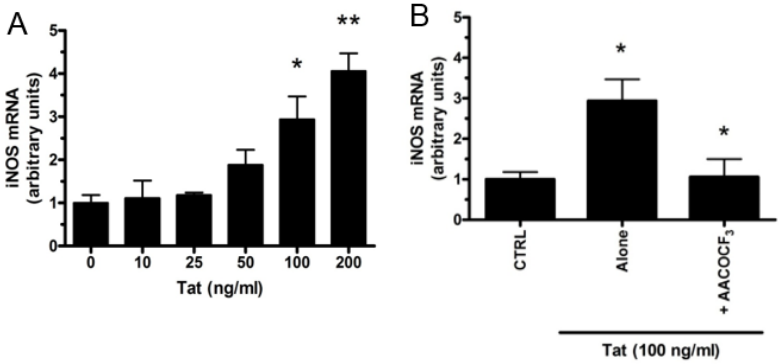

Fig 28: Role for the cPLA2 pathway in the Tat-induced iNOS mRNA accumulation. (A) Cells were treated for 4h with increasing concentrations (0–200 ng/ml) of Tat. (B) Cells were incubated for 5 min in the absence or presence of AACOCF3 (75 mM) and then treated for 4h with Tat (100 ng/ml). The PCR product for the GAPDH gene was taken as the reference cellular transcript. Estimates of the relative iNOS mRNA amounts were obtained by dividing the peak densitometry of the iNOS band by the peak densitometry of the GAPDH band. Setting the value of iNOS mRNA from untreated cells equal to 1 unit, values for the other samples were calculated relative to it. Data points are the means ± SEM from three separate experiments,each performed in duplicate.(A)*p ≤ 0.05 vs untreated cells,**p ≤ 0.001 vs untreated cells (B)*p ≤ 0.001 vs CTRL.

Altogether, these results suggest that the viral proteins gp120 and Tat induce iNOS mRNA expression through a mechanism involving the cPLA2–AA pathway. In detail, gp120 and Tat could inhibit nNOS by activating cPLA2 and promoting AA production. Consequently, basal levels of NO decrease, thus promoting NF-κB activation and iNOS transcriptional induction.
Furthermore, we investigated whether this mechanism could be applied to other NF-κB-dependent genes coding for related proinflammatory proteins. In this respect, we analyzed the role of the cPLA2–AA pathway on IL-1β mRNA induction by stimulating NHA cells with gp120 for 4h. As shown in figure 29 A,the treatment of cells with various concentrations of gp120 (1–100nM) induced IL-1β transcription in a dose-dependent manner, with a maximum observed at 30nM. Similar to iNOS transcriptional induction, pretreatment with the cPLA2 inhibitor AACOCF3 (75 μM) slashed IL-1β mRNA expression (Fig. 29 B).

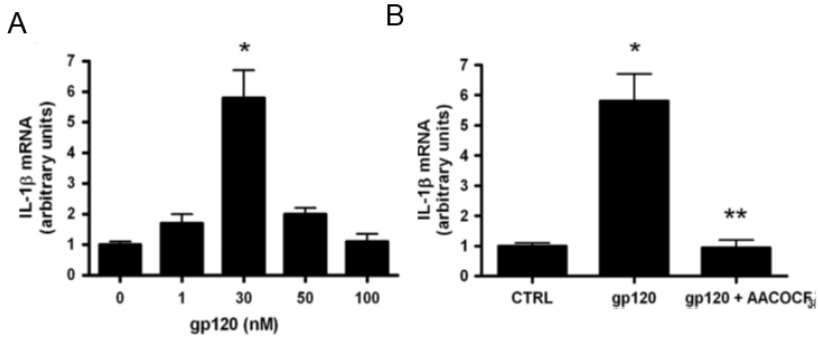

Fig 29: Role of cPLA2 pathway in the gp120-induced IL-1β mRNA accumulation. (A) Cells were treated for 4h with increasing concentrations (0–100nM) of gp120. (B) Cells were incubated for 5 min in the absence or presence of AACOCF3 (75 mM) and then treated for 4h with gp120 (30nM). The PCR product for the GAPDH gene was taken as the reference cellular transcript. Estimates of the relative IL-1β mRNA amounts were obtained by dividing the peak densitometry of the IL-1β band by the peak densitometry of the GAPDH band. Setting the value of Inos mRNA from untreated cells equal to 1unit, values for the other samples were calculated relative to it. Data points are the means ± SEM from three separate experiments, each performed in duplicate. (A)*p ≤ 0.001 vs untreated cells; (B)*p ≤ 0.001 vs CTRL; **p ≤ 0.001 vs gp120.

In the same manner, we treated NHA cells with Tat for 4h. As shown in figure 30 A, the treatment of cells with various concentrations of Tat (0–200nM) induced IL-1β transcription in a dose-dependent manner, with a maximum observed at 100/200 ng/ml. Similar to iNOS transcriptional induction, pretreatment with the cPLA2 inhibitor AACOCF3 (75 μM) decrease IL-1β mRNA expression (Fig. 30 B).

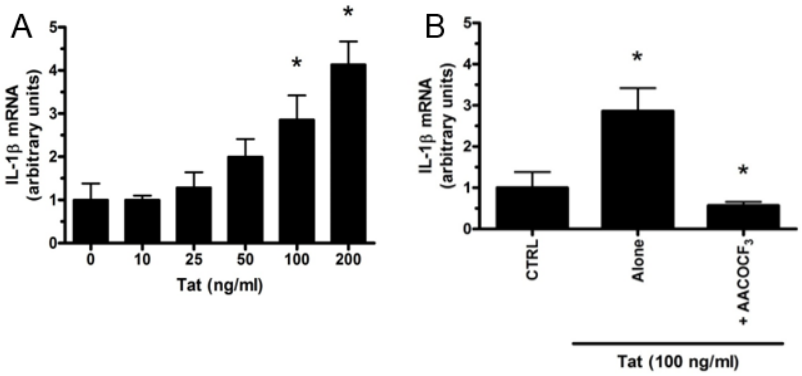

Fig 30: Role of cPLA2 pathway in the Tat-induced IL-1β mRNA accumulation. (A) Cells were treated for 4h with increasing concentrations (0–200 ng/ml) of Tat. (B) Cells were incubated for 5 min in the absence or presence of AACOCF3 (75 mM) and then treated for 4h with Tat (100 ng/ml). The PCR product for the GAPDH gene was taken as the reference cellular transcript. Estimates of the relative IL-1β mRNA amounts were obtained by dividing the peak densitometry of the IL-1β band by the peak densitometry of the GAPDH band. Setting the value of IL-1β mRNA from untreated cells equal to 1unit, values for the other samples were calculated relative to it. Data points are the means ± SEM from three separate experiments, each performed in duplicate. (A)*$p \leq 0.05$ vs untreated cells; (B)*$p \leq 0.001$ vs CTRL and Tat.

Results obtained on IL-1β transcriptional induction confirm the idea that cPLA2 and AA are involved in widespread pathways activated under proinflammatory conditions, including HAND.

In regards of these results, the hypothesis is that HIV-1 proteins (e.g.,gp120 and Tat) induce an increase in intracellular calcium concentration by interacting with several cellular receptors (Medina et al., 1999) (Fig. 31). The activation of these receptors on astrocytes membrane promotes the hydrolysis of phosphatidyl inositol bisphosphate to diacyl glycerol and a soluble messenger IP3 that triggers the release of Ca^{2+} from IP3-sensitive endoplasmic reticulum (Hoke et al., 2009; Xu et al., 2011). We speculate that, as previously demonstrated in astrocytes stimulated with LPS plus IFN-γ, the mechanism of nNOS inhibition involves phosphorylation of a critical tyrosine residue(s) of the enzyme (Palomba et al., 2004). Under these conditions, NO levels decrease, thereby leading to NF-κB activation and to the ensuing expression of a variety of genes, including iNOS and IL-1β. Note that NF-κB, in turn, is able to induce cPLA2 mRNA expression with a subsequent AA release, thus establishing a positive feedback (Hernandez et al., 1999). The expression of these genes promotes a wide production of inflammatory effectors (e.g., NO, AA, cytokines), which could contribute to HAND pathogenesis. In conclusion, these results have a great importance for the comprehension of the mechanisms involved in HAND pathogenesis, especially because interactions between cPLA2 and NOS could be involved

in neural death, chronic inflammation, and oxidative stress, conditions found in a large percentage of adults infected with HIV.

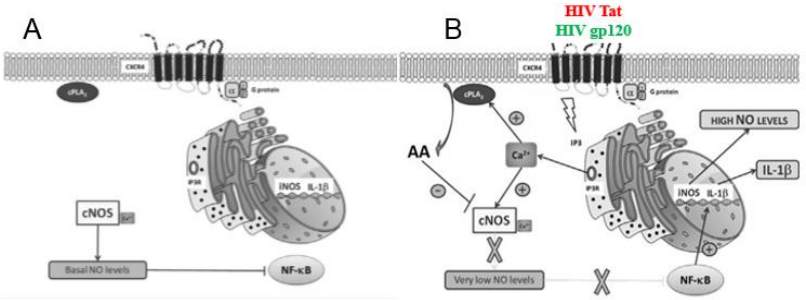

Fig 31: Proposed model of the role of AA in the crosstalk between nNOS and iNOS in astroglial cells under HIV gp120 and Tat stimulation. (A) Under normal conditions, basal amounts of NO generated by cNOS (i.e.,nNOS) keep NF-κB activity and NF-κB-dependent gene expression inhibited. (B) HIV gp120 and Tat promotes a sudden increase in the intracellular Ca^{2+} concentration. A hypothesis is that gp120 and Tat bind cell membrane chemokine coreceptors (e.g.,CXCR4), thus promoting the release of IP3 that triggers the release of Ca^{2+} from IP3-sensitive endoplasmic reticulum Ca^{2+} stores (for alternative mechanisms see the text). The influx of calcium leads to a sustained activation of cPLA2 and extensive release of AA that causes inactivation of cNOS (although the Ca^{2+} increases), thereby lowering the intracellular NO concentration. Thus, gp120 and Tat can activate NF-κB and promote iNOS and IL-1β gene expression, an event associated with the release of large amounts of NO and IL-1β, respectively.

Antioxidant response in astrocytes: activation of Nrf2/ARE pathway

Oxidative stress has been proposed as one of the primary causes of most neuropathies, not only through the structural and functional alterations that ROS produce to cell biomolecules, but also because they are potential mediators of cell death by either necrosis or apoptosis (Friedlander, 2003). Astrocytes are known to be important modulators of brain physiology, particularly during regenerative or protective processes, by producing and releasing several antioxidant enzymes like superoxide dismutase and glutathione precursors, which in turn support neuronal survival and stability (Takuma et al., 2004). Modifications in redox state are known to modulate transcription factors (Jones, 2008), such as Nrf2. In response to modifications in cellular redox state, Nrf2 is released from its repressor and translocated into the nucleus, where it binds to the ARE sequence and induces the expression of several enzymes such as GCL, GPX, SystemXc, which in turn are related to GSH metabolism (Kraft et al., 2004). GSH is one of the most intensively studied intracellular non-protein thiols because of the critical role it plays in cell biochemistry and physiology. Through maintenance of protein sulfhydryls in the appropriate redox state, GSH regulates important death and/or survival pathways. Redox changes, induced by an altered GSH and/or GSSG balance, also modulate Nrf2 release from Keap-1, and changes in GSH

homeostasis have been implicated in the etiology and progression of a number of human diseases (Darlington, 2005).
In this part of my PhD project, we investigated whether Tat was able to activate Nrf2 in human astrocytoma cells. To this aim, we examined Nrf2 translocation into the nucleus, after treatment with Tat (200 ng/ml) for 15 minutes, 2 hours and 16 hours. Nrf2 levels were measured in nuclear extracts by using Western blot analysis. As shown in figure 32, Tat induced a 2.3-fold increase of nuclear Nrf2 levels already at 15 min post-treatment, that returned to nearly control levels after 16 h. The same result was obtained by a TransAm kit based on ELISA method (see figure 33)

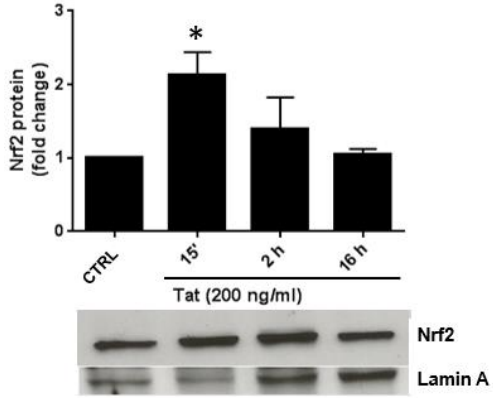

Fig 32: Effects of Tat on Nrf2 nuclear translocation in U373 cells. Cells were treated with Tat (200 ng/ml) for the indicated time points. After incubation at 37 °C, cells were mechanically harvested, and the nuclear extracts were prepared as specified in the Materials and Methods section to assess Nrf2 levels by western blot analysis with anti-Nrf2 1:1000 (Abcam) and anti-laminA 1:2000 (Abcam).. 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. * $p \leq 0.05$ vs CTRL

Since NO is an endogenous inducer of Nrf2-dependent phase-2 enzymes, both *in vitro* and *in vivo*, we analyzed the role of NO in the activation of the Nrf2-ARE pathway in Tat-stimulated astroglial cells. To this purpose, we pretreated U373 cells for 30 min with L-NAME and prepared nuclear extracts. We evaluated the Nrf2 translocation in the nucleus by a TransAm kit based on ELISA method. As shown in figure 33 A, Tat induced a 1.5-fold increase of Nrf2 activation that was restored to nearly control levels by L-NAME pretreatment. The treatment with H_2O_2 was performed as a positive control for Nrf2 activation. The graph 33 B shows a 2-fold increase of Nrf2 traslocation into the nucleus as demonstrated by Western Blot analysis; the pretreatment with 1mM L-NAME for 30 minutes restored the Nrf2 activation to control levels.

These results indicate the involvement of NO in Nrf2 regulation probably related to modification of its inhibitor Keap1.

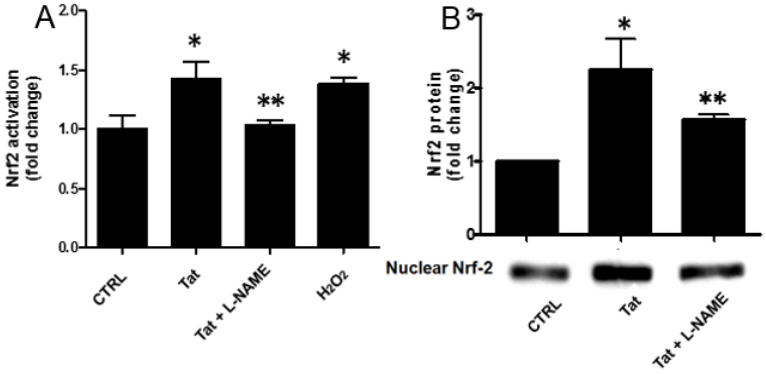

Fig 33: Role of NO in Tat-induced Nrf2 activation. (A) U373 cells were pretreated for 30 min with L-NAME (1 mM), or medium alone before the addition of Tat (200 ng/ml) for 15 min, and with H_2O_2 (0.25 Mm) for 15 min. After incubation at 37 °C, the cells were homogenized, and Nrf2 activation was quantified by TransAM assay as detailed in the Materials and Methods section. (B) U373 cells were pretreated for 30 min with L-NAME (1 mM), or medium alone before the addition of Tat (200 ng/ml) for 15 min. After incubation at 37 °C, the cells were homogenized, and Nrf2 activation was quantified by Western Blot assay as detailed in the Materials and Methods section with anti-Nrf2 1:1000 (Abcam). Data points are the means ± S.E.M. from 3 separate experiments, each performed in triplicate. One-way ANOVA, followed by Bonferroni's test, was used to determine significant differences. (A) * $p \leq 0.05$ vs CTRL, ** $p \leq 0.05$ vs Tat (B) * $p \leq 0.01$ vs CTRL, ** $p \leq 0.05$ vs Tat.

Next, we wanted to better understand if Nrf2 activation was able to induce an effective antioxidant response in U373 cells. As shown in figure 34, Tat induced SOD1, SOD2, CAT, GPX3, NQO1 mRNA up-regulation after 8h and 16 h post-treatment.

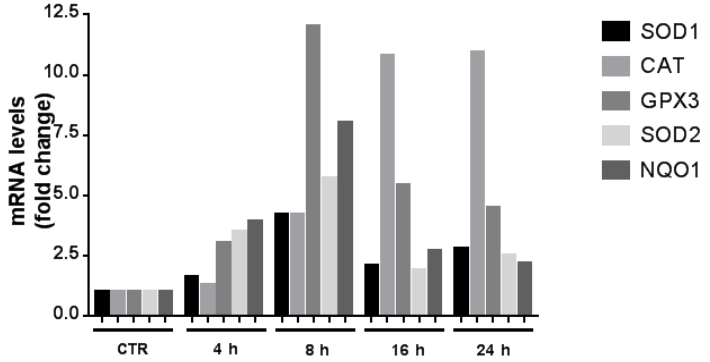

Fig 34: Effects of Tat on ARE-driven gene expression in U373 cells. U373 were treated with Tat (200 ng/ml) for 4, 8, 16 and 24 h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of several genes (NQO1, CAT, SOD1, SOD2, GPX3) by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed as the mean fold change compared with control.

Then, we wanted to focus our attention on others ARE genes, that were associated to GSH synthesis, as the glutamate-cysteine ligase GCL. The figure 35 shows a significant increase at 8h and 16 h Tat post-treatment.

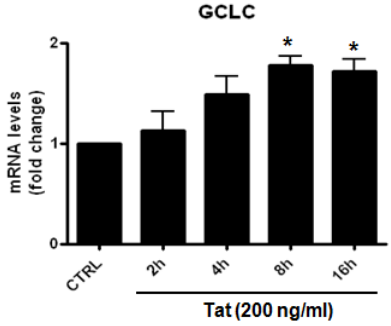

Fig 35: Effects of Tat on GCLC gene expression in U373 cells. U373 were treated with Tat (200 ng/ml) for 2, 4, 8 and 16h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of GCLC gene by RT-qPCR. Data are calculated relative to the internal housekeeping 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. * $p \leq 0.05$ vs CTRL

Among others, the SystemX_C gene, coding for a glutamate/cystine antiporter, plays a pivotal role in astrocytes. Under physiological conditions, cystine is imported and intracellularly reduced to cysteine, a building block of the antioxidant GSH. While cystine is imported, glutamate is obligatorily exported; glutamate released physiologically modulates synaptic

transmission via activation of pre- and post-synaptic glutamate receptors located in the vicinity of the synaptic cleft (Backer et al., 2002). Here, we wondered if Tat was able to induce SystemX_C transcription. As shown in figure 36, the treatment with Tat (200 ng/ml) for 8 h significantly increased SystemX_C mRNA levels.

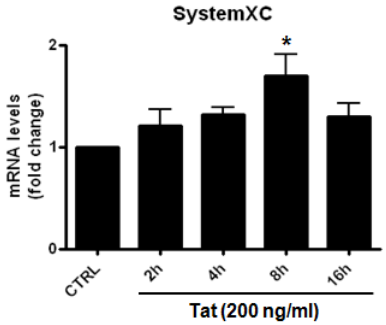

Fig 36: Effects of Tat on SystemX_C gene expression in U373 cells. U373 were treated with Tat (200 ng/ml) for 2, 4, 8 and 16h. After incubation at 37 °C, the cells were homogenized and total RNA has been purified to assess mRNA levels of SystemX_C gene by RT-qPCR. Data are calculated relative to the 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.* $p \leq 0.05$ vs CTRL

To the same purpose we performed a western blot analysis to evaluate the expression of SystemX_C protein. The figure 37 shows a significant increase (20%) of SystemX_C protein after treatment with 200 ng/ml Tat for 24 h.

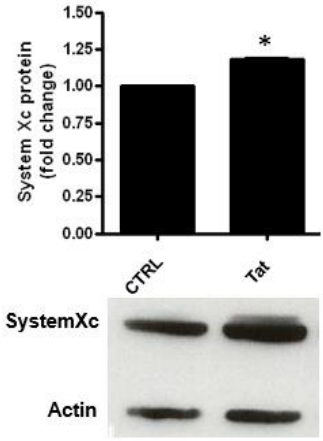

Fig 37: Effects of Tat on SystemX_C protein expression in U373 cells. U373 were treated with Tat (200 ng/ml) for 24h. After incubation at 37 °C, the cells were harvested and total protein extract were performed to assess protein levels of SystemX_C by a Western Blot analysis with anti-SystemXc 1:5000 (OriGene) and anti-actin 1:2000 (Sigma). 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.* $p \leq 0.001$ vs CTRL

Altogether these results suggest that also in astrocytes the expression of several ARE genes can be up-regulated by Tat; in particular Tat was able to increase SystemX_C expression that, on the one hand, could have a protective role for astrocytes increasing their cysteine internalization, and on the other hand, could be detrimental for neurons, leading to excessive glutamate release.

Astrocytes are one of the primary target for HIV-1 infection and elicit a severe neuronal pathology, due to secretion of viral particles, such as Tat. Thus, we wondered if Tat production within the cells and the consequent Tat secretion, were able to induce antioxidant response in astrocytes. Therefore, we stably transfected U373 astrocytoma cells with pcDNA3.1 HIV-Tat vector for Tat overexpression. In this cell culture model, we analyzed the activation of antioxidant response, simulating a situation closer to reality.

The figure 38 shows two control experiments (RT-PCR and Western Blot) to confirm the expression of pcDNA3.1 HIV Tat vector in U373 cell line;

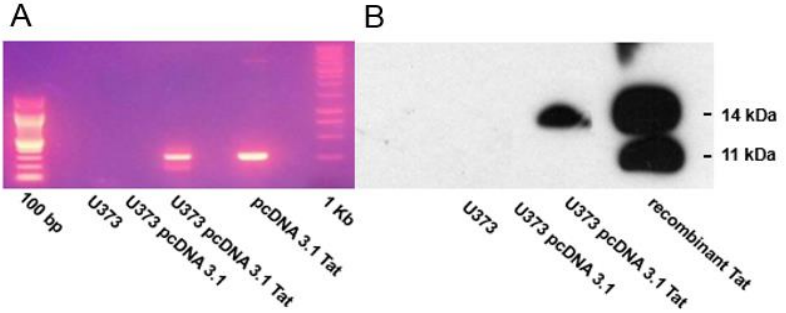

Fig 38: Control experiments of pcDNA3.1 HIV-1 Tat vector transfection in U373 cells. (A) Cells were homogenized and total RNA has been purified to assess mRNA levels of Tat gene by RT-PCR homogenates The PCR product of the GAPDH gene was taken as the reference cellular transcript. (B) Cells were mechanically harvested, and the total extracts were prepared as specified in the Materials and Methods section to assess Tat levels by western blot analysis with anti-Tat 1:1000 (Fit Biotech). 100 bp/1Kb: molecular weight.

The following experiments were aimed to evaluate Nrf2 levels in nuclear extracts by using Western blot analysis. As shown in figure 39, Tat induced a 1.6-fold increase of nuclear Nrf2 levels.

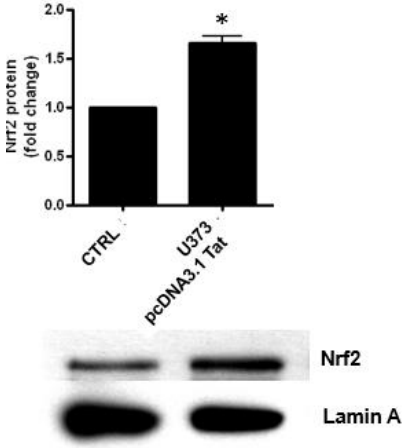

Fig 39: Effects of Tat on Nrf2 nuclear translocation in U373 pcDNA3.1Tat cells. Stably transfected cells were mechanically harvested, and the nuclear extracts were prepared as specified in the Materials and Methods section to assess Nrf2 levels by western blot analysis with anti-Nrf2 1:1000 (Abcam) and anti-laminA 1:2000 (Abcam). 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. * $p \leq 0.001$ vs CTRL

Considering that ARE genes are mainly regulated by Nrf2, we investigated whether Tat was able to induce some ARE genes in human astrocytoma cells stably transfected with Tat vector. To this aim, we examined CAT, GCLC and SystemX_C mRNA levels by using RT-qPCR analysis (Fig 40). All the three genes examined show a 2-fold increase of mRNA expression in cells stably transfected with Tat vector, respect to control cells (cells not transfected and transfected with empty vector).

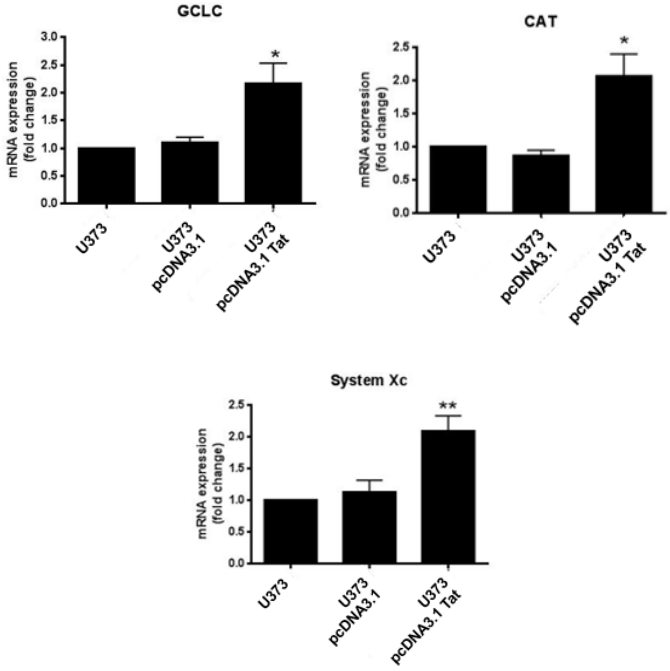

Fig 40: Effects of Tat on ARE genes expression in U373 cells stably transfected with Tat. U373 cells were homogenized and total RNA has been purified to assess mRNA levels of SystemX_C, CAT, GCLC genes by RT-qPCR. Data are calculated relative to the internal housekeeping gene (GAPDH) and are expressed 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.* $p \leq 0.05$ vs U373 and U373 pcDNA3.1; ** $p \leq 0.01$ vs U373 and U373 pcDNA3.1.

Then, we evaluated if the endogenously produced Tat was able to induce SystemX_C protein expression, thus we performed western blot analysis and we found out a 50% increase of SystemX_C protein levels in stably transfected cells overexpressing Tat compared with empty vector-transfected and non-transfected cells as shown in figure 41.

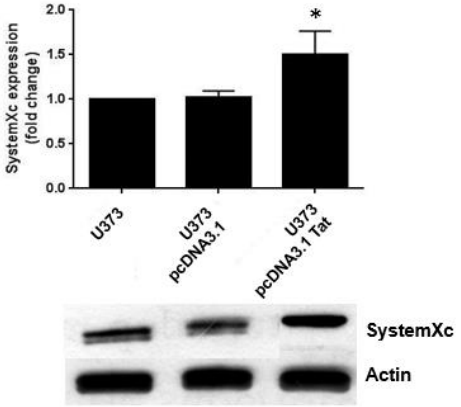

Fig 41: Effects of Tat on SystemX_C protein expression in U373 pcDNA3.1Tat cells. U373 cells were mechanically harvested, and the total extracts were prepared as specified in the Materials and Methods section to assess SystemXc levels by western blot analysis with anti-SystemXc 1:5000 (OriGene) and anti-actin 1:2000 (Sigma). The histogram shows the densitometric analysis of the western blots for each sample. Values are calculated relative to the 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. * $p \leq 0.001$ vs U373 and U373 pcDNA3.1.

These results indicate a significant increase of several ARE genes, such as SystemX_C, in both cells treated with Tat and stably transfected with Tat plasmid. This induction could have a dual role; in fact, the activation of SystemX_C could have a protective role for astrocytes, enhancing cystine uptake, followed by an increased production of GSH, but on the other hand could have a detrimental action for surrounding neurons, since the enhanced activity of SystemX_C could increase the extracellular glutamate levels, leading to excitotoxicity.

Effects of astrocyte-secreted Tat on Golgi complex and viability of co-cultured neuronal cells.

When oxidative stress occurs, all organelles show adaptive responses to oxidative stress, such as activation of genes in the nucleus that encode defensive enzymes, transcription factors, and structural proteins. With regard to Golgi apparatus (GA), it is conceivable that the steady-state structure and proper physiological function of the GA are also affected during oxidative stress; these alterations in the GA may trigger and propagate downstream stress signals that result in adaptation or, if the oxidative stress is too severe, GA fragmentation and even apoptosis (Jiang et al., 2011).

To this purpose, we treated SH-SY5Y cells with Tat for different times 15 min, 2h and 24h and we demonstrated that neuroblastoma cells treated with

Tat after 24 h, show a significant increase of fragmented and dispersal GA, as shown in the graph 42.

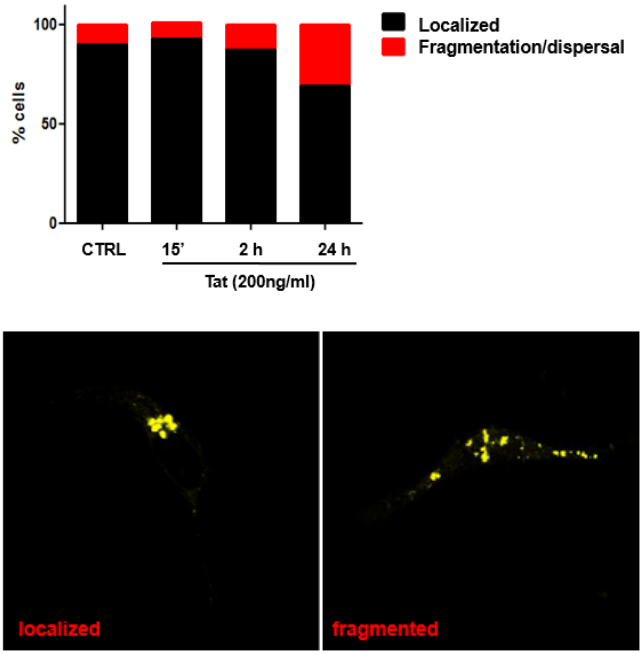

Fig 42: Effects of Tat on GA in SH-SY5Y. SH-SY5Y cells were treated with Tat (200 ng/ml) for 15 min, 2h and 24h, and transfected with a vector expressing a GA marker, were fixed with PFA 4% and analyzed by confocal microscopy as specified in the Materials and Methods section. The histogram shows the percentage of cells with localized and fragmented/dispersal Golgi. Below, two sample images to show the GA structure.

Next, we evaluated the effects of transfected astrocytes on neurons, performing co-cultures experiments (as specified in the Materials and Methods section).
We first analyzed the GA structure in neurons co-cultured with astrocytes stably transfected with Tat, compared with neurons treated with exogenous Tat. The figure 43 indicate that both Tat treatment and astrocyte-secreted Tat were able to induce Golgi dispersal in neuronal cells. This effect was more evident in co-cultured neuronal cells with respect to Tat treated cells.

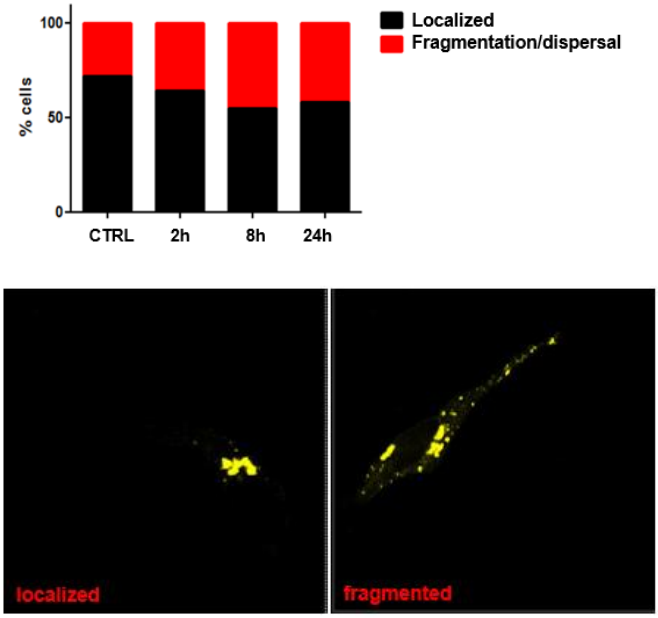

Fig 43: Effects of Tat on GA in SH-SY5Y co-cultured with U373 pcDNA3.1 Tat cells. SH-SY5Y cells were transfected with a vector expressing a GA marker, were fixed with PFA 4% and analyzed by confocal microscopy as specified in the Materials and Methods section. The histogram shows the percentage of cells with localized and fragmented/dispersal Golgi. Below, two sample images to show the GA structure.

This difference in GA fragmentation/dispersal between SH-SY5Y treated with Tat and co-cultured with astrocytes stably transfected with Tat can be explained by the release of various cellular factors, including pro-inflammatory molecules and cytokines from astrocytes.

Next, we wondered if the constant secretion of Tat in stably transfected cells was able to induce reduced viability or antioxidant response activation in co-cultured neuronal cells.

To this purpose, we performed MTT assays on SH-SY5Y cells at different time points to evaluate the cytotoxicity of Tat produced by transfected U373. The figure 44 shows that the viability of SH-SY5Ycells co-cultured with stably trasfected astrocytes was reduced of approximately 20 %.

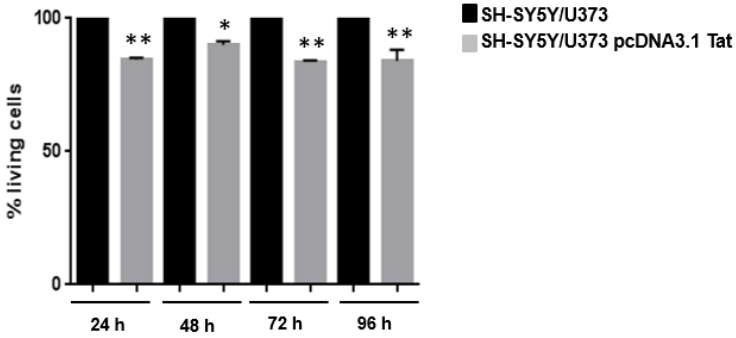

Fig 44: Effects of Tat on SH-SY5Y viability co-cultured with U373 pcDNA3.1 Tat cells. SH-SY5Y cells were co-cultured with U373 stably transfected with Tat and the MTT assays were performed as specified in the Materials and Methods section. The histogram shows the percentage of living cells. Values are calculated relative to the neuronal cells co-cultured with astrocytes not transfected with Tat 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. * $p \leq 0.01$ vs SH-SY5Y/ U373; ** $p \leq 0.001$ vs SH-SY5Y/U373 pcDNA3.1.

This mild mortality, could be due to the activation of antioxidant response in co-cultured SH-SY5Y cells. Therefore, we evaluated ARE gene expression in co-cultured SH-SY5Y with stably transfected U373 cells. The Figure 45 shows the increased levels of NQO1 and CAT genes of about 50-60% after 4 h, 8h and 24 h of co-cultures.

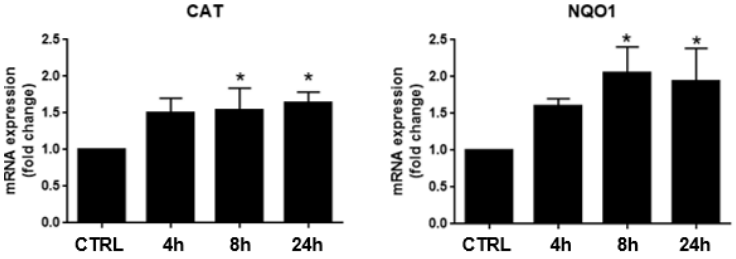

Fig 45: Effects of Tat on ARE genes expression in SH-SY5Y cells co-cultured with U373 cells stably transfected with Tat. SH-SY5Y cells were homogenized and total RNA has been purified to assess mRNA levels of CAT and NQO1 genes by RT-qPCR. Data are calculated relative to the internal housekeeping gene (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. * $p \leq 0.05$ vs CTRL.

As demonstrated in this section, Tat was able to induce both reduced viability and antioxidant response activation in neuronal cells. Although these results appear to be paradoxical findings, they could be explained by deeply analysing the role of ROS/RNS as key mediators in the regulation of cell

death and survival. Excessive ROS production can damage cellular components such as DNA, proteins and lipids, resulting in oxidative stress, which is an important inducer of different cellular processes (Filomeni et al., 2014). Previous studies have reported that HIV-1 proteins, including gp120 and Tat, lead to a dose-dependent increase in oxidative stress and to a decrease in the intracellular GSH levels in brain endothelial cells and various other cell types (Toborek et al., 2003). Note that antioxidant levels in HIV-infected patients are altered, a situation that can lead to an increased oxidative stress. In particular, serum GSH levels and GPX activity are significantly lower in HIV patients than in controls (Staal, 1998). Besides acting as cytotoxic effectors, ROS/RNS also function as signalling molecules; critical levels of ROS play important roles in regulating different cellular pathways, therefore controlling cell death and survival. As reported by Navarro-Yepes et al., a "mild" oxidative stress can act as a signalling mechanism leading to adaptive stress responses. Oxidative damage can be repaired to a certain extent, and oxidized biomolecules, such as proteins, can be degraded and recycled by distinct processes, including autophagy. Recent studies have begun to uncover the role of reversible oxidative post-translational modifications in protein cysteines in the regulation of specific signalling cascades that regulate adaptive cell response and autophagy. (Navarro-Yepes et al., 2014). As previously demonstrated in our lab, Tat was found to induce ROS production and to affect cell viability in SH-SY5Y cells, these effects being mediated by SMO activity and NMDAR stimulation (Capone et al., 2013). Here, we reported a 20% reduction of viability of SH-SY5Y cells co-cultured with Tat-expressing astrocytes. Besides this detrimental action, we also demonstrated the induction of an antioxidant response in neuronal cells as elicited by astrocyte-released Tat, this effect being due to Nrf2 activation. Tat-induced ROS/RNS generation may play crucial role in the canonical pathway of Nrf2 activation since they can directly modify the stress sensor protein Keap1.

4) CONCLUSIONS

Over the past 10 years, there has been a significant progress in our understanding of the mechanisms and the risk factors involved in the development of HAND. Neurons are vulnerable to direct damage by some viral proteins that can interact with several neuronal cell surface receptors, such as NMDAR. Astrocytes can mediates neurotoxicity through the release of several host- or viral-derived neurotoxic factor but they are also involved in neuroprotection. The two major HIV proteins that cause neuronal injury are gp120 and Tat.

The results reported in this PhD thesis indicate that both in astroglial and neuronal cells, Tat can elicit an antioxidant response. As previously reported by our group, Tat induced a SMO-mediated ROS generation in SH-SY5Y (Capone et al., 2013). Here, we demonstrated that the up-regulation of SMO induced by Tat was able to increase Nrf2 activation and ARE-driven gene expression in neuronal cells. However, despite the activation of Nrf2-dependent gene expression we observed a reduced cell viability in neuronal cells treated with Tat. Currently, the Nrf2-ARE pathway is a high-value therapeutic target for several neurodegenerative diseases, as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis, and numerous works have identified novel Nrf2-activating compounds (Johnsonet l., 2015). In this respect, DMF is able to attenuate neurotoxicity in SH-SY5Y cells and in an animal model of Parkinson's disease by enhancing Nrf2 activity as recently reported (Jing et al., 2015).

With respect to the antioxidant response induced by Tat in astroglial cells we demonstrated a significant increase of SystemX_C expression in both Tat-treated and Tat-expressing cells. The up-regulation of this transporter may have important implications in the maintenance of glutamate homeostasis and redox state. In particular, higher expression of SystemX_C leads to an increased cysteine internalization in astrocytes enabling them to counteract oxidative stress thus playing a protective role for this cell population. On the contrary, augmented glutamate release by astrocytes could be detrimental for neurons. Then, we studied the role of Tat-releasing astroglial cells on neuronal function. Intriguingly, we found out both an increased Golgi dispersal/fragmentation and a reduced neuronal viability in SH-SY5Y cells co-cultured with Tat-expressing astrocytes. These results strongly suggest that also host factors are involved in detrimental effects observed in co-cultured neuronal cells. We also demonstrated that these detrimental effects are partially counteracted by the activation of antioxidant response in neuronal cells suggesting a role for ROS/RNS-mediated Nrf2 activation.

The relationships between the GA fragmentation and oxidative stress is still to be clarified and more studies are needed to better understand their role in neurodegenerative diseases.
Our data also indicate the involvement of endogenously produced NO in both SMO activation and antioxidant response induction; however, its precise role still remains to be further investigated.
Besides oxidative stress, chronic inflammation plays a critical role in neurodegeneration associated with HIV. We found out that gp120 and Tat were able to inhibit neuronal NOS through the production of AA by cPLA2, this response being critical for allowing activation of NF-κB transcription factor and subsequent iNOS and interleukin-1β expression in astroglial cells. These results strongly suggest that gp120 and Tat can induce an increase in intracellular calcium concentration by activating receptors on astrocytes membrane, promoting several events that lead to nNOS inhibition, through phosphorylation of critical tyrosine residue(s) of the enzyme. Under these conditions, NO levels decrease, leading to NF-κB activation and expression of several pro-inflammatory genes, including iNOS and IL-1β. Note that NF-κB, in turn, was able to induce cPLA2 mRNA expression with a subsequent AA release, thus establishing a positive feedback (Hernandez et al., 1999). A large production of inflammatory effectors, as NO, AA and cytokines by the expression of NF-kB driven-genes, could contribute to HAND pathogenesis.
In summary, our findings provide evidence of an antioxidant response activation and may help our understanding of the mechanism by which Nrf2 can mediate protection against HAND and other neurodegenerative diseases associated with HIV infection. A goal of future research may be to spatially and temporally modulate the molecular pathways, involved in the potentiation of the antioxidant responses versus oxidative stress. Noteworthy SMO upregulation can be neurotoxic in the brain, not only generating ROS but also producing spermidine and reactive aldehydes. Indeed, it has been reported the hypothesis that agents that can chemically neutralize reactive aldehydes should demonstrate neuroprotective synergic actions with the antioxidant response (Wood et al., 2006).
In conclusion, it should be also reminded that the in vitro cell models used in this work may have some limitations such as a lower sensitivity to Tat-induced cytotoxicity than normal or primary cells. Therefore, further studies are needed to confirm the results in other cell models thus allowing a broader extrapolation about the molecular mechanisms involved in HAND pathogenesis.

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MATERIALS AND METHODS

Materials

Chlorhexidine digluconate (CHL) solution, MK-801 hydrogen maleate (MK-801), N methyl D-aspartic acid (NMDA), N-acetylcysteine (NAC), arachidonic acid (AA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% Trypsin–EDTA solution, and gentamicin solution 50 mg/ml were obtained from Sigma–Aldrich (Milan, Italy), Bradford reagent was obtained from Bio-Rad Italia (Milan, Italy); Arachidonyl trifluoromethyl ketone (AACOCF3) was purchased from Calbiochem (Milan,Italy). All chemicals were of analytical or reagent grade and were used without further purification.

The Kit Go Taq 2-Step RT- qPCR System and the kit GO Taq G2 DNA polymerase were obtained from Promega; the transfection kit Lipofectamine 3000 was obtained from Invitrogen. The ARP697 HIV-1 Tat-B protein and HIV gp120 protein 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, and was donated by FIT Biotech, Estonia, Dr. J. Karn.

For Western Blot analysis and immunofluorescence, the following primary antibodies were used: anti-actin 1:2000 (Sigma), anti-Nrf2 1:1000 (Abcam), anti-laminA 1:2000 (Abcam), anti-laminB 1:4000 (Abcam), anti-SystemXc 1:5000 (OriGene), anti-iNOS 1:1000 (Santa Cruz Biothecnology), anti- HIV1 Tat 1:1000 (Fit Biotech), anti-p62 1:200 for IP 1:4000 for WB (MLB), anti-ubiquitin 1:500 (Millipore), anti-SMO 1:1000 (Proteintech), anti-Vinculin 1:4000 (Sigma), anti-LC3 1:4000 (CellSignaling). For western blot, secondary peroxidase-labeled anti-mouse and anti-rabbit IgG antibodies were from Bio-Rad Italia (Milan, Italy); for immunoprecipitation, goat anti-mouse IgG Alexa Fluor 488 and goat anti-rabbit IgG (H+L) Alexa Fluor 568 (ThermoFisher).

Methods

Cell cultures and treatments

SH-SY5Y human neuroblastoma cells and U373-MG astrocytoma cells were purchased from ATCC (Manassas, VA, USA). SH-SY5Y is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor; these cells have a population doubling time of 48 h (Biedler et al., 1978). U373 cells is a human glioblastoma astrocytoma derived from a malignant tumour by explant technique; the population doubling time is 36 h (Pontén and Macintyre, 1968). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal

Bovine Serum (FBS) and 40 µg/ml gentamicin at 37 °C in a humidified 5% CO_2 incubator. Confluent monolayers of SH-SY5Y and U373 cells were sub cultured by conventional trypsinization. For the experiments, $3x10^5$ or $2x10^6$ cells were seeded in 35 -or 100-mm tissue culture dishes, respectively, and grown up to 80% confluence for 18–24 h before treatments. Working solutions of Tat and gp120 were freshly prepared in culture medium from stock solutions stored at -80 °C. Where indicated, cells were treated with 200 ng/ml HIV-1 Tat recombinant protein or NMDA agonist (1 mM) in serum-free DMEM. For the pre-treatment experiments, either CHL (0.01 µM for 16 h) or MK-801 (10 µM for 2 h) or L-NAME (1mM for 30 min) added in DMEM supplemented with 10% FBS and 40 µg/ml gentamicin.

NHAs were cultured in ABM (Astrocyte Basal Medium) supplemented with recombinant human epidermal growth factor, insulin, ascorbic acid, GA-1000 (gentamicin sulfate, amphotericin-B), L-glutamine, and fetal bovine serum, at 37°C inT-25 tissue culture flasks gassed with an atmosphere of 95% air – 5% CO2. NHAs and ABM were purchased from Cambrex Bio Science (Milan,Italy). For experiments, NHAs were used between the third and the fourth passage.

To verify cell culture purity, NHA cells at the second passage were immunostained for the glial fibrillary acidic protein (GFAP), an intermediate filament protein that is highly specific for the astroglial lineage (data not shown). In particular, fixed cells were incubated for 1h at 37°C with a polyclonal anti-GFAP antibody diluted 1:500 in phosphate-buffered saline complemented with 3% bovine serum albumin (BSA),0.1%Tween20. After being washed, cells were incubated for 1h at 37 °C with a FITC-conjugated secondary antibody diluted 1:1000. Cells were then closed with a preserving fluorescence medium (Vectashield) and observed under a fluorescence microscope.

Embryonic Telencephalic Nai¨ve Apaf (ETNA) cells (Cozzolino et al., 2004) were gently gifted by Prof. Francesco Cecconi, from Danish Cancer Society Research Centre (Copenhagen, Denmark).

Co-Coltures SHSY5Y/U373 and SHSY5Y/U373-Tat co-cultures were performed. U373 cells and U373-Tat cells were seeded in 24-well plates ($3x10^5$) and SHSY5Y in cell culture insert (1.5×10^5).

Neuronal viability in co-cultures

The cellular viability in SHSY5Y/U373 and SHSY5Y/U373-Tat co-cultures were evaluated by the MTT assay. After the treatment, 10 % MTT (5 mg/ml of MTT dissolved in PBS) was added to medium for 4h at 37°C. After incubation, the MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) was added in equal volume of medium for 30 min at 37°C. The absorbance was read at 550 nm in the Victor 3V 1420 multilabel camper (Perkin Elmer).

Determination of SMO enzyme activity

The SMO polyamine oxidase activity was assayed using a modification of the chemiluminescence analysis reported by Wang et al. 2005 Luminol-dependent chemiluminescence was determined using a Lumat LB 9507 G&G Berthold luminometer. Luminol was prepared as a 100 mM stock solution in DMSO and diluted to 100 μM with H2O immediately before use. Cell extracts (1×106 cells/sample) were assayed in 83 mM glycine buffer (pH 8.3), 20 μg/ml horseradish peroxidase, 0.2 mM 2-bromoethylamine (catalase inhibitor), 15 μM deprenyl (copper-containing amine oxidase inhibitor), 0.15 μM clorgyline (mitochondrial oxidase inhibitor), and 500 μM Spm as substrate to determine SMO activity. All reagents, with the exception of substrate, were combined and incubated for 5 min at 37°C, then 5 nmol luminol was added and incubated again at 37 °C for 2 min. The sample was then transferred to the luminometer, Spm was added, and the resulting chemiluminescence was integrated over 40 s. Polyamine concentration was determined as described in Mates et al. 1992.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-qPCR)

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 NQO1 gene (fwd 5'- ATG TAT GAC AAA GGA CCC TTC C -3' rev 5'-TCC CTT GCA GAG AGT ACA TGG - 3'), CAT gene (fwd 5'-TCA GGT TTC TTT CTT GTT CAG-3' rev 5'-CTG GTC AGT CTT ATA ATG GAA TT -3'), SOD1 gene (fwd 5'-AGT AAT GGA CGA GTG AAG G-3' rev 5'- GGA TAG AGG ATT AAA GTG AGG A-3'), SOD2 gene (fwd 5'-AAT GGT GGT GGT CAT ATC A-3' rev CCC GTT CCT TAT TGA AAC C-3'), HO-1 gene (fwd 5'-CGG GCC AGC AAC AAA GTG-3' rev 5'-AGT GTA AGG ACC CAT CGG AGA A-3') and p62 (fwd 5'-GGGAAAGGGCTTGCACCGGG-3' rev 5'-CTGGCCACCCGAAGTGTCCG-3'). GAPDH mRNA (fwd 5'-TTG TTG CCA TCA ATG ACC C -3' rev 5'- CTT CCC GTT CTC AGC CTT G-3') was examined as the reference cellular transcript. PCR product quantification was calculated by applying the SYBR-Green method. Reactions were performed in a Rotor gene 6000 machine (Corbett research) using the following program: 45 cycles of 95 °C for 15 sec, 60 °C for 60 sec, 72 °C for 20 sec. 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-□□CT) method).

Analysis of gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to verify gp120 and Tat effects on iNOS and IL-1β transcriptional induction. For these experiments, $1.5x10^5$ cells were treated with several concentrations (1–100nM) of gp120 and (1-200 ng/ml) of Tat for 4h with or without the cPLA2 inhibitor AACOCF3 (75 mM). Total RNA was then purified and reverse transcribed into cDNA as previously reported in "Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-qPCR)" section. cDNA was amplified for the iNOS gene using iNOS-specific primers (Colasanti et al., 1995) and forIL-1β and glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) using specific primers, as described elsewhere (Geller et al., 1993). The mRNA for the constitutive GAPDH enzyme was examined as the reference cellular transcript. GAPDH mRNA amplification products were present at equivalent levels in all cell lysates. Estimates of the relative Inos or IL-1β mRNA amounts were obtained by dividing the area of the iNOS or IL-1β bands, respectively, by the area of the GAPDH band (Bio-Rad Multi-Analyst).

Preparation of nuclear 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 12000 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.

Preparation of 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 TEEN (10 Mm Tris HCl Ph 7.4, 1mM EGTA, 1mM EDTA, 150 mM NaCl, 1% triton X100, protease inhibitor cocktail) to the cell pellets.. After incubation for 20 min on ice and subsequent centrifugation at 14000 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.

Western blot analysis

Equal amounts (40 µg proteins/sample for Nrf2, 5 µg proteins/sample for SystemXc, 25 µg proteins/sample for p62, 21 µg proteins/sample for iNOS) of extracts were subjected to electrophoresis in an 8% polyacrylamide gel (Nrf2, SystemXc and p62) and 7.5% polyacrylamide gel (iNOS) and transferred to nitrocellulose membranes.

Membranes were blocked with 5% non-fat dry milk for 1 hour and incubated overnight at 4°C with specific antibody. Detection was performed using ECL Western blotting detection reagents (GE Healthcare, Milan, Italy).

Immunoprecipitation

$4x10^6$ cells were lysate as specified in "preparation of total extract" section with a TEEN buffer, which was added 0.5% Triton X100. for the pre-clearing step, 1mg of cell lysate was diluted in 500 µl and was added Protein A agarose beads (20 µl). After the incubation at 4°C for 30 minutes, the supernatant was transferred to a fresh tube. Next, primary antibody was added (2 µg/ml) to supernatant and incubated with gentle rocking t at 4°C for 4h. Next, protein A agarose beads (20 µl) were added and incubated with gentle rocking for 1h at 4°C. The pellets were washed tree times with 500 µl of RIPA buffer 1X with Triton (1 mM EGTA, 1mM EDTA, 10 mM NaH2PO4, 1.5 M NaCl, 0.1 % Triton X100) and one time with RIPA buffer 1X (1 mM EGTA, 1mM EDTA, 10 mM NaH2PO4, 1.5 M NaCl). The pellets were resuspended in 40 µl of sample buffer (250 mM Tris HCl ph 7.4, 40 % glycerol, 20% SDS, 0.01% bromophenol blue, 40 mM DTT). Vortex, then microcentrifuge for 30 seconds. Heat the sample to 95–100°C for 5-10 minutes and microcentrifuge for 1 minute at 14,000 rpm. Samples were loaded on SDS-PAGE gel (8–15%) and analyzed by Western blotting (see Western Blot analysis section).

ELISA-Based Measurement of Nrf2 Activity

The TransAM Nrf2 Kit (Active Motif, Vinci-Biochem, Firenze, Italy) was used to assay the DNA-binding activity of Nrf2 in the nuclear extracts. Ten micrograms of nuclear extracts from each sample in duplicate were incubated in a 96-well plate that was coated with oligonucleotide containing a consensus binding site (5'- GTCACAGTGACTCAGCAGAATCTG-3') for Nrf2. For competitive binding experiments, which measure the specificity of the assay, 10 µg of nuclear extracts was assayed in the presence of wild-type or mutated competitor oligonucleotides. After 1 h of incubation, the wells were washed and incubated with Nrf2 antibody (1:1000) for 1 h at room temperature without agitation. Afterwards, the wells were incubated with

HRP-conjugated antibody (1:1000) for 1 h at room temperature. Afterwards, a developing solution was added for 10 min. Finally, a stop solution was added into the wells, and the absorbance was read at 450 nm with a reference wavelength of 655 nm using a plate reader.

ELISA-Based Measurement of NF-kB Activity

To quantify NF-κB activation, a TransAm kit based on the ELISA method was used. The kit contained a 96-well plate to which oligonucleotides containing the NF-κB consensus binding site (50- GGGACTTTCC-30) had been immobilized. The activated NF-κB specifically bound to these oligonucleotides was detected by using an antibody directed against the NF-κB p65subunit.

Protein extracts from each specimen were incubated in a 96-well plate for 1h, and wells were then washed and incubated with NF-κB antibody(1:1000) for 1h at room temperature with- out agitation. Afterward, the wells were incubated with horse- radish peroxidase-conjugated antibody (1:1000) for 1h at room temperature and a developing solution was added for 10 min and protected from direct light. Finally, a stop solution was added into the wells and absorbance was read at 450 nm with a reference wavelength of 655nm.

Stable tranfection of U373 cells

To induce the Tat protein expression in astrocytoma cells, the pcDNA3.1 HIV-Tat expression vector produced in our lab as previously described in Capone et al., 2013, was used. To this purpose, 4 x 10^5 cells were seeded in 6 cm dishes and transfected with Lipofectamine 3000 (Invitrogen) and with 1 μg of DNA (pcDNA3.1 HIV-Tat and pcDNA3.1). 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 colture, we used 200 μg/ml of G418.

Trasfection of SH-SY5Y cells and fluorescence analysis

To evaluate the Golgi fragmentation in astrocytoma cells, the p-YFP Golgi vector was used. To this purpose, 4 x 10^5 cells were seeded in 6 cm dishes and transfected with Lipofectamine 3000 (Invitrogen) and with 1 μg of DNA (pYFP Golgi). After 24 h the transfected cells were washed two times with PBS, fixed with PFA 4% and washed with PBS. Cells were then closed with a preserving fluorescence medium (Vectashield) and observed under a fluorescence microscope.

Immunofluorescence analysis

1 x 10^5 SH-SY5Y cells were seeded on poli L-lysined cover glass. After suitable treatments, cells were washed in PBS and fixed in PFA 4%. The

permealization was performed in methanol for 10 min at -20°C. Cells were washed and incubated for 1h at RT with a blocking solution (5% FBS, 1% BSA in PBS). After cells were incubated overnight at 4°C with polyclonal anti-p62 antibody diluted 1:200 in blocking solution and with polyclonal anti-ubiquitine 1:500 in blocking solution. After being washed, cells were incubated for 1h at 37 °C with a Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate and with Goat anti-Mouse Secondary Antibody, Alexa Fluor® 488 conjugate diluted 1:1000. 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-tests. Differences are considered statistically significant at $p \leq 0.05$.

APPENDIX

Publications during Ph.D.:

Persichini T, **Mastrantonio R**, Del Matto S, Palomba L, Cantoni O, Colasanti M. Free Radic Biol Med. 2014 Sep;74:14-20. The role of arachidonic acid in the regulation of nitric oxide synthase isoforms by HIV gp120 protein in astroglial cells.

Mastrantonio R, Cervelli M, Pietropaoli S, Mariottini P, Colasanti M, Persichini T. HIV-Tat induces Nrf2/ARE pathway through NMDA receptor-elicited spermine oxidase activation in human neuroblastoma cells. PLoS ONE (in press)

Manuscript in preparation during Ph.D.:

Mastrantonio R, Pietropaoli S, Cervelli M, Mariottini P, Colasanti M, Persichini T. The role of nitric oxide in SMO-mediated activation of Nrf2 by HIV-Tat in human neuronal cells.

Mastrantonio R, D'Ezio V, Colasanti M, Persichini T. System Xc up-regulation by HIV-Tat in human astroglial cells.

ABBREVIATIONS INDEX

arachidonic acid **(AA);** American Academy of Neurology **(AAN);** acquired immunodeficiency syndrome **(AIDS);** Alzheimer disease **(AD);** Asymptomatic neurocognitive impairment **(ANI);** antioxidant response element **(ARE);** CNC homology 1/2 **(BACH(1/2));** blood brain barrier **(BBB);** broad complex–tramtrack–bric-a-brac **(BTB);** basic leucine zipper **(bZIP);** catalase **(CAT);** Chlorhexidine **(CHL);** cap'n'collar **(CNC)** central nervous system **(CNS);** cytosolic phospholipase A_2 **($cPLA_2$);** double glycine repeat **(DGR);** endothelial nitric oxide synthase **(eNOS);** excitatory amino acid transporters **($EAAT_S$);** glutamylcysteine ligase **(GCL);** glutathione peroxidase (**GPx);** g-glutamate–cysteine–glycine, glutathione **(GSH);** Oxidised glutathione (**GSSG**); glutathione S-transferase **(GST);** highly active antiretroviral therapy **(HAART);** HIV associated dementia **(HAD);** HIV-1 associated neurocognitive disorders **(HAND);** hepatocellular carcinoma **(HCC);** human immunodeficiency virus **(HIV);** heme oxigenase -1 **(HO-1);** hydrogen peroxide **(H_2O_2);** interleukin 1β **(IL-1β);** inducible nitric oxide synthase **(iNOS);** intervening region **(IVR);** Kelch-like ECH-associated protein-1 **(KEAP1);** L-Nitro-Arginine Methyl Ester **(L-NAME);** bacterial lipopolysaccharide **(LPS);** low density lipoprotein receptor related protein (**LRP);** long terminal repeats **(LTR);** MAF recognition element **(MARE);** Mallory bodies **(MB);** HIV-associated mild neurocognitive disorder **(MND);** Dizocilpine **(MK801);** N-acetyl-L-cysteine **(NAC);** NRF2-ECH homology **(NEH);** nuclear factor kappa-light-chain-enhancer of activated B cells **(NF-kB);** N-methyl-D-aspartate receptor **(NMDAR);** neuronal nitric oxide synthase **(nNOS);** nitric oxide **(NO);** NADP(H):quinone oxidoreductase 1 **(NQO1);** NF-E2 related factor-2 **(Nrf2);** Parkinson disease **(PD);** peroxiredoxin (**PRDX);** postsynaptic density protein-95 **(PSD95);** protein transduction domain **(PTD);** reactive nitrogen species **(RNS);** reactive oxygen species **(ROS);** spermine oxidase (**SMO**); superoxide dismutase (**SOD);** spermidine **(Spd);** spermine **(Spm);** sequestrosome 1 **(SQSTM1);** sulfiredoxin (**Srx);** transactivation responsive element **(TAR);** transactivator protein **(Tat);** thioredoxin reductase (**Txnrd);** ubiquitin-proteasome system **(UPS).**