Chapter 4

Involvement of P2X7R in Tat mediated direct and indirect neuronal damage: Implication in HIV neuropathogenesis

4.1. Introduction

Brain autopsy studies of HIV/AIDS patients demonstrate severe neuronal injury and apoptosis (Shi et al., 1996). Perhaps neuronal injury is more striking than the neuronal loss in HIV patients and closely correlates with the presence and severity of cognitive impairment in HIV-associated dementia. The neuronal injury majorly includes axonal disruption and dendritic and synaptic pruning resulting in dysfunctional neuronal circuits in HIV dementia. The synaptodendritic damage and neuronal loss primarily occur in the hippocampus, basal ganglia and frontal cortex leading to cognitive, motor and behavioral abnormalities (Adle-Biassette et al., 1997; Ellis et al., 2007). Interestingly, HIV infects macrophage, microglia and astrocytes but not neurons, although neurons are affected and die by apoptosis. Currently, there is no specific treatment for HAD, mainly as a clear understanding of how HIV infection causes neuronal injury and apoptosis remains elusive.

HIV infects primarily non-neuronal cells and it is, therefore, likely that the virus exerts its neurodegenerative effect via indirect mechanisms. The release of toxic viral proteins and neurotoxins from HIV-infected cells like microglia and astrocytes are the key mediators that indirectly but adversely affect the neurons (Minagar et al., 2002). Astrocytes serve as a potential viral reservoir in the CNS protecting the virus from antiretroviral attacks. However, in the case of rapid progression of HIV dementia, infected astrocytes create a microenvironment ensuing significant astrocytic apoptosis and the higher level of neurotoxins (Thompson et al., 2001). Viral protein gp120 and HIV-transactivator of transcription (Tat) are two major viral proteins that are released from infected cells and have been detected in the central nervous system of HIV-infected individuals (Hudson et al., 2000; Mocchetti et al., 2012; Johnson et al., 2013). Astrocytes being non-productively infected by the virus do not produce full virions. Infected astrocytes mainly express non-structural viral proteins such as Rev, Nef and Tat with limited expression of structural proteins like gp120 and gp41. Tat can be released from astrocytes upon activation (Nath, 2002). Once released, the extracellular Tat can directly be taken up by neurons causing excessive calcium influx, ROS

generation, mitochondrial dysfunction, dendritic loss, caspase activation and subsequent death of neurons at concentrations lower than those required to support viral replication (Kruman et al., 1998).

Viral protein Tat in astrocytes results in significant increase in glial fibrillary acidic protein (GFAP) expression, a cellular marker for astrocyte activation and astrocytosis. Tat expression also impairs glutamate uptake by astrocyte thus increasing extracellular glutamate concentration. Importantly, cell culture supernatants from Tat-expressing astrocytes have been shown to cause dramatic neuronal death (Zhou et al., 2004). These observations strongly suggest a direct link between Tat expression in astrocytes, astrocyte activation/dysfunction and subsequent neuronal death. In contrast to HIV-1 virus, Tat protein promotes the survival of infected astrocytes inducing neuronal loss at distant sites (Chauhan et al., 2003). Though above-mentioned studies demonstrate the role of Tat-expressing astrocytes in mediating indirect neuronal damage, the underlying cellular and molecular pathways are not fully elucidated and hence the study was initiated.

Increased expression and activation of purinergic receptor P2X7R have been shown to contribute to the pathology of Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, status epilepticus and spinal cord injury. The common disease mechanisms involving P2X7R mediated pathways are excessive calcium influx, astrocyte activation, glutamate release, oxidative stress, neuroinflammation and neuronal damage. For example, increased levels of P2X7 receptors are reported in the striatum of Huntington's disease mouse models resulting in altered calcium permeability in synaptic terminals and increased neuronal apoptosis in response to stimulation of P2X7 receptors (Diaz-Hernandez et al., 2009). P2X7R antagonist oxATP prevent neuronal apoptosis and protect against the damage in spinal cord injury (Peng et al., 2009). Very recently, purinergic receptors have been implicated in HIV-1 infection in macrophages (Seror et al., 2011; Hazleton et al., 2012). ATP released from HIV-infected macrophage has been shown to decrease the dendritic spine density on neurons by purinergic receptor-mediated modulation of

glutamatergic tone. Recent studies have also documented the role of purinergic receptor family (P2X) in Tat and morphine-related neuronal injury in rat striatal neuron-glia coculture (Tovar et al., 2013; Sorrell and Hauser, 2014). Till this study, detailed investigations into the role of the astrocytic purinergic receptor in HIV-1 mediated neuronal apoptosis were lacking. As astrocytes robustly express purinergic receptors, therefore, it is likely that activation of the purinergic receptor may cause astrocyte activation and subsequent neuronal apoptosis in the present scenario. In chapter 3, we provide compelling evidence suggesting that astrocytic P2X7R activation indeed contributes to Tat-induced neuroinflammation by enhancing release of monocyte chemoattractant protein MCP-1/CCL2. In this chapter, we further extended our study and tried to investigate how P2X7R activation in astrocytes contributes to Tat-induced indirect neuronal apoptosis. For our work, we utilized pure human neuron or astrocyte culture model. Treating the neurons either directly with Tat or with supernatants from Tat-treated astrocytes we focused on delineating the role of P2X7R receptors in Tat-induced neurotoxicity via direct and indirect mechanisms.

4.2. Material and methods

4.2.1. Materials

Opti-MEM, Lipofectamine RNA iMax, Fluo-3AM were purchased from Invitrogen (San Diego, CA, USA). Culture reagents, tetrazolium salt MTT, endoribonuclease-prepared siRNA (esiRNA) against human P2X7R and mission siRNA universal negative control 1 (scramble siRNA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 7'-Dichlorodihydrofluorescein Diacetate (DCFDA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye were purchased from Millipore (Billerica, USA), TUNEL kit was purchased from Roche (Mannheim, Germany) and antibody for β-III tubulin (Tuj-1) was purchased from Promega (Madison, USA). HRP conjugated secondary antibodies were purchased from Vector laboratories. Sources for all other reagents have been specified in 3.2.1.

4.2.2. Methods

4.2.2.1. Cell culture and treatments

4.2.2.1.1. Primary human astrocytes

Human astrocytes were differentiated from primary human NPCs as described before (3.2.2.2.2). Astrocytes were treated with BzATP (100 µM) for 24 hr for assessment of apoptosis. To study the effect of astrocyte conditioned media on neurons, astrocytes were treated with BzATP (100 µM) or Tat (100 ng/ml) for 24 hr with or without pretreatment with oxATP (100 µM), A438079 (20 µM) or BBG (1 µM) and the supernatant was collected. For knockdown experiments, astrocytes were transfected with scrambled or P2X7R siRNA using lipofectamine RNAiMAX for 24 hr as described in 4.2.2.4 followed by 100 ng/ml Tat treatment for 24 hr. After 24 hr, the cell supernatant was collected and immediately used for treating pure neurons grown in parallel cultures.

4.2.2.1.2. Primary human neurons

The hNPCs were differentiated into neuronal lineage using neuronal medium in which the mitogenic factors bFGF and EGF of neural precursor cell medium were replaced with BDNF (10 ng/ml) and PDGF (10 ng/ml); the rest of the components of progenitor media remained same. Differentiation of hNPCs into neuronal cells was continued for 3 weeks replacing half of neuronal media with fresh media every alternating day. The neurons so differentiated were > 99% pure, as determined by immunocytochemistry using the anti-β-III tubulin (Tuj-1) antibody (Promega, Madison, USA), a neuronal marker. These neurons were further used for studies described in this chapter.

4.2.2.1.2.1. Direct treatment of neurons

To determine the direct effect of BzATP or Tat on neurons, neurons were cultured on poly-D-lysine coated 8 well chamber slide at a density of 10,000 cells/well. After 24 hr BzATP (100 µM) or Tat (100 ng/ml) was administered directly to the neuronal culture. To assess the effect of various P2X7R antagonists, cells were pre-treated for 1 hr with oxATP (100 µM), A438079 (20 µM) or BBG (1 µM) prior to exposure with BzATP or Tat. Assessment of apoptotic death by TUNEL assay was done 24 hr after exposure to Tat or BzATP.

4.2.2.1.2.2. Exposure of neurons to astrocyte conditioned media (ACM)

To study the indirect effect of P2X7R activation or Tat on neurons, neurons were cultured on poly-D-lysine coated 8 well chamber slide or 24 well culture plates at a density of 10,000 & 30,000 cells/well, respectively. After 24 hr, full neuronal media was aspirated. Neurons were now treated with supernatants collected from treated astrocytes as described in section 4.2.2.1.1. For each treatment, fresh neuronal media and astrocyte conditioned media (supernatant) were added in 1:1 for specified durations. Assessment of indirect neuronal apoptosis by TUNEL assay was performed 24 hr after supernatant treatment. Reactive oxygen species formation was assessed after 3hr and mitochondrial membrane depolarization after 12 hr of supernatant treatment with the subsequent addition of DCFDA and JC-1, respectively.

4.2.2.2. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay

In order to check the toxicity of different doses of P2X7R agonist BzATP on cell viability, MTT assay was performed on astrocytes. This assay is based on the reduction of the tetrazolium salt MTT (Sigma-Aldrich, Saint Louis, MO, USA) into a crystalline blue formazan product by the cellular oxidoreductase, present in living cells. The amount of formazan produced is considered as a reliable representation of viable cell number. Briefly, cells were seeded in 96 well plates at a density of 10,000 cells/well and grown in culture for 24 hr. Cells were then exposed to two different doses (100 and

200 µM) of BzATP. After 24 hr, the culture medium was removed and replaced with fresh culture medium containing MTT (0.5 mg/ml). After 4 hr incubation at 37^{o}C, this solution was removed, and the resulting blue formazan was solubilized in the solution containing 20% sodium dodecyl sulfate (SDS) and 50% dimethyl formamide and optical density was read at 570 nm using microplate reader (Benchmark plus, Biorad, Oregon, United States). Percentage MTT reduction was calculated for each treatment group, control was considered as 100 % viable and test groups were compared with control samples.

4.2.2.3. Cell toxicity assay or Terminal Deoxynucleotidyl transferase-Mediated dUTP Nick End Labelling (TUNEL) Assay

In both the direct and indirect toxicity measurements, after 24 hr treatment, neurons were fixed with 4% PFA for 20 min. Cells were blocked and permeabilized with 4% BSA containing 0.5% Triton-X-100 for 30 min and neurons undergoing apoptosis were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay using *In Situ* Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany), as per manufacturer's protocol. Neurons were labelled with the neuronal marker using anti-β-III tubulin antibody (Tuj-1) and nuclei were stained with DAPI. Images were acquired from at least 7 random fields using a Zeiss Axioplan microscope (Carl Zeiss Company, Heidenheim, Germany) with the charge-coupled device. Apoptotic neurons were expressed as percentage TUNEL positive cells/DAPI.

4.2.2.4. P2X7R knockdown by small interfering RNA (siRNA)

siRNA mediated knockdown of P2X7R was performed in primary human astrocytes using Lipofectamine RNAiMAX (Life Technologies, Invitrogen, San Diego, CA, USA). 1.6pmol of esiRNA (endoribonuclease-prepared siRNA) against human P2X7R or MISSION siRNA universal negative control (Sigma-Aldrich, Saint Louis, MO, USA) were used and transfection was carried out in 8 well chamber slides according to the manufacturer's protocol. Astrocytes were harvested 24 hr following transfection

with siRNA. The specific silencing of P2X7R was confirmed by Western blot analysis where astrocytes were treated with 4, 8 and 16 pmole of P2X7 siRNA for 24 hr in T-25 flasks. The, 16 pmole of concentration was selected for further experiments as it showed the highest level of P2X7R knockdown and was scaled down 10 times and 4 times accordingly for experiments that were carried out in 8 well chamber slides and 24 well plate respectively.

4.2.2.5. Neuronal mitochondrial membrane depolarization measurement

After treatment of neurons with astrocyte conditioned media for 24 hr, neurons were incubated with JC-1 dye at a concentration of 1 µg/ml. The fluorescence emission pattern of JC-1 was assessed 10 min after incubation using a fluorescent microscope (Zeiss, Heidenheim, Germany) in both red and green portions of the spectrum, and images of neurons were captured using a high-resolution with charge-coupled device camera. For quantitation of JC-1 red and green aggregates, parallel cultures of neurons were treated under similar conditions. Neurons were detached and JC-1 dye was added to the cell suspension. Cell suspensions were then read with fluorimeter using excitation wavelength 490 nm and emission wavelength at 530 and 590 nm using a spectrophotometer (Tecan, Infinite 200 pro Männedorf, Switzerland), and ratios of red-to-green (590/530 nm) optical density were calculated. The reduction in red to green ratio indicated mitochondrial depolarization. Similar experimental procedure was also applied for measurement of mitochondrial membrane depolarization in cultured astrocytes.

4.2.2.6. Measurement of Reactive Oxygen Species (ROS)

To monitor the level of intracellular reactive oxygen species (ROS) in astrocytes or neurons subjected to exposure to astrocyte conditioned media, we used the cell-permeable, nonpolar, H_2O_2-sensitive probe 5 (and 6)-chlromethyl-2', 7'-dichlorodihydro fluorescein diacetate (DCFDA) as described previously (Mishra et al., 2011). Control and treated astrocytes or neuronal cultures were incubated with culture

media containing 5 µM DCFDA for 1 hr at 37°C, washed twice with PBS and lysed in lysis buffer (10 mM Tris pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM Na3VO4, 0.5% NP-40 and 1% Triton X-100). The lysate was centrifuged at 10,000 g for 15 min. A 10-µl aliquot of supernatant was mixed with 90 µl of PBS in a 96-well black plate and fluorescence was measured using Tecan Infinite 200 pro spectrophotometer (Männedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The readings obtained were normalized with the amount of protein in each sample. Data are expressed as a percentage of control cultures.

4.2.2.7. Western blotting

After treatments, protein were isolated from human astrocytes and Western blotting was performed (as described in 3.2.2.8) for determining the decrease in expression of P2X7R after knockdown with different doses of specific human P2X7RsiRNA. Briefly, the blots were incubated overnight at 4^{o}C with rabbit P2X7R antibody (1:1000). After incubating with HRP labelled anti-rabbit secondary antibody (1:3000) for 1 hr, the signal was detected using chemiluminescence reagent (Millipore, Billerica, USA). Images were captured using Chemi Genius Bioimaging System (Syngene, Cambridge, UK) using Gene Snap software. Blots were stripped for 30 min at 50^{o}C in stripping buffer which contained 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS and 100 mmol/L β-mercaptoethanol. The blots were then reprobed with anti-β-tubulin (1:3000) as loading control. Blot images were analyzed using Image J software (NIH, USA) and percentage change were calculated after normalization with respective β-tubulin, taking control as 100%.

4.2.2.8. Intracellular calcium $[Ca^{2+}]_i$ measurement

Increase in intracellular calcium upon P2X7R activation in neurons was analyzed using time-lapse calcium imaging as described in section 3.2.2.3. Briefly, neurons were seeded in PDL-coated 8 well chamber slide at density of 10,000 cells/well. Cells were

loaded with fluo-3 AM. The baseline intensity of neurons was recorded for 1 min in HEPES buffer and then 100 µM BzATP was added to static bath solution. The fluorescence intensity was measured for additional 4 minutes after BzATP stimulation. Fields containing 50-60 cells were randomly selected for imaging. Fluorescence intensity in individual astrocyte was measured by selecting region of interest (ROI). The data acquisition and analysis was done with NIS elements AR software 3.2.64 bit. The change in fluorescence intensity of fluo-3 within each astrocyte was plotted on Y axis with respect to time (in sec) on X-axis.

4.2.2.9. Statistical Analysis

Experiments for each condition were repeated three to five times with an exact number of experiments mentioned under legends to the respective figure. Results were analyzed and represented as mean ± standard deviation (SD). Statistical significance between groups was calculated using student-'t' test. All values of $p < 0.05$ were taken as significant.

4.3. Results

4.3.1. Activation of P2X7R does not exert any toxic effect on astrocytes

We first checked whether activation of P2X7R had any toxic effect on astrocytes. As shown in Fig. 4.1, MTT assay analysis suggests that P2X7R activation did not lead to astrocyte apoptosis upon exposure to two different doses of BzATP (100 and 200 µM) for 24 hr. In fact, we found an increase in cell viability with both the doses (116±14 and 128±21 respectively) although not significant. The increase in cell viability might be due to increase in astrocytic proliferation.

4.3.2. Human neurons express functional P2X7R

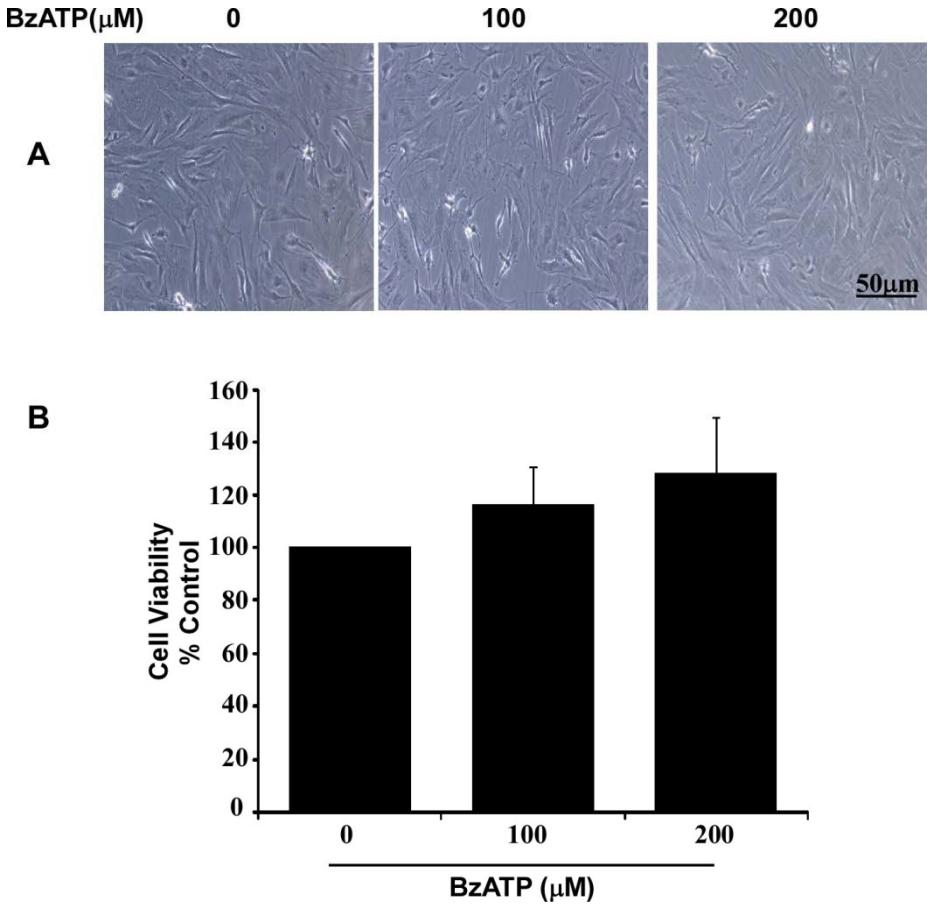

Figure 4.1. Effect of P2X7R activation on the viability of cultured human astrocytes. To assess the effect of P2X7R activation on astrocytes, cells were serum starved for 3 hrs and then treated with two different doses (100 and 200 μM) of BzATP for additional 24 hr. **(A)** Phase contrast images of cells were captured to visualize the morphology of cells. **(B)** Cell viability of astrocytes was assessed by MTT assay. BzATP did not cause astrocyte apoptosis, in fact, there was an increase in cell viability upon exposure with BzATP although the increase was not significant.

As shown by RT-PCR and Western blot analysis (Fig.3.2., Chapter 3), P2X7Rs are present in human neurons also, although at a lower level as compared to astrocytes. Furthermore, to assess whether P2X7Rs present on cultured human neurons are functional, we evaluated the alteration in intracellular calcium upon stimulation of neurons with 100 µM BzATP. We found a significant increase in fluorescence intensity of Fluo-3 loaded cells upon stimulation with BzATP (Fig. 4.2.) and similar to astrocytes the increase in fluorescence intensity in neurons remained sustained over time. This suggests that P2X7R present on neurons are indeed functional.

4.3.3. Direct activation of P2X7R on neuron leads to neuronal death

P2X7R activation did not exert any toxic effect on cultured astrocytes as shown in figure 4.1. We further checked whether the direct activation of P2X7R on primary culture of human neuron has any effect on neuronal survival. We observed significant neuronal apoptosis after exposure of neurons to P2X7 receptor agonist BzATP for 24 hr. Results from TUNEL assay revealed that approximately 40% of human neurons undergo apoptosis upon exposure of cells with BzATP (100 µM) as compared to control cells. To further confirm if the neuronal damage is due to stimulation of P2X7Rs, we pharmacologically blocked P2X7R with oxATP pre-treatment. The deleterious effect of P2X7R stimulation was significantly attenuated in the presence of oxATP, indicating that P2X7R activation is indeed toxic to neurons (Fig. 4.3.A & B).

4.3.4. P2X7R activation on astrocytes mediates indirect neuronal death

We also performed few experiments to assess astrocyte-mediated neuronal death or indirect death pathway using TUNEL assay. P2X7R mediated cell apoptosis was studied in neurons that were exposed to conditioned media collected from astrocytes after 24 hr treatment with BzATP in the presence or absence of P2X7R antagonist, oxATP. We observed that following BzATP treatment to astrocytes, the conditioned media induced significant neuronal death. However, the neuronal apoptosis was reduced up to half (15 ± 4%) when conditioned media from astrocytes treated with

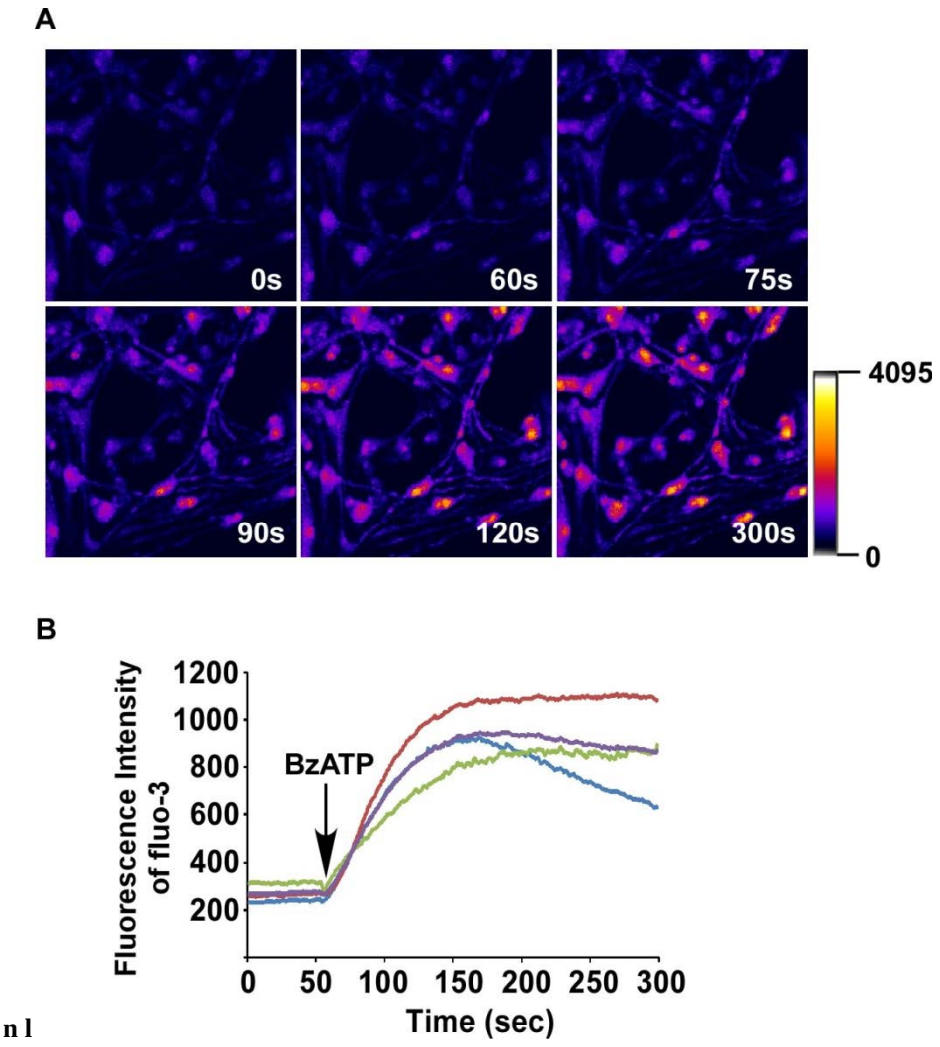

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Figure 4.2. BzATP induced [Ca2+]i response in cultured human neurons. (A) Representative pseudocolor images of Fluo-3 loaded neurons before and after stimulation with 100 µM BzATP at indicated time points. The color scale indicates linear pseudocolor representation of fluorescence intensity ranging from 0 to 4095 for the images. **(B)** Represents the temporal plot of increase in fluorescence intensity of fluo-3 recorded from four different neurons. Baseline fluorescence intensity in neurons were recorded for initial 60 sec and then 100 µM BzATP was added to static bath solution (indicated by the black arrow in the graph) and recordings were performed for 5 min. The experiment was repeated three times and similar temporal pattern of increase in fluorescence intensity in neurons was observed in each case.

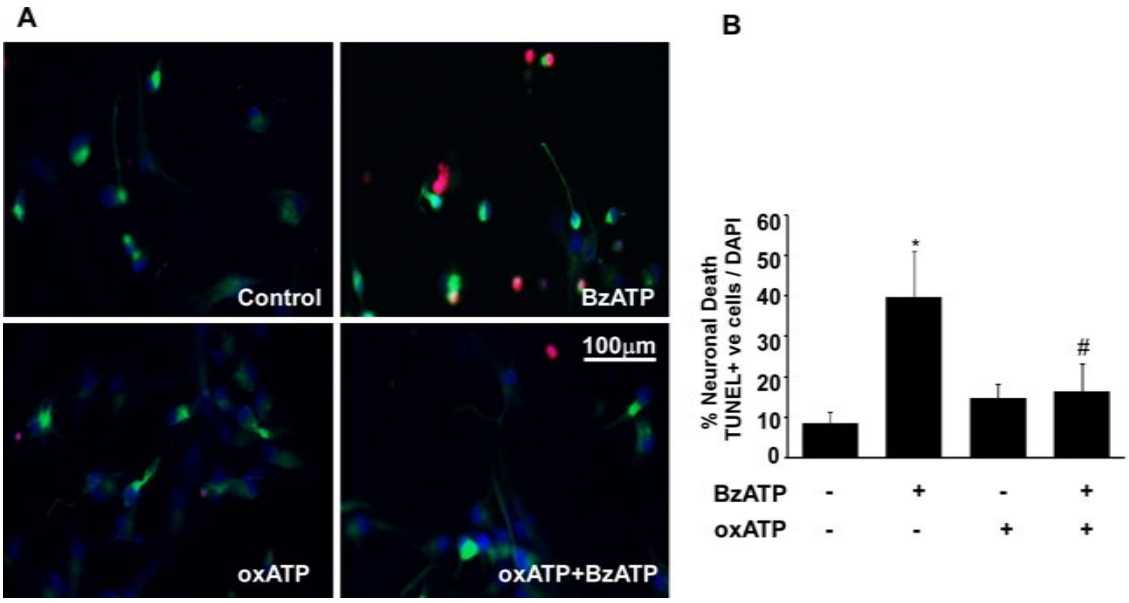

Figure 4.3. Activation of P2X7Rs on the neurons leads to neuronal apoptosis. (A) Neurons were treated with 100 µM BzATP either alone or in presence of 100 µM oxATP for 24 hr and neuronal apoptosis was detected by TUNEL assay, any red/pink nuclei indicated apoptotic cells. Neurons were immunostained with β III-tubulin, a neuronal marker and labelled with Alexa fluor 488 secondary antibody (green). Cell nuclei were stained with DAPI (blue). **(B)** Represents quantitative assessment of images shown in (A) as percentage neuronal death, determined by counting the number of TUNEL positive (red/pink) and DAPI positive cells (blue) and taking the ratio of TUNEL +ve cells/DAPI for each treatment. Pre-treatment with oxATP significantly reduced BzATP induced neuronal apoptosis indicating P2X7R involvement. The values represent the mean $\pm$ SD from five experiments performed on separate days. * indicates $p<0.05$ as compared to control, # indicate $p<0.05$ as compared to BzATP alone. Scale bar denotes 100 µm.

BzATP was used on neurons (Fig. 4.4.A & B) as compared to direct neuronal death on neuronal P2X7R activation.

4.3.5. Antagonist of P2X7R prevents direct and indirect neuronal death induced upon Tat treatment

To investigate the link between P2X7R activation and Tat-mediated neuronal damage, primary cultures of neurons were directly exposed to Tat in the presence or absence of various P2X7R antagonists. Direct exposure of primary neurons to Tat resulted in the significant death of neurons (36% ± 6%) after 24 hr as compared to control. Tat-induced neuronal death was abrogated in the presence of P2X7R inhibitors oxATP, BBG and A438079 (Fig. 4.5.), suggesting P2X7R is pivotal to cell death. Furthermore, to mimic the indirect or astrocyte-mediated neuronal death, astrocytes were exposed to HIV-1 Tat in presence or absence of P2X7R antagonists (oxATP, BBG and A438079), astrocytic media was collected 24 hr post treatments and immediately applied to neuronal cultures (Fig.4.6.). It was observed that pre-treatment of astrocytes with P2X7R antagonists prevented astrocyte-mediated Tat-induced neuronal damage, thereby confirming the involvement of P2X7R in Tat-induced indirect neurotoxicity.

4.3.6. P2X7R knockdown on astrocytes attenuates the Tat-mediated indirect neuronal apoptosis

Given that the chemical inhibitors are often non-specific, we employed a specific approach and used siRNA against human P2X7R to further ascertain the involvement of the astrocytic P2X7R in mediating the Tat-induced neuronal apoptosis. To ask, if the knockdown of P2X7R on astrocytes affected apoptosis in neurons, we transfected the astrocytes with human P2X7R siRNA as described in section 4.2.2.4. Transfection with the hP2X7R siRNA (16 pmole) significantly reduced the expression of P2X7R protein in the primary human astrocytes up to 50 ± 4.7 % as observed using Western blot (Fig. 4.7.A & B). The astrocytes transfected with the hP2X7R siRNA or scrambled siRNA were cultured in parallel and the conditioned media were collected 24 hr following the

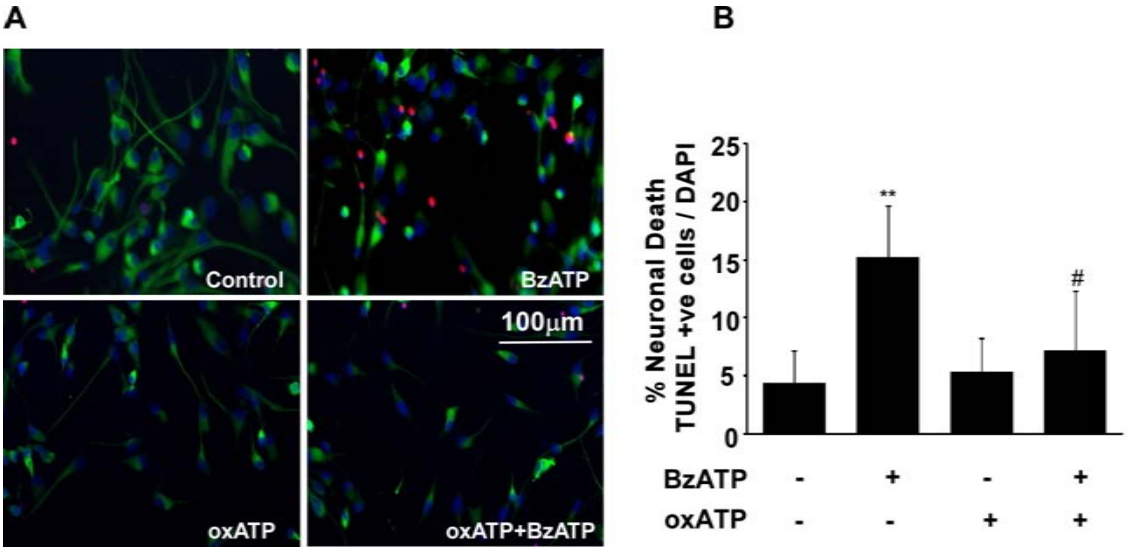

Figure 4.4. P2X7R activation on astrocytes leads to indirect neuronal damage. (A) Neurons were treated with conditioned media collected after treatment of astrocytes with 100 µM BzATP either alone or in the presence of 100 µM oxATP for 24 hr. Neurons undergoing apoptosis after 24 hr treatment with astrocyte conditioned media were identified using highly sensitive TUNEL assay. TUNEL positive cells were shown in red/pink. Neurons were immunostained with β III-tubulin and labelled with Alexa fluor 488 secondary antibody (green). Cell nuclei were stained with DAPI (blue). **(B)** Represents quantitative assessment of images shown in (A). Percentage neuronal death was determined by counting the number of TUNEL positive (red/pink) and DAPI positive cells (blue) from images shown in (A) and taking the ratio of TUNEL +ve cells/DAPI for each treatment. Pre-treatment of astrocytes with oxATP significantly inhibited BzATP induced indirect neuronal toxicity. Data represents mean $\pm$ SD from four experiments performed on separate days. ** indicates $p<0.005$ as compared to control, # indicates $p<0.05$ as compared to BzATP alone. Scale bar denotes 100 µm.

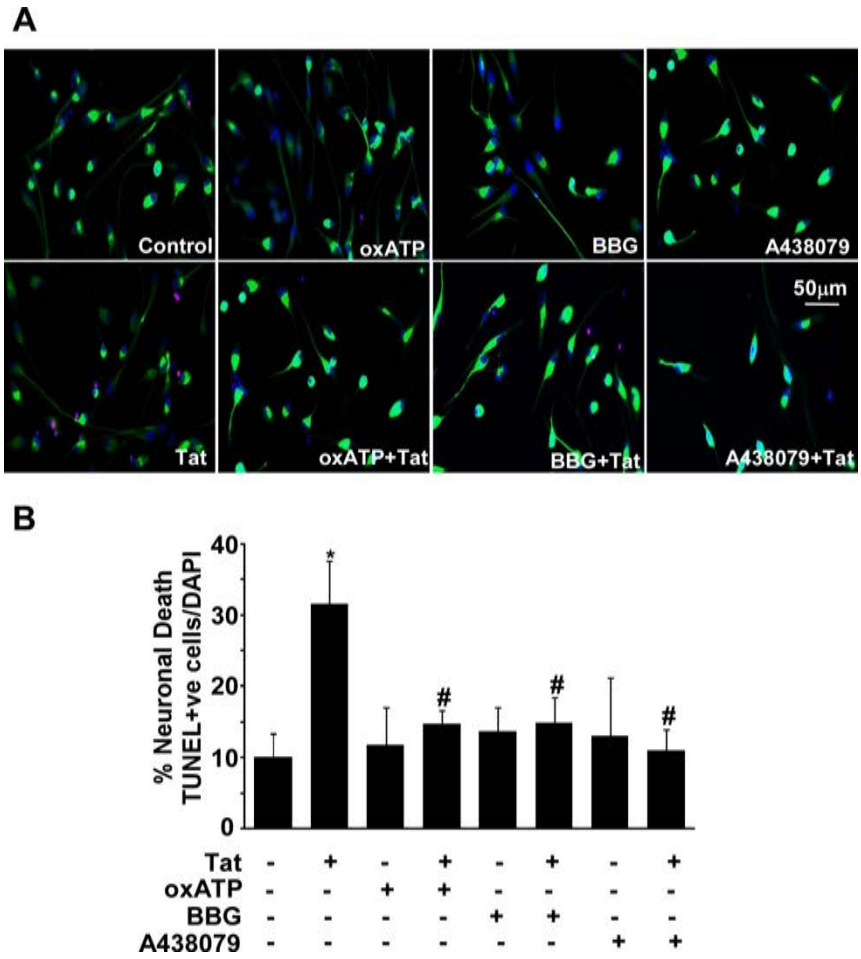

Figure 4.5. P2X7Rs are involved in Tat-mediated direct neuronal death. (A) To determine the involvement of P2X7R in Tat-induced direct neurotoxicity, cultured neurons were treated with different P2X7R antagonists oxATP (100 µM), BBG (1 µM) and A438079 (20 µM) for 1hr, prior to the addition of 100 ng/ml Tat. After 24 hr, apoptosis was detected with TUNEL assay. TUNEL positive cells were shown in pink. Neurons were immunostained with β III-tubulin and labelled with Alexa fluor 488 secondary antibody (green). Cell nuclei were stained with DAPI (blue). **(B)** Represents quantitative assessment of images shown in (A). Percentage neuronal death was determined taking the ratio of TUNEL +ve cells/DAPI for each treatment and percentage neuronal death was quantified. All P2X7R antagonists significantly inhibited Tat-induced neuronal death suggesting the involvement of P2X7R. The values represent the mean $\pm$ SD from three experiments performed on separate days. * indicates $p<0.05$ as compared to control, # indicate $p<0.05$ as compared to Tat alone. Scale bar denotes 50µm.

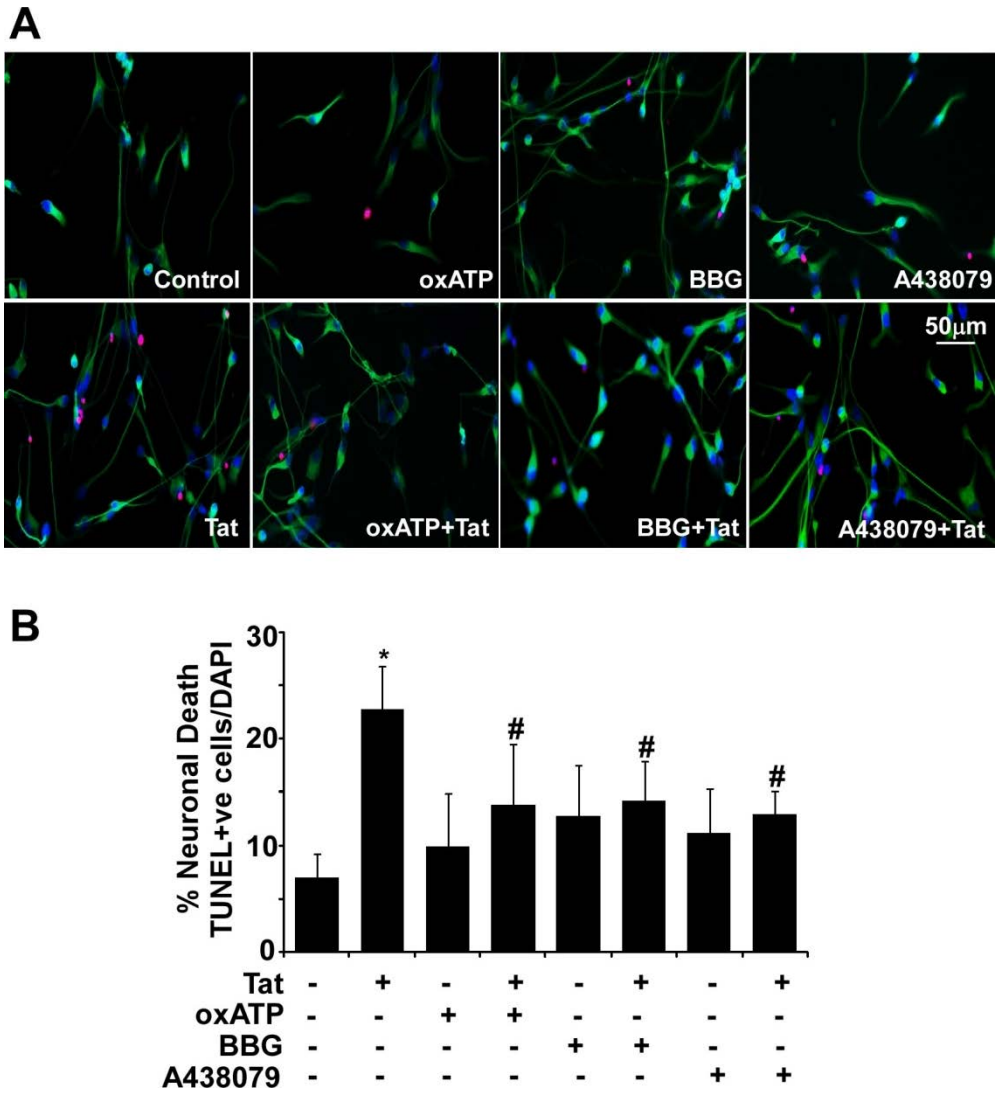

Figure 4.6. P2X7R activation on astrocytes leads to Tat-mediated indirect neuronal damage. Neurons were treated with conditioned media collected after treatment of astrocytes with Tat alone or in the presence of various P2X7R antagonists for 24 hr. All the antagonists were given 1hr prior to exposure with Tat. Neuronal death is assessed using TUNEL assay. **(A)** TUNEL positive cells under different treatment conditions were shown in red/pink. Neurons were immunostained with β III-tubulin and labelled with Alexa fluor 488 secondary antibody (green). Cell nuclei were stained with DAPI (blue). **(B)** Represents quantitative assessment of images shown in (A). Percentage neuronal death was determined by taking the ratio of TUNEL +ve cells/DAPI for each treatment. The values represent the mean $\pm$ SD of at least 3 experiments performed on separate days. * indicates $p<0.05$ as compared to controls, while # represents $p<0.05$ as compared to Tat.

exposure to Tat. The neurons were exposed to the conditioned media for 24 hr and the neuronal apoptosis was determined using the TUNEL assay. A quantitative assessment of the TUNEL positive cells from the respective treatments revealed that the P2X7R knockdown in astrocytes resulted in a significant attenuation of Tat-induced neuronal apoptosis (from 28 ± 6% to 6.88 ± 6.1%), strengthening our findings that P2X7R present on the astrocytes mediates the Tat-induced indirect neuronal death (Fig. 4.7.C & D).

4.3.7. P2X7R knockdown on astrocytes averts Tat-induced indirect oxidative stress in human neurons

Several studies in the past have confirmed that direct treatment of Tat to neurons lead to excessive calcium influx, reactive oxygen species (ROS) generation, and subsequent neuronal death (Kruman et al., 1998; Mishra et al., 2008). Tat protein also induces neuronal death via indirect pathway (Chauhan et al., 2003; Zhou et al., 2004). In accordance with the existing literature, our studies also demonstrated significant indirect neuronal apoptosis mediated via astrocytes. Additionally, we have shown that Tat-induced indirect neuronal death is prevented by inhibiting P2X7R activation or by P2X7R knockdown in astrocytes. To further investigate whether P2X7R knockdown also negatively affect other Tat-mediated pathways that may be involved in Tat-induced indirect neuronal damage, we measured ROS production in neurons exposed to astrocyte conditioned media. We observed a substantial augmentation in ROS generation in neurons after exposure with supernatant from Tat-treated astrocytes. P2X7R knockdown on astrocytes does not induce ROS generation in neurons as such; rather it prevented generation of ROS due to Tat treatment (Fig.4.8.B). We also performed similar experiments on astrocytes where astrocytes were treated with P2X7R siRNA or scrambled siRNA for 24 hr and ROS generation was measured 3 hr after Tat treatment. Significant increase in ROS was observed in Tat-treated cells as compared to control, although, the increase was lesser than that in Tat-ACM treated neurons. Contrary to neuron, P2X7R knockdown does not reduce Tat-induced ROS in astrocytes

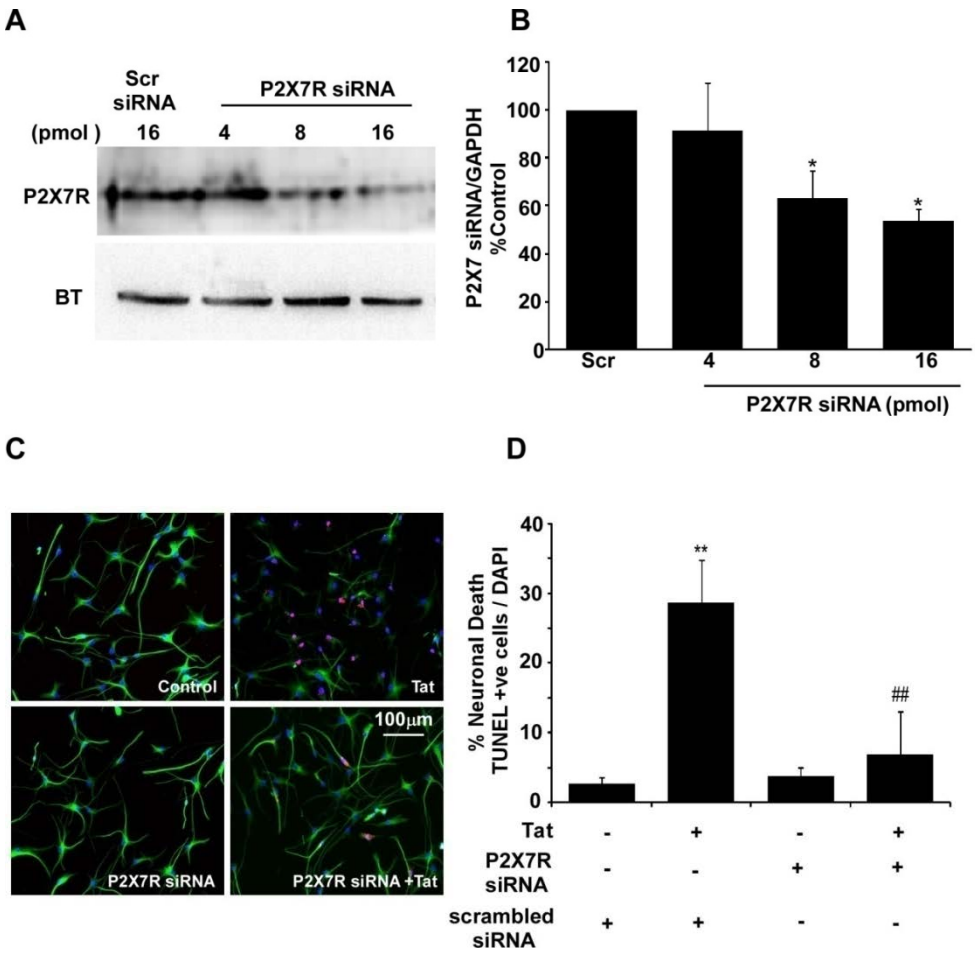

Figure 4.7. Knockdown of P2X7R on astrocytes decrease Tat-induced indirect neuronal death. (A) Astrocytes were transfected with scrambled siRNA (16pmol, control) or different doses of P2X7R siRNA (4, 8 and 16 pmole) for 24 hr and the silencing of P2X7R is determined by Western blotting. **(B)** Quantitative analysis of blot shown in (A) indicates a significant decrease with 8 pmol and 16 pmol P2X7siRNA compared to scrambled siRNA. **(C)** For apoptotic assay astrocytes were seeded in 8well chamber slide and transfected with 1.6 pmol of scrambled or P2X7 siRNA for 24 hr and then kept for additional 24 hr in presence or absence of 100 ng/ml Tat. Neuronal media was half replaced with conditioned media collected from transfected astrocytes and neuronal death was observed using TUNEL assay. **(D)** Represents quantitative assessment of percentage neuronal death of images shown in (C). Quantitative data represents effect of P2X7R knockdown on astrocytes mediated neuronal death after 24 hr Tat treatment as assessed using TUNEL assay. Silencing P2X7R in astrocytes significantly decreased Tat-induced indirect neuronal apoptosis. The values represent the mean $\pm$ SD of at least 3 experiments performed on separate days. * and ** indicates $p<0.05$ and 0.005, respectively, as compared to respective controls, while ## represents $p<0.005$ as compared to treatment with Tat alone. Scale bar denotes 100µm.

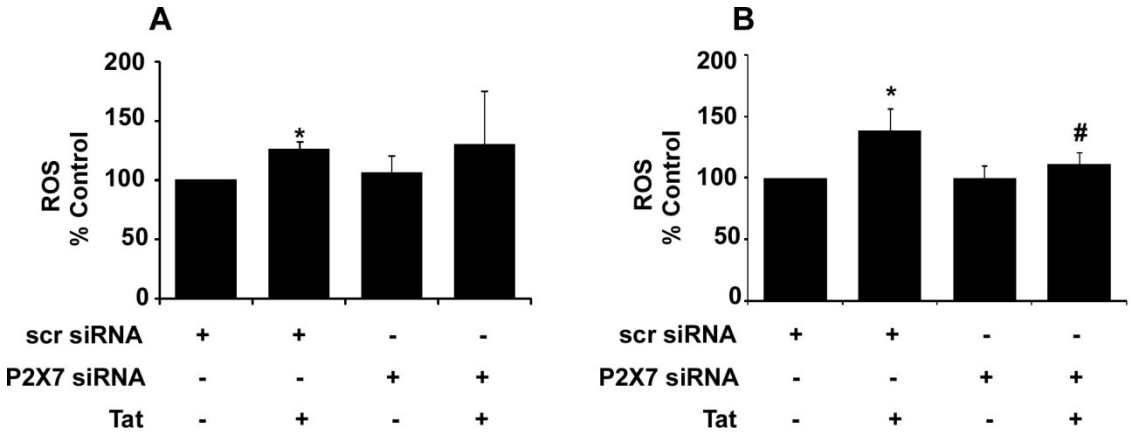

Figure 4.8. P2X7R knockdown in astrocytes prevents Tat-induced indirect reactive oxygen species formation in human neurons with no effect on ROS formation in astrocytes. Human astrocytes were treated with 1.6 pmol of scrambled siRNA (control) or hP2X7R siRNA for 24 hr and further exposed to 100 ng/ml Tat for additional 3 hr for measurement of ROS in astrocytes. For ROS measurement in cultured neurons treated with astrocyte conditioned media (ACM), Tat treatment was given for 24 hr. following P2X7R knockdown. After 24hr, astrocyte conditioned media was collected and immediately replaced with half of neuronal media in parallel cultures of neurons. After 3 hr of treatment with ACM, oxidative stress in neurons or astrocytes was determined fluorimetrically by DCFDA oxidation assay. **(A)** Quantitative assessment indicates significant elevation in oxidative stress levels (ROS) in Tat treated astrocytes as compared to control. P2X7R knockdown showed no effect on Tat-induced ROS generation in astrocytes. **(B)** Similar to astrocytes significant ROS generation was also observed in neurons treated with Tat-ACM. Tat-ACM induced oxidative stress was significantly reduced in neurons upon treatment with Tat-ACM from P2X7R knockdown astrocytes suggesting that P2X7R knockdown on astrocytes reduces the generation of oxidative stress in neurons in response to Tat exposure while having no effect on Tat treated astrocytes. Data represents mean ± SD from 4 independent experiments. * $p<0.05$ as compared to respective control and # represents $p<0.05$ as compared to Tat-ACM treated neurons.

(Fig.4.8.A) suggesting other mechanisms might be involved in Tat-mediated ROS generation in astrocytes.

4.3.8. Tat-induced mitochondrial membrane depolarization is rescued by P2X7R knockdown in astrocytes

Tat is known to cause indirect neuronal death upon astrocyte activation. It also leads to depolarization of mitochondrial membrane when exposed to astrocyte conditioned media (Mishra et al., 2008). Significant abrogation of Tat-induced indirect neuronal death by P2X7R knockdown on astrocytes prompted us to examine the neuronal mitochondrial membrane depolarization under similar treatment conditions used for apoptosis. To assess the mitochondrial membrane potential, we used JC-1 dye. JC-1 is a novel cationic dye that accumulates in the mitochondria. It is used for detecting mitochondrial depolarization occurring in the early stages of apoptosis. We added JC-1 dye to human neuronal cultures after they were exposed to conditioned media from Tat-treated astrocytes for 12 hr. We found that exposure to Tat resulted in significant depolarization of the mitochondrial membrane as seen by the substantial decrease in the red/green fluorescence, clearly indicating that Tat treatments induce cell death by mitochondrial membrane depolarization and possibly resulting in the release of cytochrome *c* from mitochondria. However, treatment of neuronal cultures with conditioned media from Tat-treated astrocytes after P2X7R knockdown, prevented the increase in depolarization of the mitochondrial membrane of the neuron as shown in Figure 4.10. We also observed the change in mitochondrial membrane depolarization of astrocytes under similar treatment conditions and found no significant change (Fig.4.9.) with Tat. This observation further supports the earlier reports that Tat mediates astrocyte activation, generates oxidative stress in astrocytes but not exerts the severe toxic effect on astrocytes.

4.4. Discussion

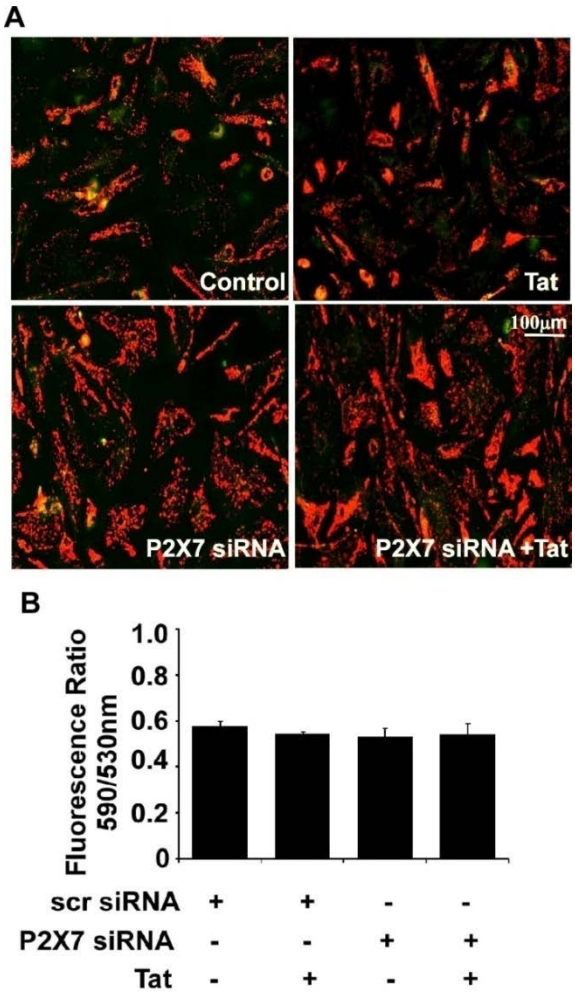

Figure 4.9. Tat and P2X7R knockdown has no effect on mitochondrial membrane depolarization in astrocytes: (A) Human astrocytes were treated with 1.6pmol scrambled siRNA (control) or P2X7R siRNA for 24 hr and then exposed with 100 ng/ml of Tat. After 12 hr, mitochondrial membrane depolarization was studied by JC-1 dye assay. No change in mitochondrial membrane depolarization was observed with Tat in astrocytes pretreated with scrambled or P2X7R siRNA. **(B)** From parallel experiments, quantitative assessment of mitochondrial membrane depolarization was done in human astrocytes spectrofluorimeter and the ratio of JC-1 aggregates/monomers (590/530nm) was calculated and represented. Data represents mean ± SD from 3 independent experiments.

The current study offers novel insights into astrocyte-mediated neuronal damage via a ligand-gated purinergic receptor, P2X7 receptor (P2X7R), following exposure to HIV-1 viral protein Tat. Using human astrocyte and neuron cell culture model our findings also unravel possible cellular and molecular events that may culminate into neuronal apoptosis. These observations have far-reaching clinical implications in events that occur subsequent to trafficking of Human Immunodeficiency Virus (HIV) to brain that leads to devastating neurological consequences in most HIV/AIDS patients that are termed as HIV-associated neurocognitive disorders (HAND). Recent advances in antiretroviral therapy have limited the systemic viral replication quite effectively, but complete eradication of the virus from patient remains a challenge till date. Brain serves as a sanctuary or "safe haven" for HIV-1 as most cART drugs have poor penetration in CNS (Churchill and Nath, 2013). Failures in complete removal of the HIV virus from sites of latent infections in the brain is the biggest stumbling block in the battle against HIV cure. Therefore, studies focusing on underlying mechanisms for subtle neuronal cell death by virotoxins; like HIV-1 Tat, were warranted. Although HIV-1 virus rarely infects neurons, interestingly axonal or dendritic damage are the major pathological events occurring during HIV dementia. This raises the possibility that majority of neuronal loss occurs either via indirect pathway or is mediated via neighboring astrocytes that harbor HIV-1. The mechanisms of neurotoxicity are thought to include altered calcium homeostasis, oxidative stress, glutamate excitotoxicity, stimulation of tumor necrosis factor-alpha (TNF-α) and stimulation of nitric oxide production. These mechanisms are likely acting in concert. Astrocyte infection by HIV-1 has substantial consequences in perturbing the brain microenvironment as they represent the most abundant cell type of brain (Churchill et al., 2009). Neuron-astrocyte interactions and astrocytic support for neurons are important for normal CNS function. Astrocytes support neurons by providing factors essential for neuronal growth, mediate uptake and release of extracellular glutamate thus maintaining optimal glutamate concentration in the extracellular fluid which can otherwise be toxic to neurons. Astrocytes also serve other important function like maintenance of pH, ionic and water homeostasis and provide metabolic support to the neuron (Montgomery, 1994; Tsacopoulos and Magistretti, 1996; Butt and Kalsi, 2006). Glial cell dysfunction, therefore, plays an

important role in various psychiatric disorders and infections. Nucleotide released by glial cells play an important role in neuron-glia communication acting via purinergic receptors (De Keyser et al., 2008). Any alteration in astrocyte functions or capabilities will hence affect neighboring neuronal cells.

Studies from brain tissue of HIV-infected patients revealed that 5-19% of astrocytes contain HIV-1 DNA and amplitude of infection correlates with the severity of neuropathological changes. Infection frequency of astrocytes is found to be maximum at the perivascular region where the astrocytes lie in close proximity to macrophages (Churchill et al., 2009). Integration of HIV virus in astrocytes makes them an important reservoir for the virus (Churchill et al., 2006).

Various soluble factors released by glial cells attribute to CNS injury in HIV-associated neurocognitive disorders. Among these, extracellular ATP binds to purinergic receptors present on macrophage and these receptors have been shown to be necessary for HIV infection of primary human macrophage. In fact, inhibition of purinergic receptors led to significant reduction in HIV infection in immune cells (Hazleton et al., 2012). At higher concentrations, ATP activates ligand-gated cation channel receptors, P2X7R that has been implicated in various neurological disorders (Volonte et al., 2012), however, the role of the P2X7 receptor in HIV-1 neuropathogenesis was not well described. A very recent study provides evidence that P2X receptors (P2X4 and P2X7) present on hippocampal neurons may mediate neuronal injury when activated via ATP released from HIV-infected macrophage (Tovar et al., 2013). Another study by Sorrell and Hauser (2014) in primary mouse neuron-glia co-cultures suggests that purinergic receptor, P2X4R, but not P2X7R, on mouse microglial cells and neurons is implicated in HIV-1 Tat and morphine-induced neuronal damage (Sorrell and Hauser, 2014). Surprisingly, this study did not find the involvement of P2X7R in Tat and morphine-mediated neuronal damage via microglial cells. Given that the astrocytes out-number all other cell types in the brain and that up to 19% of the astrocytes are infected by HIV-1, the effect of P2X7R on the astrocytes in

mediating the neuronal damage warranted an extensive examination, hence the present study was initiated.

In Alzheimer's disease, increased levels of P2X7R contribute towards the activation of the microglia by Aβ and the progressive death of the neurons (Parvathenani et al., 2003; Sanz et al., 2009). The activation of P2X7R-Panx1 is also responsible for the enteric neuron death during colitis (Gulbransen et al., 2012). We, therefore, focused our efforts to explore the effect of the activated P2X7R on the neuronal death following the application of Tat.

With our primary set of experiments, we found that P2X7R activation has no toxic effect on astrocytes (Figure 4.1.). We then performed the experiments with the pure culture of neurons and found that neurons express functional P2X7 receptors as they responded to BzATP stimulation by a huge rise in intracellular calcium which remained sustained over time (Figure 4.2.). We observed significant neuronal death attributed to direct or indirect P2X7R activation; however, the direct pathway where P2X7R agonist was added on to neurons had more negative impact on the neuronal survival (Fig 4.3. & 4.4.). Confirmation of the role of P2X7R in the astrocyte-mediated neuronal death following the Tat exposure was substantiated by the observation that the pre-treatment with the P2X7R antagonist abrogated neuronal death (Fig. 4.5. & 4.6.). As the specificity of chemical blockers is often debated, we chose molecular tools (knockdown of P2X7R using siRNA) to investigate the role of P2X7R in the astrocyte-mediated neuronal injury. Consistent with this idea, following the Tat treatment, the astrocytes lacking P2X7R showed a significant attenuation in the neuronal cell death (Fig. 4.7.).

Oxidative stress is implicated in the pathogenesis of several diseases including AIDS dementia complex (ADC), amyotrophic lateral sclerosis, Alzheimer's disease Huntington's, and Parkinson's disease and retinal degenerative disorders (Emerit et al., 2004). Extracellular Tat protein induces ROS formation in astrocytes and neurons. In astrocytes, ROS formation leads to increase in expression of adhesion molecules like

VCAM-1 and ICAM-1 via NFκB pathway enhancing monocyte adhesion (Song et al., 2007). In neurons, direct treatment of Tat induces neuronal apoptosis by a mechanism that involves disruption of calcium homeostasis, caspase activation, mitochondrial calcium uptake and ROS accumulation (Kruman et al., 1998). In our experiments, measurement of ROS in neurons exposed to conditioned media from Tat-treated astrocytes showed that Tat treatment in astrocytes augments ROS formation in neurons. Interestingly, using siRNA against P2X7R in astrocytes attenuates the Tat-induced oxidative stress in neurons indicating the importance of P2X7R in ROS formation in neurons (Figure 4.8.B.). Under similar knockdown conditions, Tat also induced significant ROS generation in human astrocytes although in a lesser extent. Unlike neuron, P2X7R knockdown did not alter Tat-induced ROS formation in astrocytes (Fig. 4.8.A.) suggesting that other mechanisms might be involved in generating oxidative stress in astrocytes.

Mitochondrial dysfunction can lead to the release of pro-caspases, cytochrome C, apoptosis-inducing factor (AIF), and apoptotic protease-activating factor-1 (APAF-1) into the cytosol. The cytochrome-c, APAF-1 and caspase-9 form multimeric complex activating downstream caspases leading to apoptotic cell death (Kannan and Jain, 2000). We checked the integrity of neuronal mitochondria membrane using JC-1 dye and observed significant mitochondrial membrane depolarization in neurons exposed to conditioned media from Tat-treated astrocytes which were reduced by knockdown of P2X7R in astrocytes (Figure 4.10). Hence, we conclude that extracellular Tat treatment in astrocytes can enhance ROS formation and mitochondrial membrane depolarization in neurons which can be possible inducers of indirect neuronal apoptosis. P2X7R knockout in astrocytes results in increased membrane potential and decreased ROS generation in neurons suggesting that activation of P2X7R in astrocytes are also involved in early events of neuronal apoptosis.

In conclusion, the findings presented in this study establish the involvement of P2X7R in Tat-induced direct and indirect neuronal damage. Our study strongly suggests that astrocytes play an important role in Tat-induced indirect neurotoxicity which has

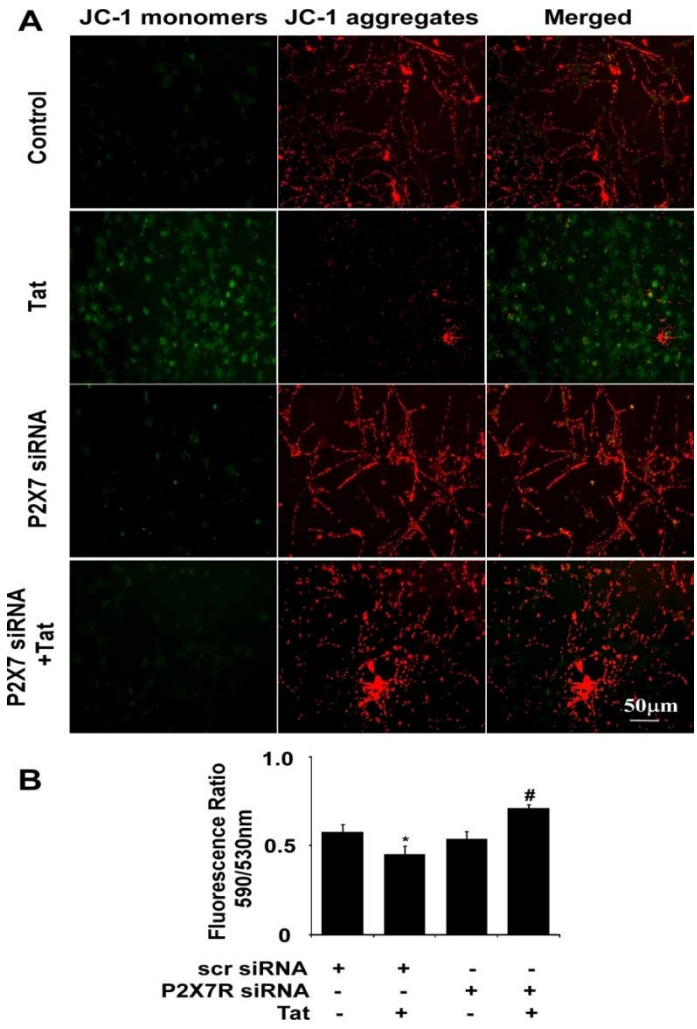

Figure 4.10. Tat-induced decrease in mitochondrial membrane depolarization in neurons is rescued by the P2X7R knockdown in astrocytes. (A) Human astrocytes were treated with 1.6 pmole scrambled siRNA (control) or P2X7R siRNA for 24 hr and then exposed with 100 ng/ml of Tat for additional 24 hr. After 24 hr, astrocyte conditioned media was collected and replaced with half of the neuronal media in pure human neuronal cultures. After 12 hr mitochondrial membrane depolarization of neurons was studied by JC-1 dye assay. Significant depolarization of the mitochondrial membrane was observed in neurons exposed with Tat treated ACM. The mitochondrial membrane depolarization induced by Tat was abrogated by P2X7R knockdown in astrocytes **(B)** From parallel experiments, quantitative assessment of mitochondrial membrane depolarization of neurons was done and the ratio of JC-1 aggregates/monomers (590/530nm) was calculated and represented. Data represents mean ± SD from 3 independent experiments. * represents $p<0.05$ as compared to control and # represents $p<0.05$ as compared to Tat alone.

profound implications in HIV dementia cases. New strategies targeting the infected astrocytes will, therefore, be of utmost importance in HIV eradication. In this backdrop, pharmacological targeting or the inhibition of the purinergic receptors present on the neuronal and non-neuronal cell, represents a novel therapeutic strategy for the control of the HIV infection and its deleterious effects on the CNS especially the neurons.