Summary

Human immunodeficiency virus-1 (HIV-1) invades human brain in early stages of infection and causes a wide range of neurological complications collectively known as HIV-associated neurocognitive disorders (HAND). HAND in its severe forms gives rise to the condition called HIV encephalitis (HIVE) and HIV-associated dementia (HAD). HAD occurs due to intense infiltration of monocytes into the brain, activation of astrocytes, microglia and macrophage and subsequent neuronal damage. In HAD, the damage to neurons is mostly through the bystander effect as neurons lack CD4+ receptors and hence rarely gets infected by the virus. Glial cells, especially astrocytes, are the major cells responsible for indirect damage to neurons in HAND. As astrocytes serve a plethora of functions critical for brain homeostasis and proper neuronal functioning, astrocyte dysfunction in human brain poses profound impact on the overall health of neurons, affecting cognitive, motor and behavioral abilities culminating in various neurological disorders.

Using *in situ* polymerase chain reaction (PCR) assays and immunohistochemistry analysis, studies on post-mortem brain tissue samples from individuals with HIV infection reported infection of astrocytes to be only around 1-3%. Being a very small percentage as compared to other infected cell types like microglia and macrophages this was ignored in earlier days. However, an *in vivo* study combining double immunohistochemistry, laser capture microdissection and highly sensitive multiplex PCR methods revealed that almost 19% of astrocytes were infected with HIV in contrast to the previous report. More importantly, the extent of astrocyte infection positively correlated with severity of HIVE, suggesting astrocyte infection as an important contributor in the progression of HIV neuropathology. *In vitro* studies performed on astrocytoma tumor cell lines and primary astrocytes cultured from human fetal brain also demonstrated that astrocytes are permissive to infection by various HIV-1 strains. Infected astrocytes predominantly express non-structural proteins like Tat, Nef and Rev while structural proteins are almost absent. This suggests a poorly productive but persistent infection in astrocytes, with no detectable increase in viral load. Nonetheless, the latent infection leads to global change in astrocyte gene expression resulting in astrocyte activation which is further intensified by the

extracellular factors released from the other HIV-infected cells like microglia, monocytes and T-lymphocytes. Microglia, monocytes and T lymphocytes further induce the infected astrocytes to synthesize and secrete several viral proteins such as HIV-1 Transactivator of transcription (Tat); a potent neurotoxicant. It drives the regulatory region of the virus and is actively released from infected cells. Once released, Tat is consequently taken up by non-infected cells propagating cellular dysfunction. Astrocyte activation by Tat leads to blood-brain barrier compromise and impairment in glutamate uptake by the astrocytes resulting in excitotoxic neuronal death. A subpopulation of astrocytes also undergoes apoptosis that correlates with severity of HAD suggesting that astrocytes are important mediators for HIV neuropathogenesis.

Highly active antiretroviral therapy (HAART) has been very successful in controlling the systemic viral load, recovering the immune system and reducing the mortality and morbidity in HIV patients. However, the poor penetrance of the anti-retroviral drug into the brain through the blood-brain barrier (BBB) limits the optimal effect of these drugs in CNS. Moreover, the anti-retroviral drugs that successfully penetrate the BBB, target only the replicating virus produced by microglia and macrophages while the infected astrocytes remain unaffected, as HIV-1 resides mostly in latent forms in astrocytes. The infected astrocytes in brain serve as a viral sanctuary and continue to release viral proteins. Neurotoxins like Tat amplify neuroinflammation and neuronal damage. The presence of the latent viral reservoirs explains the prevalence of HAD in patients on HAART despite undetectable viral load in their CSF. Therefore, the astrocyte-mediated neuronal injury is one of the major pathways for HIV neuropathogenesis and cannot be ignored. Therefore, the main objective of this research work was to evaluate the neurotoxic and neuroinflammatory potential of astrocytes and to delineate the underlying cellular and molecular mechanisms.

Elevated levels of ATP release from activated glial cells are common in several neurological disorders. Once released, ATP binds and stimulates various purinergic receptors including P2X and P2Y receptors. Purinergic receptors have recently emerged

as important candidates for various stages of HIV-1 life cycle and infection. Most of the studies regarding the role of purinergic receptors in HIV neuropathogenesis have been performed on productively infected macrophages and microglia while studies focused on their role in latently infected astrocytes and subsequent neuronal dysfunction was lacking until recently, this incited us to initiate the current study.

To delineate the cellular and molecular basis of purinergic receptor activation, in HIV Tat-induced neuroinflammation and neurodegeneration, following research aims were pursued:

1. To study the effect of Tat on P2X7 receptors in astrocytes and its role in enhanced CCL2 release from astrocytes.
2. To investigate the role of P2X7R in Tat-induced indirect neuronal death.
3. To determine the important soluble factors released from astrocyte upon exposure to Tat that promote indirect neuronal toxicity through P2X7R activation with an emphasis on the role of hemichannels in P2X7R associated mechanisms.

Enhanced monocyte or lymphocyte infiltration into the brain is one of the pathological hallmarks of HIV neuropathogenesis. The infected monocytes/lymphocytes enter the brain through "*Trojan Horse mechanism*" facilitated by chemokines present in the extracellular milieu. Once inside the brain, the infected monocytes release various HIV proteins, toxic metabolites and proinflammatory cytokines that further aggravate astrocyte activation. The activated astrocytes, in turn, release several chemokines including CCL2, CCL3 and CCL5. CCL2/MCP-1 is one of the most potent chemoattractant upregulated during the course of HAD and plays a major role in increased trafficking of infected monocytes into the brain. The levels of CCL2 positively correlate with the amount of viral load or extent of dementia. Surprisingly, elevated CSF-CCL2 levels are even reported in patients on HAART without any clinical manifestations. Hence, it is considered as a prognostic biomarker for chronic neuroinflammation in clinical practice. *In vitro* model of BBB using astrocytes and endothelial cells has demonstrated that HIV-1 Tat protein released from astrocytes also transactivates and releases CCL2, inducing transmigration of peripheral

blood monocytes and lymphocytes. These observations were confirmed on human fetal astrocytes. Given that the severity of HAND does not correlate with viral load but with the number of activated glial cells in the CNS and CCL-2 levels in CSF, it prompted us to study the role of P2X7R in human fetal astrocytes and to find out whether it plays any role in Tat-induced MCP-1 release from astrocytes. In an attempt to understand the process in more detail, we meticulously attempted to find out the signaling pathway behind the process.

Using 2'(3')-O-(4-benzoylbenzoyl) ATP (BzATP), an agonist for P2X7R (member of P2X receptor family), it has been shown that P2X7 receptor plays a role in the CCL2 release from normal astrocytes. Considering the fact that Tat also enhances CCL2 production in astrocytes, we hypothesized that activation of P2X7R might be playing a crucial role in this phenomenon. To probe into this, an *in vitro* model system of fully differentiated human fetal astrocytes or neurons was employed. These astrocytes or neurons were differentiated from human fetal-brain-derived neural precursor cells. The purity of astrocyte and neuronal culture was confirmed using GFAP and Tuj-1 and 99% of cells were found to be positive for respective markers. To study the effect on HIV neuropathogenesis, HIV protein Tat from HIV-1 subtype B was used throughout the study.

Brief stimulation of human astrocytes with potent P2X7R agonist BzATP led to sustained increase in intracellular calcium and prolonged stimulation induced pore formation, the two major characteristics of P2X7R, suggesting that P2X7R present on astrocytes are indeed functional. These two assessments were made using live cell calcium imaging in time-lapsed microscopy after incubating the cells with Fluo-3AM and the Lucifer yellow dye uptake tests, respectively. Exposure of primary cultures of human astrocytes with HIV-1 Tat significantly upregulated the expression of P2X7R in human fetal astrocytes. In accordance with existing literature, exposure to Tat led to significant CCL2 release from human fetal astrocytes. Using various P2X7R antagonists, such as oxATP, BBG, A438079 and broad spectrum P2R antagonist suramin, our study revealed significant down-regulation of Tat-induced CCL2

production, suggesting P2X7R as an important candidate for the mechanism. The CCL2 release was also abrogated by calcium chelators 1,2-bis(o-amino-phenoxy)ethane-N,N,N',N'-tetraacetic acid aceto-methyl ester (BAPTA) and ethylene glycol tetraacetic acid (EGTA) and ERK1/2 pathway inhibitor U0126, indicating that Tat mediates CCL2 release occurs via P2X7R-Ca^{2+}-ERK1/2 signaling mechanism. This is the first report that reveals the role of purinergic receptors in Tat-induced CCL2 release from astrocytes. Considering the fact that dysregulated CCL2 production plays a key role in HIV-infected lymphocyte infiltration into the CNS and the subsequent pathological characteristics of HAND, our investigation provides important insights for using P2X7R as a potential therapeutic target to treat neuroinflammation in the CNS.

In our experiments, BzATP treatment did not result in apoptosis of astrocytes, whereas a significant percentage of neurons underwent apoptosis, through both the direct (when cultures were treated with BzATP) and indirect pathways (when neurons were treated with astrocyte conditioned media). During HIV neuropathogenesis, Tat-induced neuronal apoptosis occurs via direct pathway or through bystander killing of neurons via glial cells, especially astrocytes. Since the underlying mechanisms involved in indirect neuronal injury through glial cells have remained elusive, we extended our study to investigate if astrocytic P2X7R activation plays any role in HIV Tat-induced indirect neuronal death. Exposure of human neuronal culture to astrocyte conditioned media revealed that P2X7R antagonists significantly reduced human astrocytes mediated HIV-Tat-induced neuronal death. Interestingly, giving similar treatment directly over neurons, we observed that P2X7R are also involved in Tat-induced direct neuronal apoptosis. Silencing P2X7R in astrocytes using specific P2X7R siRNA rescued neurons from Tat-induced elevation in membrane depolarization, ROS production and neuronal apoptosis. Collectively, this part of the study demonstrates that astrocytic purinergic receptor P2X7R is at the seat of astrocyte-mediated neuronal damage following HIV-Tat exposure.

In our study significant neuronal apoptosis via indirect mechanism indicates that the neuronal death is induced by the soluble factors released by activated astrocytes and

needs to be identified. The calcium oscillations in astrocytes are rapid means of reciprocal communication within the neuron-astrocyte network. The release of calcium-dependent glutamate and ATP from astrocytes govern the activity of neurons. Reports also suggest that enhanced level of ATP and glutamate can be toxic to neurons and can be limiting factors for normal neuronal functions thus affecting neuronal health. Therefore, we further focused on to study the alteration in ATP and glutamate release from astrocytes upon exposure to Tat. Our data suggests that Tat enhances ATP and glutamate release from astrocytes and P2X7R are involved in the release of both the gliotransmitters. In an attempt to understand the P2X7 receptor-Pannexin-1 hemichannel association in the scenario, dye uptake and ATP release was analyzed in the presence of Panx-1 hemichannel blocker probenecid, connexin-43 mimetic peptide Gap-26 and specific Cx43 siRNA. Our results indicated that dye uptake was significantly reduced with P2X7R antagonists and probenecid, this clearly indicated that pore formation in astrocytes may occur through P2X7R activation or Panx-1 hemichannel opening. Tat-induced ATP release was significantly blocked by pretreatment with Gap-26 which was further validated by using specific siRNA for Cx43. With probenecid, the ATP release was not altered significantly. This suggested that P2X7R activation and Cx43 hemichannels play an important role in ATP release. Furthermore, blockade of purinergic and glutamatergic signaling in neurons followed by exposure to Tat-treated astrocyte conditioned media identified ATP and glutamate as important factors released from astrocytes to cause neuronal toxicity. Further emphasis is required to fully understand the role of other purinergic receptors and their effects on HIV neuropathogenesis. Interrogating P2X7R-hemichannel interaction in context to HIV neuropathogenesis is an intriguing area that needs to be critically analyzed to gain better insights into the disease mechanism.

Collectively, the present study paves the way for a better understanding of astrocyte-mediated neuronal damage in HIV-1 neuropathogenesis and suggests P2X7R as a novel target for the therapeutic management of neuroAIDS/HAND. Targeting the astrocytes viral reservoir or preventing the astrocyte dysfunction may, therefore, have far-reaching implications for treating HIV-related disorders.