Chapter 1

Review of Literature

1.1. Human Immunodeficiency Virus-1 infection: An introduction

Since the first case of Acquired Immune Deficiency Syndrome (AIDS) in 1981, this disease has grown to a dimension of world pandemic and continues to haunt basic researchers and clinicians due to widespread death and disability caused by the disease. Human Immunodeficiency Virus (HIV) is the etiologic agent of AIDS and belongs to genus lentivirus of *Retroviridae* family (O'Brien and Goedert, 1996). Human retrovirus family includes human oncovirus strains namely T-lymphotropic virus type I (HTLV-I) and HTLV-II and lentivirus strain which includes HIV-1 (previously termed HTLV-III, LAV) and HTLV-IV/HIV-2 (Barre-Sinoussi et al., 1983; Gurgo and Gallo, 1987; Kurth et al., 1991; Karpas, 2004). HIV is a highly variable virus which mutates readily, and hence, complete eradication of the disease remains an undaunted task.

The three stages of HIV infection are acute infection stage, clinical latency stage and AIDS. HIV can be transmitted during any stage of the infection but the risk is higher during acute infection stage. Acute infection stage develops within 2-4 weeks of HIV infection during which a large number of the virus is produced in the body of the infected persons and many but not all affected individuals develop the flu-like symptoms. The virus within the body utilizes the cells of the immune system such as helper T cells (CD4+), macrophages and dendritic cells to replicate itself and spread throughout the body. In this process, it kills the CD4+ T cells, decreasing CD4 count in the blood (Schroder et al., 1994). The immune system of the infected person attempts to restore the CD4 level but often fails to acquire the pre-infection level. It is advised to initiate the antiretroviral therapy at this stage as it might prove beneficial for the health of the infected individual (Tebas et al., 2001). Acute infection stage is followed by clinical latency stage during which the virus replicates in the body although at a very low-level, mostly asymptomatically. If left untreated, the clinical latency stage lasts an average upto 10 years with increased viral load and decreased CD4 count, thus progressing towards AIDS; the final stage of HIV infection. AIDS is characterized by compromised immune system and occurrence of one or more opportunistic infection in infected persons. At this stage, the CD4 counts falls to 200 cells per cubic millimetre

(mm^3) of blood (Lloyd, 1996; Sudharshan and Biswas, 2008). Without treatment persons having AIDS typically survive for 3 years which decreases up to 1 year if the person also suffers from one or more opportunistic infections (http://www.cdc.gov, ; https://www.aids.gov, ; Deeks et al., 2013).

According to the UNAIDS global report factsheet 2015, approximately 36.9 million (34.3 million–41.4 million) people were living with HIV by the end of 2014. HIV has the most devastating effect in Sub-Saharan Africa accounting for 70% of all people living with HIV. India with 2.1 million HIV-positive individuals ranks second to Sub-Saharan Africa. Heterosexual transmission, intravenous drug use, infected blood transfusion and mother to child transmission are the major causes for the spread of HIV infection. In the mid 1990's, the advent of highly active antiretroviral therapy (HAART) for chronic suppression of HIV replication turned out to be the major achievement for the researchers and clinicians working in the field of AIDS research. As of June 2015, 15.8 million people living with HIV were accessing antiretroviral therapy globally, up from 13.6 million in June 2014. With early diagnosis and effective antiretroviral treatment the survival benefits has now increased and a number of newly infected individuals and HIV-related deaths has fallen significantly. Worldwide, 2.0 (1.9–2.2) million people became newly infected with HIV in 2014, down from 3.1 million (3.0 million–3.3 million) in 2000. Similarly, 1.2 million (1.0–1.5 million) people died from HIV-related sickness in 2014 compared to 2 million (1.7 million–2.7 million) in 2005 (UNAIDS, 2015). These statistics demonstrate that there is increased awareness and better availability of effective antiretroviral drugs, which is an encouraging indication for combating this life-threatening disease.

HIV strains are classified into types, groups and subtypes. There are two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to the child at the time of birth but they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, has less CD4+ viral loads and the period between initial infection and illness is longer and the mortality rate from HIV-2 infection is only two-thirds that of HIV-1. This may

explain the limited spread of HIV-2, both in West African countries and elsewhere. Worldwide, the predominant virus is HIV-1. Generally, when people refer to HIV without specifying the type of virus, it is mostly HIV-1 (Weber et al., 1992) as would be the case in future mention of HIV in this thesis as well.

HIV is transmitted as a single-stranded positive-sense enveloped RNA virus. The HIV life cycle is divided into early and late phase. In the early phase, the HIV infects cells through the interaction of the viral gp120 envelope (Env) protein to the host cell membrane CD4 receptor and/or co-receptors such as C-X-C chemokine receptor type 4 (CXCR4; X4) and C-C chemokine receptor type 5 (CCR5; R5) or it directly enters the cell through endocytosis. Once inside the cell, the viral RNA genome is reverse transcribed into a double-stranded DNA by host's reverse transcriptase enzyme. The viral DNA thus produced crosses the nuclear membrane, converts into functional provirus and integrates with the host DNA. This process is mediated by viral encoded integrase enzyme and host co-factors. In the late phase, the integrated virus either becomes latent or it may be transcribed producing new RNA genomes and viral protein that are packaged and released from the cells as new virus particles (Barre-Sinoussi et al., 2013; Freed, 2015). The viral DNA in its latent form (silent provirus) acts as a viral reservoir and often escapes detection by HAART. The latent virus from the viral reservoirs reappears, whenever the body defense system is compromised or if HAART is stopped for some reason.

The HIV genome is 9 kilobase in length and contains 9 different genes - *gag, pol, vif, vpu, vpr, env, nef, rev and tat.* These nine genes encode 15 different proteins that are categorized into structural, regulatory and accessory proteins. *Env, pol and gag* code for structural proteins that form the envelope, core and matrix of the virus. Tat and rev code for viral regulatory proteins that regulate viral transcription and *nef, vpu, vpr, vif* encode for viral accessory proteins (Frankel and Young, 1998; Seelamgari et al., 2004; Engelman and Cherepanov, 2012). Among all viral proteins, the viral coat protein gp120 and the transcription regulator Tat have been studied more extensively and both are considered as potent neurotoxins. Other viral proteins like gp41, Nef, Vpr and Rev

also show some deleterious effect in the CNS but the studies are very limited and need to be explored in greater detail.

The current antiretroviral drugs are able to suppress HIV-1 replication in infected patients, but they fail to eradicate the virus from the body, making life-long treatment necessary. Patients on lifelong antiretroviral therapy (ART) partially restore the immune system and do not suffer from AIDS-related illness as a primary threat. Adversely, prolonged antiretroviral treatment is associated with risk of viral resistance and drug toxicity (Palella et al., 2014; Pinoges et al., 2015). In addition, the presence of viral reservoirs also renders the ability of potent antiretroviral therapy to effectively eradicate the virus from the body (Timilsina and Gaur, 2016). Therefore, understanding the cause of viral latency and developing effective approaches that can target the viral reservoirs will be of utmost importance for HIV cure. In addition, targeting the steps in viral replication that are not disrupted by currently available drugs also might prove more fruitful for achieving complete eradication of HIV infection. The major concern with currently available antiretroviral therapy is the arousal of a new set of HIV-associated complications, resulting in novel chronic diseases like cancers, cardiovascular disease and inflammation-associated diseases (Steinhart and Emons, 2004; Piketty et al., 2008; Deeks et al., 2013). In some patients, the dysregulated immune response after initiation of cART leads to the condition called immune reconstitution inflammatory syndrome (IRIS). IRIS majorly occurs due to unbalanced reconstitution of effectors and regulatory T-cells, leading to excessive inflammatory response in patients receiving ART (Tappuni, 2011). IRIS is considered as a group of disorders, including diseases resulting from pathological inflammation to pathogens, immune-mediated inflammatory disease and autoimmune disease. The clinical outcomes of IRIS range from mild illness to severe morbidity and mortality. IRIS has been reported in 10-32% of patients starting ART (Jevtovic et al., 2005; Sharma and Soneja, 2011; Boulougoura and Sereti, 2016). Therefore, besides the worldwide success of HIV prevention campaign, more improved strategies are required to overcome the present therapeutic drawbacks.

Till date, the CCR5 mutation and CCR5R antagonists have been proved fairly promising for HIV therapy. It has been shown that individuals who were homozygous for a 32 base pair deletion in the CCR5 gene (CCR5Δ32) do not express functional cell surface co-receptor CCR5, which is a predominant co-receptor for HIV-viral entry. This confers resistance to infection with CCR5-tropic HIV strains thus preventing HIV from entering the body. Clinically, transplantation of hematopoietic stem cells from a CCR5Δ32/Δ32 donor has been proved quite successful in eliminating HIV from the recipient's immune system, suggesting that targeted CCR5 disruption can lead to HIV cure without having any adverse effect on individual's health. Thus, researchers are now putting serious efforts in designing genetic tools for CCR5 gene editing to develop curative HIV therapy (Allers and Schneider, 2015; Gates et al., 2016; Kuritzkes, 2016). Recently, CCR5 antagonist maraviroc (MVC) has been approved by many countries for the treatment of patients infected with R5-tropic HIV-1. It has potent antiretroviral activity against all CCR5-tropic HIV-1 viruses (Dorr et al., 2005) and has been shown to be effective at inhibiting HIV-1 entry into cells and is also well tolerated (Van Der Ryst, 2015; Gates et al., 2016).

1.1.1. HIV-1 infection of the central nervous system

The initial symptoms of HIV infection include immune-suppression, but it also has neurological sequels targeting the central nervous system (CNS). Once HIV enters the brain it persists there for many years, presumably until the death of the individual. HIV can cause atrophy of the brain within 3 months of infection (Nath, 2015). The virus mainly affects basal ganglia, hippocampus and prefrontal cortex leading to cognitive disabilities, motor abnormalities and behavioral changes in affected individuals (McArthur et al., 2005). The neurological complications due to HIV infection are collectively termed as HIV-associated neurocognitive disorders (HAND) and are categorized into asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorder (MND) and in its severe form as HIV-associated dementia (HAD). With HAART, the immune restoration can now be achieved in patients. As a consequence, the severity and incidence of neurological complications in affected individuals have

remarkably decreased. As a result, in the post-HAART era, HAD cases have decreased while its prevalence has increased due to increased life expectancy. Currently, patients on HAART suffer mostly with minor and asymptomatic neurological disorders (Spudich and Gonzalez-Scarano, 2012; Elbirt et al., 2015; Sheppard et al., 2015) which still occurs in 35-50% of HIV-1 infected individuals despite successful combined antiretroviral therapy (cART).

As an indirect effect, HIV renders a person immune-deficient, leading to opportunistic infections like neurotuberculosis, oral candidiasis, toxoplasmosis, cryptococcosis, herpes zoster, fulminant pyogenic meningitis and neurosyphilis (Kovacs and Masur, 2000; Ioannidis and Wilkinson, 2003; Legido-Quigley et al., 2013). Meningitis is the most common CNS infection in patients with HIV followed by mass lesion (Park et al., 2009). In addition, virus-induced polyclonal hypergammaglobulinemia results in demyelinating disease of central nervous system (CNS) and peripheral nervous system (PNS). With antiretroviral therapy HIV patients are now living longer although with increased age they are also experiencing the cumulative effects of HIV infection and aging on brain structure and function, resulting in premature age-associated cognitive decline. In older HIV-infected patients, immunosenescence combined with HIV viral loads also worsen the co-morbidity (Cohen et al., 2015; DeVaughn et al., 2015). For example; HIV infection causes imbalance of Aβ biogenesis and clearance, accounting for accelerated beta-amyloidosis observed in HAND patients making them more susceptible to Alzheimer's disease morbidity (Green et al., 2005; Mothapo et al., 2015).

Since AIDS affects persons of all age groups, dementia due to HIV/AIDS can happen to children as well as adults; although, the frequency is higher in older individuals (Valcour et al., 2004). HAD affects behavior, memory, thinking, and movement in advanced stages of AIDS. At first, symptoms are subtle and may be overlooked, but they gradually become troublesome. The symptoms vary widely from person to person. Symptoms of early dementia include reduced productivity at work, poor concentration, mental slowness, difficulty in learning new things, changes in

behavior, decreased libido, forgetfulness, confusion, word-finding difficulty, apathy (indifference), withdrawal from hobbies, and loss of reasoning. Consequently, the patient loses confidence, avoids social activities which ultimately forces him into depression (Maj et al., 1994; Rourke et al., 1999; Bassel et al., 2002; Ammassari et al., 2004; Gorman et al., 2009; Cysique et al., 2016). Symptoms of worsening dementia include speech problems, balance problems, clumsiness, muscle weakness, vision problems, loss of bladder control and occasionally bowel control. Other rarer symptoms include sleep disturbances, psychosis, mania and seizures (Navia et al., 1986; Heaton et al., 2011; Tedaldi et al., 2015).

1.1.2. HIV neuroinvasion

The blood-brain barrier (BBB) limits the entry of blood-borne elements into the CNS due to the presence of tight junction between brain microvascular endothelial cells (BMEC) and astrocytic foot processes. To gain entry into CNS, HIV must cross the blood-brain barrier (Zhang et al., 2015). Three mechanisms have been proposed for viral entry into brain: 1) HIV may directly infect endothelial cells and enter the CNS as cell-free virus (Moses and Nelson, 1994; Kanmogne et al., 2002). 2) HIV may enter the brain via infected monocytes or leukocytes facilitated by increased expression of adhesion molecules by endothelial cells (Gras and Kaul, 2010; Roberts et al., 2010). Increased levels of ICAM-1, VCAM-1, E-selectin and P-selectin found in BMEC of HIV encephalitis (HIVE) patients aid HIV-infected monocytes to adhere to endothelial cells. Transmigration of infected leukocytes causes reduction of tight junction protein expression and up-regulation of matrix metalloproteinase. 3) HIV-infected macrophage/monocytes, microglia and reactive astrocyte may stimulate up-regulation of inflammatory cytokines (TNF-α, IL-6, IL-1) which in turn increase the permeability of BBB by release of vasodilator like nitric oxide (Minagar et al., 2002). In addition, the release of Monocyte Chemoattractant Protein (MCP-1/CCL2) by astrocytes augments the transmigration of infected cells across BBB. (Dhillon et al., 2008; Covino et al., 2016).

HIV can cross the BBB via infected CD4+ T cells, macrophage or monocytes referred as ‘Trojan horse hypothesis” or in a cell independent manner (Peluso et al., 1985). HIV-1 infects CD4+ T cells due to the presence of major HIV-1 receptors CD4 and co-receptors such as CXCR4 and CCR5 in these cells. CXCR4 is the major co-receptor for lymphocytes and CCR5 for monocytes, macrophages and microglia (Peluso et al., 1985; Simmons et al., 1998; Zayyad and Spudich, 2015).

In the later stage of disease, neuroinflammation occurs due to weakened immune system and activation of immune competent cells. Increased inflammatory cytokines in the CSF easily breach the BBB and accelerate the disease process. Viral factors like Tat and Nef also disrupt the blood-brain barrier facilitating either cell-free or cell-associated virus to enter the CNS (Sporer et al., 2000; Toborek et al., 2003; Atluri et al., 2015).

1.1.3. Neurotoxicity of HIV

The neuropathological changes associated with HAD include the formation of multinucleated giant cells, proliferation of astrocytes, microgliosis, neural degeneration, myelin pallor, and breakdown of the blood-brain barrier (Gray et al., 1996).

Monocytes migrate across the blood–brain barrier to replenish the population of perivascular macrophages. Almost all the viruses produced in the brain are initially derived from monocytes that have differentiated into perivascular macrophages. Subsequently, microglia is productively infected and contributes to the production of multiple copies of the virus. In the brain, infected macrophages and microglial cells fuse with each other due to HIV envelop glycoprotein resulting in the formation of large multinucleated giant cells, or syncytia, which also produce virus before they eventually die (Williams et al., 2012). Astrocytes are also infected, but only at a low-level due to restricted HIV-1 gene expression (Gorry et al., 2003). Interestingly, HIV rarely infects neurons (the functional units of the brain), however, it leads to an indirect damage to

neurons, eventually leading to cognitive and other neurological deficits (Shi et al., 1996; Mbita et al., 2014).

Productive HIV replication in brain macrophages and microglia results in the release of progeny virion or shed gp-120 (nonvirion associated). The viral gp-120 can activate uninfected macrophage and/or astrocytes to release neurotoxins that indirectly lead to further inflammation in neighboring cells. The macrophage respond to exogenous gp120 by releasing proinflammatory cytokines (IL-1β and TNF-α), chemokines, viral proteins (Tat, Nef, Vpr, gp120 and gp41), nitric oxide, quinolinic acid, superoxide ions, platelet activated factor (PAF), arachidonic acid, and cysteine (Xu et al., 2004). gp120 also interferes with the beta-adrenergic regulation of astrocytes and microglia (Levi et al., 1993), thus altering astroglial "reactivity" and upsets the delicate cytokine network responsible for the defense against viral and opportunistic infections. Injury to astrocytes by HIV infection or HIV gene products also results in increased Na^+/H^+ exchange thus increasing intracellular pH. Intracellular alkalization activates K^+ channel that extrudes K^+. Increased extracellular K^+ impairs Na^+ dependent glutamate uptake thus enhancing glutamate release by astrocytes. Increased extracellular glutamate, in turn, activates neuronal NMDA (N-acetyl-D-aspartic acid receptor) receptors leading to excitotoxicity (Benos et al., 1994; Xiong et al., 2014). Additionally, activated astrocytes over-express MCP-1/CCL2, thereby recruiting inflammatory cells into the brain (Conant et al., 1998). The elevated MCP-1/CCL2 levels in the cerebrospinal fluid (CSF) positively correlate with HIV dementia (Cinque et al., 1998). Besides this, the secreted viral proteins and cytokines stimulate nitric oxide synthase (iNOS) in astrocytes, thereby adding on to the neurotoxicity. Other than this, HIV-1 infected macrophages stimulate NMDAR thereby inducing the deleterious intracellular signal, which causes neuronal death, by apoptosis or necrosis (Lannuzel et al., 1995; Yang et al., 2013) that can be ameliorated by using the NMDAR antagonists (Toggas et al., 1996). Additionally, the secreted neurotoxic factors which include excitatory amino acids (EAAs), triggers neuronal apoptosis through a process called excitotoxicity. This process involves excessive Ca^{2+} influx and free radical formation due to the over stimulation of glutamate receptors (Epstein and Gelbard, 1999; Bonavia

et al., 2001; Haughey et al., 2001). Thereby, this bystander mechanism may act in concert to promote neuroinflammation and neurodegeneration, even in the absence of extensive viral invasion of the brain (Figure 1.1.).

The viral protein Tat released by infected macrophage/microglia or astrocytes in brain induces uninfected macrophage to release cytokines and other toxins including matrix metalloproteinases (MMPs), IL-6, IL-8, RANTES, MCP-1 and TNF-α which adversely affect neuronal function (Rumbaugh et al., 2006; Turchan-Cholewo et al., 2009; Ben Haij et al., 2015). Tat also directly interacts with neuron causing neurodegeneration. It transports along neuronal pathways leading to synaptic injury and glial cell activation at distant sites. Tat alone triggers formation of a macromolecular complex involving the low-density lipoprotein receptor-related protein (LRP), postsynaptic density protein-95 (PSD-95), NMDA receptors, and neuronal nitric oxide synthase (nNOS) at the neuronal plasma membrane, which leads to apoptosis of neurons and astrocytes (Eugenin et al., 2007). In addition, Tat synergies with gp-120 (the other neurotropic protein) to cause calcium dysregulation and neuronal apoptosis (Haughey and Mattson, 2002). Several other studies demonstrating the role of Tat in direct and indirect neuronal death are discussed in detail in Chapter 4.

Another viral protein Nef also contributes in AIDS neuropathogenesis by enhancing replication and survival of the virus within infected cells. Nef incorporates itself into viral particle and enhances virus infectivity (Qi and Aiken, 2008). *In vitro*, Nef is lethal to neurons and glial cells in nanomolar concentrations and can increase the expression of MMPs, thus potentially modifying the permeability of the blood–brain barrier. Vpr has been shown to increase viral transcription and production in cells of the monocyte/macrophage lineage (Levy et al., 1995). Extracellular Vpr has also been demonstrated to induce apoptosis in undifferentiated and mature neuronal precursor cells via activation of caspase-8 (Guha et al., 2012).

HIV-1 also interferes with the biological function of neural stem cells and progenitor cells as these cells are positive for CXCR4 and CCR5. Treatment with HIV-

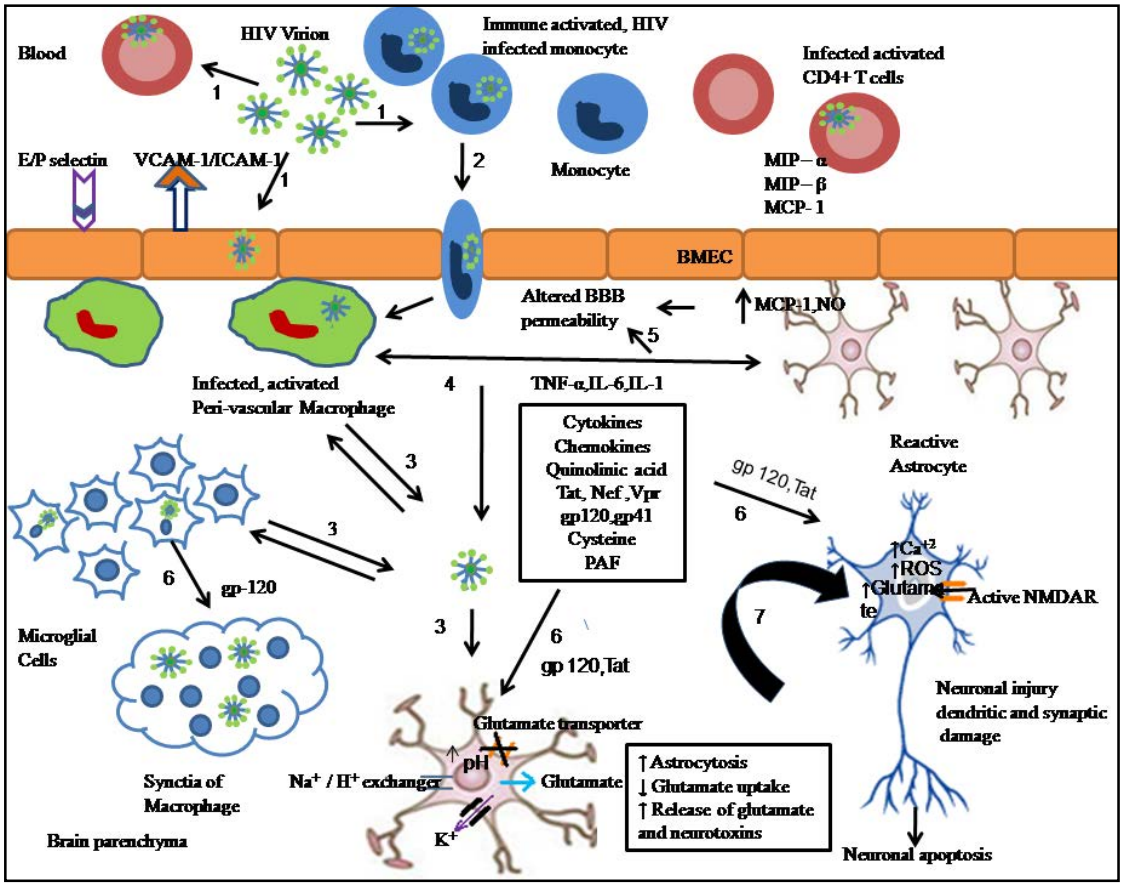

Figure 1.1. Cellular and molecular mechanisms for HIV-1 mediated neurotoxicity: 1) HIV gains entry into the brain by directly infecting brain microvascular endothelial cells (BMEC) or by infecting monocytes and CD4+ T cells in the brain capillaries. **2)** Infected monocytes migrate through the blood-brain barrier (BBB) via increased expression of members of selectin family (P-selectin, E-selectin) and cadherin family (ICAM-1, VCAM-1) and subsequently switches to perivascular macrophage. **3)** Virus released from infected macrophage further invades the uninfected macrophage, microglia and astrocytes. **4)** Reactive astrocytes and macrophages in turn release inflammatory cytokines and chemokines such as TNF-α , IL-6, IL-1, MCP-1, nitric oxide, platelet activating factor, excitatory amino acids, and viral proteins like Vpr, Tat, Nef, gp-120 and gp-41. **5)** The secreted viral and host factors further enhance the permeability of blood brain barrier. **6)** Additionally, gp-120 aids in fusion of infected and uninfected microglial cells to form the multinucleated giant cell which is a hallmark of HIV infection. gp- 120 also synergize with Tat to alter astrocytic homeostasis by increasing Na^+/H exchanger and K^+ conductance. This further impairs glutamate uptake by astrocytes simultaneously enhancing its release. Their synergistic action also leads to direct neurotoxicity. **7)** The increase in extracellular glutamate stimulates glutamate receptors in neurons leading to excessive calcium influx, and free radical formation causing neuronal injury or death (Pant et al., 2012).

1 gp-120 inhibits the proliferation of neural progenitor cell via activation of the p38 MAPK-MAPKAPK2-Cdc25B/C cascade without causing apoptosis (Okamoto et al., 2007). Hence, fewer progenitors may be left to differentiate into neurons thus impairing neurogenesis. Chemokines also promote the quiescence and survival of neural progenitor cells via CXCR4 and CCR3. This involves downregulation of ERK1/2 and stimulation of reelin in isolated neurosphere and hippocampal slice cultures (Krathwohl and Kaiser, 2004). Inhibition of neural progenitor cell proliferation by HIV-1 may result in impairment in new memories formation and difficulty in learning new tasks. Recent studies from National Brain Research Centre (NBRC), India, elegantly demonstrated that HIV-1 Tat affects the human neural stem/precursor (hNPCs) cell properties. Although, hNPCs are susceptible to HIV infection, these are usually not killed by the virus; though some morphological and physiological changes are seen in the hNPCs treated with the viral neurotoxic protein Tat. Our laboratory has shown that a decrease in cell cycle regulatory protein cyclin D1, upon exposure to Tat, severely affects their proliferation and self-renewal capacity, an important property of neural stem cells. In addition to the effect of HIV-1 Tat on proliferation, Tat induces a marked reduction in their potential to differentiate into neurons (Mishra et al., 2010). Hence, HIV-1 Tat further accelerates the neurodegeneration caused due to HIV invasion in the human brain. The tripartite containing motif 32 (TRIM32), has been found to be responsible for HIV-1 Tat-induced quiescence of neural precursor cells (Fatima et al., 2015). The proliferation ability of neural progenitor cells further decreases upon the synergistic action of Tat and drug of abuse (Malik et al., 2014). Similar effects were also seen in the murine model of gp120 transgenic animals that results in the reduction in neural stem cells (Okamoto et al., 2007).

1.1.4. Role of HIV clades in neurologic disorders

Human immunodeficiency virus type 1 is characterized by an unusually high degree of genetic variability *in vivo*. Analysis of HIV-1 virus strains from different geographic locales has revealed that HIV-1 can be divided into three main groups, M (major), O (outlier), and N (new). Group O appears to be restricted to west-central Africa and

group N is extremely rare. More than 90% of HIV-1 infections belong to HIV-1 group M. Within group M there are at least nine genetically distinct subtypes (or clades) of HIV-1. These are subtypes A, B, C, D, F, G, H, J and K and are unequally distributed around the world. Subtype A is most prevalent in Africa, subtype B in Europe and America, subtype C in India and South Africa, subtype D generally limited to East and Central Africa, and subtype F in Romania, Brazil, and Argentina (Geretti, 2006). Group O is centered in Cameroon and its neighboring countries, such as Equatorial Guinea and Gabon, Europe and the United States (Gurtler et al., 1996). The new group (N) is mainly found in Cameroon (Simon et al., 1998). Because of the genetic variability viruses in each clade encompass the different level of virulence and susceptibility for the drug (Wainberg, 2004).

In India, the infection may be due to both HIV-1 and the less pathogenic HIV-2 (preferentially subtype A). HIV-1 subtype C is predominant (Siddappa et al., 2004; Pant M. et al., 2012), while HIV-1 A/C recombinants have been described in Maharashtra. In the north-eastern states, other subtypes B, E, C and B/C recombinants are present (Tripathy et al., 2005). Clade C is responsible for causing more than 50% infection worldwide but its neurological manifestations are less severe than other subtypes. The psychiatric disorders in HIV may range from adjustment disorders, depression and anxiety to state of dementia. Although, the rate of depression in Indian HIV cases is far higher (30-40%) than that reported elsewhere. However, several studies have reported that only 1-2% of seropositive Indians develop dementia as compared to 30-40% of the clade B infected population in the U.S. and Western Europe. Reports from infected HIV seropositive patients in South India have documented the severity of neuropsychological deficits due to clade C based on neuropsychological test performance. They showed that among the seropositive subjects, 60.5% had mild to moderate cognitive deficits characterized by deficits in the domains of fluency, working memory and learning and memory (Gupta et al., 2007) although none of the subjects had severe cognitive deficits. Another group has used International HIV Dementia Scale (IHDS) to screen a well-characterized cohort of HIV-infected discordant couples in Pune, India. In their study, 48 HIV+ subjects with CD4 cell count <200 cells/mm^3

and 48 HIV- subjects were studied. The HIV+ subjects were found to have significantly lower IHDS scores compared to the HIV- subjects. 35% of the HIV+ subjects and 15% of the HIV- subjects scored < 10 on the IHDS. More surveys like this one are required to give a clearer picture of NeuroAIDS in India (Riedel et al., 2006).

The behavioral and neuropathological differences between HIV-1 clades are due to their genotypic variations. Current studies have shown that Tat contributes to the clade-specific differences in HIV neuropathogenesis. The Tat protein differs in its amino acid sequence in the clades B and C. The subtype C has serine in position 31 in contrast to cystine in subtype B. Due to this C31 S mutation clade-C Tat cause reduced neurodegeneration (Mishra et al., 2008). In recent years various *in vivo* and *in vitro* studies using Tat protein and HIV-induced models have shown that subtype C, is unable to produce NMDA receptor-mediated neurotoxicity (Li et al., 2008). Additionally, the influx of intracellular calcium and release of inflammatory cytokine like TNF-α has also been documented to be decreased in clade C Tat cases (Campbell et al., 2007). Using human fetal brain-derived neural precursor cells; it has been shown that relative to clade C, clade B enhances neuronal apoptosis via reactive oxygen species (ROS) induction and increasing mitochondrial membrane potential (Mishra et al., 2008). Elaborated *in vivo* experiments from another independent group revealed an increase in secretion of CCL2 by clade B infected astrocytes and monocyte-derived macrophages (MDM), thereby, inviting more monocytes into the brain (Rao et al., 2008). Also, type 1 Tat B and not Tat C renders resting CD4+ T cells more prone to CXCR4 mediated HIV-1 infection. This effect is brought by an increase in the expression of CXCR4 on the surface of resting CD4+ T cells (Campbell et al., 2010). In addition to this, clade B induces IL-10 production in monocytes. This, in turn, suppresses the pro-inflammatory cytokines and immune response thereby leading to rapid progression from HIV-1 infection to AIDS (Wong et al., 2010). These studies were further supported by clade B specific increase in proinflammatory cytokines; IL-6, TNF-α and IL-33 as compared to Tat C-treated monocyte cultures. However, expression of anti-inflammatory molecules IL-4 and IL-10 was found to be higher in Tat C-treated compared to Tat B-treated cultures (Gandhi et al., 2009; Yndart et al., 2015). The same group has further extended

these studies by showing an increase in serotonin dysfunction in clade B treated immature dendritic cells mediated by indolamine 2-3 dioxygenase (Samikkannu et al., 2010). This further enhances production of neurotoxins altering immune tolerance and balance adding on to another factor in impairment of cognitive function. These findings provide further insights into the differences in the biological properties of HIV-1 clades and their functional relevance in HAND.

1.2. Glial cells

In the central nervous system, glial cells outnumber neurons by a margin of 10:1. Glial cells are comprised of four subtypes: microglia, the innate immune cells of the brain; glial neuroprogenitors, that give rise to the neurons; oligodendrocytes, the myelinating cells; and the astrocytes, performing an array of functions that include neurotransmitter release and uptake, synaptic transmission, K^+ and water homeostasis, and control of blood flow (Kettenmann and Verkhratsky, 2008).

1.2.1. Astrocytes

Astrocytes are the most abundant amongst all glial cells. They are star shaped cells that possess long process but lack axons and dendrites like neurons. In contrast to most neurons, astrocytes are characterized by small soma (< 10 μm diameter) with numerous highly branched processes, extending up to 100 μm distance. The astrocyte processes contact the neurons and endothelial cells forming the tripartite synapse and the blood-brain barrier, respectively. Astrocytes are majorly divided into two subtypes: protoplasmic astrocytes and fibrous astrocytes. Protoplasmic astrocytes are found in the grey matter and posses several branches whereas the fibrous astrocytes are localized in the white matter and have the long fiber-like process (Oberheim et al., 2006).

Traditionally, astrocytes were looked upon as passive, non-excitable and non-neuronal cells that provide structural and trophic support to neuron. Over last decade, the list has been expanded several folds and researchers in the field of glial biology

strongly believe that astrocytes are indispensable cells for neuronal function. Astrocytes reside in all regions of the brain in a well organized and non-overlapping manner. They interact with all other cell types present in the brain and regulate their functions in several ways as shown in Figure 1.2. They interact with endothelial cells and act as a blood-nervous system interface thus playing an important role in maintaining the integrity of the blood-brain barrier. Astrocytic processes envelope the neuronal synapse (made by pre-synaptic and post-synaptic neuron) and form a structure called tripartite synapse where they maintain pH and ion and water homeostasis during normal neuronal activity. Astrocytes communicate to nearby astrocytes through gap junction channel by sending their signals in the form of calcium waves (Kimelberg and Nedergaard, 2010; Sofroniew and Vinters, 2010). Astrocytes interact with oligodendrocytes and promote the myelination activity of these cells by releasing cytokine leukaemia inhibiting factor (LIF) (Cohen and Fields, 2008). In addition, various factors released by astrocytes e.g. PDGF, LIF, NT-3, NT-4, CNTF and IGF-1 promotes differentiation, proliferation and survival of oligodendrocyte precursor cells. These factors also help in myelin formation and remyelination following injury (Gard et al., 1995).

1.2.2. Astrocyte-astrocyte communication

In the brain, astrocytes form an intricate network of the astrocyte-astrocyte or astrocyte-neuron synapse. Astrocyte contacts the nearby astrocyte through gap junction channel forming an electrical synapse. The gap junctions mediate propagation of astrocytic signals in the form of calcium waves (Giaume and Venance, 1998). Ca^{+2} oscillations in astrocytes represent a highly plastic signaling system underlying the reciprocal communication between astrocytes-astrocytes and astrocytes-neurons (Pasti et al., 1997). Calcium waves spread within astrocytes network through gap junction channel and through the release of ATP from astrocytes. ATP released from astrocytes into the extracellular space binds to P2Y1 receptor on nearby astrocytes and elevate intracellular calcium which triggers the release of glutamate and ATP from the cell. Thus, ATP acts as an extracellular signal propagating information in the form of calcium wave to

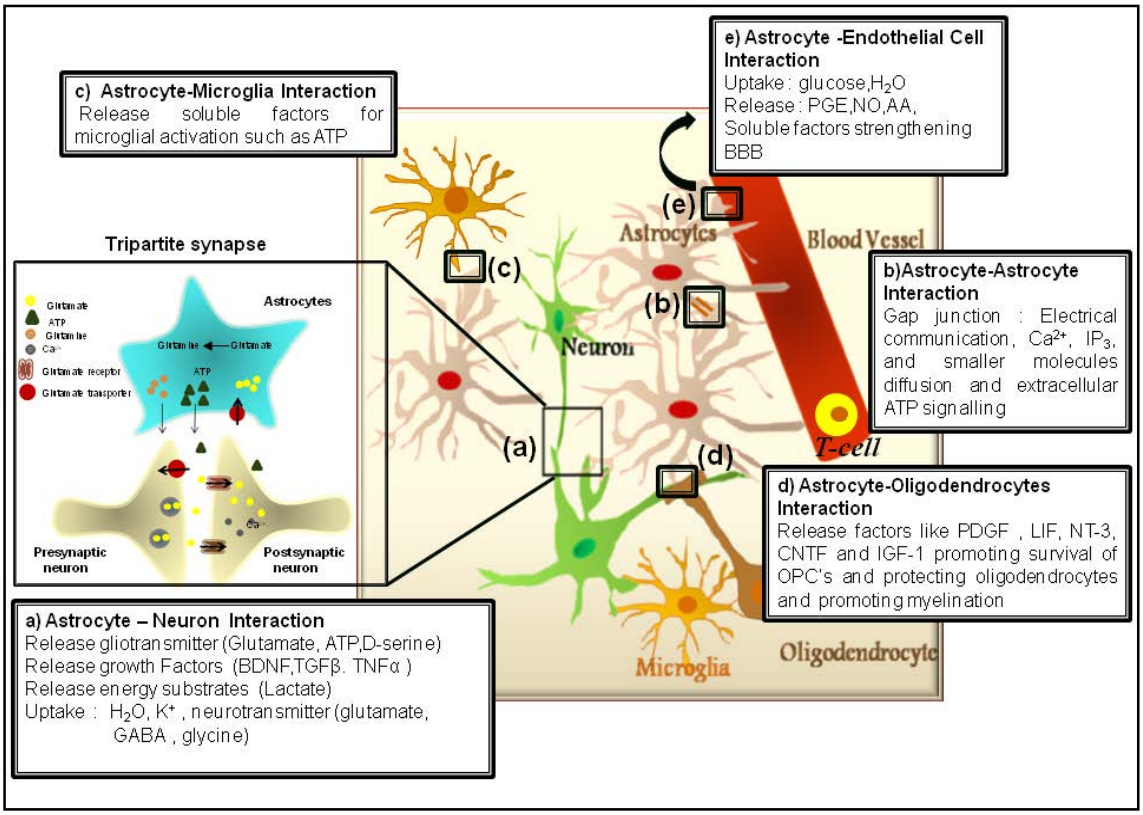

Figure 1.2. Astrocytes interaction with other brain cells: (a) Astrocyte-neuron interaction: Astrocytes in close proximity with the presynaptic and postsynaptic neuron form the tripartite synapse. Bi-directional communication occurs between astrocytes and neurons at the tripartite synapse. During neuronal activity, the astrocyte detects the neurotransmitter released from neurons and, in turn, releases gliotransmitters (glutamate, ATP, D-serine) that modulate synaptic activity. Astrocytes also release growth factors (BDNF, TGF-β) and energy substrates (lactate) to provide trophic and metabolic support to neurons. Astrocytes maintain the ionic and water homeostasis by removing H_2O and K^+ ion from the extracellular space. **(b)** Astrocyte-astrocyte interaction: Astrocytes send their signals to nearby astrocytes in the form of calcium waves. Short range calcium signaling occurs as a gap junction mediated metabolic coupling in the form of IP_3. However, the long range calcium signaling occurs through the release of ATP from astrocytes. **(c)** Astrocyte-microglia interaction: Microglial cells express purinergic receptors which activated upon stimulation with ATP that is released from astrocytes. ATP mediated calcium signaling act as a mode of communication between astrocytes and microglia. Astrocytes also play a regulatory role for differentiation and deactivation of microglial cells. **(d)** Astrocyte-oligodendrocyte interaction: Astrocytes release leukaemia inhibitory factors (LIF) which promote myelination activity of oligodendrocytes. Various factors released by astrocytes like PDGF, CNTF, IGF promote survival and differentiation of oligodendrocyte precursor cells. **(e)** Astrocyte-endothelial cells interaction: Astrocytes send their endfeet to enwrap the endothelial cells at the blood brain barrier. Various soluble factors released from astrocytes help in development and strengthening of tight junction between endothelial cells and regulate the entry and exit of various factors of the brain

distant astrocytes in the range of 100 to 250 µm from the point of wave initiation (Bowser and Khakh, 2007). Calcium waves also generate by nitric oxide-G kinase-dependent pathway, independent of ATP signaling (Willmott et al., 2000).

1.2.2.1. Connexin and hemichannels

Connexins are transmembrane proteins that belong to a gene family of 21 members in human and 20 members in mouse (Willecke et al., 2002). The different members of the connexin family are designated as Cx followed by the molecular mass of the protein in kDa, eg. Cx43 for connexin-43. Six connexin subunits oligomerize to form a connexon or hemichannel. Connexons can either be expressed as heteromeric hemichannels containing more than one type of connexin (Jiang and Goodenough, 1996; Berthoud et al., 2001; Beyer et al., 2001) or homomeric hemichannels (Evans and Martin, 2002). Connexins are synthesized in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and inserted into the cell membrane (Yeager et al., 1998). In adult CNS, different cell types including neurons, astrocytes, microglia and oligodendrocytes express different sets of connexins (Nagy et al., 2004; Kamasawa et al., 2005; Orthmann-Murphy et al., 2008; Theis and Giaume, 2012; Nualart-Marti et al., 2013a). Astrocytes primarily express Cx43 and Cx30. Other connexin subtypes such as Cx26, Cx40, Cx45 and Cx46 are also found in astrocytes but to a lower extent. In cultured astrocytes, the predominant connexin hemichannel type is Cx43 (Dermietzel et al., 1991; Giaume, 1996; Brand-Schieber et al., 2005). Microglia express Cx43, Cx36, and Cx32 while neurons mostly express Cx36, Cx30, and Cx45 with a little expression of Cx43 (Rash et al., 2000; Rouach et al., 2002; Mika and Prochnow, 2012). Hemichannels allow the exchange of ions and small molecules including NAD^+, ATP and IP_3 between cell cytoplasm and extracellular space (Orellana and Stehberg, 2014; Retamal and Saez, 2014) which play a role in autocrine/paracrine signaling. The amount of ionic exchange between the two compartments depends upon the number of open hemichannels present on the cell membrane and their open time. Most of the hemichannels including Cx43 remain closed under physiological conditions and their opening probability can be increased with positive membrane potential and low

extracellular [Ca^{2+}]. The permeability of hemichannels has been evaluated using a number of fluorescent dyes and it was found that Cx43 hemichannels are permeable to ionic dyes such as Lucifer yellow, ethidium bromide, carboxyfluorescein and 7-hydroxycoumarin-3-carboxylic acid (Saez et al., 2003; Giaume et al., 2013; Wang et al., 2013; Hansen et al., 2014a; Hansen et al., 2014b). The half life of most of the connexins is only a few hrs (Laird, 2006).

1.2.2.1.1. Gap junction

Gap junction channels are intercellular membrane channels composed of two hemichannels one contributed by each of the coupled cells. In mammals, gap junctions are composed of hemichannels on opposing membranes of the same type (homocellular gap junction) or different type (heterocellular gap junction) of cells. Both homomeric and heteromeric hemichannels exist *in vitro* and *in vivo*. Gap junctions are formed by docking of hemichannels on opposing membrane, thus allowing the direct transfer of ions, second messengers and small molecules upto 1 kDa between the cytoplasm of adjacent cells (Beyer et al., 1990; Yeager and Harris, 2007; Nielsen et al., 2012). Astrocyte gap junctions allow direct intracellular propagation of second messengers (e.g. Ca^{2+}, IP3, cAMP, and cGMP), metabolites (e.g. glutamate, glucose, and glutathione) and nucleotides (e.g. ATP, ADP, and RNA) between adjacent cells thus enabling metabolic support, spatial buffering and electrical syncytium between adjacent cells, especially neurons and vascular endothelial cells (Bruzzone et al., 1996; Goodenough et al., 1996). Extensive coupling between astrocytes and other brain cells play pivotal roles in modulating neuronal activities and maintaining CNS homeostasis. Gap-junction coupling is also crucial for neuronal differentiation. Astrocytes coupled via gap junctions primarily express Cx43 and Cx30 (Nagy and Rash, 2003; Gosejacob et al., 2011). Cx43/Cx30 double-knockout mice exhibit minimal gap-junction communication between astrocytes suggesting that functional astrocytic gap junctions are composed predominantly of these two connexins. Furthermore, single Cx30 knockout mice show only mild abnormalities, which indicates that other astrocytic connexin subtypes do not compensate for a lack of Cx43, although they express other

connexins including Cx26, Cx40, Cx45, and Cx46 (Wallraff et al., 2006; Rouach et al., 2008). Gap junction also contributes to initiation and propagation of pathological conditions as evident in stroke, trauma, ischemia and neuroAIDS, where the toxic signals from the injured cells spread to healthier cells through the intercellular gap junction (Takeuchi and Suzumura, 2014; Xie et al., 2015).

1.2.2.1.2. Pannexin hemichannels

Pannexin (Panx) is another class of protein family that form hemichannels. The pannexin family consists of Pannexin-1, Pannexin-2, and Pannexin-3 that are homologous to the invertebrate gap junction, innexins (Bruzzone et al., 2003; Penuela et al., 2013). The single-membrane channel formed by pannexins is termed as pannexon. Pannexin-1 is ubiquitously expressed in human tissues, such as the brain, heart, lung, liver, small intestine, skeletal muscle, skin and placenta. In the central nervous system, it is expressed in the cerebellum, cortex, retina, hippocampus, substantia nigra, amygdala and olfactory bulb. The Panx-1 expression has been reported in neurons, astrocytes, and more recently microglia. The expressions of Panx-2 and Panx-3 are mostly constrained to organs and tissues for example Panx-2 expression is restricted to the CNS and Panx-3 in osteoclast, fibroblast and in cultured cell lines (Cahoy et al., 2008; Dahl and Keane, 2012). Similar to connexons, pannexons are permeable to Ca^{2+}, glutamate, glucose, and glutathione thus enabling CNS homeostasis by maintaining ionic and metabolic gradients and can also control cell to cell signaling. Pannexin-1 is recognized as major conduits for ATP efflux that contributes to purinergic signaling and regulate cellular inflammasomes in various cell types under normal and disease conditions (Makarenkova and Shestopalov, 2014; Penuela et al., 2014; Wicki-Stordeur and Swayne, 2014; Cisneros-Mejorado et al., 2015a; Dahl, 2015; Jackson, 2015). Pathologically, ATP release via pannexin hemichannels from various cells have been studied under different conditions, including ischemia and neuroAIDS (Iwabuchi and Kawahara, 2011; Paoletti et al., 2013; Velasquez and Eugenin, 2014; Davidson et al., 2015; Dong et al., 2016). The released ATP from injured cells act as a “find-me” signal for its clearance (Elliott et al., 2009; Chekeni et al., 2010). Unlike

connexin hemichannels, pannexins generally do not form gap junctions (Sosinsky et al., 2011; Beckmann et al., 2016) and exist as hemichannel only.

While functions of connexin gap junction channels have been extensively investigated and characterized, the properties of connexins and pannexins as single membrane channels, or hemichannels, have only recently been given attention and explored. Hemichannel activities are assessed by evaluating dye uptake or efflux (e.g. ethidium bromide and calcein) (Luo and Turnbull, 2011), efflux of biological active molecules e.g., NAD^+, ATP and glutamate (Ye et al., 2003; Song et al., 2011; Dahl, 2015) or by measuring electrical currents through electrophysiological recordings (Patel et al., 2014). Paracrine signaling mediated by connexin or pannexin hemichannel is one of the most widely used mechanism for brain synchronization, hence, it is believed that impairments in permeability properties of connexin or pannexin hemichannel might lead to disruption of cellular and tissue homeostasis observed during various neurological diseases (Bosch and Kielian, 2014; Boyce et al., 2014).

Various experimental evidence suggest that connexons and pannexons remain active under physiological conditions but exhibit lower activity in normal conditions as compared to pathological states. But, still the extent of the channel opening is sufficient to ensure cellular signaling in the nervous system under normal conditions. Dysregulated hemichannel activity has been linked to neuroinflammation characterized by microglial and astrocyte activation and the release of inflammatory cytokines from activated cells. This suggests that hemichannel plays a dual role in regulating molecular homeostasis in the context of neurodegenerative diseases (Bennett et al., 2012). On one hand, transient hemichannel activity has been suggested to be protective during normal physiologic states as well as acute insults or inflammation. On the other hand, sustained hemichannel opening during chronic neurodegenerative diseases may promote disease progression by perturbing metabolic gradients and the exaggerated the release of toxic molecules from activated glial cells and neurons to induce cell death. This can result in the cognitive decline or motor abnormalities in the affected individuals depending

which area of the brain is affected (Bosch and Kielian, 2014; Shestopalov and Slepak, 2014).

During HIV infection, gap junction channels spread toxic signals from infected astrocytes to distant astrocytes, neurons or endothelial cells. Additionally, connexin-43 hemichannel opening upon HIV-1 infection leads to the release of dickkoff (DKK1) protein from astrocytes that lead to neuronal damage (Orellana et al., 2014). The pannexins also play the key important role in HIV infection as reviewed by (Paoletti et al., 2013) and is also discussed in more detail in chapter 5.

1.2.3. Neuron-astrocyte communication

The traditional view of functional synapse consisting of the presynaptic neuronal terminal, release of neurotransmitter from nerve terminal and its binding to the neurotransmitter receptor on the adjacent neuron has been modified over years by adding a third functionally important component - the astrocyte. The synapse thus form is termed as the tripartite synapse, in which the presynaptic terminal, the postsynaptic neuronal membrane and the surrounding astrocytes are three equally important components that mediate and modulate synaptic signaling (Araque et al., 1999; Bezzi and Volterra, 2001; Haydon, 2001). Neurotransmitters such as glutamate and ATP, released from the presynaptic terminal not only activate the glutamatergic and purinergic receptors in the postsynaptic neuronal membrane but also in the perisynaptic astroglial membranes. This, in turn, generates the postsynaptic potential in the neurons and a Ca^{2+} signal in the astrocyte. The astrocytic calcium rise may propagate through the gap junction and, in turn, trigger the release of glutamate, D-serine, and ATP from astrocytes, which may signal back to both pre and post-synaptic neurons thereby affecting neuronal activity at synapses (Santello et al., 2012) as shown in Figure 1.3. This two-way communication between neuron and astrocytes is pivotal for signal transmission, synaptic activity and thus in maintaining normal functioning of the brain (Eroglu and Barres, 2010; Nedergaard and Verkhratsky, 2012; Tanaka et al., 2013;

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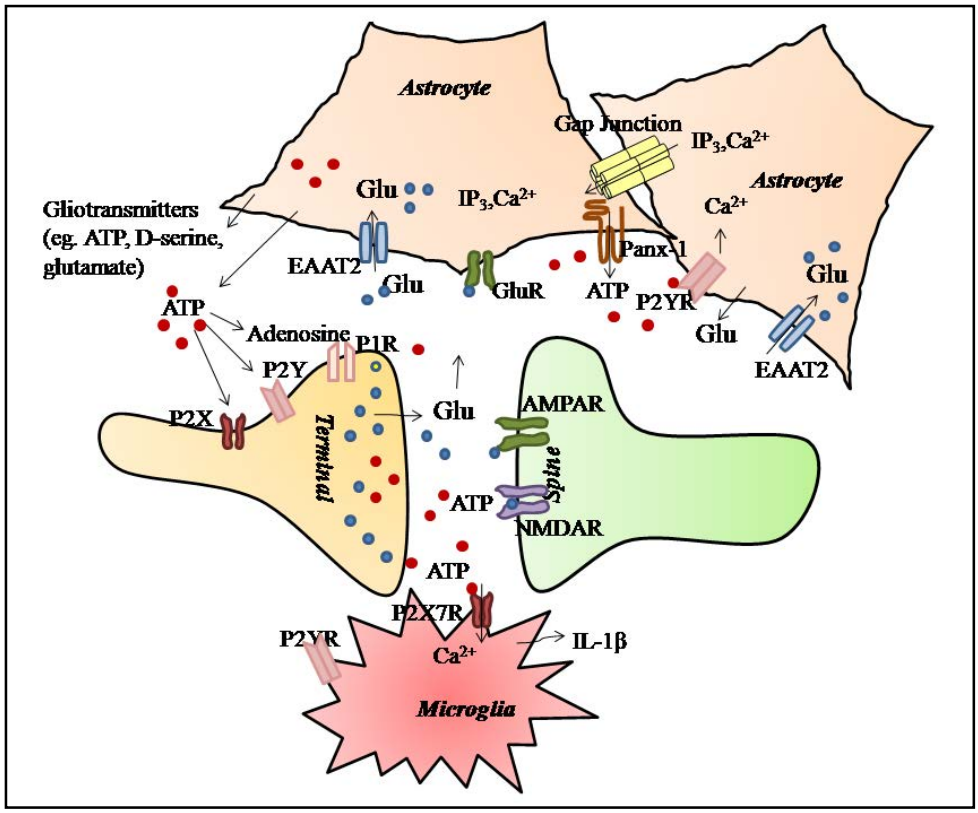


Figure 1.3. Neuron-glia crosstalk in central nervous system: ATP and glutamate are released as neuronal transmitters during normal neuronal activity. The released ATP is hydrolyzed to ADP by ectonucleotidase in the extracellular media. ATP and ADP bind to various purinergic receptors present on neurons, astrocytes and microglia. Binding of ATP to the purinergic receptors (P2) on nearby astrocytes activates the purinergic receptors leading to increase in intracellular calcium. The intracellular calcium ions and other small molecules like IP_3 pass to the adjacent cells via gap junction channel and lead to further release of ATP via pannexin-1 hemichannels. Other gliotransmitter like glutamate, D-serine are also released from astrocytes which further modulate neuronal activity by acting on the presynaptic neuron. In microglia ATP binds to various P2 receptors (P2X4, P2X7 and P2YR) and activates them, however, microglia also releases ATP through exocytosis in a calcium-dependent manner. Activation of P2X7R in microglia leads to the release of IL-1β contributing to inflammation. Thus ATP serves as a paracrine signaling molecule in the neuron-glia network (Tewari and Seth, 2015).

Araque et al., 2014). For these reasons, measurement of intracellular Ca^{2+} ion concentration has been of great functional importance for assessing astrocytic activity.

Several lines of evidence suggest the role of astrocytes in modulating neuronal activity; one such example came from the fact that the synaptic activity of neurons increased by 10 folds in the presence of astrocytes or astrocyte conditioned media (ACM). Various soluble factors released by astrocytes promote the formation and maturation of excitatory and inhibitory synapses (Hughes et al., 2010; Chung et al., 2015; Hahn et al., 2015). Astrocytes also provide metabolic and trophic support to neuron, participate in neurotransmitter uptake and release, and are key mediators of brain information processing, synaptic function, plasticity and integration (Haydon, 2001). In addition, astrocytes can mediate long-range signaling events by interacting with adjoining cells in the form of Ca^{2+} waves (Bazargani and Attwell, 2016). Astrocyte calcium oscillations are required for modulating important brain functions e.g. neurite outgrowth during development, release of gliotransmitters, long-term potentiation (LTP) and regulation of cerebral blood flow to active neurons (Fiacco and McCarthy, 2006; Kimelberg and Nedergaard, 2010; Sofroniew and Vinters, 2010). To bring about these functions astrocytes express various ion channels, receptors and transporters on its membranes. Astrocytes maintain a dynamic crosstalk with neurons to serve important brain function at the tripartite synapse, hence, it is obvious that astrocytes dysfunction may lead to neuronal impairment (Markiewicz and Lukomska, 2006; De Keyser et al., 2008). Various functions of astrocytes have been explored; however, the precise cellular and molecular mechanisms that contribute to astrocyte functions are still not well understood. A better understanding of astrocyte functions with detailed cellular and molecular insights will help approach the astrocytes for pharmacological targeting. Identifying the role of astrocytes in CNS pathologies is currently an area of active research and preventing astrocyte damage might prove an important strategy to save the dying neuron in neurological disorders.

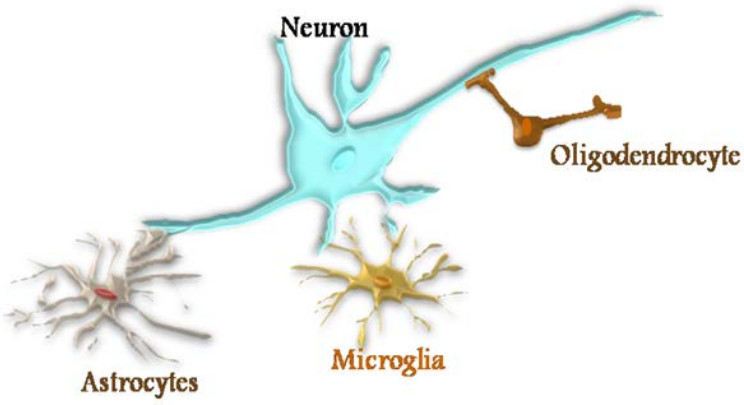
1.3. Astrocyte infection with the virus

Glial cells like microglia, oligodendrocytes and astrocytes get infected by HIV (Albright et al., 1996), but the viral tropism is severely restricted to microglia only. The pathological response of various glial cells to HIV infection is shown in Figure 1.4.

Post-mortem studies from HIV-infected brain tissue revealed that a small proportion of astrocytes are non-productively infected by the HIV virus that contributes to its neuropathogenesis (Gorry et al., 2003). Various HIV strains are permissive to astrocytes *in vivo* as well as in cultures of human fetal brain-derived astrocytes and astrocytoma cell lines (Nath et al., 1995). However, highly sensitive techniques are required to detect HIV RNA and proviral DNA. Combining double immunohistochemistry, laser capture microdissection and multiplex PCR assay, more than 19% of GFAP+ cells in patients with HIV-1 associated dementia are found to be infected with the virus. The frequency of astrocyte infection correlates with severity of HIV encephalitis and was more in cells surrounding perivascular macrophages (Churchill et al., 2009) suggesting that infected astrocyte contributes for HIV neuropathogenesis.

1.3.1. Route for HIV entry in astrocytes

Astrocytes lack cell surface CD4 expression, however, they express CXCR4, CCR5 and other co-receptors that are required for successful HIV-1 infection in addition to CD4 receptors. Some strains of HIV-1 have been shown to infect CD4 negative cells via CXCR4 and CCR5 co-receptors (Edinger et al., 1997; Picard et al., 1997). The various possible routes for entry of virus in astrocytes are mentioned in Table 1.1. Chronically infected astrocytes show a very low-level of viral replication as compared to fibroblasts and T lymphocyte cells. Limited viral replication in astrocytes results from the low-level of viral entry, transcription, viral protein processing, and virion maturation (Schweighardt and Atwood, 2001). The virus thus remains in the latent state in the astrocytes and serves as a viral sanctuary. Astrocytes produce a very low amount of virus due to the inefficient translation of HIV structural proteins Gag, Env, and Nef. However, the expression of Tat and Rev proteins occurs efficiently (Gorry et al., 1999). Some of the possible reasons that limit viral production are listed in Table 1.2.



Astrocyte in HIV

- Restricted Infection
- Increased astrocyte activation / astrocytosis
- Release cytokines and other inflammatory mediators
- Release viral proteins and neurotoxins
- Decrease in growth factors release
- Decrease glutamate uptake
- Increase glutamate secretion
- Increase BBB permeability
- Astrocyte apoptosis
- Astrocyte dysfunction leads to indirect neuronal death

Microglia in HIV

- Productive Infection
- Increased microglia activation
- Release virion and viral protein
- Increase in release of inflammatory cytokines
- Microglial nodule formation

Oligodendrocytes in HIV

- Productive Infection
- Reactive hyperplasia
- Reduced myelin synthesis
- Increased intracellular calcium levels
- Apoptosis of oligodendrocytes

Figure 1.4. Role of various glial cells during HIV neuropathogenesis.

Table 1.1.

Route for HIV entry in astrocytes
• CD4+ independent • Receptor-mediated endocytosis • Galactosyl Ceramide receptor • Mannose receptor • Cell to cell contact between $CD4^+$ T cells and $CD4^-$ astrocytes • Direct interaction by viral envelope protein (Env)

Table 1.2.

Reasons for limited viral production/ replication in astrocytes
• Inefficient translation of HIV structural proteins gag, env, and nef • Nef-mediated suppression of exogenous HIV-LTR activity via negative regulatory element (NRE) • Block in Rev RRE function and sequestration of Rev toward cytoplasm

1.3.2. Astrocyte response to HIV infection

During acute stages of HIV-1 infection, astrocytes and other glial cells exert neuroprotective mechanisms. For example; HIV-1 infected astrocytes express tissue inhibitor of metalloproteinase-1 (TIMP-1) which protects the neurons from apoptosis (Ashutosh et al., 2012). Upon gp120 exposure, the astrocytes upregulate their antioxidant defense mechanisms by increasing the expression of the nuclear factor erythroid-derived 2-related factor 2 (Nrf2) thus providing another mechanism for protection of neurons. However, during chronic inflammation the astrocyte protective

machinery fails and dysfunction of astrocytes results in neuronal death. Astrocytes respond to HIV-1 infection by astrogliosis and undergo morphological changes along with an increase in levels of GFAP, the astrocyte marker (Repunte-Canonigo et al., 2014). These changes also occur in response to HIV viral proteins and soluble factors secreted by infected macrophages and microglia. Astrogliosis is considered as one of the major pathological hallmarks of the HIV-infected brain. Early HIV infection in astrocytes augments telomerase activity and telomerase length, whereas during advanced stages HIV telomerase activity reduces to the level of uninfected cells which is one of the reasons of increased GFAP expression showing astrocytes activation (Ojeda et al., 2014). In astrocytes, the entry of the virus is limited and also the astrocytes are minimal viral productive cells hence, they do not contribute to increasing viral load during HIV-1 infection. However, they still cause excessive neuronal damage via the release of several viral proteins, neurotoxins cytokines and chemokines.

1.4. Extracellular ATP and purinergic receptor

The concept of ATP as an extracellular molecule came into limelight in 1972 by the pioneering work of Burnstock when he recognized ATP as a neurotransmitter in non-adrenergic and non-cholinergic inhibitory nerves in the guinea pig; Taenia Coli (Burnstock, 1972; Khakh and Burnstock, 2009). Two decades later, the discovery of purinergic receptors shed light on the mechanism underlying purinergic signaling and demonstrated that ATP mediates the autocrine and paracrine signaling actions via specific purinoreceptors located in the plasma membrane (Burnstock, 2012, 2014). It was found to be a co-transmitter in all the nerves of peripheral and central nervous system (Burnstock, 2004, 2009). Since then, immense interest has grown in the field of purinergic transmission and remarkable progress has been made in unravelling the role of purinergic signaling in brain development and disorders. The critical functions of ATP and its subsequent hydrolysis are initiated upon binding to purinergic receptors such as the P1 adenosine and the P2 nucleotide receptors.

P1 receptors

Based on molecular cloning and characterization, P1 receptors are subdivided as A1, A2A, A2B and A3 which are G-protein-coupled receptors, usually activated upon binding of adenosine and are inhibitory in action.

P2 receptors

P2 receptors are activated by purines ADP or ATP; and pyrimidines UTP or UDP (Abbracchio and Burnstock, 1994; Hynie, 1995; Jacobson and Gao, 2006). Purines and pyrimidines have major roles in the activities of neuronal and non-neuronal cells (Cusack and Hourani, 1990; Burnstock, 2007). Based on molecular structure and pharmacological profile, P2 receptors belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G-protein-coupled receptors.

P2Y receptors

P2Y receptors belong to the family of G-protein-coupled receptor and consist eight members - P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 (Gever et al., 2006; Fischer and Krugel, 2007; Jarvis and Khakh, 2009). Pharmacologically, P2Y receptors have been categorized into four groups.

1. Adenine nucleotide preferring receptors which include P2Y1, P2Y12, P2Y13 and human P2Y11.
2. Uracil nucleotides preferring receptors which include P2Y2, P2Y4 and P2Y6.
3. Receptors of mixed selectivity such as the P2Y2 receptor which is activated equipotently by ATP and UTP.
4. Receptors responding solely to sugar nucleotides such as the recently cloned P2Y14 receptor which responds to sugar nucleotide UDP-glucose or UDP-galactose. It is the only known P2Y subtype to be activated by nucleotide sugars.

1.4.1. P2X receptors and its subtypes

P2X receptors are non-selective ligand-gated ion channels that mediate sodium influx, potassium efflux and to varying extent calcium influx that lead to depolarization of the cell membrane. Till date, seven human P2X receptor subunits (P2X1-7) have been cloned (Koles et al., 2007). Broadly six homomers (P2X1, P2X2, P2X3, P2X4, P2X5, P2X7) and six heteromers (P2X1/P2X2, P2X1/P2X4, P2X1/P2X5, P2X2/P2X3, P2X2/P2X6, P2X4/P2X7) have been functionally characterized (Dubyak, 2007). Heteromultimers are well in existence for P2X2/3 receptors in nodose ganglia (Brederson and Jarvis, 2008), P2X4/6 receptors in CNS neurons, P2X1/5 receptors in blood vessels and P2X2/6 receptors in the brain stem. Amongst all P2X receptors, P2X6 receptors do not form a functional homomultimer (Barrera et al., 2005; Brederson and Jarvis, 2008; Alves et al., 2014). Even though P2X receptor subunits are expressed in the nervous system, their expression varies in different regions of peripheral and central nervous system (Burnstock, 2013). P2X receptors carry out many important functions in the central and peripheral nervous system such as slow neuromodulatory function, rapid synaptic transmission, neurotransmitter release and the generation of pain signals (Bardoni et al., 1997; Nakatsuka and Gu, 2001; Donnelly-Roberts et al., 2008; Koles et al., 2011; Khakh and North, 2012; Giniatullin and Nistri, 2013; Franceschini and Adinolfi, 2014). Moreover, they also have a pathophysiological role in injury, inflammation, anxiety, dementia and neurodegenerative disorders like Alzheimer's and Huntington's disease (Burnstock, 2015).

1.4.1.1. P2X7 receptor

P2X7 receptor, originally classified as the P2Z receptor (Surprenant et al., 1996) was cloned from rat brain in 1996 (Yu et al., 2008). It is an ATP-sensitive ligand-gated cation channel, which is expressed predominantly in cells of hematopoietic and immunological origin such as mast cells, monocytes, macrophages, and microglia (Collo et al., 1997). It is also present in glial cells of central and peripheral nervous system like astrocytes, microglia, oligodendrocytes, ependymal cells, Schwann cells, radial glia and satellite cells, however, its presence in neuron is a matter of debate due to unavailability of specific antibodies against P2X7 receptor (Deuchars et al., 2001;

Anderson and Nedergaard, 2006; Sperlagh et al., 2006). The signaling between astrocytes and neurons is predominantly mediated through ATP release from these cells. As this receptor is present in immune cells and brain cells, it also plays an important role in neuroinflammation (Ferrero, 2009; Fiebich et al., 2014) and neurodegeneration (Volonte et al., 2003) and provides a communicating bridge between immune system and nervous system.

1.4.1.2. Basic properties of P2X7 receptor

P2X7 receptor is a 595 amino acid protein (Figure 1.5) with a structure comprising-

- Two transmembrane domains TM1 and TM2,
- An extracellular loop (containing the ATP-binding site) joining the two transmembrane domains and
- Intracellular N-terminal and C-terminal domains (Jiang et al., 2013).

P2X7 receptor possess several characteristics that are strikingly distinct from all other members of P2X receptor family, hence, deserves special attention. Some of the structural and functional properties of P2X7R are discussed below:

1. It comprises of 3 to 6 homomeric subunits of P2X7.
2. It contains a long cytoplasmic C-terminus having protein-protein interaction motifs. This long C-terminus is responsible for pore-forming activity of receptor and it also triggers activation of caspase (Smart et al., 2003; Costa-Junior et al., 2011).
3. It has low sensitivity for ATP. Unlike other members of P2X receptor family, P2X7R requires submillimolar to millimolar concentration of ATP for its activation, which is far greater than the nanomolar concentration required for other P2X receptor (EC_{50} of ATP for P2X7R = 2-4 mM and for other P2XR = 1-10 µM. where EC_{50} indicates concentration of ATP that gives half-maximal response) (Rodrigues et al., 2015; Soares-Bezerra et al., 2015). Moreover, it has a higher affinity for 2'(3')-O-(4-benzoylbenzoyl) ATP (BzATP) than ATP (Klapperstuck et al., 2001; Young et al., 2007).

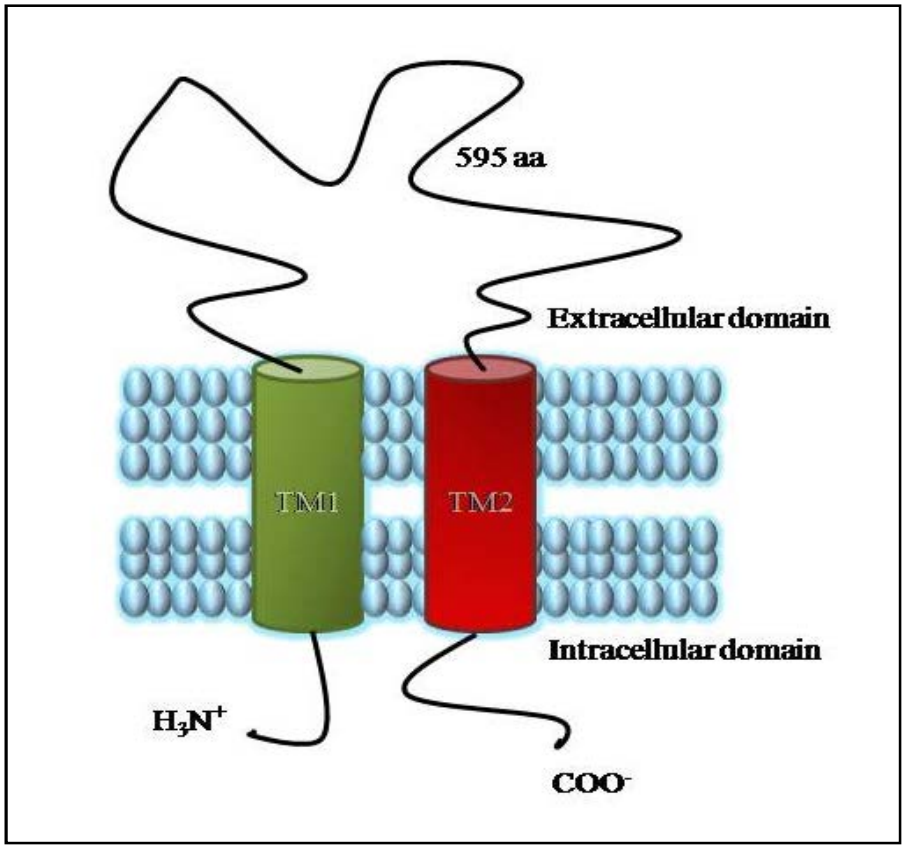


Figure 1.5. Schematic representation of a typical P2X7 receptor subunit: P2X7R is a 595 amino acid protein. It consists of intracellular N-terminal and C-terminal domain and two transmembrane (TM) domains which are connected through an extracellular loop (Tewari and Seth, 2015).

4. It is a non-selective cation channel. It shows different responses to the agonist binding depending upon its concentration and application time. Brief exposure to agonist adenosine-5'-triphosphate (ATP), or the most potent P2X7 agonist,-2'(3')-O-(4-benzoylbenzoyl)ATP (BzATP), leads to the opening of cation channel allowing K^+ efflux and Ca^{2+} and Na^+ influx into the cells whereas prolonged activation of the agonist on P2X7 receptor results in the formation of a large aqueous pore. These pores are permeable to molecules up to the molecular mass of 900 Da such as Lucifer yellow (457Da), Propidium (414Da), Ethidium (314Da) and Methyl glucamine (190Da). The pore-forming characteristic is also found in the P2X2 and P2X4 receptor (Yan et al., 2008; Yan et al., 2010).

5. Activity of the receptor is kept low under normal physiological conditions by the extracellular concentration of divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{+2} and Cu^{+2} (Michel et al., 1999; Acuna-Castillo et al., 2007; Dutot et al., 2008), protons (Flittiger et al., 2010) and anions (Kubick et al., 2011).

6. The P2X7R shows potential functional response when the concentration of extracellular Ca^{2+} and Mg^{2+} are reduced (Alloisio et al., 2010; Yan et al., 2011).

7. Its current conducting properties are also different. Other P2X receptors upon activation show a rapid transient response that decay within seconds whereas there is a little decline in the response of P2X7 receptors. Once activated they allow continuous Ca^{+2} influx which reflects their non-desensitizing behavior (Smart et al., 2003; Dutot et al., 2008).

1.4.1.3. P2X7R and pore formation: P2X7R-Pannexin-1 complex

Brief stimulation of P2X7R leads to increase in intracellular calcium influx whereas repeated or prolonged stimulation of P2X7R induces the formation of a non-selective pore allowing the entry of solutes up to 900Da in size, which eventually leads to membrane blebbing, release of cytokines and cell death (Verhoef et al., 2003; Roger et al., 2008). Studies by Sayar *et al* using rat P2X7R transfected HEK 293 cell and macrophage-derived cell line, RAW 264.7 demonstrated that P2X7 receptors activate two different permeation pathways, one exclusively permeable for cationic dye (YO-

PRO-1 and TO-TO-1) and the other for anionic dyes (Lucifer yellow and calcein). These permeation pathways are either dependent on an increase in intracellular calcium or are independent of it thus exhibiting different activation properties (Schachter et al., 2008; Cankurtaran-Sayar et al., 2009). Two hypotheses are proposed for the conversion of a non-selective cation channel to a cytolytic pore. One hypothesis suggests that the formation of large pore occurs due to the dilation of the cation channel itself (an intrinsic property of P2X7R) and the second one suggests that an additional component is required for the opening of non-selective membrane pore.

Pelegrin & Surprenant for the first time demonstrated that ATP-induced pore formation activity of P2X7R is mediated by Pannexin-1 hemichannel, a membrane protein physically associated with P2X7R (Pelegrin and Surprenant, 2006) as represented in the schematic diagram in Figure 1.6. Pharmacological blockade or siRNA targeting of Panx-1 markedly inhibited the rate and amplitude of YO-PRO1 and ethidium uptake without altering membrane current. This study was further supported by several other studies with strong evidence for the role of Pannexin-1 as a part of pore-forming unit of the P2X7 receptor. The dye uptake and electrophysiological data suggest that the proline-rich segment of the C-terminus region, of the P2X7R, is likely to be involved in the signal transduction pathway and leads to Panx1 activation following P2X7 receptor activation independent of extracellular calcium (Locovei et al., 2007; Iglesias et al., 2008). However, the extracellular calcium-dependent coupling of P2X7R-Panx1 complex and subsequent pore formation also occurs as removal of extracellular calcium increased the membrane permeability due to P2X7R stimulation in the neuron, glia and progenitor cells (Poornima et al., 2012). Currently, Panx1-P2X7R interaction is gathering due attention as several P2X7R functions have now been proved to be mediated by opening of Panx-1 hemichannel, and Panx-1 opening serves as a downstream effector for P2X7R activation (Hung et al., 2013; Orellana et al., 2013; Shoji et al., 2014; Velasquez and Eugenin, 2014; Pan et al., 2015). More importantly, the Panx-1-purinergic receptor interaction has been shown as the basic underlying mechanism for the pathogenesis of various human diseases and thus warranted further investigations (Gulbransen et al., 2012; Xia et al., 2012; Beckel et al.,

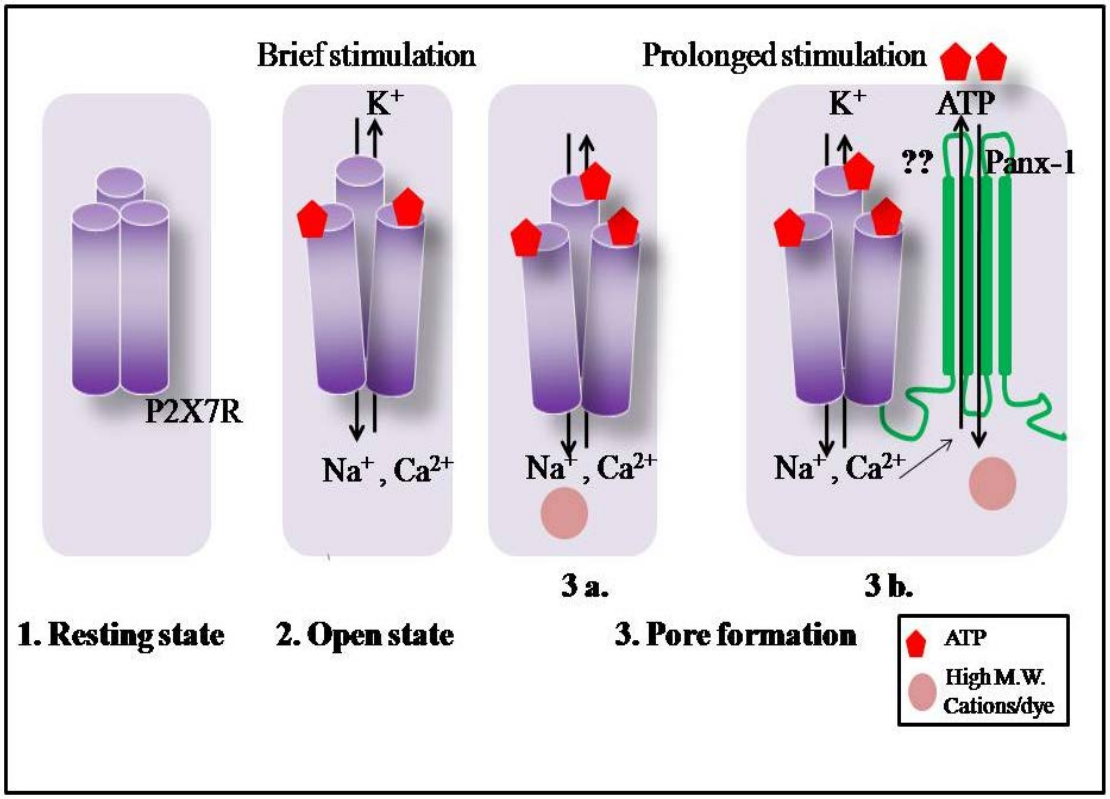


Figure 1.6. Schematic representation of resting and active state of P2X7 receptor: The schematic diagram represents the conformation of P2X7R in resting state **(1)**, open state (2) and during pore formation **(3a and 3b)**. Brief stimulation and binding of ATP on two out of three agonist binding sites allows the opening of the channel leading to sodium and calcium influx and potassium efflux **(2)**. Prolonged and/or repeated stimulation and ATP occupying all the three agonist binding sites causes pore formation allowing higher molecular weight molecules or dyes to enter the cells. One mechanism proposed for pore formation involves the dilation of the P2X7 channel itself **(3a)**. Another mechanism suggests pore formation through physical interaction of P2X7R with pannexin-1 (Panx-1) hemichannel where pannexin-1 acts as a pore forming unit (**3b)**. The opening of Panx-1 hemichannel also leads to ATP release from cells which in turn bind to and activate purinergic receptor potentiating purinergic signaling (Tewari and Seth, 2015).

2014; Velasquez and Eugenin, 2014; Cisneros-Mejorado et al., 2015b). Controversially, some important studies have suggested that Panx-1 hemichannel is not essential for P2X7R mediated pore formation (Bhaskaracharya et al., 2014). By performing patch-clamp recordings *in situ* using a whole mouse embryonic spinal cord preparation, Rigato et al. has shown that embryonic microglia express functional P2X7R and Panx-1. Also, P2X7R activation evoked a biphasic current in embryonic microglia, which is supposed to reflect membrane pore opening through Panx-1 hemichannel. Surprisingly, the biphasic current is observed even in Panx-1 knockout macrophages indicating that it rather reflected P2X7R intrinsic pore dilatation (Rigato et al., 2012). Somewhat similar results were also observed in mouse macrophages where the blockade of hemichannels using Probenecid or CBX or interference RNA (RNAi) targeting Panx-1 did not affect P2X7R induced inward currents or dye uptake. These findings strongly suggest that the high-permeability pore evident after prolonged P2X7R activation does not occur through hemichannels and some other membrane protein may be involved in P2X7R pore formation in murine macrophage (Alberto et al., 2013). This idea was supported by another study in which the authors used time-lapse microscopy and compared the rates of ATP-induced dye (Yo-Pro-1; Mr 375) uptake in single wild-type (WT) and Panx1 knockout ($Panx1^{-/-}$) macrophages. The mean rates of ATP-induced Yo-Pro-1 uptake by WT and ($Panx1^{-/-}$) macrophages were found to be almost identical suggesting that Panx1 is not the large pore pathway in native macrophages. Also, the pannexin-1 deficiency did not protect the macrophages from ATP-induced cell death (Hanley et al., 2012). Hence, the P2X7R-Panx-1 interaction needs a close and careful examination as the complex formation and its functional responses can differ in varying cell types.

The pore formation abilities differ in different areas of the brain as studies by Bianco *et. al* have demonstrated that the P2X7 receptor induces pore formation in cortical astrocytes but not in hippocampal astrocytes and the downstream pathways activated by P2X7 receptors also differ in different astrocytes (Bianco et al., 2009). These results raise the possibility that activation of P2X7 receptors differentially influences the neuroinflammatory processes in distinct brain regions. Thus, further studies are warranted to understand the functional importance of the P2X7-pannexin-1

interaction with better mechanistic insights which will unravel the mysteries of P2X7R pore formation and its functional relevance in various disorders.

1.4.1.4. Agonist potency for P2X7 receptors

BzATP is the most potent agonist for P2X7R evoking calcium influx, pore formation and IL-1β release in mouse, rat and human receptor. It is extensively used in research as the agonist of choice for P2X7R, although, it is not specific for P2X7R as it also binds and activates P2X1 and P2X2 but with lower efficiency (EC_{50} = 0.003 and 0.8 µM, respectively, as compared to P2X7R). In term of agonist potency, BzATP is 10-30 time more potent (EC_{50} = 9 µM) than ATP (EC_{50} > 300 µM) in its ability to activate P2X7R. 2MeSATP, ATPγS and ADP are less effective than ATP whereas αβ-MeATP, βγ-MeATP, ADP and UTP are ineffective (Jacobson and Gao, 2006; Jarvis and Khakh, 2009).

1.4.1.5. P2X7 receptor antagonists

A number of studies performed on the P2X7R have suggested that the response of P2X7R differs with different pharmacological blockers in different species which is previously reviewed in detail (Arulkumaran et al., 2011; Volonte et al., 2012). A list of P2X7 receptor antagonists is provided in Table 1.3 which demonstrates species-dependent sensitivities to the receptor.

Table 1.3. Various antagonists of P2X7 receptor (Tewari and Seth, 2015)

Name of Antagonist	Specificity	References
Divalent cations	Allosteric modulators of P2X7R and reduce the functionality of P2X7R.	(Acuna-Castillo et al., 2007)
PPADS tetra-sodium salt	Non-competitive, non-selective P2X antagonist with low potency and little	(Lambrecht et al., 1992)

	subtype selectivity.	
Suramin	Non-competitive and non-selective P2 antagonist. Inhibits P2X7R at 100 µM concentration.	(Dunn and Blakeley, 1988)
oxidized ATP (oxATP)	Irreversible, less potent antagonist for P2X7R, require long pre-incubation period (1-2hr) to be effective. Reversibly blocks P2X1 and P2X2 receptors.	(Evans et al., 1995; Donnelly-Roberts and Jarvis, 2007)
KN-62	Non-competitive and selective blocker of P2X7R, inhibits current evoked by BzATP in hP2X7R but not in rat P2X7R.	(Gargett and Wiley, 1997; Donnelly-Roberts and Jarvis, 2007)
Analogues of KN-62 (N-aryl piperazine, 1,2,3,4-tetrahydroisoquinoline derivatives	Agonist potency for the P2X7R receptor is lower than KN-62.	(Baraldi et al., 2002; Baraldi et al., 2003; Park et al., 2015)
Brilliant Blue G	Moderately selective antagonist for P2X7R at nanomolar concentration. Potency of rat P2X7R (10 nM) > human P2X7R (100 nM).	(Donnelly-Roberts and Jarvis, 2007; Peng et al., 2009)
A-740003 (Abbott laboratory)	Competitive, highly specific and potent antagonist for rat & human P2X7R. Completed pre-clinical trial phase.	(Honore et al., 2006)
A-438079 (Abbott laboratory)	Competitive antagonist, equal potency against human and rat P2X7R. Completed pre-clinical trial phase.	(Nelson et al., 2006; Donnelly-Roberts and Jarvis, 2007; McGaraughty et al., 2007)

A-804598 (Abbott laboratory)	Highly potent and selective on human, rat and mouse P2X7R. Completed pre-clinical trial phase.	(Donnelly-Roberts et al., 2009)
AZ 116453743 (AstraZeneca laboratory)	Highly selective and potent antagonist for P2X7R at nM concentration. The potency of human > rat P2X7R. Completed pre-clinical trial phase.	(Stokes et al., 2006; Donnelly-Roberts and Jarvis, 2007)
AZ10606120 (AstraZeneca laboratory)	The negative allosteric modulator of P2X7R. Antagonists potency for rat (nM) > human P2X7R (µM). Completed pre-clinical trial phase.	(Michel et al., 2008)
Pyridoxal-5-phosphate (Patented by Medicure Inc.)	Antagonists potency for human and rat > mouse P2X7R.	(Hibell et al., 2001)
HMA	Blocks human and mouse, but not rat P2X7R.	(Hibell et al., 2001)
Coomassie Brilliant Blue (CBB)	Highly selective for P2X7R.	(Hibell et al., 2001)
Adamantane carboxamide	More potent for human than rat P2X7R.	(Furber et al., 2007)
Arylhydrazide A-847227 (Abbott laboratory)	Highly potent and selective antagonist of human and rat P2X7R.	(Carroll et al., 2009)
Aryltetrazole and aryltriazole	Potency for human > rat P2X7R.	(Carroll et al., 2009)
GSK314181A (Glaxo Smith Kline)	Potency to inhibit human P2X7R (nM) > rat P2X7R (mM). Completed pre-clinical trial phase.	(Broom et al., 2008; Alves et al., 2013)
GSK1482160 (Glaxo Smith Kline)	The allosteric modulator of P2X7R. Tested in human subjects.	(Ali et al., 2013; Gao et al., 2015)
Evotec (EVT 401)	Completed the phase I of clinical	(Arulkumaran et al.,

	trials.	2011)
AZD9056 (AstraZeneca laboratory)	Potent, selective and orally bio available P2X7 receptor antagonist. Failed in phase II clinical trials for the treatment of rheumatoid arthritis (RA).	(Keystone et al., 2012)
CE-224,535 (Pfizer)	Selective P2X7R antagonist. Failed in phase IIA clinical trials for the treatment of rheumatoid arthritis.	(Stock et al., 2012)

Several other compounds have also been developed and patented by Pfizer, Abbott, AstraZeneca, Glaxo Smith Kline, Evotec and other pharmaceutical groups. These include cyanoguanidines, triazoles, polycyclic benzamides and natural products. Several of them are under clinical trials and some have successfully passed phase I and II of clinical trials, however, few have failed in clinical trials as reviewed by (Friedle et al., 2010; Gunosewoyo and Kassiou, 2010). For more information on the list of patents for novel P2X7R antagonists please refer to the link below- https://www.google.com/patents/WO2012163456A1

1.4.1.6. Signal transduction mechanism induced by P2X7 receptors

P2X7 receptors mediate various signaling pathways in different cell type as discussed in Table 1.4.

Table 1.4. Signaling pathways activated by P2X7R in different cell types (Tewari and Seth, 2015)

Pathway	Cell type	References
Rho-dependent pathway	MG6 cells	(Takenouchi et al., 2008)

Akt	Cortical astrocytes, 1321N1 astrocytoma cell line	(Jacques-Silva et al., 2004)
ERK1/2	1321N1 astrocytoma cell line, cerebellar granule neuron, human astrocytes, neural progenitor cells, microglia, RBA-2, macrophage	(Gendron et al., 2003; Ortega et al., 2010; Ortega et al., 2011; Martel-Gallegos et al., 2013; Tsao et al., 2013)
PKC	RBA-2 astrocytes, neural progenitor cells, macrophage, microglia	(Sun et al., 1999; Shiratori et al., 2010; Tsao et al., 2013)
p38MAPK	Microglia, astrocytes, PC-12 cells, neurons	(Panenka et al., 2001; Shiratori et al., 2010; Chen et al., 2013; Xu et al., 2015)
JNK	Microglia	(Suzuki et al., 2004)
GSK3	Cerebellar granule neurons	(Ortega et al., 2009)

1.4.1.7. P2X7 Receptor: Dual role in cell death or survival

The P2X7 receptor can induce cell death and cell survival, depending on the intracellular pathways activated by the receptor. Its low-level activation promotes cell growth, whereas the massive stimulation leads to cell death.

1.4.1.7.1. P2X7 receptor as a protective agent

In most of the cells, the P2X7R overexpression acts as a “danger” signal, but it has protective effects as well. For example, the P2X7R-Panx-1 complex decreases the muscarinic acetylcholine receptor-mediated seizure susceptibility in mice. The seizure susceptibility induced by pilocarpine was enhanced in the case of wild-type mice as compared to P2X7 $^{-/-}$ mice. Blockade of Pannexin-1 function or inhibition of P2X7R

antagonizes this effect. The negative modulation of muscarinic receptor (M1) activity is reported to be mediated via inositol-triphosphate (IP3) clearance and ATP release (Kim and Kang, 2011). These receptors also play an important role in development by controlling microglial proliferation in the mammalian spinal cord (SC) at an early embryonic stage. The proliferation of microglia occurs at the onset of motor neuron developmental cell death and during synaptogenesis in mouse embryo (E13.5). More importantly, the proliferation of embryonic spinal cord microglia, but not their activation state, depends almost entirely on P2X7R (Rigato et al., 2012). Increased level of P2X7R and its activation in the absence of any insult also drives the activation and proliferation of cultured microglial cells, supporting the notion that P2X7R pore formation has a trophic role in the brain (Monif et al., 2009). However, P2X7R driven microglial proliferation and activation has also been observed in many neurological and neuroinflammatory diseases (Rigato et al., 2012). It induces the proliferation of mouse embryonic cells during development, however, its activity needs to be suppressed for neuroectodermal differentiation and neuronal fate determination (Glaser et al., 2014). P2X7 activation leads to PKC and ERK1/2 induced neuronal differentiation in rat brain embryonic cells (Tsao et al., 2013). P2X7R is crucial to manage pain induction as it is observed that P2X7Rs present on satellite glial cells (SGC) exert the inhibitory control on the P2X3R expression and function in dorsal root ganglion (DRG) sensory neurons. Therefore, by regulating P2X7R expression, P2X3R activity can be effectively controlled and will be helpful in managing pain relief (Chen et al., 2012). In HEK293 basal activation of P2X7 receptor increases basal mitochondrial potential, mitochondrial calcium and intracellular ATP content providing the ability to the cell to grow even in the absence of serum. Basal level of P2X7R activation leads to increase in endoplasmic reticulum calcium, activates nuclear factor of activated T cells complex 1 (NFATc1) and prevent the cells against apoptosis (Adinolfi et al., 2009). Usually, P2X7R requires its pore-forming activity to activate cell growth, however, sustained stimulation leads to decrease in mitochondrial membrane potential, a huge rise in mitochondrial calcium and cell death (Adinolfi et al., 2005). These observations infer that other than a pro-apoptotic signal, P2X7R also acts as a growth-promoting factor and its dual role depends on the duration of stimulation of receptor (tonic or sustained). During bacterial

infections, activation of P2X7R prevents apoptosis of neutrophils acting as a host defense mechanism (Nagaoka et al., 2006). Its protective role is very well established during brain injury and inflammation. It is shown that activation of P2X7R on microglia activates the Toll-like receptor-4 (TLR4) which helps in clearance of cellular debris promoting recovery by increasing axonal outgrowth. This report is an evidence for the role of P2X7R in repairing mechanism after brain injury (Rajbhandari et al., 2014). Another crucial aspect to be studied is the role of P2X7R in neuron-glia communication in the brain via the release of ATP and other neurotransmitters.

1.4.1.7.2. P2X7R in neuron-glia communication

ATP acts as an extracellular signaling molecule for astrocytes-astrocyte or astrocyte-neuron crosstalk. It is released from cells by several mechanisms which include vesicular release, diffusion through plasma membrane channels (hemichannels such as pannexins, connexins and volume sensitive channel or P2X7 receptors), transporters or lysosomes (Lazarowski et al., 2003). Once released from the cell, ATP is rapidly degraded by numerous ectonucleotidase producing its derivatives ADP, adenosine monophosphate (AMP) and adenosine, which in turn also act as signaling molecules upon binding to various purinergic receptors (Yegutkin, 2008). Within neuron-glia network, ATP acts as a homomeric (neuron-neuron or glia-glia) or heteromeric (neuron-glia) neurotransmitter as shown in Figure 1.3 generating calcium waves (Guthrie et al., 1999). Within astrocyte network the calcium wave propagation occurs from stimulated astrocytes to the adjacent region by two pathways- 1) through the diffusion of the second messengers such as inositol-tri-phosphate (IP_3) via open gap junction channels (connexins) that connect adjacent astrocytes, or 2) through the release of ATP from stimulated astrocytes and the activation of purinergic (P2) receptors on adjacent astrocytes (Giaume and Venance, 1998). Binding of ATP to the P2Y receptors increases IP_3 which in turn releases Ca^{2+} from the endoplasmic reticulum (ER). Once released, Ca^{2+} activates pannexin-1 hemichannels allowing further release of ATP and propagation of signals to neighboring cells (Barbe et al., 2006). The gap junction and P2 receptor-mediated mechanisms are not mutually exclusive and are functionally

interconnected as demonstrated by Scemes et al. They demonstrated that in connexin knock-out mice the decrease in intracellular calcium is compensated by a switch in P2Y receptor subtype from P2Y1 to P2Y2 (Scemes et al., 2000). In fact, gap junction/connexin expression on astrocytes potentiate calcium wave propagation by enhancing ATP release (Cotrina et al., 1998) and integrating the calcium signals generated by activation of P2Y receptors (Suadicani et al., 2004). Within neuron-glia network, the synaptically active neuron release ATP either alone or with other neurotransmitters like glutamate, GABA and Acetylcholine (Jo and Schlichter, 1999; Burnstock, 2006; Zimmermann, 2008). The released glutamate binds to and activates the non-NMDA receptor on nearby astrocytes, increase intracellular calcium and triggers the release of ATP and other gliotransmitters from astrocytes (Butt, 2011). Once released, ATP binds to the purinergic receptors (A1, A2a and P2Y) at the pre-synaptic neurons and modulates synaptic transmission by decreasing excitatory glutamatergic transmission (Zhang et al., 2003; Koizumi and Inoue, 2004). In astrocytes, ATP is majorly released via the opening of Panx-1 to generate astrocytic Ca^{+2} waves. ATP-mediated Ca^{+2} waves are not only restricted to astrocytes but they also activate microglia by further release of ATP. The ATP thus released binds to and activates P2X7R on nearby microglia eventually leading to microglial apoptosis which was further potentiated by IFN-γ. The P2X7R activation also modulates the maturation and release of biologically active IL-1β from microglia cells (Guthrie et al., 1999; Verderio and Matteoli, 2001; Takenouchi et al., 2009). An evidence for the role of ATP in neuron-astrocyte-microglia signaling is demonstrated in dorsal root ganglion (DRG) where the electrical stimulation of DRG sensory neuron leads to the robust release of ATP from cell somata activating the P2X7 receptor in surrounding satellite cells (Zhang et al., 2007a). This subsequently leads to the release of TNF-α from astrocytes potentiating the P2X3 receptors mediated responses and enhances the excitability of DRG neurons (Chen et al., 2012). A smaller amount of ATP is also released by the activated microglial cell which is further amplified by astrocytes. Released ATP binds to neurons increasing excitatory postsynaptic current frequency through a metabotropic glutamate receptor-dependent mechanism. Thus, it is suggested that pathological

activation of microglia or astrocytes may influence synaptic transmission in neurological diseases (Pascual et al., 2012).

P2X7R has been associated with the release of glutamate, ATP, and GABA from the astrocytes (Sperlagh et al., 2002; Suadicani et al., 2006; Cho et al., 2010). Evidence suggest that P2X7R mediate the release of D-serine from astrocytes in a calcium-independent manner via pannexin -1 hemichannels providing the mechanism for activity-dependent neuron-glia interaction (Pan et al., 2015). Microglial P2X7R induces long-term potentiation of nociceptive response in the spinal neuron by up-regulation of GluR1 as inhibition of P2X7R by oxATP and BBG abrogate LTP induction and up-regulation of GluR1 *in vivo* and *in vitro* in spinal neurons (Chu et al., 2010) providing another mechanism for neuron-glia interaction.

1.4.1.7.3. P2X7 as a death receptor

ATP is released from damaged cells thus behaving as a danger signal. The prolonged activation of P2X7R results in cytoskeletal rearrangement, membrane blebbing, IL-1β release and activation of caspase- 3 ultimately leading to cell death. The P2X7 receptor is associated with the death of many cell types. It mediates apoptosis in response to elevations in intracellular calcium or mitochondrial dysfunction in different cell types including human leukaemia, neurons, neural progenitor cells and retinal ganglion cells (Hu et al., ; Parvathenani et al., 2003; Delarasse et al., 2009; Zhang et al., 2009; Hu et al., 2010; Sugiyama et al., 2010; Nishida et al., 2012). The role of the P2X7 receptor in mediating apoptosis makes it a major target for therapeutic intervention for neuroprotection.

1.4.1.8. P2X7R and NeuroAIDS:

Recently, purinergic receptors are emerging as the potential candidate for regulating different steps of HIV virus infection in the central nervous system. They are differentially involved in various stages of virus life cycle, ultimately causing neuronal

death thus participating in HIV neuropathogenesis. Various studies have been performed to explore the role of purinergic signaling in HIV infection in multiple cell types like CD4+ lymphocytes, macrophages and microglia which are known to be infected by HIV virus. Initial studies performed to investigate the role of purinergic receptors in neuroAIDS reported the essential role of ATP release for initial HIV infection. Studies by Seror *et al* showed that the HIV-1 infection leads to the release of ATP from the cells via pannexin hemichannels and this release is a pre-requisite for virus infection. Treatment with the ATP-degrading enzyme apyrase or inhibition of pannexin-1 hemichannels prevents HIV-1 infection and HIV-mediated cell death. Their study suggests that ATP released from infected cells acts on the P2Y2 receptor and activates pyk2 pathway thus providing the molecular basis for the role of ATP and its receptor in initial HIV infection (Seror et al., 2011). Another study recently reported by Schachter *et al* also supports the findings of Seror *et al* demonstrating impairment in viral infection, in the presence of ectoATPases which hydrolyze ectonucleotides thus reducing their concentration (Schachter et al., 2015). Apart from P2Y receptors, P2X receptors are also involved in HIV neuropathogenesis. It has been shown that P2X7R along with P2X1 and P2Y1 promotes viral replication in macrophage during HIV infection, however, only P2X1 receptor facilitates the viral entry in these immune cells suggesting the role of P2X7R in later stages of viral life cycle involving viral transcription and budding (Hazleton et al., 2012). Studies performed on another immune cell (CD4+ T-lymphocytes) also report the importance of P2X signaling in HIV infection but at a different stage, where the inhibition of purinergic receptors block the HIV infection at the stage of viral membrane fusion through cell-free or cell to cell contact in a dose-dependent manner (Swartz et al., 2014). ATP released from the infected macrophage regulates the glutamatergic tone and spine density on neuron indicating an effect of purinergic signaling on neuronal functions (Tovar et al., 2013). Neuronal functions are also altered by HIV viral protein Tat and drug abuse by activating microglia and consequently releasing extracellular ATP, thus, leading to synaptodendritic injury by acting on P2X4 receptor. Furthermore, the activation of P2X7 and P2X1 also leads to decreased neuronal survival (Sorrell and Hauser, 2014).

The role of purinergic receptors has been studied in monocytes, macrophages, microglia and neurons in HIV neuropathogenesis. It is striking that while the majority of the neuronal damage has been attributed to be through astrocytes and perturbation of neuron-glia interactions, till date investigations into the role of P2X7R in HIV-1 neuropathogenesis are lacking. Moreover, the functional interaction between neuron and astrocytes for neuronal survival and its important role in enhancing HIV-1 mediated toxicity makes it a major cell type to be studied in HIV neuropathogenesis. Given the background of the thesis, for this research endeavor, the role of astrocytic purinergic receptor P2X7R in HIV Tat-induced neuroinflammation and indirect neuronal damage were investigated in detail, using a novel primary culture system of human fetal brain-derived astrocytes and neurons.