The Dissertation Committee for Tetyana Buzhdygan Certifies that this is the approved version of the following dissertation:

Anomalies of GABAergic system associated with HIV-1 infection

Committee:

Benjamin Gelman, M.D., Ph.D.

Kenneth Johnson, Ph.D.

Volker Neugebauer, M.D., Ph.D.

Slobodan Paessler, D.V.M., Ph.D.

Walter Royal, III, M.D.

Dean, Graduate School

Anomalies of GABAergic system associated with HIV-1 infection

by

Tetyana Buzhdygan, M.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

2015

Anomalies of GABAergic system associated with HIV-1 infection

Publication No._____

Tetyana Buzhdygan, PhD

The University of Texas Medical Branch, 2015

Supervisor: Benjamin B. Gelman

Human immunodeficiency virus-1 (HIV) infection of the central nervous system results in cognitive and behavioral dysfunctions diagnosed as HIV-associated dementia. Findings of lowered concentrations of γ-Aminobutyric acid (GABA) synthesizing enzymes in brains of HIV-infected patients suggest impaired GABAergic inhibitory neurotransmission analogous to neurological and psychiatric disorders such as Parkinson's disease and schizophrenia. In this study I performed investigations into the neurochemical characteristics and neuropathological, neurocognitive and virological associations of dysfunctions of GABAergic inhibitory neurotransmission in HIV-infected brains using the resources of the National NeuroAIDS Tissue Consortium, including clinical data and brain tissue from 515 autopsy cases. Lowered GABAergic transcripts were linked to the higher expression of immune and endothelial cell type markers and were associated with the decrease of astroglial enzyme glutamine synthetase. No evidences were found that GABAergic anomalies are driven by replicating virus or associated with the highly active antiretroviral treatment, drug abuse history, or neuropathology of HIV encephalitis. Concentrations of GABA synthesizing enzymes were substantially reduced in all three populations of viable interneurons and associated with higher interneuronal dopamine receptor 2 (DRD2) expression. Investigation into regional distribution of GABAergic anomaly showed decrease of GABAergic transcripts in all cortical and subcortical brain regions. Low inhibitory tone in the prefrontal cortex was strongly linked to poor verbal fluency tasking, while in the anterior cingulate cortex it was associated with worse performance in broad spectrum of functional tests. Reduced concentration of GABA synthesizing enzymes in the anterior cingulate cortex results in the upregulation of astroglial-expressed GABA transporter 1 in glia limitans and abnormal increase of regional blood flow. Further investigations into the association between impaired inhibitory transmission and higher expression of endothelial cell markers revealed that damage to basal lamina and loss of pericytes observed in HIV-infected brain specimens was associated with the loss of GABAergic innervations of cortical microvessels. This dissertation study demonstrated that HIV infection is associated with abnormal GABAergic inhibitory transmission that likely represents a

process of synaptic plasticity. HIV-associated neurocognitive impairments were attributed to abnormal cognitive functionality that results from a complex failure to properly synchronize frontocortical output and to deactivate anterior cingulate region.

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ACC	anterior cingulate cortex
AIDS	acquired immunodeficiency syndrome
BBB	blood-brain barrier
BOLD-fMRI	blood oxygenation level-dependent functional magnetic resonance imaging
CALB2 mRNA	calbindin 2 mRNA (encodes for calretinin)
CBF	cerebral blood flow
CIDI	Composite International Diagnostic Interview
CNS	central nervous system
CR-IR	calretinin-immunoreacive
CSF	cerebrospinal fluid
cx36	connexin 36
DLPFC	dorsolateral prefrontal cortex
DMN	default mode network
DRD1	dopamine receptor type 1
DRD2	dopamine receptor type 2
DRD2L mRNA	dopamine receptor type 2 long isoform mRNA
FSTC	fronto-striato-thalamo-cortical circuit
GABA	γ-Aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAT-1	GABA transporter 1
GFAP	glial fibrillary acidic protein
GJD2 mRNA	gap junctions delta 2 mRNA (encodes for connexin 36)
gp120	glycoprotein 120
GS	glutamine synthetase
GZMB	granzyme B
HAART	highly active antiretroviral therapy

HAND	HIV-associated neurocognitive disorders
HIV-1	human immunodeficiency virus type 1
HIVE	HIV encephalitis
IRF1	interferon regulatory factor 1
ISG15	interferon stimulated gene 15
L-DOPA	(S)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid
nNOS	neuronal nitric oxide synthetase
NNTC	National NeuroAIDS Tissue Consortium
NO	nitric oxide
NPI	neuropsychological impairment
PARV mRNA	parvalbumin mRNA
PDGFRb	platelet-derived growth factor receptor beta
PECAM1	platelet/endothelial cell adhesion molecule 1
PENK mRNA	preenkephalin mRNA
PRISM	Psychiatric Research Interview for Substance and Mental Disorders
PV-IR	parvalbumin-immunoreactive
qRT-PCR	quantitative real-time polymerase chain reaction
SIV	simian immunodeficiency virus
SLC6A1 mRNA	solute carrier family 6 member 1mRNA (encodes for GAT-1)
SOM-IR	somatostatin-immunoreactive
tat protein	trans-activator of transcription protein
UNAIDS	Joint United Nations Programme on HIV and AIDS
VGAT	vesicular GABA transporter
vpr	viral protein R
VWF	Von Willebrand factor

Chapter 1: Introduction

1.1 NEUROAIDS AND HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

Neuropsychiatric complications of HIV-1 (human immunodeficiency virus) infection have become a major problem among population of HIV-positive patients. According to 2012 UNAIDS report, more than 36,2 million people are living with HIV-1 and more that 50% of them experience neuropsychiatric complications. Central nervous system (CNS) is protected by the blood-brain barrier (BBB), but HIV-1 virus enters the CNS early in the course of infection by means of infected peripheral monocytes (Kaul et al. 2001, Gonzales-Scarano et al. 2005), which then provide a source of further infection for brain macrophages, microglia, and astrocytes. Infected macrophages and microglia support high rates of viral replication and are main reservoirs of HIV-1 in the brain (Kaul et al. 2001). Astrocytes also are infected by HIV-1, although astroglial infection is not productive due to the lack of expression of HIV-1 structural proteins (Gorry et al. 2003). Nevertheless, infection of astrocytes could be detrimental because model systems have suggested that they are involved in damage to the neurovascular unit and blood brain barrier (Eugenin et al. 2006). Evidence of oligodendrocytes infection in vivo has been contradictory and inconclusive (Kramer-Hammerle et al. 2005). Neurons are not infected by HIV-1 but are susceptible to HIV-induced damage in the CNS. Viral proteins (gp120, tat, and vpr) and inflammatory cytokines that are released by infected macrophages, astrocytes and microglia, trigger abnormal regulation of neurotransmission and synaptic loss (Nath and Grieger 1998). Published reports suggest that protein turnover in the synapses (Nguyen and Gelman 2010, Gelman and Nguyen 2010) and specific neurotransmitter systems (GABAergic (Koutsilieri et al. 2001, Gelman et al. 2012b), dopaminergic (Nath et al. 2000, Koutsilieri et al. 2002, Gelman et al. 2006, Gelman et al. 2012a, Gelman et al. 2012b), cholinergic (Koutsilieri et al. 2001), glutamatergic (Ernst et

al. 2010), serotonergic (Murray 2003)) are impaired in the various brain regions of HIV infected patients.

Late stages of HIV infection are often accompanied by HIV encephalitis (HIVE) and mild to severe HIV-associated neurocognitive disorders (HAND) characterized by cognitive, behavioral and motor dysfunction (McArthur et al. 2005). The relationships between HIVE and HAND are complex. Almost 30% of HIV-infected patients with a diagnosis of HIVE at autopsy were cognitively unimpaired yet had no evidence of neuronal injury. Approximately 35% of HIV-infected patients without HIVE are cognitively impaired (Everall et al. 2009). Despite the fact that the introduction of highly active antiretroviral therapy (HAART) led to the successful suppression of virus replication, decreased HIVE and decreased incidence and severity of HAND (Kaul et al. 2001, Gonzales-Scarano et al. 2005, McArthur et al. 2005, Sacktor et al. 2002), the prevalence of HIV-associated neurocognitive impairments remains high. Brain viral load is not considered a pathological correlate of dementia, as association between the brain viral load and the severity of dementia is weak (Gelman et al. 2013). Elevated concentrations of neurofilament protein in the cerebrospinal fluid (CSF) that indicates axonal injury, was reported to predict the development of acquired immunodeficiency syndrome (AIDS) dementia complex (Gisslen et al. 2007).

Loss of dendrites in HIV-1 is observed widely throughout the brain. However, some brain circuits have selective vulnerability to HIV-induced damage and synaptodendritic injury of these circuits is specifically associated with poor performance in distinct cognitive tasks. The most vulnerable brain circuits in HIV-1 infection are cortico-cortical, fronto-striato-thalamo-cortical (FSTC), and limbic intrinsic/inhibitory circuit (Archibald et al. 2004, Ellis et al. 2007). Synaptodendritic loss in the FSTC was considered to be the biological correlate of HIV-induced neurocognitive impairment and is characterized by the impairments of speed of information processing, abstractive operations, decision making, attention deficit and verbal fluency (Masliah et al. 1998,

Ellis et al. 2007, Melrose et al. 2007). Notably, verbal fluency testing is an especially valid approach to examining the effect of HIV infection on brain functioning. It was shown that more than 40% of HIV infected individuals have abnormal functioning in verbal fluency tasking driven by switching impairments of two components – phonemic and semantic (Iudicello et al. 2007). Phonemic switching is associated with executive function and relies on frontal functioning; semantic switching is related to semantic memory and relies on medial temporal lobes function (Baldo et al. 2006). In HAND the semantic and phonemic fluency deficits are of similar magnitude which implies that frontal and temporal lobe functions both are impaired (Ludicello et al. 2007).

1.2 GABAERGIC INHIBITORY SYSTEM

Extensive evidence has accumulated that the GABAergic neural transmission is abnormal in people infected with HIV-1. γ-Aminobutyric acid (GABA) that is the major inhibitory neurotransmitter in the brain, is synthesized in the interneurons by the rate limiting enzyme glutamic acid decarboxylase (GAD) (Sibille et al. 2011, Soghomonian and Martin 1998). GAD has two isoforms – GAD65 and GAD67, which are products of two independently regulated genes GAD2, located on chromosomes 2, and GAD1, located on chromosome 10 respectively (Soghomonian and Martin 1998). *GAD2* mRNA is less expressed than *GAD1* mRNA; but GAD65 protein is 350% more abundant than GAD67 protein (Dracheva et al. 2004, Oh et al. 2012). GADs activity is regulated by phosphorylation – GAD65 is active when phosphorylated, whereas GAD67 is inactive in phosphorylated state (Wei and Wu 2008). Each isoform has a distinct pattern of intracellular and cellular localization: GAD67 is widely distributed in the cytoplasm of tonic or regular spiking neurons and synthesizes cytoplasmic GABA; GAD65 is located in axon terminals of phasic or bursting neurons and synthesizes GABA for vesicular

release (Soghomonian and Martin 1998, Akbarian and Huang 2006, Wei and Wu 2008, Oh et al. 2012).

The primary precursor for interneuronal GABA synthesis is glutamine (Sonnewald et al. 1993, Rae et al. 2003). Astrocyte processes remove glutamate from the synaptic cleft and convert it to glutamine by the enzyme glutamine synthetase (GS). Then glutamine is transported to presynaptic terminals, and is used as the main precursor to interneuronal GABA synthesis by glutamic acid decarboxylase (Sonnewald et al. 1993, Rae et al. 2003). Dysregulation of GS and a deficit of glutamine supplies both can produce a shutting down of GABA expression (Ortinski et al. 2010) and a lower concentration of interneuronal GAD enzymes (Struzynska et al. 2004). The expression, distribution, and activity of brain GS is altered in several brain disorders, including Alzheimer's disease, schizophrenia, depression, and temporal lobe epilepsy (Papageorgiou et al. 2011, Eid et al 2004, Hasler et al. 2007, Ortinski et al. 2010, Eid et al. 2011, Eid et al. 2012). Overproduction of oxygen or nitrogen-reactive species and inflammatory stimuli (e.g. lipopolysaccharide, interferon γ, HIV-1 coat protein gp120) were reported to result in the dysregulation of astroglial GS (Muscoli et al. 2005, Janda et al. 2011).

The cortical inhibitory system consists of various types of interneurons, which differ in their axonal and dendritic morphology, neuropeptide contents, laminar distribution, electrophysiological properties and synaptic targeting (Wei and Wu 2008, Xu et al. 2004, Blatow et al. 2005). The most widely accepted division of interneurons is based on expression of one of the Ca2+ binding proteins (Gonchar and Burkhalter 1997, Kubota et al. 1994, Kubota et al. 2011, Blatow et al. 2005). Three non-overlapping populations of interneurons are parvalbumin-, calretinin- and somatostatin-immunoreactive cells (Sibille et al. 2011, Gonchar and Burkhalter 1997, Kubota et al. 1994). Each of these populations has distinct morphological and physiological features (Benes and Berretta 2001, Blatow et al. 2005, Kawaguchi and Kondo 2002).

Parvalbumin-immunoreactive (PV-IR) interneurons ("fast spiking cells") regulate the generation of γ and θ oscillations and play a crucial role in cognitive functioning (Priest et al. 2001, Curley and Lewis 2012, Sohal et al. 2009). PV-IR interneurons consist of two large morphologically distinct types of interneurons – chandelier and basket cells. Chandelier cells are found mostly in neocortical layers II and III (Benes and Berretta 2001, Inda et al. 2007), where they form symmetric synapses exclusively with the axon initial segment (AIS) of pyramidal cells. Terminal portions of their axons contain GABA transporter 1 (GAT-1, Conti et al. 1998, Inda et al. 2007) which rapidly removes extracellular GABA, terminates inhibitory currents and prevents neurotransmitter spillover. Blocking of GAT-1 activity experimentally leads to stronger phasic inhibition and reduced neocortical recurrent activity (Razik et al. 2013). Alteration of GAT-1 mRNA and protein expression is linked to cognitive impairment associated with schizophrenia (Lewis et al. 2001, Woo et al. 1998, Volk et al. 2001, Sundman-Eriksson et al. 2002) and aging (Cruz et al. 2003, Sundman-Eriksson and Allard 2006). The basket cells are PV-IR neurons found mostly in neocortical layers III-V. They form very dense vesicular GABA transporter (VGAT)-immunoreactive terminals ("Complex basket formations" or "Cbk-formations") on the somata and proximal dendrites of pyramidal cells (Markram et al. 2004, Blazquez-Llorca et al. 2010). Neocortical Cbk formations are the primary source of lateral inhibition (Melchitzky et al. 1999). Dysfunction of basket cells is associated with the clinical manifestations of schizophrenia (Beasel and Reynolds 1997, Beasley et al. 2002, Lewis et al. 2005, Lewis et al. 2012). A reduced level of mRNA encoding for parvalbumin, without evidence of interneuron cell loss, was reported in the frontal neocortex of patients with schizophrenia (Woo et al. 1997, Cotter et al. 2000, Hashimoto et al. 2003) and bipolar disorder (Sibille et al. 2011). In other studies, total cortical density of parvalbumin-expressing interneurons was reduced in the same brain sectors of schizophrenics (Beasley and Reynolds 1997, Beasley et al. 2002).

Calretinin-immunoreactive interneurons have two distinct morphological types – bipolar and double bouquet cells. Bipolar cells are mostly localized in layers II-VI and form synapses with the basal dendrites of pyramidal neurons. Double bouquet cells are located in the supragranular layers and form synapses with spines and shafts of pyramidal neurons (Markram et al. 2004, DeFelipe et al. 2006). Like chandelier cells double bouquet cells are not reached by thalamo-cortical afferents and their main source of excitatory input is from cortico-cortical fibers (Benes and Berretta 2001). Calretinin-immunoreactive interneurons can exert a significant disinhibitory effect on the output of pyramidal neurons (Gonchar and Burkhalter 1999), and function as a gate that switches the flow of informational processing between several pathways. Loss or damage to the calretinin-expressing population of interneurons is not a characteristic feature for neurocognitive or psychiatric diseases. Populations of calretinin-expressing interneurons are not abnormal in the dorsolateral prefrontal and anterior cingulate cortices of patients with schizophrenia (Cotter et al. 2002, Beasley et al. 2002) or major depressive disorder (Oh et al. 2012). The invulnerability of this type of interneuron, which also was observed in my study, may be due to properties of the calretinin molecule itself, which was reported to be potentially neuroprotective (Diop et al. 1996).

Somatostatin-immunoreactive interneurons also have two morphological types – bitufted cells and Martinotti cells. Bitufted cells are found in layers II-VI. Their axons project to proximal columns in wide horizontal spans and form synapses with the dendrites of pyramidal neurons. Martinotti cells are unique in that they provide the only source of cross-columnar inhibition via layer I from layers II-VI. They form synapses with the distal, proximal and perisomatic dendrites, as well as with somata of pyramidal neurons (Markram et al. 2004). Somatostatin was found to be downregulated at the level of mRNA and protein expression in the DLPFC (Sibille et al. 2011) and in the ACC (Tripp et al. 2011) of subjects with major depressive disorder. Lowered somatostatin

concentration in the cerebrospinal fluid is also associated with the episodes of depression (Post et al. 1988).

The heterogeneous populations of GABAergic interneurons outlined above are extensively interconnected by electrical synapses (also called gap junctions), which provide circuit-level regulation of synchronized oscillatory currents. These neocortical oscillations can be fast – like θ (4-12 Hz) and γ (20-70 Hz) or ultrafast (in the range of 100-600 Hz). Neocortical fast and ultrafast rhythms underlie neuronal processing, sensory perception, motor performance, learning, attention, and memory consolidation (Bennett and Zukin 2004). Gap junctions or electrical synapses are specialized membrane regions that contain transmembrane channels called connexons, which directly connect the cytoplasm of adjacent cells (Bennett and Zukin 2004) and form synchronized networks of interneurons. The proteins that form the connexons are called connexins. Connexin 36 (cx36) is of great importance to the inhibitory circuitry because it is expressed exclusively by the PV-IR interneurons (Galarreta and Hestrin 1999, Galarreta and Hestrin 2001, Hestrin and Galarreta 2005, Cruikshank et al. 2005), which form gap junctions exclusively with the interneuron belonging to the same type (Bennett and Zukin 2004). Abnormal connexin 36 expression results in the loss of electrical coupling between interneurons and leads to the disruption of cortical synchronized oscillations (Ma et al. 2011).

1.3 GABAERGIC NEUROTRANSMISSION IN DISEASE

Neurocognitive and behavioral impairments in several diseases have been associated with abnormal GABAergic neurotransmission, including Parkinson's disease, epilepsy, bipolar disorder, schizophrenia, and drug abuse (Volk et al. 2000, Wong et al. 2003, Dracheva et al. 2004, Akbarian and Huang 2006, Thompson et al. 2009, Sibille et al. 2011, Rossignol 2011, Chattopadhyaya and Di Cristo 2012, Oh et al. 2012). Several research reports have suggested that expression of GABAergic transcripts also is

abnormal in brain specimens from patients who were infected with HIV-1 (Koutsilieri et al. 2001, Gelman et al. 2012b). To date, few studies attempted to elucidate the fate of GABAergic cells in HIV infected patients. A study in patients with end-stage AIDS prior to the era of HAART showed that the neocortical cell bodies and neuronal endings of parvalbumin-immunoreactive interneurons were fragmented, although the density of neurons in tissue sections was not significantly reduced (Masliah et al. 1992). Other studies prior to HAART reported decreased densities of somatostatin– and calbindin-immunoreactive interneurons in the frontal neocortex of HIVE patients (Masliah et al. 1995, Fox et al. 1997).

1.4 GABAERGIC AND DOPAMINERGIC INTERPLAY

GABAergic and dopaminergic neurotransmitter systems overlap with each other and are potentially interactive (Seamans et al. 2001). Dopamine receptors are present in neocortical layers II-VI, with the highest density found on large interneurons and small pyramidal neurons in layers V and VI (Vincent et al. 1993). Among all interneuronal subpopulations, the fast spiking PV-IR population of interneurons has been shown to be most highly dopaminoceptive (Gorelova et al. 2002). Dopamine modulation of GABAergic inhibition of cortical pyramidal neurons can occur both pre- and postsynaptically. Presynaptically, dopamine activates either dopamine receptor type 1 (DRD1) or DRD2. Acting on the DRD1, dopamine induces excitation of inhibitory interneurons followed by increased inhibition of the pyramidal cells (Seamans et al. 2001). Only higher concentrations of dopamine activate DRD2, inducing the inhibition of inhibitory interneurons followed by decreased inhibition (disinhibition) of pyramidal cells (Seamans and Yang 2004). Disinhibition of pyramidal cells leads to spontaneous activation of unstable cortical networks, disrupted information processing, production of prefrontal noise and cognitive dysfunction (Trantham-Davidson et al. 2004, Li et al. 2011). Postsynaptically, dopamine can modulate GABAergic responses in cortical

pyramidal neurons by the regulation of GABAa receptor excitability on pyramidal neurons (Seamans and Yang 2004). Disruption of dopaminergic neurons in the midbrain is associated with the altered *GAD1* mRNA expression in the frontal cortex (Gorelova et al. 2002). Abnormal cortical and mesocortical dopaminergic innervations of GABAergic interneurons often occurs in neuropsychological disorders such as schizophrenia and Parkinson's disease (Akil et al. 1999, Gorelova et al. 2002, Seamans and Yang 2004). Although, patients with HAND have many clinical signs similar to those in patients with Parkinson's disease, which is the prototypal disturbance of dopaminergic neurotransmission: apathy, bradyphrenia, poorly articulated speech (Koutsilieri et al. 2002); the interactions between GABAergic and dopaminergic neurotransmitter systems in HIV remains unknown. Increased dopamine availability (using L-DOPA) in the presence of retroviral infection (shown on SIV-infected monkeys) is often associated with the induction of neuropathology, rather than cognitive rescue (Koutsilieri et al. 2002). Downregulation of *DRD2L* mRNA expression in the prefrontal cortex of HIV-1 infected patients is associated with better neurocognitive and neuropathological outcome, while those with unchanged or a slightly upregulated level of DRD2L performed worse in neurocognitive testing (Gelman et al. 2012).

1.5 GABAERGIC SYSTEM AND CEREBRAL BLOOD FLOW

Neurovascular coupling or functional hyperemia is the process of adjustment of blood supply to neurons, depending on their activity level, to appropriately respond to changing metabolic demands. GABAergic interneurons play a central role in regulating neurovascular coupling (Choi et al. 2006, Kocharyan et al. 2008, Radhakrishnan et al. 2011). Axons, dendrites and somata of interneurons establish direct contact with blood vessels (Duchemin et al. 2012) and perivascular astrocytes (Vaucher et al. 2000). They regulate cerebral blood flow by releasing nitrous oxide (NO) or GABA, which diffuses to the blood vessel walls and produces vasodilation (Duchemin et al. 2012). Direct

stimulation of the basal forebrain activates interneurons in the layers I to VI and produces increased blood flow (Kocharyan et al. 2008). At the circuit level, GABAergic inhibition plays important role in the regulation of blood flow associated with the functioning of the default mode network (DMN) (Northoff et al. 2007, Hu et al. 2013, Krause et al. 2014). The DMN is a network of brain regions (anterior and posterior cingulate, medial prefrontal cortices, amygdala and hippocampus) that is deactivated during task performance and is activated in the resting brain (Raichle et al. 2001). Failure to properly deactivate DMN while on task is associated with abnormal global blood flow and worse task performance (Weissman et al. 2006) in patients with Alzheimer disease (Rombouts et al. 2005), schizophrenia (Garrity et al. 2007, Pomarol-Clotet et al. 2008), and aging (Sambatoro et al. 2010). Deactivation of the DMN is directly dependent on the regional concentration of GABA (Northoff et al. 2007, Hu et al. 2013, Krause et al. 2014). As in the other diseases mentioned above, blood flow measured using fMRI BOLD cerebral blood flow also has been found to be abnormal in HIV infected patients. The mechanism of the abnormal cerebral blood flow in HIV-infected patients, which occurs with or without HAND, is not known (Cauli et al. 2004, Ances et al. 2011, Towgood et al. 2013). In my studies I explored the possibility that anomalies of GABAergic transmission (Giourard and Iadecola 2006, Del Zoppo 2010) may be associated with HIV-associated changes in cerebral blood flow (Persidsky et al. 2006).

1.6 HYPOTHESIS AND EXPERIMENTAL DESIGN

Neuronal loss, synaptodendritic simplification (Masliah et al. 1997, Masliah et al. 2004), and alterations in synaptic protein turnover (Nguyen and Gelman 2010, Gelman and Nguyen 2010) have been suggested to occur in later stages of HIV-induced neuropathology. Several neurotransmitter systems are impaired including dopaminergic (Nath et al. 2000, Koutsilieri et al. 2002, Gelman et al. 2006, Gelman et al. 2012a, Gelman et al. 2012b), cholinergic (Koutsilieri et al. 2001), glutamatergic (Ernst et al.

2010), serotonergic (Murray 2003), and inhibitory GABAergic (Koutsilieri et al. 2001, Gelman et al. 2012b). The neurochemical mechanisms of GABAergic anomalies and their impact on brain circuits of in HIV-infected patients remain poorly understood. The overall objective of my thesis work was to fully characterize the GABAergic system in the CNS of HIV-infected patients in order to gain better understanding of the mechanism of neurocognitive impairment associated with HIV-1 infection. The main hypothesis was that HIV infection is associated with the decreased expression of GABA synthesizing enzymes in a viable population of interneurons, resulting in neuronal dysfunction and dysregulation of cerebral blood flow. To test this hypothesis I evaluated biochemical and microanatomical features of GABAergic system in the neocortex of HIV-infected and control subjects and correlated these differences with neuropsychological, neuropathological, neurovirological, neuroimmune and neurovascular effects of HIV infection. To accomplish this I made extensive use of human brain specimen resources of the National NeuroAIDS Tissue Consortium. The following hypotheses were addressed:

Hypothesis 1: HIV-1 infection is associated with the downregulation of GABAergic transcripts expression in the cortical and subcortical regions.

Brain gene arrays showed that transcripts of the GABAergic system are abnormal in the patients with HIV encephalitis and HAND (Gelman et al. 2013). However, many variables, which can affect GABAergic anomalies in HIV-infected patients, were not accounted for (such as, HIVE-neuropathology that often alters synaptic transmission (Masliah et al. 1997, Gelman et al. 2004, Gelman et al. 2012); HAART medications, which affect neurochemistry directly by interacting with neurotransmitter systems and indirectly by suppressing viral replication; and substance abuse that often leads to altered GABAergic neurotransmission (Zhang et al. 2006, Wadleigh et al. 2012). Therefore, I proposed to characterize the relationships between GABAergic anomalies and several

aspects of HIV infection including clinical virology, neuropathology, endothelial and immune cell markers, drug abuse history, and neurocognitive impairment. I further proposed to determine whether there is a characteristic anatomical pattern or regional distribution of abnormal GABAergic transmission in the CNS. To test this hypothesis, I examined the level of multiple GABAergic transcripts and proteins expressed in brain specimens from a robust sampling of HIV infected patients and uninfected comparison patients. The studies illustrated that lower expression of GABAergic transcripts occurs widely in the CNS and is related to the elevated concentrations of neuroimmune and neurovascular markers.

Hypothesis 2: HIV-associated downregulation of expression of GABA synthesizing enzymes occurs in the population of viable cortical interneurons.

Different neuronal populations show selective vulnerability to HIV-induced damage and the fate of GABAergic interneurons in HIV-infected brain remains controversial. The question whether low GABA synthesis reflects the death of inhibitory interneurons due to classical neurodegeneration remains unknown. I have suggested the alternative view and hypothesized, that GABAergic mRNAs are transcriptionally downregulated in viable inhibitory neurons that undergo changes mediated by the process of synaptic plasticity. To test this hypothesis, I proposed to determine whether particular subpopulation of interneurons is selectively vulnerable in HIV and to assess the GAD67 expression in specific types of interneuronal subpopulations. I examined each subpopulation of interneurons by assessing the concentration of transcripts and the intensity of immunostaining of the specific interneuronal markers, and determined GAD67 expression in interneurons by dual staining using GAD67 and subpopulational markers simultaneously. The studies illustrated that lower staining for GAD67 the HIV infected patients occurs in viable interneurons.

Hypothesis 3: The concentration and subcellular distribution of glutamine synthetase in the cortical astrocytes is altered in the HIV-1.

Astroglial enzyme glutamine synthetase plays a critical role in the metabolism of GABA and glutamate. Dysregulation of GS results in reduced synthesis of GABA (Ortinski et al. 2010) and lower interneuronal GAD concentrations (Struzynska et al. 2004). The expression, distribution and activity of brain GS in HIV remains unknown. I proposed to examine the concentration and subcellular distribution of the astroglial glutamine synthetase and to examine whether it is involved in the lowering of GADs concentration in HIV-infected patients. To address that question I measured the concentration and analyzed the intracellular distribution of GS in the brain specimens of HIV-positives and compared the results to their lower *GAD1* expression. The studies illustrated that decreased level of interneuronal *GAD1* mRNA is associated with the lower astroglial GS expression in the HIV-infected brains.

Hypothesis 4: Low GABA is related to the altered plasticity of dopaminergic transmission in HIV infection.

GABAergic and dopaminergic neurotransmitter systems overlap with each other and are functionally interactive (Seamans et al. 2001). Heightened expression of *DRD2L* mRNA was reported in prefrontal cortex of neurocognitively impaired HIV infected patients (Gelman et al. 2012b). Those neurochemical findings are thought to represent altered synaptic plasticity. To further explore the concept that synaptic plasticity is the driving force of neurochemical changes in HIV infection, I proposed to examine whether dopamine-activated DRD2 on local GABAergic interneurons are potential cause of decreased *GAD1* mRNA expression. To address this hypothesis, I determined whether overexpression of DRD2 observed in the cohort of cognitively impaired HIV-patients is associated with higher expression of DRD2 in GABAergic interneurons and whether it is related to downregulation of GABA synthesizing enzymes. The studies illustrated that

DRD2 immunoreactivity is substantially higher on small pear-shaped interneurons in HIV-infected patients and that higher *DRD2L* mRNA expression was associated with lower level of *GAD1* mRNA.

Hypothesis 5: Low GABA is linked with altered regulation of GABA transport by astrocytes.

Neocortical GABA transporter 1 (GAT-1) powerfully controls inhibitory synaptic tone by rapidly removing extracellular GABA, terminating inhibitory current and preventing neurotransmitter "spillover" from the synaptic cleft. Upregulation of glial GAT-1 expression was reported in several disorders and is thought to be the compensatory mechanism for the loss of inhibitory neurons (Sundman-Eriksson et al. 2002). Loss of function of inhibitory neurons is likely to occur in HIV-associated cognitive impairment, but the question whether GAT-1 transport is abnormal in the cortical regions of patients with HIV-associated neurocognitive impairments has not been addressed yet. I proposed to examine whether the reduction of neuronal GADs expression is associated with changes in GABA transport. To address that question I examined the concentrations, cellular distribution, association with the *GAD1* mRNA, and clinical relevance of GAT-1 mRNA and protein expression in cortical regions of HIV-infected patients. The studies revealed that lower *GAD1* mRNA concentration was linked to the higher level of mRNA encoding for GAT-1 and that heightened GAT-1 expression co-localized within GFAP-stained astroglial processes in the glia limitans.

Hypothesis 6: Low GABA is related to changes in key components of the neurovascular unit in HIV infected patients

HIV-1 infection in CNS often results in the damage to the neurovascular unit and blood brain barrier, elevated concentrations of endothelial markers in blood plasma (Eugenin et al. 2006), and abnormal cerebral flood flow (Ances et al. 2009, Towgood et al. 2013) GABAergic interneurons are critically involved in the regulation of

neurovascular unit (Vaucher et al. 2000, Cauli et al. 2004). My preliminary data suggest that lower GABAergic mRNAs in brain cortex are strongly related to abnormally high concentrations of endothelial type mRNAs in the brain. The associations between lowered concentration of GABAergic transcripts and abnormal regulation of the neurovascular unit in HIV have not been described yet. Therefore, I proposed to examine whether low GABA is related to changes in components of the neurovascular unit in HIV infected patients. To address that issue I examined the GABAergic innervations of blood vessels and morphology of capillaries and pericytes in the selected cortical regions of HIV-infected patients. The studies revealed the loss of GABAergic innervations, the damage of the basal lamina and loss of pericytes on the cerebral microvessels in the specimens from HIV-infected patients.

Chapter 2: Materials and Methods

2.1 HUMAN SPECIMEN ACCRUAL

Postmortem brain tissue was obtained from HIV-1 infected subjects who volunteered to participate in the National NeuroAIDS Tissue Consortium (NNTC). The NNTC is composed of four academic medical centers in the USA (Morgello et al. 2001), engaged in an HIV brain banking project, as follows: The University of Texas Medical Branch (UTMB, Galveston, TX), Mount Sinai Medical Center (New York, NY), The University of California, Los Angeles (UCLA), The University of California, San Diego (UCSD). The HIV infected subjects in this study underwent longitudinal testing of neuropsychological and neurological functions, clinical neurovirological and immunological monitoring, documentation of substance use, and collection of basic neuromedical information at six months intervals. Blood, cerebrospinal fluid (in consenting patients), and urine samples were collected. The blood plasma and CSF both were assayed for the HIV-1 RNA concentration using methods described below. A CD4 lymphocyte count was done using an automated analyzer in the clinical laboratories of the four participating medical centers of the NNTC.

2.2 STUDY SUBJECTS

Total of 515 frozen human brain samples were obtained from the National NeuroAIDS Tissue Consortium (NNTC) (Morgello et al. 2001, Gelman et al. 2012a). 449 out of 515 were HIV-1 infected patients and 66 out of 515 were demographically matched HIV-1 seronegative controls. 357 out of 449 HIV-1 infected patients had HIV encephalitis (HIVE). 161 out of 449 HIV-1 infected patients had HIV-associated neurocognitive disorders (HAND) (Table 2.1). Dissected dorsolateral prefrontal cortical

tissue (DLPFC) was analyzed to measure GABAergic transcripts expression and to correlate it with brain, CSF and plasma viral load, immune and endothelial cell transcripts, and cognitive tests normalized T scores.

Detailed examination of GABAergic neurotransmission in the brain regions of interest, as indicated in the figures legends and tables, was performed on smaller subset of 48 brain specimens selected from larger NNTC cohort described above. 12 out of 48 were HIV-1 seronegative controls and 36 out of 48 were HIV-1 infected subjects. 36 HIV-1 infected subjects were selected from the described above group of 449 HIV-1 positives. HIV-1 infected subjects were included based on the following selection criteria: age, gender, postmortem interval, and concentration of *GAD1* transcript expressed in the DLPFC. The averages for all of these selection criteria reflect those of larger group of 449. An additional criterion was HIVE diagnosis, in order to examine how GABAergic neurotransmission changes in the brain with HIV-encephalitis. 12 out of 36 selected HIV-positives had neuropathological features characteristic of HIV-encephalitis. Special emphasis was also placed on HIV-1 infected subjects with neuropsychological impairment (NPI) or HIV-associated neurocognitive disorders (HAND) in the small panel. 24 out of 36 selected HIV-positives were diagnosed with NPI or HAND; 12 out of 36 were HIV-1 infected without signs of neurocognitive impairment. 12 controls were selected from the described above group of 66 HIV-1 seronegative subjects. Control subjects were included based on the same selection criteria: age, gender, postmortem interval, and concentration of *GAD1* transcript expressed in the DLPFC. The averages for every selection criteria represent those of larger group of 66 controls (Table 2.2).

2.3 NEUROCOGNITIVE TESTING AND SUBSTANCE USE SURVEY.

318 out of 449 HIV-positives died in the Highly Active Antiretroviral Therapy (HAART) era (from 1998 to 2011). 219 out of those 318 were participants of the

longitudinal study where neuropsychological evaluations and substance use surveys using NNTC protocol were performed every 6 months to document the development and progression of HIV-associated neurocognitive decline. The NNTC neurocognitive test battery included assessment of: 1) speed of information processing (Wechsler adult intelligence scale); 2) abstract and executive functioning (Wisconsin card sorting test-64; 3) verbal fluency (F-A-S test); 4) motor functioning; 5) learning and memory functioning (Hopkins verbal learning and brief visuospatial memory tests; 6) attention and working memory (paced auditory serial addition test); 7) memory and intellectual level (wide range assessment test-3) (Woods et al. 2004). Seven component neurocognitive T scores and a composite T score were obtained and normalized according to the age-adjusted norms (Woods et al. 2004). Annually patients were given the Psychiatric Research Interview for Substance and Mental Disorders (PRISM) or the Composite International Diagnostic Interview (CIDI) used to obtain self-reported lifetime histories of substance abuse and dependence (Morgello et al. 2006, Robins et al. 1988).

2.4 HANDLING AND STORAGE OF BRAIN TISSUE

Postmortem brain was collected during the autopsy procedure according to the NNTC protocol. The whole brain was sliced in the sagittal plane into two hemispheres. One hemisphere was then cut in the coronal plane in 1 cm thick slices. Fresh cut slices were frozen flat on a copper plate at – 80 °C. After freezing the slices were placed in labeled plastic bags and stored at – 80 °C. The opposite hemisphere was immersed in 20 % formalin solution at 7 °C for 10 days and then was sliced into coronal sections. Selected brain tissue blocks from predetermined regions were cut from formalin-fixed tissue, trimmed, embedded in paraffin wax and stored in ambient temperature. The neuropathological diagnoses were obtained using the brain sampling and staining protocols of the NNTC (Morgello et al. 2001). The neuropathologists at the four NNTC clinic sites obtained diagnoses, and intrasite variation was monitored over a period of 16

years in a periodic quality control program that was maintained by the NNTC, The diagnosis of HIV encephalitis (HIVE) was made according to established criteria (Budka et al. 1991, Gelman et al. 2013).

2.5 ASSAY OF HIV RNA IN HUMAN BRAIN SPECIMENS

For RNA extraction 100-150 mg of grey matter from the dorsolateral prefrontal cortex in Brodmann area 9 or 8 was dissected from fresh frozen brain tissue and RNAeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) was used to isolate RNA. Protocol by Palmer (Palmer et al. 2003) was used for single copy detection of the HIV-1 RNA. Single strand cDNA was prepared using Bio-Rad iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). For cDNA preparation, 1 ug of brain mRNA was mixed with 4 ul of 5x iScript reaction mix and 1 ul of iScript reverse transcriptase. Total volume was adjusted to 20 ul with nuclease-free water. The reaction mixture was incubated in the Bio-Rad I-cycler programmed for 5 min at 25 ^{0}C, 30 min at 42 ^{0}C, 5 min at 85 ^{0}C and held at 4 ^{0}C. For real-time quantitative PCR, 4 µl of cDNA were added to 25 ul JumpStart Taq ReadyMix (Sigma Aldrich, Saint Louis, MO) containing primer-probe set for HIV-1 quantification designed to bind to a conserved region of gag: 84R (5-TGCTTGATGTCCCCCCACT-3) (1 uM) and FAM probe. PCR reactions were performed on Mastercycler RealPlex (Eppendorf, Germany) under the following conditions: activation (10 min at 95 ^{0}C), and 40 cycles of amplification (each cycle consisting of 15 sec at 95 ^{0}C for annealing and 60 sec at 60 ^{0}C for extension). Each specimen was run in duplicates. To build standard curve threshold cycle (Ct) values from diluted HIV-1 standards were plotted on the ordinate as a function of the input transcript copy number and linear regression was performed. The number of copies of HIV-1 in the test specimen was determined by interpolation of experimentally obtained Ct to the standard curve. Number of copies in the specimen was expressed as the log10 of copies per mg of wet weight of brain tissue.

2.6 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION OF MRNAS FROM HUMAN BRAIN SPECIMENS

For studies that focused on frontal lobe neocortex, about 100 mg of frozen grey matter from the dorsolateral prefrontal cortex in Brodmann area 9 or 8 was dissected for mRNA extraction. Other brain regions were dissected for regional comparisons as indicated in the figures legends and tables. Tissue specimens from all the regions were homogenized in 1 ml of QIAzol reagent (RNeasy Mini Kit, Qiagen, Valencia, CA) and isolation of total RNA was performed according to the manufacturer's protocol. Single strand cDNA was prepared using Bio-Rad iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). 2 ug of brain mRNA were mixed with 8 ul of 5x iScript reaction mix, 2 ul of iScript reverse transcriptase and total volume was adjusted to 40 ul with nuclease-free water. The reaction mixture was incubated in the Bio-Rad I-cycler programmed for 5 min at 25 ^{0}C, 30 min at 42 ^{0}C, 5 min at 85 ^{0}C and held at 4 ^{0}C. For quantitation of the mRNA primers and FAM probes were obtained for *GAD1* (Cat. No. Hs01065893_m1), *GAD2* (Cat. No. Hs00609534_m1), *GJD2* (Cat. No. Hs00706940_s1), *PVALB* (Cat.No.Hs00161045_m1), *CALB2* (Cat. No. Hs00242371_m1), *SST* (Cat.No. Hs00356144_m1), *SLC6A1* (Cat.No. Hs01104475_m1), *DRD2L* (Cat.No. Hs01024460_m1,), *ISG15* (Cat. No. Hs00192713_m1), *MX1* (Cat.No. HS00182073_m1), *IRF1* (Cat.No. Hs00971959_m1), *GZMB* (Cat.No. Hs01554355_m1), *CD4* (Cat.No. Hs01058407_m1), *CD8A* (Cat.No. Hs01555600_m1), *CD19* (Cat.No. Hs99999192_m1), *CD68* (Cat.No. Hs00154355_m1), *CD163* (Cat.No. Hs01016661_m1), *VWF* (Cat.No. Hs00169795_m1), *PECAM* (Cat.No. Hs00169777_m1), from Applied Biosystems, Foster City, CA, USA), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, Cat. No. Hs99999905_m1) was used as the normalizing gene. For a 20 ul reaction mixture 1 ul of cDNA was mixed with 10 ul of TaqMan 2x PCR Master Mix (Applied Biosystems), 8 ul H_2O and 1 ul ABI 20x primer (gene of interest or *GAPDH*). PCR reactions were performed on Mastercycler RealPlex (Eppendorf, Germany) under the following

conditions: activation (10 min at 95 ^{0}C), and 40 cycles of denaturation (15 sec at 95 ^{0}C) – annealing/extension (60 sec at 60 ^{0}C). Experiments were performed on 96-well plates, each sample was run in duplicates, triple negative controls and standard calibrators were run on each plate. Duplicate C_T values were analyzed using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems). The amount of targets ($2^{-\Delta\Delta C_T}$) was obtained by normalizing to endogenous reference (*GAPDH*).

2.7 WESTERN BLOTTING OF HUMAN BRAIN PROTEINS

200-300 mg of frozen grey matter from the dorsolateral prefrontal cortex in Brodmann area 9 or 8 was dissected for protein extraction. Other brain regions were dissected for regional comparisons as indicated in the figure legends and tables. Dissection was performed on dry ice. Frozen samples maintained on dry ice were placed in pre-weighed and pre-cooled vials and stored at – 80 ^{0}C. Tissue weight was measured and recorded. Specimens were assayed in batches that were freshly thawed and homogenized in 3x volume of buffer (10 mM Tris-HCl, 5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 0.03 % Triton X-100, pH 7.8) by silica bead beating (2 times for 20 seconds) and sonication (2 times for 20 seconds) with tubes kept on ice for 5 minutes between sessions. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) standards. 15 ug of total protein in 15 µL volume was added to 15 µL of 2x Laemmli Sample Buffer (Bio-Rad Laboratories) with 5 % beta-mercaptoethanol in 0.5 mL microcentrifuge tubes and boiled for 5 min. Samples were loaded into Criterion Precast 18-well 4-20 % gradient Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and run at 180 volts for 45 minutes. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ) in 10 mM Tris-glycine buffer containing 10% methanol at 75W for 1 hour at 4 ^{0}C. Membrane was blocked in 5 % non-fat milk in Tris-

buffer saline with Tween-20 (TBS-T; 50 mM Tris-HCl, 150 mM NaCl, 0.01 % Tween-20) for 1 hour and incubated with mouse anti-GAD67 (Cat. No. ab22050, Abcam, Cambridge Science Park, Cambridge, England) diluted 1:1000, mouse anti-GAD65 (Cat.No. sc-73650, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted 1:500, rabbit anti-glutamine synthetase (Cat.No. ab49873, abcam) diluted 1:10,000, rabbit anti-GAT1 (Cat.No. ab426, Abcam) diluted 1:2,000, rabbit anti-connexin 36 (Cat.no. ab48814, Abcam) diluted 1:800, rabbit anti-parvalbumin (Cat.No. ab11427, Abcam) diluted 1:1000, rabbit anti-somatostatin (Cat.no. sc-13099, Santa Cruz biotechnologies) diluted 1:200, rabbit anti-calretinin (Cat.No. 180211, Invitrogen Life Technologies, Grand Island, NY, USA) diluted 1:500 and mouse anti-GAPDH (Cat.No. sc-47724, Santa Cruz Biotechnologies) diluted in 1:20,000 in blocking solution for overnight at 4 ^{0}C. The membrane was washed three times in TBS-T and incubated with sheep anti-mouse IgG antibodies (Cat. No. Na931V, GE Healthcare, UK) diluted 1:1000 in blocking solution, for 2 hours. Then the membrane was incubated with Enhanced Chemiluminescence Detection Reagent (Amersham Biosciences, Piscataway, NJ) for 2 minutes and exposed to Kodak BioMax XAR film (Kodak, Rochester, NY). Film was developed and scanned and bands densities were measured using ImageJ software (NIH, Bethesda, MD, USA).

2.8 IMMUNOPEROXIDASE IMMUNOHISTOCHEMISTRY OF HUMAN BRAIN SPECIMENS

Sections of tissue of uniform thickness (6-8 um) were cut from archival paraffin-embedded tissue block and mounted on glass slides (Superfrost Plus Gold, Erie Scientific, Portsmouth, NJ) by the UTMB Histopathology Core Lab. The sections were baked at 60 °C for 2 hours to increase adherence of the tissue to the glass slides. Then sections were deparaffinized in 3 baths of 100 % xylene and rehydrated in baths of 100 %, 95 % and 80 % C_2H_5OH. For antigen retrieval slides were immersed in 0.01 M sodium citrate solution with 0.1 % Tween-20 (pH 6.0) and kept at boiling point in a microwave oven for 20 minutes. Unspecific binding was blocked for 1 hour with 1%

normal serum and then the sections were incubated overnight at 4 ^{0}C with primary antibodies diluted in blocking solution. Following primary antibodies were used: rabbit anti-GAD67 (Cat. No. ab26116, Abcam) diluted 1:1000, rabbit anti-GAT1 (Cat.No. ab1570, EMD Milipore, Merck KGaA, Darmstadt, Germany) diluted 1:100, rabbit anti-parvalbumin (Cat.No. ab11427, Abcam) diluted 1:1000, rabbit anti-calretinin (Cat.No. 180211, Invitrogen) dilted 1:100, rabbit anti-somatostatin (Cat.No. ab22682, Abcam) diluted 1:500, mouse anti-DRD2 (Cat.No. sc 5303, Santa Cruz Biotechnologies) diluted 1:1000, mouse anti-PDGFb (Cat.No. sc-19995) diluted 1:100, mouse anti-CD31 (Cat. No. M0823, Dako, Via Real, Carpinteria, CA, USA) diluted 1:30, mouse anti-granzyme B (Cat.No. GB-7, Sanquin, Sanquin Blood Supply, Amsterdam, Netherlands) diluted 1:50, mouse anti-CD8A (Cat.No. M710301-2, Dako) diluted 1:50, mouse anti-CD68 (Cat.No. M0814, Dako) diluted 1:50. Next day tissue was treated with 3 % hydrogen peroxide for 10 minutes to block endogenous peroxidase activity and rinsed with TBS-T (50mM Tris-HCl, 150mM NaCl, 0.05% Tween-20). Appropriate anti-mouse or anti-rabbit Vectastain secondary antibodies were applied for 1 hour, followed by Vectastain ABC and Vectastain DAB color development using peroxidase-diaminobenzidine reactions (Vector Laboratories, Burlingame, CA). Stained sections were dehydrated in graded ethanol and xylene. Coverslips were mounted using Permount (Fisher Scientific, Hampton, NH). Composite microscopic fields were acquired by combining contiguous images taken using an Olympus DP71 camera and Olympus DP controller software (Olympus America Inc, Center Valley, PA, USA).

2.9 IMMUNOFLUORESCENCE IMMUNOHISTOCHEMISTRY OF HUMAN BRAIN SPECIMENS

Sections for immunofluorescence microscopy using confocal imaging underwent the same deparaffinization and antigen retrieval treatment as described for immunoperoxidase immunohistochemistry. After antigen retrieval, the tissue sections were permeabilized for 15 min with 0.1 % Triton-X in Phosphate buffered saline (PBS)

and treated with ImageT FX signal enhancer (Invitrogen Molecular Probes, Eugene, Oregon) for 30 min to block unspecific staining, followed by blocking with 1% normal goat serum applied for 1 hour. Primary antibodies were diluted in normal blocking serum and applied overnight at 4 ^{0}C. Following primary antibodies were used: mouse anti-GAD67 (Cat. No. mab5406, EMD Millipore Corporation, Billerica, MA, USA) diluted 1:100, rabbit anti-parvalbumin (Cat. No. ab11427, abcam) diluted 1:1000, rabbit anti-calretinin (Cat.No. 180211, Invitrogen) diluted 1:100, rabbit anti-somatostatin (Cat.No. ab22682, abcam) diluted 1:500, rabbit anti-glutamine synthetase (Cat.No. ab49873, abcam) diluted 1:1000, mouse anti-GFAP (Cat.No. 3670, Cell Signaling Technology, Inc. Danvers, MA, USA) diluted 1:1000, rabbit anti-GAT1 (Cat.No. ab1570, EMD Milipore, Merck KGaA, Darmstadt, Germany) diluted 1:100, mouse anti-DRD2 (Cat.No. sc-5303, Santa Cruz biotechnologies) diluted 1:50, mouse anti-CD13 (Cat.No. sc-166105, Santa Cruz Biotechnologies) diluted 1:50. Next day sections were rinsed in with TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween-20) and mixture of secondary antibodies diluted 1:500 in PBS were applied for 3 hours (AlexaFluor 488 goat anti-mouse (Cat.No.A11029) and AlexaFluor 594 goat anti-rabbit (Cat.No.A11037), Life Technologies, Grand Island, NY, USA). After incubation with secondary antibodies, sections were rinsed with TBS-T and treated with Sudan Black B (0.5 % in 70 % ethanol) for 20min to block tissue autofluorescence caused by endogenous lipofuscin deposits and tissue fixation. Then sections were rinsed in ddH2O, air-dried and coverslips were mounted using ProLong Gold with DAPI mounting media (Life Technologies). Clear nail polish was used to seal the edges of the coverslips. Slides were then labeled and stored in slide holders at - 20 °C. Confocal images were acquired with a Zeiss LSM-510 Meta confocal microscope with 63x/1.40 Oil DIC (WD=0.19 mm) objective (Carl Zeiss Microscopy, Peabody, MA, USA) and processed using LSM Image Browser (Zeiss).

2.10 STATISTICAL ANALYSIS

Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, DC, USA) and GraphPad 6 (GraphPad Software, Inc, La Jolla, CA, USA) were used for data organizing, performing of statistical tests and preparing the figures. Two-tailed Student's t-test was used to compare two groups. One-way and two-way analyses of variance with post hoc Scheffé's, Tukey's multiple comparisons or Fisher's LSD (least significant difference) tests were used to compare three groups or more. Pearson's correlation was used to analyze correlations between GABAergic mRNAs level and neurovirological, neuroimmunological and neurocognitive measurements. Where indicated, data were log10 transformed to normalize the distribution. Significance of correlation was corrected for the false discovery rate using the Bonferroni correction. Significance was set at $\alpha = 0.05$. Multiple regression models were used to determine whether potentially confounding factors such as age, gender, race and drugs of abuse were associated with GABAergic and other mRNAs. Fisher z-transformation was used to estimate the significance of the difference between two correlation coefficients found in two independent samples.

Table 2.1Demographics of study subjects (I)

Characteristic	HIV infection		p-value	HIV encephalitis		p-value	HIV associated dementia		p-value
	HIV-	HIV+	HIV- vs HIV+	HIVE-	HIVE+	HIVE- vs HIVE+	no HAND	HAND	no HAND vs HAND
Number of subjects	63	449	N/A	357	92	N/A	43	161	N/A
Age (years, mean ± stdev)	44.9 ± 11.4	42.6 ± 9.6	0.0782	42.9 ± 10	41.3 ± 7.7	0.139	48 ± 8.1	43.4 ± 12.2	0.003
Gender (male/female)	49/ 14	387 / 62	0.113	302/55	85/7	0.053	39/4	129/32	0.106
Race (W/B/A/O)	39/18/0/6	271/149/6/23	0.431	221/114/6/16	53/32/0/7	0.341	35/7/0/1	105/40/3/13	0.186
Hispanic or Latino (yes/no)	16/47	87/362	0.356	64/293	23/69	0.126	37/ 6	35/126	<0.001
Postmortem interval (hours, mean ± stdev)	15.5 ± 11.7	14.5 ± 14.7	0.6683	14.2 ± 13.5	15.6 ± 18.8	0.426	21 ± 27.6	13 ± 12.2	0.006
Log10 Plasma HIV RNA (copies/ml, mean ± stdev)	N/A	(n = 258) 4.3 ± 1.4	N/A	(n = 217) 4 ± 1.6	(n = 47) 5.1 ± 0.9	p < 0.001	(n = 39) 3.6 ± 1.8	(n = 136) 4.3 ± 1.5	0.023
Log10 CSF HIV RNA (copies/ml, mean ± stdev)	N/A	(n = 181) 2.9 ± 1.5	N/A	(n = 163) 2.6 ± 1.3	(n = 28) 4.2 ± 1.7	p < 0.001	(n = 32) 2.5 ± 1.4	(n=90) 2.8 ± 1.3	0.391
Blood CD4+ lymphocyte count (cells/mm3, mean ± stdev)	N/A	(n = 274) 106.5 ± 162	N/A	(n = 222) 119.4 ± 174.9	(n = 52) 51.5 ± 64.1	0.006	(n = 41) 158.7 ± 177.7	(n = 139) 103.1 ± 176.0	0.078
HAART treatment (yes/no)	N/A	131/318	N/A	249/108	69/23	0.323	43/0	147/14	0.045
a HIV-, Human Immunodeficiency Virus seronegative; HIV+, seropositive									
b HIVE, HIV encephalitis									
c HAND, HIV associated neurocognitive disorders									
d mean ± standard deviation, Student's t-test									
e M/F, Male/female									
f Chi-square									
g B/W/A/O, Black/White/Asian/Other									
h N/A, not applicable									

Table 2.2 Demographics of study subjects (II)

Characteristic	HIV infection		p-value	HIV encephalitis		p-value	HIV associated dementia		p-value
	HIV-	HIV+	HIV- vs HIV+	HIVE-	HIVE+	HIVE- vs HIVE+	no HAND	HAND	no HAND vs HAND
Number of subjects	12	36	N/A	24	12	N/A	12	24	N/A
Age (years, mean ± stdev)	38.3 ± 5.9	42.5 ± 9.3	0.163	45.6 ± 9.7	36.4 ± 7.0	0.006	52.7 ± 7.5	37.5 ± 6.3	10-3
Gender (male/female)	11/1	33/3	1.000	22/2	11/1	1.000	11/1	22/2	1.000
Race (W/B/A/O)	8/4/0/0	24/11/0/1	0.837	19/5/0/0	5/6/0/1	0.046	9/3/0/0	16/7/0/1	0.203
Hispanic or Latino (yes/no)	1/11	1/35	0.404	0/24	1/11	0.152	0/12	1/23	0.473
Postmortem interval (hours, mean ± stdev)	12.4 ± 8.4	17.1 ± 13.0	0.285	15.3 ± 10.5	20.6 ± 16.9	0.264	20.5 ± 11.2	15.4 ± 13.8	0.279
Log10 Plasma HIV RNA (copies/ml, mean ± stdev)	N/A	(n = 23) 4.1 ± 1.6	N/A	(n = 17) 3.8 ± 1.6	(n = 6) 5.2 ± 0.9	0.049	(n = 11) 3.9 ± 1.8	(n = 12) 4.4 ± 1.4	0.422
Log10 CSF HIV RNA (copies/ml, mean ± stdev)	N/A	(n = 22) 3.2 ± 1.6	N/A	(n = 16) 2.7 ± 1.0	(n = 6) 4.5 ± 2.1	0.009	(n = 10) 2.7 ± 1.1	(n = 12) 3.6 ± 1.8	0.238
Blood CD4+ lymphocyte count (cells/mm3, mean ± stdev)	N/A	(n = 23) 99.2 ± 115.5	N/A	(n = 17) 117.2 ± 129.7	(n = 6) 48 ± 25.4	0.214	(n = 11) 114.3 ± 129	(n = 12) 85.3 ± 105.5	0.561
a HIV-, Human Immunodeficiency Virus seronegative; HIV+, seropositive									
b HIVE, HIV encephalitis									
c HAND, HIV associated neurocognitive disorders									
d mean ± standard deviation, Student's t-test									
e M/F, Male/female									
f Chi-square									
g B/W/A/O, Black/White/Asian/Other									
h N/A, not applicable									

Chapter 3: Three GABAergic transcripts are downregulated in HIV infected patients

3.1 INTRODUCTION

3.1.1 Rationale

Neuropsychological impairment in several diseases has been linked with abnormal GABAergic neurotransmission, including Parkinson's disease, epilepsy, bipolar disorder, schizophrenia, and drug abuse (Volk et al. 2000, Wong et al. 2003, Dracheva et al. 2004, Akbarian and Huang 2006, Thompson et al. 2009, Sibille et al. 2011, Chattopadhyaya and Di Cristo 2012). Several research reports have suggested that neurotransmitter systems also are abnormal in brain specimens from patients with HAND. Systems thus far implicated in HAND include GABAergic (Koutsilieri et al. 2001, Gelman et al. 2012b), dopaminergic (Nath et al. 2000, Koutsilieri et al. 2002, Gelman et al. 2006, Gelman et al. 2012a, Gelman et al. 2012b), cholinergic (Koutsilieri et al. 2001), glutamatergic (Ernst et al. 2010) and serotonergic (Murray 2003). Independently performed brain gene arrays showed that transcripts of the GABAergic system are especially abnormal in patients with HIV encephalitis and HAND. The neurochemical mechanisms and its impact on circuit level function of these GABAergic anomalies in HIV infected people remain poorly understood. The main goal of my investigation was to further elucidate the characteristics and clinicopathological associations of GABAergic dysfunction using brain specimens from HIV-infected patients.

In my investigation I have measured several aspects of GABAergic neurotransmission in HIV-infected patients, including those with and without neuropsychological impairment. My first goal was to determine whether there is specific

anatomical pattern or regional distribution of abnormal GABAergic transmission in the CNS, and then to characterize relationships with clinical virology, neuropathology, endothelial and immune cell markers, drug abuse history, and neurocognitive impairment. To address that I chose to examined the level of multiple GABAergic transcripts and proteins expressed in brain specimens from a robust sampling of HIV infected patients and uninfected comparison patients.

3.1.2 Experimental design

The analysis was performed using samples of postmortem brain tissue collected from 449 HIV-positive subjects and 66 demographically matched HIV-negative controls of the National NeuroAIDS Tissue Consortium. The large sample size was necessary because of the wide biological variation in neurotransmitter measurements that is present when using postmortem brain tissue. As well, there are several comorbid conditions that can occur in HIV-infected populations that affect neural transmission, and they also can impart substantial variability. Neurocognitive testing T scores were available for 219 HIV-positives, evaluated using NNTC neurocognitive test battery. Self-reported lifetime histories of substance abuse and dependence were available for 191 HIV-positives, evaluated using The Psychiatric Research Interview for Substance and Mental Disorders (PRISM). Dorsolateral prefrontal cortex (DLPFC) was selected as main region of interest because functional output of DLPFC was reported to be specifically abnormal in HAND (Thompson et al. 2005, Woods et al. 2009). The concentrations of GABAergic type transcripts in the dissected postmortem brain tissue were measured by quantitative real-time polymerase chain reaction. To enhance the robustness of the readout, three different elements of the GABAergic system were examined at the level of mRNA. The concentrations of GAD67, GAD65 and connexin 36, were also measured at the protein level using immunoblotting. The cellular localization of GAD67 and potential co-localization with immune and endothelial cell markers were examined using

immunohistochemistry. Statistical analyses were performed to determine if groups of patients differed with respect to GABAergic neurochemical features. Potential independent variables that were examined included HIV infection, HIV neuropathology, HIV-associated neuropsychological impairment, and selected biologically relevant co-morbid conditions. Neurovirological, neuroimmunological and neurocognitive correlates were evaluated using Pearson's correlation analysis. Log transformation was used to normalize certain outcome distributions.

3.2 RESULTS

3.2.1 GABAergic transcripts expression in the dorsolateral prefrontal cortex

Three markers of GABAergic neurotransmission were abnormally downregulated in the DLPFC of HIV-infected patients compared to normal seronegative controls. mRNAs encoding for GAD67, GAD65, and connexin 36 proteins all were significantly lower in the HIV positive subjects versus the HIV negative controls (Figs 3.1.A, 3.1.B, and 3.1.C) by 26 %, 22 % and 21 %, respectively ($p < 10^{-4}$, $p < 0.001$, $p = 0.004$).

Relationship of GABAergic mRNAs to HAART medications

HIV infected patients prior to the HAART era (about 1995) could not attain effective suppression of HIV replication, whereas during the HAART era, effective suppression of viral replication is common. HAART drugs probably affect neurochemistry indirectly by suppressing viral replication, but also could directly interact with neurotransmitter systems upon entering the CNS. When HIV-positives were sorted into subjects who died before and after the introduction of highly active antiretroviral therapy (HAART), all three tested GABAergic type mRNAs were significantly lower in both groups of HIV-positives when compared to the HIV-negatives ($F = 13.27$, $p < 10^{-4}$; $F = 5.957$, $p = 0.0028$; $F = 4.425$; $p = 0.0124$). No differences were found between pre- and post-HAART groups (Figures 3.1.D, 3.1.E., and 3.1.F).

Relationship of GABAergic mRNAs to HIV neuropathology

Synaptodendritic simplification followed by altered synaptic transmission is a neuropathological change that occurs in HIVE (Masliah et al. 1997). Presynaptic and postsynaptic proteins were changed at the level of transcriptional regulation in the DLPFC of HIVE patients (Gelman et al. 2004, Gelman et al. 2012). To examine the role of HIVE in the development of GABAergic anomalies, HIV-positive subjects were sorted according to the neuropathological diagnosis of HIV-encephalitis (HIVE) at autopsy, the mRNAs were significantly lower in patients with or without HIVE in comparison with HIV-negative controls (Figure 3.1.g, 3.1.H, and 3.1.I; $F = 12.55$, $p < 0.0001$, $F = 5.960$, $p = 0.0028$, $F = 4.313$, $p = 0.014$, respectively). No difference between HIV-positives with and without HIVE was present.

Relationship of GABAergic mRNAs to substance use history

Substance abuse can result in altered GABAergic neurotransmission (Zhang et al. 2006, Wadleigh et al. 2012). Abnormal expression of *GAD1* and *GAD2* mRNAs were reported to occur after cocaine (Enoch et al. 2012), nicotine (Satta et al. 2008), morphine (Sultana et al. 2010), and methamphetamine (Anneken et al. 2013) consumption. Population of HIV-infected subjects includes high-risk behavior individuals, therefore it is important to control for possible correlates between the decrease of GABAergic transcripts and drug abuse history. When sorted according to self-reported history of any drug abuse, all three GABAergic markers mRNAs in both groups of HIV-infected subjects with and without drug abuse history were significantly lower compared to controls (Figure 3.2). Post hoc comparison using Scheffé's test revealed no difference between infected patients with and without substance use histories.

3.2.2 GAD67 and GAD65 protein expression in the DLPFC

Level of gene expression can be modified by post-transcriptional (mRNA transport), translational (initiation of protein translation), and post-translational

(modification of protein) mechanisms. Concentration of mRNA is not always predictive for the level of protein. To identify the relationship between gene expression at the mRNA and protein levels I performed immunoblotting for GAD67 and GAD65 proteins, which are GABA synthesizing enzymes, and showed that both GAD67 and GAD65 concentrations in the DLPFC of 36 HIV-positives were significantly lower when compared to 12 seronegative controls (Figure 3.3.C and F). A total of 48 brain samples were analyzed. Results indicate that protein expression in the DLPFC of 36 HIV-infected patients was significantly lower for GAD67 (-51.2 %, $p < 10^{-4}$) and GAD65 (- 25.7 %, $p = 0.043$) when compared to 12 uninfected comparison patients. When *GAD1* and *GAD2* mRNAs from these patients was measured, *GAD1* mRNA in the DLPFC of HIV infected patients was lower by 39.8 % ($p = 0.0004$) and *GAD2* mRNA was lower by 36.9 % ($p = 0.009$) when compared to uninfected comparison patients (Figure 3.3.B and E). Figures 3.3.A and 3.3.D show fragments of membrane immunoblotted against GAD67 and GAD65, or GAPDH, which was used as a loading control.

3.2.3 GAD67 immunostaining of brain tissue specimens

Immunostaining of GAD67 protein in the DLPFC showed that stain intensity was lower in the neocortex of HIV-infected patients (Figure 3.4). Lower stain intensity was present diffusely in the cell bodies and neural processes in all cortical laminae. GAD67 immunostaining of the basal ganglia from the same subjects also showed lower staining intensity in paleo- and neostriatum of the HIV infected patients, but the number of stained neurons was similar between groups.

3.2.4 Relationship between GABAergic mRNAs, clinical virology and neurovirology

To examine whether GABAergic anomalies are driven by replicating HIV-1, three GABAergic mRNAs were correlated with the HIV RNA copy number in different body compartments: cerebrospinal fluid, plasma and grey matter of frontal neocortex. For

HIV-positive subjects correlation coefficients between the GABAergic mRNAs in the DLPFC and HIV RNA copy number in brain tissue were not significant (Table 3.2). When three GABAergic mRNAs each were correlated with the HIV RNA copy number in blood plasma, the correlation coefficient was negative and significant only for *GAD2* mRNA (n = 264, r = -0.1372, p = 0.0258). When the three GABAergic mRNAs were correlated with the HIV RNA copy number in the cerebrospinal fluid (CSF) samples that were available, the coefficients were negative and significant for *GAD2* mRNA (n = 191, r = -0.1582, p = 0.0286) and *GJD2* mRNA (n = 191, r = -0.1801, p = 0.0127). Neurovirological correlations with brain function in patients with HIVE often differ from those without HIVE (Gelman et al. 2012a, Gelman et al. 2013). To analyze whether HIVE affected correlations between GABAergic mRNAs and the concentration of HIV RNA in brain tissue specimens, the patients with and without HIVE were evaluated separately. *GAD1* mRNA was negatively and significantly correlated with HIV RNA copy number in the brain in the HIV infected patients without HIVE (n = 355, r = -0.1045, p = 0.0491). In contrast, the same correlation in patients with HIVE was positive and almost reached statistical significance (n = 92, r = 0.1544, p = 0.1417). Fisher z-transformation showed that the two oppositely sloped regression lines were statistically different from each other (two-tailed probability p = 0.0278). This suggested that HIVE, which is characterized by high virus replication rates and increased host inflammatory responses in the brain, might blunt GABAergic downregulation.

3.2.5 Relationships between GABAergic mRNAs and endothelial or immune cell markers

HIV-1 infection of astrocytes and perivascular macrophages results in the damage to the neurovascular unit and in the loss of the blood brain barrier integrity, contributing to pathological changes in the brain and increased concentration of inflammatory markers. To examine whether GABAergic anomalies were related to the immune and

vascular changes, neurovascular and neuroimmune mRNAs were correlated with GABAergic mRNAs. The results showed that having lower GABAergic mRNA expression was most strongly and significantly correlated with high expression of *PECAM1* and *VWF*, which are two frequently used biological markers that are selectively expressed by endothelial cells (Table 3.2). Low GABAergic mRNAs also were significantly, but less strongly correlated with high expression of neuroimmune type markers in the brain, including a type 1 interferon response gene (MX1), and a type 2 interferon response gene (*IRF1*). Several immune cell marker mRNAs also were correlated significantly with low GABAergic mRNAs including those expressed selectively by macrophages (*CD163* and *CD68*), CD8+ cytotoxic T lymphocytes (*CD8A*), and natural killer cells (*GZMB*). In contrast, mRNA markers for B lymphocytes (*CD19*) and CD4+ T lymphocytes (*CD4*) were not significantly correlated with low GABAergic mRNAs. Immune and endothelial cell immunostaining in the DLPFC confirmed that low GAD67 expression in the HIV infected patients was linked with higher expression of immune and endothelial cell type markers proteins (Figure 3.5).

3.2.6 Regional distribution of low GABAergic mRNA expression in the CNS

To determine whether the results of lowered GABAergic mRNAs obtained for the DLPFC are representative for other CNS regions, I measured *GAD1* mRNA in a total of 16 sectors of the human CNS, including 7 neocortical samples, 6 subcortical samples, cerebellum and spinal cord (Figure 3.6). Six HIV negative controls and 6 HIV-infected subjects were selected for study based upon their GABAergic mRNA values in the DLPFC. There was significant variation in *GAD1* mRNA expression between brain regions ($F(16, 218) = 7.17$, $p < 10^{-4}$), two way ANOVA). In almost all sectors *GAD1* mRNA was lower in the HIV infected subjects, including neocortex, neostriatum and cerebellar lobule. Exceptions were spinal cord, paleostriatum (globus pallidus) and

hippocampus which had weaker non-significant lowering of *GAD1* expression in the HIV infected patients.

3.2.7 Relationship of GABAergic mRNAs to neurocognitive impairment

Abnormal GABAergic transmission occurs in diseases that can adversely affect neurocognitive test performance, especially for tasking that is driven by the DLPFC (Menzies et al. 2007). To address whether the GABA disturbance was related to HIV-associated neurocognitive disorders (HAND), HIV-1 infected subjects were sorted according to the diagnosis of HAND and then GABAergic mRNAs were compared between the groups. All three GABAergic mRNAs were significantly lower in HIV-infected patients with HAND and without HAND when compared to the uninfected patients (Figure 3.1.J, 3.1.K, and 3.1.L; $F = 8.420$, $p = 0.0003$; $F = 10.51$, $p < 10^{-4}$, respectively). No difference between HIV-positives with and without HAND was found with respect to these mRNAs. Pearson's correlation analysis showed that there was no significant correlation between global T score (a measure of composite performance on neuropsychological testing that is normalized for age) and GABAergic mRNAs in the DLPFC. However, when GABAergic mRNAs were compared to distinct functional domains that were assessed in the test battery, there was a significant correlation between low GABAergic mRNAs and worse performance on verbal fluency tasking ($r = 0.1974$, $p = 0.0036$ for *GAD1*, $r = 0.2358$, $p = 0.0005$ for *GAD2*, and $r = 0.2171$, $p = 0.0013$ for *GJD2*) after correction for multiple comparisons (Table 3.3). To determine if GABAergic neurotransmission in specific brain regions is related to neurocognitive test performance in the same way that I observed for DLPFC, I examined whether low GABAergic mRNA in other brain sectors was correlated with neurocognitive test scores in a manner similar to the DLPFC. Using 27 HIV-infected brain specimens, *GAD1* mRNA was measured in anterior cingulate cortex, dorsolateral prefrontal cortex, somatosensory cortex and superior temporal cortex and then was compared to neuropsychological T scores. The

results showed that low *GAD1* mRNA concentration in the anterior cingulate cortex was strongly and significantly correlated with normalized T scores for composite performance, abstract executive and learning domains (Table 3.4). For speed of information processing, attention and working memory, memory, and verbal fluency domains, correlation was also positive and almost reached the level of statistical significance. The significant relationships between low *GAD1* in the anterior cingulate cortex and neuropsychological test performance were generally specific to anterior cingulate cortex as they were not observed for the other brain cortex sectors that were sampled.

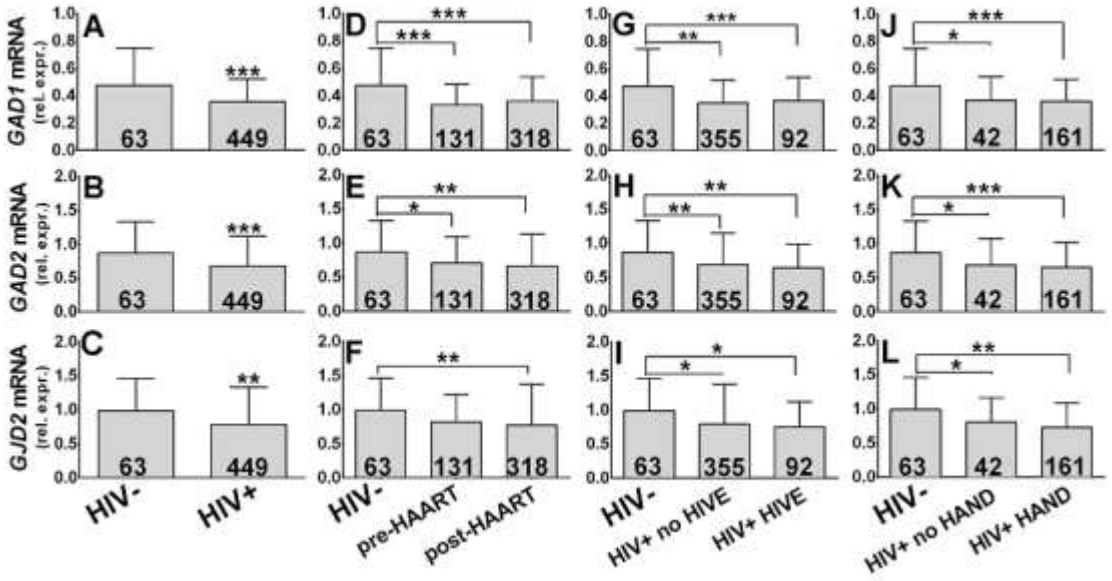

Figure 3.1 GABAergic transcripts expression in the dorsolateral prefrontal cortex. Glutamate decarboxylase 1 (GAD1), glutamate decarboxylase 2 (GAD2), and connexin 36 (GJD2) mRNAs were measured in the dorsolateral prefrontal cortex of 449 HIV infected and 66 HIV seronegative patients (Panels A - L). "Rel. exp." on the ordinates denotes mRNA expression relative to GAPDH mRNA. Panels A-C. On the abscissa HIV-seronegative controls were compared to HIV-positive subjects. GABAergic markers mRNA expression was significantly lower in the HIV infected patients by 26 % for GAD1, 22 % for GAD2 and 21 % for GJD2. Between group analyses using two-tailed Student's t-test produced p values of < 10-4 for GAD1, 0.001 for GAD2, and 0.0043 for GJD2. Panels D-F. On the abscissa HIV-positive subjects were divided into subjects who died before and after the introduction of highly active antiretroviral therapy (HAART). mRNA expression was significantly lower in pre-HAART and post-HAART HIV patients by 30 % and 24 % for GAD1, 19 % and 24 % for GAD2, 17 % and 22 % for GJD2 as compared to the uninfected comparison group. Between group analysis using one-way analysis of variance (ANOVA) yielded F = 13.27 and p < 10-4) for GAD1, F = 5.957 and p = 0.0028 for GAD2, and F = 4.425 and p = 0.012 for GJD2. There were no significant differences between pre- and post-HAART groups. Panels G-I. On the abscissa HIV-positive subjects were divided according to neuropathological diagnosis of HIV-encephalitis (HIVE). Compared to the uninfected comparison group, all three mRNAs were significantly lower in patients without HIVE and with HIVE by 26 % and 23 % for GAD1, 21 % and 27 % for GAD2, 20 % and 24 % for GJD2. Between group analysis using one-way analysis of variance (ANOVA) and post hoc Scheffé's test yielded F = 12.55 and p < 10-4 for GAD1, F = 5.960 and p = 0.0028 for GAD2 and F = 4.313 and p = 0.014 for GJD2. Panels J-L. On the abscissa HIV-positive subjects were divided according to the diagnosis of HIV-associated neurocognitive disorders (HAND). Compared to the uninfected comparison group, all three mRNAs were significantly lower in patients without HAND and with HAND by 23 % and 25 % for GAD1, 21 % and 25 % for GAD2, 19 % and 26 % for GJD2. Between group analysis using one-way analysis of variance (ANOVA) and post hoc Scheffe's test yielded GAD1 F = 8.420 and p = 0.0003, F = 7.384 and p < 0.0008 for GAD2, and F = 10.51 and p < 10-4 for GJD2. Relative expression of mRNAs was normalized to GAPDH mRNA. The number of subjects in each group is given in the bars. Mean ± standard deviation.

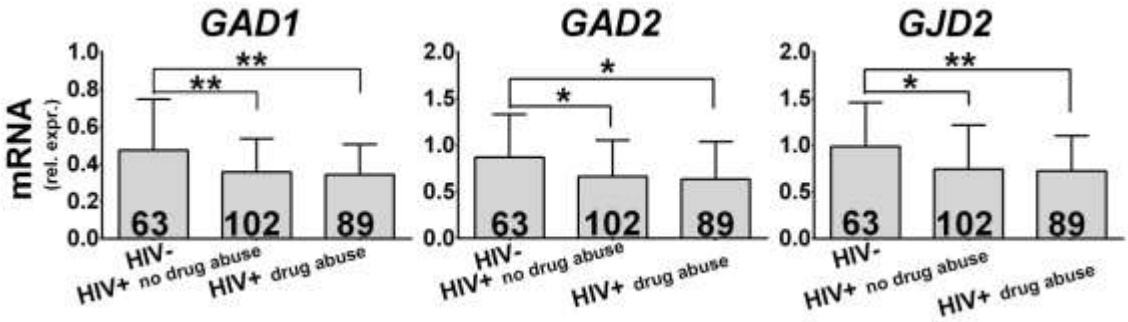

Figure 3.2 GABAergic transcripts expression in the dorsolateral prefrontal cortex with and without drug abuse. Glutamate decarboxylase 1 (GAD1), glutamate decarboxylase 2 (GAD2), and connexin 36 (GJD2) mRNAs were measured in the dorsolateral prefrontal cortex of 102 HIV-infected patients with self-reported history of drug abuse, 89 HIV-infected subjects with history of no drug abuse and 66 seronegative patients (Panels A - C). "Rel. exp." on the ordinates denotes mRNA expression relative to GAPDH mRNA. On the abscissa HIV-positive subjects were divided according to the self-reported history of drug abuse. On the abscissa HIV-positive subjects were divided according to the self-reported history of drug abuse. Compared to the uninfected comparison group, all three mRNAs were significantly lower in patients without drug abuse and with drug abuse by 25 % and 28 % for GAD1, 24 % and 27 % for GAD2, 25 % and 27 % for GJD2. Between group analysis using one-way analysis of variance (ANOVA) and post hoc Scheffé's test yielded GAD1 $F = 9.242$ and $p < 10\text{-}4$, $F = 7.124$ and $p = 0.001$ for GAD2, and $F = 8.12$ and $p = 0.0004$ for GJD2. Relative expression of mRNAs was normalized to GAPDH mRNA. The number of subjects in each group is given in the bars. Mean ± standard deviation**.**

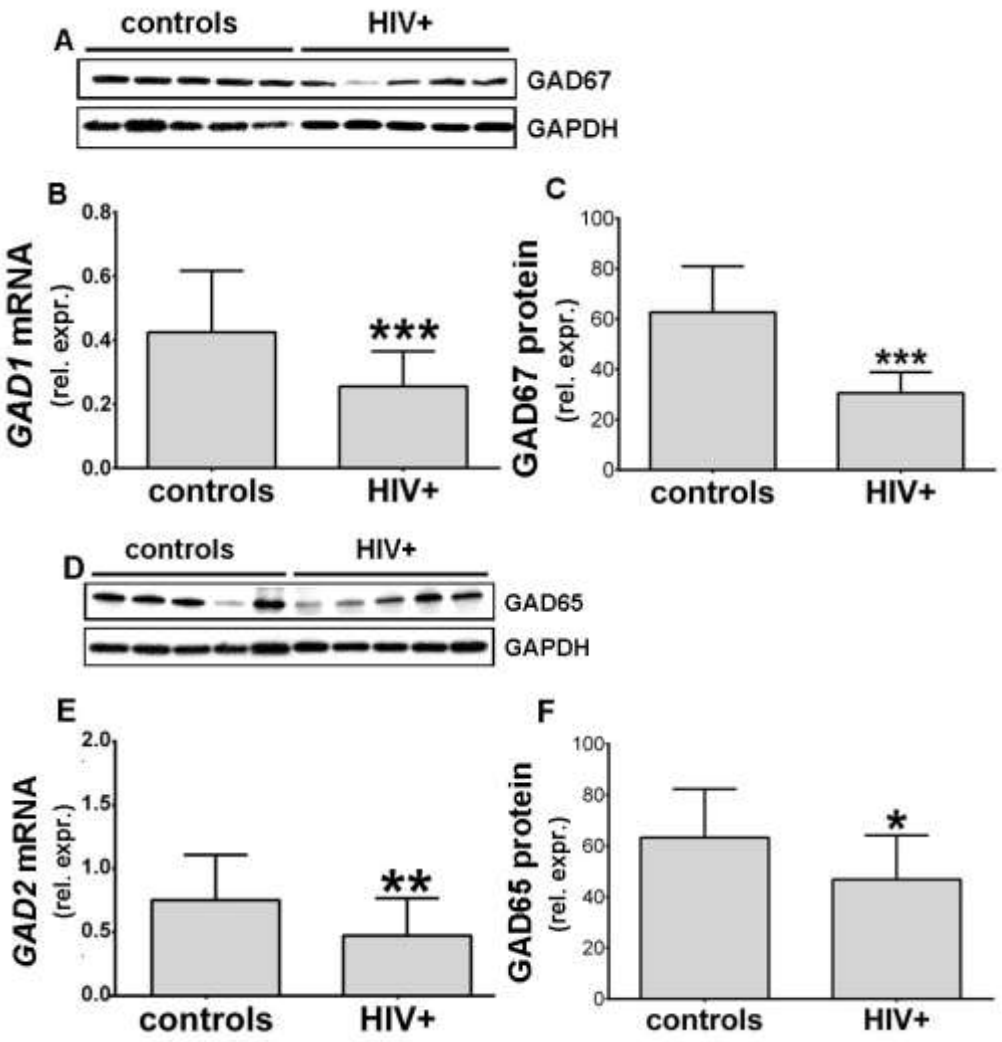

Figure 3.3 Glutamate decarboxylase 67 (GAD67) and glutamate decarboxylase 65 (GAD65) protein and mRNA expression in the dorsolateral prefrontal cortex (DLPFC). GAD67 and GAD65 immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (C, F). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. The band intensities were significantly lower in HIV-positive group for GAD67 (-51.2 %, p = 0.0001) and for GAD65 (-25.7 %, p = 0.043). Band intensities for ten of the patients are illustrated in A and D. The concentration of *GAD1* and *GAD2* mRNAs is shown for comparison (B and E), mean GAD1 mRNA decrease was -39.8 % (p = 0.0004) and mean *GAD2* mRNA decrease was -36.9 % (p = 0.0009) in the HIV+ group. GAD67 and GAD65 are expressed relative to the GAPDH band intensity. Relative expression of *GAD1* and *GAD2* mRNAs was normalized to *GAPDH* mRNA. Mean ± standard deviation is shown. P values were obtained using the Student's t test.

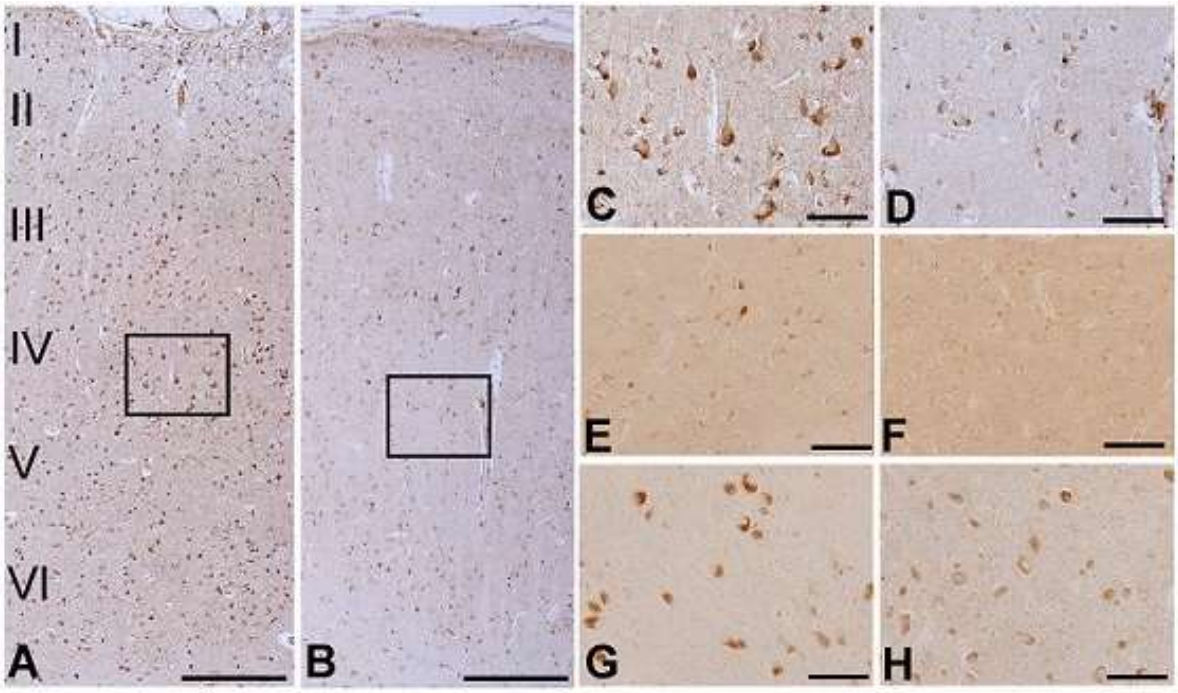

Figure 3.4 Glutamate decarboxylase 67 (GAD67) immunostaining of a brain tissue specimen obtained from a HIV infected patient with comparison to an uninfected patient. Panels A and B illustrate dorsolateral prefrontal cortex (DLPFC) with neocortical laminae labeled I through VI at left. The specimen from the HIV infected patient (B) contains less intense staining in all of the laminae compared to the seronegative patient (A). Boxed areas in A and B are magnified in C and D, which illustrate less intense immunostaining in pyramidal cell cytoplasm, in pear-shaped interneurons and in neuritic processes. In the neostriatum (caudate nucleus), the lower GAD67 staining intensity in the HIV-infected patient was not as clear-cut but still was present (E vs F). Neurons in globus pallidus also were stained less intensely in the HIV infected patient. The number of stained neurons in the HIV infected patient is similar to the uninfected patient (G vs H). Scale bars are 200 um in A and B, and 100 um in C through F.

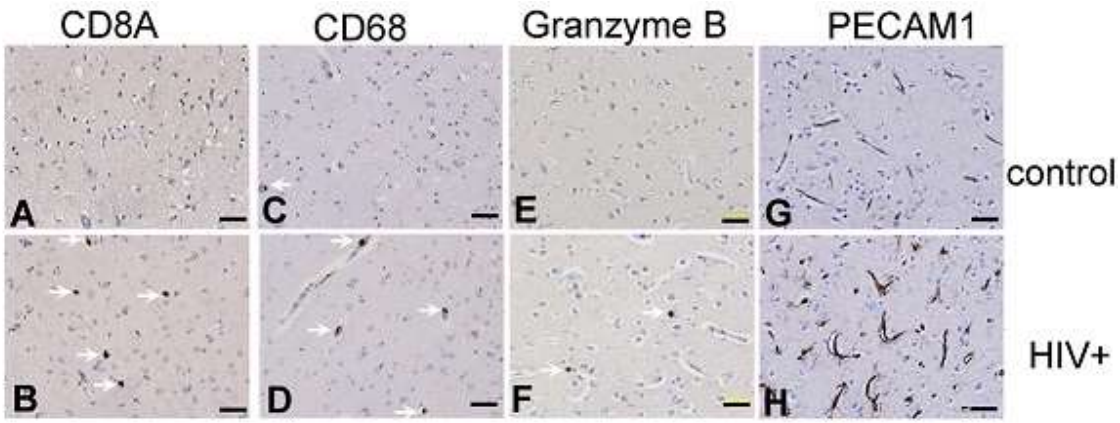

Figure 3.5 Immune and endothelial cell immunostaining in the dorsolateral prefrontal cortex of HIV-seronegative and HIV-infected patients. Immune and endothelial cells were immunostained in the dorsolateral prefrontal cortex of the HIV-seronegative control (panels A, C, E, and G) and HIV-infected subjects (B, D, F, and H) from Figure 3.4. CD8+ cytotoxic T cells (arrows, A vs B) and CD68+ macrophages (arrows, C vs D) were more numerous in the HIV infected patients with low *GAD1* expression, as suggested by higher concentrations of the corresponding mRNAs as shown in Table 2. Granzyme B-containing inflammatory cells are likely to be natural killer cells, and they also were more numerous with low GAD1 expression (arrows, E vs F). Staining for the endothelial cell marker CD31 (PECAM1) produced more intense marking of blood vessel walls in subjects with low *GAD1* (G vs H), and is consistent with the higher expression of these mRNAs. Bars represent 50 um.

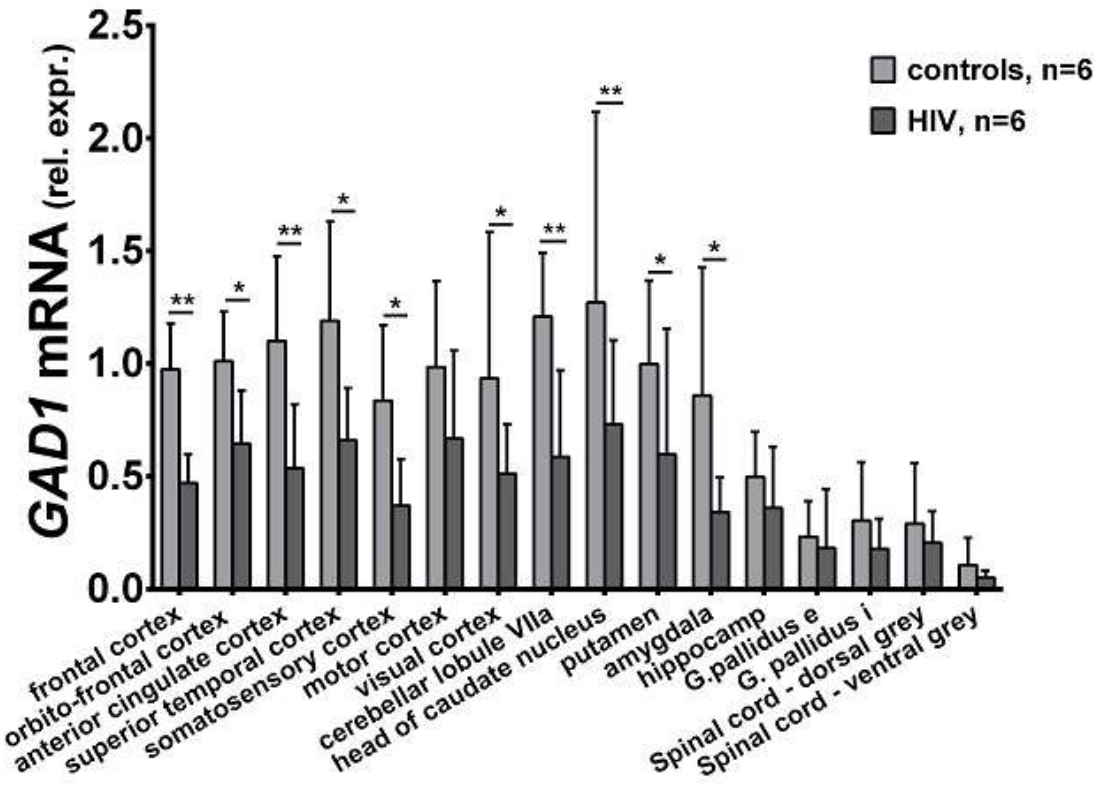

Figure 3.6 *GAD1* mRNA expression in the brain regions. Glutamate decarboxylase 1 mRNA (*GAD1*) expression was measured in 16 different brain regions obtained from 6 HIV-infected patients and 6 uninfected patients. Using two-way ANOVA, only variation in *GAD1* mRNA concentrations between brain regions was significant ($F(16, 218) = 7.17$, $p < \mathbf{10^{-4}}$). *GAD1* was lower in the HIV infected brain specimens in most regions including neocortex, cerebellum and neostriatum (caudate nucleus and putamen). Uncorrected Fisher's least significant difference (LSD) tests were significant for dorsolateral prefrontal (-51.7 %, p = 0.010), orbitofrontal (-36.6%, p=0.049), anterior cingulate (-51.2 %, p = 0.009), superior temporal (-44.5 %, p = 0.013), somatosensory (-55.6 %, p = 0.042), and visual cortices (-45.4 %, p = 0.038), cerebellar lobule VIIa (-51.5 %, p = 0.003), head of caudate nucleus (-42.5 %, p = 0.009), putamen (-40.1 %, p = 0.049), and amygdala (-60 %, p = 0.012). Spinal cord, paleostriatum, and hippocampus also had slightly lower *GAD1* in the HIV infected brain specimens, but those differences were not significant statistically. Relative expression of *GAD1* mRNA was normalized to *GAPDH* mRNA. Mean ± standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001, two way ANOVA.

Table 3.1 GABAergic transcripts correlated with HIV-1 replication and CD4+ lymphocyte count in blood plasma

		GAD1 mRNA		*GAD2* mRNA		*GJD2* mRNA	
HIV+, all	n	R	p	r	p	r	p
log brain VL	447	-0.0021	0.9647	-0.0661	0.1630	-0.0017	0.9714
log blood VL	264	-0.1067	0.0836	-0.1372	0.0258*	-0.0949	0.1240
log CSF VL	191	-0.1250	0.0849	-0.1584	0.0286*	-0.1801	0.0127*
Blood CD4 cell count	274	0.0414	0.4950	0.0661	0.2756	0.1163	0.0545
HIV+, no HIVE							
log brain VL	355	-0.1045	0.0491*	-0.0926	0.0815	-0.0311	0.5592
log blood VL	217	-0.1340	0.0487*	-0.1502	0.0269*	-0.1123	0.0990
log CSF VL	163	-0.1426	0.0694	-0.1642	0.0362*	-0.2077	0.0078*
Blood CD4 cell count	222	0.0468	0.4878	0.0701	0.2984	0.1368	0.0417*
HIV+, HIVE							
log brain VL	92	0.1544	0.1417	0.0864	0.4128	0.2188	0.0361*
log blood VL	47	-0.0053	0.9718	0.0124	0.9341	0.1244	0.4048
log CSF VL	28	-0.1284	0.5149	-0.0918	0.6422	-0.0050	0.9799
Blood CD4 cell count	52	0.1289	0.3624	0.0767	0.5889	-0.0580	0.6830
*Asterisk denotes statistically significant p value. Brain HIV-1 RNA and GABAergic transcripts were measured in the frontal neocortex							
HIV - human immunodeficiency virus type 1, log VL – log HIV RNA copies per gram of tissue, CSF - cerebrospinal fluid, Blood CD4 cell count – CD4+ lymphocyte count in blood plasma expressed as cells per ml, HIVE - HIV encephalitis, r - correlation coefficient, p - two-tailed probability							

Table 3.2 GABAergic transcripts correlated with neuroimmunological and endothelial markers in 449 HIV infected patients

Marker type	Marker	*GAD1* mRNA		*GAD2* mRNA		*GJD2* mRNA	
		r	p	r	p	r	p
Interferon response	*MX1* mRNA	-0.1375	0.0035*	-0.1721	0.0002*	-0.0465	0.3256
	ISG15 mRNA	-0.0306	0.5178	-0.0510	0.2809	-0.0186	0.6943
	IRF1 mRNA	-0.1574	0.0008*	-0.1504	0.0014*	-0.0804	0.0888
M1 macrophage	*CD68* mRNA	-0.1946	0.0000*	-0.1732	0.0002*	-0.1181	0.0123*
M2 macrophage	*CD163* mRNA	-0.1950	0.0000*	-0.1081	0.0221*	-0.0750	0.0563
lymphocyte	*CD8A* mRNA	-0.1197	0.0112*	-0.1821	0.0001*	-0.0369	0.4354
T lymphocyte	*CD4* mRNA	-0.0808	0.3426	-0.0647	0.4476	0.0854	0.3157
B lymphocyte	*CD19* mRNA	-0.0501	0.2895	-0.0661	0.1620	-0.0173	0.7145
Natural killer cell	*GZMB* mRNA	-0.1450	0.0021*	-0.1553	0.0010*	-0.1018	0.0310*
Endothelial cell	*VWF* mRNA	-0.2769	0.0000*	-0.2902	0.0000*	-0.0501	0.2895
Endothelial cell	*PECAM1* mRNA	-0.3419	0.0000*	-0.3529	0.0000*	-0.1980	0.0000*
Preenkephalinergic presynaptic	*PENK* mRNA	0.2968	0.0000*	0.3534	0.0000*	0.2018	0.0000*
*Asterisk denotes statistically significant p value. GABAergic, neuroimmunological and endothelial transcripts were measured in the frontal neocortex							
HIV human immunodeficiency virus type 1, r Pearson's correlation coefficient, p two-tailed probability							

Table 3.3 GABAergic transcripts correlated with normalized neurocognitive T-scores in HIV infected patients

HIV+		*GAD1* mRNA		*GAD2* mRNA		*GJD2* mRNA	
Neurocognitive test domain	n	r	p	r	p	r	p
Memory	218	0.0322	0.6363	0.0256	0.7070	0.0528	0.438
Attention and working memory	212	0.0229	0.7403	0.0791	0.2515	0.0493	0.475
Learning	219	0.0073	0.9145	0.0653	0.3361	0.0539	0.4274
Motor	202	0.0353	0.6180	0.0306	0.6650	0.1124	0.1112
Verbal fluency	216	0.1974	0.0036*	0.2358	0.0005*	0.2171	0.0013*
Abstract executive	209	0.0614	0.3771	0.0974	0.1606	0.0918	0.1861
Speed of information processing	217	0.0377	0.5810	0.0375	0.5827	0.0461	0.4993
Global T-Score	196	0.0793	0.2690	0.1003	0.1619	0.1072	0.135
*Asterisk denotes statistically significant p value. GABAergic transcripts were measured in the frontal neocortex							
HIV human immunodeficiency virus type 1, r correlation coefficient							

Table 3.4 *GAD1* mRNA in different cortical regions correlated with normalized neurocognitive T-scores in 27 HIV infected patients

Neurocognitive test	Anterior cingulate		Dorsolateral prefrontal		Somato-sensory		Superior Temporal	
	r	p	r	p	r	p	r	p
Abstract Executive	0.501	0.008*	0.449	0.019*	0.401	0.038*	0.380	0.050*
Speed of Info Processing	0.365	0.061	0.326	0.097	0.103	0.601	0.108	0.592
Attention and Working	0.360	0.065	0.289	0.144	0.313	0.112	0.208	0.297
Learning Domain	0.391	0.044*	0.274	0.167	0.244	0.220	0.170	0.397
Memory Domain	0.328	0.094	0.279	0.159	0.180	0.367	0.171	0.394
Verbal Fluency	0.315	0.109	0.385	0.047*	0.102	0.613	-0.039	0.847
Motor Domain	0.160	0.425	0.106	0.599	0.001	0.996	0.024	0.905
Global T-Score Standardized	0.478	0.012*	0.403	0.037*	0.267	0.178	0.233	0.242
*Asterisk denotes statistically significant p value.								
HIV human immunodeficiency virus type 1, r correlation coefficient, p two-tailed probability								

Chapter 4: GABAergic abnormalities occur in viable interneurons of HIV-infected patients

4.1 INTRODUCTION

In Chapter 3 I provided robust data, which supports the hypothesis that the concentration of GABA synthesizing enzymes GAD65 and GAD67 is lower in the CNS of HIV-infected patients at the level of mRNA and protein expression both. A critical follow up question to address was whether the difference from controls was produced by the death and dropping out of inhibitory interneurons (classical neurodegeneration), or alternatively, was produced by altered expression of the genes and/or proteins in viable interneurons. I hypothesized that the number of interneurons would not be decreased in these HIV-infected brains, and that GABA synthesizing enzymes would be transcriptionally downregulated in viable interneurons.

4.1.1 Rationale

Different neuronal populations show selective vulnerability to HIV induced damage. In HIVE it has been reported that pyramidal neurons sustain a significant loss in number (Everall et al 2009). The fate of inhibitory GABAergic interneurons in HIV infected patients remains controversial. The density of parvalbumin-immunoreactive neurons in neocortex was not changed significantly in HIV-positives, but morphological abnormalities such as cell body shrinkage and fragmentation of neuron endings were observed (Masliah et al. 1992). The number of calbindin-immunoreactive interneurons in the frontal neocortex of HIVE subjects was lower in laminae 2-3 and 5-6, where the decrease was reported be up to 50% in HIVE (Masliah et al. 1995). Less intense somatostatin immunoreactivity, a marker produced by inhibitory neurons primarily, also

was observed in frontal neocortex of HIVE cases (Fox et al. 1997). Brain gene arrays have repeatedly shown that gene transcripts pertaining to GABAergic neural transmission are abnormally low in patients with HIVE. All the prior observations leave open the question regarding whether the lower staining intensity of GAD65 and GAD67, and the lower mRNA concentrations reflect the death of inhibitory interneurons due to classical neurodegeneration. A highly plausible alternative view that has never been excluded is that the expression of GABAergic cell markers is regulated in viable inhibitory neurons that have not undergone neurodegeneration. Such changes are well known to occur in the CNS during the nonlethal and normal process of synaptic plasticity (Feldman 2009). To pursue that question, I examined whether the loss of immunostaining of the GABA synthetic enzymes was associated with a reduced number of neocortical interneurons as judged by their staining using other cellular markers simultaneously. The GAD67 expression was examined in specific types of interneuronal subpopulations to determine whether a subpopulation of interneurons was selectively vulnerable. The studies will illustrate that lower staining for certain GABAergic type cell markers in the HIV infected patients occurred in viable interneurons that did not exhibit degenerative type morphological changes. As well, the tissue staining did not suggest a reduction in neuronal cell density and/or the dropping out of neurons.

4.1.2 Experimental design

This analysis was performed using samples of postmortem brain tissue collected from 48 brain specimens (Table 2.2). 12 out of 48 were HIV-1 seronegative controls and 36 out of 48 were HIV-1 infected subjects. 12 out of 36 selected HIV-positives had neuropathological features characteristic of HIV-encephalitis as determined by a neuropathologist. HIVE diagnosis was used as additional selection criterion in order to examine GABAergic anomalies in the brains with HIV-encephalitis in comparison to the HIV-positives without encephalitis. Emphasis was also placed on HIV-1 infected subjects

with neuropsychological impairment (NPI) or HIV-associated neurocognitive disorders (HAND), 24 out of 36 selected HIV-positives were diagnosed with NPI or HAND. 12 out of 36 were HIV-1 infected were clinically diagnosed as having normal neurocognitive function (Table 2.2).

The concentrations of calretinin, parvalbumin and somatostatin mRNAs in the dissected postmortem brain tissue were measured by quantitative real-time polymerase chain reaction. The concentrations of CR, PV, SOM and connexin 36 proteins were measured using immunoblotting. The individual interneuronal subpopulations were visualized by immunostaining with specific antibodies to these neuronal markers. Cellular co-localization of GAD67, which was used as the model GABAergic marker, and other antibodies that mark specific interneuronal populations was done using confocal microscopy and fluorescence immunohistochemistry. As well co-localization of connexin 36 in PV-positive interneurons was examined. The statistical analyses were performed to determine if these groups of patients differed with respect to their expression of these GABAergic neurochemical markers.

4.2 RESULTS

4.2.1 Calretinin, parvalbumin and somatostatin protein and mRNA expression in the DLPFC and ACC

Several approaches may be used to classify interneuronal subpopulations (e.g. classification based on neurochemical, morphological, physiological or synaptic features of interneurons). I used a general neurochemical approach described by Markram that is based on the fact that interneurons express either calretinin, or parvalbumin, or somatostatin to mutual exclusion. Hence, these proteins can be used to distinguish three discrete non-overlapping subpopulations of neocortical interneurons that is inclusive of almost 100 % of the GABAergic neurons in human neocortex (Markram et al 2004, Kubota et al. 2011). I analyzed GABAergic interneurons in two different brain sectors: 1)

the dorsolateral prefrontal and 2) the anterior cingulate cortices. The reason that I chose to examine these two sectors is that *GAD1* mRNA concentrations in these regions were strongly and specifically correlated with neurocognitive test scores, as highlighted in Table 3.4. The latter findings raised the possibility that cell loss in a particular neural circuit might drive worse performance specifically on tasks driven by the output of that circuit.

Calretinin mRNA and protein expression

Calretinin-Immunoreactive (CR-IR) interneurons innervate preferentially other GABAergic interneurons, exerting disinhibitory action on the principal cells (Melchitzky and Lewis 2008). The cognitive deficits observed in HAND could, therefore, result from cortical disinhibition mediated by a change in the number, morphology or laminar distribution of CR-IR neurons. To address that, I measured the concentrations of *CALB2* mRNA and CR protein in the DLPFC and ACC (Figure 4.1). No significant difference was found between the concentrations of mRNA and protein in HIV-positive patients and HIV-negative controls in these two neocortical brain sectors. To determine whether the number, morphology, or laminar distribution of CR-IR interneurons was changed I immunostained CR in the frontal neocortex of HIV-positive and HIV-negative subjects. The results showed that stain intensities, number and laminar distribution of stained neurons was similar between HIV-positive and HIV-negative specimens (Figure 4.2 A and B). Intracellular distribution of calretinin within cytoplasm and neuritic processes were similar between groups (Figure 4.2 C and D).

Parvalbumin mRNA and protein expression

Parvalbumin-immunoreactive (PV-IR) interneurons are the most powerful source of cortical inhibition. These fast-spiking neurons are responsible for the generation of γ and θ oscillations and play crucial role in cognitive function (Priest et al. 2001, Curley and Lewis 2012, Sohal et al. 2009). Loss of staining of cortical PV-IR interneurons was reported for schizophrenics, which exhibit symptoms of cognitive dysfunction similar to

those observed in HAND. To determine whether PV-IN was abnormal in the HIV-infected patients, I measured the levels of *PARV* mRNA and parvalbumin protein in the DLPFC and ACC (Figure 4.3). No significant difference was found between the levels of *PARV* mRNA and protein in HIV-positive patients and HIV-negative controls. To confirm that the number, morphology, or laminar distribution of PV-IR interneurons was not changed in HIV-infected brain, I immunostained PV in the frontal neocortex and observed that the stain intensities, number and laminar distribution of stained neurons all were similar between HIV-positive and HIV-negative specimens (Figure 4.4 A and B). The intracellular distribution of PV staining within cytoplasm and neuritic processes also was similar between groups (Figure 4.4 C and D).

Somatostatin mRNA and protein expression

Somatostatin-immunoreactive (SOM-IR) interneurons provide the only source of cross-columnar (horizontal) inhibition of impulse spread. Neuronal nitric oxide synthase (nNOS)-expressing SOM-IR interneurons also form extensive contacts with the basal lamina of capillaries and with perivascular astrocytes, which permits them to exercise control over blood flow through cerebral microcirculation (Duchemin et al. 2012). The latter type of regulation is potentially important because there are reports that cerebral blood flow, measured using blood oxygenation level-dependent functional magnetic resonance imaging (BOLD-fMRI, Ances et al. 2011, Towgood et al. 2013) is abnormal in HIV infected patients. That in turn could suggest that abnormal SOM-IR neurons in the HIV infected patients could be involved in abnormal cerebral blood flow. To address that question I measured concentrations of *SOM* mRNA and protein in the DLPFC and ACC (Figure 4.5). In both brain regions *SOM* mRNA concentration was significantly lower in the HIV-positives (-56.6 % and $p = 0.0003$ in the DLPFC and -54.7 % and $p = 0.002$ in the ACC; Figures 4.5 A and B). SOM protein expression was significantly lower in DLPFC (-26.2 %, $p = 0.012$, Figure 4.5 C), but not in ACC (+5.8 %, $p = 0.761$, Figure 4.5 D). To examine whether the decrease in SOM protein concentration was associated

with neuronal loss of this cell population, I immunostained SOM and observed that the stain intensity of small pear-shaper interneurons was similar between the HIV-positive and HIV-negative specimens, but staining of larger pyramidal shaped neurons was substantially lower in the HIV infected patients (Figure 4.6 A and B). It is noteworthy that the cellular distribution of somatostatin within the cytoplasm and neuritic processes of interneurons situated near blood vessels did not appear abnormal in the HIV infected patients (Figure 4.6 C-F).

4.2.2 GAD67 localization in the populations of calretinin-, parvalbumin-, and somatostatin-immunoreactive interneurons

Low concentration of GABA synthesizing enzymes in the brain homogenate as reported in chapter 3 could be explained by GABAergic cell drop off or by low level of GADs expression in the preserved population of interneurons. As described above, I did not found any considerable changes neither in the concentrations of protein markers specific for interneuronal subpopulation, nor in the intensity of markers immunostaining in the interneurons. Here I addressed the question whether GAD67 protein expression in the viable interneurons is lowered in the frontal neocortex of HIV-positive patients. I performed dual-immunostaining of GAD67 and each subpopulational marker (PV, CR or SOM) to localize GAD67 in each subpopulation of interneurons. Staining intensity of subpopulational markers present in the cell bodies and neural processes was of similar intensity in HIV-infected and control specimens. GAD67 staining intensity was lower in all three subpopulations of cortical interneurons of HIV-infected patients (Figure 4.7).

4.2.3 Connexin 36 protein expression in parvalbumin-immunoreactive interneurons

Morphological abnormalities such as cell bodies shrinkage and fragmentation of neuron endings were reported to occur in neocortical parvalbumin interneurons in HIV (Masliah et al. 1992). I did not find any difference in the PV-IR interneurons morphology

between HIV-positive and control subjects at the cellular level using bright field microscopy (Figure 4.4) or laser scanning confocal microscopy (Figure 4.7). To augment my findings that neuritic processes of the cortical PV-IR interneurons are intact, I measured the concentration of connexin 36 and localized gap junctions on these cells. Connexin 36 is expressed exclusively by PV-IR neurons (Galarreta and Hestrin 2001) where it forms gap junctions between neuron endings and the cell bodies of adjacent cells (Bennett and Zukin 2004). Connexin 36 is often used as marker of neuritic processes of PV-IR interneurons. I found that the concentration of *GJD2* mRNA, which codes for connexin 36, was significantly lower in the DLPFC of HIV-positives (as shown in large scale experiment, Figure 3.1), but the corresponding protein concentration was not significantly different. (Figure 4.8 C). mRNA expression for these subjects is illustrated in panel B. Localization of connexin 36 confirmed that concentration of connexin 36 in HIV-positive specimen was not lower (Figure 4.9). Immunostained connexin 36 was present as dot-like structures of similar density and intensity in the periphery of cell bodies where gap junctions from neural processes of adjacent cells are located. This suggests that the number of neural ending of PV-IR interneurons is not abnormal in the HIV infected subjects.

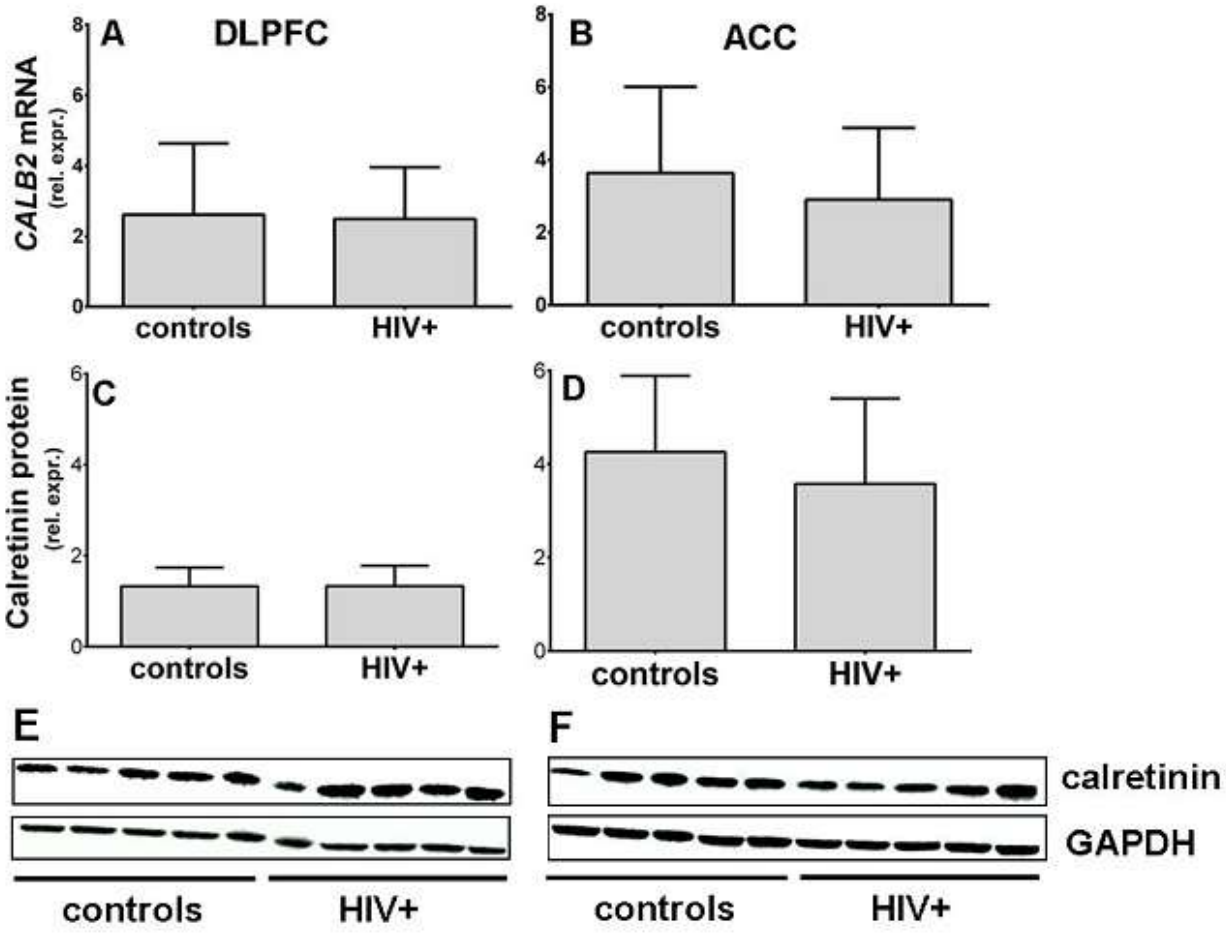

Figure 4.1 Calretinin protein and mRNA expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) cortices.. Calretinin immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (C, D). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. The band intensities did not differ significantly between the groups in DLPFC (+0.3 %, p = 0.977) and ACC (-16 %, p = 0.312). Band intensities for ten of the patients are illustrated in E and F. The concentration of *CALB2* mRNA also was not significantly different in the HIV infected patients (A, B) (-4.6 % and p = 0.824 in the DLPFC and -20.2 % and p = 0.299 in the ACC). Calretinin is expressed relative to the GAPDH band intensity. *CALB2* mRNA is expressed relative to the *GAPDH* mRNA. Mean ± standard deviation is shown. P values were obtained using the Student's t test.

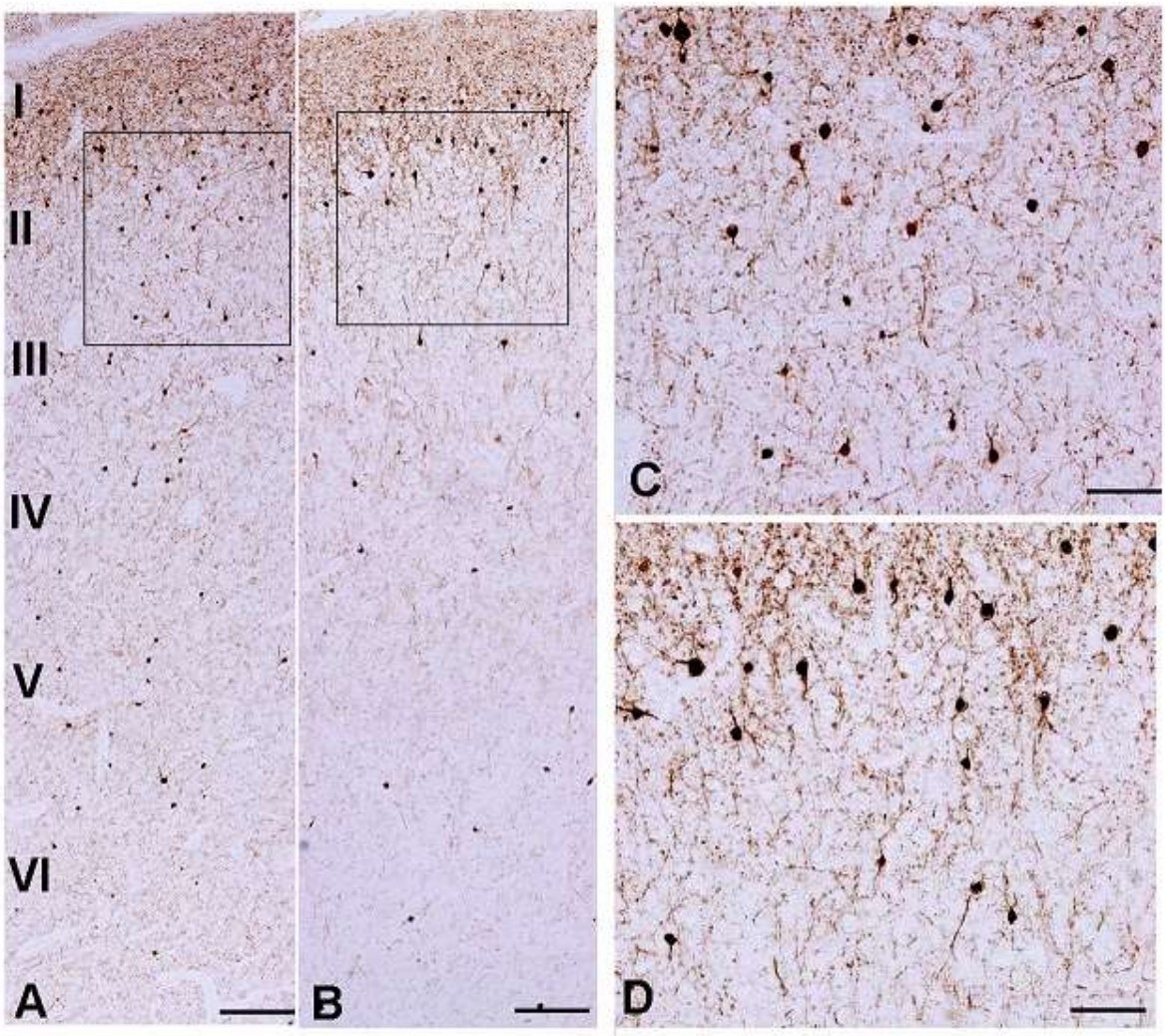

Figure 4.2 Calretinin immunostaining of a brain tissue specimen obtained from an HIV infected patient with comparison to an uninfected patient. Dorsolateral prefrontal cortex from an HIV- control patient (A) and an HIV infected patient (B) were immunostained for calretinin. Neocortical laminae are labeled I through VI at left. The general intensity of the staining is equivalent comparing the two specimens. Boxed areas are magnified in C and D, which also show equivalent stain intensities in neuronal cytoplasm and the neuritic processes of bipolar and double bouquet types of interneurons. The number of stained neurons also appears equivalent between the two specimens. Scale bars are 200 um in A and B, and 100 um in C and D.

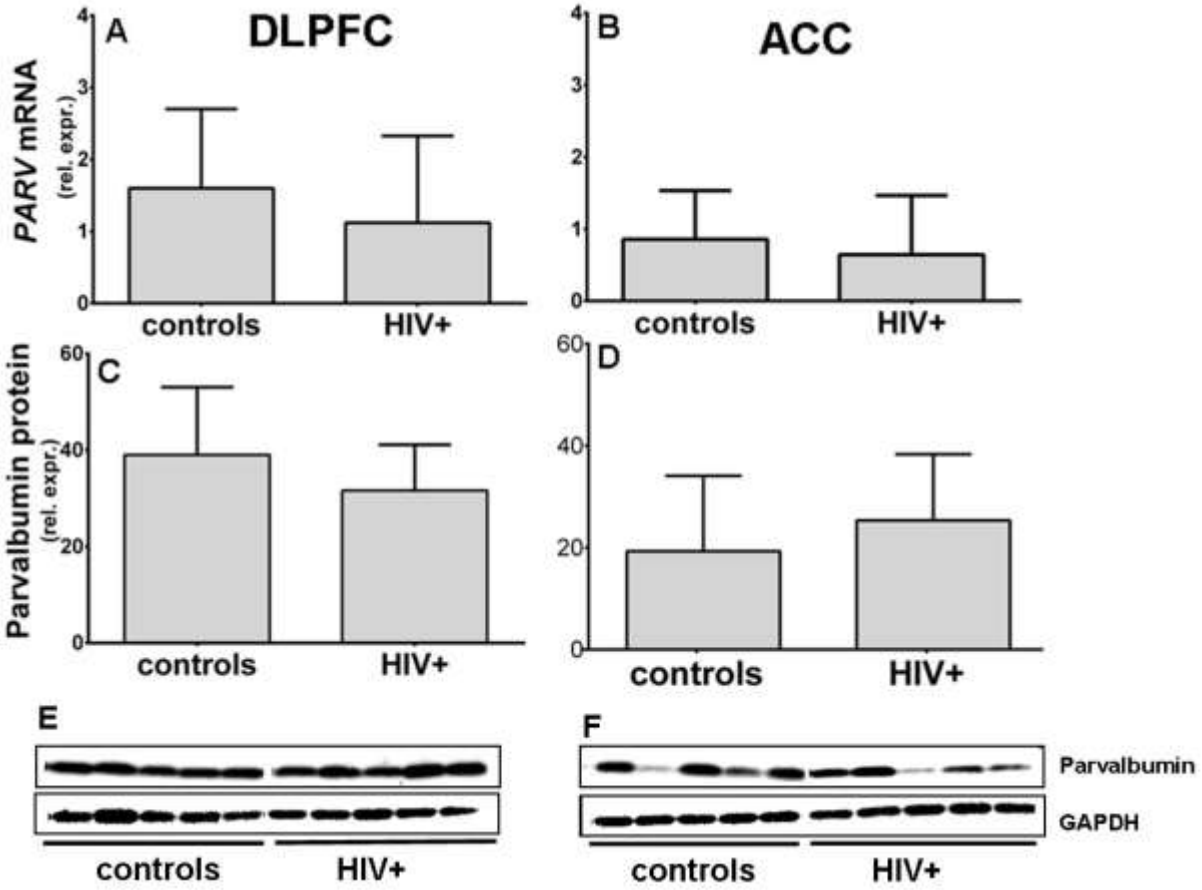

Figure 4.3 Parvalbumin protein and mRNA expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) cortices. Parvalbumin immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (C, D). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. The band intensities did not differ significantly between the groups in DLPFC (-18.9 %, p = 0.079) and ACC (+31.5 %, p = 0.253). Band intensities for ten of the patients are illustrated in E and F. The concentration of *PARV* mRNA also was not significantly different in the HIV infected patients (A, B) (-30 % and p = 0.230 in the DLPFC and -25 % and p = 0.425 in the ACC). Parvalbumin is expressed relative to the GAPDH band intensity. *PARV* mRNA is expressed relative to the *GAPDH* mRNA. Mean ± standard deviation is shown. P values were obtained using the Student's t test.

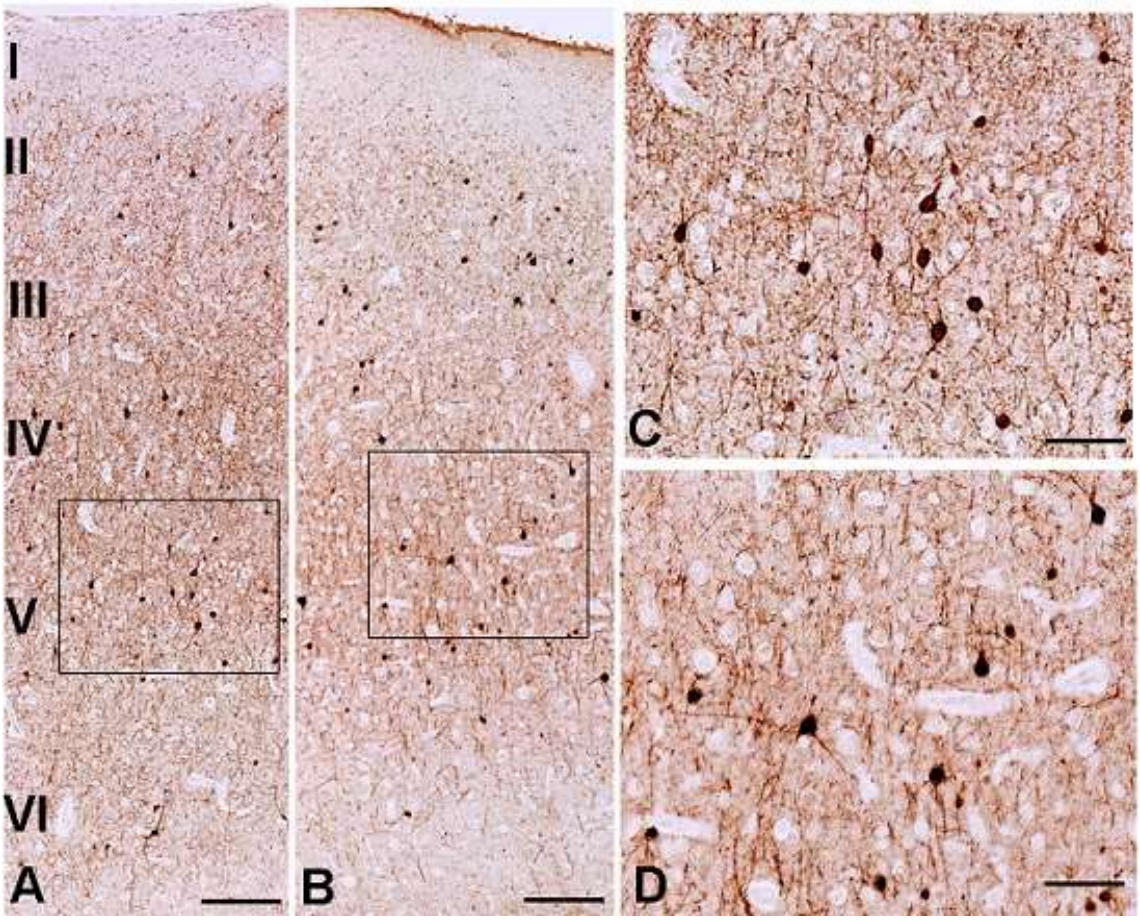

Figure 4.4 Parvalbumin immunostaining of a brain tissue specimen obtained from an HIV infected patient with comparison to an uninfected patient. Dorsolateral prefrontal cortex from an HIV- control patient (A) and an HIV infected patient (B) were immunostained for parvalbumin. Neocortical laminae are labeled I through VI at left. The general intensity of the staining is equivalent comparing the two specimens. Boxed areas are magnified in C and D, which also show equivalent stain intensities in neuronal cytoplasm and the neuritic processes of large basket and chandelier types of interneurons. The number of stained neurons also appears equivalent between the two specimens. Scale bars are 200 um in A and B, and 100 um in C and D.

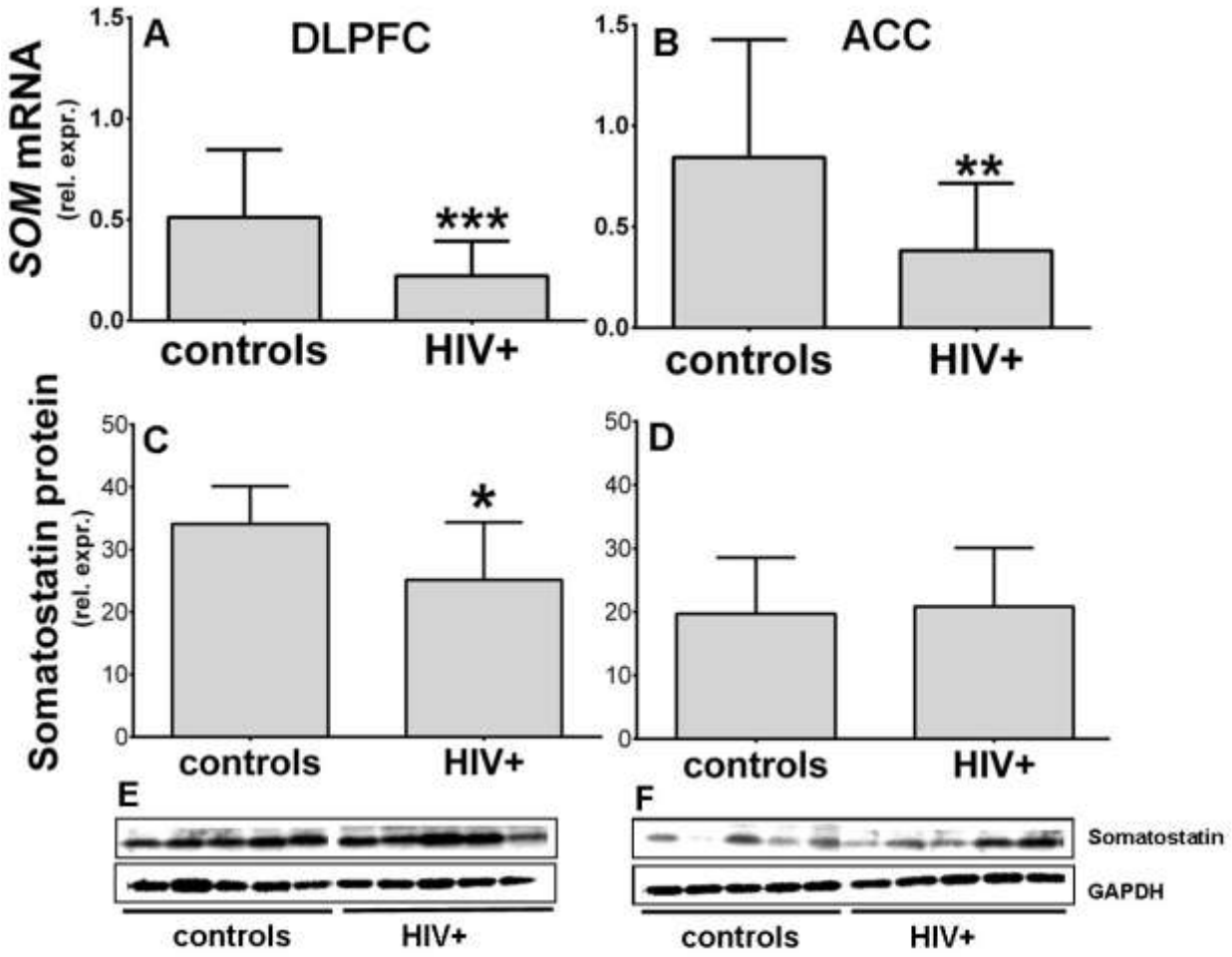

Figure 4.5 Somatostatin protein and mRNA expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) cortices. Somatostatin immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (C, D). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. The band intensities were significantly lower for the HIV-positive group in DLPFC (-26.2 %, p = 0.012) but not in ACC (+5.8 %, p = 0.761). Band intensities for ten of the patients are illustrated in E and F. The concentration of *SOM* mRNA was significantly lower in the HIV infected patients (A, B) (-56.6 % and p = 0.0003 in the DLPFC and -54.7 % and p = 0.002 in the ACC). Somatostatin is expressed relative to the GAPDH band intensity. *SOM* mRNA is expressed relative to the *GAPDH* mRNA. Mean ± standard deviation is shown. P values were obtained using the Student's t-test.

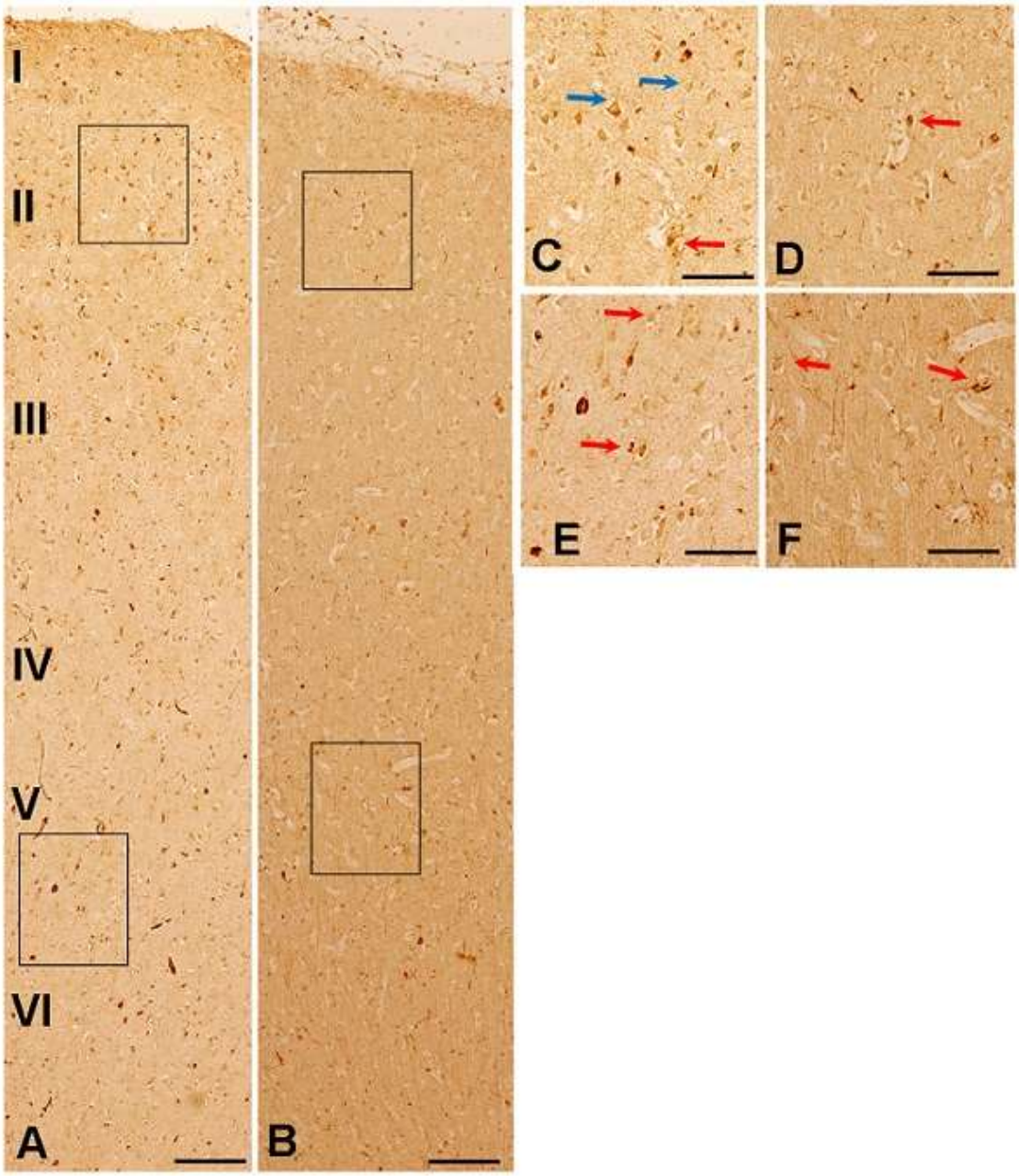

Figure 4.6 Somatostatin immunostaining of a brain tissue specimen obtained from an HIV infected patient with comparision to an uninfected patient. Dorsolateral prefrontal cortex from an HIV- control patient (A) and an HIV infected patient (B) were immunostained for somatostatin. Neocortical laminae are labeled I through VI at left. The intensity of the staining in large pyramidal cells is lower in the specimen from HIV infected patient. Boxed areas are magnified in C and D, which also show lower intensity of stain in large pyramidal neurons (blue arrows), but equivalent stain intensities in neuronal cytoplasm and the neuritic processes of pear-shaped interneurons (red arrows) near blood vessels. The number of stained pear-shaped interneurons also appears equivalent between the two specimens. Scale bars are 200 um in A and B, and 100 um in C and D.

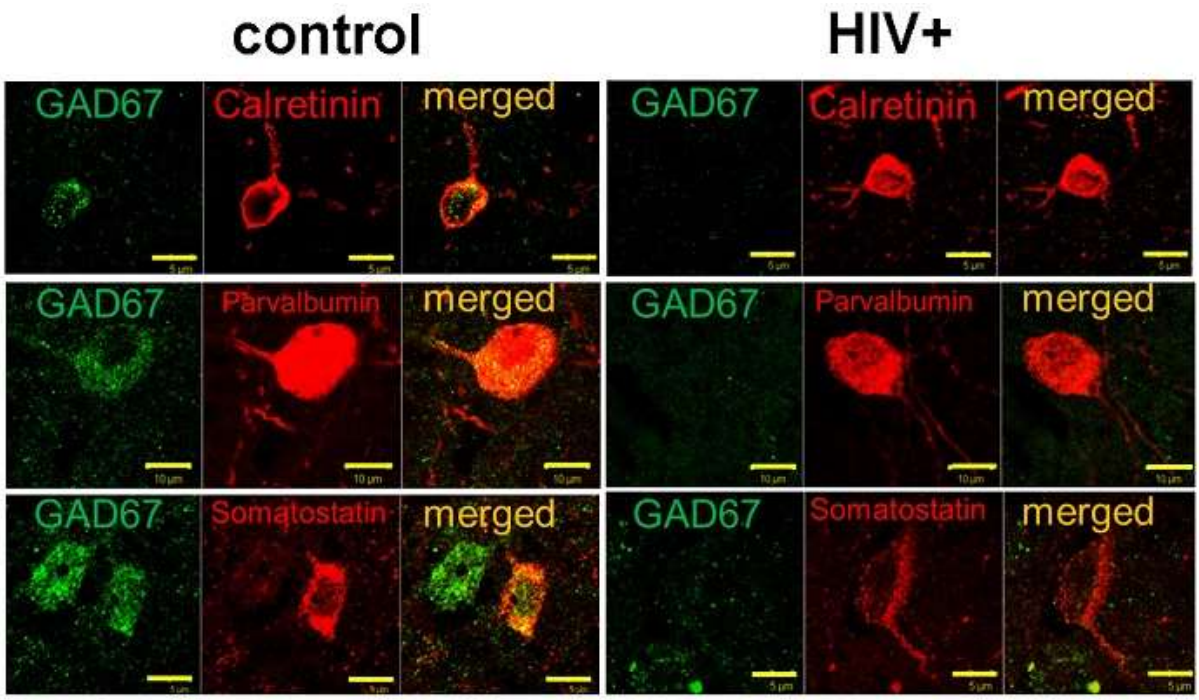

Figure 4.7 Loss of GAD67 immunostaining in viable, morphologically typical interneurons. Left panel illustrates GAD67 localization with CR, PV, and SOM in the DLPFC of control subject. Right panel illustrates GAD67 localization with markers of interneuronal subpopulation in the DLPFC of HIV-positive patient. Note that staining intensities of markers of subpopulations in the cell bodies and neural processes and was of comparable intensity in HIV-infected and control specimens. GAD67 staining intensity was lower in all three subpopulations of cortical interneurons of HIV-infected patients. Scale bars are 10um.

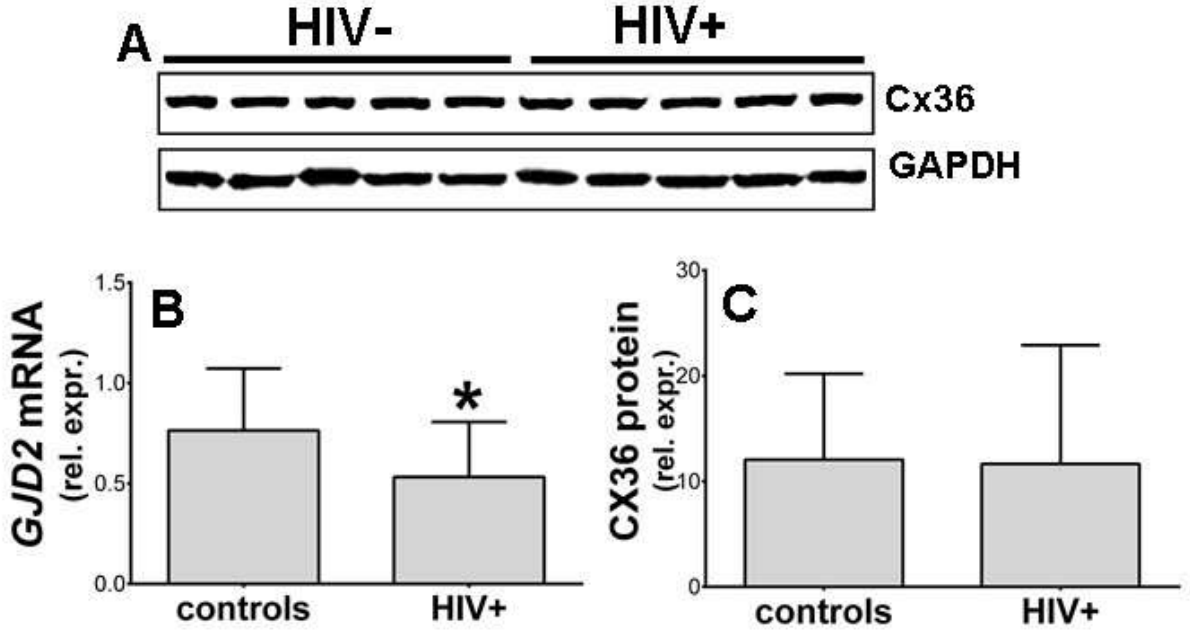

Figure 4.8 Connexin 36 protein and mRNA expression in the dorsolateral prefrontal cortex. Connexin 36 immunoblots from the DLPFC of 10 subjects, and GAPDH loading controls (panel A). Equal amounts of protein were loaded in each well. Band intensities did not differ significantly (-3.3 %, p = 0.92) in 36 HIV+ subjects compared to 12 HIV- subjects (panel C). Expression of *GJD2* mRNA encoding for connexin 36 is shown for comparison (panel B), mean *GJD2* mRNA decrease is -30.3 % (p = 0.03). Connexin 36 expression is shown relative to the GAPDH band intensity, and *GJD2* mRNA expression is shown relative to the *GAPDH* mRNA. Mean ± standard deviation. P values were obtained using the Student's t test.

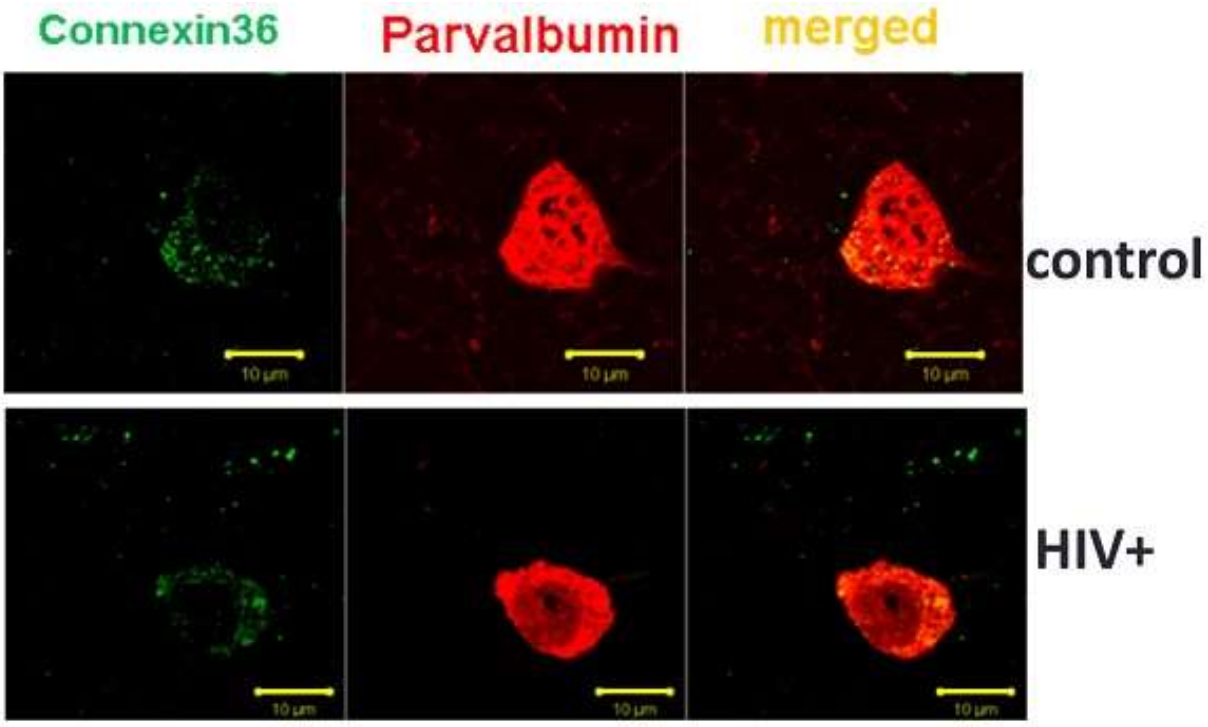

Figure 4.9 Connexin 36 co-localization with parvalbumin in the frontal cortex from an HIV infected patient and a seronegative control. The brain specimen from the HIV infected patient (bottom panels) contains a parvalbumin expressing interneuron that exhibits connexin 36 staining of equivalent intensity to an interneuron from the uninfected control patient. Scale bars are 10 um.

Chapter 5: Relationship of low GABA expression to astroglial synthesis of glutamine synthetase

5.1 Introduction and Rationale

A critical observation that I made in the work described previously is that the HIV RNA copy number in brain tissue was not related significantly to the lower expression of GABAergic mRNAs. Instead I found that the lower GABAergic mRNA was significantly linked to higher expression of immune and endothelial cell type markers. One implication of those observations was that the cells that synthesized those markers, which would include activated astrocytes and CNS macrophage and microglial cells, could be involved in GABAergic anomalies.

Astrocytes are known to be critically involved in synaptic transmission. The delicate cytoplasmic processes of these cells are an integral component of the "tripartite synapse". One way that they regulate synaptic function is by terminating neurotransmitter action. Astrocytes can remove glutamate from the synaptic cleft and then convert it to glutamine via the enzyme glutamine synthetase (GS). In turn, the glutamine is transported to presynaptic terminals, and is converted back to glutamate to be reused. In addition to recycling glutamate, the glutamine synthesized by astroglial cells is the main precursor to interneuronal GABA synthesis via glutamic acid decarboxylase (Sonnewald et al. 1993, Rae et al. 2003). As a result, dysregulation of GS is an important mechanism that can reduce the synthesis of GABA (Ortinski et al. 2010) and lower interneuronal GAD concentrations (Struzynska et al. 2004). The expression, distribution and activity of brain GS is altered in several brain disorders including Alzheimer's disease, schizophrenia, depression and temporal lobe epilepsy (Eid et al 2004, Eid et al. 2011, Papageorgiou et al. 2011, Eid et al. 2012). Overproduction of oxygen or nitrogen-reactive species and other inflammatory type stimuli that are associated with HIV infection can lead to

dysregulation of astrocyte GS in vitro (Muscoli et al. 2005, Janda et al. 2011). Since astroglial cell GS plays a critical role in GABAergic and glutamatergic neurotransmitter metabolism, I hypothesized that the dysregulation of astroglial glutamine synthetase might be involved in the lowering of GABA synthesis in HIV-infected patients. To address that question I measured the concentration and analyzed the intracellular distribution of GS in the brain specimens of HIV-positives and compared the results to their lower GAD67 expression.

5.1.2 Experimental design

The analysis was performed using samples of postmortem brain tissue as described in chapter 4.1.2. Dorsolateral prefrontal and anterior cingulate cortices were regions of interest for this study, because *GAD1* mRNA concentrations specifically in these regions were strongly and significantly correlated with neurocognitive test scores (Table 3.4).

The concentrations of glutamine synthetase in the dissected postmortem brain tissue were measured by immunoblotting. Localization of GS and glial fibrillary acidic protein (GFAP) were done using immunofluorescence microscopy and confocal imaging. Student's t-test was performed to determine if groups of patients differed with respect to GS expression. Correlation between levels of *GAD1* and GS expression was evaluated using Pearson's correlation analysis.

5.2 RESULTS

5.2.1 Concentration of glutamine synthetase protein in the ACC and DLPFC

Elevated concentrations of inflammatory factors, which are an essential feature of HIV-infected brain, were shown to cause the decrease of GS in astroglial cell culture (Muscoli et al. 2005, Janda et al. 2011). Nevertheless the question whether GS concentration is changed in HIV-infected brain has not been addressed yet. To fill that

gap, I measured the levels of GS protein in the DLPFC and ACC (Figure 5.1). In both brain sectors GS was significantly lower in HIV-positive patients (-23.9 % and $p = 0.038$ in the DLPFC, and -30.8 % and $p = 0.043$ in the ACC).

5.2.2 Correlation between the level of GS and *GAD1*

Lower expression of interneuronal GABA synthesizing enzyme was observed as result of diminished glutamine transport to nerve endings (Struzynska et al. 2004). To investigate whether lower astroglial GS expression is related to the decreased level of interneuronal *GAD1* mRNA in the HIV-infected brains, I performed Pearson's correlation analysis. For both regions the correlation coefficients were significant with a positive slope (in the DLPFC $r = 0.374$ and $p = 0.029$); in the ACC $r = 0.462$ and $p = 0.050$). Using bootstrapping and Fisher z-transformations these correlations were not significantly different from each other (two-tailed $p = 0.664$).

5.2.3 Distribution of GS within the cortical astrocytes in HIV-1

Redistribution of GS from distal to proximal astrocytic branches and cell bodies was reported in pathological states (e.g. epilepsy) and indicates lowered GS activity (Eid et al. 2012). Such changes in intracellular localization GS often is followed by decreased synthesis of neuronal GABA (Sonnewald et al. 1993). To analyze the distribution pattern of GS within cortical astrocytes, I performed dual immunofluorescence staining of GS and the astroglial marker glial fibrillary acidic protein (GFAP). Staining intensity of GS was lower in the cell bodies and proximal processes and predominantly lost in the distal processes of astrocytes of the HIV-infected patients. The results from two neocortical sectors examined were equivalent (Figure 5.3).

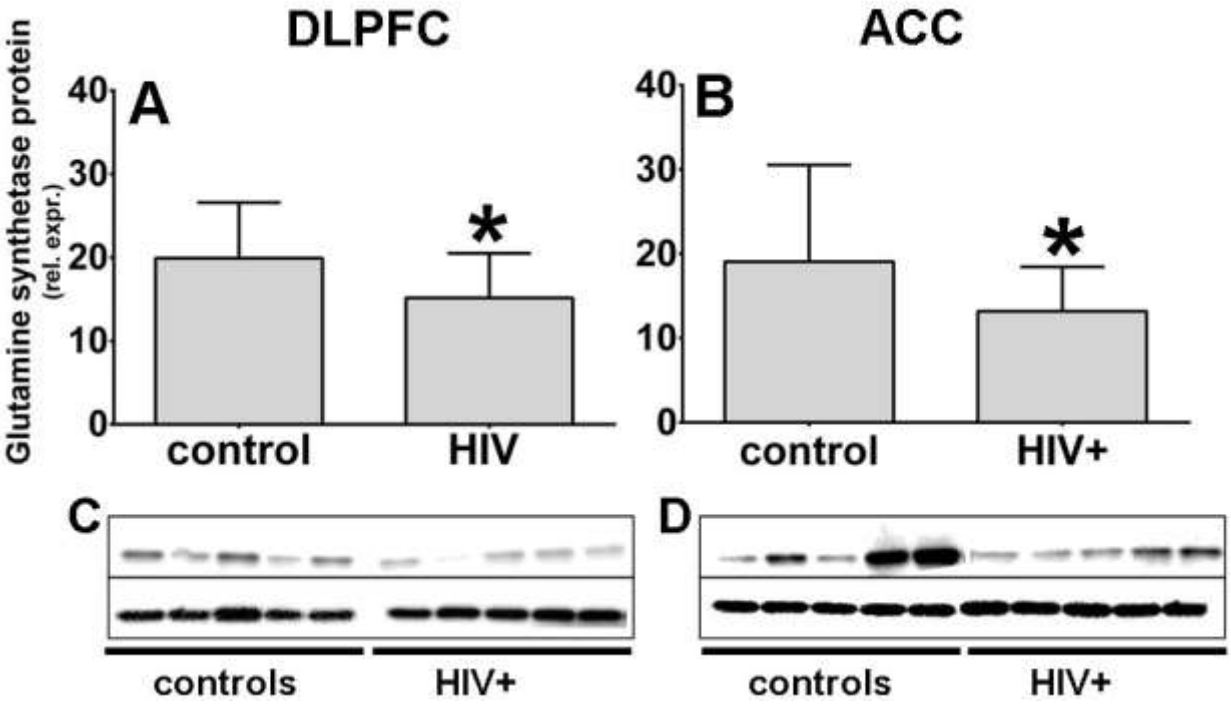

Figure 5.1 Glutamine synthetase protein expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) cortices. GS immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (A and B). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. The band intensities were significantly lower for the HIV-positive group in DLPFC (-23.9 %, p = 0.038) and ACC (-30.8 %, p = 0.043). Band intensities for ten of the patients are illustrated in C and D. GS is expressed relative to the GAPDH band intensity. Mean ± standard deviation is shown. P values were obtained using the Student's t test.

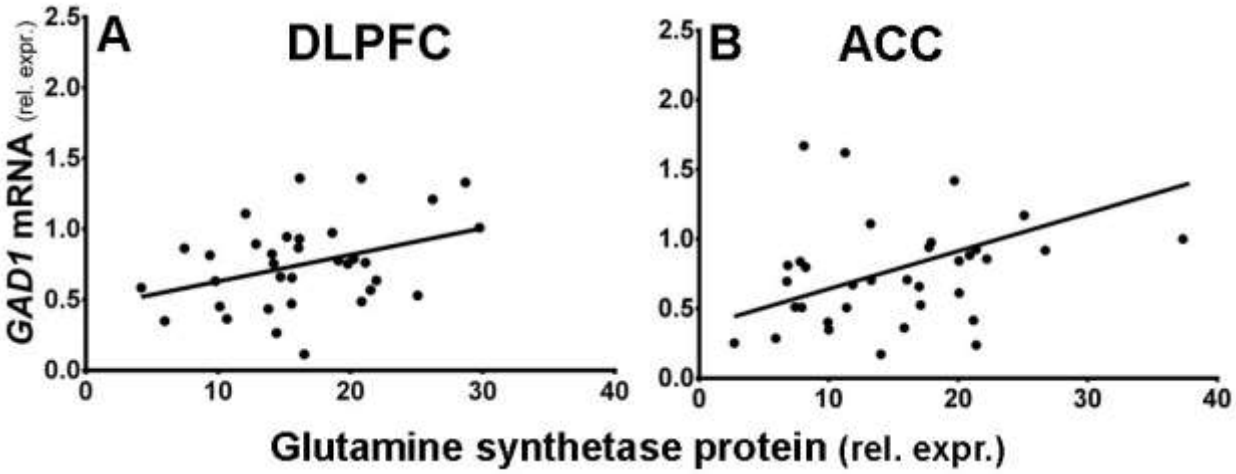

Figure 5.2 Correlation between the levels of GS and *GAD1* mRNA expression in the dorsolateral prefrontal and anterior cingulate cortices. In both neocortical regions examined glutamine synthetase protein and *GAD1* mRNA concentrations tended to run in the same direction; the correlation coefficients were significant with positive slopes in the DLPFC r = 0.374 (p = 0.029) and the ACC r = 0.462 (p = 0.050). The two correlation coefficients illustrated were not significantly different from each other (Fisher's z-score -0.434, p = 0.664). Expression of the mRNAs was normalized to *GAPDH* mRNA**.**

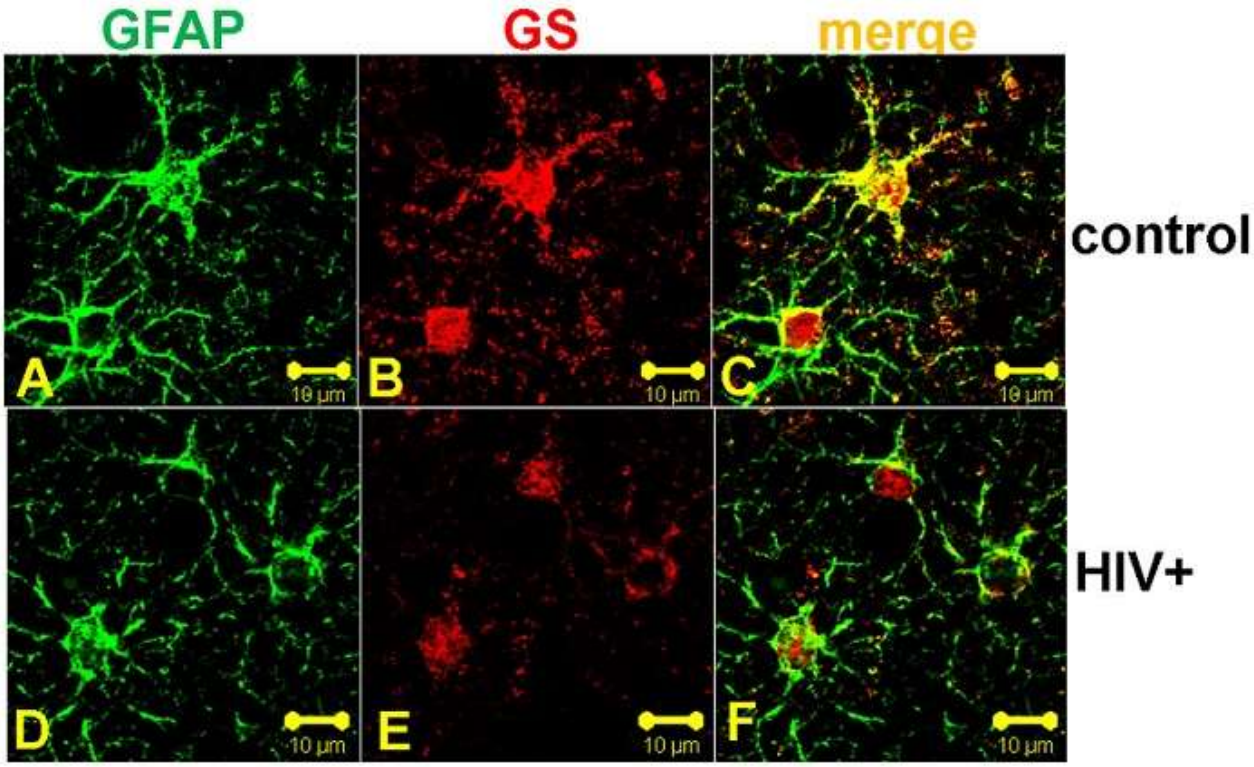

Figure 5.3 Loss of glutamine synthetase immunostaining in neocortical astrocytes of an HIV infected patient. Confocal immunofluorescence microscopy for GS (red) and the astroglial marker GFAP (green) was performed in frontal neocortex samples of an HIV-infected patient (D, E, and F) and an uninfected control patient (A, B, and C). The intensity of astrocyte GFAP staining in these two patients r is equivalent (A and D). Immunostaining for GS is much lower in the HIV-infected patient (B and E). In the merged images note the complete loss of punctate deposits of GS protein in the cell processes (C and F). Scale bars are 10 um.

Chapter 6: Low GABA is interrelated with the regulation of dopaminergic transmission

6.1 INTRODUCTION

6.1.1 Rationale

Several neurotransmitter systems have been reported to be abnormal in HIV infected people with HAND. Foremost among them is the dopamine neurotransmitter system (Nath et al. 2000, Koutsilieri et al. 2002, Gelman et al. 2006, Gelman et al. 2012a, Gelman et al. 2012b), but the exact nature of the disturbance remains unclear. Clinical reports of an unusual Parkinsonian syndrome in HIV infected patients suggested that there may be worsening of neurodegenerative changes of dopaminergic brainstem neurons in certain people (Berger and Arendt 2000, Koutsilieri et al. 2002). De novo neurodegeneration of dopaminergic neurons has been suggested as a potential explanation of these case reports (Berger and Arendt 2000, Koutsilieri et al. 2002). However, the dropping out and degeneration of dopaminergic neurons to an extent that would be severe enough to produce Parkinsonian syndrome (at least 60 % of the neurons) has not generally been confirmed pathologically. Instead, neurochemical studies of HIV infected patients have suggested that dopaminergic tone is higher in patients that perform worse on neuropsychological tests. In a study of over 500 human brain specimens, the failure to suppress the expression of dopamine receptor type 2 long isoform mRNA (*DRD2L*) was associated with worse neurocognitive performance in HIV infected patients (Gelman et al. 2012b). These neurochemical changes were suggested to be attributable generally to altered synaptic plasticity, and more specifically, to a failure to accommodate to higher presynaptic tone. While these data were quite robust statistically, the fact that postmortem data are retrospective means that the higher *DRD2L* expression

could reflect a pre-existing trait of patients that performed worse on neuropsychological tests (versus being driven by HIV infection).

To further explore the prevailing concept that synaptic plasticity is what drives neurochemical changes in HIV infection, I decided to explore whether lower GABAergic tone is interrelated with changes in other neurotransmitter systems including dopamine. Studies were conducted to compare the GABAergic system to a key anomaly observed in the dopaminergic system. This was a highly plausible avenue to explore because it has been established experimentally that these two neurotransmitter systems overlap with each other, and they are potentially interactive (Seamans et al. 2001). My working hypothesis was that dopamine-activated DRD2s on local GABAergic interneurons are one potential cause of decreased *GAD1* mRNA expression (Lindefors 1993, Kalkman and Loetscher 2003). If so, the net result in these patients would be that inhibitory transmission to principal cells would become suppressed in part by higher dopaminergic tone (Seamans et al. 2001, Gorelova et al. 2002, Xu et al. 2010). Such a disinhibition of cortical output is believed to be a key characteristic of HIV-associated cognitive disorders (Archibald et al 2004). I addressed that general hypothesis by performing studies to determine whether overexpression of DRD2 observed in the cohort of cognitively impaired HIV-patients (Gelman et al. 2012b) is associated with higher expression of DRD2 in GABAergic interneurons, and also, whether interneuronal DRD2 is related to downregulation of GABA synthesizing enzymes in the impaired HIV-infected patients First I examined whether there is a specific neuroanatomical pattern or regional distribution of abnormal DRD2 expression in the cortex of HIV-infected patients. I then examined DRD2 expression specifically in cortical interneurons and characterized the relationship between overexpression of DRD2 and lower *GAD1* mRNA concentration in these patients.

6.1.2 Experimental design

The analysis was performed using samples described in chapter 4.1.2. Dorsolateral prefrontal and anterior cingulate cortices were regions of interest for this study, because *GAD1* mRNA concentrations specifically in these regions were strongly and significantly correlated with neurocognitive test scores (Table 3.4). The concentration of *DRD2L* mRNA in the dissected postmortem brain tissue was measured by quantitative real-time polymerase chain reaction. The cellular localization of DRD2 was examined using immunohistochemistry. Cellular co-localization of DRD2 and parvalbumin that mark specific interneuronal populations was done using confocal microscopy and fluorescence immunohistochemistry. Statistical analyses were performed to determine if groups of patients differed with respect to *DRD2L* mRNA expression. Potential independent variables that were examined included HIV infection, HIV neuropathology, HIV-associated neuropsychological impairment. Relationships between the concentrations of *DRD2L* and *GAD1* mRNAs were evaluated using Pearson's correlation analysis.

6.2 RESULTS

6.2.1 Regional distribution of *DRD2L* anomaly in the CNS

Overexpression of dopamine receptor type 2 long isoform mRNA (*DRD2L*) was reported in the DLPFC of the HIV-infected patients with worse neurocognitive performance (Gelman et al. 2012b). To determine if only DLPFC was selectively vulnerable to having abnormal *DRD2L* mRNA expression, I measured *DRD2L* mRNA in 4 cortical regions including dorsolateral prefrontal, anterior cingulate, superior temporal, somatosensory cortices (Figure 6.1). Eight HIV negative controls, nine HIV-infected subjects without HIV-neuropathology and 3 subjects with HIVE were selected for study based upon their *GAD1* mRNA values in the DLPFC. There was substantial variation in *DRD2L* mRNA expression between brain regions ($F = 8.431$, $p < 10^{-4}$ two way ANOVA)

and between groups of subjects ($F = 17.38$, $p < 10^{-4}$, two way ANOVA). In all cortical sectors *DRD2L* mRNA was higher in the HIV infected subjects with HIVE.

6.2.2 DRD2-immunoreactivity in brain tissue

In the primate neocortex *DRD2* mRNA is expressed by approximately 54% of pyramidal neurons and 15-20% of GABAergic parvalbumin-immunoreactive interneurons (De Almeida and Mengod 2010). DRD2-expressing neurons are primarily concentrated in lamina V, where they control cortical output (Lidow et al 1991, Wang et al. 2004). Overexpression of *DRD2L* mRNA linked to worse cognitive outcome was reported for DLPFC of HIV-infected patients (Gelman et al. 2012). To address the question whether cortical interneurons contribute to increase in *DRD2* mRNA levels, I performed immunostaining of the brain neocortex from HIV-infected and HIV-negative comparison patients. Stain intensities, number and laminar distribution of DRD2 immunoreactive large pyramidal neurons were similar between HIV-positive and HIV-negative specimens (Figure 6.2 A-F). Number and stain intensities of small pear-shaped interneurons were substantially higher in HIV-infected patient (Figure 6.2 B, D, and F). To confirm that the DRD2 staining occurred in the interneurons I performed dual-immunostaining of DRD2 and parvalbumin (PV), which is a marker of a subpopulation of GABAergic interneurons. The staining intensity of PV present in the cell bodies and neural processes was of similar intensity in HIV-infected and control specimens (Figure 6.3 A and D). DRD2 staining intensity was higher in interneurons of the HIV-infected patients (Figure 6.3 B and E).

6.2.3 Relationships between *DRD2L* and *GAD1* mRNAs in the DLPFC.

Abnormal *DRD2L* mRNA is related to abnormal GABAergic transmission and synaptic plasticity. GAD67 expression is decreased by chronic stimulation of DRD2 receptors (Kalkman and Loetscher 2003). To examine the relationship between *GAD1*

and *DRD2L* mRNAs expression in the HIV-infected patients, I compared *DRD2L* gene expression to the results for *GAD1* mRNA (Figure 6.4). In seronegative controls *GAD1* mRNA was positively and significantly correlated with *DRD2L* ($n = 66$, $r = 0.267$, $p = 0.03$). In contrast, the same correlation analysis in the HIV infected subjects produced a significant negative correlation coefficient ($n = 449$, $r = -0.157$, $p = 0.0008$). Using bootstrapping and Fisher z-transformations these correlations were significantly different from each other (Fisher's z-score 3.209, two-tailed $p = 0.001$). Thus, lower GABAergic transmission is significantly related to higher *DRD2L* expression in the HIV infected subjects but not in the seronegative controls. When HIV-positive subjects were grouped according to the clinical diagnosis of HAND versus no HAND there was a significant correlation between *GAD1* and *DRD2L* mRNA in the subjects with HAND ($n = 197$, $r = -0.183$, $p = 0.01$). Infected subjects without HAND also exhibited a negatively sloped regression line, but the correlation was not significant statistically ($n = 43$, $r = -0.159$, $p = 0.306$), likely due to the more limited number of available subjects in that subgroup.

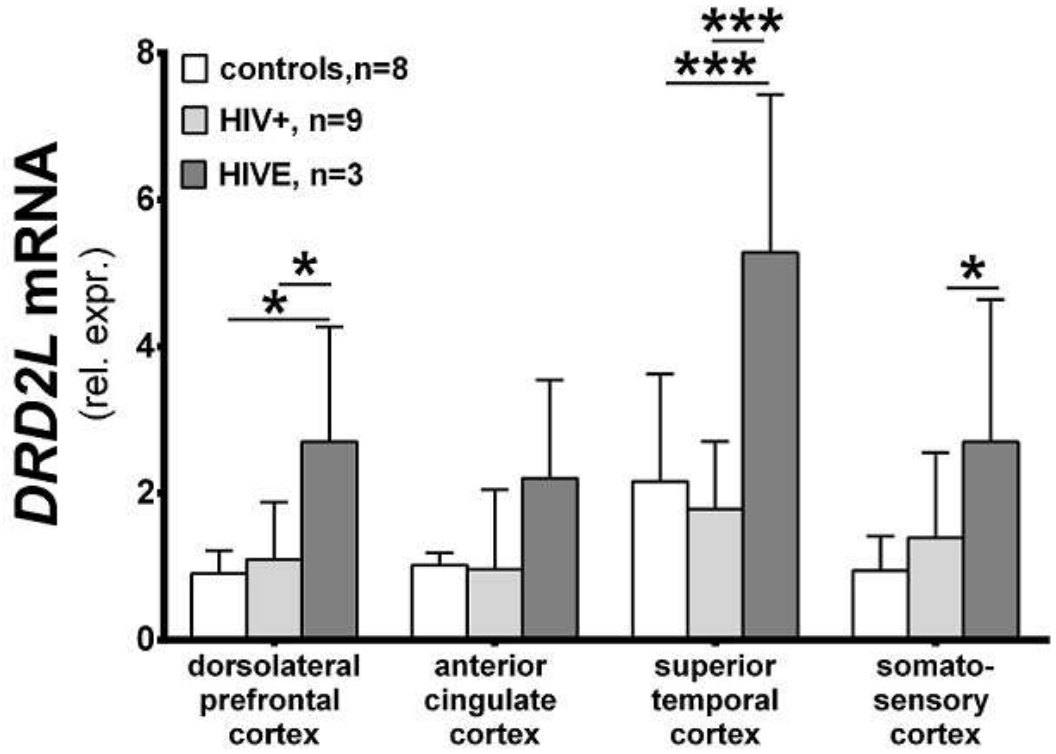

Figure 6.1 *DRD2L* mRNA expression in four different sectors of human neocortex. Dopamine receptor 2 long isoform 1 mRNA (*DRD2L*) expression was measured in 12 HIV-infected patients that included 3 with HIVE, and in 8 uninfected patients. Significant variations in DRD2L mRNA concentrations between groups of subjects ($F(2, 66) = 17.38$, $p < 10^{-4}$) and between brain regions ($F(3, 66) = 8.431$, $p < 10^{-4}$, two way ANOVA) are shown. Interaction between the factors was not significant ($p = 0.297$). *DRD2L* mRNA concentration in the superior temporal cortex was significantly different when compared to prefrontal, anterior cingulate, and somatosensory cortices ($p = 0.002$, $p = 0.001$, and $p = 0.04$ respectively, Tukey's multiple comparisons tests). *DRD2L* was higher in the HIV infected brain specimens with HIVE pathology in all cortical regions. Post-hoc Tukey's multiple comparisons tests were significant for dorsolateral prefrontal (+198 %, $p = 0.013$, when compared to controls; + 146%, $p = 0.025$ when compared to HIV+ without HIVE), superior temporal (+ 145%, $p < 10^{-4}$, when compared to controls; + 196%, $p < 10^{-4}$, when compared to HIV+ without HIVE), and somatosensory cortices (+ 186%, $p = 0.016$ when compared to controls). In the anterior cingulate cortex *DRD2L* mRNA expression was also higher in HIVE group, but did not reached significance (+ 116%, $p = 0.099$). *DRD2L* mRNA is expressed relative to *GAPDH* mRNA. Mean ± standard deviation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, two-way ANOVA.

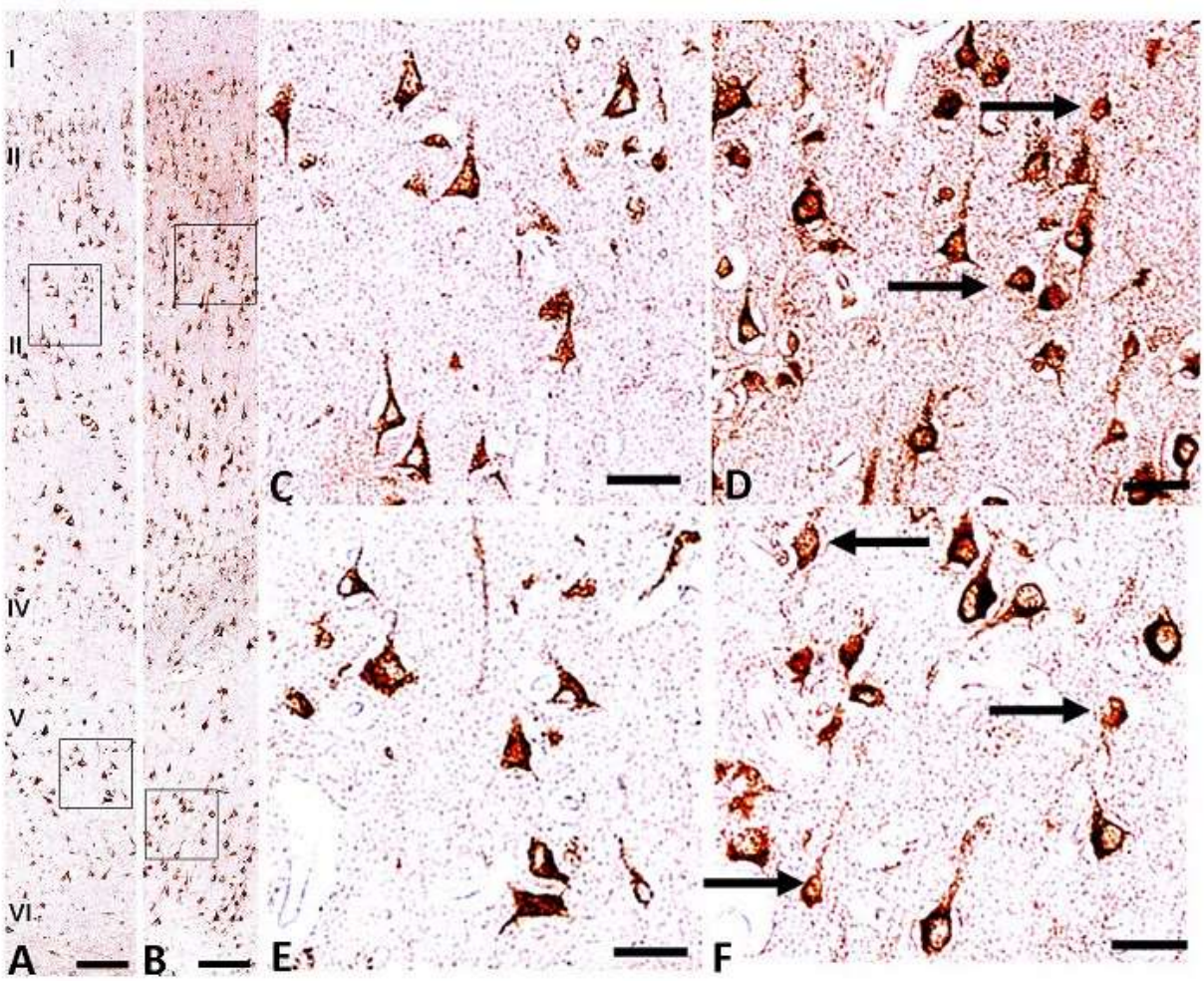

Figure 6.2 DRD2 immunostaining of a brain tissue specimen obtained from an HIV infected patient with comparison to an uninfected patient. Dorsolateral prefrontal cortex from an HIV- control patient (A) and an HIV infected patient (B) were immunostained for DRD2. Neocortical laminae are labeled I through VI at left. The intensity of the staining in large pyramidal cells is similar between the specimens. Note the number of stained small pear-shaped neurons (black arrows) is increased in the HIV-positive tissue (B, D, and F). Boxed areas are magnified in C, D, E, and F, respectively. Scale bars are 200 um in A and B, and 100 um in C, D, E, and F.

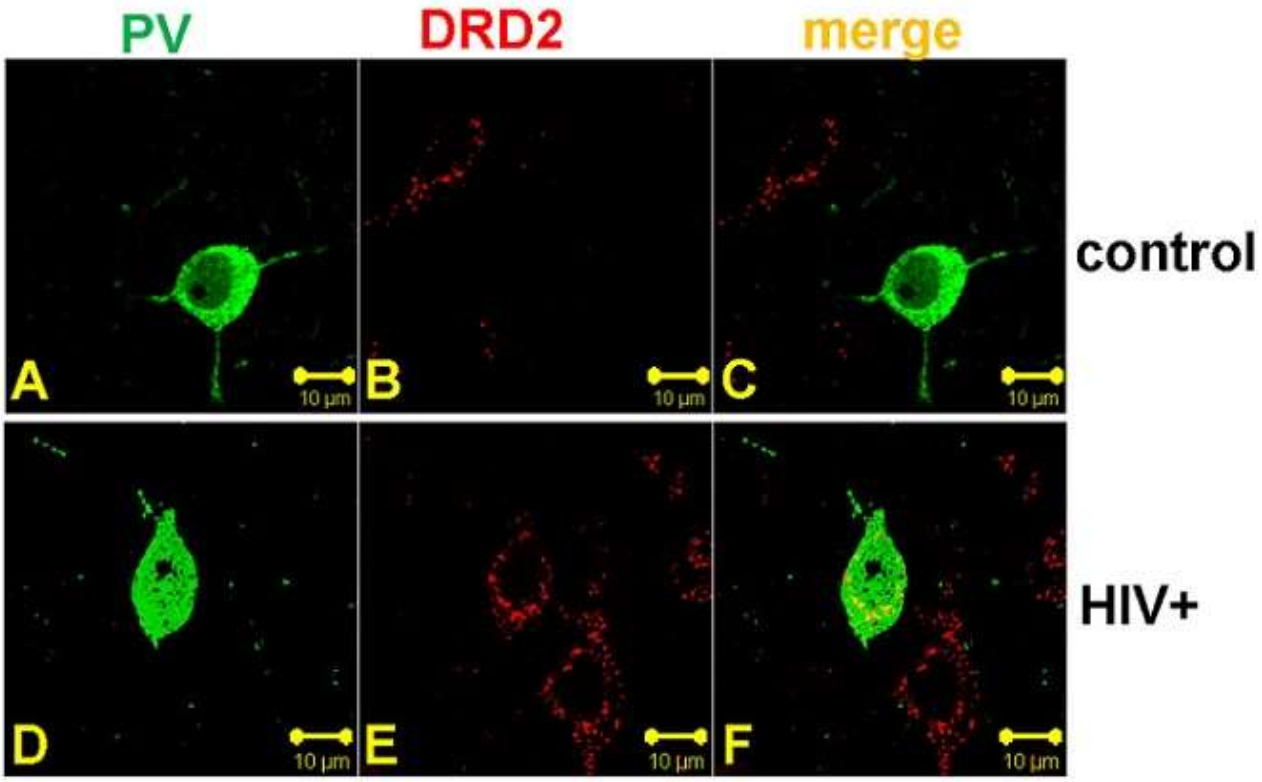

Figure 6.3 Dopamine receptor 2 (DRD2) staining is increased in parvalbumin-stained interneurons. Confocal immunofluorescence microscopy of the interneuron marker parvalbumin (PV, green, A and D) and DRD2 (red, B and E) is shown in the dorsolateral prefrontal cortex of an uninfected patient (top) and a patient with HIV infection and HAND (bottom). Note that the HIV-infected patient with HAND has more DRD2 expression than the uninfected control patient (E versus B). DRD2 was more frequently localized within PV-stained interneurons in the HIV-infected patients with HAND as compared to the uninfected patients (orange, F versus C). Immunostaining for PV was of similar intensity in these two patients (A and D). The fact that the HIV infected patients often do not downregulate their neocortical DRD2 expression, as illustrated here, was previously shown to be associated with worse performance on neuropsychological testing. Scale bars are 10 um.

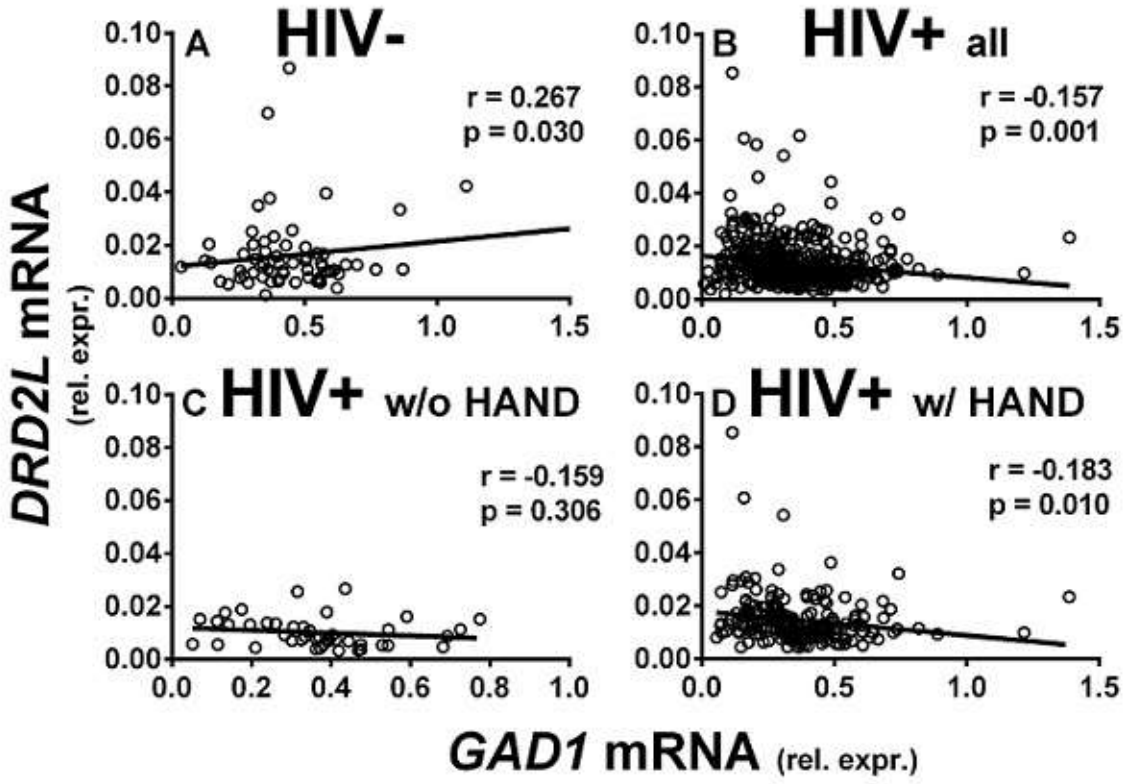

Figure 6.4 Low *GAD1* mRNA is correlated with high dopamine receptor type 2 long isoform (*DRD2L*) in HIV infected patients. *GAD1* and *DRD2L* mRNAs in the dorsolateral prefrontal cortex were correlated with each other significantly in HIV-infected and uninfected patients. The regression line was positively sloped and Pearson's coefficient was significant (n = 66, r = 0.267, p = 0.030) in the uninfected patients (A). In sharp contrast, the regression line was negatively sloped and significant (n = 449, r = -0.157, p = 0.0008) in the HIV-positive subjects (B). When the HIV-positive subjects were sorted according to whether or not HAND was diagnosed when data were available (C and D), the negatively sloped regression line and significance remained in the subjects with HAND (n = 230, r = -0.183, p = 0.01), but significance was not retained in the subjects without HAND (n = 43, r = -0.159, p = 0.305), likely because few patients without impairment were available. Relative expression of the mRNAs was normalized to *GAPDH* mRNA. HAND, HIV-associated neurocognitive disorders.

Chapter 7: Low GABA is linked with altered regulation of GABA uptake by astroglial cell processes in the synaptic cleft

7.1 Introduction and Rationale

In brain neocortex GABA transporter 1 (GAT-1) accumulates within presynaptic boutons of parvalbumin-immunoreactive interneurons, which are predominantly chandelier cells. These cells contact the axon initial segments of pyramidal neurons and to a lesser extent are in contact with distal branches of astroglial processes (Inda et al. 2007, Beenhakker et al. 2010). GAT-1 can exert strong control over inhibitory synaptic tone by rapidly removing extracellular GABA, which terminates inhibitory current and prevents neurotransmitter "spillover" from the synaptic cleft. Suppression of GAT-1 activity experimentally leads to stronger phasic inhibition and reduced neocortical recurrent activity (Razik et al. 2013). *SLC6A1* mRNA (encoding for GAT-1 protein) and GAT-1 protein expression are decreased in the prefrontal cortex of patients with schizophrenia (Lewis et al. 2001, Woo et al. 1998, Volk et al. 2001) and in brain specimens from elderly people (Cruz et al. 2003, Sundman-Eriksson and Allard 2006). Disease-associated changes in GAT-1 expression appear to exhibit some specificity for certain anatomical regions and circuits of the brain. Thus, GAT-1 protein was found to be elevated in the anterior cingulate cortex of patients with schizophrenia (versus a decrease in prefrontal cortex). The suggested mechanism for the change is that it represents a compensatory reduction in the strength of inhibitory networking within particular brain circuits (Sundman-Eriksson et al. 2002), presumably via synaptic plasticity. Astrocytes were also known to express GABA transporters, with GAT-1 primarily found on distal processes enwrapping synapses and GAT-3 on the soma and proximal processes (Minelli et al. 1995, Beenhakker et al. 2010). Dysfunction or abnormal regulation of astrocytic

GATs was reported for seizures (Pirttimaki et al. 2013) and schizophrenia, where upregulation of glial GAT-1 expression was attributed to the compensatory mechanism for the loss of inhibitory neurons (Sundman-Eriksson et al. 2002). The fact that the predominantly "subcortical" neurocognitive impairment in HIV-infected patients can strongly resemble what occurs in schizophrenic patients (McArthur et al. 2010) suggested to me that GABAergic transport also could be abnormal in patients with HAND. To address that question I measured the concentrations of *SLC6A1* mRNA and GAT-1 protein in selected sectors of brain neocortex, and then examined the laminar and cellular distributions of GAT-1 protein in brain tissue sections. All these measurements were then subjected to a clinicopathological analysis as in previous chapters.

7.2 EXPERIMENTAL DESIGN

The analysis was performed using samples of postmortem brain tissue described in chapter 4.1.2. Dorsolateral prefrontal and anterior cingulate cortices were regions of interest for this study, because *GAD1* mRNA concentrations specifically in these regions were strongly and significantly correlated with neurocognitive test scores (Table 3.4). The concentration of *SLC6A1* mRNA in the dissected postmortem brain tissue was measured by quantitative real time polymerase chain reaction. The concentration of GAT-1 protein was measured by immunoblotting. Laminar distribution of GAT-1 was evaluated by immunostaining with specific antibody and bright-field microscopy. Localization of GAT-1 and glial fibrillary acidic protein (GFAP) were done using immunofluorescence microscopy and confocal imaging. Student's t-test was performed to determine if groups of patients differed with respect to *SLC6A1* mRNA or protein expression. Correlations were evaluated using Pearson's correlation analysis.

7.3 RESULTS

7.3.1 *SLC6A1* mRNA and protein expression in the DLPFC and ACC

Lowered concentrations of neocortical *SLC6A1* mRNA and GAT-1 protein were reported in neurocognitive and psychiatric disorders (Woo et al. 1998, Volk et al. 2001, Cruz et al. 2003, Sundman-Eriksson and Allard 2006). The question whether GAT-1 concentration is changed in the cortical regions of patients with HIV-associated neurocognitive impairments has not been addressed yet. To address that question, I measured the concentrations of *SLC6A1* mRNA and GAT-1 protein in the DLPFC and ACC (Figure 7.1). *SLC6A1* mRNA was significantly higher in the group of HIV-infected patients with HIVE when compared to HIV-positives without HIVE (Fig. 7.1 panels A and B). Higher *SLC6A1* in the patients with HIVE was much more pronounced in the samples of anterior cingulate cortex (ACC) relative to dorsolateral prefrontal cortex (DLPFC) (+50.1 % and $p < 0.05$ in the DLPFC versus +90.8 % and $p < 0.01$ in the ACC). When GAT-1 protein concentration was estimated using the band densities in Western blots, I did not observe a corresponding increase in the patients with HIVE in the extracts from DLPFC (Fig. 7.1 panel C). However, the GAT-1 protein concentration was significantly lower in patients with HIVE in the extracts from the ACC (Fig. 7.1 panel D).

7.3.2 Clinicopathological relevance of high *SLC6A1* mRNA concentration in the anterior cingulate cortex

Impaired GABA transport from synaptic cleft leads to enhanced inhibition and altered spontaneous neocortical activity (Razik et al. 2013), resulting in worse cognitive functioning. To examine whether the changes in GABA uptake in the ACC is associated with the poor cognitive performance in region-specific functional domains, I correlated *SLC6A1* mRNA concentration in the ACC with the normalized T scores for composite test performance and for performance on tests in individual functional domains. A high T score (strong performance) on tests of abstract executive function was significantly and

negatively associated with *SLC6A1* mRNA expression in the ACC (r = -0.570, p = 0.007, Figure 7.2.B).

7.3.3 Relations between *GAD1* and *SLC6A1* mRNAs in the ACC of HIV infected patients

Concentration of cortical GAT-1 protein can be tuned to compensate the reduction in the inhibitory GABAergic transmission in disease (Sundman-Eriksson et al. 2002). To examine whether HIV-1 infection affects the association between *SLC6A1* and *GAD1*, one of the main GABAergic transcripts, I correlated *SLC6A1* and *GAD1* mRNAs concentration in the ACC of HIV-infected patients and compared to seronegative controls. The comparison between *SLC6A1* and *GAD1* mRNAs produced a regression line with a positive slope and a significant correlation coefficient in the uninfected control patients (r = 0.754, p = 0.011, Figure 7.3.A). In the HIV-infected patients the slope was negative and almost reached the level of statistical significance (r = -0.374, p = 0.065, Figure 7.3.B).

7.3.4 GAT-1 protein expression in brain neocortex

GAT-1 is predominantly expressed by presynaptic boutons of parvalbumin-immunoreactive interneurons (chandelier cells), which contact axon initial segment of pyramidal neurons. In pathological condition (e.g. schizophrenia) compensatory over-expression of GAT-1 was reported on distal processes of astroglial cell (Sundman-Eriksson et al. 2002). I found that GAT-1 protein concentration is significantly decreased in the cortical regions of HIV-infected patients. In order to characterize cellular localization and laminar distribution of GAT-1 expressing cells, I did immunostaining for GAT-1 in the ACC of HIV-infected patients. Representative images of GAT-1 immunostaining of human brain neocortex are provided in Figure 7.4. GAT-1 immunostaining was lower generally in specimens from HIV-infected patients relative to

uninfected patients (Panels A versus B, and C versus D). When present the tissue markings appear as candle-like structures that have been shown to represent axo-axonal synapses between chandelier cells (a subtype of GABAergic interneuron) and pyramidal neurons. Those markings often were sharply diminished in the tissue sections obtained from the HIV-infected patients. I also observed a very interesting and novel anomaly in the laminar distribution of GAT-1 immunostaining in the HIV infected patients. GAT-1 stain intensity in glia limitans of many HIV-positive patients was much more intense than normal brain tissue (Figure 7.4.B). I further observed that that these intensely stained markings in glia limitans were not candle-like structures described above. Instead the markings strongly resembled cellular processes. Since glia limitans does not contain neuronal cell bodies or proximal axon segments, and is composed of astrocytic processes primarily, I hypothesized that these markings represented accumulation in astroglial cell processes. That hypothesis was consistent with the fact that astrocytes are capable of expressing GAT-1 (Beenhakker et al. 2010). Also consistent with that concept was the fact that astroglial processes engage in the buffering of GABA accumulation in the synaptic cleft (Beenhakker et al. 2010, Razik et al. 2013). To pursue my hypothesis I performed dual staining with antibodies against GAT-1 and the astroglial marker GFAP, and then examined the tissue sections using confocal microscopy (Figure 7.5). I observed that GFAP-stained astroglial processes were indeed quite prominent in glia limitans and were of generally equivalent intensity in HIV infected and uninfected patients (Figure 7.5, panel A versus D). As observed previously immunostaining for GAT-1 was much more intense in the HIV-infected specimens (Figure 7.5, panel B versus E). The merged images confirmed that a substantial proportion of the heightened GAT-1 expression co-localized within GFAP-stained astroglial processes (Fig. 7.5, panel C versus F).

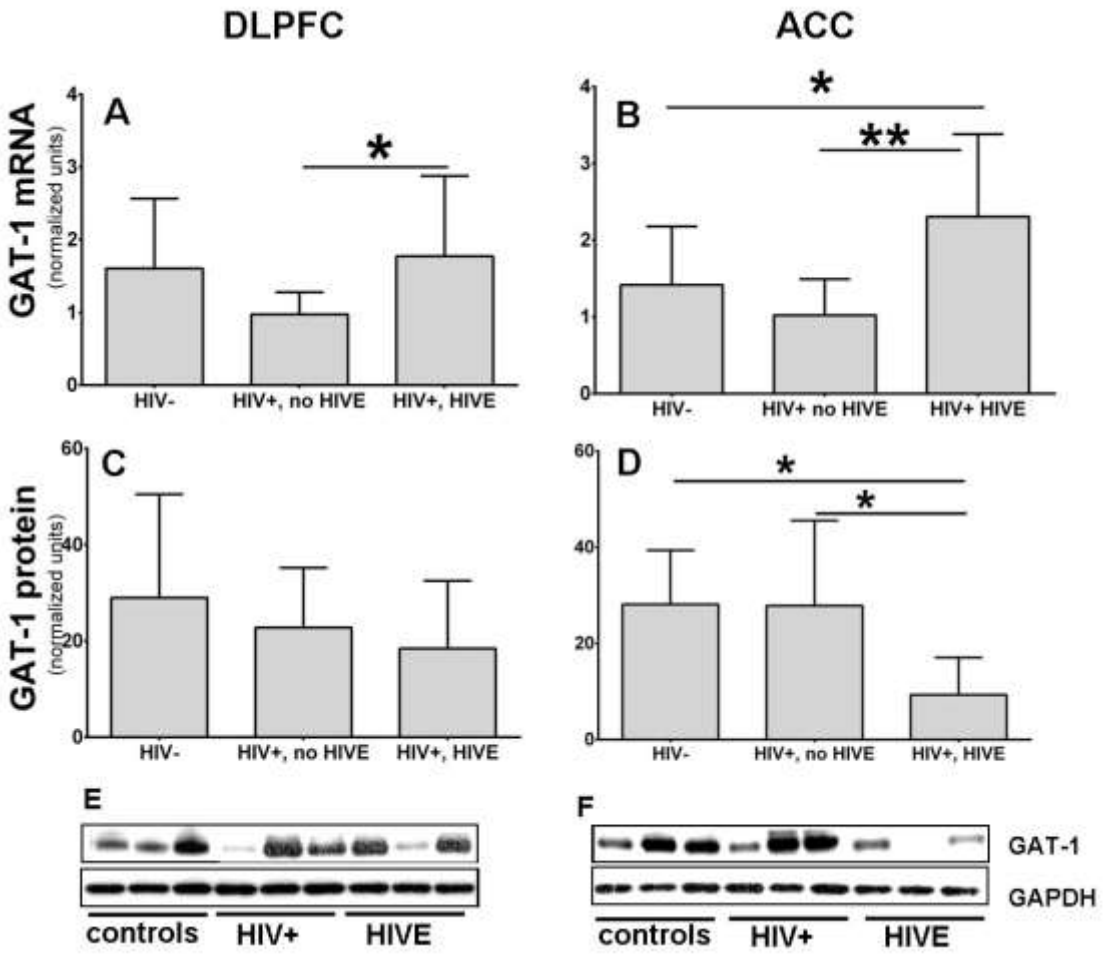

Figure 7.1 GAT-1 protein and *SLC6A1* mRNA expression in the dorsolateral prefrontal (DLPFC) and anterior cingulate (ACC) cortices. *SLC6A1* mRNA expression was significantly higher in the group of HIV patients with HIVE by 50.1 % and by 90.8 % in the DLPFC and ACC respectively, when compared to the group of HIV-positives without HIVE (panels A and B respectively). The between group analysis using one-way analysis of variance (ANOVA) yielded F = 3.176, p = 0.035 for DLPFC, F = 8.105, p = 0.0015 for ACC. GAT-1 immunoblotting was done on 36 HIV infected patients and 12 uninfected controls (panels C and D)). GAPDH blots were done for loading controls. Equal amounts of protein were added to each well. GAT-1 protein expression was not significantly different between groups in the DLPFC. In the ACC GAT-1 protein was significantly lower in patients with HIVE by 67.0% compared to the uninfected comparison group, and by 66.7%, when compared to HIV-positives without HIVE. Between group analysis using one-way analysis of variance (ANOVA) yielded F = 4.49 and p = 0.019. Representative band intensities for nine of the patients in this series are illustrated (panels E and F). GAT-1 is expressed relative to the GAPDH band intensity. Relative expression of *SLC6A1* mRNA was normalized to *GAPDH* mRNA. Mean ± standard deviation is shown. P values were obtained using the post-hoc Tukey's test.

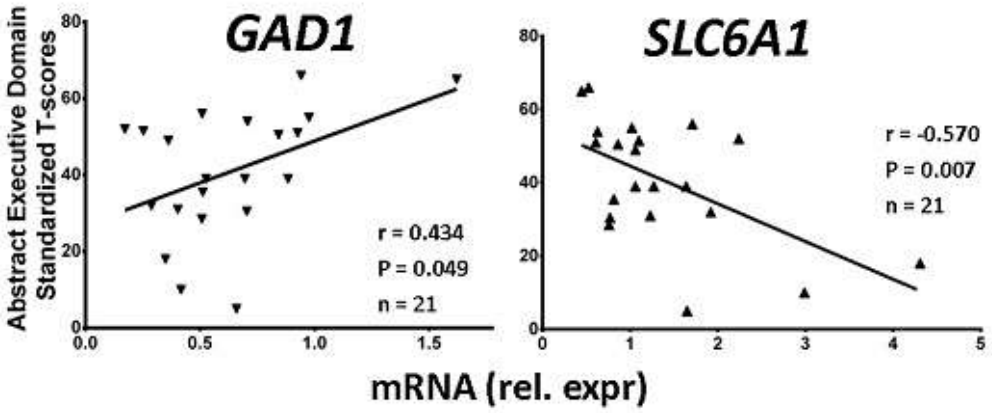

Figure 7.2 Higher expression of the *SLC6A1* mRNA corresponding to GAT-1 in the anterior cingulate cortex was correlated with worse abstract executive function in HIV infected patients. *SLC6A1* mRNA corresponds to GAT-1 protein. Its concentration was strongly and negatively correlated with performance on tasks of abstract executive function ($r = -0.570$ and $p = 0.007$). When the clinicopathological relevance of *GAD1* mRNA was compared to *SLC6A1* mRNA, the slope of the regression line was significant and positive ($r = 0.434$, $p = 0.049$). These two correlations are significantly different from each other (Fisher's z-score 3.34, two-tail $p = 0.00085$). The relative expression of these mRNAs was normalized to *GAPDH* mRNA.

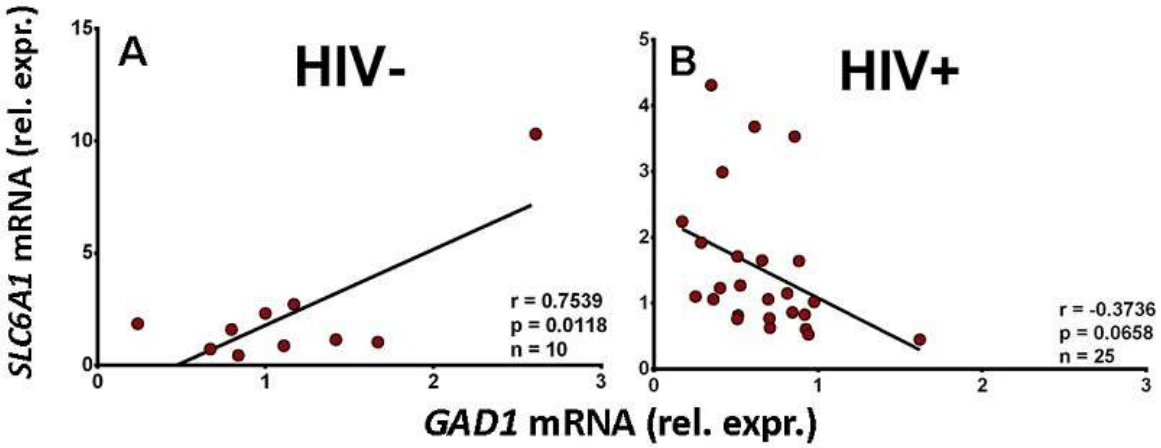

Figure 7.3 Correlation between *SLC6A1* and *GAD1* mRNA expression in the anterior cingulate cortex. Expression of *SLC6A1* mRNA (encodes for GAT-1 protein) was positively and significantly correlated with *GAD1* mRNA expression (r = 0.754 and p = 0.012) in the uninfected patients. In sharp contrast to control patients, the correlation coefficient was negative and approached statistical significance in the HIV-infected patients (r = -0.374, p = 0.065). The two correlations shown are significantly different from each other (Fisher's z-score 3.167, two tail p = 0.0015). Relative expression of mRNAs was normalized to *GAPDH* mRNA.

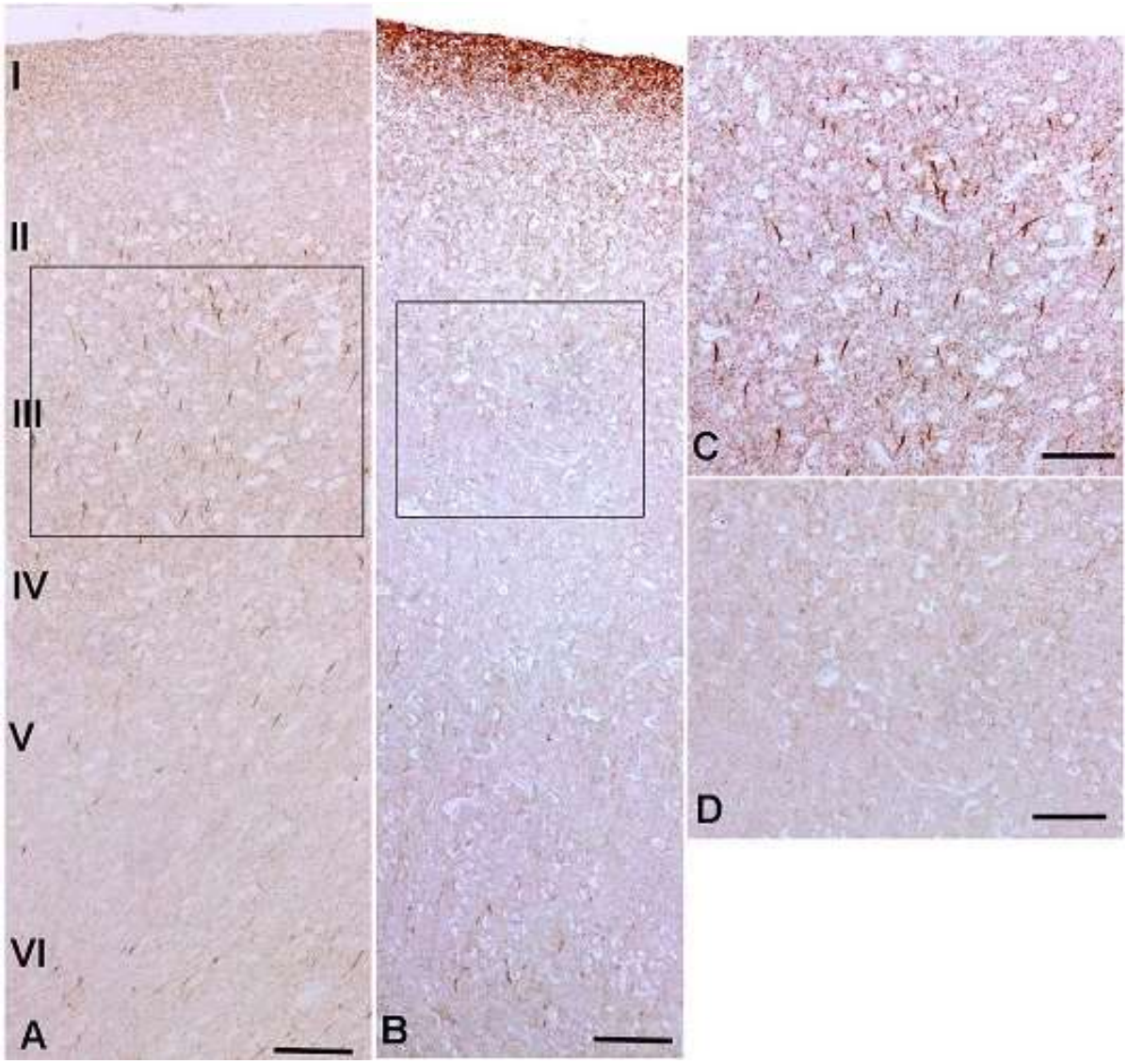

Figure 7.4 GAT-1 immunostaining of brain neocortex of an HIV-infected patient and an uninfected patient. Anterior cingulate cortex from an uninfected patient (A) and an HIV-infected patient (B) were immunostained for GAT-1. Neocortical laminae are labeled I through VI at left. The general intensity of the staining is lower in the specimen from HIV-infected patient. Boxed areas are magnified in C and D and show less of stain intensity of the candle-like processes that represent axo-axonal contacts of axon initial segments. Note the very strong increase in stain intensity in glia limitans and lamina I of the HIV-infected patient (panel B). Scale bars are 200 um in A and B, and 100 um in C and D.

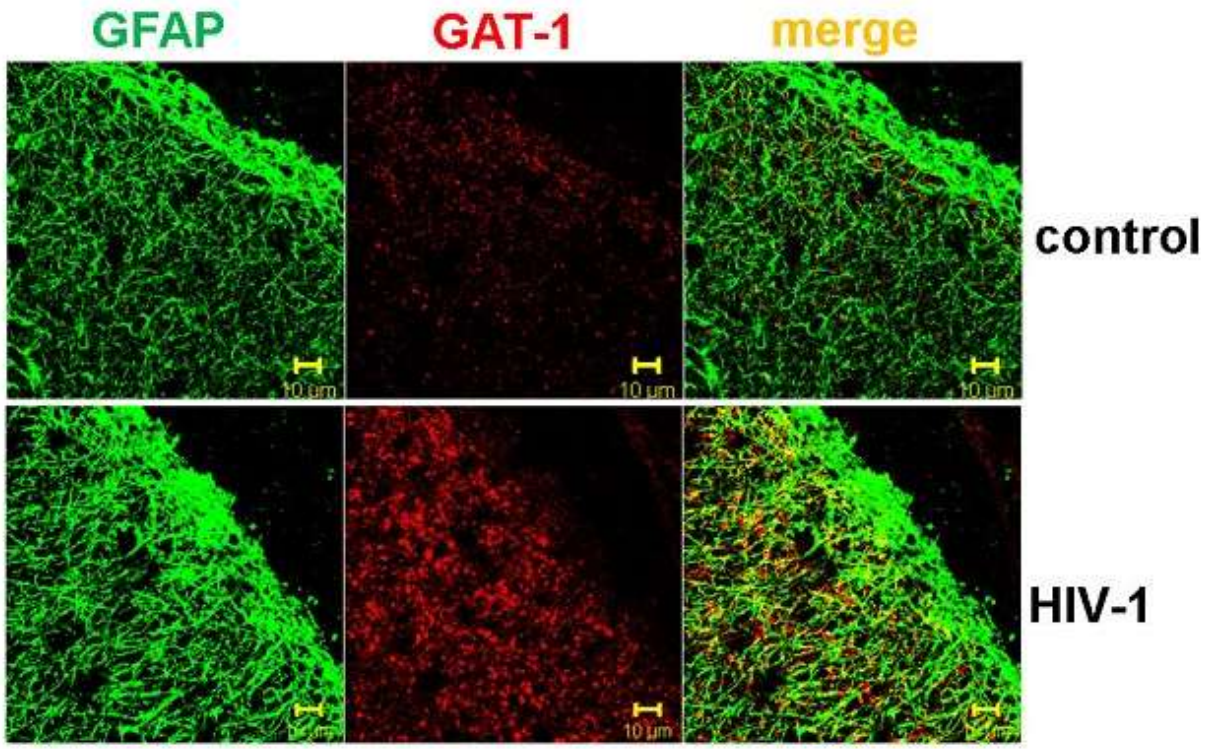

Figure 7.5 Increased GAT-1 immunostaining in astroglial processes in neocortical glia limitans. Glia limitans in the anterior cingulate cortex was dually stained for the astroglial marker GFAP (panels A and D, green) and GAT-1 (B and E, red) and examined using confocal immunofluorescence microscopy. An uninfected control patient (A – C) is compared to an HIV infected patient (D – F). . The HIV infected patient has more intense GAT-1 staining (B versus E). GFAP staining appears generally equivalent between these two patients (A versus D). The merged images show that there is abundant co-localization of GAT-1 with GFAP-stained astroglial processes in the HIV infected patient, but not in the uninfected patient (C versus F, yellow). Scale bars are 10 um.

Chapter 8: Low GABA and the neurovascular unit in HIV-infected patients

8.1 Introduction and Rationale

Normal brain function relies on high consumption of energy to maintain ionic gradients and produce membrane potentials in excitable cells. Within seconds after the onset of a wave of depolarization, arterioles and capillaries in the affected brain region become dilated in order to increase blood flow. This reaction is referred to generally as neurovascular coupling or functional hyperemia. Neurovascular coupling involves a coordinated interaction among inhibitory interneurons, glial and vascular cells. GABAergic interneurons play a critical role in the regulation of cerebral blood flow (CBF) in concert with their function as the local integrators of cortical activity (Markram et al. 2004). They powerfully innervate local arterioles, capillaries, perivascular astrocytes and pericytes (Vaucher et al. 2000), which play a critical part in rapidly adapting CBF in response to neuronal activity (Cauli et al. 2004). In neuropathological conditions, reduction of CBF has been associated with lowered concentration of GABA (Donahue et al. 2014, Krause et al. 2014). Disruption of the blood brain barrier and elevated concentrations of endothelial markers in blood plasma such as Von Willebrandt Factor; VWF) also can occur in neuropathological conditions (Sabayan et al. 2014). HIV-1 infection in CNS also can produce damage to the neurovascular unit and the blood brain barrier. These changes are often associated with elevated concentrations of endothelial markers in blood plasma (Eugenin et al. 2006) and also with localized anomalies in the CBF (Ances et al. 2009, Towgood et al. 2013). In my initial work I showed that lower GABAergic mRNAs in brain cortex were strongly related to abnormally high concentrations of endothelial type mRNAs in the brain, including VWF

(Table 3.2). This suggested the hypothesis that lower GABA might have an influence on the abnormal CBF that has been described in HIV-infected patients. To test that hypothesis I examined the GABAergic innervations of blood vessels and morphology of capillaries and pericytes in the selected cortical regions of HIV-infected patients.

8.2 EXPERIMENTAL DESIGN

The analysis was performed using samples of postmortem brain tissue described in chapter 4.1.2. Anterior cingulate cortex was region of interest for this study, because CBF in this region was abnormally low in HIV-infected patients (Towgood et al. 2013) and *GAD1* mRNA concentration in the ACC was related to the whole brain cerebral blood flow (Krause et al. 2014). To evaluate GABAergic innervations of cortical microvessels, I performed dual immunostaining of GAD67 and PDGFRb followed by bright field microscopy. Examination of CD13 immunoreactivity in the cortical sectors was done using immunofluorescence staining and confocal microscopy.

8.3 RESULTS

8.3.1 Loss of GABAergic innervations of cortical microvessels in the anterior cingulate cortex of HIV-infected patient

GABAergic interneurons innervate cerebral capillaries and pericytes (Vaucher et al. 2000), rapidly adapting CBF upon neuronal activity (Cauli et al. 2004). GABA concentration was reciprocally related to the blood flow in the same region (Donahue et al. 2014, Krause et al. 2014). Cortical GABAergic anomalies reported for HIV-infected patients can lead to the lowered inhibitory signaling to the brain microvessels. I performed dual staining of GAD67, a marker of GABAergic fibers, and platelet-derived growth factor receptor beta (PDGFRb), a marker of pericytes, to evaluate the density and the number of contacts between the GABAergic interneurons and capillaries in the ACC of HIV-infected patient. The staining intensity for GAD67 and the number of GAD67-

immunoreactive fibers in contact with blood vessel was substantially lower in the specimen from HIV-infected patients when compared to control tissue (Figure 8.1).

8.3.2 Shedding of basal lamina protein and loss of pericytes in the ACC of HIV-infected patients with low concentration of *GAD1* mRNA.

To analyze the relations between the low GABAergic tone and the morphology of cortical pericytes and capillaries, I performed immunostaining of the ACC from HIV-infected patients for alanine aminopeptidase (CD13), a marker for basal lamina of brain microvessels (Figure 8.2). Specimens from HIV-infected patients with abnormally low level of *GAD1* mRNA had less compact staining suggestive of damage to basal lamina and loss of pericytes (Figure 8.2. E and F). Surprisingly, staining of specimens from HIV-infected patients with normal *GAD1* mRNA concentration was similar to control specimens (Figure 8.2. A-D).

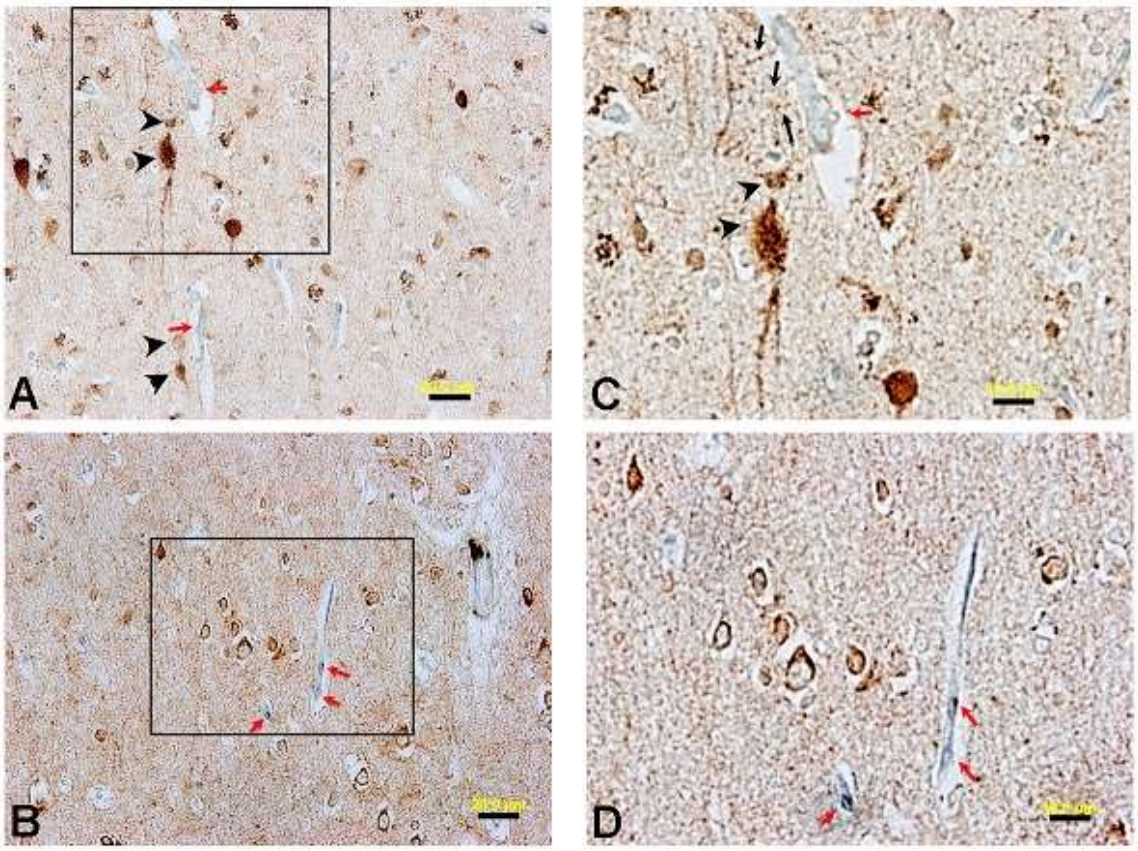

Figure 8.1 Loss of GABAergic innervation of cortical microvessels in the anterior cingulate cortex of an HIV-infected patient. The intensity of GAD67 immunostaining (brown) is lower in the specimen from the HIV-infected patient (B) compared to the uninfected patient (A). The immunoreactivity of PDGFRb (dark blue stained objects with red arrows), which is a marker of pericytes, is not different between the two patients. Boxed areas are scaled in C and D, respectively. Note the absence of stained GABAergic fibers in contact with microvessel in the tissue from HIV-infected patient (panel D) when compared to rich innervations observed in control specimen (panel C). The pericyte staining is not different in this particular exhibit using this technique. Black arrowheads point at GABAergic cell bodies; black arrows – GABAergic nerve fibers; red arrows – pericytes. Scale bars are 20 um in A and B, and 10 um in C and D.

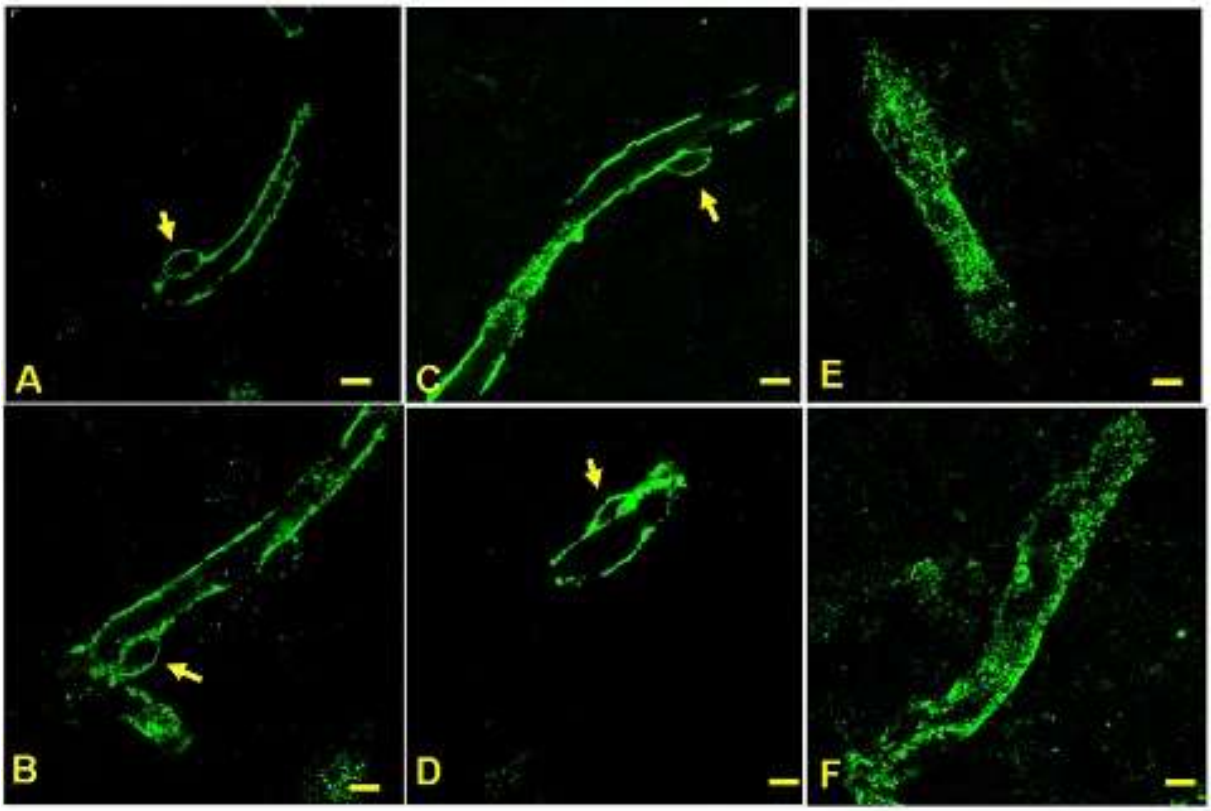

Figure 8.2 CD13 staining shows loss of pericytes in a patient with low neocortical *GAD1*. Panels C and D illustrate CD13 immunostaining in the ACC of an HIV-infected patient with normal *GAD1* mRNA concentration. Note the thin, sharply demarcated lining of the basal lamina that surrounds he pericytes (yellow arrows). The CD13 immunostaining of an HIV-infected patient with abnormally low *GAD1* mRNA level (E and F) shows poorly demarcated basal lamina staining of the vessel wall. A relative absence of pericytes also is evident in the HIV infected patient. A and B show a specimen from a normal uninfected patient for comparison (A and B). Scale bars are 10 um.

Chapter 9: Discussion

FAILURE TO DEACTIVATE THE ANTERIOR CINGULATE CORTEX LEADS TO ABNORMAL TASK-INDUCED ACTIVATION OF THE DLPFC

My thesis work demonstrated that the lowered inhibitory tone in the DLPFC was correlated specifically with worse performance in verbal fluency domain, while abnormal GABAergic neurotransmission in the ACC was associated with broad spectrum of neurocognitive dysfunction. Further investigation revealed the anomalous regulation of GABA transport in the ACC of HIV-infected patients. With these evidences, I hypothesize a novel concept that in the ACC low inhibitory tone together with the impaired GABA uptake results in permanent disinhibition of the principal cells. Signal of abnormally high metabolic demand is sensed by astroglial syncytium and results in the reversal of GABA transport in astrocytes of glia limitans in order to induce GABA-dependent relaxation of pial arterioles and to adjust the blood supply accordingly. At the brain circuit level, low GABAergic synaptic tone in the ACC would ultimately lead to the failure to deactivate the default mode network, resulting in impaired task-induced activation of prefrontal cortex and underlying HIV-associated neuropsychological impairment (Figure 9.1).

Blood flow in the ACC is abnormally increased in HIV infected patients (Ances et al. 2011, Towgood et al. 2013). My concept substantially extends those studies and provides a cause and a mechanism of abnormal adjustment of blood supply in the ACC of HIV-infected patients. My concept is suggestive that the nature of abnormal prefrontal functionality observed in HIV-infected patients is not simple hypofrontality, but rather represents a complex failure to properly synchronize frontocortical output and to deactivate anterior cingulate region, as it is necessary for normal cognitive performance.

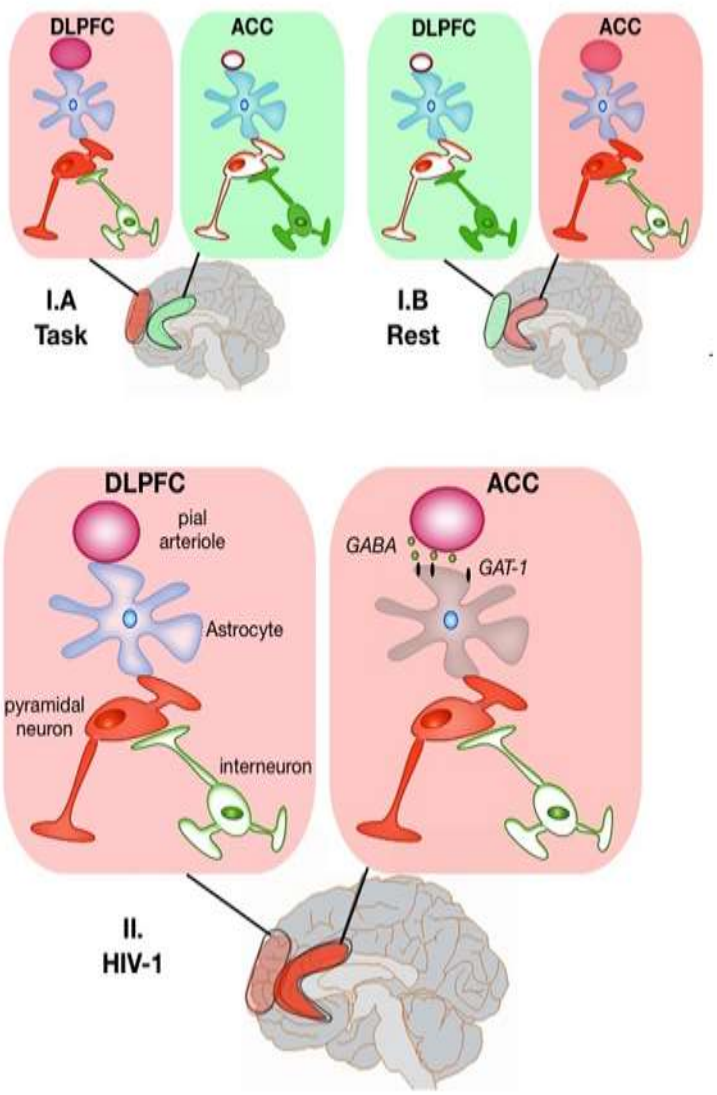

Illustration 9.1 Failure to deactivate the anterior cingulate cortex (ACC) leads to abnormal task-induced activation of the DLPFC. I. Normal conditions (A) Task-induced activation of the DLPFC: pyramidal neurons in the DLPFC are disinhibited and regional blood flow is increased; Task-induced deactivation of the ACC: pyramidal neurons in the ACC are inhibited and regional blood flow is decreased. (B) In rest, in the DLPFC the activity of principal cells is inhibited and blood flow is decreased; in the ACC the activity of pyramidal neurons is disinhibited and regional blood flow increases. II. In HIV-infected brains, low GABAergic tone results in permanent disinhibition of pyramidal neurons in both cortical regions. In the ACC loss of inhibitory control leads to failure of task-induced deactivation. Signal of pathologically high neuronal activity in the ACC is transmitted via the astroglial syncytium to the glia limitans, where astrocytes release GABA to pial arterioles to increase regional blood flow.

Abnormal GABAergic transmission in the DLPFC is associated specifically with poor performance in verbal fluency task. Clinicopathological features of HAND involve slowed thinking, mental rigidity and word-finding difficulties, which greatly affect everyday living. Verbal fluency tasking is thought to be a valid approach of quantification of these difficulties and provides a way to assess the effects of HIV-infection on brain functioning. It has been reported that verbal fluency tasking relies on the frontal-striatal circuitry (Woods et al. 2004). Prior investigations into frontocortical neurotransmitter systems in HIV-1 showed that abnormal enkephalinergic transmission (low *PENK* mRNA) had no clinical relevance and defective dopaminergic tone (Gelman et al. 2012b) was related to a broad spectrum of neurocognitive dysfunctions (Seamans and Yang 2004). Specific neurotransmitter system in the DLPFC that is associated with the functional deficit in verbal fluency domain has not been identified yet. In this study I found that lowered GABAergic transmission in the DLPFC was strongly linked to the worse performance in verbal fluency task. Despite the fact that abnormal GABAergic transmission per se could not be used as biomarker for HAND, because both groups of HIV-infected patients with and without neurocognitive impairment had lowered levels of GABAergic mRNAs when compared to controls, but were not significantly different when compared to each other, my findings endorse clinicopathological relevance of anomalous inhibitory tone in the development and progression of HIV-associated neurocognitive impairment. Highly complex picture emerges that involves shifting of multiple neurotransmitter systems, each having implications regarding particular aspects of abnormal neurocognitive function in HAND.

Abnormal GABAergic transmission in the ACC has strongest associations with worse neuropsychological outcome. Brain gene array data was suggestive that the abnormal GABAergic transmission might occur selectively in the neocortex (Gelman et

al. 2012b). Regional survey performed in this study showed that decrease in GABAergic inhibitory tone in HIV-infected patients is not restricted to the specific brain region or circuit. Lowered levels of GABAergic transcripts were reported for all cortical and subcortical regions examined. Abnormally low *GAD1* mRNA concentration in the anterior cingulate cortex showed the strongest associations with the neuropsychological outcome. This observation can be attributed to the ACC being an essential part of default-mode network (DMN, McKiernan et al. 2003), which is a network of brain regions deactivated during the task performance and active in the resting brain (Raichle et al. 2001, Buckner et al. 2008). Deactivation of ACC is necessary for proper task-oriented activity of prefrontal cortex. The more energy-demanding the task is - the stronger deactivation needs to be (McKiernan et al. 2003). Reduced deactivation of the ACC depends on the regional concentration of GABA (Northoff et al. 2007, Hu et al. 2013) and is associated with worse cognitive performance (Weissman et al. 2006) in patients with Alzheimer disease (Rombouts et al. 2005), schizophrenia (Garrity et al. 2007, Pomarol-Clotet et al. 2008), and elderly (Sambatoro et al. 2010). My findings strongly suggest that cognitive impairments observed in HIV-infected patients represent a complex failure to properly synchronize frontocortical output and to deactivate anterior cingulate region (Figure 9.1).

Low GABA is linked to altered regulation of GABA transport in the ACC. GABA transporter 1 (GAT-1) is predominantly expressed by the axonal terminals of chandelier cells (Conti et al. 1998, Inda et al. 2007) and plays critical role in the regulation of activity of pyramidal neurons (Kawaguchi and Kubota 1997, Markram et al. 2004) by synchronizing the firing of multiple principle cells (Cobb et al. 1995). GABA is recycled upon uptake (Conti et al. 1998) therefore loss of GAT-1 often leads to a decrease in the presynaptic GABA concentration (Conti et al. 2011). Abnormally low concentrations of GABA in the synapses differentially activate GABA receptors (Jensen et al. 2003), increasing pathological tonic vs physiological phasic inhibitory conductance. Reduction

of GAT-1 protein and loss of axo-axonal inhibition was previously attributed exclusively to the schizophrenia, as other psychiatric disorders, suicide, and substance abuse were not correlated to the loss of GAT-1 terminals (Woo et al. 1998, Pierri et al. 1999, Volk et al. 2001). Here I report the loss of GAT-1 immunoreactivity in all cortical layers of the ACC of HIV infected patients. The loss of GAT-1 immunoreactive terminals does not seem to be due to the dropout of chandelier cells because parvalbumin mRNA and protein, and the density of parvalbumin-expressing neurons in the same neocortical laminae all were not changed. Therefore, the lower density of interneuronal GAT-1 terminals is more likely to reflect synaptic accommodation to the decrease of synaptic GABA release.

Reversal of GABA transport in astrocytes may be linked to increased regional blood flow. Under physiological conditions astrocytes express mostly GABA transporter type 3 (GAT-3), localized on the soma and proximal processes, with only small quantities of GAT-1 found on the distal processes of astrocytes, that enwrap synapses (Conti et al. 1998, Beenhakker et al. 2010). In pathological brains however, upregulation of astrocytic GAT-1 is widely evident (e.g. in seizures (Pirttimaki et al. 2013), drug abuse (Zink et al. 2004), and schizophrenia (Sundman-Eriksson et al. 2002). Astrocytes are capable to reverse GAT-1-mediated GABA transport to release GABA and regulate tonic and phasic inhibition (Allen et al. 2004, Wu et al. 2007, Lee et al. 2011, Le Meur et al. 2012) and in highly precise manner compensate for the loss of inhibitory tone (Sundman-Eriksson et al. 2002, Heja et al. 2012, Yoon et al. 2012). In this study I found the upregulation of astroglial GAT-1 expression specifically in the outermost cortical layer of the ACC, the glia limitans. Glia limitans is the thick sheath of astrocytic processes overlying the cortex, which form close contacts with the pial arterioles and exert powerful control over their dilation (Cauli et al. 2004, Xu et al. 2004) and cerebral blood perfusion. It is already known that predominant hemodynamic adjustments originate upstream of capillaries in the pial arterioles (Iadecola and Nedergaard 2007, Fernandez-Klett et al. 2010), as pericytes were capable of modulating capillary diameter in vitro (Peppiatt et al. 2006),

but in vivo evidences were not found (Hamilton et al. 2010). I hypothesized, that in HIV low inhibitory tone results in local increase of neuronal activity, which is sensed by parenchymal astrocytes (Gordon et al. 2007, Kowiansky et al. 2013). All parenchymal and glia limitans astrocytes are coupled by the means of connexin 43 gap junctions into functional syncytium (Xu et al. 2004), that allows signal to be immediately transmitted to the astrocytic endfeet in the glia limitans, which contact pial arterioles (Iadecola and Nedergaard 2007, Xu et al. 2008, Filosa et al. 2013). Upon stimulation, astrocytes in glia limitans release GABA via the GAT-1 that results in the dilation of the upstream arterioles (Cauli et al. 2004, Mulligan and MacVicar 2004, Doengi et al. 2009, Velez-Fort et al. 2012) and increase of regional blood flow in the ACC as it was reported in HIV infected patients (Ances et al. 2011, Towgood et al. 2013). (Figure 9.1).

SYNAPTIC ACCOMMODATIONS LEAD TO THE DEFICIT OF INHIBITORY TONE.

My thesis study demonstrated that lower concentrations of GABA-synthesizing enzymes in HIV-infected brains occurs without evident loss of interneurons and are associated with 1) increased levels of inflammatory and neurovascular markers, 2) decreased concentrations of astroglial enzyme glutamine synthetase, and 3) increased expression of DRD2 on interneurons in HIVE patients. With these findings, I propose a novel concept that the persistent induction of astrocytosis by inflammatory stimuli and viral coat proteins in HIV-infected brain alters the synaptic plasticity and diminishes the concentration of GABA in viable interneurons (Figure 9.2).

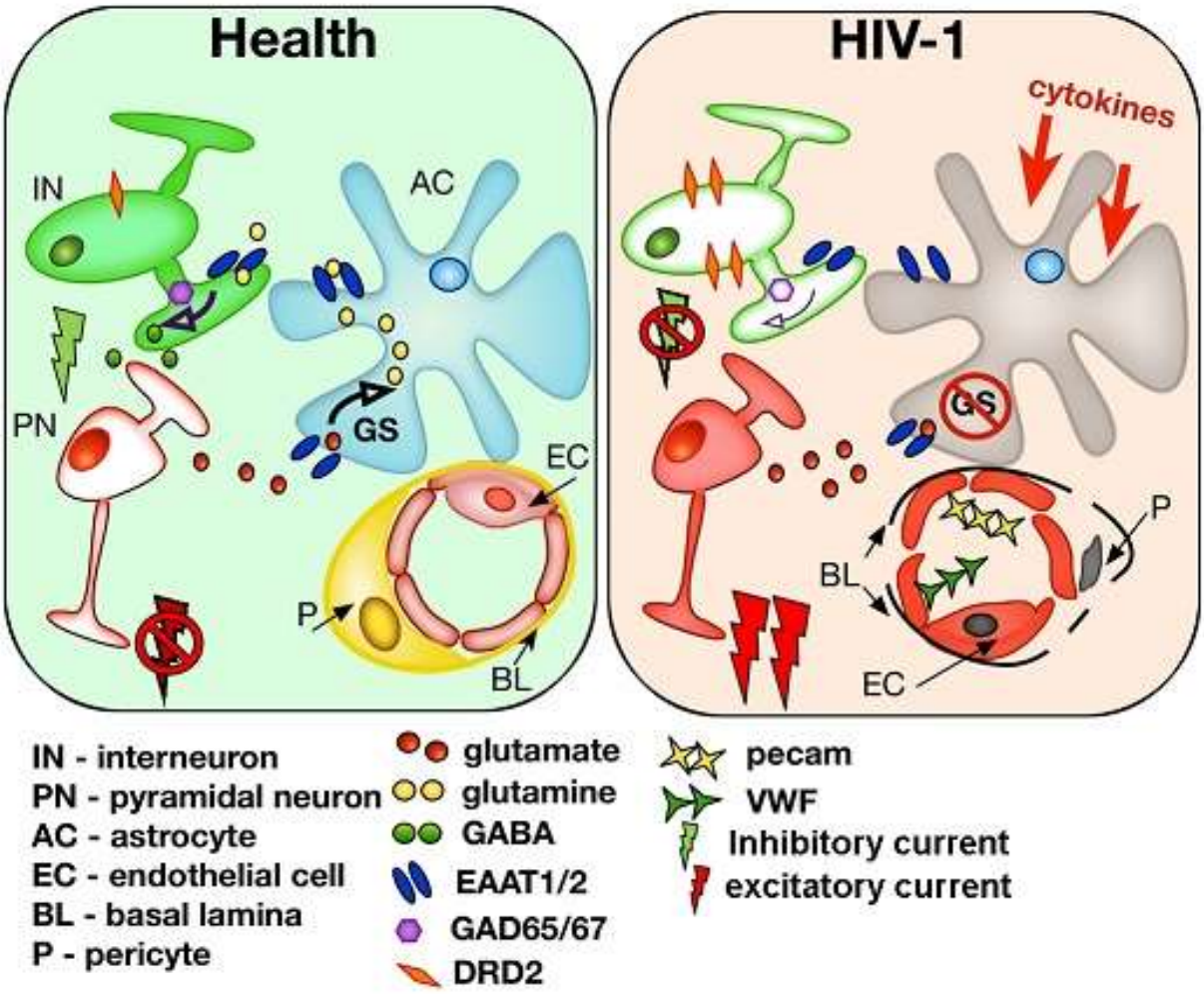

Illustration 9.2 Synaptic accommodations lead to the deficit of inhibitory tone. In health, enzyme glutamine synthetase (GS) is constitutively active and concentrated in the distal processes of astrocytes, where GS metabolizes glutamate to glutamine and provides constant supply of glutamine, a precursor for GABA synthesis, to interneurons. In HIV, a) elevated levels of cytokines, inflammatory stimuli, and gp120 lead to the astroglial dysfunction characterized by loss of GS in the distal processes, that results in the decrease of GADs expression and shutdown of GABA synthesis, reduced inhibitory currents and disinhibition of pyramidal neurons. Astroglial dysfunction is also accompanied by loss of pericytes, elevated levels of endothelial markers and damage to the BBB. b) Overexpression of interneuronal DRD2, observed in HIV, also contributes to loss of GADs and reduced inhibitory currents.

Deficit of GABA inhibitory currents have profound effects on neuronal functioning, resulting in shift toward excitation within local microcircuits, formed by inhibitory interneuron and principal cell. This leads to uncontrolled spread of activation within the local cortical network and decreased signal-to-noise ratio. At the behavioral level, clinical manifestations include impaired working memory, planning and executive functions, slowed thinking, word finding difficulties - a features characteristic for neuropsychological findings in HIV-1. Decreased expression of GABAergic transcripts was also described in studies of schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, stroke, traumatic brain injury, and conditions characterized by neuroinflammation (Eid et al. 2004, Hasler et al. 2007, Ortinski et al. 2010). Here I suggest that low GABAergic tone could be secondary to neuroinflammation that accompanies HIV-infection and can induce astroglia-mediated anomalies of synaptic transmission.

Loss of GABAergic immunostaining is not likely to reflect neurodegeneration. A critical issue that was addressed in this analysis is whether the loss of immunostaining of GABAergic cells reflects the degeneration of inhibitory neurons in the HIV infected brain tissue, versus reduced expression of the cell marker in viable neurons (Benes et al. 1991, Masliah et al. 1995). I found that the reduced concentrations of GABAergic mRNAs and proteins occur without evidence of significant dropout of inhibitory interneurons. For example, inhibitory neurons, which completely lacked GAD67 expression in the HIV-infected patients were virtually invisible with confocal imaging, but appeared morphologically typical when visualized by staining with an alternative cell marker such as calretinin, parvalbumin and somatostatin. Clarifying this issue in HIV infected patients is important because reports obtained prior to the era of HAART often have suggested that HAND is a classical neurodegenerative disease that includes the dropping

out of nonviable inhibitory interneurons. The validity of that suggestion as it pertains to HAART-era brain specimens with HAND remains doubtful (Gelman and Moore 2011), and that concept also has been challenged in patients with schizophrenia and a GABAergic disturbance (Benes et al. 1991).

My morphological examination illustrates that DLPFC specimens from HIV-infected patients can have decreased of GAD67 staining intensity across the cortical lamina, but still contain viable small pear-shaped interneurons expressing calretinin, parvalbumin or somatostatin, which are alternative markers expressed by GABAergic neurons. Specimens from HIV-infected patients contained GAD67-negative neuronal cell bodies of normal size, often with typical pear-shaped profiles, with nuclear profiles that were not suggestive of necrosis or apoptosis. The normal control specimens were usually well stained with granular GAD67 cytoplasmic markings, which is in agreement with what has been described previously (Bubar et al. 2011). My overall conclusion is that I find little or no support for the hypothesis that the abnormal GABAergic neural transmission in HIV infected brain specimens is reflective of neuronal dropout or classical neurodegeneration. Instead I suggest the alternate interpretation that the transcription of genes that control the rate limiting enzymes of GABA synthesis is downregulated in viable interneurons. Such a change is within the range of changes that are known to drive synaptic plasticity and accommodation to stress (Gelman et al. 2012b). That interpretation is compatible to results from schizophrenics, patients with mood disorders and Parkinson's disease, in which *GAD1* mRNA is lower without GABAergic cell loss in the frontal cortex (Akbarian et al. 1995, Volk et al. 2000, Benes and Berretta 2001, Guidotti et al. 2005, Lanoue et al. 2010). My findings also corroborate with the reports, which show no evidences suggestive of dropout of somatostatin- (Fox et al. 1997) or parvalbumin- (Masliah et al. 1992) expressing interneurons in the neocortex of patients with HIVE. Taken together, my results indicate that the GABAergic changes in HIV infected brain specimens resemble those that are present in other types of

neuropsychiatric diseases. As with these other diseases, lower GABAergic transmission is unlikely to reflect classical neurodegeneration neuropathologically.

Abnormal GABAergic transmission is related to dopaminergic transmission in the DLPFC. Using the same cohort of HIV infected patients, it was shown previously that failure to suppress the expression of dopamine receptor type 2 long isoform mRNA (*DRD2L*) in the DLPFC was associated with worse neurocognitive performance (Gelman et al. 2012a). The implied interpretation is that lower expression of *DRD2L* is a beneficial accommodation driven by synaptic plasticity. The same report showed that enkephalinergic neural transmission also exhibits an accommodative type of decrease in many of these HIV infected brain specimens (lower preproenkephalin mRNA expression). When abnormal GABAergic transmission was compared to these other systems I found that changes typically associated with higher dopaminergic tone and lower enkephalinergic synaptic tone both were linked significantly to abnormally low GABAergic transmission. I visualized some of these interactions histologically in tissue sections of the DLPFC and found that higher expression of DRD2 often occurs in typical interneurons lacking GAD67 immunoreactivity. My findings in HIV infected patients are consistent with reports that DRD2 are mostly expressed by large parvalbumin-expressing interneurons and small pyramidal neurons (Vincent et al. 1993, Seasack et al. 1996, Khan et al. 1998). Dopamine stimulation of DRD2 or overexpression of DRD2 on inhibitory interneurons was reported to result in decreased GABA expression (Steulet et al. 1990, Lindefors et al. 1993, Seamans et al. 2001, Trantham-Davidson et al. 2004). My findings suggest a functional link between the dopaminergic and GABAergic neurotransmitter systems in the DLPFC of HIV infected patients, that likely represents a most commonly observed form of synaptic plasticity – presynaptic inhibition of transmitter release. Dopamine activation of interneuronal DRD2 produces an inhibition of GABA release (Seamans et al. 2001) and result in short term modulation of synaptic inhibitory transmission. In the long run high frequency stimulation of DRD2 leads to the long-term

decreases in synaptic release, known as long-term depression (LTD). GABAergic LTD combined with the deficit of GADs expression result in shift toward excitation within local microcircuits formed by inhibitory interneurons and the principal cells. In turn, this results in uncontrolled spread of activation within the local cortical network and decreased signal-to-noise ratio. At the behavioral level the clinical manifestations include impaired working memory, planning and executive functions, slowed thinking, word finding difficulties: these features are characteristic neuropsychological findings in schizophrenia and HIV (Seamans et al. 2001, Winterer and Weinberger 2004, De Almeida and Mengold 2010).

GABAergic anomaly is linked to the loss of astrocytic enzyme glutamine synthetase. Main precursor to interneuronal GABA synthesis is a glutamine synthesized by astroglial enzyme glutamine synthetase (Sonnewald et al. 1993, Rae et al. 2003). Dysregulation of GS is an important mechanism that can reduce the expression of interneuronal GADs (Struzynska et al. 2004) and shutdown the synthesis of GABA (Ortinski et al. 2010), like it was observed in several brain disorders including Alzheimer's disease, schizophrenia, depression and temporal lobe epilepsy (Papageorgiou et al. 2011, Eid et al 2004, Eid et al. 2011, Eid et al. 2012). In this study I showed that expression of *GAD1* was downregulated when concentration and subcellular distribution of astrocytic glutamine synthetase was altered in HIV-infected brain. The mechanism of GS dysregulation includes the nitration of tyrosine residues in the active sites that occurs upon the exposure to HIV-1 protein gp120 and elevated levels of inflammatory stimuli (Muscoli et al. 2005). Notably, that the deficiency of glutamine synthetase apart from causing seizures as result of abnormal inhibitory tone (Coulter et al. 2012, Thrane et al. 2013), often leads to increased cerebral flow (Master et al. 1999) that is another characteristic feature of HIV-1 infection.

Abnormal GABAergic transmission is related to neuroimmune responses. This and many other studies have shown that several aspects pertaining to the neurobiology of

HIV infection in humans do not appear to be under the control of HIV replication in the CNS (Gelman et al. 2012b, Gelman et al. 2013). Indeed, there is abundant evidences that host immune responses in the CNS may play a critical role in driving HAND in a manner that is at least partly independent of HIV replication (Glass et al. 1995, Gartner and Liu 2002). Consistent with that, I found that low GABAergic transcripts were significantly correlated with higher neuroimmune type markers in the brain including interferon response genes. These findings are in agreement with published reports, where host immune response in the CNS (e.g. activation of brain macrophages and microglia), but not the concentration of HIV RNA, drove the development and progression of neurocognitive impairment in patients without HIVE (Glass et al. 1995, McArthur et al. 2004, Yadav et al. 2009, Gelman et al. 2013) and GABAergic anomaly was suggested as possible neuropathological substrate for cognitive dysfunction in HIV (Gelman et al. 2013). The fact that patients without encephalitis often had abnormal GABAergic transmission remains unexplained at this time, which is seemingly analogous to the fact that HAND also is highly prevalent in patients without encephalitis.

The neurovascular unit may be related to abnormal GABAergic transmission. Brain gene array data suggested that patients with HAND who are taking HAART but do not have high viral replication rates in the CNS or encephalitis might harbor a disturbance in neurovascular biology (Gelman et al. 2012b). That suggestion could be especially important for abnormal GABAergic transmission that plays a critical role in controlling cerebral blood flow (Fergus and Lee 1997, Northoff et al. 2007). The hypothesis that anomalous inhibitory tone is associated with the disturbance of neurovascularity in HIV was endorsed by neurochemical analysis performed in this study that showed strongest associations between abnormal GABAergic transmission and higher expression of the endothelial cell markers. Capillaries, pericytes and perivascular astrocytes, which are the components of the neurovascular unit (NVU, Abbott, 2002, Ballabh et al. 2004), all are innervated by NOS- (NO synthetase) expressing subpopulation of SOM-IR interneurons

(Fernandez-Klett et al. 2010, Duchemin et al. 2012, Hall et al. 2014). Under normal conditions pericytes should receive inhibitory input and maintain relaxed resting state to allow the minimal blood supply to the neurons (Peppiatt et al. 2006, Yao et al. 2013). Whether in HIV-infected brain pericytes remain intact and receive inhibitory input has not been clarified yet. To clarify that issue, I performed immunohistochemical evaluation and observed a loss of GABAergic fibers in contact with capillaries accompanied by degenerative changes of basal lamina and loss of pericytes that was especially profound in the ACC of HIV-infected patients with low level of *GAD1* mRNA. Those similar anomalies of neurovascularity were previously reported for only few pathological conditions characterized by inflammatory responses in brain: epilepsy (Liwnicz et al. 1993), hypoxia (Gonul et al. 2002), and traumatic brain injury (Dore-Duffy et al. 2000, Ozen et al. 2014). My findings of complex associations between low GABAergic transmission, higher expression of endothelial type markers, and abnormalities of capillaries and pericytes observed in HIV could be linked to reactive astrocytosis that destabilize the neurovascular unit and BBB, contributing to abnormal blood flow. Elevated concentrations of inflammatory stimuli and cytokines produced by infected immune cells cause astrocytes activation. Activated astrocytes upregulate expression of endothelial markers and cell adhesion molecules (Seigneur et al. 1997, Woodman et al. 1999, Eugenin et al. 2006), and suppress the expression of glutamine synthetase that results in shutdown of GABA synthesis and loss of GADs. Abnormal GABAergic innervations of microvasculature result in migration of pericytes and degeneration of basal lamina that was observed in this study. Degeneration of basal lamina compromises integrity of BBB that results in leakage of infected and activated macrophages and lymphocytes into brain tissue (Louboutin and Strayer 2012) and persistent induction of astroglial dysfunction.

The amount of replicating HIV-1 in the CNS is not a major driving force behind abnormal GABAergic transmission. The data on brain virus replication shows that

GABAergic anomaly is not correlated with the amount of replicating HIV-1 in the CNS. Further, my findings show a lack of association with HIVE, which is a neuropathology that is driven by replicating HIV-1 in the CNS. Also, the anomaly was not normalized in patients taking HAART, which is a treatment that lowers viral replication in the CNS and systemically. All of these observations argue strongly that low GABAergic transmission is not driven directly by the amount of replicating HIV-1 in the CNS compartment or systemically. The lack of linkage with replicating HIV-1 as demonstrated here is striking because it runs contrary to many conclusions suggested by prior work done using far fewer subjects (Masliah et al. 1995). My conclusion regarding the lack of a direct role of HIV-1, while potentially counterintuitive and not in agreement with prior suggestions, is strongly supported by the highly robust patient sample and the rigorous neurochemical analysis that was conducted. This conclusion also is consistent with recent data showing that the neurovirological correlation between brain viral replication and HAND is not significant in most patients taking HAART (Gelman et al. 2013).

Substance use history was not related to abnormal GABAergic transmission. Drug addiction was common in this HIV-infected patient cohort and could have influenced synaptic transmission; 89 out of 449 HIV-infected patients self-reported current or past history of substance abuse. That rate is comparable generally to other HIV-infected populations in which lifetime prevalence of any substance use ranges from 23 to 56 percent (Klinkenberg and Sacks 2004). In our cohort GABAergic expression was low in subjects with and without a history of drug dependence, and the two groups of subjects were not statistically different from each other. This result was somewhat unexpected because substance abuse often does result in the altered GABAergic neurotransmission (Zhang et al. 2006, Wadleigh et al. 2012). Altered expression of GABAergic mRNAs and proteins were reported in cocaine (Enoch et al. 2012), nicotine (Satta et al. 2008), methamphetamine (Hanson et al. 2004, Anneken et al. 2013), and morphine users (Sultana et al. 2010). These discrepancies are likely to be related to the

choice of model, as studies of substance abuse predominantly utilize animal models, where in humans the effect could be less pronounced. The combined influence of substance abuse and HIV-1 on inhibitory system was studied in very few prior studies (Langford et al. 2003, Chana et al. 2006). Some of those studies implied that the calbindin-expressing interneurons were selectively susceptible to neurodegeneration in methamphetamine-using patients with HIVE. Potential reasons for the divergence include the fact that those studies used widely different approaches including smaller cohort sizes, highly restrictive inclusion and exclusion criteria and a lack of neurochemical outcome measures.

Chapter 10. Conclusions and future directions

Conclusions

1. *Abnormal lowering of cortical GABAergic inhibitory transmission is not driven by HIV-1 replication.* The neurochemical results I have obtained showed that three markers of GABAergic neurotransmission were altered in the DLPFC of HIV-infected patients. Increasing evidences for the role of inhibitory neurotransmission in the neurocognitive and psychiatric disorders along with the recent reports of GABAergic anomaly in HIV/HIVE patients (Gelman et al. 2013) prompted the investigation into anomalies of the GABAergic transmission in HIV-infected brains. My analysis of GABAergic transcripts expression revealed that correlations between the GABAergic mRNAs in the DLPFC and HIV RNA copy number in brain tissue were not significant; suggesting that observed anomalies of inhibitory transmission are not driven by virus replication. Further analysis revealed that low concentrations of mRNAs encoding for GAD67, GAD65, and connexin 36 in the frontal cortex of HIV positive subjects could not be attributed to the HAART therapy, neuropathological diagnosis of HIVE, or drug abuse history.

2. *GABAergic dysfunction is linked to activated astrocytes and host neuroimmune response.* I demonstrated that GABAergic dysfunction is associated with abnormal neurovascular regulation and host neuroimmune response in the HIV. Loss of astroglial enzyme glutamine synthetase in HIV-positive patients was related to the decreased level of interneuronal *GAD1* mRNA, suggesting that HIV-induced dysfunction of astrocytes might represent a possible link between viral infection and GABAergic anomaly observed in this study.

3. *Lowered expression of GABA synthesizing enzymes occurs in viable populations of interneurons throughout the CNS.* Investigations into the expression of

interneuronal markers lead me to conclusion that concentration of GADs was lowered in all three subpopulations of viable cortical interneurons. Findings that lower GABAergic transmission was significantly related to higher *DRD2L* mRNA expression further support the hypothesis that lowered GABAergic tone represents the synaptic accommodation rather than neurodegeneration. I also found that expression of *GAD1* mRNA was lowered in all brain sectors, including neocortex, neostriatum and cerebellar lobule. However, only two cortical regions showed significant correlation between low inhibitory tone and cognitive outcome. In the prefrontal cortex low *GAD1* was linked to poor verbal fluency tasking and in the anterior cingulate low *GAD1* was associated with worse performance in broad spectrum of functional tests. These findings suggested that abnormal inhibitory transmission in the DLPFC and the ACC is associated with the neuronal dysfunction underlying HIV-neurocognitive impairment.

4. *Upregulation of astroglial GAT-1 in the glia limitans of the ACC might represent a mechanism of regional blood flow regulation.* Using immunohistochemical and immunofluorescence analysis I observed a very interesting anomaly of the laminar and cellular distribution of GAT-1 immunostaining in the ACC of HIV infected patients. GAT-1 stain intensity was predominantly absent from cortical laminae 2-5, but instead was much more intense in glia limitans, where it co-localized within GFAP-stained astroglial processes. These novel findings lead me to conclusion that upregulation of astroglial-expressed GAT-1 represents an adaptation mechanism to the loss of neuronal inhibition and could be linked to abnormal regulation of regional blood flow observed in the ACC of HIV-infected patients.

5. *Lowered GABAergic transmission is linked to the abnormal regulation of neurovascular blood flow.* Investigations into the relations between the low GABAergic tone and the morphology of cortical pericytes and capillaries revealed damage to basal lamina and loss of pericytes in the specimens from HIV-infected patients with abnormally low level of *GAD1* mRNA. Findings that GABAergic innervations of cortical

microvessels are predominantly lost in HIV further support the hypothesis that anomalous GABAergic inhibition is linked to the abnormal regulation of neurovascular blood flow in HIV-infected brain.

Recommendations for future research

Downregulation of glutamine synthetase expression could result from low level of the substrate (glutamate) availability due to decreased expression of glutamate transporters (Wang et al. 2003). To further explore the role of astroglial dysfunction in the abnormal regulation of inhibitory tone in HIV-infected brains the assessment of expression of glutamate transporters EAAT1 and EAAT2 and glutamate uptake in HIV-1 and gp120 treated astrocytes in vitro should be performed. As well, it would be of great interest to examine the expression of EAAT1 and EAAT2 in postmortem brain tissue from HIV-infected patients. Cell culture experiments combined with animal studies are also required to investigate to what extent other components of the glutamate/glutamine cycle are affected.

Under normal conditions, GABA is synthesized from glutamate by enzymes GAD65 and GAD67. Under pathological conditions GABA can also be produced in reactive astrocytes by the degradation of polyamine putrescine (Yoon et al. 2014, Jo et al. 2014). A key enzyme involved in the conversion of putrescine to GABA is monoamine oxidase B (MAOB, Yoon et al. 2014, Jo et al. 2014). It would be of great interest to determine whether alternative pathway of GABA synthesis plays a neuroprotective role in HIV-neuropathology. Multidisciplinary approaches including immunoblot analysis, immunohistochemistry, and colorimetric enzyme assay could be utilized to demonstrate that the concentration and the activity of MAOB in the postmortem brain samples from HIV-infected patients are altered.

Single nucleotide polymorphisms (SNPs) of the genes that regulate the metabolism and activity of GABAergic interneurons can critically affect the predisposition to HIV-associated anomalies of inhibitory transmission. Gene variants

often induce abnormal mRNA structure, affecting its stability, splicing, transcription and translation, which would lead to lower GABA synthesis. Several *GAD1* SNPs have been associated with low *GAD1* mRNA levels (Marenco et al. 2010), risk of predisposition to psychiatric diseases characterized by GABAergic anomalies, e.g. schizophrenia (De Luca et al. 2004, Straub et al. 2007, Marenco et al. 2010), and with measures of prefrontal cortical function (Straub et al. 2007). It would be of great importance to study if HIV-associated decrease of *GAD1* mRNA expression is more pronounced in *GAD1* SNPs carriers. In future, screening for genetic variants of GABAergic genes would help to early determine HIV-infected patients who are genetically more susceptible to anomalies of inhibitory transmission.

Altered connectivity of the default mode network and underlying anomalies in the cerebral blood flow may result in dysfunctional associations between the cognitive processes. Decreased DMN activity was already reported for many neurocognitive disorders (e.g. epilepsy, autistic spectrum, attention deficit, Alzheimer's disorder, depression, anxiety, and schizophrenia (Buckner et al. 2008, Broyd et al. 2009). Noteworthy, that altered patterns of DMN activity usually precede the onset of disorder (Broyd et al. 2009). Future studies that utilize functional magnetic resonance (fMRI) and magnetoencephalography (MEG) to detect low frequency activities in the brain regions of HIV-infected patients with and without HIV-associated neurocognitive impairments should be performed to examine the pattern of neuronal activation in task-negative and task-positive components of the DMN and its relations to GABA concentrations in corresponding brain regions. That would open up the possibility of early classification of pre-clinical HIV-associated neurocognitive impairment.

Further studies could also be focused at abnormal cerebral microcirculation in HIV-infected brain. This pathological condition is widely observed in many neurological disorders such as epilepsy (Liwnicz et al. 1993), hypoxia (Gonul et al. 2002), and traumatic brain injury (Dore-Duffy et al. 2000). Loss of pericytes and thickening of the

basal lamina that was especially profound in the ACC of HIV-infected patients with low levels of *GAD1* mRNA is a novel finding for HIV-1. Observed thickening could represent the residues left after degenerated pericytes. The viability and contractile properties of pericytes in contact with capillary remain unknown. Studies of in vitro capillaries treated with HIV-1 and gp120 could be used to further examine degenerative processes of microvessels associated with HIV-infection.

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Vita

Tetyana Pylypivna Buzhdygan was born on October 10, 1981 to Pylyp and Olexandra Buzhdygan in Bogorodchany, Ivano-Frankivska oblast, Ukraine. She graduated with B.S in Biology in 2003. Then she continued her education in same university and graduated in 2004 with Specialist diploma (equivalent to Master in US system) in Molecular biology and in 2007 with Specialist diploma in Psychology. She started her graduate studies in UTMB in August 2009.

Education

B.S in Biology, 2003, Taras Shevchenko National University of Kyiv

Specialist in Molecular Biology, 2004, Taras Shevchenko National University of Kyiv

Specialist in Psychology, 2007, Taras Shevchenko National University of Kyiv

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Alexander S. Shavkunov, Norelle C. Wildburger, Miroslav N. Nenov, Thomas F. James, Tetyana P. Buzhdygan, Neli I. Panova-Elektronova, Thomas A. Green, Ronald L. Veselenak, Nigel Bourne, and Fernanda Laezza. "The Fibroblast Growth Factor 14•Voltage-gated Sodium Channel Complex Is a New Target of Glycogen Synthase Kinase 3 (GSK3)". The Journal of Biological Chemistry, 288, 19370-19385. doi: 10.1074/jbc.M112.445924

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Permanent address: 10 Tychyny str, apt 23.

Kyiv 02098, Ukraine