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Expression of substance P, neurokinin-1 receptor and immune markers in the brains of individuals with HIV associated neuropathology

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Abstract

The tachykinin neuropeptide substance P (SP) has an important signaling role in both the nervous and the immune systems. Two naturally occurring variants of the Neurokinin-1 receptor (NK1R) mediate the effects of SP, full-length receptor (NK1R-F) and a truncated form (NK1R-T) that lacks 96 amino acid residues at the C-terminus. We previously reported decreased expression of the NK1R-F in the CNS of HIV-positive individuals in comparison to HIV-negative control subjects. There were no differences in the expression of the NK1R-T in the same groups. In the current study, we quantified the expression of SP precursor mRNA preprotachykinin (TAC1), NK1R (full and truncated forms), viral load (HIV-gag) and several proinflammatory and immune markers (CD4, CCR5, CXCR4, Fractalkine, IL-6, IL-10, CCL2, CCL20, and CD163) in the frontal cortex of autopsied brains from HIV-1 positive individuals with or without HIV associated neuropathology. The expression of SP and, to lesser extent, NK1R-F, were decreased while the expression of CXCR4, CCR5 and CCL2 were increased in CNS of individuals with HIV associated neuropathology. There was no change in HIV loads associated with neuropathology; however, we found a positive correlation between viral loads and the expression of haptoglobin-hemoglobin scavenger receptor CD163. Analysis of CSF from corresponding samples demonstrated an increase in proinflammatory markers (CCL2 MIP-1α and MIP1β) associated with neuropathology. Although our data confirm the overall inflammatory nature of HIV associated neuropathology, we observed a decrease in the expression of SP and NK1R-F, which is also associated with other forms of neuroinflammation.

Keywords

Substance P; Neurokinin-1 receptor; HIV; HIVE; neuropathology; brain

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Conflict of interest

The authors declare that they have no competing interests

1. Introduction

HIV neuropathology is characterized by changes directly attributed to HIV infection of the central nervous system (CNS) as well as the subsequent changes associated with the inflammatory conditions of the brain, which include the presence of HIV infected macrophages and microglial cells [1–3]. HIV associated neuropathology is the main cause of neurocognitive decline associated with HIV-1 infection [4, 5]. Though prevalence of HIV-associated dementia (HAD) and, to lesser extent, HIV encephalitis (HIVE) has declined in the era of combination antiretroviral therapy (cART) [5–8], HIV replication in CNS during early infection as well as in some individuals treated with cART is likely to contribute to neuropathological and neurocognitive disorders [2]. Neuropathological manifestations of HIV in the CNS include the increased production of multiple pro-inflammatory markers including; the chemokines CCL2 and fractalkine [9–11]; the cytokines TNFα, IL-1, IL-6, GM-CSF [12–16]; the CCR5 and CD163 cellular receptors [17, 18]; free radical nitric oxide [19, 20]; and increased matrix metalloproteinase activity [21, 22].

We previously compared the expression of the two neurokinin-1 receptor (NK1R) isoforms in cingulate cortex of HIV positive individuals and non-infected controls [23]. We demonstrated a decrease in the expression of full-length NK1R (NK1R-F) but not the truncated form of the receptor (NK1R-T) in HIV-positive individuals in comparison to HIV-negative controls. NK1R is a member of the G-protein coupled receptor superfamily and is present in the cells of the nervous system and immune system [24]. A splice variant of the human NK-1R mRNA with a truncated C-terminus has been cloned and identified [24]. The NK1R-F is the predominant form expressed at sites in the human brain, whereas NK1R-T occurs throughout the central nervous system and in peripheral tissues [25]. While it appears that both receptors have equal SP binding properties [26], there are significant differences in the signaling properties between the two isoforms [24, 27–30]. Activation of the SP-NK1R signaling pathway is linked to neurogenic inflammation and neuroinflammation [31–34] with few exceptions [33, 35]. Our previous finding in which NK1R-F expression was decreased in the cingulate cortex of HIV infected individuals was somewhat surprising and led to the hypothesis that the reduced expression of full-length form of NK1R may be significant in deficiencies in cognitive functions associated with neuroAIDS.

In the current study we extend our research on the role of SP-NK1R interactions in neuroAIDS by comparing its expression in CNS of individuals with or without HIV associated neuropathology.

2. Methods

2.1. Subjects and Specimens

Autopsy tissue from the frontal cortex of the brain and cerebrospinal fluid (CSF) specimens from HIV positive individuals were made available through a request to the National NeuroAIDS Tissue Consortium R-187. Histological assessment of brain specimens for diagnoses of HIV-associated neuropathology were made at each of the four NNTC collection sites by board-certified neuropathologists. Standardized protocols and data collection forms were utilized to increase diagnostic reliability between different pathologists. Histological findings for study participants were dichotomized as follows: HIV-NP−: No parenchymal HIV-associated neuropathology. HIV-NP+: Parenchymal HIV-associated neuropathology, including HIV encephalopathy, HIV leukoencephalopathy, and microglial nodular encephalitis. Diagnostic criteria were based upon the 1991 consensus report by Budka et al [1]. Diagnosis of HIVE was based upon histological findings of astrocytosis, multiple disseminated foci of microgliosis, the presence of multinucleated giant

cells, and immunohistochemistry for HIV p24 antigen. Diagnosis of HIV leukoencephalopathy included histological findings of microgliosis, astrocytosis, and myelin loss, with or without a presence of multinucleated giant cells. Table 1.

2.2. RNA extraction and real-time RT PCR

Total RNA was extracted from brain frontal cortex tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH), treated with RNAse free DNAse (Ambion/Life Technologies, Grand Island, NY) and reverse transcribed (1μg) using an AffinityScript qPCR cDNA synthesis kit (Agilent, Santa Clara, CA) with random primer, all as instructed by the manufacturer. One-tenth of the resulting cDNA was used as a template for real-time PCR amplification using MyiQ iCycler system (Bio-Rad Laboratories, Inc., Hercules, CA). The sequences of the primers and probes used in this study are listed in Table 2.

All primers and probes were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR fragments were cloned into pGEM-T vector (Promega, Madison, WI) and used to generate standard curves for corresponding genes.

The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression. All amplification reactions were performed in duplicate and an average mRNA quantity is expressed as copy number per 1,000 copies of GAPDH. The specificity of the amplification with SYBR was confirmed by dye dissociation curve.

2.3. Cytometric Bead Array Assay

A flow cytometry-based Cytometric Bead Array (CBA; BD Biosciences, San Diego, CA) assay was used to measure MIP-1α, MIP-1β, MCP-1, IL-6, CCL5 (RANTES), TNFα, γIFN and GM-CSF in CSF. Aliquots of 70μl of CSF were frozen at −70°C until evaluated by Cytometric Bead Array assay according to the manufacturer's instructions.

2.4. Substance P assay

A modified commercially available antigen competition enzyme immunoassay (EIA) from Cayman Chemical Company (Ann Arbor, MI) was used to quantitate SP, as previously described [36].

2.5. Statistical analysis

The results are expressed as Scatter dot plot and Mean of $\log_{10}$-adjusted values for each individual. Shapiro-Wilkes tests were performed to assess normality of distribution. Evaluation of significant differences between parameters was performed with independent sample t-tests or Mann-Whitney, as appropriate. In all cases, $p<0.05$ was considered significant. Correlation was determined using Pearson correlation test.

3. Results

The mRNA expression of both SP and NK1R-F was decreased in the frontal cortex of individuals with HIV associated neuropathology, though only the decrease in SP was significant. We also observed an increase in the mRNA expression of the HIV co-receptors CXCR4 and CCR5 as well as the chemokine CCL2. HIV viral load, determined by quantitating HIV-1-gag RNA [37], was unchanged between two groups (Fig. 1). In addition, the mRNA expression levels for NK1R-T, CD4, fractalkine, IL-6, IL-10, CCL20 and CD163 were unchanged (not shown).

Analysis of CSF available for selected donors revealed increased levels of MIP-1α, MIP-1β and CCL2 in HIV-NP+ group (Fig. 2). While there was an increasing trend in IL-6

expression, this change was not significant. The levels of SP, TNFα (Fig. 2), RANTES, IFNγ and GM-CSF were unchanged (not shown).

Correlation analysis was performed between markers of inflammation and viral loads in CNS and CSF samples (Fig. 3). A significant correlation in the expression levels was detected between the HIV coreceptors CCR5 and CXCR4 as well as between SP and NK1R-F in CNS. The only marker showing significant correlation with viral load was the haptoglobin-hemoglobin scavenger receptor CD163. A weak negative correlation was detected between the expression of SP and either CXCR4 orCCR5 (only CXCR4 is shown) in CNS (Fig. 3A). A significant correlation was also detected between CCL2 and IL-6 and between MIP-1α and MIP-1β in CSF. (Fig. 3B).

4. Discussion

We found evidence of inflammation in the frontal cortex and the CSF of individuals with HIV-associated neuropathology which is consistent with previous reports (reviewed [38]). In our cohort, we detected an increased level of CCL2 in both the CNS and the CSF of individuals with HIV-associated neuropathology. The CCL2 chemokine is most commonly associated with HIV infection, CNS viral invasion and viral neuropathology [9, 11, 39]. We also detected a neuropathology-associated increase in the expression of the HIV-1 co-receptors CCR5 and CXCR4 but not CD4. There was a significant correlation between an increase in CCR5 and an increase CXCR4 in the CNS suggesting a common mechanism driving the expression of both HIV co-receptors in individual samples.

Previous studies suggest that macrophage infiltration and viral replication correlate with brain dysfunction in HIV infected individuals [2, 40]. We were unable to detect an increase in either the viral load or CD163 receptor expression, a cell surface marker expressed exclusively on cells of monocyte-macrophage lineage, associated with HIV neuropathology. We did, however, observe a correlation between viral RNA and the expression of CD163 in brain samples. CD163 plays a significant role in HIV infection. Elevated expression of CD163 is associated with HIV infection of monocytes and macrophages *in vivo* and *in vitro* and the plasma levels of soluble CD163 may serve as a marker of chronic HIV infection and NeuroAIDS [41–44]. This finding supports previous observations that HIV replicates in the CNS in cells predominantly of monocytes-macrophage origin.

Contrary to proinflammatory markers, the levels of SP and, to the lesser extent, NK1R-F were reduced in brain tissue from donors with HIV associated neuropathology. A significant correlation between the expressions of those two molecules suggests a coordinated decrease in their expression in individual samples. Although there is no direct proof that SP-NK1R interactions are involved in neuroAIDS several lines of evidence indicate that disruption of SP-NK1R signaling may contribute to HIV infection of the CNS. The present finding is consistent with our previous results which demonstrate a decrease in the expression of the NK1R-F but not in NK1R-T in HIV positive individuals in comparison to HIV negative controls [23]. It is also consistent with a report that the expression of the SP precursor, TAC1, is decreased in frontal cortex of patients with HIVE in comparison to HIV negative controls (B. B. Gelman, personal communication CROI, Poster E167, Boston, MA, 2-28-2011). In another study involving an SIV model, we demonstrated an increased expression of SP and NK1-R in SIVE lesions, with macrophages positive for CD163 and SIV being the principal cells expressing NK1-R [45]. The apparent discrepancy between the Rhesus model and human patients may be explained by the fact that in the Rhesus model, we assayed tissue from animals with acute HIVE while the human samples reflect a broader, chronic neuropathology. We were unable to detect any increase in either HIV RNA or CD163 in samples with HIV associated neuropathology in comparison to HIV positive

individuals without neuropathology consistent with absence of acute infection/inflammation in HIV-NP+ group. The accumulation of damage to neurons and other CNS resident cells may result in the loss of both NK1R and SP expression, which negates any increases associated with chronic CNS inflammation.

Recently, we demonstrated that SP enhances HIV-1 infection in human fetal brain cell cultures expressing full-length neurokinin-1 receptor [46]. Previously, we also reported an increase in blood SP levels in men and women associated with HIV infection [47, 48]. This increase may be related to elevated SP production by both peripheral neurons and cells of non-neuronal origin in response to the chronic inflammation associated with HIV infection. It was also suggested that substance P plays a major pathogenetic role in HIV infection of the CNS by increasing permeability of brain endothelium exposed to HIV-1 gp120 [49, 50].

Decreased SP levels in the CNS may be related to neuronal damage and dysfunction possibly contributing to the cognitive deficiencies associated with neuroAIDS. SP-NK1R interactions may play an important role in stress, depression and drug-induced cognitive dysfunction (reviewed [51]. One possible mechanism by which the expression of SP could be regulated involves the transcription factor ΔFosB [52–54]. Additional evidence suggesting that tachykinins may be involved in the control of psychiatric disorders was provided by Bardelli et al. [55]. This study demonstrated that the protein levels of NK1R were significantly decreased while the levels of NK2R were increased compared to healthy subjects in monocytes from patients with major depressive disorder under stable antidepressant therapy.

Overall this study demonstrates that, despite clear evidence that inflammation is present in CNS of patients with HIV associated neuropathology, the expression of SP and, to lesser extent, NK1R-F are decreased. Our results justify further research of interaction between HIV, neuropeptides and proinflammatory factors which may lead to future therapeutic strategies targeting neurokinin receptors in neuroAIDS.

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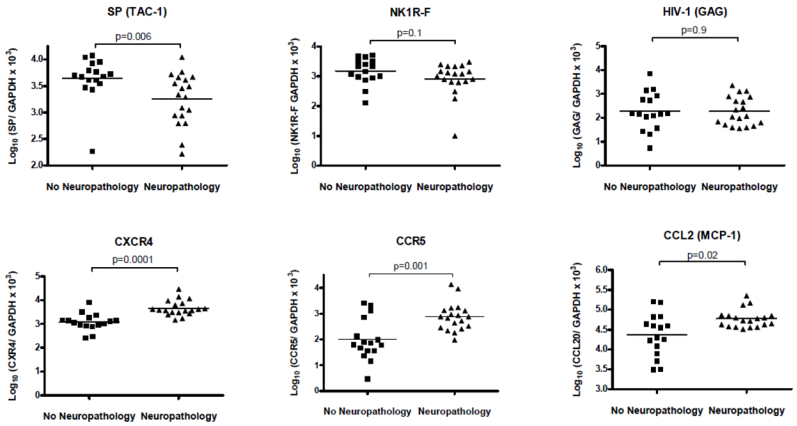

Figure 1. RNA levels of SP, NK1R, HIV and immune markers in frontal cortex of subjects from HIV-NP− and HIV-NP+ groups

Total RNA was extracted from frontal cortex of HIV-1 infected subjects with (n=19) or without (n=16) HIV-associated neuropathology and assayed for specific RNA levels by real-time RT PCR. RNA levels are expressed as $\log_{10}$ copies number relative to GAPDH mRNA. Results are presented as data for individual samples and mean. Differences in levels of CXCR4 and CCR5 were compared by independent sample t-tests between the two groups. Mann-Whitney tests were performed to compare differences between SP, NK1R-Full, CCL2 and HIV-1 GAG in the two groups.

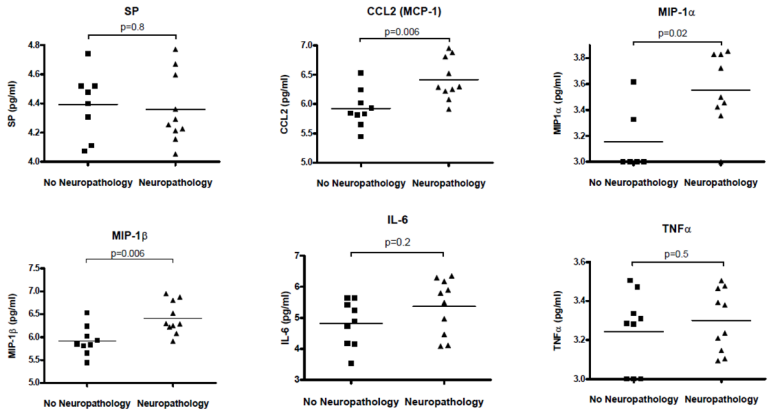

Figure 2. Protein levels of SP and immune markers in CSF of subjects from HIV-NP− and HIV-NP+ groups

Levels of immune markers were analyzed in CSF of HIV-1 infected subjects with (n=10) or without (n=9) HIV-associated neuropathology using flow cytometry-based Cytometric Bead Array or antigen competition enzyme immunoassay for SP. Data are expressed as $\log_{10}$ pg/ml. Differences between the two groups were assessed for all markers using Mann-Whitney tests.

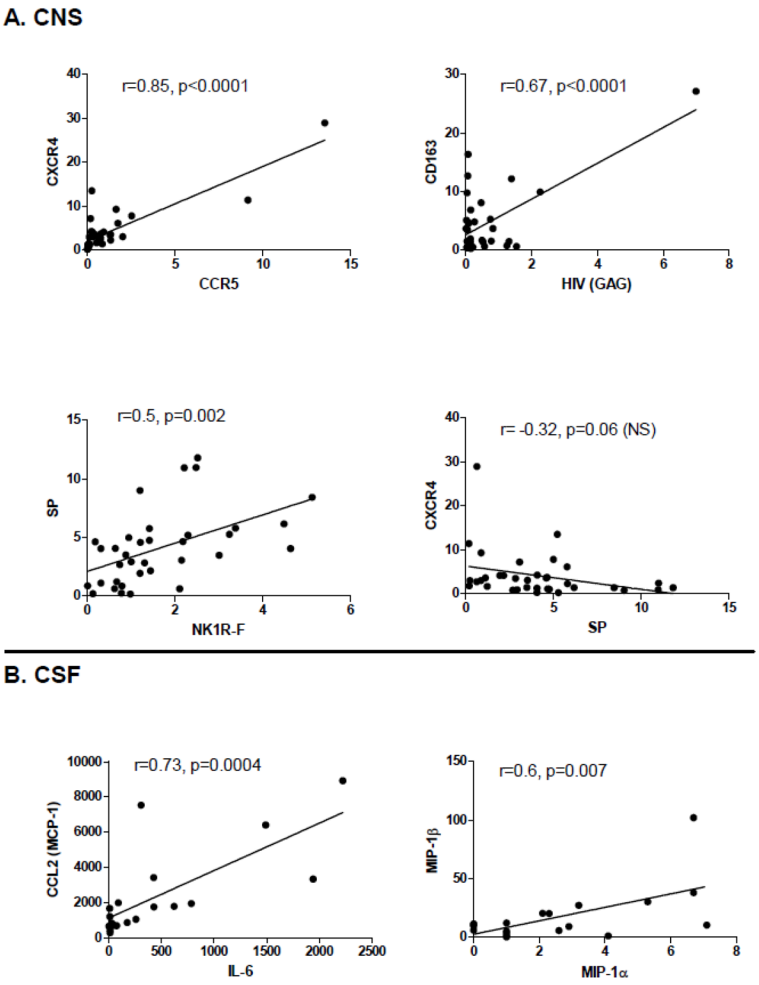

Figure 3. Correlations of gene expression in CNS and CSF of HIV-1 infected subjects

Correlation analysis between markers in CNS (n=35) and CSF (n=19) of HIV-1 infected subjects was performed using Pearson correlation test. Correlation coefficient (r) and p value are shown, along with a linear regression line.

Table 1

Sample information

	Number of samples (brain)	Number of samples (CSF)	Age at death, y, mean (SD)	Gender M/F	Duration of HIV since diagnosis, mean years (SD)	Nadir CD4 mean cells/μl(SD)
HIV-NP−	16	9	48(9)	14/2	10(6)	146(153)
HIV-NP+	19	10	42(8)	16/3	12(5)	108(118)

Table 2

Primers used for real-time PCR Assays

Gene	Primers (forward/reverse, 5′-3′)	Detection
NK1R-F	TCTTCTTCCTCCTGCCCTACATC GCCCAGACGGAACCTGTCAT	56-FAM-CCAGATCTCTACCTGAAGAAGTTTATCCAGCA-3BHQ1
NK1R-T	TCTTCTTCCTCCTGCCCTACATC TGG AGAGCTCATGGGGTTGGGATCCT	5′ FAM-CCAGATCTCTACCTGAAGAAGTTTATCCAGCA-3′ BHQ1
TAC-1 (SP)	CGGACCAGTAATTCAGATCATCA GAGGAACCAGAGAAACTCAGC	56-FAM-CATGTTGGA/ZEN/TTTTGCGACGGACAGT/3IABkFQ
CCR5	CAAAAAGAAGGTCTTCATTACACC CCTGTGCCTCTTCTTCTCATTTCG	SYBR
CD4	GGAAAGAAAGTGGTGCTGGGCAAA TCCTTGGTCCCAAAGGCTTCTTCT	SYBR
CXCR4	TGGCCTTATCCTGCCTGGTATTGT AGGAGTCGATGCTGATCCCAATGT	SYBR
Fractalkine	GAGCCGACTCCTTCTTCC CCTCCATCCTGAGCCTTT	SYBR
IL-6	AGTGAGGAACAAGCCAGAGC GTCAGGGGTGGTTATTGCAT	SYBR
CCL2 (MCP-1)	CTCGCTCAGCCAGATGCAATCAAT TGGAATCCTGAACCCACTTCTGCT	SYBR
CCL20 (MIP3A)	AGTTTGCTCCTGGCTGCTTTGATG CTGCCGTGTGAAGCCCACAATAAA	SYBR
IL-10	GTGATGCCCCAAGCTGAGA CACGGCCTTGCTCTTGTTTT	56-FAM-CCAAGACCCAGACATCAAGGCGCA-3BHQ1
CD163	AAAAGCGAAGACAGAGACAGC ATCATCTGCATTCAGGCAAG	SYBR
HIV-gag [37]	CATGTTTTCAGCATTATCAGAAGGA TGCTTGATGTCCCCCCACT	56-FAM-CACCCCACAAGATTTAAACACCATGCTAA-3BHQ1
GAPDH	GTGGTCTCCTCTGACTTCAACA TGCTGTAGCCAAATTCGTTG	56-FAM-CTGGCATTGCCCTCAACGACC-3BHQ1