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Toll-like receptor pathway gene expression is associated with human immunodeficiency virus–associated neurodegeneration

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The innate immune system is a significant component of the brain's defense against infection, especially as the blood-brain barrier restricts access of the members of the adaptive immune system, such as T and B cells. The innate immune system includes Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns. Within the central nervous system, they are expressed on glial cells and their expression can be modulated by pathological states. Although their function is to recognize foreign pathogens and stimulate a protective immune response through the production of cytokines and interferons, there is emerging evidence that activation of these receptors can result in neurodegeneration. In the current study, the authors assessed the expression of TLR-related genes, using a customized Superarray gene chip, and correlated the expression findings with indices of neurodegeneration. We found that, using a stringent threshold for statistical significance to overcome the potential problem of multiple statistical testing, there were significant correlations between the expression of nine TLR-related genes and reduction in dendritic and synaptic staining. Two of these genes, TLR4 and SIGIRR, were validated by quantitative real-time polymerase chain reaction. Additionally, the authors demonstrated *in vitro* at the protein level that human primary astrocytes exposed to the toxic human immunodeficiency virus (HIV) envelope protein gp120 had a significant increase in TLR4 protein expression. In conclusion, these findings indicate that TLR-related gene expression may contribute to the development of HIV-related neurodegeneration. *Journal of NeuroVirology* (2007) **13,** 496–503.

Keywords: brain; HIV; innate immunity; neurodegeneration; toll-like receptors

Introduction

Within the central nervous system (CNS), microglia and astrocytes are the major cellular components of the immune response. The stimuli that result in glial cell activation are numerous and include pathogen-associated molecular patterns (PAMPs), which are molecules recognized as nonself that characterize invading pathogens. PAMPs are recognized by the Toll-like receptor (TLR) family, and the peptidoglycan recognition protein receptors (PGRPs). PGRPs are a recently described family of PAMP receptors, in humans comprising a long form (PGRP-L), two intermediate length forms (PGRP-Iα and -Iβ), and a short form (PGRP-S) (Liu *et al*, 2001). PGRP-L is proposed to be membrane spanning and with a possible role in signaling activity. All PGRPs are thought to be secreted with amidase activity to catalyze the binding and cleaving of bacterial peptidoglycans (PGNs), thus removing immunostimulatory PGNs and down-regulating the immune response (Steiner, 2004). To date, the TLR family consists of 11 members (TLR1

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to TLR11). TLR10 occurs mainly in lymphoid tissue and TLR11 in the urinary tract (Zhang *et al*, 2004). TLR2 forms a heterodimer with either TLR1 or TLR6. Viral and bacterial ligands are being identified for various TLRs. Activation of a TLR facilitates interaction with downstream adaptor proteins, with recruitment of various adaptor proteins to the TLRs defining the specificity of a particular TLR signal. The most important adaptor appears to be MyD88, because accumulating evidence indicates that in all TLRs there is a common MyD88-dependent pathway. This pathway results in activation of interleukin-1 receptor (IL-1R)–activated kinase (IRAK) and nuclear factor (NF)-κB with the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and IL-8. There is also a MyD88-independent pathway that is peculiar to TLR3 and TLR4, stimulation of which results in activation of interferon regulatory factor-3 (IRF-3) and production of interferon-β (IFN-β) and chemokines such as RANTES (O'Neill *et al*, 2003).

Within the human CNS, expression of TLR1 through TLR9 has been characterized. TLR1 through TLR8 have been detected in microglial cells, with TLR1, TLR2, TLR4, and TLR7 being expressed at intermediate levels with the other receptors at low levels when compared to β-actin mRNA expression (Bsibsi *et al*, 2002). In human astrocyte cultures TLR3 has been found to be expressed at an intermediate level with lower levels found for TLR1, TLR4, TLR5, and TLR9 (Jack *et al*, 2005). TLR expression in the CNS can be modulated by pathological states, including neurodegeneration and infections, for example, there is increased astrocytic expression of TLR3 and TLR4 in multiple sclerosis lesions (Bsibsi *et al*, 2002) and there is up-regulation of TLR expression following viral infection in the brain (McKimmie *et al*, 2005). TLR activation can be pathogenic, for example, stimulation of microglial TLR9 results in dendritic damage and neurodegeneration (Iliev *et al*, 2004), with activation of microglial TLR4 receptors, resulting in apoptosis (Jung *et al*, 2005). Furthermore, stimulation of TLR2, TLR4, or TLR9 in latently infected mast cells has been found to trigger human immunodeficiency virus (HIV)-1 replication (Sundstrom *et al*, 2004).

The brain is a major target of HIV infection, resulting in a spectrum of inflammatory and neurodegenerative changes (Budka, 1991). Clinically, these can manifest as HIV-related neurocognitive disorder (Everall *et al*, 1999). Within the brain, perivascular macrophages and microglial cells represent the major cellular reservoirs of infection and maintenance of viral replication. HIV-associated neurodegeneration results from both the production of neurotoxic viral products and the production of proinflammatory cytokines as a response to the infection (Trillo-Pazos and Everall, 1997). Due to the existence of the blood-brain barrier (BBB), the brain is relatively shielded from the components of the adaptive immune system, such as B and T cells. Thus the response of the innate immune system, which includes brain TLRs, becomes more prominent. To date, however, little attention has focused on the interaction of HIV and TLRs. In the current study, we investigated gene expression of TLRs and their downstream signaling pathways in HIV-infected brain tissue and compared this profile to indices of neurodegeneration. We observed highly significant correlations between TLR pathway gene expression and HIV-associated neurodegeneration using microarray, quantitative real-time polymerase chain reaction (qRT-PCR). As we could not determine which cells were involved in differential gene regulation, we also assessed the protein expression of TLR4 in human primary astrocytes exposed to gp120 by immunocytochemistry.

Results

Microarray data

Of the 102 genes assessed on the Superarray genechip, 9 TLR-related genes showed at least one statistically significant association with the neuropathological cellular parameters tested, with a P value of less than 0.0005. In addition, a further six almost reached the adjusted level of statistical significance, with a P value of less than 0.001. These genes are summarized in Table 1, including the relevant Pearson correlation coefficient (r^2) and significance level. Of the nine genes, two were significantly associated with dendritic MAP2 staining, these were SIGIRR and SITPEC, whereas the other seven (TLR3, TLR4, LY86, MAPK8IP3, PGRP-S, PGRP-Iα, PGRP-L, and IκBb) were associated with synaptophysin staining. The expression of a further six genes revealed a trend ($P < 0.001$) towards a correlation with MAP2 staining (MAPK8IP3), synaptophysin staining (PGRP-S, SARM, NFκB2, and IκBL), or the marker of astrocytosis, GFAP (IκK-b). The Pearson correlation coefficient revealed that for all the significantly correlated TLR-related genes increasing gene expression was associated with decreased MAP2 or synaptophysin staining. The presence of HIV encephalitis (HIVE) had no association with TLR gene expression, neither did the density of either calbindin- or parvalbumin-immunopositive layer II/III neurons or cd45-immunopositive microglia ($p > 0.05$).

qRT-PCR data

Seven genes were selected for validation by qRT-PCR and their fold change assessed. These changes were then correlated to the neuropathological and clinical variables. As shown in Table 2, four genes demonstrated statistically significant correlation with indices of neurodegeneration: TLR4, SIGIRR, RIP1, and IL-8. TLR4 was inversely correlated with MAP2 staining ($r^2 = -0.53$, $P = 0.0068$), with a trend for synaptophysin staining ($r^2 = 0.25$, $P = 0.09$). SIGIRR gene

Table 1 TLR-related genes and cellular indices of neurodegeneration and inflammation

		Neudegeneration		
Cell parameter	HIVE *P*	MAP2 (r^2) *P*	Syn (r^2) *P*	GFAP (r^2) *P*
Toll-like receptors				
SIGIRR (TIR8)		(.73) .0004		
TLR3			(−.72) .0005	
TLR4			(.72) .0005	
Adaptors and TLR-interacting proteins				
LY86 (MD-1)			(−.74) .0003	
MAPK8IP3 (JIP3)		(−.69) .0009	(−.74) .0003	
PGLYRP (PGRP-S)			(−.68) .001	
PGLYRPIalpha (PGRP-Ialpha)			(−.81) <.0001	
PGRP-L			(−.81) <.0001	
SARM			(−.71) .0006	
Effectors				
SITPEC (ECSIT)		(−.89) <.0001		
Downstream pathways and target Genes				
NFκB pathway				
IKBKB (IκK-b)				(.71) .0006
NFκB2			(−.68) .0009	
NFκBIB (IκBb)/TRIP9			(−.78) .0001	
NFκBIL1 (IκBL)			(−.69) .0009	

Relationship between the presence of HIVE, indices of neurodegeneration and astrocytosis, and 14 TLR-related genes. For HIVE the *P* value was derived from a Student's *t* test with the appropriate log transformation where necessary. For MAP2, synaptophysin, and G FAP correlation was by linear regression with the pearson correlation coefficient, r^2, shown in parentheses followed by the *P* value.

expression was directly correlated with MAP2 staining ($r^2 = 0.38$, $P = 0.042$) and a trend for synaptophysin staining ($r^2 = 0.35$, $P = 0.057$). RIP2 was not correlated with either MAP2 or synaptophysin staining but directly correlated with the parvalbumin-immunopositive cell density in layers II to III ($r^2 = 0.58$, $P = 0.043$) and trends for correlation with parvalbumin-immunopositive cell density in layers V to VI and calbindin-immunopositive neurons (for all $r^2 = 0.26$, $P = 0.09$). Finally, there was a trend for IL-8 to correlate with the cellular density of cd45-immunopositive microglia ($r^2 = 0.32$, $P = 0.055$).

Immunocytochemistry

As the gene expression studies were performed in postmortem brain tissue, it is not possible to determine which glial cell population contributes to the alteration in TLR pathway gene expression. Thus to complement these experiments, we carried out work on the expression of TLR4 protein in human primary astrocytes exposed to gp120. Qualitative assessment of the immunocytochemical staining revealed a clear immunoreactive signal for TLR4 in the gp120-exposed astrocyte cultures, whereas the signal intensity was notably lower in the nonexposed control astrocyte cultures (Figure 1). These observations were then confirmed by intensity measurements from a representative sample of individual astrocytes in randomly selected fields. In the control condition, on the 256 grey scale in which the lower the value the darker the staining, the mean optical density was 172 (SD 4), whereas in the gp120-treated condition, the density of the staining had increase to 76 (SD 9). This difference in optical staining was statistically significant ($t = 19.49$, $df = 6$, $P = 0.0011$), demonstrating that TLR-4 expression was significantly up-regulated in gp120-exposed astrocyte cultures when compared to controls.

Discussion

In this study, we have assessed the relationship between expression of TLR-related genes and indices

Table 2 Correlation of four TLR RT-PCR–verified gene expression with indices of neurodegeneration

Gene	MAP2 r^2 (*P*)	Synaptophysin r^2 (*P*)	PV 2–3 r^2 (*P*)	PV 5–6 r^2 (*P*)	CB np r^2 (*P*)	CB p r^2 (*P*)	Cd45 cells
TLR4	−.53 (.0068)	.25 (.09)	n.s.	n.s.	n.s.	n.s.	
SIGIRR	.38 (.042)	.35 (.057)	n.s.	n.s.	n.s.	n.s.	
RIP2	n.s.	n.s.	.58 (.0043)	.26 (.09)	.26 (.09)	.26 (.09)	
IL-8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	.32 (.055)

Pearson correlation coefficients and their level of statistical significance for four TLR-related genes validated by QRT-PCR against measures of neurodegeneration: MAP2 for microtubule-associate protein-2 dendritic staining; synaptophysin synaptic staining; PV for parvalbumin neuronal staining; CB for calbindin neuronal staining; cd45 for cd45 immunopositive microglial cell staining.

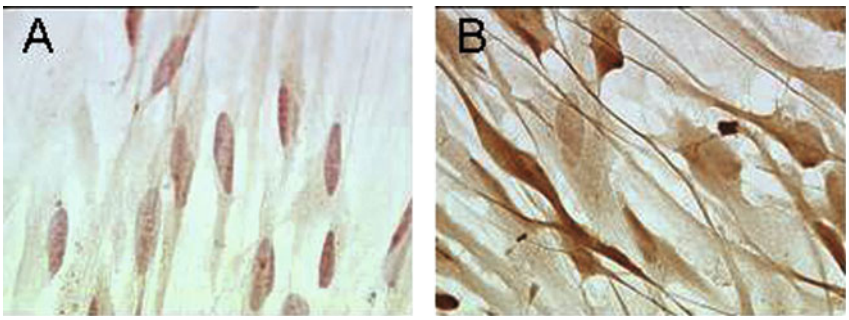

Figure 1 TLR-4 immunocytochemistry in astrocyte cultures. Immunocytochemistry demonstrating increased signal strength of TLR-4 in astrocyte cultures exposed to Gp120. (**A**) Nonexposed astrocyte controls. (**B**) Gp120-exposed astrocyte cultures. Images captures at ×1000 objective.

of neurodegeneration in HIV. Data derived from the original microarray chip may have been subject to statistical type I errors. This has been a general criticism of using whole-genome microarray chips in which, depending on the type of bioinformatic analysis undertaken, the type I error rate can vary from 45% to 29% (Sasik *et al*, 2002). However, it is unlikely that such a potential problem relates to our data as the Superarray chip utilized had only 102 genes and we applied a Bonferroni correction to the observed level of statistical significance. Out of the 102 genes represented on the Superarray genechip, 9 demonstrated a statistically significant correlation with MAP2 and synaptophysin markers of neurodegeneration, when applying a Bonferroni correction ($P < 0.0005$) to account for multiple comparisons. These nine genes were SIGIRR, TLR3, TLR4, LY86, MAPK8I3, PGRP-Iα, and PGRP-L. A further five genes revealed a trend to being correlated with neurodegeneration and one with a marker for astrocytosis: No gene expression correlated with either the presence of HIVE, the density of calbindin- or parvalbumin-immunopositive neurons, or cd45-immunopositive microglia. Although it may appear unusual that the none of the TLR-related genes correlated with the presence of either HIVE or cd45-immunopositive microglia, it may be that brain RNA viral load would have been a more sensitive correlate if it was available. As the microarray and qRT-PCR experiments were performed with postmortem brain tissue, it is not possible to identify which glial cell population was contributing the altered TLR pathway gene expression. To investigate this, we performed an additional experiment to quantitate the cellular immunopositivity of TLR4 protein expression in human primary astrocytes exposed to the viral envelope protein gp120. We observed that for TLR4 protein, there was up-regulation following exposure to gp120, indicating that astroyctes may be involved in at least part of the dysregulation of genes in the TLR pathway. Further studies should assess the other significantly dysregulated TLR genes that we noted in this study, in both human primary astrocytes and human primary microglial cultures. It would also be informative to assess TLR pathway gene expression in control brain tissue. The latter tissue processed in the same manner or with similar clinical and neuropathological quantification was not available.

TLRs and PGRPs are major components of the innate immune system and are activated by pathogens. The PGRP class of molecules are an emerging mammalian class of proteins with bactericidal activity. Within the brain, TLRs are expressed on microglia and astrocytes, with their expression levels being modulated by pathological processes and stimulation of TLRs resulting in neurodegeneration (Bottcher *et al*, 2003). In the current study, three receptors, four adaptor genes, one effector gene, and only downstream genes belonging to the NFκB pathway were correlated with neurodegeneration. None of the JNK/p38MAPK, NF/IL6κB, or IRF pathways were implicated in neurodegeneration.

On a small number of genes we verified our genechip findings by qRTPCR. The qRT-PCR data confirmed that TLR4 gene expression was negatively correlated with MAP2 and synaptophysin as indices of neurodegeneration. SIGIRR was also noted to be positively correlated with both MAP2 and synaptophysin indices of neurodegeneration, whereas only a direct correlation with MAP2 staining was observed on the Superarray genechip. As already mentioned, TLR4 activation in microglia is associated with neurodegeneration (Lehnardt *et al*, 2003). Furthermore, it has been observed that IFN-β production following TLR4 activation determines the apoptotic potential of this TLR signaling in microglia (Jung *et al*, 2005). Interestingly, TLR4 signaling is negatively regulated by SIGIRR (Bottcher *et al*, 2003), and on our microarray data it was observed that as levels of TLR4 gene expression rose, indices of neurodegeneration worsened. This trend was reversed for SIGIRR where the opposite relationship to neurodegeneration was seen. This latter relationship maybe related to SIGIRR attempting to diminish the consequences of TLR4 signaling through increased expression. Astrocytic TLR3 can be activated by double-stranded RNA signaling and is part of the antiviral and inflammatory responses. TLR3 can also reduce gene

expression of the EAAT/GLAST astrocytic glutamate transporter, which can result in dysfunctional handling of synaptic glutamate, thereby increasing the potential for excitotoxicity (Scumpia *et al*, 2005). The TLR-interacting protein MAPK8IP3/JIP3 in the CNS is expressed exclusively in neurons and mediates axonal transport of synaptic components (Akechi *et al*, 2001), with increased gene expression of MAPK8IP3/JIP3 once again correlating with exacerbation of neurodegeneration. However, it is unclear whether this change in gene expression relates directly to neurodegenerative effects or is in an attempt to rectify MAPK8IP3/JIP3 protein levels, which has been observed in the neuronal cytoplasm and growth cones of developing dendrites (Kelkar *et al*, 2000). Similarly, it is difficult to interpret the consequences of dysregulation of these genes and pathways as their effects appear to be context dependent. For example, both NF-κB or IFN-β can exacerbate glutamate excitotoxicity but they can also be neuroprotective (Barger *et al*, 2005). However, in this study, gene expression of NFKBIB/IkBb is significantly correlated with synaptic degeneration. Furthermore, events in one cell population can be detrimental for another population as in the cases of activation of the JNK/p38 pathway in activated glia, which can cause neurodegeneration (Bonny *et al*, 2005). However, gene expression of JNK/p38 pathway was not associated with neurodegeneration in this study. The expression of three PGRP genes were also significantly associated with neurodegeneration. The functions of PGRPs in mammals is still being elucidated, and it is as yet not clear if they have signaling capability or whether the secreted forms, which bind to PGNs, can act as an off switch for the innate immune response (Steiner, 2004).

In the present study, we observed an increase in TLR4 expression in primary human astrocyte monolayer cultures exposed to the HIV envelope protein gp120. The complexities of TLR activation and the subsequent intracellular signaling and its effects is increasingly being recognized (Barton and Medzhitov, 2003), with the role of TLRs in the HIV-specific immune response slowly being unraveled. Previous research has demonstrated that freshly isolated human monocytes displayed significantly enhanced expression of TLRs upon stimulation of HIV type 1 gp120. Alongside this, it has also been observed that TLR2 stimulation in HIV-infected patients induces increased viral replication (Heggelund *et al*, 2004). In addition, stimulation by ligands for TLR2, TLR4, and TLR9 have been demonstrated to significantly enhance viral replication in a dose- and time-dependent manner in HIV-1–infected mast cells (Sundstrom *et al*, 2004), with microbes and microbial antigens having been shown to promote HIV replication in infected patients via activation of TLR pathways (Lawn *et al*, 2001). Our increased TLR4 observations in astrocytes exposed to gp120 could indicate that in the case of HIV and astroglia, the TLR4 pathway could play a role in this augmented viral replication.

Further *in vitro* and postmortem studies are required prior to the validation of the relationship of TLRs to neurodegenerative indices observed in this study, and also in order to fully understand the consequences of activation and enhanced gene expression of TLR-related genes. Due to protease inhibitors being found to inhibit TLR2 and TLR4 (Equils *et al*, 2004), a further investigation into the role of these compounds in possibly preventing TLR activation and neurodegeneration we have observed here would be of relevance. Finally, if our initial findings of TLR activation leading to exacerbation of HIV-mediated neurotoxicity are confirmed, it will be worthwhile to assess TLR expression patterns in other neurodegenerative disorders to ascertain whether the effects of TLRs affect a common pathway or maybe specific to HIV.

Materials and methods

Materials

Fresh-frozen frontal cortical brain tissue from 13 individuals with HIV disease were assessed in this study. The pathological specimens were provided by the Californian NeuroAIDS Tissue Network (CNTN), with clinical and demographic information provided from individuals enrolled in a longitudinal prospective study at the University of California, San Diego, HIV Neurobehavioral Research Center. The clinical and demographic details have been described previously (Everall *et al*, 2005). On clinical neuropathological examination, five of the individuals had no discernable HIV-associated brain pathology, whereas the other eight cases had HIV encephalitis (HIVE). No other opportunistic infection or lymphoma was present.

Morphometric analysis

Paraffin-embedded glass mounted tissue sections were prepared and morphometric analysis performed for the area of microscopic field stained by the neuronal dendritic marker microtubule-associated protein-2 (MAP2) or the synaptic vesicle–associated protein synaptophysin. The number of glial fibrillary acidic protein (GFAP)-immunopositive astrocytes, CD45-immunopositive microglia, calbindin (CB)-immunopositive pyramidal or nonpyramidal neurons, and parvalbumin (PV)-immunopositive neurons in cortical layers II/III and V/VI were also calculated in the same region as previously described (Masliah *et al*, 2004).

RNA extraction

As previously described (Masliah *et al*, 2004), approximately 1 cm^3 of fresh-frozen tissue was lyzed in 1 ml TRIzol for total RNA extraction as per manufacturer's protocol (Invitrogen, CA). The RNA was purified using RNeasy columns (Qiagen, CA), quantified (OD 260 nm/280 nm), and checked for quality on a

denaturing 1% agarose gel. Ten micrograms of total RNA was used to generate double-stranded cDNA according to published protocols (Affymetrix, CA).

Hybridization and microarray
The cDNA was then hybridized against the GEArray Q Series Human Toll-Like Receptor Signaling Pathway Gene Array HS-059 as per the manufacturer's instructions. This array contains probes for 102 genes in the following categories:

Toll-like receptors. LY64 (RP105/CD180), SIGIRR (TIR8), TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10.
Adaptors and TLR-interacting proteins. BTK, CD14, GPC1 (SP-A), HMGB1, HRAS, HSPA4, HSPD1, LY86 (MD-1), LY96 (MD2), MAL, MAPK8IP3 (JIP3), MYD88, PELI1 (Pellino 1), PELI2 (Pellino 2), PGLYRP (PGRP-S), PGLYRPIalpha (PGRP-Ialpha), PGRP-L, RIPK2 (RIP2), SARM1, TIRAP, TIRP (TICAM2), TOLLIP, TRIF (TICAM1).
Effectors. CASP8, FADD, IRAK1, IRAK2, IRAK3 (IRAK-M), IRAK4, MAP3K7IP1 (TAB1), MAP3K 7IP2 (TAB2), NR2C2 (TAK1), PRKRA (PKR), SITPEC (ECSIT), TRAF6, UBE2N (Ubc13), UBE2V1 (Uev1A).
Downstream pathways and target genes NFκB pathway. CCL2 (MCP-1), CHUK (IKK-a), CSF2 (GM-CSF), CSF3 (G-CSF), IFNB1, IFNG, IKBKB (IKK-b), IKBKG (IKK-g), IL1A, IL1B, IL2, IL6, IL8, IL12 A, IL12B, LTA (TNF-b), MAP3K1 (MEKK1), MAP3K14, MAP4K4 (NIK), NFKB1, NFKB2, NFK BIA (IkBa / mad3), NFKBIB (IkBb), NFKBIL1, NF RKB, RELA, RELB, TNF (TNFa).
JNK/p38 pathway. ELK1, FOS, JUN, MAP2K3 (MKK3), MAP2K4 (MKK4), MAP2K6 (MKK6), MA P3K1 (MEKK1), MAPK8 (JNK1), MAPK9 (JNK2), MAPK11 (p38bMAPK), MAPK12 (p38gMAPK), MAPK13, MAPK14 (p38 MAPK).
NF/IL6 pathway. CLECSF9, PTGS2 (Cox-2).
IRF pathway. CXCL10 (IP-10), IFNB1, IFNG, IRF1, IRF3.
Regulation of adaptive immunity. CD80, CD86, RIPK2 (RIP2), TRAF6.

Quantitative real-time polymerase chain reaction (qRT-PCR)
As previously described (Sheeter *et al*, 2003; http://genomics.ucsd.edu), the level of expression of specific transcripts of seven TLR-related candidate genes were determined using the Perkin-Elmer ABI Prism 7700 (ABI, USA). Briefly, total RNA from the autopsy brain tissue was extracted using the TRIzol method (Gibco-BRL) followed by digestions with a deoxyribonuclease to remove any contaminating genomic DNA. Equal amounts of cDNA were then used in triplicate and amplified with the TaqMan Master Mix provided by Perkin-Elmer. Amplification efficiencies were then validated and normalized against 18S RNA and fold change calculated using the standard curve method. The primer sequences for the candidate TLR genes are:

TLR4. [TLR4-av-(NM_003266)-2511F ATC CAG AGC CGC TGG TGT AT; TLR4-av-(NM_003266)-2561R CTG CCA GGT CTG AGC AAT CTC]
IL-2. [IL-2-(NM_000586)-457F AAA CTC ACC AGG ATG CTC ACA TT; IL-2-(NM_000586)-507R TGT GGC CTT CTT GGG CA]
TLR3. [TLR3-(NM_003265)-2735F CTG GCC AGT TCA GAA AGA ACG; TLR3-(NM_003265)-2785R ACT TGC AAT TTA TGA CGA AAG GC]
IFNB1. [IFNB1-(NM_002176)-252F TGA CAT CCC TGA GGA GAT TAA GC; IFNB1-(NM_002176)-302R GCG TCC TCC TTC TGG AAC TG]
PGRP1A. [PGRP1A-(NM_052891)-713F (aka PGLYRP3) CCA GAC TGT CGT CCG AAA CA; PGRP1A-(NM_052891)-763R (aka PGLYRP3) AAG TTC CGT GTG TCC ATG TGA A]
RIPK2. [RIP2-(NM_003821)-1537F (CARD3, RICK, RIPK2) AAC GTC TGC AGC CTG GTA TAG C; RIP2-(NM_003821)-1587R (CARD3, RICK, RIPK2) CTT CCC TTT TGC TCT GGA TCC];
SIGIRR. [SIGIRR-(NM_021805)-1193F CAG CTG CAG GAC GAC AAG G; SIGIRR-(NM_021805)-1243R AGG GAC TCG GCC TCG AAG];
IL-8. [IL-8-(NM_000584)-131F GGC AGC CTT CCT GAT TTC TG; IL-8-(NM_000584)-210R TGC ACT GAC ATC TAA GTT CTT TAG CA].

Statistical analysis
Regression analysis was performed for gene expression and the quantitation of MAP2, synaptophysin, GFAP-immunopositive astrocytes, CD45-immunopositive microglia, CB- and PV-immunopositive pyramidal or nonpyramidal neurons. A Student's *t* test was performed for gene expression changes in the presence and absence of HIVE, with log transformation of the data where appropriate; to account for this multiple testing and the possibility of a type I error, we applied a Bonferroni correction to reduce the level of statistical significance to a value of $P = 0.0005$ or less.

Human fetal astrocytes and HIV gp120 exposure
Human fetal brain tissue of gestational age 14–18 weeks was provided in a clinically anonymous manner in accordance with University of California, San Diego, Institutional Review Board approval. Brain tissue was collected and prepared as previously described (Trillo-Pazos *et al*, 2004). Tissue was processed to a single-cell suspension, followed by seeding into T-175 flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal calf serum, 0.5% glutamine (200 mM), and 0.1% gentamycin sulphate (all solutions from Sigma, UK). Flasks were then placed in an incubator set at 5% $CO^2$and 37°C. Following a 2-week incubation period, flasks were confluent and astrocyte cultures were passaged down and reseeded into flasks. After a further 2 weeks, astrocytes were seeded at 1×10^5 cells/ml onto glass coverslips coated with

poly-l-ornithine in 12-well plates. Medium was changed routinely every 3 days for 1 week, after which the coverslips were confluent with astrocytes. For experiments, the astrocytes were treated with the HIV envelope protein gp120 for 24 h at a concentration of 100 ng/ml in medium. Control cultures consisted of astrocytes exposed to untreated medium.

Immunocytochemistry
Cell cultures were immunostained according to standardized protocols (Vectorlabs, USA). Briefly, culture medium was removed and cells washed in phosphate-buffered saline (PBS) × 3, followed by fixing cells in 10% paraformaldehyde for 30 min. Cells were then washed in PBS × 3, permeabilized, and blocked for 1 h using a 0.1% triton in blocking antibody solution. Following this, the blocker was removed and the TLR4 primary antibody (R&D systems; 1:500) applied and left overnight at 4°C. Coverslips were then exposed to a biotinylatyed secondary antibody, followed by incubation with Vectastain ABC–horseradish peroxidase complex, according to standard protocols (Vector Labs, USA). A hemotoxylin counterstain was then applied and the coverslips mounted permanently onto superfrost plus slides (VWR, USA) using a permanent mountant.

Optical density counts
Gray scale–based cell intensity measurements were obtained using four coverslips per condition. For each coverslip, four randomly selected fields were sampled, with a total of 96-cell intensities per condition being measured to provide a representative mean cell densitometry value per condition. The color intensity values were calculated using Image Pro Plus 4.0 software (Media Cybernetics, USA). The scale for color intensity values was mapped to a black and white intensity scale in Image Pro Plus, which ranges from 0 to 255 (where 0 = black and 255 = white). An independent-sampled *t* test was implemented using SPSS version 11.0 (SPSS, USA) to compare the means of controls to the gp120-exposed group for TLR4 immunocytochemistry expression.

References

Akechi M, Ito M, Uemura K, Takamatsu N, Yamashita S, Uchiyama K, Yoshioka K, Shiba T (2001). Expression of JNK cascade scaffold protein JSAP1 in the mouse nervous system. *Neurosci Res* **39:** 391–400.

Barger SW, Moerman AM, Mao X (2005). Molecular mechanisms of cytokine-induced neuroprotection: NFkappaB and neuroplasticity. *Curr Pharm Des* **11:** 985–998.

Barton GM, Medzhitov R (2003). Toll-like receptor signaling pathways. *Science* **300:** 1524–1525.

Bonny C, Borsello T, Zine A (2005). Targeting the JNK pathway as a therapeutic protective strategy for nervous system diseases. *Rev Neurosci* **16:** 57–67.

Bottcher T, Von Mering M, Ebert S, Meyding-Lamade U, Kuhnt U, Gerber J, Nau R (2003). Differential regulation of Toll-like receptor mRNAs in experimental murine central nervous system infections. *Neurosci Lett* **344:** 17–20.

Bsibsi M, Ravid R, Gveric D, Van Noort JM (2002). Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol* **61:** 1013–1021.

Budka H (1991). Neuropathology of human immunodeficiency virus infection. *Brain Pathol* **1:** 163–175.

Equils O, Shapiro A, Madak Z, Liu C, Lu D (2004). Human immunodeficiency virus type 1 protease inhibitors block toll-like receptor 2 (TLR2)- and TLR4-Induced NF-kappaB activation. *Antimicrob Agents Chemother* **48:** 3905–3911.

Everall I, Luthert P, Lantos P (1993). A review of neuronal damage in human immunodeficiency virus infection: its assessment, possible mechanism and relationship to dementia. *J Neuropathol Exp Neurol.* **52:** 561–566.

Everall IP, Heaton RK, Marcotte TD, Ellis RJ, Mccutchan JA, Atkinson JH, Grant I, Mallory M, Masliah E (1999). Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center. *Brain Pathol* **9:** 209–217.

Everall I, Salaria S, Roberts E, Corbeil J, Sasik R, Fox H, Grant I, Masliah E; Hnrc GROUP (2005). Methamphetamine stimulates interferon inducible genes in HIV infected brain. *J Neuroimmunol* **170**(1–2): 158–71

Heggelund L, Muller F, Lien E, Yndestad A, Ueland T, Kristiansen KI, Espevik T, Aukrust P, Froland SS (2004). Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. *Clin Infect Dis* **39:** 264–269.

Iliev AI, Stringaris AK, Nau R, Neumann H (2004). Neuronal injury mediated via stimulation of microglial toll-like receptor-9 (TLR9). *FASEB J* **18:** 412–414.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, Mccrea E, Shapiro A, Antel JP (2005). TLR signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol* **175:** 4320–4330.

Jung DY, Lee H, Jung BY, Ock J, Lee MS, Lee WH, Suk K (2005). TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-beta as a decision maker. *J Immunol* **174:** 6467–6476.

Kelkar N, Gupta S, Dickens M, Davis RJ (2000). Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Mol Cell Biol* **20:** 1030–1043.

Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW (2004). Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc Natl Acad Sci U S A* **101:** 1315–1320.

Lawn SD, Butera ST, Folks TM (2001). Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev* **14:** 753–777, table.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T (2003). Activation of innate immunity in the CNS triggers neurodegeneration

through a Toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A* **100:** 8514–8519.

Liu C, Xu Z, Gupta D, Dziarski R (2001). Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J Biol Chem* **276:** 34686–34694.

Masliah E, Roberts ES, Langford D, Everall I, Crews L, Adame A, Rockenstein E, Fox HS (2004). Patterns of gene dysregulation in the frontal cortex of patients with HIV encephalitis. *J Neuroimmunol* **157:** 163–175.

Mckimmie CS, Johnson N, Fooks AR, Fazakerley JK (2005). Viruses selectively upregulate Toll-like receptors in the central nervous system. *Biochem Biophys Res Commun* **336:** 925–933.

O'Neill LA, Fitzgerald KA, Bowie AG (2003). The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol.* **24:** 286–290.

Sasik R, Calvo E, Corbeil J (2002). Statistical analysis of high-density oligonucleotide arrays: a multiplicative noise model. *Bioinformatics* **18:** 1633–1640.

Scumpia PO, Kelly KM, Reeves WH, Stevens BR (2005). Double-stranded RNA signals antiviral and inflammatory programs and dysfunctional glutamate transport in TLR3-expressing astrocytes. *Glia* **52:** 153–162.

Sheeter D, Du P, Rought S, Richman D, Corbeil J (2003). Surface CD4 expression modulated by a cellular factor induced by HIV type 1 infection. *AIDS Res Hum Retroviruses* **19:** 117–123.

Steiner H (2004). Peptidoglycan recognition proteins: on and off switches for innate immunity. *Immunol Rev* **198:** 83–96.

Sundstrom JB, Little DM, Villinger F, Ellis JE, Ansari AA (2004). Signaling through Toll-like receptors triggers HIV-1 replication in latently infected mast cells. *J Immunol* **172:** 4391–4401.

Trillo-Pazos G, Everall IP (1997). From human immunodeficiency virus (HIV) infection of the brain to dementia. *Genitourin Med* **73:** 343–347.

Trillo-Pazos G, Kandanearatchi A, Eyeson J, King D, Vyakarnam A, Everall IP (2004). Infection of stationary human brain aggregates with HIV-1 SF162 and IIIB results in transient neuronal damage and neurotoxicity. *Neuropathol Appl Neurobiol* **30:** 136–147.

Zeinstra E, Wilczak N, Streefland C, De Keyser J (2000). Astrocytes in chronic active multiple sclerosis plaques express MHC class II molecules. *Neuroreport* **11:** 89–91.

Zeinstra EM, Wilczak N, Wilschut JC, Glazenburg L, Chesik D, Kroese FG, De Keyser J (2006). 5HT(4) agonists inhibit interferon-gamma-induced MHC class II and B7 costimulatory molecules expression on cultured astrocytes. *J Neuroimmunol.*

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* **303:** 1522–1526.