Available online at www.sciencedirect.com
ScienceDirect
Brain, Behavior, and Immunity 22 (2008) 185–194
BRAIN, BEHAVIOR, and IMMUNITY
www.elsevier.com/locate/ybrbi

SIV infection decreases sympathetic innervation of primate lymph nodes: The role of neurotrophins

Erica K. Sloan [a,*], Christina T. Nguyen [a], Benjamin F. Cox [a], Ross P. Tarara [b], John P. Capitanio [b,c], Steve W. Cole [a,d]

[a] University of California Los Angeles, Department of Medicine, Division of Hematology-Oncology, UCLA School of Medicine, UCLA AIDS Institute, and the Cousins Center for Psychoneuroimmunology at the UCLA Semel Institute for Neuroscience and Human Behavior, USA
[b] California National Primate Research Center, University of California Davis, USA
[c] Department of Psychology, University of California Davis, USA
[d] Jonsson Comprehensive Cancer Center and the UCLA Molecular Biology Institute, USA

Received 21 May 2007; received in revised form 12 July 2007; accepted 13 July 2007
Available online 17 September 2007

Abstract

The sympathetic nervous system regulates immune responses in part through direct innervation of lymphoid organs. Recent data indicate that viral infections can alter the structure of lymph node innervation. To determine the molecular mechanisms underlying sympathetic denervation during Simian Immunodeficiency Virus (SIV) infection, we assessed the expression of neurotrophic factors and neuromodulatory cytokines within lymph nodes from experimentally infected rhesus macaques. Transcription of nerve growth factor (NGF), brain-derived neurotropic factor (BDNF) and neurotrophin-4 (NT4) decreased significantly *in vivo* during chronic SIV infection, whereas expression of the neuro-inhibitory cytokine interferon-γ (IFNγ) was up-regulated. Acute SIV infection of macaque leukocytes *in vitro* induced similar changes in the expression of neurotrophic and neuro-inhibitory factors, indicative of an innate immune response. Statistical mediation analyses of data from *in vivo* lymph node gene expression suggested that coordinated changes in expression of multiple neuromodulatory factors may contribute to SIV-induced depletion of catecholaminergic varicosities within lymphoid tissue. Given previous evidence that lymph node catecholaminergic varicosities can enhance SIV replication *in vivo*, these results are consistent with the hypothesis that reduced expression of neurotrophic factors during infection could constitute a neurobiological component of the innate immune response to viral infection.

Keywords: Neurotrophin; SIV; Denervation; Sympathetic nervous system; Nerve growth factor (NGF)

1. Introduction

All primary and secondary lymphoid organs are innervated by sympathetic fibers of the autonomic nervous system (ANS) (Felten et al., 1985). Following activation, catecholamine neurotransmitters are released from varicosities along the length of the nerve fiber and bind adrenergic receptors on immune cells, thereby modulating immune function (Sanders et al., 2001; Sanders, 2006). Catecholamine signaling has also been found to promote replication of Human Immunodeficiency Virus Type 1 (HIV-1) and the closely related Simian Immunodeficiency Virus (SIV) (Cole et al., 1998, 1999; Sloan et al., 2006). Recent work has shown that sympathetic innervation of peripheral lymphoid organs is dynamic, with altered patterns of innervation observed during aging (Bellinger et al., 2005), inflammation (Kelley et al., 2003) and viral infection (Sloan et al., 2006). Recently, we showed that experimental infection of primates with SIV led to a significant reduction in the density of sympathetic innervation in lymph nodes of rhesus macaques (Sloan et al., 2006). Loss of lymphoid

* Corresponding author. Fax: +1 310 794 9247.
E-mail address: esloan@ucla.edu (E.K. Sloan).

0889-1591/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbi.2007.07.008

innervation has also been observed in the gastrointestinal tract of individuals infected with HIV-1 (Batman et al., 1991) and the spleen of mice infected with the LM-BP5 mixture of retroviruses (Kelley et al., 2003). These results suggest that sympathetic nervous system (SNS) regulation of immune function may change during the course of infection. However, the molecular mechanisms that mediate these effects are currently unknown, and it is unclear what their functional significance might be for viral pathogenesis.

Several neuromodulatory factors are known to regulate the density of sympathetic innervation under physiologic conditions. Members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4), are critical in shaping patterns and density of innervation, both centrally and in the periphery. NGF was first characterized as an essential factor for neuronal development and survival (Levi-Montalcini, 1987) and is expressed in a wide range of neuronal and non-neuronal celltypes (Levi-Montalcini, 1987; Frossard et al., 2004). Target-derived NGF is essential for guiding developing sympathetic neurons to peripheral organs (Kuruvilla et al., 2004). NT3 has a complementary role in neuronal guidance, stimulating outgrowth of sympathetic neurons from ganglia and intermediate targets including vasculature (Belliveau et al., 1997; Francis et al., 1999; Kuruvilla et al., 2004). The biological activity of neurotrophins is mediated through, (1) the p75 neurotrophin receptor ($p75^{NTR}$), which binds all neurotrophins with similar avidity, and, (2) the family of Trophomyosin receptor kinases (TrkA, B and C), which differ in neurotrophin specificity. Sympathetic neurons express $p75^{NTR}$, and TrkA, which binds NGF and NT3 (Reichardt, 2006). BDNF is produced by both sympathetic neurons and target-organ cells including leukocytes (Kuruvilla et al., 2004; Bronzetti et al., 2006; Edling et al., 2004). On sympathetic neurons, which lack Trk B, BDNF acts via $p75^{NTR}$ to antagonize NGF-mediated support for innervation (Kohn et al., 1999). Expression of $p75^{NTR}$ is upregulated by high levels of NGF in the target organ, providing a feedback loop to ensure tight regulation of peripheral innervation density (Kuruvilla et al., 2004). The role of NT4 in peripheral innervation of tissues is unclear, although some evidence indicates that NT4 can regulate the development of the dorsal root ganglia (Liebl et al., 2000), and may help protect central nervous system (CNS) neurons against injury (Cheng et al., 1994).

Sympathetic neural networks are also influenced by negative guidance cues that deflect innervation (Chen et al., 1998). Cytokines, including leukemia inhibitory factor (LIF) and interferon-γ (IFN-γ) have been shown to induce dendritic retraction in cultured sympathetic neurons (Guo et al., 1997; Kim et al., 2002). Consistent with a neural-inhibitory role for IFN-γ, intestinal denervation occurs following infection with the protozoan *Trypanosoma cruzi* in wildtype mice but not in IFN-γ knock-out mice (Arantes et al., 2004). Semaphorins are locally-acting nerve repellents (Huber et al., 2003) that also influence the development and function of other systems including cardiovascular, endocrine, gastrointestinal (Yazdani and Terman, 2006). Increased expression of semaphorin 3C is associated with loss of sympathetic nerve fibers in synovial tissue from rheumatoid arthritis patients (Miller et al., 2004).

To determine whether effects of viral infection on lymphoid tissue innervation might be driven by altered expression of the same neuromodulatory factors that regulate sympathetic innervation density under normal physiologic conditions, we examined the expression of genes encoding NGF, BDNF, NT3, NT4, LIF, IFN-γ and semaphorin 3C in lymph nodes sampled from rhesus macaques experimentally infected with SIV. Results show a marked reduction in the expression of key neurotrophic factors relative to non-infected controls. Additional analyses of macaque leukocytes infected with SIV *in vitro* show similar changes in the absence of any adaptive immune response to virus, and in the absence of neural or stromal cells. The results are consistent with the hypothesis that cells of the immune system have evolved a coordinated gene-regulation program that may act to alter local neural architecture during chronic viral infection.

2. Materials and methods

2.1. Subjects

This study investigated 26 lymph nodes that were randomly selected from 7 non-infected rhesus macaques (2 nodes from each of 5 animals, 1 node from 1 animal, 3 nodes from 1 animal) and from 9 macaques infected with Simian Immunodeficiency Virus (SIV) (1 node from each of 7 animals, 2 nodes from 1 animal , 3 nodes from 1 animal). Animals came from a larger study comprising 36 male rhesus macaques aged 7–10 years, that were individually housed for the duration of the study. At week 0, twenty-four animals were experimentally infected with SIV ($10^{2.66}$ $TCID_{50}$ SIV_{mac251}), and all became infected as documented by RT-PCR detection of plasma viral genomes (Sloan et al., 2006). At week 37, four axillary lymph nodes were biopsied from each of 20 macaques, two of which were fresh frozen and the remaining two of which were fixed in OCT. Lymph nodes for the current study were selected from the pool of 40 fresh frozen lymph nodes. SIV replication within lymph node tissue was confirmed by *in situ* hybridization using probes to detect SIV *env, gag* and *nef* mRNA as previously described (Sloan et al., 2006; Canto-Nogues et al., 2001). All procedures were carried out under protocols approved by the Institutional Animal Use and Care and University Institutional Review Boards at the University of California, Davis and Los Angeles campuses.

2.2. Lymph node innervation

16 μm fresh-frozen tissue sections were mapped for the distribution of catecholaminergic varicosities using glyoxylic acid chemofluorescence as previously described (Sloan et al., 2006; de la Torre and Surgeon, 1976). Glyoxylic acid reacts with catecholamines to cyclize their ethylamine side chain. This may be visualized as blue-white fluorescence under ultraviolet illumination, thereby distinguishing catecholaminergic from peptidergic nerve fibers (Guidry, 1999). The density of parenchymal- and blood vessel-associated variocosities per 250 μm^2 tissue unit was assessed using a stereologically-based statistical analysis (Sloan et al., 2006). Briefly, each

lymph node section was digitally imaged over its entire surface at 200× magnification using an Axioskop 2 microscope equipped with an AxioCam HRc color camera and AxioVision 4.2 software (Zeiss, Thornwood NY). Individual images were assembled to compose a single digital file representing the entire organ section, and a grid of 250 μm^2 tissue units was superimposed. Within each tissue unit, the number of catecholaminergic varicosities was counted, and the tissue unit was allocated to a functionally distinct subregion (cortex, paracortex, medulla) by a veterinary pathologist (RPT) based on an adjacent section stained by hematoxylin and eosin.

2.3. Statistical analysis

To determine whether SIV infection might influence lymph node innervation, generalized linear model analyses were carried out using SAS v9.1 (SAS Institute, Cary NC) to assess the frequency of catecholaminergic varicosities in tissue units of lymph nodes biopsied from SIV-infected animals vs. non-infected controls. To ensure independence of observations, all analyses included indicator variables controlling for differences in innervation density across individual lymph nodes (treated as a random factor nested within the fixed experimental factor of SIV infection vs. control) (Miller, 1986). The frequency of SIV replication sites varied substantially across lymph nodes from SIV-inoculated animals, and Spearman correlations were computed to determine whether increasing prevalence of SIV replication within a given lymph node might relate to the prevalence of catecholaminergic varicosities.

Statistical mediation analyses (Hoyle and Kenny, 1999; Baron and Kenny, 1986) were conducted in the context of generalized linear model analyses to determine whether the effects of SIV infection on lymph node innervation density might be attributable to variations in the expression of neurotrophic or neuro-inhibitory gene products (as quantified below). Briefly, a ''total effects'' analysis first quantified the entire relationship between SIV infection and lymph node innervation density (i.e., the effect stemming from all sources: neurotrophic factors + other influences). Subsequent analyses controlled for any variation in innervation density that could be attributed specifically to neurotrophic factors, and quantified the residual effect of SIV infection (i.e., that attributable to other effects besides changes in neurotrophic factor expression). Mediation was quantified as the percent of total SIV-induced denervation attributable specifically to changes in neurotrophic factors. To determine whether changes in neurotrophin expression might potentially account for all systematic effects of SIV infection on innervation density, residual (non-neurotrophin) effects of SIV infection were tested for statistical significance (i.e., non-significance of residual effects indicating that neurotrophins are sufficient to account for all systematic effects of SIV on innervation) (Hoyle and Kenny, 1999; Baron and Kenny, 1986).

2.4. Expression of neurotrophic factors and cytokines

Real-time RT-PCR was used to quantify *NGFB*, *BDNF*, *NT3*, *NT4* (*NT4/5*), *IFNG, LIF,* and *SEMA3C* mRNA in total RNA extracted from 3 mg of lymph node tissue (or 10^6 rhesus peripheral blood leukocytes in *in vitro* infection studies outlined below) using an RNeasy Kit (Qiagen, Valencia CA). cDNA was synthesized with iScript reverse transcriptase according to the manufacturer's protocol (Bio-Rad, Hercules CA). Real-time RT-PCR utilized iQ SYBR Green Supermix (Bio-Rad) and 45 amplification cycles of 95 °C for 15 s followed by 60 °C for 60 s on an iCycler instrument (Bio-Rad). Assays were completed in triplicate and quantitated by threshold cycle analysis of SYBR Green fluorescence. Values were normalized to *GAPDH* and *ACTB* mRNA amplified in parallel. Triplicate determinations of normalized threshold cycle data were averaged for *t*-test analysis of differences between SIV-infected vs. non-infected tissues. Primer sequences and gene regions that were amplified are as follows:

NGF: Genbank: XM_001100522, bp: 434–601
F 5′-GTTTTACCAAGGGAGCAGCTTTC-3′
R 5′-TAGTCCAGTGGGCTTGGGGGA-3′
BDNF: Genbank: AF208982, bp: 33–294
F 5′-TAACGGCGGCAGACAAAAAGACT-3′
R 5′-GTGTCTATCCTTATGAATCGCCAGCCAA-3′
NT3: Genbank: XM_001118191.1, bp: 254–515
F 5′-GCATCCAAGGTAACAACATGGATC -3′
R 5′- GGTGAGTTGTAGCGTCTCTGTTG -3′
NT4: Genbank: XM_001114610.1, bp: 655–716
F 5′- CCTCCGCCAGTACTTCTTTGA-3′
R 5′- CCGGGCCACCTTCCTC-3′
IFNG: Genbank: A4376145, bp: 346–423
F 5′-GAAAAGCTGACCAATTATTCGGTAA-3′
R 5′-AGCCATCACTTGGATGAGTTCA-3′
LIF: Genbank: BC093733, bp: 222–297
F 5′-TGCCAATGCCCTCTTTATTC-3′
R 5′-GCCACATAGCTTGTCCAGGT-3′
SEMA3C: Genbank: XM_001108385.1, bp: 863–1139
F 5′- GCAAAATGGCTGGCAAAGATCC-3′
R 5′- CCCATGAAATCTATATACATTCC-3′
GAPDH: Genbank: BC083511, bp 58–226
F 5′-GAAGGTGAAGGTCGGAGTC-3′
R 5′-GAAGATGGTGATGGGATTC-3′
ACTB: Genbank: NM_001101, bp: 549–573
F 5′-TCACCCACACTGTGCCCATCTACGA-3′
R 5′-CAGCGGAACCGCTCATTGCCAATGG-3′

2.5. In vitro SIV infection

10^7 rhesus macaque peripheral blood mononuclear cells (PBMC) were cultured under standard conditions (5% CO_2, RPMI + 10% fetal bovine serum), activated with 25 μg/mL phytohemagglutinin (PHA, Sigma, St. Louis MO) and 50 U/mL recombinant human interleukin-2 (University of California, Davis), and incubated for 45 min in 1 mL of cell culture supernatant containing $10^{2.66}$ $TCID_{50}$ SIV_{mac251} (grown in CEMx174 cells) for infection, or 1 mL uninfected cell culture supernatant for a non-infected control. PBMC were then washed, and cultured for 6 days in RPMI + 10% fetal bovine serum. At 2, 4, and 6 days post infection, viral replication was assessed by ELISA for SIV p27 (Coulter, Miami FL) within cell-free culture supernatants, and by real-time RT-PCR detection of SIV RNA in 10^6 PBMC using previously published primer sequences against SIV *gag* and *env* (Canto-Nogues et al., 2001) and the protocol outlined above (*Expression of neurotrophic factors and cytokines*). Threshold cycle data were normalized to *GAPDH* mRNA as described above, and triplicate determinations were averaged for statistical analysis by *t*-test.

3. Results

3.1. SIV replication in lymph nodes

To assess the effects of SIV infection on sympathetic innervation of peripheral lymphoid tissue, we biopsied axillary lymph nodes from adult rhesus macaques 37 weeks after experimental inoculation with SIV_{mac251} and parallel non-infected controls. At this time, SIV-positive animals were chronically infected but had not progressed to end-stage disease (e.g., no evidence of opportunistic infection). *In situ* hybridization for SIV *nef*, *gag* and *env* mRNA (Fig. 1c, inset) confirmed active viral replication in all but one lymph node sampled from SIV-infected animals.

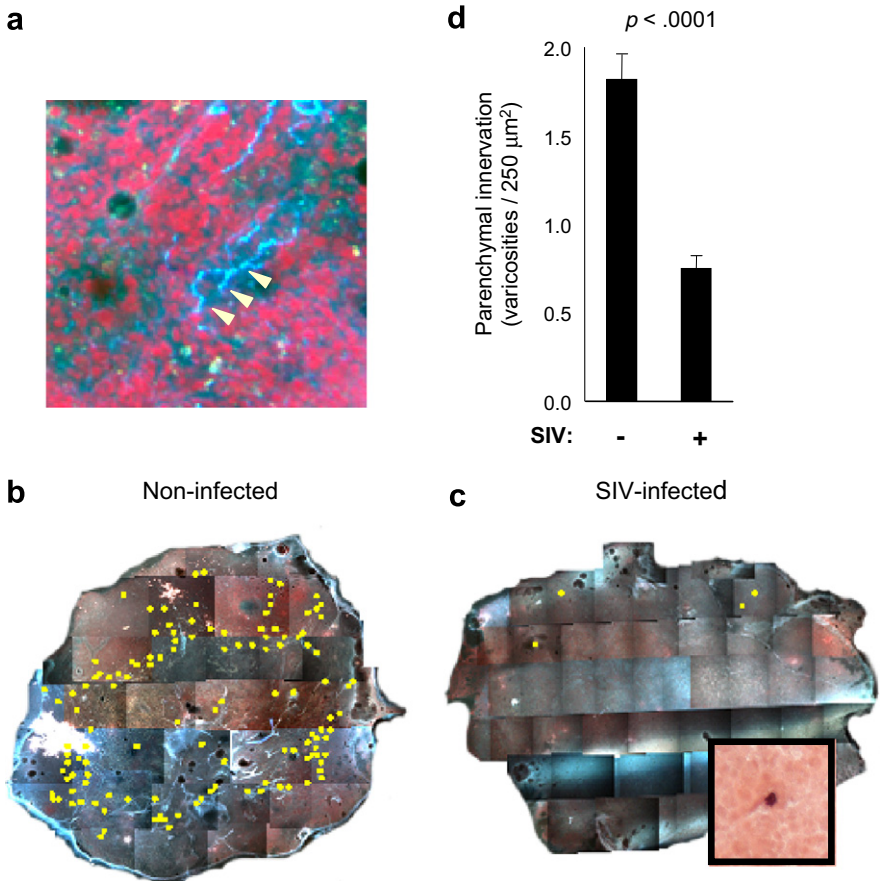

Fig. 1. Effect of SIV infection on lymph node sympathetic innervation. Parenchymal catecholaminergic varicosities (arrowheads) were localized in lymph node sections using glyoxylic acid chemofluorescence (a). Immune cells are counterstained red. Sites of innervation were mapped (yellow dots) onto whole lymph node sections from non-infected (b) and SIV-infected rhesus macaques (c). SIV-infection was confirmed by *in situ* hybridization against viral *gag*, *pol*, and *env* genes (c, inset). Spatial stereological techniques quantified the density of innervation in non-infected and SIV-infected lymph nodes (d).

Active SIV replication was detected in two other lymph nodes biopsied from the same animal, confirming that this animal had contracted systemic infection. Primary analyses focused on lymphoid tissues harboring active viral replication and so excluded the lymph node lacking active replication. However, parallel analyses that retained this sample showed substantively identical results. SIV mRNA was not detected in any tissue obtained from non-infected animals.

3.2. Lymph node innervation

Catecholaminergic neural varicosities were localized using glyoxylic acid chemofluorescence (Fig. 1a). Varicosities within the lymph node parenchyma were distinguished from perivascular innervation, and mapped across entire lymph node sections from non-infected and SIV-infected rhesus macaques (Fig. 1b and c). Spatial stereological analyses showed that the density of parenchymal catecholaminergic varicosities was reduced by 59% in lymph nodes harboring active SIV replication (vs. non-infected tissues; Fig. 1d; $p < .0001$). In non-infected macaques, the density of innervation ranged from .45 to 17.43 varicosities/250 μm^2 tissue unit (mean = 1.82 ± .14 standard error). In contrast, innervation density ranged from ranged from .00 to 3.18 varicosities/250 μm^2 in lymph nodes harboring active SIV replication (mean = .75 ± .07). SIV-induced denervation was most pronounced in the paracortex, which showed a 72% decrease in the density of parenchymal catecholaminergic varicosities ($p < .0001$). SIV infection decreased the density of cortical innervation by 17% ($p = .011$), and had no significant impact on the density of medullary innervation (16% decrease, $p = .264$). These results suggest that SIV-induced depletion of catecholaminergic innervation within the lymph node parenchyma is most pronounced in regions populated by T lymphocytes, macrophages and antigen presenting cells, and minimal in proximity to B-lymphocyte-rich follicular zones.

Although comparisons between SIV-infected animals and non-infected controls showed substantial effects of experimental SIV infection on the density of lymph node innervation, quantitative analyses of variation among the SIV-infected samples did not identify a significant correlation between the magnitude of sympathetic denervation and systemic measures of disease progression (i.e., correlation of innervation density with plasma SIV viral load and

CD4+ T lymphocyte levels, $r = +.24$ and $-.25$, respectively, both $p > .509$, with individual viral load and CD4 values published previously in Table 1 of (Sloan et al., 2006)). Moreover, analyses of 3 lymph nodes sampled from a single SIV-infected animal varied greatly in their quantitative magnitude of denervation (>10-fold range in density), suggesting that local biological dynamics (rather than systemic virologic parameters they shared in common) exert a dominant influence on the density of lymph node sympathetic denervation. Consistent with that hypothesis, differences in the density of sympathetic innervation across those three lymph nodes were inversely proportional to the density of SIV gene expression within each lymph node ($r = -.79$, $p < .001$). A similar inverse correlation between lymph node-specific levels of SIV gene expression and innervation density was observed across all SIV+ tissues examined ($r = -.47$, $p < .001$).

Table 1
Statistical mediation analyses assessing the role of neurotrophic and neuro-repellant factors as mediators of SIV effects on lymph node sympathetic innervation density

Model	Mediator	Type III SS	Residual p value
1	None (total effect attributed to SIV)	7002.4950	Not applicable
2a	*NGF* alone	233.4324	.008
2b	*BDNF* alone	124.5549	.052
2c	*NT4* alone	.0517	.965
2d	*IFNG* alone	42.6872	.120
3	*NGF* + *BDNF* + *NT4* (trophic factors)	.0208	.978
4	*NGF* + *BDNF* + *NT4* + *IFNG*	1.1017	.720

Models 2a–d examine individual factors as potential mediators of SIV effects on lymph node innervation density. Model 3 examines mediational potential of all 3 neurotrophic factors simultaneously. Model 4 examines mediational potential of 3 neurotrophic factors + neuro-repellant *IFNG*. Type III SS = sum of squares for residual effect of SIV infection on innervation density after controlling for listed mediator(s).

3.3. Neurotrophin levels in lymph nodes

To determine whether changes in tissue expression of neuro-trophic or neuro-repellant factors might mediate the effects of SIV infection on sympathetic denervation, we assayed levels of *NGF*, *BDNF*, *NT3* and *NT4* mRNA by quantitative realtime RT-PCR. Consistent with the reduced sympathetic innervation of SIV-infected lymph nodes, *NGF* mRNA levels were reduced by an average of 73% relative to non-infected controls ($p < .001$) (Fig. 2). *BDNF* and *NT4* mRNA levels also showed substantial reduction in SIV-replicating tissues (81% and 80%, respectively, $p < .0001$ and $p = .004$). In contrast, levels of *NT3* were not significantly altered (difference <1.29-fold, $p = .327$). These results indicate that SIV infection can selectively inhibit the local tissue expression of key neurotrophic factors known to support peripheral sympathetic innervation.

3.4. Expression of neural-repulsive factors in lymph nodes

To investigate whether SIV infection might also diminish the density of lymphoid sympathetic innervation by increasing local expression of sympathetic nerve repellants, we assayed expression *IFNG*, *LIF* and *SEMA3C* by quantitative RT-PCR. *IFNG* expression increased by an average 2.8-fold in SIV-positive lymph nodes ($p = .011$) (Fig. 3), consistent with the hypothesis that localized increases in IFN-γ might promote sympathetic denervation. Expression of *LIF* was not significantly altered ($p = .127$), and expression of *SEMA3C* was reduced by 56% in SIV-positive tissues ($p = .029$). The latter result is inconsistent with a role for increased semaphorin 3c expression as a mechanism for SIV-induced sympathetic denervation. *IFNG* was the only neural-repulsive gene product showing changes consistent with SIV-induced denervation, and this factor was thus retained along with the neurotrophic factors in subsequent analyses.

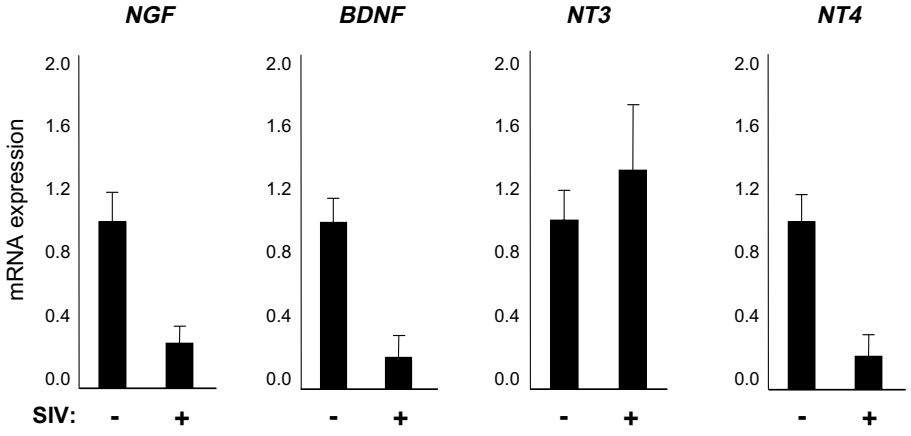

Fig. 2. Effect of SIV infection on neurotrophin mRNA expression in lymph nodes. Real-time RT-PCR quantification of neurotrophin mRNAs in lymph nodes biopsied from uninfected (−) and SIV-infected (+) macaques. mRNA levels have been normalized to expression of housekeeping genes.

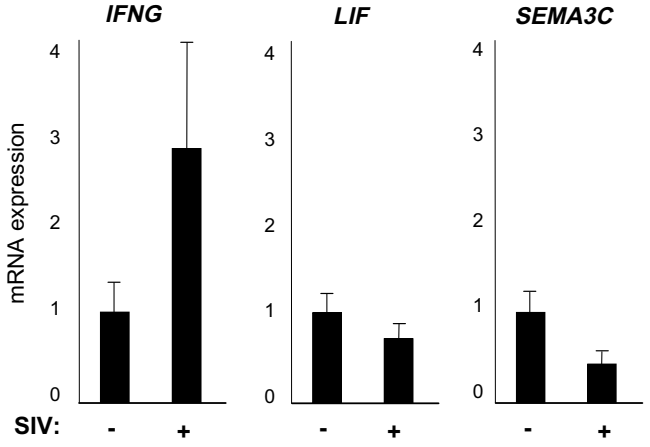

Fig. 3. Effect of SIV infection on neural inhibitor mRNA expression in lymph nodes. Expression of *IFNG*, *LIF* and *SEMA3C* mRNA in lymph nodes from non-infected (−) and SIV-infected (+) macaques was quantified by real-time RT-PCR. mRNA levels have been normalized to expression of housekeeping genes.

3.5. Effect of SIV infection on neurotrophin expression by leukocytes

To determine whether lymphoid cells are a source of sympathetic modulators that may be regulated by SIV infection, we experimentally infected (SIV-naïve) rhesus macaque PBMC with SIV_{mac251} and analyzed expression of *NGF*, *BDNF*, *NT3*, *NT4* and *IFNG* six days afterward, at the peak of infection *in vitro* (infection data not shown). SIV infection altered leukocyte expression of *NGF*, *BDNF*, *NT4* and *IFNG* in ways that were consistent with sympathetic denervation and paralleled the changes observed in lymphoid tissue *in vivo*. Levels of *NGF*, *BDNF*, *NT3* and *NT4* mRNA all decreased significantly (76%, 62%, 88% and 80% decrease, respectively; all $p < .005$) (Fig. 4a), and SIV infection significantly enhanced expression of *IFNG* (>4000-fold increase, $p < .001$). These results suggest that cells of the immune system might constitute a target for infection-induced regulation of lymphoid sympathetic innervation.

3.6. Mediation of in vivo denervation

To determine whether the observed changes in *NGF*, *BDNF*, *NT4* and *IFNG* expression might be sufficient to account for the effects of SIV infection on lymph node sympathetic denervation, we conducted statistical mediation analyses in the context of generalized linear models (Baron and Kenny, 1986). (*In vitro* analyses showed that SIV infection is capable of reducing NT3 expression (Fig. 4), but *in vivo* analyses showed that NT3 expression was not likely to contribute to denervation because its levels were maintained in lymph nodes of SIV-infected animals (Fig. 2). Therefore, NT3 was not retained in analyses of *in vivo* mediation.) The total effect of SIV infection accounted for 20.7% of the total *systematic* variation in the density of parenchymal catecholaminergic varicosities (i.e., variations in varicosity density specifically attributable to differences among individual lymph nodes, and across lymph nodes from SIV-infected vs. non-infected animals). To determine whether specific individual neuro-modulatory factors (i.e. *NGF*, *BDNF*, *NT4*, or *IFNG* alone) might function as mediators of that total effect of SIV infection on innervation density, we carried out a series of analyses controlling for each factor alone and examined the residual relationship between SIV infection and innervation density (i.e., that not attributable to the measured factor). Results showed that variations in *NGF* expression alone could potentially account for up to 96.7% of the total effect of SIV on innervation density (Table 1, Model 2a, column 3). However, the residual effect of SIV infection on innervation density remained statistically significant ($p = .008$ in Table 1, Model 2a, column 4), suggesting that variation

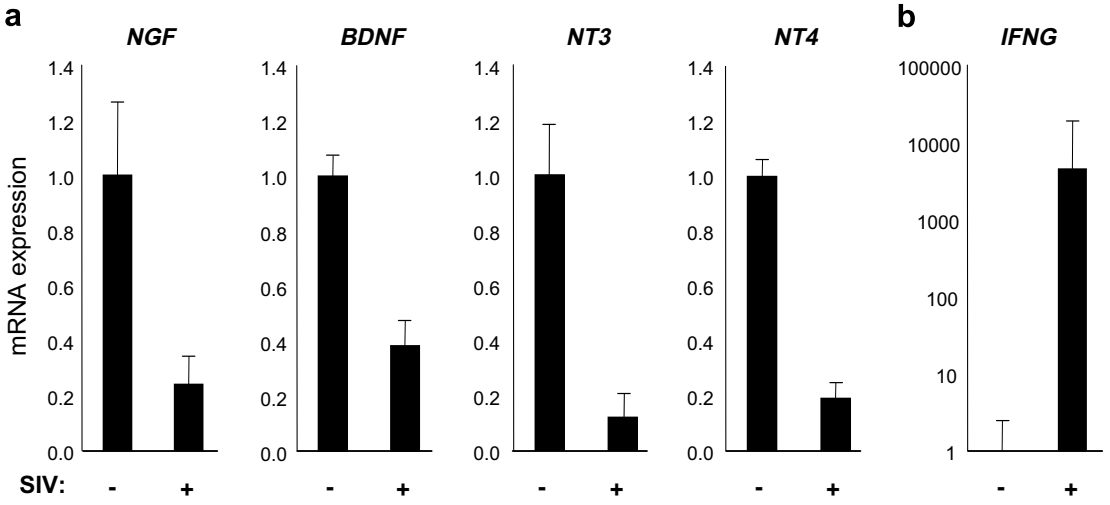

Fig. 4. Effect of *in vitro* SIV infection on neurotrophin expression by leukocytes. Expression of *NGF*, *BDNF*, *NT4* (a) and *IFNG* (b) in PBMC were quantified by real time RT-PCR. mRNA levels have been normalized to expression of housekeeping genes.

Table 2
Correlations among neuro-modulators and SIV infection across lymph nodes

	NGF	*BDNF*	*NT4*	*IFNG*	*SIV*
NGF	—	.577	.667	.234	.446
BDNF	.578	—	.713	.219	.522
NT4	.736	.767	—	.214	.596
IFNG	.245	.214	.165	—	−.401
SIV	.382	.544	.554	−.441	—

Upper diagonal values = Pearson correlation coefficient; Lower diagonal values = Spearman rank correlation coefficient. $r > .229 = p < .05$; $r > .363 = p < .01$; $r > .456 = p < .001$.

in other neurotrophic factors might also contribute to SIV's effects. Control for *BDNF* alone, *NT4* alone, or *IFNG* alone indicated that variations in each factor's expression could potentially account for more than 98% of the total effect of SIV on innervation density (all $p < .0001$; residual SIV effects on innervation density, all $p > .052$). However, it is unclear whether the effects attributed to each individual neurotrophic factor represent truly distinct influences, as there was a high degree of correlation in their pattern of variation across lymph nodes (Table 2).

The highly correlated expression of neurotrophic and neuro-inhibitory factors suggested potential co-ordinate regulation of these genes in the lymph node environment. This led us to consider multivariate models assessing the simultaneous effects of multiple factors. As shown in Table 1 (Model 3), results suggested that coordinate variation in the neurotrophic factors *NGF*, *BDNF*, and *NT4* could account for more than 99% of the total effect of SIV infection on innervation density (residual effect of SIV, $p = .978$). Analysis of *p*-values for individual model coefficients failed to identify any single neurotrophic factor as individually significant above and beyond the effect of others, suggesting that the effects of SIV infection on innervation density are most likely mediated by the joint effect of multiple neurotrophic factors (i.e., the overlapping co-variance among factors). Similar results emerged in analyses that included the neuro-inhibitory *IFNG* in addition to the multiple neurotrophic factors (Table 1, Model 4). These results suggest that the effects of experimental SIV infection on lymph node innervation by catecholaminergic neural fibers may involve the simultaneous influence of multiple factors that jointly regulate neural structure and plasticity (Fig. 5).

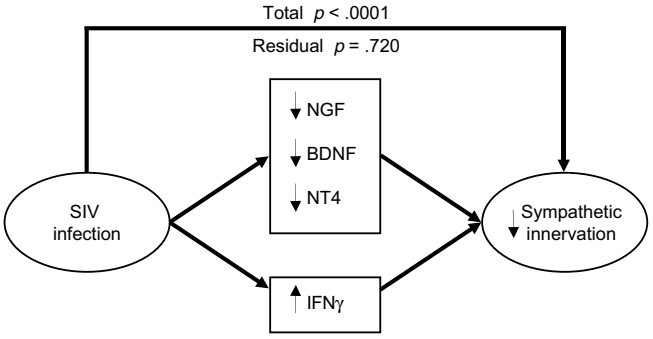

Fig. 5. Model evaluating neuro-modulatory factors as mediators of SIV-induced denervation of lymphoid tissue. Top *p*-value evaluates statistical significance of SIV effects on innervation density. Bottom *p*-value indicates statistical significance of residual effect of SIV infection on innervation density after controlling for variations in postulated mediators *NGF*, *BDNF*, *NT4*, and *IFNG*.

4. Discussion

The present study showed that chronic infection with SIV can substantially reduce the sympathetic innervation of lymphoid tissue in adult primates, and that such effects are associated with marked alterations in tissue expression of key neurotrophic factors (NGF, BDNF, and NT4), and the neuro-repellant cytokine, IFN-γ. Quantitative mediation analyses suggested that much of the SIV-induced denervation of lymphoid tissues observed *in vivo* could be attributed to changes in the expression of those neuro-modulatory factors. Additional *in vitro* analyses showed the ability of leukocytes to modulate expression of neuro-regulatory genes in response to viral infection. This may constitute a major cellular pathway for remodeling lymphoid tissue innervation in response to viral infection. The teleologic basis for these effects requires further exploration, but the present findings suggest that cells of the immune system may have evolved the capacity to dynamically regulate their exposure to sympathetic neural regulation during viral infections and/or inflammatory responses. This could provide a form of immune-to-nervous system feedback in the lymphoid tissue microenvironment that parallels the systemic feedback circuit in which circulating pro-inflammatory cytokines regulate activity of the hypothalamic-pituitary-adrenal axis (Woloski et al., 1985; Besedovsky et al., 1986; Turnbull and Rivier, 1999). Indeed, the strong coordinate regulation of multiple neuro-modulatory factors in lymphoid tissue suggests that redundant neuro-regulatory systems may have evolved to facilitate functional denervation of lymphoid organs during immune or inflammatory reactions.

Reduced innervation of lymphoid tissues following infection may result from direct viral effects (Xu et al., 2004; Eugenin et al., 2007), in addition to changes in neurotrophin expression. This raises the possibility that reduced neurotrophin expression following SIV infection might be a consequence of denervation, rather than a cause, since neurotrophins may derive from both immune and neural sources (Kuruvilla et al., 2004; Bronzetti et al., 2006; Edling et al., 2004; Frossard et al., 2004; Levi-Montalcini, 1987). However, two findings argue against the hypothesis that changes in neurotrophin expression are solely a consequence of denervation. First, the changes in neurotrophin expression observed here were selective to several key regulatory molecules (NGF, BDNF, and NT4), and did not affect all neurotrophins in parallel (e.g., *in vivo* NT3 expression was not significantly altered). This suggests that some stable source of neurotrophins must exist in addition to dynamic neural structures.

In addition, experimental infection of isolated leukocytes showed that SIV infection can causally alter the expression of neurotrophic factors in the absence of any neural source. These results do not rule out the possibility that sympathetic nerve fibers are also a source of lymph node neurotrophins *in vivo*, but they do suggest that other sources of neurotrophic factors are available within the lymphoid tissue environment. In addition, the present data show that neurotrophic factor expression by lymphoid cells is empirically responsive to viral infection.

The present study has identified several candidate molecular mediators of SIV-induced denervation, and a clear understanding of their individual roles will require experimental manipulation of individual mediators (e.g., restoring virally-induced diminution of lymph node NGF, BDNF, or NT4). Plausible mechanisms of influence on innervation density have already been identified for several of the neuro-modulatory factors altered by SIV in the present study. Target organ-derived NGF plays a key role in guiding nerve fibers into peripheral organs and supporting survival of peripheral sympathetic neurons (Kuruvilla et al., 2004). SIV-induced reduction of lymph node *NGF* expression might diminish NGF support for neuronal growth and survival. Evidence suggests that NT4 may be neuroprotective in the central nervous system (Han and Holtzman, 2000; Cheng et al., 1994), and any similar effect on peripheral sympathetic neurons could explain the strong quantitative profile of reduced *NT4* expression as a potential mediator of SIV effects on sympathetic innervation (i.e., Table 1, Model 2c). IFN-γ can contribute to denervation by up-regulating inducible nitric oxide synthase (iNOS) and enhancing neuro-destructive nitric oxide (Arantes et al., 2004). The specific functional role for individual neurotrophins in infection-induced modulation of sympathetic innervation represents an important topic for further research. Moreover, the strong correlation in expression of multiple neuro-modulators (Table 2) raises the possibility that no single neuro-modulatory mediator may be solely responsible for the observed dynamics.

Another question raised by the present findings involves the mechanism by which viral infection influences the expression of neurotrophic factors. Analyses of isolated leukocytes suggest that SIV infection can alter the expression of multiple neurotrophic factors independently of the influence of neural or stromal factors. Moreover, the use of SIV-naïve donor cells in acute *in vitro* infection studies implies that leukocyte-mediated reduction in the expression of neurotrophic factors is an innate immune response to infection, rather than an antigen-specific adaptive immune response (because no adaptive immune response is present in SIV-naïve cells *a priori*, and the culture duration is too short to permit its *de novo* development) (Kabelitz and Medzhitov, 2007). It is conceivable that innate immune recognition of viral replication, and the subsequent production of pro-inflammatory cytokines and Type I interferons (Collado-Hidalgo et al., 2006) might selectively impair the transcription of neurotrophic factors. Indeed, a localized innate immune response to infection would be consistent with the spatially localized denervation observed in this study (with the most pronounced denervation occurring in the T cell-rich paracortex in which SIV predominately replicates). This hypothesized link between innate antiviral responses and sympathetic denervation is particularly intriguing given recent indications that sympathetic neurotransmitters can inhibit innate antiviral responses (Collado-Hidalgo et al., 2006). From this perspective, the localized sympathetic denervation observed here may serve to functionally de-repress Type I interferons (Collado-Hidalgo et al., 2006) and withdraw catcholaminergic support for viral replication (Prosch et al., 2000; Bloom et al., 1994; Leib et al., 1991; Willey et al., 1984; Chang et al., 2005; Turgeman and Aboud, 1998; Cole et al., 1998, 1999, 2001; Sloan et al., 2006). If true, this hypothesis implies that the dynamic denervation of lymphoid organs in response to viral infection may constitute a neurobiological component of the broader innate immune response to infection.

Acknowledgments

We thank Suzanne Stevens and Srinivasan ThyagaRajan for protocols for glyoxylic acid chemofluorescence; Ding Lu and the Immunlogy Core Lab at the California National Primate Research Center (CNPRC) for SIV_{mac251} stock, Harry Vinters and the UCLA AIDS Institute for access to BSL2+ cryostat; Christine Brennan, Erna Tarara, Greg Vicinio, Carmel Stanko, Laura Del Rosso, Kristen Cooman, and the Veterinary, Animal Care, and Research Services staff of the CNPRC for study conduct; Jesusa Arevalo, Caroline Sung, and Cathey Heron for administrative support; and Michael Irwin, Jerome Zack and members of the Zack laboratory for their thoughtful discussions of this research. These studies were supported by the National Institute of Mental Health (MH049033), the National Institutes of Allergy and Infectious Disease and Neurological Disorders and Stroke (AI/NS052737), the National Cancer Institute (CA116778), the University of California Universitywide AIDS Research Program (CC99-LA-02), the Cousins Center for Psychoneuroimmunology in the Semel Institute for Neuroscience and Human Behavior at UCLA, the UCLA AIDS Institute and the James B. Pendelton Charitable Trust.

References

Arantes, R.M., Marche, H.H., Bahia, M.T., Cunha, F.Q., Rossi, M.A., Silva, J.S., 2004. Interferon-gamma-induced nitric oxide causes intrinsic intestinal denervation in *Trypanosoma cruzi*-infected mice. Am. J. Pathol. 164, 1361–1368.

Baron, R.M., Kenny, D.A., 1986. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. J. Pers. Soc. Psychol. 51, 1173–1182.

Batman, P.A., Miller, A.R., Sedgwick, P.M., Griffin, G.E., 1991. Autonomic denervation in jejunal mucosa of homosexual men infected with HIV. AIDS 5, 1247–1252.

Bellinger, D.L., Stevens, S.Y., Thyaga Rajan, S., Lorton, D., Madden, K.S., 2005. Aging and sympathetic modulation of immune function in Fischer 344 rats: effects of chemical sympathectomy on primary antibody response. J. Neuroimmunol. 165, 21–32.

Belliveau, D.J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C., Rusakov, D., Kaplan, D., Miller, F.D., 1997. NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J. Cell Biol. 136, 375–388.

Besedovsky, H., del Rey, A., Sorkin, E., Dinarello, C.A., 1986. Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. Science 233, 652–654.

Bloom, D.C., Devi-Rao, G.B., Hill, J.M., Stevens, J.G., Wagner, E.K., 1994. Molecular analysis of herpes simplex virus type 1 during epinephrine-induced reactivation of latently infected rabbits in vivo. J. Virol. 68, 1283–1292.

Bronzetti, E., Artico, M., Pompili, E., Felici, L.M., Stringaro, A., Bosco, S., Magliulo, G., Colone, M., Arancia, G., Vitale, M., Fumagalli, L., 2006. Neurotrophins and neurotransmitters in human palatine tonsils: an immunohistochemical and RT-PCR analysis. Int. J. Mol. Med. 18, 49–58.

Canto-Nogues, C., Jones, S., Sangster, R., Silvera, P., Hull, R., Cook, R., Hall, G., Walker, B., Stott, E.J., Hockley, D., Almond, N., 2001. In situ hybridization and immunolabelling study of the early replication of simian immunodeficiency virus (SIVmacJ5) in vivo. J. Gen. Virol. 82, 2225–2234.

Chang, M., Brown, H.J., Collado-Hidalgo, A., Arevalo, J.M., Galic, Z., Symensma, T.L., Tanaka, L., Deng, H., Zack, J.A., Sun, R., Cole, S.W., 2005. beta-Adrenoreceptors reactivate Kaposi's sarcoma-associated herpesvirus lytic replication via PKA-dependent control of viral RTA. J. Virol. 79, 13538–13547.

Chen, H., He, Z., Tessier-Lavigne, M., 1998. Axon guidance mechanisms: semaphorins as simultaneous repellents and anti-repellents. Nat. Neurosci. 1, 436–439.

Cheng, B., Goodman, Y., Begley, J.G., Mattson, M.P., 1994. Neurotrophin-4/5 protects hippocampal and cortical neurons against energy deprivation- and excitatory amino acid-induced injury. Brain Res. 650, 331–335.

Cole, S.W., Jamieson, B.D., Zack, J.A., 1999. cAMP up-regulates cell surface expression of lymphocyte CXCR4: implications for chemotaxis and HIV-1 infection. J. Immunol. 162, 1392–1400.

Cole, S.W., Korin, Y.D., Fahey, J.L., Zack, J.A., 1998. Norepinephrine accelerates HIV replication via protein kinase A-dependent effects on cytokine production. J. Immunol. 161, 610–616.

Cole, S.W., Naliboff, B.D., Kemeny, M.E., Griswold, M.P., Fahey, J.L., Zack, J.A., 2001. Impaired response to HAART in HIV-infected individuals with high autonomic nervous system activity. Proc. Natl. Acad. Sci. USA 98, 12695–12700.

Collado-Hidalgo, A., Sung, C., Cole, S., 2006. Adrenergic inhibition of innate anti-viral response: PKA blockade of Type I interferon gene transcription mediates catecholamine support for HIV-1 replication. Brain Behav. Immun.

de la Torre, J.C., Surgeon, J.W., 1976. Histochemical fluorescence of tissue and brain monoamines: results in 18 minutes using the sucrose-phosphate-glyoxylic acid (SPG) method. Neuroscience 1, 451–453.

Edling, A.E., Nanavati, T., Johnson, J.M., Tuohy, V.K., 2004. Human and murine lymphocyte neurotrophin expression is confined to B cells. J. Neurosci. Res. 77, 709–717.

Eugenin, E.A., King, J.E., Nath, A., Calderon, T.M., Zukin, R.S., Bennett, M.V., Berman, J.W., 2007. HIV-tat induces formation of an LRP-PSD-95- NMDAR-nNOS complex that promotes apoptosis in neurons and astrocytes. Proc. Natl. Acad. Sci. USA 104, 3438–3443.

Felten, D.L., Felten, S.Y., Carlson, S.L., Olschowka, J.A., Livnat, S., 1985. Noradrenergic and peptidergic innervation of lymphoid tissue. J. Immunol. 135, 755s–765s.

Francis, N., Farinas, I., Brennan, C., Rivas-Plata, K., Backus, C., Reichardt, L., Landis, S., 1999. NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. Dev. Biol. 210, 411–427.

Frossard, N., Freund, V., Advenier, C., 2004. Nerve growth factor and its receptors in asthma and inflammation. Eur. J. Pharmacol. 500, 453–465.

Guidry, G., 1999. A method for counterstaining tissues in conjunction with the glyoxylic acid condensation reaction for detection of biogenic amines. J. Histochem. Cytochem. 47, 261–264.

Guo, X., Metzler-Northrup, J., Lein, P., Rueger, D., Higgins, D., 1997. Leukemia inhibitory factor and ciliary neurotrophic factor regulate dendritic growth in cultures of rat sympathetic neurons. Brain Res. Dev. Brain Res. 104, 101–110.

Han, B.H., Holtzman, D.M., 2000. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J. Neurosci. 20, 5775–5781.

Hoyle, R.H., Kenny, D.A., 1999. Statistical power and tests of mediation. In: Hoyle, R.H. (Ed.), Statistical Strategies for Small Sample Research. Sage, Newbury Park.

Huber, A.B., Kolodkin, A.L., Ginty, D.D., Cloutier, J.F., 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu. Rev. Neurosci. 26, 509–563.

Kabelitz, D., Medzhitov, R., 2007. Innate immunity—cross-talk with adaptive immunity through pattern recognition receptors and cytokines. Curr. Opin. Immunol. 19, 1–3.

Kelley, S.P., Moynihan, J.A., Stevens, S.Y., Grota, L.J., Felten, D.L., 2003. Sympathetic nerve destruction in spleen in murine AIDS. Brain Behav. Immun. 17, 94–109.

Kim, I.J., Beck, H.N., Lein, P.J., Higgins, D., 2002. Interferon gamma induces retrograde dendritic retraction and inhibits synapse formation. J. Neurosci. 22, 4530–4539.

Kohn, J., Aloyz, R.S., Toma, J.G., Haak-Frendscho, M., Miller, F.D., 1999. Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. J. Neurosci. 19, 5393–5408.

Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H., Ginty, D.D., 2004. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118, 243–255.

Leib, D.A., Nadeau, K.C., Rundle, S.A., Schaffer, P.A., 1991. The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc. Natl. Acad. Sci. U S A 88, 48–52.

Levi-Montalcini, R., 1987. The nerve growth factor 35 years later. Science 237, 1154–1162.

Liebl, D.J., Klesse, L.J., Tessarollo, L., Wohlman, T., Parada, L.F., 2000. Loss of brain-derived neurotrophic factor-dependent neural crest-derived sensory neurons in neurotrophin-4 mutant mice. Proc. Natl. Acad. Sci. U S A 97, 2297–2302.

Miller, L.E., Weidler, C., Falk, W., Angele, P., Schaumburger, J., Scholmerich, J., Straub, R.H., 2004. Increased prevalence of semaphorin 3C, a repellent of sympathetic nerve fibers, in the synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 50, 1156–1163.

Miller, R.G., 1986. Beyond ANOVABasics of Applied Statistics. Wiley, New York.

Prosch, S., Wendt, C.E., Reinke, P., Priemer, C., Oppert, M., Kruger, D.H., Volk, H.D., Docke, W.D., 2000. A novel link between stress and human cytomegalovirus (HCMV) infection: sympathetic hyperactivity stimulates HCMV activation. Virology 272, 357–365.

Reichardt, L.F., 2006. Neurotrophin-regulated signalling pathways. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 1545–1564.

Sanders, V.M., 2006. Interdisciplinary research: noradrenergic regulation of adaptive immunity. Brain Behav. Immun. 20, 1–8.

Sanders, V.M., Kasprowicz, D.J., Kohm, A.P., Swanson, M.A., 2001. Neurotransmitter receptors on lymphocytes and other lymphoid cells. In: Ader, R., Felten, D.L., Cohen, M. (Eds.), Psychoneuroimmunology. Academic Press, San Diego, p. 161.

Sloan, E.K., Tarara, R.P., Capitanio, J.P., Cole, S.W., 2006. Enhanced replication of simian immunodeficiency virus adjacent to catecholaminergic varicosities in primate lymph nodes. J. Virol. 80, 4326–4335.

Turgeman, H., Aboud, M., 1998. Evidence that protein kinase A activity is required for the basal and tax-stimulated transcriptional activity of human T-cell leukemia virus type-I long terminal repeat. FEBS Lett. 428, 183–187.

Turnbull, A.V., Rivier, C.L., 1999. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79, 1–71.

Willey, D.E., Trousdale, M.D., Nesburn, A.B., 1984. Reactivation of murine latent HSV infection by epinephrine iontophoresis. Invest. Ophthalmol. Vis. Sci. 25, 945–950.

Woloski, B.M., Smith, E.M., Meyer 3rd, W.J., Fuller, G.M., Blalock, J.E., 1985. Corticotropin-releasing activity of monokines. Science 230, 1035–1037.

Xu, Y., Kulkosky, J., Acheampong, E., Nunnari, G., Sullivan, J., Pomerantz, R.J., 2004. HIV-1-mediated apoptosis of neuronal cells: proximal molecular mechanisms of HIV-1-induced encephalopathy. Proc. Natl. Acad. Sci. USA 101, 7070–7075.

Yazdani, U., Terman, J.R., 2006. The semaphorins. Genome Biol. 7, 211.