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Chronic Binge Alcohol-Associated Differential Brain Region Modulation of Growth Factor Signaling Pathways and Neuroinflammation in Simian Immunodeficiency Virus-Infected Male Macaques

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Abstract

Aims: Microarray analysis of hippocampal tissue from chronic binge alcohol (CBA)-administered, simian immunodeficiency virus (SIV)-infected male macaques identified altered immune response and neurogenesis as potential mechanisms underlying cognitive deficits in macaques. This study investigated the differential brain region associations between markers of neuroinflammation and growth factor signaling with microtubule-associated protein 2 (MAP2) expression.

Methods: Adult male rhesus macaques were administered CBA (13–14 g EtOH/kg/week, $n = 8$) or sucrose (SUC, $n = 7$) beginning 3 months prior to SIV infection and continued until animals reached end-stage disease criteria (3–24 months post infection). Expression of inflammatory cytokines, growth factors, and viral loads were determined in the prefrontal cortex (PFC), caudate (CD), and hippocampus (HP). Brain-derived neurotrophic factor (BDNF) expression and phosphorylation of intracellular kinases downstream of BDNF were investigated in the PFC.

Results: Our results show reduced MAP2 expression in the PFC of longer-surviving, CBA/SIV macaques. BDNF expression was most closely associated with MAP2 expression in the PFC. In the caudate, significant positive associations were observed between MAP2 and BDNF, time to end-stage and set-point viral load and significant negative associations for CBA. In the hippocampus, positive associations were observed between MAP2 and inflammatory cytokines, and negative associations for brain viral load and CBA.

Conclusions: CBA differentially affects growth factor and inflammatory cytokine expression and viral load across brain regions. In the PFC, suppression of growth factor signaling may be an

important neuropathological mechanism, while inflammatory processes may play a more important role in the CD and HP.

INTRODUCTION

People living with HIV (PLWH) have a greater incidence of lifetime history of alcohol use disorder (AUD) compared to the general population (Heaton *et al.*, 2011; Grant *et al.*, 2015). Both current and past alcohol use exacerbate neurodegeneration and cognitive impairment in PLWH (Gongvatana *et al.*, 2014) and are associated with unsuppressed viral load, lower CD4 T-cell counts (Baum *et al.*, 2010), and reduced antiretroviral therapy (ART) adherence (Hendershot *et al.*, 2009). In the combination ART era, the prevalence of HIV-associated dementia (HAD) has declined (Heaton *et al.*, 2010), although neurocognitive deficits in the form of asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) are still prevalent in up to 50% of PLWH (Heaton *et al.*, 2010; Saylor *et al.*, 2016). Moreover, the domains affected have shifted, and a lower percentage of PLWH have deficits in verbal domains, information processing, and motor coordination, but have more learning and executive function impairments (Heaton *et al.*, 2011; Sacktor *et al.*, 2016). These shifts in cognitive deficits may reflect differential vulnerability of specific brain regions to HIV-associated neuropathology.

Studies from our group have shown that chronic binge alcohol (CBA) administration unmasks cognitive impairment in simian immunodeficiency virus (SIV)-infected macaques (Winsauer *et al.*, 2002). Subsequent microarray analysis of hippocampus (HP) demonstrated increased expression of genes involved in immune function and dysregulated gene expression involved in neurogenesis (Maxi *et al.*, 2016), which may result from impaired brain-derived neurotrophic factor (BDNF) signaling. Both alcohol and HIV can suppress neurotrophic factors critical for neuronal growth and survival (Bachis *et al.*, 2012; Boyadjieva and Sarkar, 2013; Fields *et al.*, 2014).

Previous work has suggested brain region differences in neuronal vulnerability to HIV neuropathology (Masliah *et al.*, 1992). In PLWH, inflammation and impaired neurogenesis are associated with hippocampal damage and cognitive impairment (Gongvatana *et al.*, 2014), and hippocampal neuropathology is an independent predictor of cognitive impairment (Moore *et al.*, 2006). The prefrontal cortex (PFC) plays an important role in executive function, decision making, memory, emotion, and language, all of which are commonly impaired in PLWH with HIV-associated neurocognitive disorders (HAND) (Heaton *et al.*, 2011). Inflammation in the caudate (CD) was a common characteristic of HIV encephalitis in the pre-ART era (Ghorpade *et al.*, 2005) and continues to affect PLWH, particularly those who are ART-naïve or older than 50 years of age (Thames *et al.*, 2012).

BDNF is a key growth factor with implications in both AUD and HIV infection (Davis, 2008) and its expression is lower in the PFC, HP, and CD of PLWH with HAND compared to uninfected controls (Bachis *et al.*, 2012). Reduced BDNF expression is associated with neuronal apoptosis in preclinical models of HIV infection (Nossheny *et al.*, 2004), and BDNF overexpression rescues HIV protein gp120-mediated decreases in neurogenesis (Avraham *et al.*, 2014). Moreover, preclinical models show reduced BDNF gene expression and phosphorylated TrkB across multiple brain areas following alcohol administration (Raivio *et al.*, 2012).

Expression of tissue pro-inflammatory cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1 β (IL-1 β), chemokine

ligand 2 (CCL2), and interferon stimulated gene 15 (ISG15) are commonly reported as positive correlates of HAND neuropathology and cognitive deficits (Mankowski *et al.*, 2004; Fields *et al.*, 2014; Festa *et al.*, 2015). Furthermore, their increased expression is thought to precede the onset of neurocognitive impairments in PLWH (Sanna *et al.*, 2017). Similarly, these cytokines have been identified as contributors to alcohol-induced neuropathology (Tiwari and Chopra, 2013). Thus, three brain regions (PFC, HP, and CD) were selected to test the hypothesis that expression of pro-inflammatory cytokine and growth factor genes exhibit a region-specific association with MAP2 (a molecular marker of neurocognitive dysfunction) in brains of non-ART-treated, SIV-infected rhesus macaques.

MATERIALS AND METHODS

Animals

Analysis was performed in tissues collected from previously completed macaque studies. All experiments were approved by the Institutional Animal Care and Use Committee at Tulane National Primate Research Center (TNPRC) in Covington, LA and Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, LA. Experiments adhered to the National Institutes of Health Guidelines for the Care and Use of Experimental Animals (2011). Detailed experimental procedures for alcohol administration, SIV inoculation, clinical evaluation of disease, and criteria for euthanasia are described elsewhere (Bagby *et al.*, 2003; Molina *et al.*, 2006, 2008).

Briefly, Indian origin, male rhesus macaques, approximately 4 years of age at the beginning of the study, were split into sucrose-administered, SIV-infected (SUC/SIV+, $n = 7$) and CBA-administered, SIV-infected (CBA/SIV+, $n = 8$) groups. CBA (13–14 g EtOH/kg/week; 30% w/v EtOH in water) was delivered via an indwelling gastric catheter. Administration began 3 months prior to SIV infection and continued through the study (Bagby *et al.*, 2003; Molina *et al.*, 2008). The chronic binge protocol of alcohol administration used in these studies results in blood alcohol concentrations ranging from 50–60 mmol/L 2 hours post-initiation of the infusion. Three months after CBA administration, macaques were inoculated intravenously with 10,000 times the infective dose of SIVmac251 (provided by Preston Marx, TNPRC). Progression of SIV disease was monitored throughout the study using biochemical, immunological, clinical, and plasma viral kinetic analysis as described elsewhere (Bagby *et al.*, 2003; Molina *et al.*, 2006, 2008). Macaques were euthanized upon reaching end-stage criteria in accordance with the Panel on Euthanasia of the American Veterinary Medical Association, defined as (1) 25% loss of body weight from the maximum weight since assignment to the protocol, (2) major organ failure or medical conditions unresponsive to treatment, (3) surgical complications unresponsive to immediate intervention, (4) complete anorexia for 4 days, or (5) clinical judgment of the attending veterinarian. Macaques in this study that reached end-stage criteria between 3–5 months were designated ‘short infection (SI)’. Those that reached euthanasia criteria between 12–24 months, were designated ‘long infection (LI)’. Four groups of

animals were studied: SUC/SI-SIV+ ($n = 4$), CBA/SI-SIV+ ($n = 5$), SUC/LI-SIV+ ($n = 3$), and CBA/LI-SIV+ ($n = 3$). Whole brains were removed during necropsy, frozen in liquid nitrogen, and stored at -80°C until analysis. Many of the SIV-infected animals had generalized inflammation of the cerebral cortex and meninges. One alcohol-administered animal had multifocal perivascular lymphomatous and granulomatous encephalitis of the gray matter and spongiosis of the hippocampus and the brain of another animal showed giant cell syncytia in cerebral cortex. The animal studies and tissue collection were completed in late 2010 and the molecular analysis was performed in 2015. An additional set of tissue samples from age-matched SIV-infected animals ($n = 5$) was acquired from TNPRC. The animals were not part of the study design, were free from any neuropathology, and served as naïve controls.

Brain region isolation

The PFC, CD, and HP tissue were isolated for qPCR, western blot, and immunohistochemical analyses. The brain regions were isolated by gross dissection using a macaque brain atlas as a guide (Paxinos, 2009). The PFC was isolated by cutting off the most rostral 5 mm of tissue. The CD was removed from resulting ~20 mm thick section of the slice made at the intersection of the inferior arcuate sulcus, the superior arcuate sulcus, and the arcuate sulcus spur, approximately 20 mm caudal from the first cut (Bregma = 0.00). The HP was exposed using a section made through the coronal plane of the brain 20 mm caudal to the previous cut (Bregma = -20.00). Brain tissue sections to be used for immunohistochemical analysis were fixed in 4% paraformaldehyde for 48 hours at room temperature and transferred to 70% ethanol prior to paraffin embedding.

Viral load quantification

RNA was extracted from frozen brain tissue using the RNeasy Universal Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. SIV RNA levels were quantified with TaqMan technology (Life Technologies, Carlsbad, CA, USA) qPCR that targets a conserved region of SIV gag as previously described (Molina *et al.*, 2014). Briefly, quantity of SIV RNA was determined by adding approximately 100 ng of sample RNA to duplicate qPCR amplification assays. The average SIV RNA copy number was then determined and normalized to micrograms of RNA that targets the housekeeping gene, ribosomal protein S13 (RPS13), with validated TaqMan primers and probe. The limit of detection in these assays is 50 copies SIV RNA per microgram of RNA.

Western blot

Frozen, pulverized tissue was prepared for western blot analysis. Samples were homogenized by sonication in lysis buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% SDS, and protease inhibitor cocktail, phosphatase inhibitor cocktails II and III diluted 1:100; Sigma, St. Louis MO, USA), and heated at 95°C for 5 minutes. Protein concentrations were determined using DC Protein Assay (Bio-Rad #5000116, Hercules, CA, USA) and stored at -80°C until use. Protein samples of 20 or 40 μg were subjected to SDS-polyacrylamide gel electrophoresis on 10%, 4–15%, or 10%–20% polyacrylamide gels (Bio-Rad), depending on the molecular weight of the target protein. Low molecular weight proteins were run using a Tris/Tricine/SDS buffer system (Bio-Rad #1610744); other targets were run using Tris/Glycine/SDS buffer system (Bio-Rad #1610772). Following gel electrophoresis, proteins were transferred to polyvinylidene

difluoride membranes (Millipore, #IPVH00010, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk (Bio-Rad, #1706404XTU) for 1 hour, followed by overnight incubation in primary antibody diluted in 5% non-fat milk in 0.1% tween-tris buffered saline (2.5% non-fat milk for phosphorylated proteins) at 4°C . Membranes were washed and incubated in secondary antibody (Cell Signaling Technology, #7074 S, Danvers, MA, USA) (1:10,000) for 1 hour. Following chemiluminescent detection (Millipore, #WBLUF0500 or #WBLUR0500), membranes were stripped for 10 minutes at room temperature (Thermo Fisher Scientific, #46430, Pittsburgh, PA, USA) and reprobed for either total protein levels of phosphorylated proteins or loading control, β -actin. Immunoreactivity was quantified using ImageJ (version 1.49b, NIH) under linear exposure conditions. Values were expressed as a fold-change of the mean control values. Downstream TrkB signaling was analyzed by comparing the relative amounts of phosphorylated signaling proteins AKT, PLC γ , and ERK1/2. Phosphorylated proteins were normalized to total protein (e.g. pTrkB/TrkB). Total protein levels were normalized to β -actin or ERK. See Supplemental Table 1 for specific running conditions of each protein target.

qPCR

qPCR was performed on selected inflammatory and growth factor genes and MAP2 in each brain region. RNA purity and concentration were determined using spectrophotometry (NanoDrop, Wilmington, DE, USA). One microgram of RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, #205313, Valencia, CA, USA). Macaque primers for TNF, IL6, ISG15, MAPK1, IGFBP5, TGFA, CCL2, BDNF, RPS13, and MAP2 were purchased from Qiagen. PCR reaction was performed in a 20 μl reaction volume as duplicates with SybrGreen Fast (Qiagen, #330513) to detect target probe amplification. The relative gene expression was quantified using the $\Delta\Delta\text{Ct}$ method. Target genes were compared to the endogenous control ribosomal protein S13 (RPS13). We have previously shown that RPS13 tissue (i.e. peripheral blood mononuclear cells, muscle, fat, liver, brain) expression is not altered with alcohol treatment in SIV-infected macaques and that it serves as a reliable reference gene for normalization of mRNA (Robichaux *et al.*, 2016). The relative fold change of the gene expression used in the regression models is shown in Supplemental Table 2.

IHC

Paraffin-embedded tissue sections were deparaffinized by three 30-minute incubations in xylene and rehydrated. Antigen retrieval was performed in citrate buffer (pH 6.0) heated in a pressure cooker for 30 minutes and endogenous peroxidase quenched in 3% hydrogen peroxide in methanol for 20 minutes. Slides were washed and blocked in 5% normal goat serum in PBS-BSA 0.1% for 2 hours. The sections were incubated in primary antibody (MAP2 1:200, Cell Signaling Inc. #4542, Danvers, MA, USA) diluted in 0.1% PBS-BSA overnight at room temperature and then for 1 hour in 0.5% (1:200) biotinylated secondary antibody (Vector #PK-6105, Burlingame, CA, USA) in PBS-BSA 0.1% at room temperature. The slides were washed and then incubated in Avidin-Biotin complex (Vector #PK-6105, Burlingame, CA, USA) for 1 hour at room temperature. The slides were developed using Vector NovaRED (Vector #SK-4800, Burlingame, CA, USA) and dehydrated. The slides were cleared in xylene (2×30 minutes) and mounted with Permount (Thermo Fisher Scientific, #SP15-100, Pittsburgh, PA, USA).

Microscopy was performed on Olympus BX51 microscope using an Olympus DP72 camera and cellSens Entry (version 1.2 Build 7533) software. Camera sensitivity was set to ISO1600, white balance was performed on the first slide analyzed, and the same light settings were used for each slide to ensure consistent image conditions. Twenty to one hundred images of the gray matter were taken per slide at 60× magnification. Quantification of the images was performed using ImageJ (version 1.49b, NIH) to determine the percent area of positive staining for each image.

Data analysis

Two-way ANOVA models were used to analyze the associations of CBA and time-to-end-stage disease and their interaction with MAP2 gene expression using GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA). PROC REG in the Statistical Analysis System (SAS, Version 9.4, Cary, NC, USA) was used to construct linear regression models for assessing associations among growth factors, pro-inflammatory genes, clinical factors, and MAP2 gene expression. Regression models for MAP2 expression included predictors for gene expression, plasma and brain tissue viral loads from each brain region, and the grouping variables CBA and time-to-end-stage disease. The all subsets regression modeling approach was used to determine the best combination of factors to explain variability in MAP2 expression levels in each brain region based on the Mallows's Cp selection criterion, and the model R^2 .

RESULTS

Factors that predict PFC MAP2 gene expression across three brain regions

Growth factor, inflammatory cytokine gene expression, and CBA predict MAP2 gene expression in the PFC

A significant interaction between CBA administration and time-to-end-stage disease was observed in relation to PFC MAP2 expression (Figure 1A) ($P < 0.05$, $F = 6.083$, $df = (1, 11)$). In the PFC, the model selected for MAP2 expression included MAPK1, TGFA, ISG15, TNF, IL6, CCL2, BDNF, set point viral load, and CBA (adjusted $r^2 = 0.99$, F -statistic = 177.9, $df = (9, 5)$, $P < 0.0001$) (Table 1). Significant positive associations were observed between MAP2 expression and TGFA, IL6, CCL2, BDNF, and CBA while significant negative associations were observed for the other predictors except TNF.

Growth factors, inflammatory cytokines, and set-point plasma viral load predict MAP2 gene expression in the caudate

A significant interaction between CBA administration and time-to-end-stage disease was observed in relation to CD MAP2 expression (Figure 1B) ($P < 0.05$, $F = 5.153$, $df = (1, 11)$). In the caudate, the model selected for MAP2 expression included IGFBP5, MAPK1, TGFA, IL6, IL1B, BDNF, TES, Set Point viral load (VL) and CBA (adjusted $r^2 = 0.963$, F -statistic = 41.2, $df = (9, 5)$, $P < 0.0004$) (Table 1). Significant positive associations between MAP2 expression and the predictor variables were observed for IGFBP5, MAPK1, BDNF, TES, Set point viral load (VL). Significant negative associations were observed for TGFA, IL6, IL1B, and CBA.

MAPK1, inflammatory cytokines, and brain tissue viral load predict MAP2 gene expression in the HP

A significant effect of CBA was observed in terms of MAP2 expression in the HP (Figure 1C). In the HP, the model selected for MAP2 expression included MAPK1, IL6, Brain VL, and CBA (adjusted $r^2 = 0.959$, F -statistic = 81.8, $df = (4, 10)$, $P < 0.0001$). Significant positive associations between MAP2 expression and MAPK1 and IL6, while significant negative associations were observed for brain VL and CBA (Table 1).

The PFC is susceptible to CBA-associated suppression of BDNF and MAP2 gene expression

Among the significant molecular predictors, BDNF expression had the strongest contribution to MAP2 ($\beta = 1.226$) within the PFC. To explore how CBA in combination with SIV infection leads to cognitive deficits, we used MAP2 as a correlate of cognitive function. Because of the significant effect of CBA and the significant interaction between the expression of BDNF and MAP2 determined by the model, BDNF signaling was selected for further analysis in the subset of longer surviving CBA/SIV+ and SUC/SIV+ animals.

Mean BDNF mRNA expression in CBA/SIV+ animals was two-fold less than that of the mean expression in SUC/SIV+ animals, but this difference was not statistically significant ($z = -1.550$, $P > 0.1$, Figure 2A). BDNF peptide levels did not differ among SIV-infected groups (Figure 2B).

However, CBA administration significantly ($P < 0.05$, $z = -1.964$) decreased TrkB phosphorylation at tyrosine residue 515 (Y515) in the PFC of CBA/SIV+ macaques compared to SUC/SIV+ macaques, with no difference between the two groups in relative levels of the full-length TrkB receptor (TrkB-FL) (Figure 2D and E). The levels of the

Fig. 1. Relative expression of microtubule associated protein 2 (MAP2) in (A) prefrontal cortex (PFC), (B) Caudate (CD), and (C) Hippocampus (HP). Mean (solid lines) and individual values shown as fold-change relative to SIV- (indicated by dashed line) for sucrose- (SUC; black circles) and chronic binge alcohol-treated (CBA; gray circles) reaching end stage after a short period of infection (SI; ~4 months post-SIV infection) or a long period of infection (LI; ~18 months post-SIV infection). Significant interaction by two-way ANOVA ($P < 0.05$) of time-to-end-stage and CBA in PFC and CD.

Table 1. Model of MAP2 expression in prefrontal cortex (PFC), caudate and hippocampus

Model of PFC MAP2 expression				
Model statistics:	Adjusted $r^2 = 0.99$		$P < 0.0001$	
Variable	Beta Coefficient	Std. Error	t-value	P-value
Intercept	0.362	0.0854	4.24	0.0081
MAPK1	-0.244	0.0456	-5.35	0.0031
TGFA	0.171	0.0264	6.51	0.0013
ISG15	-0.004	0.0003	-11.37	<0.0001
TNF	-0.007	0.0030	-2.23	0.0758
IL6	0.083	0.0051	16.09	<0.0001
CCL2	0.002	0.0003	8.66	0.0003
BDNF	1.226	0.0634	19.35	<0.0001
Set Point VL	-0.073	0.0167	-4.39	0.0071
CBA	0.210	0.0307	6.85	0.0010
Model of caudate MAP2 expression				
Model statistics	Adjusted $r^2 = 0.963$		$P = 0.0004$	
Variable	Beta Coefficient	Std. Error	t-value	P-value
Intercept	-1.563	0.234	-6.68	0.0011
IGFBP5	0.851	0.118	7.20	0.0008
TGFA	-1.003	0.344	-2.91	0.0332
IL6	-0.035	0.008	-4.37	0.0072
IL1B	-0.026	0.009	-3.07	0.0277
BDNF	0.314	0.063	4.96	0.0043
TES	0.331	0.078	4.23	0.0082
CBA	-0.289	0.084	-3.44	0.0185
Set point VL	0.239	0.040	6.02	0.0018
Model of hippocampus MAP2 expression				
Model statistics	Adjusted $r^2 = 0.959$		$P < 0.0001$	
Variable	Beta Coefficient	Std. Error	t-value	P-value
Intercept	0.109	0.071	1.53	0.1568
MAPK1	1.044	0.067	15.54	<0.0001
IL6	0.112	0.014	8.03	<0.0001
Brain VL	-0.124	0.014	-8.73	<0.0001
CBA	-0.212	0.050	-4.25	0.0017

VL—viral load; TES—time to end-stage; CBA—chronic binge alcohol.

truncated form of TrkB (TrkB-T) were not different between the SUC/SIV+ and CBA/SIV+ macaques (Figure 2F). There were no differences in PTP1B, a phosphatase that dephosphorylates TrkB receptors (data not shown).

TrkB receptors signal via the kinases AKT, PLC γ , and ERK 44/42. AKT and ERK 44/42 are both activated downstream of TrkB phosphorylation at Y515, while PLC γ is downstream of the Y816 phosphorylation site. A significant reduction in phosphorylated AKT at serine 473 in the PFC of CBA/SIV+ animals compared to SUC/SIV+ animals (Figure 3A) ($P < 0.05$, $z = -1.964$) was detected. Conversely, total AKT was significantly reduced in the PFC of SUC/SIV+ macaques compared to the CBA/SIV+ macaques (Figure 3D) ($P < 0.05$, $z = 1.964$). There was no difference in phosphorylated or total ERK 44/42 between CBA/SIV+ and SUC/SIV+ macaques (Figure 3B and E) or expression of phosphorylated PLC γ (Figure 3C and F).

CBA did not increase markers of apoptosis in PFC

The expression of Bax and Bcl-2 did not differ between the CBA/SIV+ and SUC/SIV+ macaques (Supplemental Figure 1A–B). The ratio of Bax: Bcl-2 as an apoptotic index did not show a significant difference between treatment groups (Supplemental Figure 1C). To complement these findings, the levels of poly (ADP-ribose) polymerase

(PARP) were determined. In the PFC there was no significant difference in PARP levels (Supplemental Figure 1D) ($z = -1.091$, $P > 0.1$) in CBA/SIV+ compared to SUC/SIV+ macaques.

CBA attenuates MAP2 immunoreactivity in PFC

Immunohistochemical analysis to visualize neuronal damage and apoptosis in the PFC did not show significant differences in MAP2 staining of neuronal cell bodies and dendrites between SUC/SIV+ and CBA/SIV+ macaques (Figure 4A–D). However, both SUC/SIV+ and CBA/SIV+ macaques appeared to have lower MAP2 expression than SIV- animals. Statistical analysis of this observation is precluded due to the low number of SIV- tissue samples available ($n = 2$). While there was no difference in the percent area of positive staining, visual analysis of CBA/SIV+ MAP2 staining indicates less dendritic MAP2 immunoreactivity compared to SUC/SIV+ animals.

Brain tissue viral load did not differ between CBA/SIV+ and SUC/SIV+ macaques

Mean plasma viral load of the CBA/SIV+ group was higher than that of SUC/SIV+ macaques at every time point measured, but this difference failed to reach statistical significance (Supplemental Figure 2A). The plasma viral load at set point (Supplemental Figure 2B), calculated as the mean viral load on days 28, 45, 60,

Fig. 2. Brain-derived neurotrophic factor (BDNF) mRNA (A) and protein (B) levels in the prefrontal cortex (PFC) of sucrose (SUC) and chronic binge alcohol (CBA)-treated SIV-infected macaques (SUC/SIV and CBA/SIV respectively). C) Representative western blot from SIV- (C), SUC/SIV+ (S), and CBA/SIV+ (A). Phosphorylated TrkB relative to total TrkB levels (D), full-length TrkB (E), truncated TrkB (F) in the PFC SUC/SIV and CBA/SIV respectively. G) Representative western blots from SIV- (C), SUC/SIV+ (S), and CBA/SIV+ (A). Mean (solid lines) and individual values shown as fold-change relative to SIV- (indicated by dashed line) for (SUC; black circles) and chronic binge alcohol-treated (CBA; gray circles) CBA-treated (gray circles) reaching end stage after long period of infection (~18 months post SIV infection). * $P < 0.05$ compared to SUC/SIV+ by Wilcoxon-Rank sum test ($z = -1.964$).

and 90, was higher in the CBA/SIV+ group compared to the SUC/SIV+ group, but this difference failed to achieve statistical significance. There were no statistically significant differences in PFC viral load (Supplemental Figure 2C) between CBA- and SUC-treated, SIV-infected macaques.

DISCUSSION

This study examined the brain region specificity of associations between growth factor signaling pathways, pro-inflammatory cytokines, and clinical factors in SIV-infected macaques. Our results indicate that MAP2 expression is reduced in the PFC of longer-surviving, CBA-administered, SIV-infected macaques. Furthermore, BDNF expression in this longer-surviving subset was most closely associated with MAP2 expression in the PFC, indicating a link

between the expression of BDNF and MAP2. To test the hypothesis that CBA administration suppresses BDNF signaling, we determined the activation sites of TrkB receptors, downstream kinases, and MAP2 protein expression. CBA administration significantly decreased TrkB and AKT phosphorylation in the PFC but failed to result in increased markers of apoptosis.

MAP2 expression in the PFC was associated with both growth factor gene expression and alcohol use. BDNF signals via the TrkB receptor and plays a key role in neuronal growth, survival, and synaptic plasticity (Reichardt, 2006). Mean BDNF mRNA was twofold less in the CBA/SIV+ compared to SUC/SIV+ macaques and CBA was associated with reduced TrkB Tyr⁵¹⁵ phosphorylation. Additional endogenous TrkB ligands, like neurotrophin-3 or neurotrophin-4/5 could contribute to reduced TrkB phosphorylation, which we speculate may occur primarily in neurons. The

Fig. 3. Levels of phosphoproteins involved in TrkB signaling pAKT/AKT (A), AKT (D), pERK/ERK (B), ERK (E), pPLC/PLC (C), and PLC (F) in the prefrontal cortex (PFC) of sucrose- and chronic binge alcohol-treated macaques (SUC/SIV+ and CBA/SIV+ respectively). Mean (solid lines) and individual values (normalized for β -actin) shown as fold-change relative to SIV- (indicated by dashed line) for (SUC; black circles) and chronic binge alcohol-treated (CBA; gray circles) reaching end stage after long period of infection (~18 months post-SIV infection). G) Representative western blots from SIV- (C), SUC/SIV+ (S), and CBA/SIV+ (A). * $P < 0.05$ compared to SUC/SIV+ by Wilcoxon-Rank sum test ($z = -1.964$).

phosphorylation domains are present in the full-length TrkB (TrkB-FL) receptor, which is expressed primarily on neurons *in vivo* (Frisen *et al.*, 1993) and this phosphorylation site was selected for its specific activation of AKT and ERK 44/42, critical mediators of BDNF/TrkB-mediated neuronal growth and survival (Minichiello, 2009). These outcomes were considered of primary interest based on previous findings in which expression of genes related to neuronal growth and survival were dysregulated in CBA/SIV+ macaques

compared to SUC/SIV+ macaques (Maxi *et al.*, 2016). Consistent with the reduced phosphorylation at TrkB Y515, reduced phosphorylation of the signaling enzyme AKT was detected.

Reduced phosphorylation of AKT is associated with neuronal apoptosis, and activators of AKT reduce neuronal apoptosis via mitochondrial dependent mechanisms (Liu *et al.*, 2007; Wang *et al.*, 2007). Proteins downstream of AKT involved in the mitochondrial apoptotic pathway (Bax and Bcl-2) were investigated in the PFC.

Fig. 4. Microtubule-associated protein 2 (MAP2) immunoreactivity in the prefrontal cortex (PFC) of sucrose- and chronic binge alcohol-treated macaques (SUC/SIV+ and CBA/SIV+ respectively). (A) SIV- MAP2 staining (MAP2 1:200, Cell Signaling Inc. #4542) shows defined staining of neuronal cell bodies (arrows) and dendrites (braces). (B) SUC/SIV+ MAP2 immunoreactivity shows neuronal cell body and dendrite staining, which is fainter than SIV-. (C) CBA/SIV+ MAP2 immunostaining is restricted to neuronal cell bodies. Microscope images acquired with 60x magnification with 10x objective. (D) Quantification of MAP2 staining, mean (solid lines) for SIV- (open circles) SUC (black circles) and (CBA; gray circles) reaching end stage after long period of infection (~18 months post-SIV infection). (E) Representative western blots from SIV- (C), SUC/SIV+ (S), and CBA/SIV+ (A). Mean (solid lines) and individual values (normalized for β -actin) shown as fold-change relative to SIV- (indicated by dashed line) for (SUC; black circles) and chronic binge alcohol-treated (CBA; gray circles) reaching end stage after long period of infection (~18 months post-SIV infection)

No significant differences in Bcl-2 or Bax levels were detected in CBA/SIV+ compared to SUC/SIV+ macaques. However, two of the three CBA/SIV+ macaques had an elevated Bax: Bcl-2 ratio, compared to one out of three SUC/SIV+ macaques. During apoptosis, PARP is cleaved by caspases, and cleaved PARP fragment is a marker of apoptosis. Cleaved PARP was not detected in any of the tissue samples. We believe this may be due to issues related to antibody specificity rather than the complete absence of apoptosis. Full-length PARP levels were unchanged in the PFC of CBA/SIV+ macaques compared to SUC/SIV+ macaques.

Loss of MAP2 is correlated with cognitive impairment (Moore *et al.*, 2006; Levine *et al.*, 2016). Neither immunohistochemistry nor western blot (Figure 4E) showed any significant differences in PFC MAP2 expression between SUC/SIV+ and CBA/SIV+ macaques. However, both methods showed that MAP2 expression in CBA/SIV+ was less than that of SIV-controls. Gutierrez-Vargas *et al.* (2015) demonstrated a link between BDNF/TrkB/MAP2 and cognitive function. We speculate that the decreased TrkB signaling could contribute to previously identified cognitive impairments in CBA/SIV+ macaques (Winsauer *et al.*, 2002).

The MAP2 expression in the CD and HP demonstrated significant associations with growth factors, and inflammatory cytokines

and viral load suggesting these brain regions may be more susceptible to inflammatory damage resulting from ongoing viral replication (Rostasy *et al.*, 1999). Clinical data show increased CD glial metabolites indicative of neuroinflammation (von Giesen *et al.*, 2001) in brains of persons with HIV-associated motor deficits. Overall, the integration of these findings suggests that the mechanisms of neurodegeneration in HIV are brain region-dependent, with subcortical structures at greater risk for inflammatory damage.

Previously we reported increased viral load at set point in CBA/SIV macaques (Bagby *et al.*, 2006) and this could be predictive of disease outcome and cognitive impairments (Bagby *et al.*, 2006; McCombe *et al.*, 2013). In this study, a significant univariate correlation between set point viral load and time-to-end-stage disease was detected, implying that the higher the set point viral load, the less time-to-end-stage disease. In addition, univariate correlations between set-point viral load and ISG15, CCL2, and tissue viral load in the PFC, and set-point viral load, ISG15, tissue viral load in the HP were detected. We speculate these data suggest a relationship between early peripheral viral kinetics and CNS inflammatory changes.

In summary, our results show that CBA interacts with both growth factor and inflammatory cytokine gene expression, as well

as viral load, differentially across brain regions. The strongest association was detected between BDNF and MAP2 in the PFC. The relevance of this association was validated by demonstration that the BDNF signaling pathway was suppressed in the PFC of CBA/SIV macaques. Meanwhile, CD and HP MAP2 expression showed significant negative and positive relationships with inflammatory cytokines and viral load, respectively. These results suggest differential brain region susceptibility to inflammatory, viral, neurotropic, and alcohol insults associated with neurocognitive dysfunction. Specifically, suppression of growth factor expression and signaling may be an important neuropathological mechanism, particularly in the PFC, while inflammatory processes may play a more important role in the CD and HP. Future studies will test the effectiveness of ART in ameliorating neuroinflammation or BDNF signaling deficits in CBA/SIV+ macaques.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Alcohol And Alcoholism* online

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CONFLICT OF INTEREST STATEMENT

None declared.

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