



Tyrosine metabolism during interferon-alpha administration: Association with fatigue and CSF dopamine concentrations

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ABSTRACT

Chronic exposure to interferon (IFN)-alpha, an innate immune cytokine, produces high rates of behavioral disturbances, including depression and fatigue. These effects may be mediated by the actions of IFN-alpha on dopamine (DA) metabolism in the basal ganglia. Diminished conversion of phenylalanine (Phen) to tyrosine (Tyr), the primary amino acid precursor of DA, has been associated with inflammation, and may reflect decreased activity of the enzyme phenylalanine-hydroxylase (PAH). This study investigated the peripheral Phen/Tyr ratio in relation to cerebrospinal fluid (CSF) concentrations of DA and its metabolites in subjects treated with IFN-alpha plus ribavirin for hepatitis C and controls awaiting IFN-alpha therapy. Plasma Phen/Tyr ratios were significantly increased in IFN-alpha-treated subjects ($n = 25$) compared to controls ($n = 9$), and were negatively correlated with CSF DA ($r = -0.59$, $df = 15$, $p < 0.05$) and its metabolite, homovanillic acid ($r = -0.67$, $df = 15$, $p < 0.01$), and positively correlated with fatigue ($r = 0.44$, $df = 23$, $p < .05$) in IFN-alpha-treated patients but not controls. Given the role of tetrahydrobiopterin (BH4) in the PAH conversion of Phen to Tyr, CSF concentrations of BH4 and its inactive oxidized form, dihydrobiopterin (BH2), were examined along with CSF interleukin (IL)-6 in a subset of patients. BH2 concentrations were significantly increased in IFN-alpha-treated patients ($n = 12$) compared to controls ($n = 7$), and decreased CSF BH4 concentrations correlated with increased CSF IL-6 ($r = -0.57$, $df = 12$, $p < 0.05$). These results indicate that IFN-alpha is associated with decreased peripheral conversion of Phen to Tyr, which in turn is associated with reduced DA in the brain as well as fatigue. These alterations may be related to oxidation of BH4 secondary to IFN-alpha-induced activation of a CNS inflammatory response.

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1. Introduction

Activation of the innate immune system has been shown to affect behavior through changes in neurocircuitry and neurotransmitter systems in the brain, mediated in part by effects of inflammatory cytokines on monoamine neurotransmission (Dantzer et al., 2008; Haroon et al., 2012; Miller et al., 2009). Recent evidence indicates that the basal ganglia and dopamine (DA) may be primary targets of inflammatory cytokines leading to cytokine-induced behavioral changes (Capuron et al., 2007, in press; Haroon et al., 2012; Liu and Hong, 2003; Theodore et al., 2006, 2008). The basal ganglia are key subcortical structures regulating

motivation and motor activity (Grace, 2002), and cytokine effects on basal ganglia DA may contribute to the development of depression and fatigue, as well as psychomotor disturbances, in both medically ill and medically healthy subjects.

Numerous studies have reported elevated inflammatory cytokines in depressed individuals (Maes, 1999; Sluzewska, 1999), and patients exposed to increased inflammation during chronic illness experience significantly higher rates of depression and fatigue than the general population (Yirmiya, 2000; Yirmiya et al., 2000, 1999). Evidence that inflammatory cytokines can cause behavioral alterations exists in numerous reports of the neuropsychiatric symptoms induced by chronic administration of the inflammatory cytokine, interferon (IFN)-alpha, used to treat certain cancers and viral infections. Indeed, IFN-alpha produces an array of behavioral disturbances, many of which are consistent with decreases in basal ganglia DA function including anhedonia, fatigue, and psychomotor

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slowing (Bersano et al., 2008; Capuron et al., 2009, 2002; Majer et al., 2008; Sunami et al., 2000). Interestingly, whereas anxiety and some depressive symptoms in IFN- α -treated patients are alleviated by selective serotonin reuptake inhibitor (SSRI) therapy, fatigue and psychomotor retardation are less responsive to SSRIs (Capuron et al., 2002; Morrow et al., 2003; Raison et al., 2005). Of note, fatigue is also one of the primary residual symptoms in SSRI-treated medically healthy depressed patients (Nierenberg et al., 1999; Targum and Fava, 2011), who, as noted above, have been shown to exhibit evidence of increased inflammation. These findings suggest that neurotransmitter systems other than serotonin, such as DA, may be involved in these SSRI-resistant, inflammation-related symptoms, and substantiate further investigation of cytokine effects on DA in the basal ganglia.

Accordingly, recent studies in our laboratory and others have investigated the effects of inflammatory cytokines and innate immune activation on the basal ganglia and DA function. Positron emission tomography (PET) imaging in IFN- α -treated patients revealed increased basal ganglia glucose metabolism (consistent with Parkinson's disease) that correlated with symptoms of fatigue (Capuron et al., 2007). Moreover, functional magnetic resonance imaging (fMRI) demonstrated decreased neural activation to a hedonic reward task during IFN- α treatment (Capuron et al., *in press*). Similarly, lipopolysaccharide (LPS) and typhoid vaccination have been shown to have effects on basal ganglia activity (Brydon et al., 2008; Eisenberger et al., 2010), including decreased ventral striatal activation to a reward task (Eisenberger et al., 2010), suggesting that the effects on basal ganglia function generalize to multiple inflammatory stimuli. In regard to DAergic mechanisms of cytokine effects on the basal ganglia, PET studies have also revealed increased uptake and decreased turnover of [18 F]fluorodopa (FDOPA) in the caudate and putamen of IFN- α -treated patients, both of which correlated with depression and fatigue scores (Capuron et al., *in press*). Additionally, decreased cerebrospinal fluid (CSF) concentrations of the DA metabolite, homovanillic acid (HVA), have been observed in rhesus monkeys that display anhedonic, depressive-like huddling behavior during chronic IFN- α administration (Felger et al., 2007). Together, these findings support the idea that alterations in DA neurotransmission in the basal ganglia during exposure to inflammatory cytokines contribute to the development of depression and fatigue.

Although cytokines may affect multiple aspects of DA neurotransmission, increased FDOPA uptake and decreased DA metabolites during IFN- α administration suggest that chronic exposure to inflammatory cytokines may decrease DA synthesis. One mechanism by which inflammatory cytokines may impact DA synthesis is by reducing the availability of the enzyme co-factor, tetrahydrobiopterin (BH4). BH4 is necessary for phenylalanine (Phen) hydroxylase (PAH) conversion of Phen to tyrosine (Tyr), the primary amino acid precursor of DA, as well as for the activity of Tyr hydroxylase (TH), the rate-limiting enzyme in DA synthesis. BH4 is also a co-factor for nitric oxide synthases (NOS), which convert L-arginine to NO (Cunnington and Channon, 2010). Additionally, BH4 is highly redox-sensitive and is readily oxidized to dihydrobiopterin (BH2) (Landmesser et al., 2003), which can be regenerated to BH4 by dihydrofolate reductase (Cunnington and Channon, 2010; Dumitrescu et al., 2007; Haroon et al., 2012). Although inflammation and cytokines have been shown to induce GTP-cyclohydrolase I, the enzyme necessary for BH4 synthesis, inflammation-induced increases in inducible NOS (iNOS) activity and the production of reactive oxygen and reactive nitrogen species (ROS and RNS) can ultimately lead to decreased BH4 availability (Kitagami et al., 2003; Neurauter et al., 2008b). When BH4 is readily usurped and oxidized to BH2, less BH4 is available for the conversion of Phen to Tyr and Tyr to DA. The Phen/Tyr ratio, as indication of PAH activity and Tyr metabolism, as well as BH4

and BH2 concentrations, can be assessed in blood or CSF, and serve as indirect measures of changes in DA synthesis (Capuron et al., 2011; Hashimoto et al., 2004; Neurauter et al., 2008b).

In the present study, Tyr metabolism in the periphery (the plasma Phen/Tyr ratio) was examined to determine whether it predicted reduced DA and HVA concentrations in the CSF and the development of neuropsychiatric disturbances in IFN- α -treated patients. To identify whether changes in Tyr metabolism and CSF DA/HVA concentrations were related to inflammation-mediated increases in the oxidation of BH4, BH4 (and its inactive form, BH2) was also examined in CSF from a subset of subjects and correlated with CSF IL-6. Increases in circulating IL-6 have been demonstrated to predict development of IFN- α -induced depression (Prather et al., 2009; Wichers et al., 2007, 2006), and we have previously reported IL-6 to be the inflammatory cytokine with the greatest increase in the CSF of IFN- α -treated subjects (Raison et al., 2009). Therefore, IL-6 was chosen for correlation with CSF BH4 because it is a behaviorally relevant marker of immune activation in the CNS.

2. Methods and materials

2.1. Participants

Thirty-seven HCV-positive subjects (19 males, 18 females) were enrolled. Exclusion criteria included decompensated liver disease; liver disease from any cause other than HCV; unstable cardiovascular, endocrinologic, hematologic, renal or neurologic disease (as determined by physical exam and laboratory testing); infection with HIV (as reported by the subjects' treating physician); and history of schizophrenia or bipolar disorder and/or a diagnosis of major depression or substance abuse/dependence within 6 months of study entry (determined by Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders—Fourth Edition) (First et al., 1997). Patients were required to be off all antidepressant, antipsychotic, or mood stabilizer medications for at least 4 weeks prior to study entry (8 weeks for fluoxetine). Subjects were also required to discontinue other agents that might affect study results (i.e., narcotic analgesics, benzodiazepines, and anti-inflammatory agents) at least 2 weeks prior to sample collection. The subjects reported on herein represent a subsample of subjects included in previous studies on effects of IFN- α on cognitive performance, neuroendocrine function, gene expression, and inflammatory responses (Felger et al., 2011, *in press*; Raison et al., 2009, 2010a,b). All subjects provided written informed consent, and study procedures were approved by the Emory University Institutional Review Board.

2.2. Study design

Study participants were enrolled in a longitudinal study examining immune, neuroendocrine, and neuropsychiatric variables at baseline and 12 weeks of either no treatment or treatment with IFN- α /ribavirin. For purposes of this study, plasma ($n = 34$) and CSF ($n = 24$) was obtained between 11–12 weeks from a subset of HCV + patients treated with IFN- α plus ribavirin ($n = 26$) and untreated HCV + patients awaiting IFN- α /ribavirin therapy (control subjects, $n = 11$). All subjects who underwent IFN- α treatment received either pegylated IFN- α -2b (Pegintron, Schering Plough, Kenilworth, NJ; 1.5 μ g/kg) ($n = 15$) or pegylated IFN- α -2a (Pegasys, Roche-Genentech, San Francisco, CA; 180 mg) ($n = 11$) administered subcutaneously plus ribavirin (800–1400 mg/day). Participation in the treatment versus control group was determined by patients and their physicians based on

scheduling constraints and personal preferences and was not based on standardized criteria or controlled by study protocol.

For lumbar puncture (LP), subjects in the 11th week of study participation were admitted to the Emory University General Clinical Research Center (GCRC). Lumbar puncture was performed between 4:00 PM and 5:00 PM by a trained physician. For each subject, ~10 cc of CSF was withdrawn, after discarding the initial 1 cc to avoid blood contamination. Samples were collected into chilled tubes, aliquoted into 1 cc vials, and immediately frozen at -80°C until assay. Subjects were then discharged after an overnight stay. To limit the impact of the stress of CSF sampling on peripheral blood immune parameters and assessments of depression, blood sampling and depression assessments were conducted 6 to 7 days after LP. For blood sampling, subjects in the 12th week of study participation were admitted to the Emory GCRC in the evening with lights out at 10:00 PM. The following morning, subjects were awakened at 7:15 AM and served breakfast, and neuropsychiatric assessments were conducted. For the purpose of amino acid analysis, blood was collected between 10–11:00 into chilled EDTA-coated tubes. Blood was immediately centrifuged at 1000g for 10 min at 4°C , and plasma was removed and frozen at -80°C until assay. During blood sampling, subjects were asked to rest quietly for 30 min prior to blood withdrawal. Because behavioral effects of pegylated IFN-alpha tend to be most pronounced immediately following the weekly injection, both LP and blood sampling were scheduled 4–5 days following each subject's last IFN-alpha injection. Urine drug screens were conducted at all visits to rule out substance abuse. Control subjects participated in all study procedures in parallel with IFN-alpha/ribavirin-treated patients.

2.3. Neuropsychiatric assessments

Neuropsychiatric assessments were conducted at baseline and 12 weeks of study participation. Depression was evaluated using the Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery & Asberg, 1979). The MADRS is a 10-item, clinician-administered scale that assesses the severity of depressive symptoms. Fatigue was assessed using the self-report, 20-item Multidimensional Fatigue Inventory (MFI) (Smets et al., 1995).

2.4. Sample Analysis

IL-6 was measured in duplicate by high sensitivity quantitative enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minnesota). Inter-assay and intra-assay variability were reliably ~12% and assay sensitivity was .04 pg/mL. DA, HVA, and DOPAC were measured in duplicate 20 μL CSF aliquots using high-performance liquid chromatography (HPLC) as described (O'Connor et al., 2008) using an ESA Model 530 Fluorescence detector at +320 mV. The mobile phase (pH = 3.0) consisted of 75 mM NaH_2PO_4 , 25 μM EDTA (disodium salt), 1.7 mM octanesulfonic acid, and 100 $\mu\text{L/L}$ triethylamine in acetonitrile:water (7:93 v:v). CSF was diluted 1:4 with 0.02 N HClO_4 and then samples were centrifuged at 12,000g for 5 min at 4°C . The chromatograms were integrated and quantified using ESA EZ Chrom SI software (ESA Inc., Chelmsford, MA). A standard curve was generated on each day from concentrated standards made up in 0.02 N HClO_4 and held at 4°C until a 20 μL volume was injected into the system. Standards were made using serial dilution to encompass expected levels in the CSF samples. The standard curve was created using the system software, and samples were not run unless a linear standard curve with R^2 greater than 0.98 was achieved. Phen and Tyr concentrations were determined by ion exchange chromatography by ARUP Laboratories (Salt Lake City, UT). To estimate the activity of PAH, the ratio of the substrate Phen versus the concentrations of

the enzyme product Tyr (=Phen/Tyr) was calculated. CSF biopterins (BH4 and its oxidized form, BH2) were measured by HPLC using a differential oxidation method as previously described (Fukushima and Nixon, 1980; Landmesser et al., 2003; Widder et al., 2007). Samples were exposure to 1% I_2 and 2% KI at room temperature for 1 h under dark conditions. Ascorbic acid was added to stop the reaction, and the mixture was then centrifuged for 10 min at 12,000g. Biopterins in the supernatant were quantified by HPLC on a C18 column (5 \times 250 mm, 5 μm) with 5% methanol/95% water as a solvent at a flow rate of 1.0 ml per minute. The fluorescence detector was set at 350 nm for excitation and 450 nm for emission. The amount of BH4 was determined from the difference between the total biopterins and oxidized biopterin, BH2.

2.5. Statistical analysis

Differences between groups were assessed using *t* tests, Chi-squared Tests, or Fisher's Exact Test for categorical variables. *T* tests were also used to examine the effects of treatment (IFN-alpha versus control) on plasma Phen/Tyr ratio and the percent of total biopterin comprised of BH4. Two-way analyses of variance (ANOVA) was used to assess the effects of treatment (IFN-alpha versus control) on total biopterin, BH2, and BH4 concentrations. Normality and equivalence of variance were computed using Kolmogorov-Smirnov and Levene's tests, respectively, and square root transformation procedures were used to achieve normality and equal variance. Post hoc analyses were conducted using Tukey's test. Univariate general linear model was then employed to examine the potential influence of the covariates, age, sex, body mass index (BMI), and history of major depression (hxMD) on significant relationships identified by *t* test or ANOVA. Pearson correlation coefficients were calculated to evaluate associations between the plasma Phen/Tyr ratio and depression and fatigue scores, as well as CSF concentrations of DA and metabolites, during IFN-alpha treatment. Pearson correlation was also used to evaluate relationships between CSF concentrations of BH4 and CSF IL-6, DA, and DA metabolites in all subjects. Where indicated, multivariate analyses (backward and forward multiple linear regression) were performed to assess the potential contribution of age, sex, BMI, and hxMD. All tests of significance were two-tailed with alpha <0.05, and all statistics were conducted using SPSS software (Chicago, IL, USA).

3. Results

3.1. Subject Characteristics

As shown in Table 1, no significant differences between IFN-alpha/ribavirin-treated subjects and controls were observed for rele-

Table 1
Characteristics of study participants.

Characteristic	Control (n = 11)	IFN-alpha (n = 26)	p-Value
Age (mean, SD)	45.4 (7.2)	46.9 (6.2)	0.50
Sex (n, %) Males	5 (45.5)	14 (53.8)	0.73
Race (n, %)			0.69
Caucasian	5 (45.5)	10 (38.5)	
Black	6 (54.5)	13 (50)	
Hispanic	0 (0)	2 (7.7)	
Asian American	0 (0)	1 (3.8)	
past MD (n, %)	0 (0)	7 (26.9)	0.08
Past substance abuse (n, %)	8 (72.7)	18 (69.2)	0.58
Baseline MADRS (mean, SD)	4.5 (5.2)	3.1 (3.6)	0.38
Baseline MFI (mean, SD)	42.8 (16.1)	39.6 (12.8)	0.53
BMI (mean, SD)	29.6 (6.0)	29.8 (4.9)	0.91

BMI, body mass index; MADRS, montgomery-asberg depression rating scale; MD, major depression; MFI, multidimensional fatigue inventory.

vant clinical characteristics including age, race, gender, BMI, and past history of substance abuse. However, there was a trend toward a significant difference between IFN-alpha treated subjects and controls for hxMD. Therefore, hxMD was included as a covariate in all linear regression and general linear models, along with age, sex, and BMI. It should also be noted that there were no differences between IFN-alpha-treated and control subjects in baseline depression (MADRS) and fatigue (MFI) scores. However, IFN-alpha-treated subjects displayed significant increases in depression ($t = 4.79$, $df = 35$, $p < 0.001$) and fatigue ($t = 3.34$, $df = 35$, $p < 0.01$) scores after 11–12 weeks of treatment compared to controls.

3.2. Increased Phen/Tyr ratio was associated with fatigue and decreased CSF HVA and DA during IFN-alpha administration

To evaluate the effect of IFN-alpha administration on the efficiency of PAH conversion of Phen to Tyr in the periphery, the Phen/Tyr ratio was compared in plasma samples from IFN-alpha-treated subjects ($n = 25$) and controls ($n = 9$). The Phen/Tyr ratio was significantly increased in the plasma of IFN-alpha-treated patients at 12 weeks of IFN-alpha/ribavirin administration compared to controls ($t = -2.3$, $df = 33$, $p < 0.05$) (Fig. 1(A)). Age, sex, BMI and hxMD had no significant effect on the observed difference in Phen/Tyr ratio between IFN-alpha-treated patients and controls. To examine whether the increase in plasma Phen/Tyr ratio in IFN-alpha-treated patients was associated with depression or fatigue scores during treatment, the plasma Phen/Tyr ratio was correlated with MADRS and MFI scores at 12 weeks. Increases in the plasma Phen/Tyr ratio were significantly correlated with increases in fatigue scores in IFN-alpha-treated subjects ($r = 0.44$, $df = 23$, $p < 0.05$) (Fig. 1(B)) but not controls ($r = -0.30$, $df = 7$, $p = 0.42$), whereas a trend for an association between Phen/Tyr ratio and MADRS scores ($r = 0.37$, $df = 23$, $p = 0.07$) was observed in these patients but not in controls ($r = -0.34$, $df = 7$, $p = 0.37$). To determine whether peripheral PAH activity was related to central DA and DA metabolite concentrations, plasma Phen/Tyr ratios were correlated with DA and HVA concentrations in CSF samples that were available from these patients at week 11 ($n = 17$). Interestingly, the plasma Phen/Tyr ratio was negatively correlated with CSF HVA ($r = -0.67$, $df = 15$, $p < 0.01$) (Fig. 2(A)) and DA ($r = -0.59$, $df = 15$, $p < 0.05$) (Fig. 2(B)) but not DOPAC ($r = -0.08$, $df = 15$, $p = 0.77$) concentrations during IFN-alpha administration. The covariates age, sex, BMI, and hxMD were entered into separate multiple linear regression analyses that revealed final models for Phen/Tyr ratio as the strongest predictor of MFI scores ($F[1, 23] = 5.51$, $p < 0.05$) and HVA concentrations

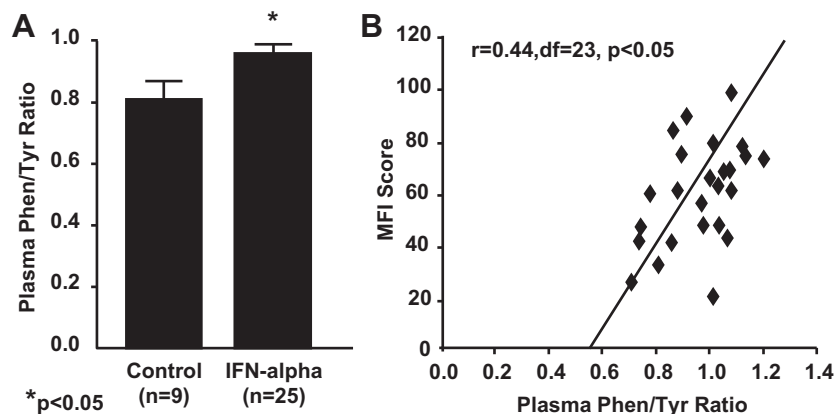


Fig. 1. Increased plasma Phen/Tyr ratio after 12 weeks IFN-alpha administration was correlated with fatigue. The mean plasma Phen/Tyr ratio was significantly increased in subjects administered 12 weeks IFN-alpha therapy ($n = 25$) compared to controls ($n = 9$) (A) Increased Phen/Tyr ratio was positively correlated with the development of fatigue, as measured by MFI, in IFN-alpha-treated subjects ($n = 25$) at 12 weeks (B) IFN-alpha = interferon-alpha, MFI = Multidimensional Fatigue Inventory, Phen = phenylalanine, Tyr = tyrosine. Data are summarized as mean \pm SE, * $p < 0.05$.

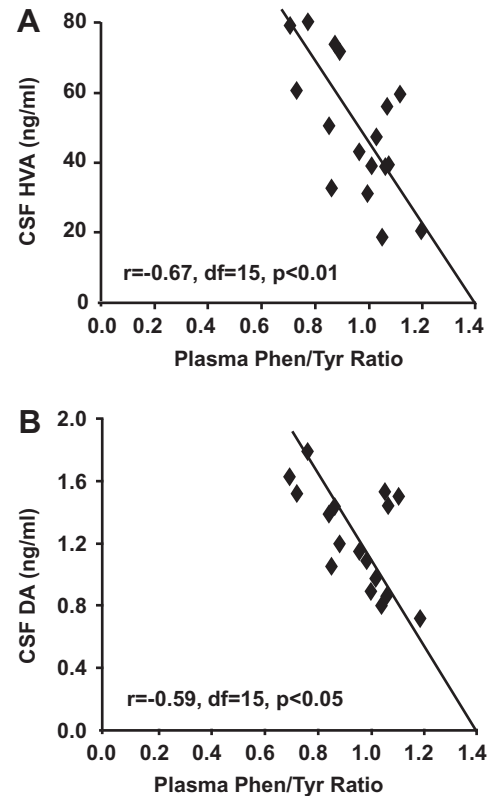


Fig. 2. Increased plasma Phen/Tyr ratio was associated with decreased CSF HVA and DA after 11–12 weeks IFN-alpha administration. Increased plasma Phen/Tyr ratio at 12 weeks was negatively correlated with decreased HVA (A) and DA (B) in the CSF of subjects after 11 weeks of IFN-alpha administration ($n = 17$). CSF = cerebrospinal fluid, DA = dopamine, HVA = homovanillic acid, IFN-alpha = interferon-alpha, Phen = phenylalanine, Tyr = tyrosine.

($F[1, 15] = 11.88$, $p < 0.01$), and the Phen/Tyr ratio and sex as the final predictors of DA concentrations ($F[2, 14] = 14.46$, $p < 0.001$).

3.3. Concentrations of BH2 were increased in the CSF during IFN-alpha administration, and decreased BH4 was correlated with increased CSF IL-6

The concentration of biopterins, BH4 and its oxidized form, BH2, were measured in CSF samples available from a subset of

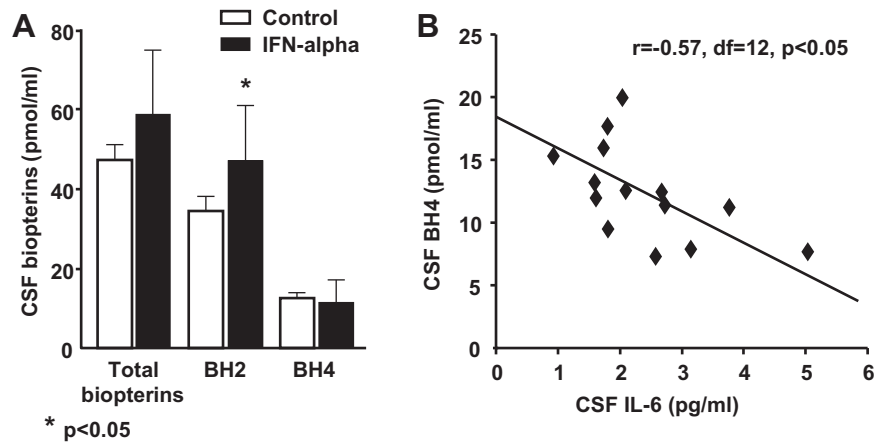


Fig. 3. Increased CSF BH2 was observed after 11 weeks IFN-alpha administration, and decreased CSF BH4 correlated with increased CSF IL-6. A treatment effect was observed for CSF concentrations of total bipterins, BH2, and BH4 in subjects administered 11 weeks IFN-alpha therapy ($n = 12$) compared to controls ($n = 7$), and post hoc tests revealed significantly increased BH2 (A) Decreased CSF BH4 concentrations were negatively correlated with increased CSF IL-6, in a subset of IFN-alpha-treated and control subjects ($n = 14$) (B) BH2 = dihydrobiopterin, BH4 = tetrahydrobiopterin, CSF = cerebrospinal fluid, IFN-alpha = interferon-alpha, IL-6 = interleukin-6. Data are summarized as mean \pm SE, * $p < 0.05$.

IFN-alpha-treated ($n = 12$) and control subjects ($n = 7$), to determine the degree of oxidation of BH4 in the central nervous system. When comparing the total bipterins, BH2, and BH4 concentrations, two-way ANOVA indicated a significant effect of treatment ($F[1, 17] = 4.49, p < 0.05$), and post hoc analysis revealed an increase in CSF BH2 at 11 weeks IFN-alpha/ribavirin treatment compared to control ($p < 0.05$) (Fig. 3(A)). This increase in BH2 also corresponded to a decrease in the percent of total bipterin comprised of BH4 (control mean = 27.4% \pm 3.1 SEM versus IFN-alpha mean = 20.0% \pm 1.6 SEM; $t = 2.31, df = 17, p < 0.05$). When age, sex, BMI and hxMD were entered into models containing treatment and either BH2 or percent BH4, sex was determined to have a potential effect on bipterin concentrations and was therefore examined individually, but did not affect the significance of the treatment effects of IFN-alpha compared to control at $p < 0.05$. Furthermore, the relationship between BH4 availability and CSF DA and HVA concentrations, as well as CSF IL-6 in a subset of patients ($n = 15$), was examined. A trend for CSF BH4 to correlate with decreased HVA was observed for IFN-alpha-treated patients ($r = 0.51, df = 9, p = 0.11$), yet there was no relationship between BH4 and DA ($r = 0.33, df = 9, p = 0.33$). However, increased CSF IL-6 was significantly correlated with decreased BH4 in all patients ($r = -0.57, df = 12, p < 0.05$) (Fig. 3(B)), and a multiple linear regression analysis containing age, sex, BMI, and hxMD revealed a final model with IL-6 as the only significant predictor of BH4 ($F[1, 12] = 5.87, p < 0.05$). Of note, no significant relationships were observed between CSF BH4 concentrations and depression, fatigue, or plasma Phen/Tyr ratio (all $p > 0.40$).

4. Discussion

The findings herein support the idea that the peripheral Phen/Tyr ratio may be a biomarker of decreased central DA synthesis, and that changes in DA metabolism may contribute to IFN-alpha-induced fatigue. The plasma Phen/Tyr ratio was increased in IFN-alpha plus ribavirin-treated subjects compared to controls. Although, the increase in Phen/Tyr ratio in response to IFN-alpha administration reflected a moderate effect size (0.37), this corresponded to $\sim 17\%$ increase in Phen/Tyr ratio. Previous studies have reported similar group differences in Phen/Tyr ratio that have been associated with behavioral alterations and markers of inflammation and oxidative stress (Capuron et al., 2011; Neurauter et al., 2008a,b;

Zangerle et al., 2010). For example, patients with advanced stages of ovarian cancer had $\sim 20\%$ increases in Phen/Tyr ratio that correlated with soluble TNF receptors and neopterin (Neurauter et al., 2008a). In another study examining inflammation and monoamine metabolism in aging, age was associated with increased immune markers and neuropsychiatric symptoms, and increased Phen concentrations and Phen/Tyr ratio were correlated with neurovegetative dimensions of altered sleep and digestive symptoms (Capuron et al., 2011). Therefore, it is reasonable to assume that slight elevations in Phen or the Phen/Tyr ratio during chronic immune activation may confer physiological relevance, particularly in terms of predicting increased oxidative stress, behavioral alterations, and decreased dopamine synthesis. Indeed, a novel finding of the current study is that increased plasma Phen/Tyr ratio correlated with decreased CSF HVA and DA concentrations. Therefore, the plasma Phen/Tyr ratio may be indicative of decreased PAH activity in the periphery that reflects inflammation-associated depletion of DA/metabolites in the central nervous system.

Activity of PAH depends on BH4, and inflammation can oxidize BH4 to inactive species (Cunnington and Channon, 2010; Haroon et al., 2012), thus decreasing its availability and potentially limiting DA synthesis in the CNS. Therefore, CSF concentrations of BH4 and its more oxidized form, BH2, where measured, and BH4 concentrations were correlated with CSF IL-6. Central BH2 concentrations were significantly elevated during IFN-alpha administration, and the percent of total bipterins comprised of BH4 was significantly reduced, indicating potential inflammatory-cytokine-induced stimulation of GTP-cyclohydrolase I activity and BH4 synthesis that is rapidly oxidized to BH2 (Cunnington and Channon, 2010). Interestingly, previous studies have reported similar findings of increased total bipterins and decreased ratio of BH4 to total bipterins in the plasma of patients with major depression (Hashimoto et al., 1990, 1994). In the present study, a significant correlation between decreased CSF BH4 and increased CSF IL-6 concentrations, and a trend for a positive association between decreased CSF BH4 and HVA concentrations, was observed in spite of a small sample size. These data suggest that inflammation-induced BH4 oxidation may be related to decreased DA synthesis in the central nervous system, yet more work is necessary to establish a causal relationship between reduced BH4 availability and changes in DA metabolism and IFN-alpha-induced fatigue.

In terms of the association between plasma Phen/Tyr ratio and behavior, only a trend for an association between the Phen/Tyr

ratio and MADRS scores was observed in this small sample size ($n = 25$), yet the Phen/Tyr ratio was significantly correlated with the development of IFN- α -induced fatigue. These data are consistent with the hypothesis that inflammation-induced decreases in DA synthesis may be specifically associated with changes in basal ganglia function and the development of fatigue symptoms. As discussed previously, the symptom spectrum likely mediated by changes in DA function, such as fatigue, is resistant to treatment with SSRIs (Capuron et al., 2002; Fava et al., 2006; Morrow et al., 2003; Nierenberg et al., 2010; Raison et al., 2005). Surprisingly, these symptoms in medically ill populations have also been difficult to treat with classical stimulant medications, such as amphetamines and DA reuptake inhibitors (Butler et al., 2007; Mar Fan et al., 2008; Moraska et al., 2010; Pucci et al., 2007; Stankoff et al., 2005), indicating cytokine effects on DA synthesis may sabotage the mechanism of action of these agents. Since stimulants act to increase DA release and block DA transporter function, these drugs may not provide long term efficacy if cytokine effects on DA function are primarily mediated through inhibitory effects on synthesis. Therefore, consideration should be given to alternative strategies such as compounds that increase DA synthesis.

Synthesis of DA may be improved by boosting BH4 activity, thus increasing the synthetic capacity of PAH and TH, and there are a number of compounds that can boost BH4 availability. For instance, inflammation-reduced BH4 concentrations can be restored through the salvage pathway with administration of synthetic pterin following conversion by sepiapterin reductase (Cunnington and Channon, 2010; Nichol et al., 1983). Sapropterin (Kuvan) is the first non-dietary, FDA-approved, synthetic form of BH4 for patients with phenylketonuria (PKU) that has been shown in randomized, double-blind trials to be effective in lowering blood phenylalanine levels (Burton et al., 2010). Several other strategies are also currently available to address deficiencies in BH4, including the use of folic acid, L-methylfolate, and S-adenosyl-methionine (SAME), all of which have a role in the synthesis and/or regeneration of BH4 (Miller, 2008a,b; Stahl, 2007). Although BH4 administration has not been studied in the context of depression or fatigue, studies examining folic acid, L-methylfolate, and SAME have been conducted in depression. For example, administration of L-methylfolate (marketed as Deplin and Zervalx) or SAME to depressed patients has been shown to augment the efficacy of standard antidepressant therapy (Ginsberg et al., 2011; Godfrey et al., 1990; Papakostas et al., 2010). Interestingly, low serum folate has been associated with increased risk of depression as well as non-response to antidepressant treatment and an increased likelihood of depression relapse (Fava et al., 1997; Gilbody et al., 2007a,b; Papakostas et al., 2004a,b).

Another strategy to augment inflammation-induced decreases in BH4 is to block the associated ROS and RNS production. One source of inflammation-mediated increases in oxidative stress is through production of neuroactive metabolites of the kynurenine pathway, such as quinolinic acid, via activation of indoleamine 2,3-dioxygenase (IDO) (Behan et al., 1999; Guillemin et al., 2005; Heyes et al., 1993; Santamaria et al., 2003). Indeed, IDO inhibition or genetic deficiency is associated with resistance to behavioral changes following infection or immune activation with LPS in animal models of immune-mediated depressive-like behavior (O'Connor et al., 2009, 2008). Finally, strategies that inhibit inflammation and/or the inflammatory cytokines themselves may, of course, be considered, and administration of the TNF- α antagonist, etanercept, has been shown to inhibit fatigue in patients with advanced cancer (Monk et al., 2006). Nevertheless, further studies are required to determine the most appropriate treatments to target basal ganglia and DA-mediated behavioral symptoms of chronic inflammation.

This study provides strong evidence that plasma Phen/Tyr ratio can serve as a possible predictor of fatigue and decreased DA/metabolites in the CSF, and future studies can examine these biomarkers in other patient populations, such as cancer patients experiencing fatigue secondary to chemotherapy and radiation. However, only a small number of CSF samples were available to investigate central BH4 availability in a subset of IFN- α -treated and control subjects. Therefore, future studies are needed to confirm that increased Phen/Tyr ratio and decreased DA/metabolites are, in fact, the result of inflammation-induced increases in BH4 oxidation.

Some limitations of this study include the small sample size, the lack of availability of CSF samples/data for all patients, and the fact that all of the IFN- α -treated patients were concomitantly treated with ribavirin. However, IFN- α mono-therapy for malignant melanoma has been associated with profound induction of depression and fatigue as well as changes in basal ganglia metabolism (Capuron et al., 2007; Musselman et al., 2001; Raison et al., 2005). Thus, the changes in Tyr metabolism, BH4 availability, and CSF DA/metabolite concentrations observed in this study that were associated with fatigue may be attributed to specific effects of IFN- α . Additionally, the present study was not a randomized experiment in which patients were experimentally allocated to treatment and control groups. Therefore, a trend for the treatment group to have a higher rate of past hxMD was observed, yet controlling for hxMD did not significantly affect observed differences between groups.

In summary, the results of this study indicate that IFN- α -induced neuroinflammation is associated with decreased BH4 availability, and that decreases in peripheral BH4-dependent PAH activity are associated with reduced central DA/metabolites and increased fatigue. These findings provided further evidence of functional effects of immune activation on the basal ganglia and DA function, and justify the use of peripheral Phen/Tyr ratio as an indicator of inflammation-induced decreases in DA synthesis and risk of fatigue. Multiple pharmacological treatment strategies to potentially improve DA synthesis exist, such as compounds to restore BH4 activity, yet future studies are needed to identify the ideal therapies for cytokine-induced fatigue. Further understanding of the mechanisms of inflammatory cytokine effects on the basal ganglia and DA function will enhance our fundamental understanding of neuropsychiatric symptoms, such as fatigue, in both medically ill and medically healthy depressed individuals, and inform new treatments strategies.

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