



## Short Communication

## Early activation of p38 mitogen activated protein kinase is associated with interferon-alpha-induced depression and fatigue

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## ABSTRACT

Cytokine-induced stimulation of p38 mitogen activated protein kinase (MAPK) has been shown to influence behaviorally-relevant pathophysiologic pathways including monoamine neurotransmission and neuroendocrine function and thus may contribute to behavioral changes that occur during chronic administration of the innate immune cytokine, interferon (IFN)-alpha. Accordingly, in the current study, phosphorylation (activation) of intracellular p38 MAPK in peripheral blood lymphocytes was analyzed by flow cytometry every 2 h for 12 h following the initial injection of IFN-alpha in eleven patients with chronic hepatitis C. Hourly assessments of plasma concentrations of adrenocorticotrophic hormone, cortisol and interleukin-6 were also obtained. Symptoms of depression and fatigue were measured at baseline and after 4 and 12 weeks of IFN-alpha treatment. Acute administration of IFN-alpha significantly increased the percentage of lymphocytes staining positive for intracellular phosphorylated p38 (p-p38). IFN-alpha-induced increases in p-p38 were significantly greater in patients that developed clinically significant depressive symptoms [Montgomery–Asberg Depression Rating Scale (MADRS) score  $\geq 15$ ] during the first 12 weeks of IFN-alpha treatment. Increases in the percentage of p-p38-positive lymphocytes following the first IFN-alpha injection also highly correlated with depression severity at weeks 4 ( $r = 0.85$ ,  $p = 0.001$ ) and 12 ( $r = 0.70$ ,  $p = 0.018$ ). Similar relationships were observed for fatigue. Examination of relationships between p-p38 induction and factors previously reported to predict IFN-alpha-induced depressive symptoms revealed strong associations of p-p38 with baseline MADRS ( $r = 0.82$ ,  $p = 0.002$ ) and cortisol responses to the initial injection of IFN-alpha ( $r = 0.91$ ,  $p = 0.000$ ). Taken together, these findings indicate that sensitivity of p38 MAPK signaling pathways to immune stimulation is associated with depressive symptoms during chronic IFN-alpha treatment.

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### 1. Introduction

Activation of the innate immune system has been shown to produce behavioral alterations that are mediated in part by changes in monoamine metabolism and the hypothalamic–pituitary–adrenal (HPA) axis (Dantzer et al., 2008; Miller et al., 2009). For example, administration of the innate immune cytokine, interferon (IFN)-alpha, for infectious diseases and cancer induces high rates of depression and fatigue, and alters both monoamines and HPA axis function. More specifically, IFN-alpha increases cerebrospinal fluid (CSF) cytokines which negatively correlate with CSF concentrations

of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), that, in turn, correlate with depression (Raison et al., 2009). In addition, polymorphisms in the serotonin transporter have been associated with IFN-alpha-induced depression severity (Bull et al., 2009; Lotrich et al., 2009). IFN-alpha has also been shown to inhibit glucocorticoid receptor (GR) function and lead to flattening of the diurnal cortisol curve, which correlates with depression and fatigue (Hu et al., 2009; Raison et al., 2010a).

One mechanism by which innate immune cytokines such as IFN-alpha may affect monoamine metabolism and neuroendocrine function is through activation of p38 mitogen activated protein kinase (MAPK). Activation of p38 MAPK has been shown to increase the activity and expression of the serotonin transporter *in vitro* and *in vivo* (Zhu et al., 2006, 2010). In addition, increased basal p38 MAPK activation in peripheral blood mononuclear cells of abused monkeys was found to correlate with decreased CSF concentrations of 5-HIAA (Sanchez et al., 2007). Cytokine-induced

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activation of p38 MAPK has also been shown to inhibit GR function through blocking translocation of GR from cytoplasm to nucleus (Wang et al., 2004).

To investigate the potential role of p38 MAPK in IFN- $\alpha$ -induced behavioral disturbance, *in vivo* p38 MAPK responses to the initial injection of IFN- $\alpha$  were examined in peripheral blood lymphocytes of patients with chronic hepatitis C virus (HCV) infection. In addition, the relationship between p38 MAPK phosphorylation (activation) and neuroendocrine (HPA axis) and immune (IL-6) responses to the initial injection of IFN- $\alpha$  as well as subsequent depression and fatigue at weeks 4 and 12 of IFN- $\alpha$  treatment were explored.

## 2. Methods and materials

### 2.1. Participants

Eleven HCV-positive subjects 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); and history of schizophrenia or bipolar disorder and/or diagnosis of major depression (MD) 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). Subjects were required to be off all psychotropic medications and prescription medications known to affect the immune system for at least 4 weeks prior to study entry. Complete blood counts were obtained at baseline, and all subjects had white blood cell counts and percentages of lymphocytes, monocytes, and granulocytes within normal limits. All patients received either pegylated IFN- $\alpha$ -2b (Pegintron, Schering Plough, Kenilworth, NJ;  $n = 5$ , 1.5  $\mu\text{g}/\text{kg}$ ) or pegylated IFN- $\alpha$ -2a (Pegasys, Roche-Genentech, San Francisco, CA;  $n = 6$ , 180 mg) administered subcutaneously weekly, plus oral ribavirin (800–1400 mg) daily. The subjects represent a subgroup of patients reported on previously (Raison et al., 2009, 2010a,b).

### 2.2. Study design

Subjects were admitted to the Emory Clinical Interactions Unit the evening prior to blood sampling. Blood was collected into EDTA-coated tubes hourly from 9AM–9PM on two separate days via an indwelling catheter inserted at 8AM. Blood was centrifuged at 1000 $\times$ g for 10 min at 4 °C, and plasma was removed and frozen at –80 °C until assay. For flow cytometry, 200  $\mu\text{l}$  of whole blood was removed prior to plasma isolation. Pegylated IFN- $\alpha$  was administered subcutaneously after the 10AM blood draw on the second day of blood sampling. Ribavirin was not administered on the day of the first IFN- $\alpha$  injection, and was initiated the day after completion of blood sampling. Subsequent administration of IFN- $\alpha$  and ribavirin was supervised by the patients' treating physician. Behavioral assessments were conducted on the first day of blood sampling and at follow-up visits on weeks 4 and 12. Depression was evaluated using the 10-item, clinician-administered Montgomery–Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979), and fatigue was assessed using the self-report, 20-item Multidimensional Fatigue Inventory (MFI) (Smets et al., 1995).

### 2.3. p38 Protocol

Fresh blood was lysed of red blood cells, and the resultant cell pellet was washed, fixed in 2% paraformaldehyde, and permeabilized

with 90% methanol at 4 °C for 30 min. Cells were incubated 1 hour at room temperature with mouse anti-phosphorylated p38 (p-p38) MAPK (T180/Y182) phycoerythrin-conjugated monoclonal antibody clone 36 (BD Biosciences, San Diego, CA), washed and resuspended in 2% paraformaldehyde for flow cytometry assessment (FACSCalibur or LSRII, BD Biosciences) (Sanchez et al., 2007). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Clone 36 and similar monoclonal antibodies to p38 MAPK (T180/Y182) are commonly used in flow cytometry and Western blot analysis to measure p-p38 (Rius et al., 2010; Zampieri et al., 2007). *In vitro* studies were conducted to demonstrate antibody specificity and the impact of IFN- $\alpha$  on intracellular p38 protein and p38 phosphorylation in specific lymphocyte subsets (Supplementary materials).

### 2.4. Hormone and cytokine assays

Commercially available immunoradiometric assay and radioimmunoassay kits were used for assessment of plasma adrenocorticotrophic hormone (ACTH; ALPCO Diagnostics, Salem, NH and Nichols Institute Diagnostics, San Juan Capistrano, CA, when available) and cortisol (DiaSorin, Stillwater, MN), respectively. Intra- and inter-assay coefficients of variation respectively were 2.8% and 5.7% (ALPCO) or 4.5% and 6.3% (Nichols) for ACTH and 8.5% and 12.7% for cortisol. Concentrations of IL-6 were measured in duplicate by high sensitivity quantitative enzyme-linked immunosorbent assays according to manufacturer's specifications (R&D Systems, Minneapolis, MN). Inter- and intra-assay variability for IL-6 was reliably < 12%.

### 2.5. Statistical analysis

Differences between groups were assessed using *t* tests or Fisher's Exact Test for categorical variables. One- and two-way repeated measures analysis of variance on log-transformed data were used to assess change in lymphocyte intracellular p-p38 across time in all subjects and in subjects with a MADRS  $\geq 15$  versus < 15. A MADRS score of 15 has been used as a cut-off score for clinically significant depressive symptoms (Kearns et al., 1982; Potter et al., 2004). Post-hoc comparisons between means of interest were performed using Fisher's Least Significant Difference test. Delta maximum p-p38 MAPK (delta max p-p38) was calculated as the highest percentage of cells staining positive for intracellular p-p38 following IFN- $\alpha$  injection minus the 9AM baseline value. Pearson correlation coefficients were calculated to evaluate associations between delta max p-p38 and depression and fatigue during IFN- $\alpha$  treatment as well as the acute response of ACTH, cortisol and IL-6 to the initial IFN- $\alpha$  injection. For non-normally-distributed data, Spearman correlation coefficients were employed. Where indicated, partial correlation coefficients were determined to control for relevant clinical covariates. All tests of significance were two-tailed with  $\alpha < 0.05$ .

## 3. Results

In the sample as a whole ( $n = 11$ ), IFN- $\alpha$  injection significantly increased the percentage of lymphocytes positive for intracellular p-p38 MAPK compared to the 9AM baseline ( $F = 4.5$ ,  $df = 6, 59$ ,  $p < 0.001$ ), with peak increases being observed 5 h after IFN- $\alpha$  injection (Supplementary materials, Fig. S1). No changes in p-p38 were observed over the diurnal cycle absent of IFN- $\alpha$  administration (data not shown). *In vitro* studies of IFN- $\alpha$ -treated peripheral blood mononuclear cells revealed the majority of lymphocytes positive for p-p38 were CD4+ T cells (Supplementary materials, Fig. S2). Moreover, *in vitro* administration of

IFN- $\alpha$  was not associated with an overall increase in p38 protein as measured by Western blot (Supplementary materials, Fig. S2).

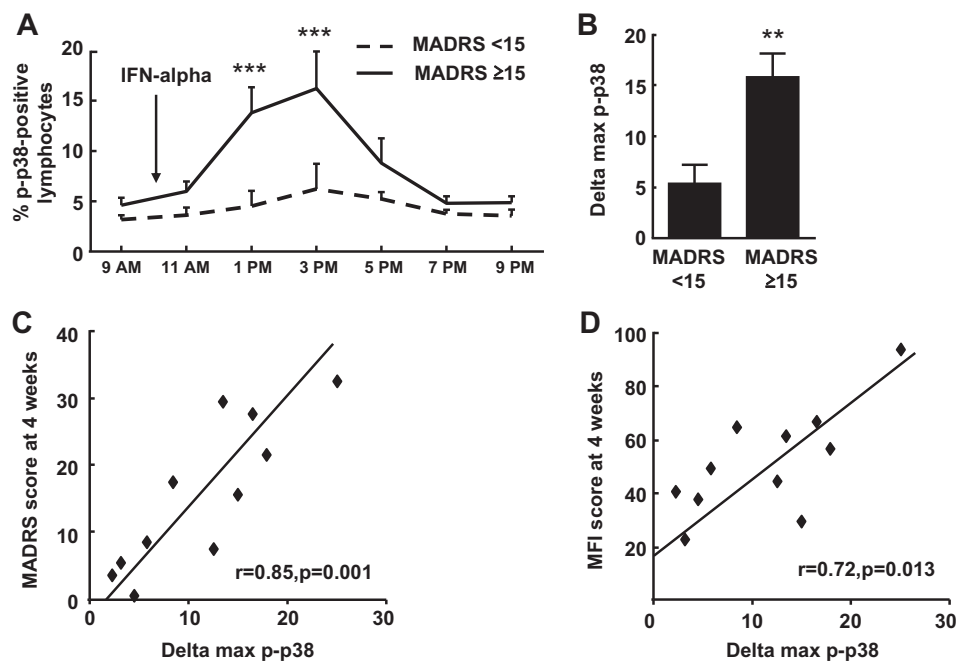
Six of 11 patients experienced significant depressive symptoms (MADRS score  $\geq 15$ ) at some point during the first 12 weeks of IFN- $\alpha$  treatment. No significant differences were found between patients with MADRS scores  $\geq 15$  versus those with MADRS scores  $< 15$  in age [48.0(SD = 4.05) versus 46.8(SD = 7.9),  $p = 0.75$ ], body mass index (BMI) [27.4(SD = 6.0) versus 30.2(SD = 5.2),  $p = 0.44$ ] or race, sex, history of MD, history of substance abuse, or type of IFN- $\alpha$  (Fisher Exact tests, all  $p > 0.50$ ).

Patients who exhibited a MADRS score  $\geq 15$  during the first 12 weeks of IFN- $\alpha$  treatment demonstrated a significantly greater percent of cells positive for intracellular p-p38 MAPK after IFN- $\alpha$  injection than did patients whose MADRS scores were consistently  $< 15$  (time by treatment interaction:  $F = 2.4$ ,  $df = 6,54$ ,  $p < 0.05$ ). Post hoc analyses revealed significant differences between groups at 3 and 5 h post-IFN- $\alpha$  injection (Fig. 1A). The mean maximal change in percent of lymphocytes positive for intracellular p-p38 MAPK (peak response minus 9AM baseline: delta max p-p38) was also significantly higher in patients with MADRS scores  $\geq 15$  versus those  $< 15$  ( $t = 3.2$ ,  $df = 9$ ,  $p < 0.05$ ) (Fig. 1B). Delta max p-p38 was positively correlated with MADRS scores at week 4 ( $r = 0.85$ ,  $df = 9$ ,  $p = 0.001$ ) (Fig. 1C) and 12 ( $r = 0.70$ ,  $df = 9$ ,  $p = 0.018$ ) of IFN- $\alpha$  therapy. Similar correlations were found between delta max p-p38 and MFI scores at week 4 ( $r = 0.72$ ,  $df = 9$ ,  $p = 0.013$ ) (Fig. 1D) and 12 ( $r = 0.74$ ,  $df = 9$ ,  $p = 0.009$ ). Of note, although cut-off scores have not been established for the MFI, individuals with MFI scores above the median ( $> 50$ ) during the first 12 weeks of IFN- $\alpha$  treatment also exhibited a significantly higher percentage of lymphocytes positive for p-p38 at 3 h following IFN- $\alpha$  administration ( $F = 3.1$ ,  $df = 6,54$ ,  $p < 0.05$ ). All correlations between delta max p-p38 and MADRS and MFI remained significant after controlling for age, sex, BMI, history of MD and type of INF- $\alpha$ .

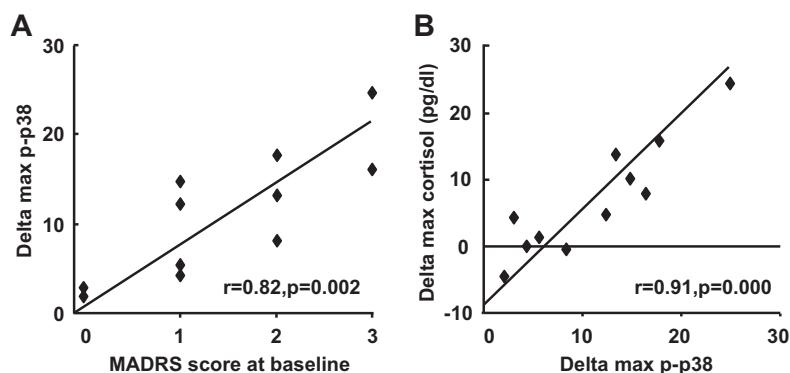
To further understand the relationship between p38 MAPK activation and development of behavioral symptoms during IFN- $\alpha$  treatment, correlations between delta max p-p38 and baseline MADRS and HPA axis responses to the first injection of IFN- $\alpha$  were explored. Both baseline depression scores and ACTH and cortisol responses to the initial injection of IFN- $\alpha$  have been found to predict development of behavioral changes during IFN- $\alpha$  treatment (Capuron et al., 2003; Lotrich et al., 2007). Consistent with previous reports, MADRS at baseline significantly correlated with depressive symptoms at week 4 ( $r = 0.88$ ,  $df = 9$ ,  $p = 0.000$ ) and 12 ( $r = 0.85$ ,  $df = 9$ ,  $p = 0.002$ ), and delta max ACTH and delta max cortisol following the initial injection of IFN- $\alpha$  were positively correlated with MADRS scores at week 4 (ACTH:  $r = 0.69$ ,  $df = 9$ ,  $p = 0.019$ , cortisol:  $r = 0.81$ ,  $df = 9$ ,  $p = 0.003$ ) but not at week 12.

Delta max p-p38 MAPK positively correlated with MADRS scores at baseline ( $r = 0.82$ ,  $df = 9$ ,  $p = 0.002$ ) (Fig. 2A) and delta max cortisol responses ( $r = 0.91$ ,  $df = 9$ ,  $p = 0.000$ ) (Fig. 2B). Both correlations remained significant after controlling for age, sex, BMI, history of MD and type of INF- $\alpha$ . A similar relationship was observed for ACTH ( $r = 0.75$ ,  $df = 9$ ,  $p = 0.008$ ), however this relationship was not significant after controlling for clinical covariates noted above ( $r = 0.73$ ,  $df = 4$ ,  $p = 0.102$ ). No significant relationship was found between delta max p-p38 and delta max IL-6 ( $r = 0.53$ ,  $df = 9$ ,  $p = 0.096$ ), and as previously reported in patients receiving IFN- $\alpha$  for cancer (Capuron et al., 2003), delta max IL-6 did not correlate with MADRS scores at week 4 ( $r = 0.16$ ,  $df = 9$ ,  $p = 0.649$ ) or 12 ( $r = -0.38$ ,  $df = 9$ ,  $p = 0.247$ ).

Given the relationship between baseline MADRS scores and MADRS scores at weeks 4 and 12, correlations between delta max p-p38 and development of depressive symptoms during IFN- $\alpha$  treatment were repeated using delta MADRS scores at week 4 and 12 (thus controlling for baseline). Delta max p-p38 was positively correlated with delta MADRS at week 4 ( $r = 0.84$ ,  $df = 9$ ,  $p = 0.001$ ) and week 12 ( $r = 0.67$ ,  $df = 9$ ,  $p = 0.025$ ) of



**Fig. 1.** Increased p-p38 MAPK in response to the initial IFN- $\alpha$  injection is associated with depression and fatigue during the first 12 weeks of IFN- $\alpha$ . The mean increase in percentage of lymphocytes positive for phosphorylated (p)-p38 MAPK at 3 and 5 h following the initial IFN- $\alpha$  injection was significantly higher in patients with MADRS scores  $\geq 15$  during the first 12 weeks of IFN- $\alpha$  treatment (A). The mean change from the 9AM baseline to the maximal percentage of lymphocytes positive for p38 MAPK (delta max p-p38) was also significantly higher in subjects with MADRS scores  $\geq 15$  versus those with scores  $< 15$  during IFN- $\alpha$  treatment (B). Delta max p-p38 was positively correlated with MADRS scores (C) and MFI scores (D) at week 4 of IFN- $\alpha$  treatment. IFN, interferon; MADRS, Montgomery-Asberg Depression Rating Scale; max, maximal; MFI, Multidimensional Fatigue Inventory; p-p38, phosphorylated-p38. Data are summarized as mean  $\pm$  SE, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Fig. 2.** The response of p-p38 MAPK to the initial IFN- $\alpha$  injection positively correlated with MADRS scores at baseline and the cortisol response to the first IFN- $\alpha$  injection. Delta max phosphorylated (p)-p38 MAPK was positively correlated with the MADRS scores at baseline (A), and with the delta max cortisol response to the initial injection of IFN- $\alpha$  (B). IFN, interferon; MADRS, Montgomery–Asberg Depression Rating Scale; max, maximal; p-p38, phosphorylated-p38.

IFN- $\alpha$  treatment. These relationships remained significant after controlling for clinical covariates noted above.

#### 4. Discussion

Activation of p38 as manifested by percent of lymphocytes positive for intracellular p-p38 following the initial injection of IFN- $\alpha$  was associated with depression and fatigue during IFN- $\alpha$  treatment. In addition, activation of p-38 was significantly correlated with previously reported predictors of IFN- $\alpha$ -induced depressive symptoms including baseline depression scores and the acute HPA axis response to the first IFN- $\alpha$  injection. These data suggest that sensitivity of p38 MAPK signaling pathways to immune stimulation *in vivo* may reflect an immunologic vulnerability to IFN- $\alpha$ -induced depressive symptoms. Moreover, these data are consistent with a potential impact of p38 pathways on behavior through effects on the serotonin transporter (Zhu et al., 2006, 2010) and the GR (Wang et al., 2004), which may contribute to previously described effects of IFN- $\alpha$  on CSF monoamine metabolites and HPA axis function (Raison et al., 2009, 2010a).

Studies have demonstrated that the initial neuroendocrine response to IFN- $\alpha$  administration can predict subsequent behavioral changes. For example, HPA axis responses following the first IFN- $\alpha$  injection have been shown to predict depressive symptom severity during the first several weeks of IFN- $\alpha$  treatment in patients with cancer (Capuron et al., 2003). The present study has replicated these results in a population of subjects with chronic hepatitis C and has extended these findings to include an inflammatory signaling pathway, p38 MAPK, which not only correlated with subsequent behavioral responses but also correlated with baseline neuroendocrine and behavioral parameters known to predict IFN- $\alpha$ -induced behavioral change. The addition of p38 MAPK responses to other known predictors of IFN- $\alpha$ -induced behavioral alterations suggests that sensitization of immune responses may contribute to a pattern of vulnerability to cytokine effects on behavior. Although exaggerated immune and neuroendocrine responses may represent a genetic predisposition, such sensitized responses may also reflect the ability of environmental factors such as stress to prime inflammatory as well as neuroendocrine responses that are then revealed under conditions of immune challenge. Indeed, studies in laboratory animals have shown that exposure to stress can increase the inflammatory response to a subsequent immune stimulus (Gibb et al., 2008; Johnson et al., 2002). The correlation of baseline depression scores and p-p38 after the first IFN- $\alpha$  injection in the current study may reflect such a relationship between exposure to stress (as manifested by

subclinical depressive symptoms) and a heightened inflammatory response to immune challenge.

Sensitization of p38 signaling pathways may be manifested by increased baseline biomarkers of inflammation previously found to predict depressive symptoms during IFN- $\alpha$  treatment. For example, baseline plasma concentrations of IL-6 and its soluble receptor have been found to be associated with depression during IFN- $\alpha$  therapy (Friebe et al., 2007; Prather et al., 2009; Wichers et al., 2006). Nevertheless, the relationship between these baseline immune measures and p-p38 has yet to be established.

Several strengths and weaknesses of the study warrant consideration. Time series data after a standardized immune stimulus allowed for a well-controlled assessment of responsiveness of p38 signaling pathways. Moreover, the longitudinal design provided comprehensive behavioral assessments during the first 3 months of IFN- $\alpha$  treatment in the absence of psychotropic medications. Nevertheless, the sample size was small, limiting generalizability of results and the ability to conduct mediational analysis. In addition, no assessments of activated p38 during IFN- $\alpha$  therapy or viral response were available. Furthermore, we only examined one inflammatory signaling pathway, and it is well-known that IFN- $\alpha$  influences numerous signal transduction pathways that may have influenced behavioral changes. The p38 pathway was chosen based on an established role in neuroendocrine and monoamine regulation (Sanchez et al., 2007; Wang et al., 2004; Zhu et al., 2006, 2010), thus increasing its potential relevance to IFN- $\alpha$ -induced behavioral change. Finally, although p38 activation was measured in the periphery, IFN- $\alpha$  (and other cytokines induced by IFN- $\alpha$ ) can access brain (Raison et al., 2009) and affect inflammatory processes in the CNS. Indeed, both IFN- $\alpha$  (as demonstrated herein) and IL-6 (Heinrich et al., 2003) can activate p38 signaling pathways, and both of these cytokines have been shown to be increased in brain during IFN- $\alpha$  treatment.

Taken together, increased sensitivity of p38 MAPK signaling pathways may represent a vulnerability to IFN- $\alpha$ -induced depression. Thus, p38 pathways may serve as a therapeutic target for reversing neuropsychiatric consequences of chronic exposure to innate immune cytokines.

#### Conflict of interest statement

All authors declare that there are no conflicts of interest, and all financial disclosures are listed for each author: Charles L. Raison serves as a consultant for PamLab LLC and Biolex Therapeutics; Andrew H. Miller has served as a consultant for Abbott Laboratories, AstraZeneca, GlaxoSmithKline, Lundbeck Research USA, F. Hoffmann-La Roche Ltd., Schering-Plough Research Institute and

Wyeth/Pfizer Inc. and has received research support from Centocor Inc., GlaxoSmithKline, and Schering-Plough Research Institute; Jennifer C. Felger, Oyetunde Alagbe, Thaddeus W.W. Pace, Bobbi J Woolwine, and Fang Hu have nothing to declare.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2011.02.015.

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