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**TITLE PAGE**

# Interleukin-6 attenuates serotonin 2A receptor signaling by activating the JAK-STAT pathway

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**RUNNING TITLE PAGE**

IL-6 attenuates 5-$HT_{2A}$ signaling by activating JAK-STAT

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This manuscript is 33 pages long and includes:

1 table

8 figures

42 references

The number of words in each section is as follows:

Abstract 249 words

Introduction 496 words

Discussion 1,537 words

The following non-standard abbreviations are used in this manuscript:

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| | |
|---|---|
| IL-6 | Interleukin 6 |
| 5-$HT_{2A}$ | Serotonin 2A receptor |
| IP | Inositol phosphate |
| JAK | Janus activated kinase |
| STAT | Signal transducer and activator of transcription |
| ERK | Extracellular signal-regulated kinase |

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## ABSTRACT

The serotonin 2A (5-$HT_{2A}$) receptor and the pro-inflammatory cytokine, interleukin-6 (IL-6), have both been implicated in psychiatric disorders. Previously, we demonstrated that these molecules both facilitate cognitive flexibility, a prefrontal cortex-mediated executive function impaired in multiple mental illnesses. In this study, we tested the hypothesis that IL-6 influences 5-$HT_{2A}$ receptor signaling, providing a potential mechanism by which this cytokine may influence behavior. We first demonstrated that 5-$HT_{2A}$ receptors and IL-6-mediated STAT3 phosphorylation co-localize in cells of the prefrontal cortex, providing the neuroanatomical substrate for a potential interaction. In the neuronally derived A1A1 cell line, which expresses both IL-6 and 5-$HT_{2A}$ receptors, we found that IL-6 attenuates inositol phosphate (IP) accumulation in response to the 5-$HT_2$ agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), suggesting that IL-6 can regulate 5-$HT_{2A}$ receptor function. To identify the signaling pathway(s) that mediate this effect, we measured DOI-mediated IP accumulation in the presence of IL-6 and either the JAK-STAT inhibitor, JSI-124, or the ERK inhibitor, PD-98059. The IL-6 effect was blocked by JSI-124, but not PD-98059. Further, siRNA knockdown of either JAK or STAT blocked the IL-6 effect, suggesting that IL-6-induced JAK-STAT activation can regulate 5-$HT_{2A}$ receptor signaling. Finally, to determine if IL-6 specifically regulates the 5-$HT_{2A}$ receptor system, we measured IP production mediated by another $G_q$-coupled receptor, bradykinin B2. IL-6 had no effect on bradykinin-mediated IP accumulation, suggesting that regulation may occur at the 5-$HT_{2A}$ receptor. These results may provide clues to the pathological mechanisms underlying certain psychiatric disorders and may suggest novel therapeutic strategies for their treatment.

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## INTRODUCTION

The serotonin 2A (5-$HT_{2A}$) receptor is a G-protein coupled receptor that is widely distributed throughout the cortex (Pompeiano et al., 1994; Willins et al., 1997). 5-$HT_{2A}$ receptors couple to multiple intracellular signaling cascades (Berg et al., 1998; Urban et al., 2007), however, $G_{q/11}$ protein-mediated activation of phospholipase C is perhaps the best characterized pathway (Leysen, 2004; Nichols and Nichols, 2008). The 5-$HT_{2A}$ receptor is thought to play an important role in regulating mood and cognition, and has been implicated in the pathology of several psychiatric disorders (Carr and Lucki, 2011; Gray and Roth, 2001). In humans, depression and schizophrenia have been associated with increased expression of 5-$HT_{2A}$ receptors in the brain (Gurevich and Joyce, 1997; Shelton et al., 2009) and effective treatment strategies have been shown to decrease 5-$HT_{2A}$ receptor expression and/or function (Gray and Roth, 2001). For example, atypical antipsychotics are thought to exert their therapeutic benefit, in part, by acting as inverse agonists that reduce 5-$HT_{2A}$ receptor constitutive activity (Meltzer, 2012). Thus, understanding the functional regulation of this receptor could have important implications for improved treatment of schizophrenia and other psychiatric disorders.

Recently, the pleiotropic cytokine, interleukin-6 (IL-6), has also received attention for its potential role in cognitive function and mental illness (Dowlati et al., 2010; Gimeno et al., 2009). IL-6 signaling is initiated by the formation of a hexameric complex comprised of two molecules of IL-6 each bound to a non-signaling IL-6 receptor α and the trans-membrane signal-transducing glycoprotein (gp130) receptor. This receptor complex primarily activates the janus activated kinase/signal transducer and activator of transcription (JAK-STAT) pathway. JAK proteins associated with the gp130 receptor

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phosphorylate STATs, which then dimerize and move to the nucleus where they can activate transcription. Alternatively, IL-6 receptor activation can also initiate the extracellular signal-regulated kinase (ERK) cascade through Ras/Raf activation (Heinrich et al., 2003).

Cognitive flexibility, the adaptive ability to modify behavior in the face of environmental change, is impaired in a variety of psychiatric disorders, including depression and schizophrenia (Kehagia et al., 2010). Cognitive inflexibility may also underlie other symptoms, including alterations in mood, and targeting this deficit is the basis for cognitive behavioral therapy, one of the most effective and enduring treatment strategies for mood and anxiety disorders (Beck, 2008). We have demonstrated that both serotonin, acting specifically at the 5-$HT_{2A}$ receptor, and IL-6 can facilitate reversal learning, a form of cognitive flexibility, in the orbitofrontal cortex (OFC) (Donegan et al., 2014; Furr et al., 2011). However, it is unknown whether these signaling molecules act independently in the OFC, or interact with each other, either sequentially or convergently, to regulate behavior. To address this question here, we first determined if IL-6-induced JAK-STAT activation in the OFC of rats occurred in cells that also co-express 5-$HT_{2A}$ receptors, providing a neuroanatomical substrate for potential interaction. Then, using a cell line derived from rat embryonic cortex (Berg et al., 1994), we tested the hypothesis that the IL-6 and 5-$HT_{2A}$ receptor signaling pathways interact to produce a functionally distinct signaling outcome.

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## MATERIALS AND METHODS

**Drugs and Reagents.** The JAK inhibitor, JSI-124 (Indofine Chemical, Hillsborough, NJ) was prepared as a 19.4 mM stock in 100% EtOH. The ERK inhibitor, PD-98059 (Tocris Biosciences, Bristol, UK) was dissolved in 100% EtOH at a concentration of 5mM. IL-6 (R&D Systems, Minneapolis, MN) was received in a stock concentration of 0.25 µg/µl in sodium acetate. DOI-HCl ((-)-1-(2.5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; Sigma, St. Louis, MO) and bradykinin (Sigma) were dissolved in water at 0.1 M concentration. ON-TARGETplus silencing constructs (GE Dharmacon; Table 1) arrived in dried pellet form, and were resuspended with 1x siRNA buffer to a 20µM stock. Stock solutions were stored in aliquots at -20°C. Experimental working solutions were diluted from stocks using 200 mM LiCl as the vehicle.

**Immunohistochemistry**. Male Sprague-Dawley rats were infected by local bilateral microinjection into the OFC of an adeno-associated viral vector, serotype 2/5, containing a constitutively-expressed functional IL-6 construct (AAV-IL6), to produce stable over-expression of IL-6 selectively in neurons in the OFC. We have shown previously that this method of IL-6 over-expression results in elevated STAT3 phosphorylation. (Donegan et al., 2014). Three weeks after infection, rats were anesthetized and perfused transcardially with 4% paraformaldehyde. Dual-fluorescence immunohistochemistry was performed sequentially on free-floating 40 µm coronal sections through the OFC. Sections were first incubated for 30 min in 3% $H_2O_2$ in phosphate buffered saline (PBS), washed (PBS containing 0.3% Triton X-100), and blocked for 1 hr in PBS containing 0.3% Triton X-100, 5% normal goat serum, and 1% BSA. Sections were incubated with a rabbit anti-5-$HT_{2A}$ receptor antibody (1:20,000,

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AbCam, Cambridge, UK) at 4°C overnight. After incubation with biotinylated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, UK), sections were incubated with an avidin-peroxidase conjugate (Vectastain ABC, Vector Laboratories, Burlingame, CA) then Tyramide Signal Amplification Cy3 reagent (Perkin Elmer, Waltham, MA). For subsequent antigen retrieval and peroxidase quenching, sections were incubated in 3% $H_2O_2$ in methanol for 10 min at -20°C, then boiled in 10 mM citric acid (pH 6.0) for 5 min. Sections were then blocked in PBS containing 0.3% Triton X-100, 5% normal sheep serum, and 1% BSA before incubating with the second primary antibody, mouse anti-phospho-STAT3 (Tyr705, 1:500, Cell Signaling, Danvers, MA) at 4°C overnight. Sections were washed, then incubated with a biotinylated anti-mouse secondary antibody (Sigma), avidin-peroxidase conjugate, and Tyramide Signal Amplification fluorescein reagent. Sections were mounted on gelatin-coated slides, coverslipped with Vectashield mounting media (Vector Labs), then imaged using an Olympus IX81 Motorized Inverted confocal microscope. Digital images were acquired using FV10-ASW software and enhanced using ImageJ. To quantify the number of cells expressing each label, 3 OFC sections from each of 3 rats were imaged for a total of 9 measurements. For each section, the OFC was defined as a triangular area with its vertices at the rhinal sulcus, the medial boundary of the forceps minor, and lateral edge of the forceps minor. A standard field of view (200 µm x 200 µm) was positioned within the center of this area. The total number of cells labeled by each marker were counted within this field, and the percentages exhibiting co-expression were calculated.

**Cell Culture.** A1A1 cells were originally derived from cortical cells cultured from embryonic day 16 rats and express nestin, a marker for neuronal precursor cells (Berg

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et al., 1994). Cells were maintained in 5% $CO_2$ at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). For each experiment, cells were seeded on poly-$_L$-ornithine-coated plates. Twenty-four hr prior to each experiment, the DMEM was replaced with serum-free media (DMEM/F12, Invitrogen) as described previously (Berg et al., 1994).

**Western Blots.** Cell lysates were prepared in RIPA Buffer (50mM Tris, 150mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS, 1% Nonident-P40) containing protease and phosphatase inhibitors. Protein content was determined using the Bradford Assay and equal amounts of protein were subjected to SDS-PAGE then transferred to PVDF membrane (Immobilon P, Millipore, Billerica, MA). Primary antibodies (Cell Signaling) were rabbit anti-phospho-STAT3 (Tyr 705; 1:10,000) and mouse anti-phospho-ERK1/2 (Thr202/Tyr204; 1:10,000). After incubation with an HRP-linked secondary antibody (Cell Signaling) and Prime ECL detection reagent (GE Healthcare), blots were stripped and re-probed with a mouse anti-STAT3 antibody (sc-8019; 1:5,000, Santa Cruz, Dallas, TX) or rabbit anti-ERK1/2 antibody (sc-94; 1:50,000, Santa Cruz), to normalize the corresponding phospho-protein signals.

**Inositol Phosphate (IP) Accumulation.** Measurement of IP accumulation was used as an index of phospholipase C activity as described previously (Berg et al., 1994). Briefly, A1A1 cells, seeded at a density of 70,000 cells per well in a 24 well dish, were incubated with 1 µCi/ml [$^3$H]*myo*-inositol (Perkin-Elmer) in serum free medium for 24 hr prior to the experiment. In the silencing RNA experiments, ON-TARGETplus siRNA constructs (25-50nM) were added 24 hr prior to the [$^3$H]*myo*-inositol, amounting to 48 hr

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total transfection time. Before the assay, cells were washed 3 times in HBSS with 20 mM HEPES. Cells were incubated with varied concentrations of DOI or bradykinin (100 nM) along with 20 mM LiCl at 37°C for 25 min. IL-6 (50 ng/ml) was added to the cells 15 min prior to either DOI or bradykinin incubation. When inhibitors of JAK/STAT or ERK were used, they were added 1 hr before IL-6. To terminate IP production, media were aspirated and cells were incubated on ice with 1 ml 10 mM formic acid for at least 20 min. Total IP (inositol monophosphate, inositol bisphosphate, inositol trisphosphate) in the formic acid extracts were isolated by ion exchange chromatography. The tritium content of eluted column fractions was measured by liquid scintillation counting (Beckman Coulter, Indianopolis, IN).

**Analyses.** In all figures, data are shown as mean ± S.E.M. and *n* is indicated in the figure legend. In the western blot experiments, protein phosphorylation was analyzed by one-way ANOVA. When main effects were significant, Dunnett's *post hoc* test was used. Concentration-response data for DOI-mediated IP accumulation were fit to a logistic equation using nonlinear regression analysis to provide estimates of maximal response ($E_{max}$), and potency ($EC_{50}$) using Prism software (GraphPad Software, Inc, San Diego, CA). Statistical differences in the $EC_{50}$ or $E_{max}$ parameters between groups were analyzed with Student's paired *t* test or by two-way ANOVA and Bonferroni *post hoc* test when interactions were significant. In the siRNA and bradykinin experiments, results were analyzed by paired t-test. All tests were two-tailed, and significance was determined at $p<0.05$.

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## RESULTS

**5-HT$_{2A}$ receptors and IL-6-induced phospho-STAT3 are co-localized in the rat OFC.** We have shown previously that both 5-HT$_{2A}$ receptor- and IL-6-mediated signaling in the orbitofrontal cortex (OFC) can facilitate reversal learning (Donegan et al., 2014; Furr et al., 2011). To determine if these signaling mechanisms exist in the same cells of the OFC, we used dual fluorescence immunohistochemistry to label 5-HT$_{2A}$ receptors and IL-6-induced STAT3 phosphorylation in rat OFC tissue. To induce STAT3 phosphorylation, we used an adeno-associated viral vector to produce stable IL-6 over-expression (Donegan et al., 2014). We found that essentially 100% of 5-HT$_{2A}$ receptor-positive cells in the OFC also showed STAT3 phosphorylation in response to overexpressed IL-6 (Figure 1). By contrast, only a subset of phospho-STAT3-positive cells, approximately 20%, also expressed 5-HT$_{2A}$ receptors.

**IL-6 induces STAT3 and ERK1/2 phosphorylation in a time- and concentration-dependent manner in A1A1 cells.** The A1A1 neuronal cell line expresses the 5-HT$_{2A}$ receptor (Berg et al., 1994), providing an excellent model to examine the potential interaction between 5-HT$_{2A}$ and IL-6 signaling. To characterize IL-6 signaling in A1A1 cells, we first established the time course for IL-6-mediated responses. Cells were treated with a concentration of IL-6 (100 ng/ml), which has been shown to produce robust JAK-STAT activation (Yasukawa et al., 2003), for 5, 15, 30, 60 or 120 min before western blot analysis to determine phosphorylation of STAT3 and ERK1/2 as an index of activation. IL-6 significantly increased phosphorylation of both STAT3 ($F_{(5,17)}$=48.58, $p<0.0001$) and ERK1/2 ($F_{(5,17)}$=6.28, $p<0.01$), indicating activation of both kinases.

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However, the effect of IL-6 on STAT3 and ERK1/2 phosphorylation was time-dependent. STAT3 phosphorylation (Figure 2A) was significantly increased over basal levels after 5, 15, or 20 min while ERK 1/2 phosphorylation (Figure 3A) was only elevated following a 15 min incubation period with IL-6. Therefore, in subsequent experiments we treated cells with IL-6 for 15 min.

We next evaluated the concentration dependence for IL-6 to activate either STAT3 or ERK1/2. As shown in figures 2B and 3B, we found that phosphorylation of both STAT3 and ERK1/2 was increased with increasing concentrations of IL-6 and maximal activation of both kinases occurred with 50 ng/ml IL-6 (for STAT3: $F_{(7,23)}$=12.49, p<0.0001; 2B; for ERK1/2: $F_{(7,15)}$=8.25, p<0.01. The $pEC_{50}$ for IL-6 was 8.25 ± 0.19 (5.6nM) for phospho-STAT3 and 8.49 ± 0.20 (3.2nM) for phospho-ERK1/2 and the Emax was 1 ± 0.08% and 12 ± 0.7% above basal for phospho-STAT3 and phospho-ERK1/2, respectively.

**IL-6-induced JAK-STAT activity attenuates DOI-induced IP accumulation in A1A1 cells.** To determine if IL-6 modulates 5-$HT_{2A}$ receptor signaling in A1A1 cells, we measured inositol phosphate (IP) accumulation in response to DOI. DOI is a well characterized, highly selective 5-$HT_2$ receptor agonist. In most cell lines expressing 5-$HT_2$ receptors, DOI has been found to have partial agonist activity relative to 5-HT (Berg et al., 1998). DOI produced a concentration-dependent increase in IP accumulation that was attenuated in the presence of a maximal concentration (50 ng/ml) of IL-6 (Figure 4A). The Emax for DOI was 46 ± 3% above basal and the DOI $pEC_{50}$ was 7.43 ± 0.17 (230 nM; n=32) in cells pretreated with vehicle. In the presence of IL-6, the maximal

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response to DOI was reduced to 25 ± 4% above basal (for $E_{max}$ $t_{(30)}$=4.653, p<0.0001; n=31. In these experiments basal IP accumulation was not significantly affected by IL-6 (500 ± 21 dpm without IL-6 and 541 ± 24 dpm in the presence of IL-6). Importantly, the $EC_{50}$ for DOI was unchanged following incubation with IL-6, suggesting that IL-6 may regulate the efficacy, but not the affinity, of agonists acting at 5-$HT_{2A}$ receptors. DOI alone did not activate JAK/STAT in our experiments (data not shown).

In the next set of experiments, we identified the signaling pathway responsible for the IL-6 effect on 5-$HT_{2A}$ signaling using the JAK-STAT inhibitor, JSI-124, JAK and STAT silencing RNA (siRNA) constructs, or the MEK inhibitor, PD-98059. First, we tested the ability of the inhibitors to block IL-6-induced signaling. We found that 50 μM JSI-124 was sufficient to prevent IL-6-induced STAT3 phosphorylation (50 ng/ml IL-6; $F_{(2,6)}$=23.25, p<0.01; Figure 5A). As shown in Figure 5B, this concentration of JSI-124 completely abolished the effect of IL-6 on DOI-induced IP accumulation. As shown before, the maximal response to DOI was reduced from 43 ± 4% under control conditions to 22 ± 5% above basal in cells incubated with IL-6. However, after pretreatment with JSI-124, IL-6 had no effect on the maximal response to DOI (45 ± 6% above basal vs 46 ± 5% above basal; JSI-124: $F_{(1,43)}$=7.418, p<0.01; IL-6: $F_{(1,43)}$=4.051, p=0.0503; Interaction: $F_{(1,43)}$=4.657, p<0.05; n=12). In these experiments basal IP accumulation was 492 ± 29 dpm for vehicle-treated cells and 534 ± 34 dpm for IL-6-treated cells. The presence of JSI-124 had no significant effect on basal IP accumulation in cells treated with or without IL-6. Pretreatment with JSI-124 alone did not alter either the Emax or EC50 for DOI-mediated IP accumulation.

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Next, we used siRNA (Table 1) to determine the role of specific JAK and STAT isoforms in IL-6-mediated regulation of the 5-$HT_{2A}$ receptor. JAK1, JAK2 and STAT3 are all expressed in the brain and preferentially respond to IL-6 (De-Fraja et al., 1998) (Stahl et al., 1995). Using siRNA targeting these isoforms, we demonstrated a robust knock-down of the target proteins. JAK1 siRNA (25nM) produced a 97% knock-down, JAK2 siRNA (50nM) produced a 77% knock-down, and STAT3 siRNA (25nM) produced a 93% knock-down of protein compared to cells treated with a non-targeting siRNA pool (scRNA; 25nM; Figure 6A). As demonstrated previously, in the presence of scRNA, IL-6 attenuated DOI-mediated IP production, reducing the maximal response from 37 ± 2% to 25 ± 5% over basal. Consistent with the JSI-124 results, the effect of IL-6 was completely abolished in the presence of all three siRNA constructs (for JAK1, effect of DOI without IL-6: 60 ± 13%; effect in the presence of IL-6: 70 ± 13%; for JAK2, effect of DOI without IL-6: 27 ± 3%, effect with IL-6: 20 ± 5%; and for STAT3, DOI without IL-6: 33 ± 10%, with IL-6: 47 ± 12%; Figure 6B). In these experiments, basal IP levels under control conditions were 944 ± 129 dpm and were not significantly altered by either IL-6 or any of the siRNA constructs tested.

We also tested the effect of the ERK 1/2 inhibitor PD-98059 on both IL-6 mediated activation of ERK and the reduction in DOI-mediated IP accumulation. We found that although 50 μM PD-98059 was sufficient to block IL-6-induced ERK activation (50 ng/ml IL-6; $F_{(2,6)}$=15.14, $p<0.05$; Figure 7A), this concentration of PD-98059, had no effect on the IL-6-mediated reduction of DOI-induced IP accumulation (Figure 7B). The Emax for DOI was reduced from 51 ± 12% above basal to 29 ± 9% above basal following IL-6 pretreatment alone and from 54 ± 7% above basal to 34 ±

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8% in the presence of IL-6 and the ERK inhibitor, PD-98059 (IL-6: $F_{(1,27)}$=5.3, $p<0.05$; n=8). In these experiments basal IP accumulation was 550 ± 22 dpm for vehicle-treated cells and was not significantly altered by IL-6 treatment with or without pretreatment with PD-98059. Further, the EC50 for DOI was not altered by IL-6 in either the presence or absence of PD-98059. Together, these results suggest that IL-6 acts through the JAK-STAT pathway to modulate 5-$HT_{2A}$ receptor signaling.

**IL-6 does not affect bradykinin-induced IP accumulation.** To determine if the effect of IL-6 is specific to the 5-$HT_{2A}$ receptor system, or if it generalizes to other $G_q$-coupled receptor systems in A1A1 cells, bradykinin (BK) B2 receptor-mediated IP accumulation was measured in the presence or absence of IL-6. There was no difference in BK-mediated IP accumulation after incubation with IL-6 (Figure 8). BK produced a 188 ± 7% above basal increase in IP accumulation in the absence of IL-6, and a 180 ± 11% increase above basal IP accumulation when IL-6 was present ($t_{(7)}$=0.5197, $p>0.05$; n=8), suggesting that IL-6 specifically modulates signaling by the 5-$HT_{2A}$ receptor system. In these experiments basal IP accumulation was 658 ± 15 dpm and 629 ± 16 dpm in the absence or presence of IL-6, respectively.

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## DISCUSSION

Both IL-6 and the 5-$HT_{2A}$ receptor have been implicated in the pathology of mental illness (Dowlati et al., 2010; Gray and Roth, 2001). We previously found that both molecules can facilitate reversal learning, a form of orbitofrontal cortex (OFC)-mediated cognitive flexibility that is impaired across psychiatric disorders (Donegan et al., 2014; Furr et al., 2011). In the current study, we found that IL-6 over-expression induced endogenous JAK-STAT activation in OFC cells that also express 5-$HT_{2A}$ receptors, providing a neuroanatomical substrate for a potential interaction between these signaling systems. Using an *in vitro* model, we then demonstrated that IL-6 reduced 5-$HT_{2A}$ agonist-mediated IP accumulation, suggesting that this cytokine can regulate 5-$HT_{2A}$ receptor signaling. While IL-6 attenuated the maximal response to DOI, it had no effect on the $EC_{50}$, suggesting that this cytokine may alter 5-$HT_{2A}$ receptor agonist efficacy without influencing ligand affinity. Further, we showed that this regulation was mediated by activation of the JAK-STAT pathway. IL-6 had no effect on BK-induced IP accumulation, suggesting that IL-6 may specifically modulate 5-$HT_{2A}$ receptor signaling.

Although IL-6 and the 5-$HT_{2A}$ receptor have been implicated in psychiatric disorders, little is known regarding the potential interaction of these two signaling pathways, especially in the brain. Current evidence suggests that JAK-STAT may be a downstream target of the 5-$HT_{2A}$ receptor. In smooth muscle cells, JAK2 can associate with the 5-$HT_{2A}$ receptor (Guillet-Deniau et al., 1997) and 5-$HT_{2A}$ agonists increase JAK1 and JAK2 phosphorylation (Banes et al., 2005). Perhaps paradoxically, three well-characterized antagonists/inverse agonists at the 5-$HT_{2A}$ receptor, olanzapine, clozapine and MDL-100,907, have also been shown to activate the JAK2-STAT3

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pathway in both the neuronally derived A1A1 cell line (Singh et al., 2009; Singh et al., 2007) and in the frontal cortex (Singh et al., 2010). Interestingly, in these systems, JAK-STAT activation was shown to mediate 5-$HT_{2A}$ receptor desensitization (Singh et al., 2010; Singh et al., 2009; Singh et al., 2007). These previous observations coincide with our demonstration that directly activating the JAK/STAT pathway, through the IL-6 receptor system, attenuates DOI induced 5-$HT_{2A}$ receptor-mediated IP accumulation, substantiating the possibility of cross-talk between these receptor systems.

The mechanism(s) by which IL-6 and JAK-STAT regulate 5-$HT_{2A}$ receptor signaling is still unknown; However, our results indicate that the regulation is specific to this $G_q$ protein- coupled receptor, as IL-6 did not influence BK B2 receptor-mediated IP accumulation. One explanation for this specificity is that IL-6-activated JAK acts directly on the 5-$HT_{2A}$ receptor, rather than on the downstream $G_{q/11}$ cascade, inducing phosphorylation-mediated conformational changes to the receptor complex. These changes could ultimately affect ligand binding to the receptor, receptor trafficking, and/ or coupling with downstream signaling molecules. Indeed, JAK2 has been shown to associate with the 5-$HT_{2A}$ receptor (Guillet-Deniau et al., 1997); Therefore, IL-6-induced activation of this pathway may lead to a physical association of JAK with the 5-$HT_{2A}$ receptor, which in turn leads to a reduction in $G_{q/11}$-mediated activation of PLC. In support of this idea, we demonstrated that the JAK1 and JAK2 isoforms are required for IL-6 regulation of 5-$HT_{2A}$ receptor signaling. Future experiments will identify the mechanism(s) by which IL-6 and JAK-STAT activation reduce the efficacy of the 5-$HT_{2A}$ receptor agonist, DOI, including measurement of potential changes in receptor expression on the cell surface.

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In both our previous behavioral studies and the current *in vitro* experiments, the effect of IL-6 is rapid (within 15 min), possibly suggesting a non-transcriptional mechanism. However, the transcription factor, STAT, is the primary downstream target of JAK and we demonstrated that down regulation of STAT3 by siRNA, which presumably will have prolonged transcriptional consequences, can block the effect of IL-6 on 5-$HT_{2A}$ receptor signaling. Therefore, STAT molecules can also influence 5-$HT_{2A}$ receptor signaling. These observations also imply that long-term activation of the JAK-STAT pathway, which occurs under chronic inflammatory conditions, such as obesity and cardiovascular disease, may have different or more permanent effects on the 5-$HT_{2A}$ receptor system. There is evidence to suggest that STAT proteins can influence expression of the 5-$HT_{2A}$ receptor itself (Singh et al., 2009). However, it is also possible that STAT proteins may affect the transcription of other components of this signaling cascade. For example, long-term treatment with olanzapine, which caused prolonged activation of the JAK-STAT pathway, increased expression of regulator of G protein signaling (RGS) proteins, which have been shown to desensitize multiple G-protein coupled receptor systems (Singh et al., 2010; Singh et al., 2009; Singh et al., 2007). Therefore, long-term interactions between the IL-6 and 5-$HT_{2A}$ receptor systems may have lasting effects, through STAT-dependent changes in gene transcription, that may influence behavior and mental health.

In addition to the duration of JAK-STAT activation, the effect of IL-6 on 5-$HT_{2A}$ receptor signaling and cognitive function may depend on the concentration of this cytokine. In preliminary experiments in A1A1 cells, we found that concentrations of IL-6 ranging from 1 pg/ml to 1 ng/ml, which are close to levels measured in healthy rat

brains, did not influence STAT3 phosphorylation, possible reflecting a lower concentration of IL-6 receptors in A1A1 cells compared to the intact brain (data not shown). It is possible that the concentration of IL-6 used in the current experiments (50 ng/ml), may result in more robust or even differential effects on 5-$HT_{2A}$ receptor function than physiological concentrations. However, in pilot studies in A1A1 cells, we found that lower concentrations of IL-6 (5 ng/ml), which produced a non-significant increase in STAT3 phosphorylation (Fig 2B) and may represent non-inflammatory conditions *in vivo*, also reduce IP accumulation in response to DOI, although to a lesser extent than higher concentrations (data not shown). Although still speculative, our preliminary findings suggest that IL-6 may regulate 5-$HT_{2A}$ receptor signaling similarly, regardless of absolute concentration. It is still possible, however, that IL-6 may have differential effects on 5-$HT_{2A}$ receptor signaling in the more complex context of inflammation. Indeed, IL-6 has been shown to have differential effects, impairing learning and memory under inflammatory conditions (Dugan et al., 2009; Sparkman et al., 2006), but proving beneficial for cognitive function under physiological conditions (Baier et al., 2009; Donegan et al., 2014; Matsuda et al., 1996). Understanding the differences in IL-6 and JAK-STAT signaling in the brain under basal vs. inflammatory conditions will be important and may provide additional insight into the regulation of 5-$HT_{2A}$ receptor signaling.

In previous studies, we demonstrated that blockade of either IL-6 or 5-$HT_{2A}$ receptor signaling in the OFC impairs cognitive function (Donegan et al., 2014; Furr et al., 2011). Because these molecules seem to have similar effects on behavior, we hypothesized that in our *in vitro* system, IL-6 would augment 5-$HT_{2A}$ signaling, yet we

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found the opposite result. One explanation for this could be the cell types involved in the *in vivo* interaction. Using immunohistochemistry, we demonstrated that IL-6-induced STAT3 phosphorylation occurs in all 5-$HT_{2A}$-positive cells in the OFC. Activation of 5-$HT_{2A}$ receptors is thought to be excitatory at the cellular level (Gellman and Aghajanian, 1994; Puig et al., 2003), but these receptors have been localized to both excitatory pyramidal cells and inhibitory interneurons in the prefrontal cortex (De Almeida and Mengod, 2007; Santana et al., 2004). This suggests that 5-$HT_{2A}$-mediated cellular excitation can have opposing effects on circuit function. Interestingly, 5-$HT_{2A}$ receptors have also been shown to have differential effects on behaviors relevant to prefrontal cortical function (e.g. facilitating cognitive flexibility but impairing attention and increasing impulsivity (Fletcher et al., 2007; Winstanley et al., 2003)). Activation of 5-$HT_{2A}$ receptors may facilitate reversal learning by directly exciting pyramidal cells and causing OFC activation, which has been associated with improved cognitive flexibility (Remijnse et al., 2006). By contrast, if IL-6 attenuates excitatory 5-$HT_{2A}$ receptor signaling specifically in inhibitory interneurons during reversal learning, the net result would also be an increase in OFC activation. Indeed, there is some evidence that IL-6 may have unique functional consequences in interneurons. For example, in the hippocampus, IL-6 selectively decreased the number of parvalbumin-positive interneurons (Dugan et al., 2009) and while cortical IL-6 had no effect on pyramidal cell excitability, it reduced inhibitory post-synaptic currents mediated by interneurons (Garcia-Oscos et al., 2012). Further, we have demonstrated that IL-6-induced STAT3 phosphorylation does occur in interneurons of the OFC (data not shown). Therefore, it is possible that IL-6 and JAK-STAT modulation of 5-$HT_{2A}$ receptor signaling occurs

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specifically in interneurons to release inhibitory control over the OFC, resulting in improved cognitive function.

Atypical anti-psychotic drugs can act as inverse agonists at 5-$HT_{2A}$ receptors leading to a reduction in constitutive (ligand-independent) receptor signaling. Further, the therapeutic benefit of anti-depressant drugs has been attributed in part to their ability to down-regulate 5-$HT_{2A}$ receptors. Therefore, understanding the regulation of this receptor may allow for more finely tuned treatment strategies. Our results suggest that IL-6 and JAK-STAT signaling, which we have shown to be beneficial for cognitive function, attenuates 5-$HT_{2A}$ receptor signaling. It is possible that other molecules that activate the JAK-STAT cascade and are less likely to stimulate an inflammatory response, such as Leukemia Inhibitory Factor and Ciliary Neurotrophic Factor, can also influence 5-$HT_{2A}$ receptor signaling. Our results provide insight into the role of JAK-STAT signaling in an un-medicated state, and suggest that IL-6 and potentially other compounds that activate JAK-STAT signaling may have clinical utility in psychiatric disorders such as depression and schizophrenia as 5-$HT_{2A}$ receptor desensitizing agents.

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## AUTHORSHIP CONTRIBUTIONS

*Participated in research design:* Donegan, Berg, Morilak, and Girotti.

*Conducted experiments:* Donegan, Patton, Chavera, and Girotti.

*Contributed new reagents or analytic tools:* Berg.

*Performed data analysis:* Donegan, Patton, Berg and Girotti.

*Wrote or contributed to the writing of the manuscript:* Donegan, Patton, Chavera, Berg, Morilak, and Girotti.

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## FOOTNOTES

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b) This work has not been previously presented.

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## FIGURE LEGENDS

**Figure 1.** STAT3 phosphorylation co-localizes to 5-$HT_{2A}$-positive cells in the OFC. A representative OFC section labeled for phospho-STAT3 (green), 5-$HT_{2A}$ (red), and Dapi (blue) indicates that IL-6-induced STAT3 phosphorylation occurs in nearly 100% of $5HT_{2A}$-positive cells (n=3 brains analyzed). Scale bar is 20 microns.

**Figure 2.** IL-6 increases STAT3 phosphorylation in a time and concentration-dependent manner in A1A1 cells. **A)** Maximal STAT3 phosphorylation occurred within 15 min treatment with 100 ng/ml IL-6 . **B) )** Maximal STAT3 phosphorylation occurred in response to 50 ng/ml IL-6 * $p<0.05$ compared to basal (no IL-6). Data are expressed as mean ± S.E.M. from 3 individual experiments.

**Figure 3.** IL-6 increases ERK1/2 phosphorylation in a time and concentration-dependent manner in A1A1 cells. **A)** Maximal ERK1/2 phosphorylation occurred within 15 min of treatment with 100 ng/ml IL-6. **B)** Maximal ERK1/2 phosphorylation was observed in response to 50ng/ml IL-6. * $p<0.05$ compared to basal. Data are expressed as mean ± S.E.M. from 3 individual experiments.

**Figure 4.** IL-6 attenuates DOI-mediated IP accumulation. Pre-treatment of A1A1 cells with 50 ng/ml IL-6 produced a significant reduction in the maximal response ($p<0.05$), but had no effect on the EC50 for DOI. In these experiments basal IP accumulation was

500 ± 21 dpm and 541 ± 24 dpm in the absence or presence of IL-6. Data are expressed as mean ± S.E.M. from 32 individual experiments.

**Figure 5.** JSI-124 prevents JAK/STAT activation and blocks the IL-6 attenuation of DOI-induced IP accumulation. **A)** IL-6-induced increases in STAT3 phosphorylation did not occur in cells pre-treated with JSI-124 (50 μM, 1 hr) . * $p<0.05$ compared to basal and JSI-124 + IL-6. Data are expressed as mean ± S.E.M. from 3 individual experiments. **B)** The effect of IL-6 (50 ng/ml) on DOI-mediated maximal IP accumulation is abolished in cells treated with JSI-124 (50 μM, 1 hr). In these experiments basal IP accumulation was 492 ± 29 dpm for vehicle-treated cells and 534 ± 34 dpm for IL-6-treated cells. The presence of JSI-124 had no effect on basal IP accumulation in cells treated with or without IL-6. Data are expressed as mean ± S.E.M. from 12 individual experiments.

**Figure 6.** Silencing RNA (siRNA) targeting JAK1, JAK2, and STAT3 attenuate target protein expression and block IL-6-mediated reduction in DOI-induced IP accumulation. **A)** JAK1 siRNA (25nM) produced a 97% knock-down, JAK2 (50nM) siRNA produced a 77% knock-down, STAT3 (25nM) siRNA produced a 93% knock-down of protein compared to cells treated with a non-targeting siRNA pool (scRNA). **B)** IL-6 (50 ng/ml) mediated reduction of DOI-mediated IP production is blocked in the presence of siRNA targeting JAK1, JAK2, or STAT3 but not scRNA. In these experiments, basal IP levels under control conditions were 944± 129 dpm and were not altered by either IL-6 or any of the siRNA tested. * $p<0.05$ compared to scRNA without IL-6. Data are expressed as mean ± S.E.M. from 4 or 5 individual experiments.

**Figure 7.** PD-98059 prevents ERK1/2 activation but does not affect the IL-6 attenuation of DOI-mediated IP accumulation. **A)** PD-98059 pretreatment (50 μM, 1 hr) blocked IL-6-induced increases in ERK1/2 phosphorylation. * $p<0.05$ compared to PD + IL-6. Data are expressed as mean ± S.E.M. from 3 individual experiments. **B)** PD-98059 had no effect on IL-6-mediated reduction in maximal responses to DOI in A1A1 cells. In these experiments basal IP accumulation was 550 ± 22 dpm for vehicle-treated cells and were not altered by IL-6 treatment with or without pretreatment with PD-98059. Data are expressed as mean ± S.E.M. from 8 individual experiments.

**Figure 8.** IL-6 does not affect bradykinin (BK) B2 receptor-mediated IP accumulation. A1A1 cells were pretreated with IL-6 (50 ng/ml) 15 min before incubation with BK (100 nM). In these experiments basal IP accumulation was 658 ± 15 dpm and 629 ± 16 dpm in the absence or presence of IL-6, respectively. Data are expressed as mean ± S.E.M. from 8 individual experiments.

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## TABLES

| siRNA Constructs | NCBI Gene ID | Target sequence |
|---|---|---|
| JAK1 | 84598 | *GCAUUAUGGAGCCGAGAUA* |
| JAK2 | 24514 | *CGGAAGCGGAUAAGGUACA* |
| STAT3 | 25125 | *CAUCAAUCCUGUGGUAUAA* |
| Non-targeting pool | N/A | *UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA* |

**Table 1.** Description of silencing RNA constructs.

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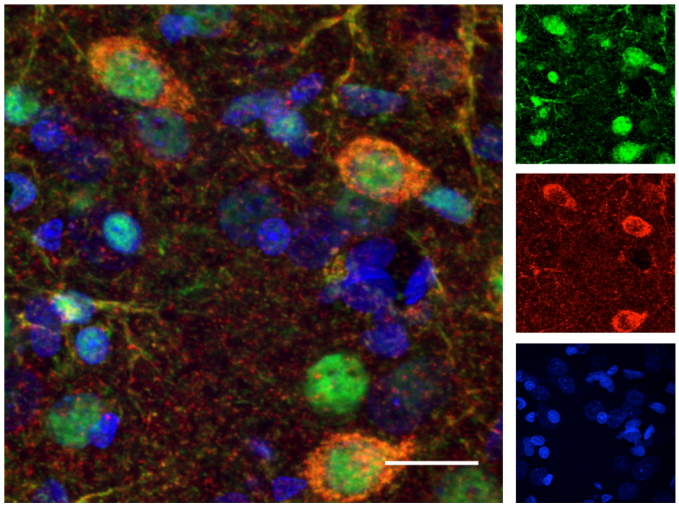

**Figure 1**

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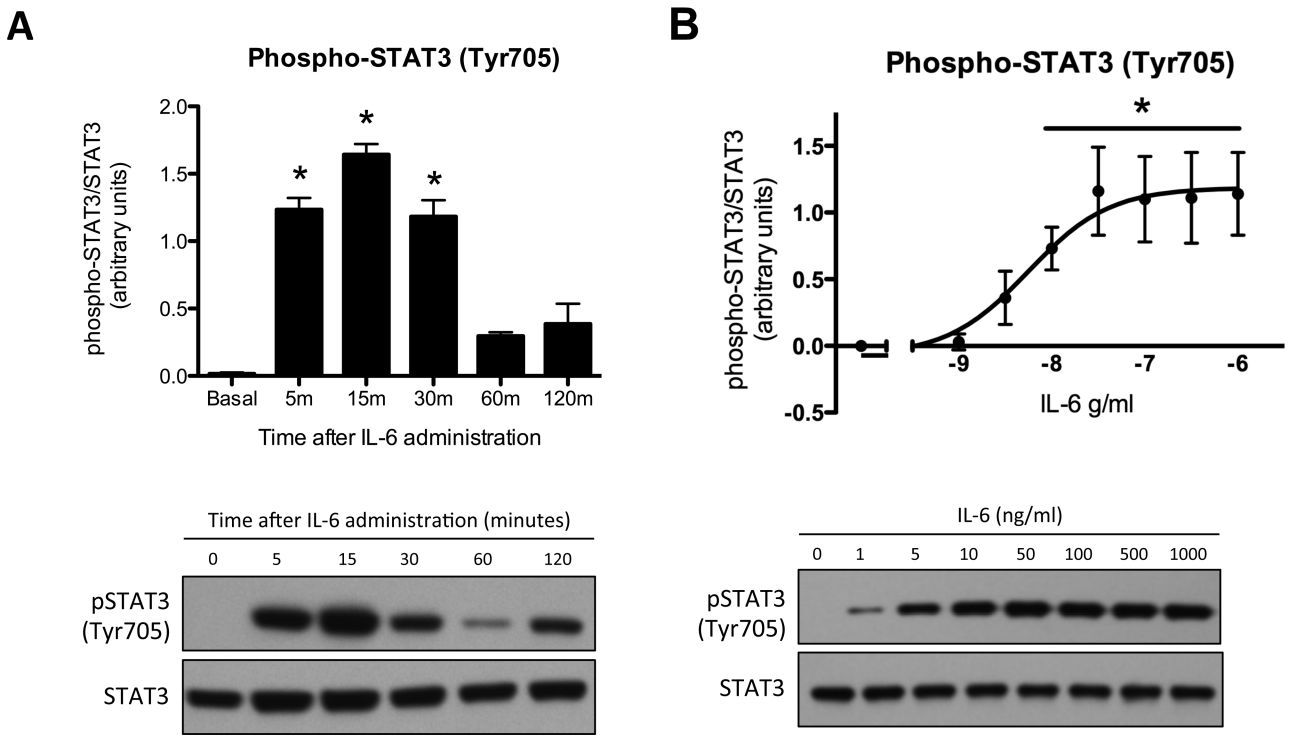


**Figure 2**

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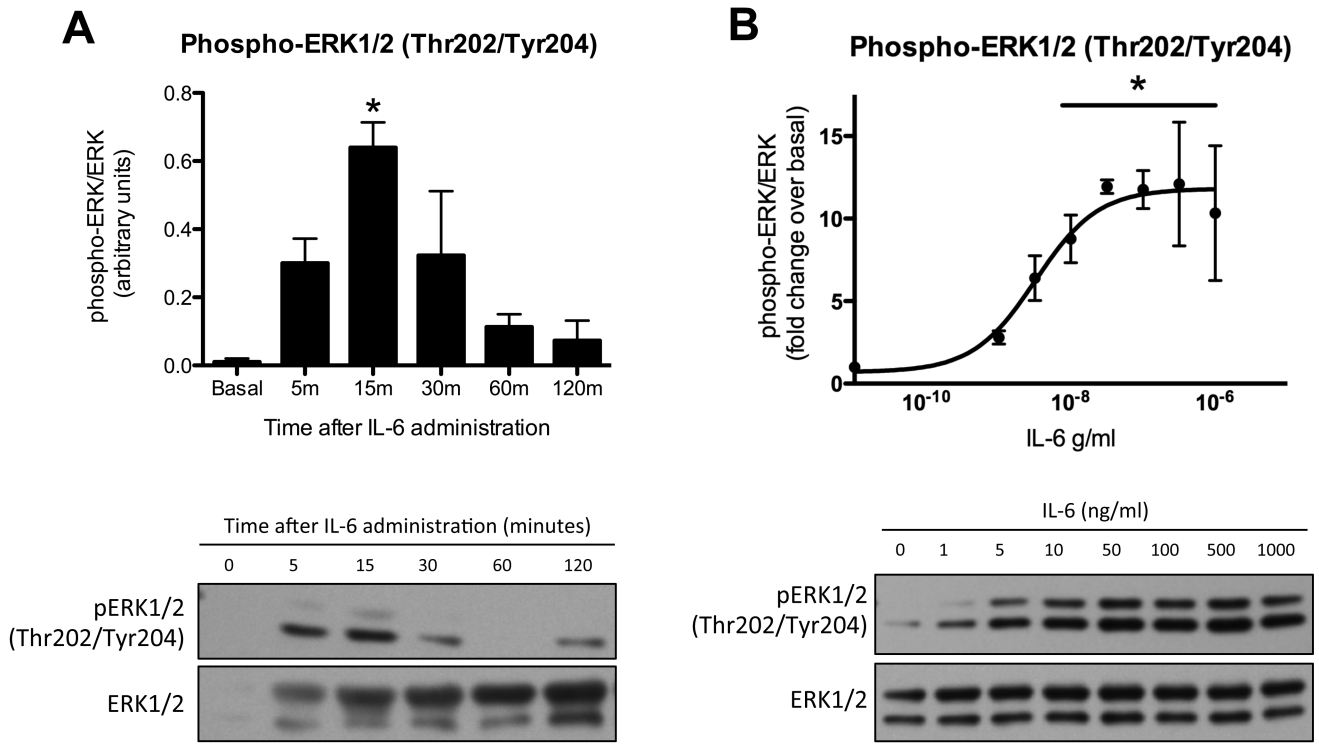


**Figure 3**

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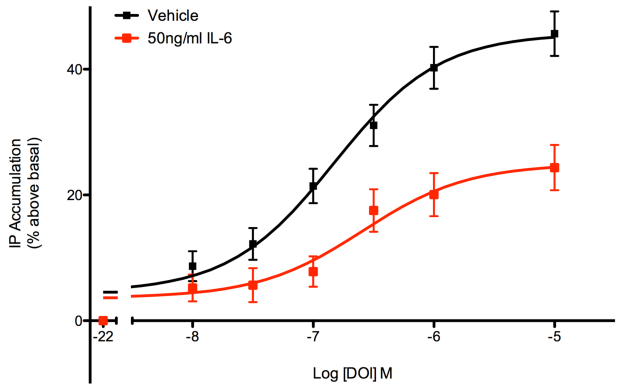


Figure 4

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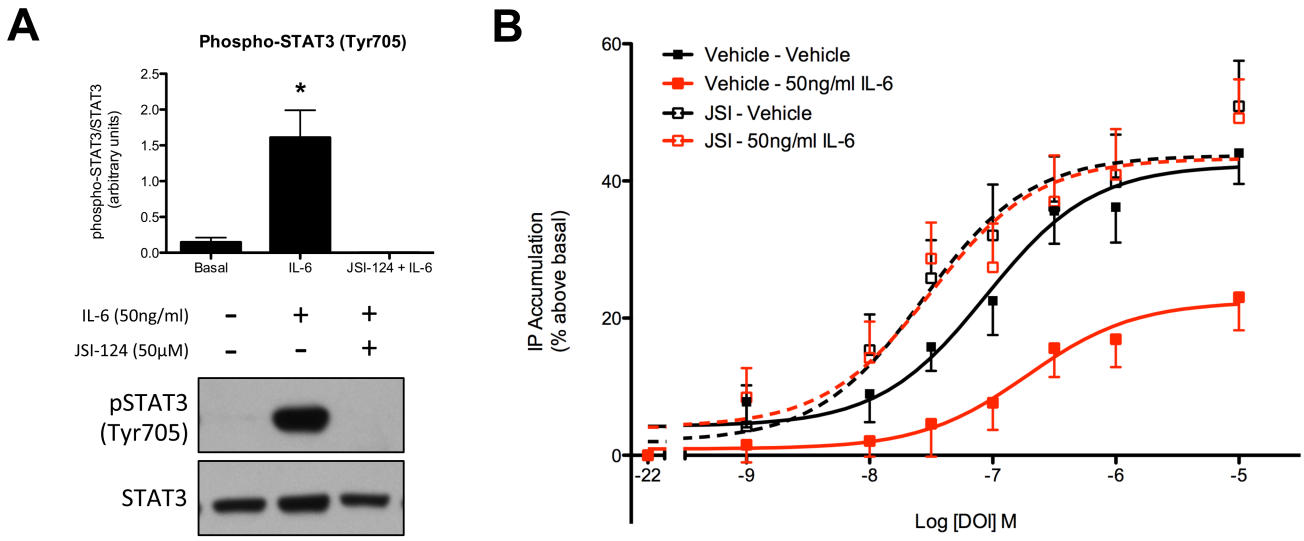


**Figure 5**

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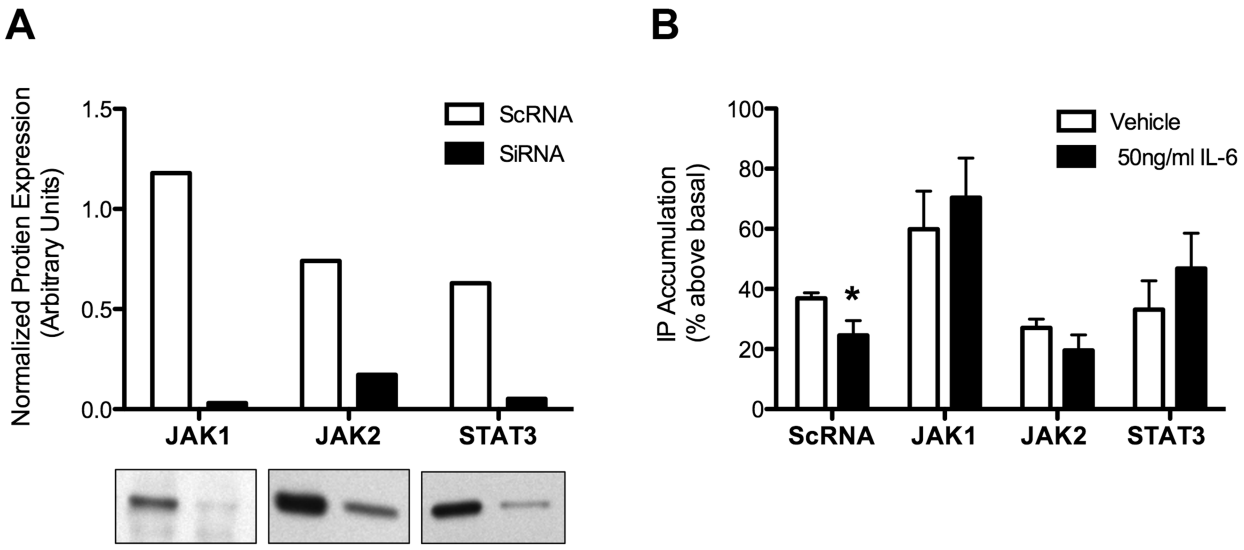


Figure 6

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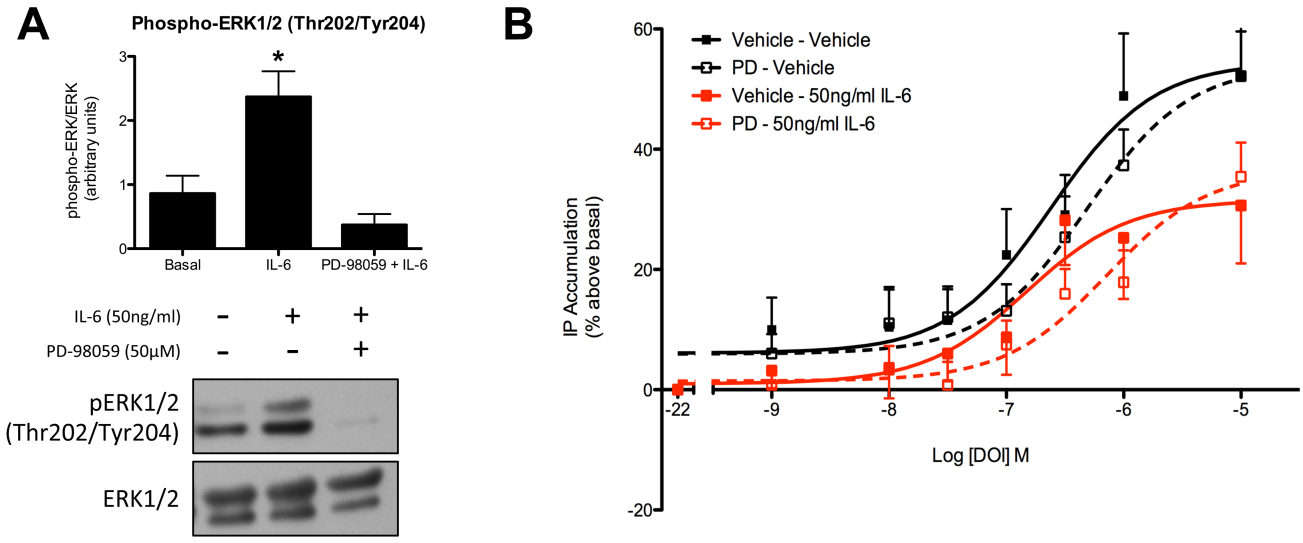


**Figure 7**

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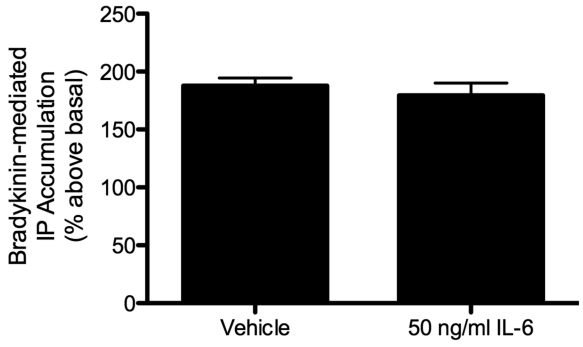


**Figure 8**