Extracellular CIRP Activates the IL-6Rα/STAT3/Cdk5 Pathway in Neurons

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Research Article

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Abstract

Extracellular cold-inducible RNA-binding protein (eCIRP) stimulates microglial inflammation causing neuronal damage during ischemic stroke and is a critical mediator of alcohol-induced cognitive impairment. However, the precise role of eCIRP in mediating neuroinflammation remains unknown. In this study, we report that eCIRP activates neurotoxic cyclin-dependent kinase-5 (Cdk5)/p25 through the induction of IL-6Rα/STAT3 pathway in neurons. Amyloid b (Aβ)-mediated neuronal stress, which is associated with Alzheimer's disease, increased levels of eCIRP released from BV2 microglial cells. The released eCIRP levels from BV2 cells increased 3.2-fold upon stimulation with conditioned medium from Neuro-2a (N2a) cells containing Aβ compared to control N2a supernatant in a time-dependent manner. Stimulation of N2a cells and primary neurons with eCIRP upregulated the neuronal Cdk5 activator p25 expression in a dose- and time-dependent manner. eCIRP directly induced neuronal STAT3 phosphorylation and p25 increase via its novel receptor IL-6Rα. Next, we showed using surface plasmon resonance that eCIRP-derived peptide C23 inhibited the binding of eCIRP to IL-6Rα at 25 mM, with a 40-fold increase in equilibrium dissociation constant (K_d) value (from 8.08 x 10^{-8} M to 3.43 x 10^{-6} M), and completely abrogated the binding at 50 mM. Finally, C23 reversed the eCIRP-induced increase in neuronal STAT3 phosphorylation and p25 levels. In conclusion, the current study demonstrates that upregulation of neuronal IL-6Rα/STAT3/Cdk5 pathway is a key mechanism of eCIRP's role in neuroinflammation and that C23 as a potent inhibitor of this pathway, has translational potential in neurodegenerative pathologies controlled by eCIRP.

Introduction

Cold-inducible RNA-binding protein (CIRP) is an 18-kDa RNA-chaperone which is constitutively expressed and acts as a nuclear regulator of protein translation [1-3]. In addition to cold-shock and hypothermia, cellular stressors such as hypoxia and ultraviolet irradiation induce CIRP expression, nuclear to cytoplasmic translocation in stress granules, and extracellular release [4,5]. The released extracellular CIRP (eCIRP) has been identified as a danger-associated molecular pattern molecule (DAMP) promoting inflammation, tissue injury and mortality in systemic and brain specific inflammation in several studies from our lab [5,6]. In particular, we have shown that cerebral ischemia and alcohol exposure induce microglial expression of CIRP and release of eCIRP, which then acts as a neuroinflammatory mediator causing neuronal damage and death [7,8]. Recently, we showed that eCIRP also mediates alcohol-induced regional metabolic hypoactivity and memory impairment [9,10] and has a potential to be a mediator of Alzheimer's disease associated with alcohol consumption [11]. But, the precise mechanism of how eCIRP causes neuronal injury is still unknown.

Amyloid b (Aβ) peptides are produced from the neurons and are derived from the proteolytic cleavage of a much larger amyloid precursor protein (APP) [12,13]. APP isoform containing 695 amino acids is mainly expressed in neurons and is the most abundant form in the human brain [14]. In Alzheimer's disease, pathogenic forms of Aβ peptides accumulate as plaques in the brain which are closely associated with microglia [15]. Pathogenic Aβ forms are known to interact with and activate resting

microglia in the brain causing release of chemokines, proinflammatory cytokines, reactive oxygen species, and cytotoxins which are detrimental for surrounding neurons inducing neurotoxicity [15,16]. Whether exposure to Aβ can also cause eCIRP release from microglia has not been shown.

Neurotoxic insults cause calpain-mediated cleavage of p35, a regulator of the serine/threonine kinase cyclin-dependent kinase-5 (Cdk5), to generate a truncated carboxy-terminal fragment p25 [17]. Being more stable, induction of p25 causes prolonged activation and mislocalization of Cdk5 resulting in tau hyperphosphorylation, neurite retraction, microtubule collapse, apoptosis and is involved in the pathology of neurodegenerative diseases [18-20]. Moreover, several studies showed elevated p25 levels in the human brain correlate with Alzheimer's disease [21,22]. Signaling via the IL-6Rα/STAT3 pathway in neurons has been previously shown to deregulate Cdk5/p35 pathway [23]. Interestingly, we have recently shown that eCIRP directly binds to IL-6Rα and activates the IL-6Rα/STAT3 pathway in macrophages [24]. Therefore, we reasoned that eCIRP may also activate the IL-6Rα/STAT3/Cdk5 pathway in neurons.

In the present study, we used physiological forms of $A\beta_{1-42}$ and $A\beta_{1-40}$ overproduced and released from N2a neuroblastoma cells stably transfected with human APP695 to stimulate BV2 microglial cells and examined the effects of Ab stress on the microglial release of eCIRP. We also evaluated the harmful effects of eCIRP on neurons by assessing the expression of neurotoxic-mediator p25 in N2a cells and primary neurons stimulated with different doses of eCIRP over time. We then examined if eCIRP stimulation results into direct activation of IL-6Rα pathway in neurons. Next, we tested Compound 23 (C23) peptide, a small 15-mer competitive antagonist of eCIRP activity, for its ability to physically block the interaction of eCIRP with IL-6Rα and its effect on IL-6Rα/STAT3 signaling and p25 expression. This approach not only further elaborated the role of eCIRP in regulating Cdk5-p25 mediated neuronal damage via IL-6Rα/STAT3 signaling but also evaluated the potential of a peptide inhibitor C23 for future therapeutic strategy targeting eCIRP in neurodegeneration in Alzheimer's disease.

Materials And Methods

Recombinant Proteins, Reagents and Antibodies

Recombinant murine (rm) CIRP with His-tag expressed in *E. coli* was prepared in-house as described previously by us [6]. The quality control assays included LPS detection by limulus amebocyte lysate (LAL) assay (Cambrex, East Rutherford, NJ) and purity evaluation by Coomassie blue staining and Western blotting. The biological activity of the purified endotoxin-free protein was assessed by measuring the TNF-α levels released from rmCIRP-challenged macrophages. All the quality control assays were performed for each lot of purified rmCIRP. Culture mediums and cell culture reagents were purchased from MilliporeSigma (Burlington, MA) and Thermo Fisher Scientific (Waltham, MA). Anti-Aβ-1–16 (clone 6E10, catalog 803001) antibody was from Biolegend (San Diego, CA). Mouse CIRP ELISA kit (catalog LS-F16777) was purchased from LifeSpan Biosciences (Seattle, WA). Goat anti-mouse IL-6R polyclonal neutralizing antibody (catalog AF1830), normal goat IgG (catalog AB-108-C) and carrier-free, tag-free recombinant human (rh) rhIL-6Rα protein (catalog 227-SR/CF) derived from *Spodoptera frugiperda*, *Sf* 21

baculovirus, were purchased from R&D Systems (Minneapolis, MN). rhCIRP with C-terminal DDK tag, transfected and expressed from human HEK293T cells was obtained from Origene (Rockville, Maryland). C23 peptide (GRGFSRGGGDRGYGG) was synthesized (>95% purity) and obtained from GenScript (Piscataway, NJ). Western blotting antibodies included anti-mouse p35/p25 (clone C64B10, **catalog** 2680), anti-mouse p-STAT3 (Tyr705, catalog 9131) and total STAT3 (catalog 9139) from Cell Signaling Technologies (Danvers, MA), β-actin antibody (clone AC-15, catalog A5441) from MilliporeSigma (Burlington, MA) and infrared dye–labeled secondary antibodies from LI-COR Biosciences (Lincoln, NE).

Cell Culture

Mouse microglial cell line BV2 and mouse neuroblastoma Neuro-2a (N2a) cells, untransfected or stably transfected with human APP695, were described before [25,26]. BV2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, and 100 U/mL penicillin-streptomycin at 37°C in 5% CO_2. N2a cells were cultured in 1:1 DMEM/Opti- minimum essential medium (MEM) supplemented with 10% FBS and 100 U/mL penicillin-streptomycin at 37°C in 5% CO_2. 0.25 % Trypsin was used for N2a cells detachment. All cell lines were tested negative for mycoplasma contaminants. Conditioned media (CM) was collected from untransfected and stably transfected N2a cells cultured in Opti-MEM overnight.

Mouse Primary Cortical Neuron Isolation and Culture

Postnatal day 1 (P1) pups obtained from pregnant female C57BL/6 mice breeding in-house or purchased from Charles River (Wilmington, MA) at timed late gestation were used in all experiments. These mice were housed in a temperature-controlled room on a 12 h light/dark cycle in the animal facility within the Feinstein Institute for Medical Research (Manhasset, NY) to acclimate to the environment before being used for experiments and fed a standard laboratory diet. All experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Feinstein Institute for Medical Research. All efforts were made to minimize suffering. Primary neurons were isolated from P1 pups as previously described [27]. Briefly, P1 pups were decapitated and the forebrains were dissected in ice-cold HBSS (Invitrogen, Carlsbad, CA) containing 0.5% D-Glucose (Sigma, St. Louis, MO) and 25 mM HEPES (Invitrogen) referred to as HDGH. Mechanical dissociation and digestion were done in dissection medium, which is HDGH containing 0.01% papain (Worthington Biochemical Corporation, Lakewood, NJ), 0.1% dispase II (Roche Applied Science, Indianapolis, IN), and 0.01% DNase I (Worthington Biochemical Corporation), first by means of sterile razor blades, then by serial pipetting, and incubation at 37°C twice for 15 minutes. Cells were then spun down at 250 ***g*** for 5 min at 4°C; resuspended in Neurobasal medium supplemented with 2% B27, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin, and 2 mM Glutamax (all from Invitrogen, Carlsbad, CA); filtered through a 40-μm cell strainer (Thermo Fisher Scientific, Waltham, MA) and counted. Cells were seeded in 12-well plates coated with 15 mg/ml poly-L-ornithine (Sigma, St. Louis, MO) and 4 mg/ml laminin (Invitrogen, Carlsbad, CA,) at a density of 4 x 10^5 cells/well and maintained at 37°C in 95%

humidity and 5% CO_2. Culture medium was completely replaced after 16–20 hours, and new medium (30% of starting volume) was added every 3 days until 10-12 days. 10–12 DIV (day *in vitro*) neurons were used for the different treatments.

In vitro Treatments

BV2 cells seeded in 24 well plate at 10^5 cells/well were left untreated or treated with 1:1 N2a CM (untransfected or stably transfected with APP) and Opti-MEM for 6 h or 24 h. Following the incubation supernatants from BV2 wells were harvested. N2a cells and DIV 10 primary neurons seeded in 12-well plates were either left untreated or pre-treated with IL-6R neutralizing antibody (catalog AF1830) (3 mg/ml), normal goat IgG (3 mg/ml) or C23 peptide (25 mg/ml) for 30 minutes, followed by the stimulation with rmCIRP at 2.5 mg/ml for 1 h for STAT phosphorylation and 16 h or 48 h for p25 levels. For dose-response experiments cells were treated with 0.1, 1 and 2.5 mg/ml rmCIRP for 1 or 48 h timepoints. After rmCIRP treatment, the cells were harvested for total protein extraction.

eCIRP ELISA

To measure the effect of neuronal Aβ stress on eCIRP release, released CIRP levels were measured in the conditioned medium samples from BV2 cells, treated for 6h and 24h with supernatants from untransfected N2a cells (Ctrl-N2a) or from N2a cells stably transfected with human APP constructs containing extracellular amyloid β (Aβ-N2a), by using ELISA kit (LifeSpan Biosciences) following manufacturer's instructions.

Immunoblotting Analysis

The conditioned medium from untransfected or stably transfected with human APP695 N2a cells was incubated with 0.02% deoxycholic acid and 10% trichloroacetic acid (TCA) at 4 °C overnight for protein precipitation, and then subjected to Western blotting. Ab levels in the TCA precipitated supernatant were determined by Western blot analysis using anti-Aβ antibody (clone 6E10). N2a cells and primary neurons treated with various concentrations of rmCIRP with or without pretreatment with IL-6Rα neutralizing antibody or C23 peptide were harvested in lysis buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitor and phosphatase inhibitor cocktail tablet (Thermo Fisher Scientific) and total protein was extracted. Levels of Cdk5 activator p25 and phosphorylation status of STAT3 were determined by Western blot analysis using p-STAT3, STAT3, p25 and β-actin antibodies. Cell lysates were fractionated on 4%–12% Bis-Tris gels and transferred to nitrocellulose membranes. After blocking with 0.1% casein in Tris-buffered saline, the membranes were incubated in respective primary antibodies overnight at 4°C. The target bands were detected by using infrared dye–labeled secondary antibodies and Odyssey Clx image system (Li-Cor Biosciences). The intensities of the bands were analyzed using Image Studio 5.2 software (Li-Cor Biosciences). The densitometric analysis of blots was done using ImageJ software.

Biacore Assay

Analysis of IL-6Rα-CIRP and C23 peptide- IL-6Rα-CIRP interactions was conducted using the surface plasmon resonance (SPR) technique on the BIAcore 3000 instrument (GE Healthcare). Binding reactions were performed in 1× HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% P20, pH 7.4.). The CM5 dextran chip (flow cell-2) was first injected with 89 µl of 0.1 M N-ethyl-N'-[3-diethylamino-propyl]-carbodiimide and 0.1 M N-hydroxysuccinimide for activation. For ligand immobilization, the recombinant human (rh) IL-6Rα protein (R&D) diluted in 10 mM sodium acetate (pH 4.5) at 5 µg/ml was injected in 200 µl volume into flow cell-2 of the CM5 chip. Next, the remaining active sites were blocked by injecting 135 µl of 1 M ethanolamine (pH 8.2). To evaluate nonspecific binding, the flow cell-1 without coating with the rhIL-6Rα protein was used as a control. The flow rate of 30 µl/min at 25 °C was used to perform the binding analyses. To evaluate the binding, the analyte rhCIRP protein (Origene) (500 nM for yes or no binding analysis or ranging from 62.5 nM to 500 nM with or without 25-50 mM C23 for the kinetics analysis) was injected into flow cell-1 and -2 and the association of analyte and ligand in the presence or absence of C23 was recorded respectively by SPR. The blank channel (flow cell-1) signal was subtracted from the ligand coated channel (flow-cell 2). Data were analyzed by the BIAcore 3000 Evaluation Software. Data were globally fitted to the Lagmuir model for a 1:1 binding.

Statistical Analysis

Data were analyzed using SigmaPlot12.5 graphing and statistical analysis software (Systat Software Inc., San Jose, CA) and presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Student-Newman-Keuls' (SNK) test were performed for multigroup analysis. All data were tested for normality. For comparison of 2 groups, we performed unpaired 2-tailed Student's *t* tests. Differences in values were considered significant if $p < 0.05$.

Results

Microglial Cells Are a Major Source of eCIRP After Exposure to Aβ

We have previously shown that hypoxic stress and alcohol exposure increases microglial cell expression of CIRP and release of eCIRP [7,8]. To determine whether microglial cells act as source of brain eCIRP under Ab-associated neuronal stress, we evaluated the effects of Ab exposure on BV2 microglial cells. We first confirmed the presence of extracellular total Aβ in the TCA precipitated conditioned medium (CM) from N2a cells stably transfected with human APP695 constructs (Aβ-N2a), and its absence in the CM from the control untransfected N2a (Ctrl-N2a) cells (Fig. 1a). We then cultured BV2 mouse microglial cells with CM from either transfected or untransfected N2a cells for 6 h and 24 h. The Ctrl-N2a CM and Aβ-N2a CM had only baseline eCIRP levels comparable to CM of control BV2 cells (Fig. 1b), suggesting negligible effects of Aβ on the neuronal release of eCIRP. Aβ-containing CM induced BV2 cells to release eCIRP in a time-dependent manner, while Aβ-free CM had no effect (Fig. 1c). The eCIRP levels in the media of BV2

cells exposed to Aβ-containing CM for 6 h were 1.8-fold higher than in the media of cells exposed to Aβ-free CM, further increasing to 3.2-fold ($p < 0.001$) after 24 h exposure to Aβ-containing CM compared to Aβ-free CM (Fig. 1c). These findings suggest that microglial cells are a key source of eCIRP in the brain during neuronal stress caused by Ab.

eCIRP Upregulates the Neuronal Cdk5 Activator p25

Cdk5 activity is regulated by unstable binding with the neuron-specific activator proteins p35. The more stable p25, generated from calpain-dependent cleavage of p35, is responsible for aberrant hyperactivation and deregulation of Cdk5 [17]. The generation of p25 is induced under neurotoxic stress conditions and Cdk5-p25 complex promotes neurodegeneration and mediates further neurotoxicity [18-20]. To determine whether eCIRP stimulation could generate p25 in neurons, we treated N2a cells and primary neurons with increasing concentrations of eCIRP (0, 0.1, 1 and 2.5 mg/ml) for 48 h and analyzed p25 protein expression by Western blotting. We found that eCIRP significantly upregulated p25 in a dose-dependent manner in both N2a cells (Fig. 2a) as well as primary neurons (Fig. 2b). After 48 h of eCIRP treatment in N2a cells, there was 1.9-fold induction of p25 at 1 mg/ml eCIRP, which further increased to 4.1-fold on stimulation with 2.5 mg/ml eCIRP (Fig. 2a). Primary neurons responded to 48 h CIRP stimulation with 49.9-fold induction of p25 at 1 mg/ml CIRP, going up to 136.2-fold with 2.5 mg/ml CIRP (Fig. 2b). Moreover, eCIRP time dependently induced upregulation of neuronal p25 in N2a cells (Fig. 2c) and primary neurons (Fig. 2d). After 16 h of CIRP treatment N2a cells expressed 12.7-fold higher p25 than control cells which increased to 22.2-fold at 48 h stimulation (Fig. 2c). Similarly, primary neurons demonstrated 11.9-fold induction in p25 levels at 16 h of CIRP stimulation compared to no CIRP stimulation, further increasing to 26.3-fold at 48 h CIRP stimulation (Fig. 2d). Of note, in the absence of CIRP stimulation there was no noticeable p25 expression in control N2a cells and primary neurons (Fig. 2a-d). These findings indicate that eCIRP upregulates the expression of neurotoxic truncated p25 form which is known to induce aberrant hyperactivation of Cdk5. Thus, induction of p25 may be the mechanism by which eCIRP induces neuronal damage.

eCIRP Activates the IL-6Rα/STAT3 Pathway

Signaling via the IL-6Rα and STAT3 has been previously shown to deregulate the Cdk5/p35 pathway in neurons [23,28]. Additionally, we have recently shown that eCIRP directly binds to IL-6Rα activating STAT3 phosphorylation in macrophages [24]. IL-6 activates STAT3 phosphorylation in both primary neurons and N2a cells [23,29,30]. To evaluate whether eCIRP could also activate the IL-6Rα/STAT3 pathway in neurons, we treated neuronal cells with increasing concentrations of eCIRP (0, 0.1, 1 and 2.5 mg/ml) for 1 h before measuring changes in the STAT3 phosphorylation by Western blotting. In N2a cells, eCIRP activated STAT3, measured by STAT3 phosphorylation (pSTAT3), in a dose-dependent manner. N2a cells stimulated with 0.1 μg/ml CIRP increased pSTAT3 levels by 1.4-fold, 1 μg/ml eCIRP by 1.9-fold and 2.5 μg/ml eCIRP by 2.3-fold when compared to unstimulated N2a cells (Fig. 3a). Likewise, in primary

neurons, 2.5 μg/ml eCIRP induced STAT3 phosphorylation by 2.5-fold (Fig. 3b). To prove whether eCIRP activated STAT3 via the IL-6R signaling pathway, we pretreated N2a cells and primary neurons with IL-6Rα neutralizing antibody or IgG control for 30 min prior to 2.5 μg/ml eCIRP stimulation. As anticipated, the eCIRP induced pSTAT3 in N2a cells by 2.6-fold and IL-6Rα neutralization decreased the pSTAT3 levels by 37.9% (Fig. 3c). Similarly, in primary neurons eCIRP stimulation upregulated pSTAT3 expression by 2–fold which was decreased by IL-6Rα neutralization to baseline levels similar to unstimulated condition (Fig. 3d). Taken together, these results reveal that eCIRP activates the IL-6Rα/STAT3 pathway in neuronal cells.

eCIRP Induces STAT3 Phosphorylation and p25 Via IL-6Rα

Next, we interrogated the role of IL-6Rα on the eCIRP-induced neuronal p25 expression. We stimulated N2a cells and primary neurons with 2.5 μg/ml eCIRP for 48 h plus either IgG or IL-6Rα neutralizing Abs. The eCIRP stimulation increased the p25 expression 19-fold which was suppressed by 38.9% on IL-6Rα neutralizing antibody pretreatment in N2a cells (Fig. 4a). Compared with the resting state, primary neurons stimulated with eCIRP induced 7.9-fold p25 which was decreased by 62.5% with IL-6Rα neutralization (Fig. 4b). These results further support the concept that eCIRP induces the neuronal p25-Cdk5 pathway via the activation of the IL-6Rα/STAT3 pathway.

C23 Inhibits the Direct Binding of eCIRP to IL-6Rα

Recently, we showed IL-6Rα to be a novel high-affinity receptor for eCIRP in macrophages [24]. We have also generated C23, a 15-amino acid eCIRP inhibitor peptide derived from human CIRP [6]. We postulated that C23 may inhibit eCIRP binding to IL-6Rα. We used SPR to determine whether different concentrations of C23 could disrupt the binding of eCIRP to IL-6Rα. As predicted, rhCIRP bound to rhIL-6Rα with high-affinity with an equilibrium dissociation constant (K_d) value of 8.08×10^{-8} M (Fig. 5a), which is similar to K_d 9.81×10^{-8} M reported in our previous study. In the presence of 25 μM C23 this binding affinity dropped by 40-fold, with the K_d value for CIRP binding to IL-6Rα increasing to 3.43×10^{-6} M (Fig. 5b). At the concentration of 50 μM, C23 completely abrogated the binding between eCIRP and IL-6Rα (Fig. 5c). These results provide direct evidence that C23 inhibits the binding of eCIRP to IL-6Rα and, thus, has the potential to also inhibit eCIRP's activation of the neuronal IL-6Rα/STAT3/Cdk5 pathway.

C23 Inhibits eCIRP's Induction of STAT3 Phosphorylation and Upregulation of p25

To evaluate C23's ability to inhibit eCIRP's biological activity in neurons, we pretreated N2a and primary neurons with C23 and then stimulated the cells with eCIRP. Stimulation with eCIRP induced STAT3 phosphorylation by 2.6-fold in N2a cells which was inhibited by 27.1% with C23 pretreatment (Fig. 6a).

Interestingly, 1.96-fold induction of pSTAT3 levels in primary neurons by eCIRP treatment was brought back to unstimulated levels by C23 (Fig. 6b). Along the lines of pathway, C23 also significantly prevented the eCIRP-induced upregulation of p25 in both N2a cells (from 19-fold to 11.5-fold, down by 39.5%; Fig. 6c), and primary neurons (from 8.9-fold to 5.4-fold, down by 40.1%; Fig. 6d). These data show that C23 not only disrupts eCIRP's binding to IL-6Rα, but also blocks eCIRP's activation of the IL-6Rα/STAT3/Cdk5 pathway. Thus, C23 is predicted to attenuate eCIRP's effect on the neuronal injury.

Discussion

In spite of advances in the management and care of patients suffering from neurodegenerative disorders including Alzheimer's disease, there is still no approved cure which poses an urgent need to identify new targets and design new therapeutic strategies. The neurodegeneration and dysfunction is essentially caused by the deregulation of multiple complex and diverse signal transduction pathways, many of which are required for normal regulated functions, which get abnormally triggered or repressed [31,32]. Studies from our and other labs have undoubtedly shown that eCIRP is a major contributing factor causing neuroinflammation, neuronal injury and memory dysfunction in various settings [7,8,9,10,33,34]. However, the mechanism and signaling pathway involved in eCIRP-mediated neuronal injury and dysfunction has not yet been elucidated. Certainly, understanding the signaling pathways activated by eCIRP in neurons causing dysregulation resulting in neuronal damage will impact the search for new targets and therapeutic strategies in treating neurodegeneration.

In the current study, we evaluated eCIRP as a key inducer of the IL-6Rα/STAT3/Cdk5 signaling pathway in neurons for exploring its potential to be a critical mediator of neurodegeneration. First, we confirmed the effect of established neurotoxic insult involved in neurodegeneration on eCIRP release and demonstrated that exposure of BV2 microglial cells to neuronal Ab stress increased the microglial eCIRP release. Next, we identified eCIRP to be a critical activator of neuronal IL-6Rα/STAT3/Cdk5 pathway. For this, we first showed that eCIRP stimulation of neurons produced Cdk5 hyperactivator p25. We then illustrated that eCIRP directly interacted with IL-6Rα, leading to activation of the downstream signal transducer STAT3 and upregulation of p25. Furthermore, we discovered that C23, a CIRP-derived 15-mer peptide, effectively inhibited eCIRP's interaction with IL-6Rα. Finally, pretreatment of neurons with C23 abrogated the eCIRP-induced downstream activation of the IL-6Rα/STAT3/Cdk5 pathway.

Alzheimer's disease pathogenesis has been associated with Ab peptides in aggregated conformation form found in the plaques as Ab fibrils, as well as non-aggregated, soluble Aβ forms which can also stimulate neuronal dysfunction [35]. The stimulation of microglia with synthetic Ab monomers, oligomers and fibrils has revealed very unique and differential activation profiles for these various forms each involving specific signaling pathway [36]. For this reason, we used conditioned medium containing Ab released from N2a cells stably transfected with human APP695 instead of synthetic Ab forms to stimulate microglial cells. This approach would include both $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides in their various physiological forms to better mimic the Ab species involved in disease condition. Ab-mediated stress and activation induced release of eCIRP from BV2 microglia, which is consistent with eCIRP being a stress

response protein. We have shown previously that eCIRP was released from the BV2 cells exposed to either hypoxia [7] or high doses of alcohol [8] which supports the current finding.

Another DAMP, HMGB1, has also been shown to be extracellularly associated with Ab plaques and released from neurons after Ab stimulation [37,38]. However, the conditioned medium from the Ab producing N2a cell lines used in this study only showed basal eCIRP levels similar to the eCIRP levels from control BV2 cells. Further studies are needed to show the mechanism by which Ab causes eCIRP release from microglia and if any other cell types in the brain could also release eCIRP on Ab-stimulation. It would be also interesting to explore whether additional neurotoxic insults involved in neurodegeneration other than Ab such as pathological Tau forms could also induce eCIRP release. Detection of increased eCIRP levels in the cerebrospinal fluid from patients with neurodegenerative diseases would further establish the association of upregulation of eCIRP release to neurodegeneration.

Variety of neurotoxic insults can cause p25 generation in neurons resulting in their damage and death [17]. This supports our finding that eCIRP stimulation of neurons lead to p25 production suggesting neurotoxic ability of eCIRP. Our initial results suggested that primary neurons might be more sensitive to eCIRP stimulation. However, the differences in stimulation scales were mostly related to differences in background of immunoblots and amount of protein loaded on gels in these set of experiments. Cdk5 activation is well-known to cause neuronal dysfunction including its role in increasing pathological tau phosphorylation and aggregation [39,40]. However, Cdk5 also plays a critical role in regulating signal transmission across neurons, synaptic plasticity, and cognition as part of the normal physiological brain function [40]. In addition, inhibition of the Cdk5/p25 complex using small molecule inhibitors has been reported to reduce neurodegeneration and improve cognition [18,20]. Of note, neurons exposed to Ab fibrils also showed deregulated Cdk5 activity associated with Cdk5-p35 complex stability regulated by Cdk5 phosphorylation [41]. Considering that other mechanisms may also exist for regulation of Cdk5 activity [42], further studies showing eCIRP-mediated Cdk5 activation, pathological tau phosphorylation and direct assessment of neuronal damage and death will be needed to confirm the indicated signaling outcome. Several proinflammatory cytokines such as IL-1b, IL-6 and TNF-α can also participate in neurodegeneration [43]. We considered IL-6 in particular since it uses the same signaling pathway. However, IL-6 levels were undetectable in the supernatants of N2a cells stimulated with eCIRP, indicating that eCIRP did not activate the IL-6Rα/STAT3/Cdk5 pathway via induction of IL-6 release. Of note, eCIRP also induces neuroinflammation causing increased proinflammatory cytokine produced from microglia which would further add to the neuronal damage as an independent factor [7,8].

C23 peptide has been demonstrated to be protective in hemorrhage, renal ischemia/reperfusion and sepsis models reducing inflammation and injury [6,44-47]. C23 has been previously reported to work by competitively inhibiting eCIRP binding via binding to TLR4/MD2 complex with high affinity [6]. Remarkably at higher concentrations, C23 also inhibited physical interaction of eCIRP with IL-6Rα. Furthermore, C23 peptide also significantly decreased the eCIRP-induced IL-6Rα/STAT3/p25 signaling. The ability of C23 peptide to abrogate the neuronal damage due to hyperactive Cdk5 signaling and pathological tau phosphorylation needs to be further evaluated to demonstrate its potential to prevent

neurodegeneration and may be potential cognitive improvement. We also need to consider that along with IL-6Rα, eCIRP binds to a variety of other receptors such as TLR-4/MD2, RAGE, TREM-1, many of which are present on neurons as well [11]. Further studies will be needed to carefully dissect whether and which additional receptors on neurons are activated by eCIRP, if a crosstalk exists among these receptors and what decides which receptor dominates the complex interplay.

In conclusion, our data provided in this study identifies that Ab stress causes microglial cells to release eCIRP, which then directly activates the IL-6Rα/STAT3/Cdk5 signaling pathway in neurons. eCIRP does so by effectively inducing the generation of Cdk hyperactivator p25. In particular the eCIRP inhibitor C23, previously known to antagonize eCIRP's binding to the TLR4-MD2 complex, also inhibits physical interaction of eCIRP with IL-6Rα and thus abrogates the eCIRP-induced increase in neuronal p25. Thus, eCIRP is a novel mediator of neuronal dysfunction via IL-6Rα/STAT3/Cdk5 activation which can be mitigated by C23 providing a novel targeting strategy in neurodegeneration in Alzheimer's disease.

Declarations

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Authors' Contributions

AS, MB, and PW conceived and designed the experiments; AS performed all the experiments and analyzed the data; AJ helped with some immunoblots; AS wrote the manuscript; MB and AJ edited and reviewed the manuscript; PM provided reagents and critical input in some experimental designs; PW and PM critically reviewed the manuscript, and PW supervised the research. All authors read and approved the final manuscript.

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Data Availability

All data generated or analyzed during this study are included in this article.

Compliance with Ethical Standards

Conflicts of Interest

PW is an inventor of patent applications (WO/2010/120726 and 61/881.798) covering targeting cold-inducible RNA-binding protein with peptides, licensed by TheraSource LLC. PW is a cofounder of TheraSource LLC.

Ethics approval

The study design of the mouse primary cortical neuron isolation experiments was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Feinstein Institute for Medical Research.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

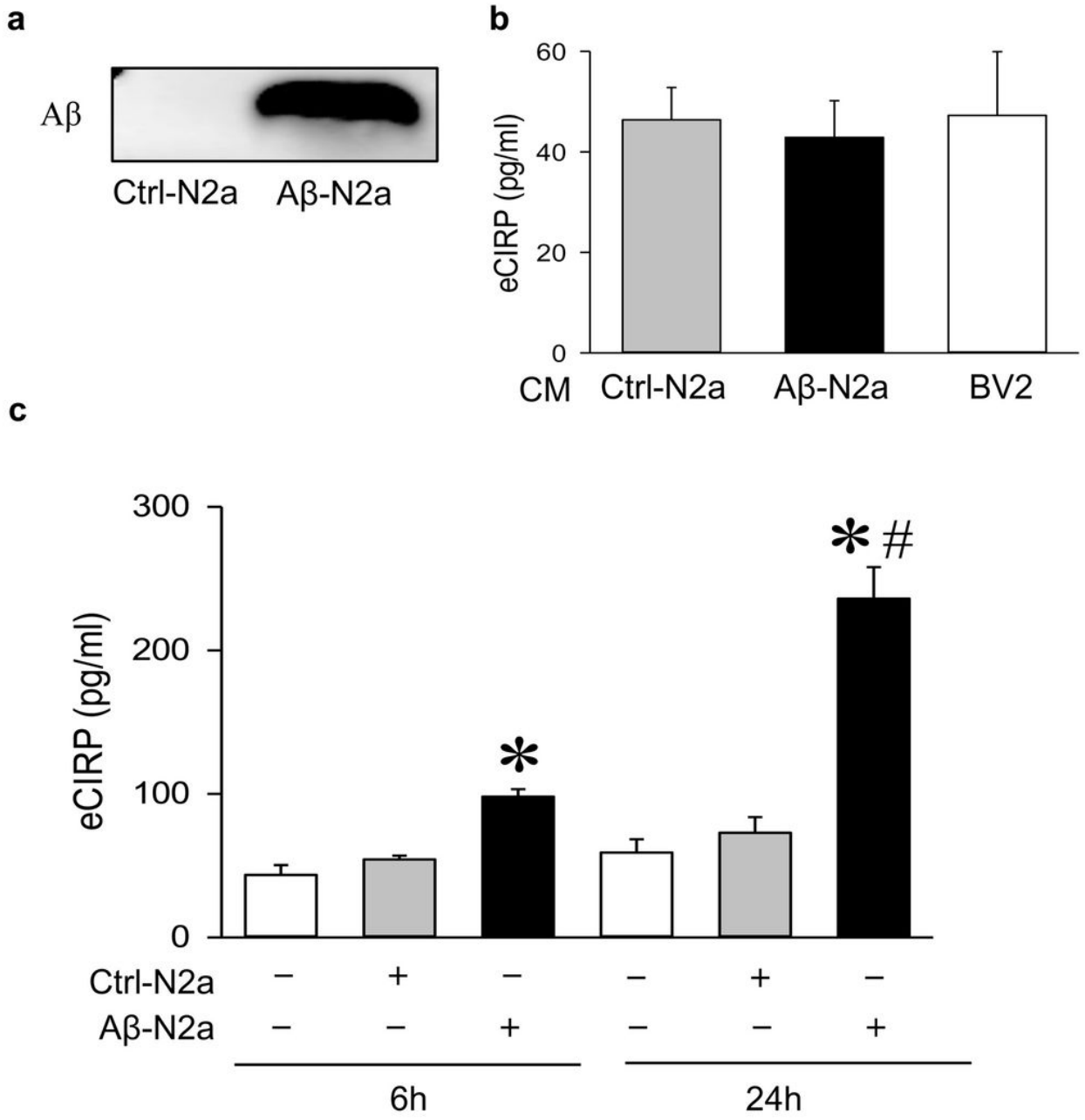

Figure 1

Amyloid β promotes the release of eCIRP by BV2 microglial cells. a Representative 6E10 Western blot image showing that Aβ was only present in the conditioned media (CM) of human APP-transfected N2a cells (Aβ-N2a) but not in that of untransfected N2a cells (Ctrl-N2a). b eCIRP levels in the CM from Ctrl-N2a or Aβ-N2a or BV2 cells (n=4 sample/group) as determined by ELISA. ANOVA analysis showing differences were not significant (N.S). c Time-dependent increase in eCIRP levels in the media of BV2 cells stimulated with CM from Ctrl-N2a or Aβ-N2a (n=10 sample/group). Data were expressed as means ±

SEM. Significance was determined by ANOVA analysis showing * for p < 0.05 vs. Ctrl-N2a and # for p < 0.05 vs. 6h Aβ-N2a

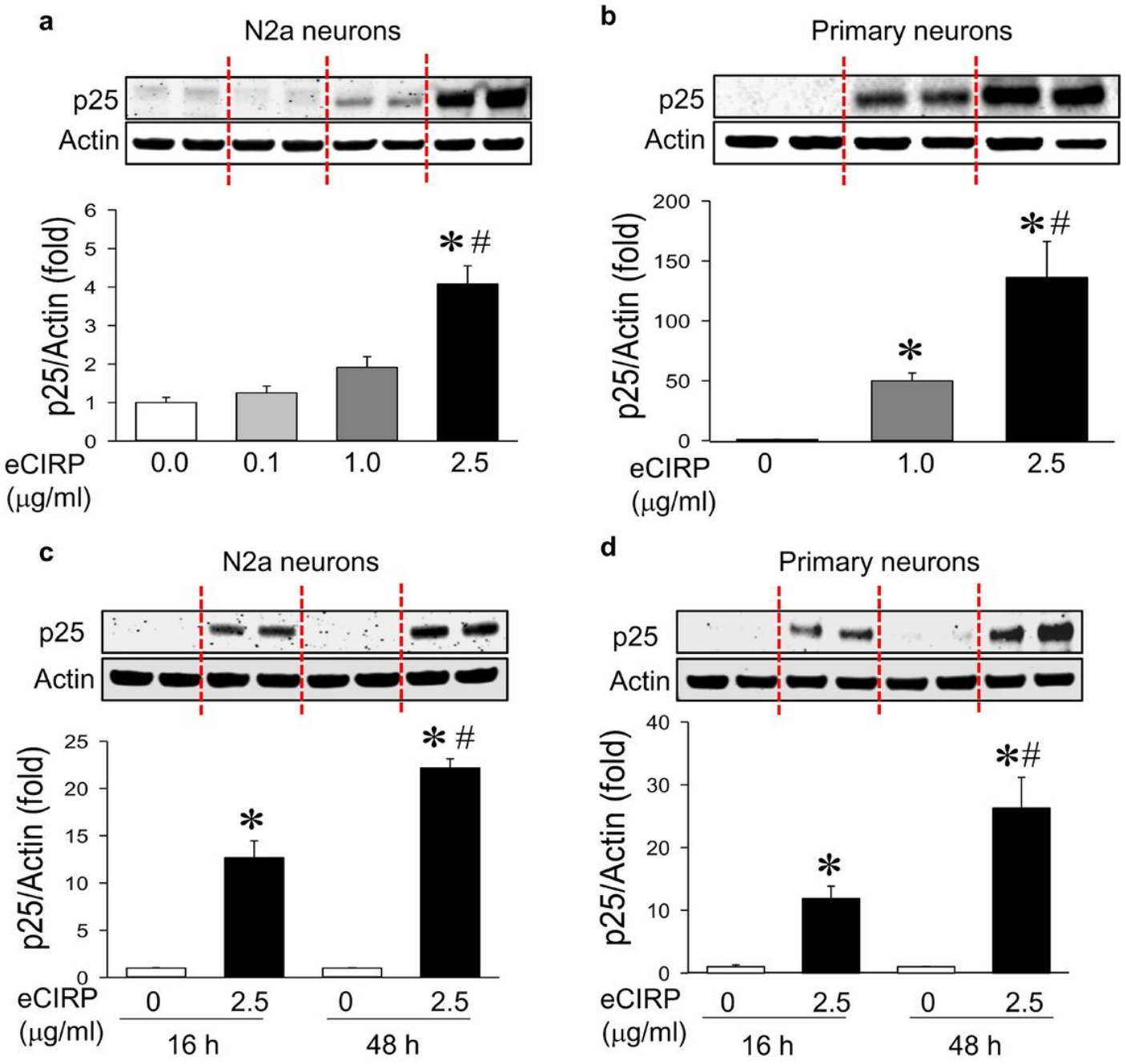

Figure 2

eCIRP upregulates the neuronal Cdk5 activator p25. eCIRP (rmCIRP) caused dose-dependent upregulation of p25 at 48 h in a N2a cells (n=8/group) and b primary neurons (n=4/group). Data shown as representative Western blot images and bar graphs from the densitometric analysis of blots expressed as means ± SEM. ANOVA analysis of data expressed as * for p < 0.05 vs. no eCIRP and # for p < 0.05 vs. 1

μg/ml eCIRP. eCIRP also caused time-dependent upregulation of p25 in c N2a cells (n=4/group) and d primary neurons (n=4/group). Data shown as representative blot images and densitometric analysis of blots expressed as means ± SEM. ANOVA analysis of data for significant differences expressed as * for $p < 0.05$ vs. no eCIRP and # for $p < 0.05$ vs. 2.5 μg/ml eCIRP for 16 h

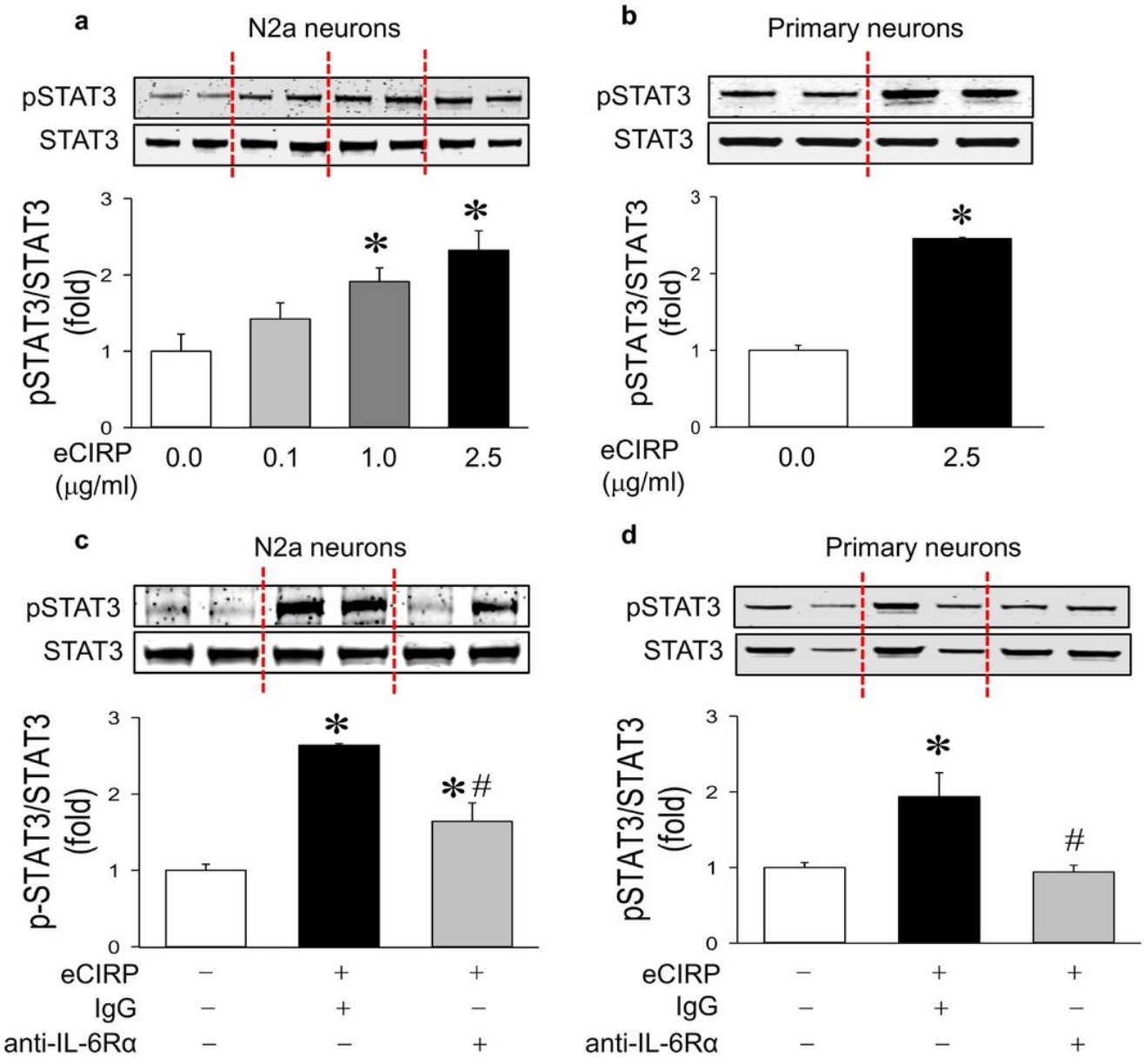

Figure 3

eCIRP activates neuronal IL-6Rα. Total cell lysates were prepared from N2a cells and primary neurons treated with eCIRP for 1 h and subjected to Western blotting. eCIRP dose-dependently induced STAT3 phosphorylation in a N2a neuronal cells (n=4/group), and b primary neurons (n=4/group). Data shown as representative Western blot images and bar graphs from the densitometric analysis of blots expressed as

means ± SEM. ANOVA analysis was done for a and Student's t test analysis was done for b showing * for $p < 0.05$ vs. no eCIRP (0). Anti-IL-6Rα antibodies impeded eCIRP-induced STAT3 phosphorylation in c N2a neuronal cells (n=4/group), and d primary neurons (n=4/group). Data shown as representative Western blot images and bar graphs from the densitometric analysis of blots expressed as means ± SEM. ANOVA analysis showing * $p < 0.05$ vs. no eCIRP (0) and # for $p < 0.05$ vs. eCIRP plus IgG

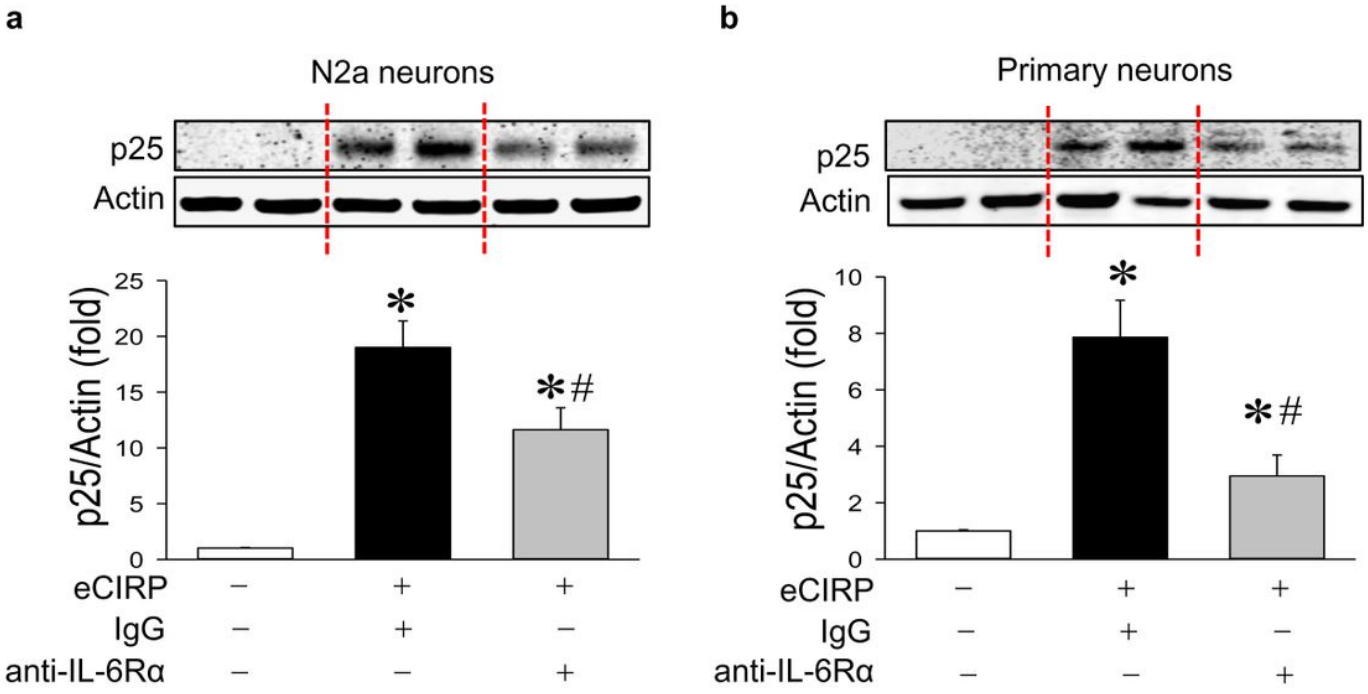

Figure 4

eCIRP induces p25 via IL-6Rα. IL 6Rα neutralizing antibody decreased eCIRP-induced p25 levels. Total cell lysates of a N2a cells (n=6/group) and b primary neurons (n=4/group) treated with eCIRP for 48 h. Data shown as representative Western blot images and bar graphs from the densitometric analysis of blots expressed as means ± SEM. ANOVA analysis showing * for $p < 0.05$ vs. no eCIRP and # for $p < 0.05$ vs. eCIRP+IgG. The no eCIRP controls in b are from the same blot but not in the continuous order

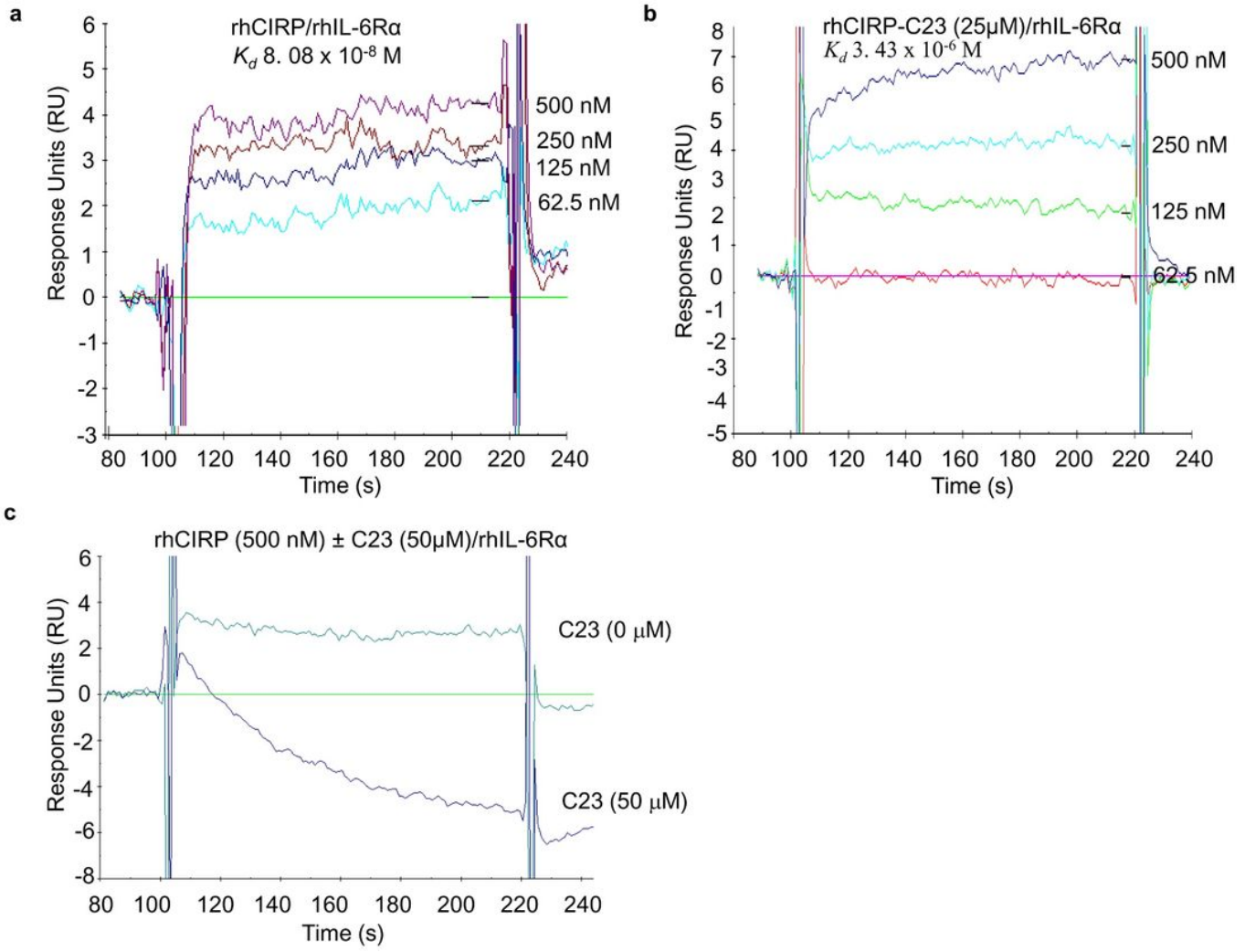

Figure 5

C23 inhibits eCIRP binding to IL-6Rα. The sensograms for binding kinetics acquired by SPR analysis on BIAcore showing association and dissociation of analyte recombinant human (rh) CIRP with and without C23 at the indicated concentrations passed over the ligand rhIL-6Rα immobilized on the sensor chip. a The binding affinity between rhCIRP and rhIL-6Rα expressed as Kd, dissociation constant. The binding affinity between eCIRP and IL-6Rα in the presence of b 25 μM C23, and c 50 μM C23. The data is representative of two to three independent experiments.

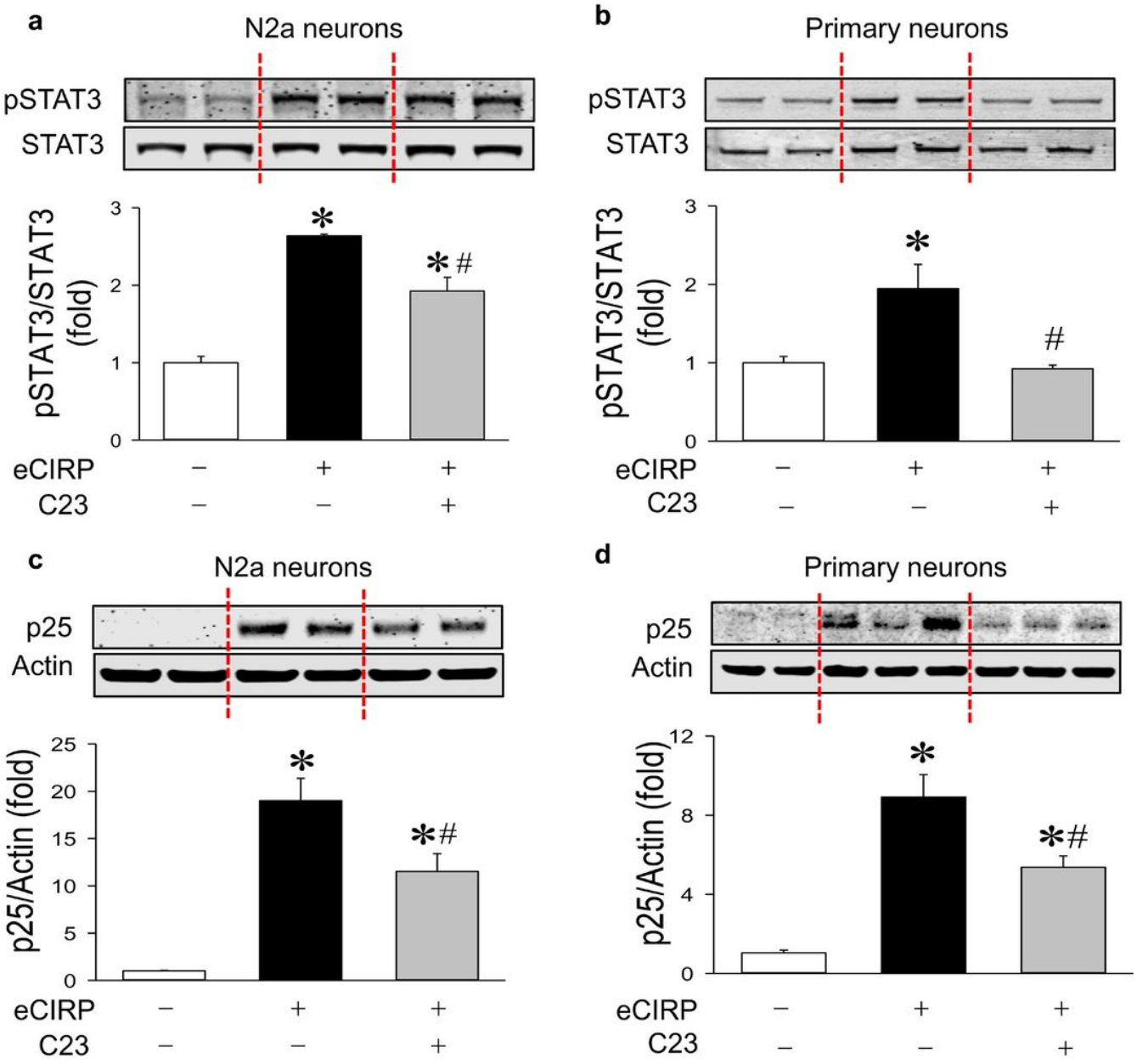

Figure 6

C23 inhibits STAT3 phosphorylation and p25 upregulation by eCIRP. N2a cells or primary neurons were pretreated with 25 μg/ml C23 and then treated with 2.5 μg/ml eCIRP for 1 h. C23 reduced eCIRP-induced STAT3 phosphorylation in a N2a neuronal cells (n=6/group) and b primary neurons (n=4/group). N2a cells or primary neurons were pretreated with 25 μg/ml C23 and then treated with 2.5 μg/ml eCIRP for 48 h. C23 reduced eCIRP-induced p25 upregulation in c N2a neuronal cells (n=6/group) and d primary neurons (n=4/group). Data shown as representative Western blot images and bar graphs from the densitometric analysis of blots expressed as means ± SEM. ANOVA analysis showing * for $p < 0.05$ vs. no eCIRP (0) and # for $p < 0.05$ vs. eCIRP alone