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Role of Sigma Receptor in cocaine-mediate induction of Glial Fibrillary Acidic Protein: Implications for HAND

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Abstract

Cocaine abuse has been shown to accelerate the progression of human immunodeficiency virus (HIV)-1-associated neurological disorders (HANDs) partially through increasing neuroinflammatory response mediated by activated astrocytes; however, the detailed molecular mechanism of cocaine mediated astrocytes activation is unclear. In the current study, we demonstrated increased astrogliosis in the cortical regions of brains from HIV^+ cocaine abusers compared with the HIV^+ group without cocaine abuse. We next sought to explore whether cocaine exposure could result in increased expression of Glial Fibrillary Acidic Protein (GFAP), a filament protein critical for astrocyte activation. Exposure of cocaine to astrocytes resulted in rapid translocation of sigma receptor to the plasma membrane with subsequent activation of downstream signaling pathways. Using a pharmacological approach, we provide evidence that cocaine-mediated upregulation of GFAP expression involved activation of mitogen-activated protein kinases (MAPKs) signaling with subsequent downstream activation of the early growth response gene 1 (Egr-1). Egr-1 activation in turn, caused transcriptional regulation of GFAP. Corroboration of these findings *in vivo* demonstrated increased expression of GFAP in the cortical region of mice treated with cocaine compared with the saline injected controls. A thorough understanding of how cocaine mediates astrogliosis could have implications for the development of therapeutic interventions aimed at HIV-infected cocaine abusers.

Keywords

cocaine; HAND; astrocyte activation; σ-1R translocation; Egr-1

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Introduction

Although the advent of combined anti-retroviral therapy has changed the phenotype of HIV infection from a death sentence to a manageable condition[1], as individuals on therapy continue to live longer, complications of the CNS such as HIV-associated neurological disorders (HAND) are on a rise[2, 3,]. Adding complexity to this is the use of illicit drugs which further increase the prevalence and severity of HAND [4, 5]. Cocaine, often abused by HIV-infected patients, has been suggested to hasten as well as worsen disease pathogenesis [6]. While the effects of HIV viral proteins have been well studied [7, 8], how drugs of abuse such as cocaine can cause glial activation and concomitant neuronal toxicity remains less clear. In the current study we sought to examine the molecular mechanisms of cocaine-mediated induction of GFAP expression in astrocyte cultures and in mice injected with cocaine. The basis for this notions stems from a previous study demonstrating that acute cocaine administration in a mouse model triggered induction of GFAP with increased astrocytic proliferation and changes in cell morphology[9].

A characteristic feature of astrocytic activation is increased expression of GFAP, an intermediary filament protein, that is specific for mature astrocytes, and is likely involved in regulating the shape and movement of astrocytes [10]. While the information on cocaine-mediated regulation of GFAP is extant [11–13], there is paucity of information on the mechanism(s) involved in this process. Originally proposed to be a subtype of opioid receptor, the sigma-1 receptor(σ-1R) is now recognized as the non-opioid receptor family that binds to diverse class of psychotropic drugs including cocaine [14]. Expression of σ-1R is strongly upregulated in the brain after cocaine administration [15, 16]. σ-1R has also been closely linked to HIV-related pathogenesis [17, 18]. Activation of this receptor in microglia has been shown to increase HIV expression in microglia.[18] Our previous studies also demonstrated that cocaine-mediated activation of σ-1R modulate the expression of MCP-1 in microglia, a pivotal chemokine critical for migration of HIV-infected mononuclear phagocytes across the blood-brain barrier [19].

Acute cocaine administration has been shown to elicit rapid and transient induction of several immediate-early genes in the brain, including the early growth response-1(Egr-1) gene [20, 21], which belongs to a zinc finger family of transcription factors. It has been suggested that these proteins function as molecular switches to coordinate changes in gene expression and regulate the cellular responses. In the present study, we explored the intracellular mechanism(s) by which cocaine induced the expression of GFAP. Such an understanding will have clinical relevance in developing therapeutics aimed at targeting astrogliosis in cocaine abusers with or without HIV infection.

Materials and Method

Animals

Eighteen 2-month-old male C57BL/6N mice (Charles River, Sulzfeld, Germany) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All the animals were housed under conditions of constant temperature and humidity on a 12-h light, 12-h dark cycle, with lights on at 0700 h. Food and water were available ad libitum. Animals were

deeply anesthetized by overdose of isoflurane followed by pneumothorax prior to perfusion. All animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Cell cultures

Primary mice astrocyte cells were obtained from 1-3-day-old C57BL/6N newborn pups. After digestion and dissociation of the dissected brain cortices in Hank's buffered salt solution supplemented with trypsin (1mg/ml), cells were pelleted, resuspended and plated, and maintained in CO_2 incubator at 37°**C** for 7d. Cells were then shaken gently to remove loosely attached cells, and the attached cells that remained and that were primarily astrocytes, were allowed to grow for 3–4d, followed by passaging and seeding them on culture dishes. Seven days post plating, cultures consisted of 97% astrocytes as assessed by immunostaining using the GFAP antibody. Mouse primary astrocytes were cultured in DMEM medium (Invitrogen, Life Technologies, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 units/ml) and streptomycin (100 μg/ml). Human astrocytic cell line A172 (ATCC no. CRL-1620; American Type Culture Collection) was cultured as described previously[22].

Semi-quantitative and real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen, CA) according to the manufacturer's instructions and our previous reports [19]. 1 μg of RNA was used for cDNA synthesis according to manufacturer's instructions (Thermo Scientific, MA). The resulting cDNA was appropriately diluted, and amplified using Titanium TaqDNA polymerase and human σ-1R primers (forward: 5'-TATCGCAGTGCTGATCCA-3', reverse: 5' TACTCCACCATCCACGTGTT-3'). PCR products were examined by running on a 1.5% agarose gel. Real Time RT2 qPCR primer sets for human GFAP primers (forward: 5'-GGCGCTCAATGCTGGCTTCA-3', reverse: 5'-TCTGCCTCCAGCCTCAGGTT-3') were obtained from SA Biosciences (Frederick, MD). Quantitative analyses of mRNA were conducted using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, CA). Data were normalized using the cycle threshold (Ct) values for 18s in each sample. To calculate relative amounts mRNA, the average Ct values were subtracted from 18s values for each target gene to provide changes in Ct value. Fold change in expression was calculated as $\log_2$ relative units.

Short Interfering (si) RNA transfection

Astrocytes were transfected with 20 nmol/L siRNAs specific for either σ-1R or Egr-1 obtained from Thermo Scientific Dharmacon RNAi Technologies (Accell SMART Pool). The knockdown efficiency of siRNAs was determined 2 days post transfection by western blotting.

Transfection with plasmid constructs

Astrocytes were transfected with plasmid constructs containing either wild type (WT) or dominant negative [23] forms of Egr-1 using Lipo2000 (Invitrogen, CA) according to the manufacturer's protocol. Knockdown efficiencies were examined by western blotting.

Western blotting

Treated cells were lysed using the Mammalian Cell Lysis kit (Sigma, MO). Equal amounts of the proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (12%) under reducing conditions followed by transfer to PVDF membranes. The blots were blocked with 5% non-fat dry milk in phosphate buffered saline. Western blots were then probed with antibodies recognizing the phosphorylated and total forms of ERK, JNK, p38 (1:800, Cell Signaling, MA), GFAP (1:10,000,Santa Cruz, CA), Egr-1 (1:800, Santa Cruz, CA), σ-1R (1:800, Cell Signaling, MA) and β-actin (1:4000, Sigma). Cells were then incubated with goat-anti-rabbit secondary Ab (1:5000; Invitrogen, CA). Signals were detected by Chemiluminescence (Pierce, IL). All experiments were repeated at least three times individually, and representative blots are presented in the figures.

Lipid raft isolation and analysis

Lipid raft were isolated from confluent A172 cells treated with cocaine according to the previous report[24]. Briefly, lysates were mixed with 1 ml of 85% (w/v) sucrose, were overlaid with 35% & 5% sucrose (5ml each) and were centrifuged for 24h at 39,000 rpm (Beckman SW 4 rotor) and 4°C. Ten 1 ml fractions were collected from the top to bottom. The concentration of cholesterol and protein in each fraction was analyzed with lipid raft maker GM-1 antibody (1:1000, Abcam, CA) and a BCA protein assay kit (Pierce, IL).

Immunocytochemistry

Prior to treatment, cells were seeded on cover slips to perform immunocytochemical analysis of Egr-1 or GFAP. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature following treatment. The cells were then permeabilized with PBS containing 0.3% Triton X-100, and incubated with 10% normal goat serum PBS blocking solution for 1h. Following blocking, cells were incubated at 4°C overnight with anti-Egr-1 (1:200, Santa Cruz ,CA) rabbit polyclonal antibody or anti-GFAP (1:200,Santa Cruz, CA) mouse polyclonal antibody. Following extensive washing goat anti-rabbit and anti-mouse Alexa Fluor 488-conjugated secondary antibodies were used for signal detection. For negative controls, cells were treated as described above, but without the primary antibody. Cells were mounted with Prolong Gold containing DAPI to stain nuclei. Images were captured with an x40 objective.

Immunohistochemistry

Animals were divided into 3 groups (n = 6). These groups were treated with (1) saline, (2) cocaine, or (3) BD1047 and cocaine. Cocaine was injected intraperitoneally at a dose of 20 mg/kg once daily. In the BD1047 plus cocaine group, BD1047 was first injected for 2 days at the concentration of 3.5ug/kg, followed by cocaine injection for subsequent 7 days. Animals were sacrificed 1h after the final dose of cocaine for isolation of brain tissue for

sectioning. Mice were perfused transcardially using chilled 4% paraformaldehyde. Free-floating sections encompassing the entire brain were sectioned at 40μm using a cryostat. For GFAP and Egr-1 co-immunostaining, tissue sections were incubated with anti-Egr-1 (1:200, Santa Cruz, CA), rabbit anti-Mouse and anti-GFAP (1:200, Sigma, MO) rabbit anti-Mouse antibodies overnight at 4°C. Fluorescent tagged goat anti-mouse (594nm, Red), goat anti-rabbit (488nm, Green) secondary antibodies were used to probe GFAP and Egr-1 expression, respectively. Immunostained floating tissue samples were gelatin mounted and examined for co-localization of GFAP and Egr-1 protein. Images were captured at wavelengths encompassing the emission spectra of the probes with a 40× objective.

For immunohistochemistry, wections (5μm thick) from paraffin-embedded human cortex (National NeuroAIDS Tissue Consortium) (Table 1) were chosen followed by deparaffinization and rehydration. Immunostaining with GFAP antibody involved boiling the tissue sections in Tris/EDTA buffer (pH-9) for about 20 minutes for efficient antigen retrieval. Sections were blocked with 10% Normal Goat Serum (NGS) for 1hr at RT and then incubated in primary anti-GFAP (1:50,, Sigma) antibody at 4°C overnight. The sections were then incubated with biotin-labeled secondary antibody (Southern Biotechnology Associates) at a 1:500 dilution for 2 hr at RT followed by incubation with streptavidin-conjugated horseradish peroxidase (HRP; Southern Biotechnology Associates) at a 1:500 dilution for 1 hr at RT. HRP-labeled areas were detected using a solution containing 0.3 mg/ml of 3-3'-diaminobenzidine (DAB) in 100 mM Tris, pH 7.4 and 0.02% hydrogen peroxide (H_2O_2). Sections were examined under bright-field light microscopy and images were captured with a 40x objective.

Statistical analysis

All the data are expressed as the mean ± SEM. Statistical significance was evaluated with Student t-test for between two groups or ANOVA followed by the Newman-Keuls' test for multiple groups. $P<0.05$ was considered as statistically significant difference.

Results

Upregulation of GFAP expression in astrocytes from HIV-infected drug (cocaine) abusers

To establish the clinical relevance of GFAP expression in the context of HIV-1 infection with drug (cocaine) abuse, we examined GFAP expression levels in the sections of postmortem brain tissues from HIV^-, HIV^+/no cocaine, and HIV^+/cocaine drug abusers (Fig.1a). Because of the inherent difficulty in finding tissues from unidrug abusers, we resorted to samples from polydrug abusers that included a history of cocaine abuse. As shown in Fig.1b, there was a significant increase in the expression of GFAP positive cells in the cortical sections of brains from HIV^+ positive patients compared with the uninfected controls due to the global activation of astrocytes in the HIV-infected brain. Interestingly, HIV+/cocaine abusers exhibited an even more increased activation of astrocytes exemplified by hypertrophic astrocytes, compared with the HIV+ individuals.

Cocaine-mediated up-regulation of GFAP mRNA and protein in astrocytes

In the present study, as an initial investigation to examine the potential modulation of GFAP expression following cocaine treatment, human astrocyte A172 cells were first serum-starved overnight followed by exposure to varying concentrations of cocaine (1–100 μM) for 3 hr. Messenger RNA levels of GFAP were determined by real-time PCR. The rationale for using the chosen concentrations of cocaine is based on the premise that in human volunteers cocaine concentrations in the plasma following intranasal administration of 1.5 mg/kg cocaine usually range from 0.4 to 1.6μM [25] , while plasma cocaine concentrations in tolerant abusers, reach levels of up to 13 μM [26]. Additionally, cocaine concentrations of up to 100μM and higher have been reported in postmortem brains of chronic human cocaine users following acute intoxication [27]. As shown in Fig.2a, GFAP mRNA was maximally up-regulated in A172 cells exposed to 10μM cocaine. A concentration of 10μM cocaine was thus used for all the subsequent experiments.

Having determined that cocaine mediated increased expression of GFAP mRNA, we next examined whether the mRNA up-regulation translated into increased protein expression. A172 cells were treated with cocaine for various time points (1 to 24 hrs), followed by cell lysis and subjected to immunoblotting. As shown in Figure.2b cocaine time-dependently up-regulated GFAP protein expression with maximal expression at 12 hr post-cocaine treatment in A172 cells. To further confirm the result in mouse primary astrocytes, cells were serum-starved overnight followed by treatment with 10μM cocaine for 1 to 24 hours. As shown in Fig.2c cocaine exposure resulted in induction of GFAP in mouse primary astrocytes. Confirmation of this finding by immunostaining also revealed increased GFAP expression with a characteristic alteration in the morphology of primary astrocytes following 10μM cocaine treatment for 6 hr (Fig.2d). As is evident in the representative image cocaine treated astrocytes displayed more hypertrophic looking cells with larger cell bodies and increased cellular size. Cumulatively, these data clearly demonstrated cocaine-mediated induction of GFAP both at the mRNA and protein levels in astrocytes.

Cocaine-induced activation of MAPK (ERK, JNK, p38) pathways

To better understand how cocaine modulated GFAP expression in astrocytes, we next sought to elucidate the signaling pathways involved in this process. Since MAPK kinase pathways play critical roles in cocaine-mediated signaling [28, 29], the next logical step was to examine the role of these pathways in cocaine-mediated induction of GFAP. As shown in Fig.3a, treatment of A172 cells with 10 μM cocaine resulted in time-dependent increase in phosphorylation of ERK, JNK, p38 kinases. Further, using a pharmacological approach, we were able to confirm activation of the respective signaling pathways following cocaine treatment. Pretreatment of cells with either the MEK (U0126), JNK (SP600125), or p38 (SB203580) inhibitor(s) followed by treatment with 10 μM cocaine for 30mins resulted in amelioration of cocaine-mediated induction of ERK, JNK and p38 (Fig.3b-d) respectively.

Involvement of Egr-1 in cocaine-mediated up-regulation of GFAP in astrocytes

Since acute cocaine administration has been shown to elicit rapid and transient induction of several immediate-early genes in the brain, including Egr-1, c-fos and junB [30] and Egr-1 has been suggested to function as a molecular switch that coordinates changes in GFAP

expression [31], we sought to examine whether cocaine-mediated induction of GFAP expression involved activation of Egr-1 in astrocytes. As shown in Figure 4a & b, cocaine up-regulated expression of Egr-1 in a time-dependent manner with peak expression elicited between 1–3h and a decline thereafter, both in A172 cells and in mouse primary astrocytes. This was further validated by immunostaining A172 cells for Egr-1 expression. As shown in Fig.4c, cells treated with 10 μM cocaine for 15 mins demonstrated increased immunostaining for Egr-1 compared with untreated cells.

We next examined the role of Egr-1 in cocaine-mediated induction of GFAP using the gene silencing approach. Briefly, A172 cells were transfected with either the short interfering RNA (siRNA) for Egr-1 or a scrambled construct and assessed for Egr-1 knockdown. As shown in Figure 3d, transfection of A172 with Egr-1 siRNA resulted in efficient knockdown of Egr-1 protein as evidenced by western blotting. Intriguingly, Egr-1 siRNA significantly abrogated cocaine-mediated up-regulation of GFAP expression (Fig. 4e). To further validate the involvement of the Egr-1 in cocaine-induced regulation of GFAP, cells were transfected with either WT or DN constructs of Egr-1, followed by exposure with 10 μM cocaine for 12 hr. Cocaine-mediated induction of GFAP was attenuated in cells transfected with the DN-Egr-1 construct, but not in cells transfected with the WT-Egr-1 construct (Fig.4f). Together these data clearly underscore the role of Egr-1 in cocaine-mediated induction of GFAP.

MAPK (ERK, JNK, p38) pathways are involved in cocaine-mediated Egr-1 and GFAP expression in astrocytes

Having determined the activation of ERK, JNK and p38 MAPKs in cocaine-treated A172 cells, the next logical step then was to examine whether there existed a link that could tie together the activation of ERK1/2, JNK and p38 MAPKs with Egr-1. Similar to the studies on signaling molecules described above, A172 cells were pretreated with inhibitors specific for the respective signaling pathways prior to stimulation with cocaine and assessed for expression of GFAP. As shown in Fig.5a, all three MAPK pathways were demonstrated to be involved in cocaine-induced activation of Egr-1. Next we wanted to address the functional role of MAPKs in cocaine-mediated induction of GFAP expression. Briefly, A172 cells were pretreated with the respective MAPK inhibitors followed by exposure to cocaine and assessed for expression of GFAP by immunoblotting. As shown in Fig.5b, all the three pharmacological inhibitors abrogated cocaine-mediated induction of GFAP expression. Collectively, these findings thus confirmed the involvement of ERK, p38 as well as the JNK MAPK cascade in cocaine-mediated induction of GFAP in A172 cells.

Cocaine-mediated activation of Sigma-1 receptor (σ-1R) and its translocation to the lipid rafts

Several studies have demonstrated the translocation of ER residing σ-1R following stimulation with ligands such as cocaine and (+) Pentazocin [32]. It has been shown that upon ligand binding σ-1Rs translocate from the mitochondrial-ER (MAM) membrane to other areas of the cell including the extended ER reticular network, the plasmalemmal area or the plasma membrane lipid raft domains where they interact and regulate the function of a variety of ion channels, receptors or kinases [19]. To examine the role of σ-1R in cocaine-mediated induction of GFAP, we first examined whether A172 cells did indeed express

σ-1R. As shown in Fig.5a, these cells expressed both the σ-1R RNA (Fig.6a) and protein (Fig.6b) as demonstrated by RT-PCR and immunoblotting, respectively. To assess the translocation of σ-1R following treatment with cocaine, stimulated A172 cells were lysed and fractionated by ultracentrifugation for isolation of the lipid rich domains of the plasma membrane. As shown in Fig.6c, there was a significant increase in σ-1R levels in the lipid raft domain of cocaine-treated cells compared with the untreated cells. Lipid raft domain was characterized by the GM-1 marker. Further validation of this phenomenon was confirmed by immunofluorescence. Briefly, A172 cells were treated with cocaine and immunostained using antibodies specific for GM-1 and σ-1R. The overlay images indicated that cocaine treatment resulted in translocation of σ-1R to the lipid raft with its colocalization with the GM-1 rich microdomains. Untreated cells exhibited a diffuse pattern of σ-1R staining in the cytoplasm (Fig.6d).

Engagement of σ-1R is critical for cocaine-mediated induction of GFAP expression

Having determined that cocaine activated σ-1R and triggered its translocation to the lipid raft microdomains, we next sought to assess the role of σ-1R in cocaine-mediated induction of GFAP in astrocytes. To examine the involvement of σ-1R in this process, we used σ-1R antagonist BD1047 (10mM) to block its function. As shown in Fig.6, cocaine-mediated induction of Egr-1 (Fig.7a) and GFAP (Fig.7b) was significantly attenuated in cells pre-treated with the known σ-1R antagonist BD1047. To further validate the role of σ-1R in cocaine-mediated regulation of GFAP expression, we used the knockdown approach by transfecting A172 cells with either the σ-1R or the scrambled siRNA construct. As shown in Figs.7c & d, σ-1R siRNA transfected cells exhibited significant abrogation of cocaine-mediated induction of Egr-1 as well as GFAP, thereby underscoring the role of σ-1R in this process.

Cocaine-mediated induction of GFAP *in vivo* involves activation of σ-1R

To validate the role of cocaine/σ-1R axis in cocaine-mediated induction of GFAP *in vivo* we resorted to treating mice with cocaine in the presence of absence of the σ-1R antagonist BD1047. Briefly, mice were divided into three groups – a) saline; b) cocaine; and c) BD1047 and cocaine treated. For the BD1047 treated group, mice were pre-treated for 2 days with IP injection of BD1047 (3.5ug/kg) prior to cocaine administration, which was given daily at a concentration of 20 mg/kg for 7days. As expected from our cell culture studies, cocaine administration in the cortex resulted in increased expression of both Egr-1 and GFAP compared with saline injected mice in the same region, as demonstrated by immunohistochemistry and, this effect was ameliorated in mice pretreated with BD1047 (Fig.8a). To further confirm our findings, cortical regions were isolated from the three groups of mice and homogenates from these regions were assessed for expression of both Egr-1 and GFAP by western blot. As shown in Figure 8b, cocaine-administered mice demonstrated increased expression of both Egr-1 and GFAP in brain tissues compared with the saline group. Mice pretreated with BD1047 demonstrated attenuated expression of both Egr-1 and GFAP compared with the cocaine-treated animals.

Discussion

Although antiviral therapies have had a profound impact on controlling systemic HIV-1 load, the poor penetrative property of these anti-retrovirus drugs coupled with increased longevity of infected individuals makes the brain a sanctuary for virus-induced toxicity, thereby leading to increased prevalence of HAND [2]. Adding fuel to this problem is the increased use of illicit drug abuse that is also a common problem in HIV-1 infected individuals [33]. Intriguingly, increased use of the psychostimulant cocaine in HIV-1 infected individuals has been linked to increased progression of HAND [6, 4]. One of the hallmark features of HAND is reactive astrocytosis, exemplified by enhanced GFAP expression, cellular hypertrophy, and astrocyte dysfunction [1]. While the role of HIV proteins such as Tat and GP120 in mediating astrocytosis is well characterized, very few studies have explored the detailed mechanism(s) by which cocaine triggers astrocyte activation exemplified by increased expression of GFAP expression, and leading to enhanced cognitive decline and disease pathogenesis in cocaine-abusers

Astrocyte activation is a finely tuned process of progressive change encompassing changes in gene expression and cellular morphology that are subtly regulated by complex intercellular and intracellular signaling pathways [34]. The extent of various molecular changes involved in this process is not well characterized. Upregulation of the intermediate filament proteins, in particular GFAP, by reactive astrocytes is perhaps the best-known hallmark feature of reactive astrogliosis [34, 35]. Since cocaine abuse has been associated with glial activation, we undertook the present study to explore the molecular pathways involved cocaine-mediated activation of astrocytes. Herein we report that cocaine exposure of both human astrocyte cell line and primary mouse astrocytes resulted in both transcriptional and translational induction of GFAP. These findings are in agreement with previous *in vivo* report indicating the induction of GFAP expression following acute cocaine injection in mice [9]. Intriguingly, using human brain cortical sections from HIV+/cocaine drug abusers, we found prominent upregulation of GFAP compared with either the HIV+ or the control uninfected groups. These findings add further validity to previous reports [22, 36] indicating cocaine-mediated exacerbation of astrocyte activation and the ensuing neuroinflammatory responses in the context of HIV infection..

σ-1R are unique drug-binding proteins that are present in the CNS, as well as in the periphery [37]. Cocaine is known to exhibit moderate affinity for σ-1R which are expressed in most neuronal cells [14]. The role of σ-1R in cocaine-induced immune alteration and HIV expression has been the focus of many studies, for example, findings from Gekker *et. al* demonstrated that σ-1R antagonist BD1047 blocked cocaine-stimulated increase of HIV-1 expression in microglial cells [18]. Moreover, σ-1R activation via alteration of lipid components is known to cause a remodeling of plasma membrane lipid rafts, thereby facilitating signal transduction triggered by growth factors and other mediators [19, 32]. In agreement with these findings, our data also demonstrated cytoplasmic localization of σ-1R in untreated cells, with a translocation of the receptor to the lipid raft domains of the plasma membrane following cocaine exposure. Our data implicated that in addition to inflicting addictive properties [15, 38], activation of σ-1R in the brain may also be involved in development of cocaine-induced activation of astrocytes and that, this in part, could be a

possible mechanism of increased neuropathogenesis observed in HIV-infected cocaine abusers.

Downstream of σ-1R activation, we also demonstrated cocaine-mediated activation of various signaling pathways, which ultimately led to increased expression of GFAP in astrocytes, and further that, this effect of cocaine was significantly abrogated in cells pretreated with either BD1037 or transfected with σ-1R siRNA. Cocaine mediated activation of MAPK signal transduction has been documented in reactive astrocytes in diverse animal models of CNS pathogenesis [28, 39]. A recent study also demonstrated a functional link between JNK activation and GFAP accumulation in astrocytes [40]. Consistent with these findings, using both pharmacologic and genetic approaches, we also demonstrated activation of ERK1/2, JNK and p38 MAPK pathways in astrocytes exposed to cocaine.

The zinc finger family of transcription factors, of which Egr-1 is a member, undergo a rapid and transient activation following growth factor(s) and/or environmental stimuli including hypoxia, physical force, or vascular injury [23]. Previous studies have suggested a correlation between increased activation of Egr-1 to increased expression of pivotal regulators of inflammation such as cytokines(IL-1β), chemokines(MIP-2, MCP-1) as well as GFAP expression [41, 42]. Our findings have demonstrated a time-dependent up-regulation of Egr-1 expression in astrocytes treated with cocaine. Further validation of the role of Egr-1 involved both the pharmacological and genetic approaches. These findings suggested that cocaine-mediated activation of ERK and JNK MAPKs pathways were upstream of Egr-1. Interestingly, our findings are in agreement with a report from Fan *et a*l demonstrating that HIV Tat-mediated astrogliosis involves activation of Egr-1 and transcriptional upregulation of GFAP [7]. It is likey that Egr-1 is one of the common signaling molecules critical for both Tat and cocaine-mediated activation of astrocytes.

In summary, our findings have demonstrated a detailed molecular pathway of cocaine-mediated induction of GFAP in astrocytes both *in vitro* and *in vivo*. Our findings suggest that in astrocytes ligation of σ-1R following exposure of cocaine triggers its translocation to the plasma membrane, leading to activation of the MAPK pathway cascade with the downstream activation of the transcription factor Egr-1, which in turn, transcriptionally upregulates GFAP expression. Our finding of increased astrogliosis in brain sections of patients with HIV and cocaine abuse compared with the HIV-infected group further corroborates the role cocaine in activating cellular pathways leading to exacerbated pathology of HAND. These findings are in agreement with those reporting the role of HIV protein Tat in upregulating GFAP in astrocyte[7]. It is intriguing that drugs of abuse such as cocaine can hijack the host signaling response similar to the virus/viral proteins. These findings have clinical ramifications for the development of therapeutic strategies aimed at dampening astrogliosis as a treatment modality for cocaine abusers with or without HIV infection.

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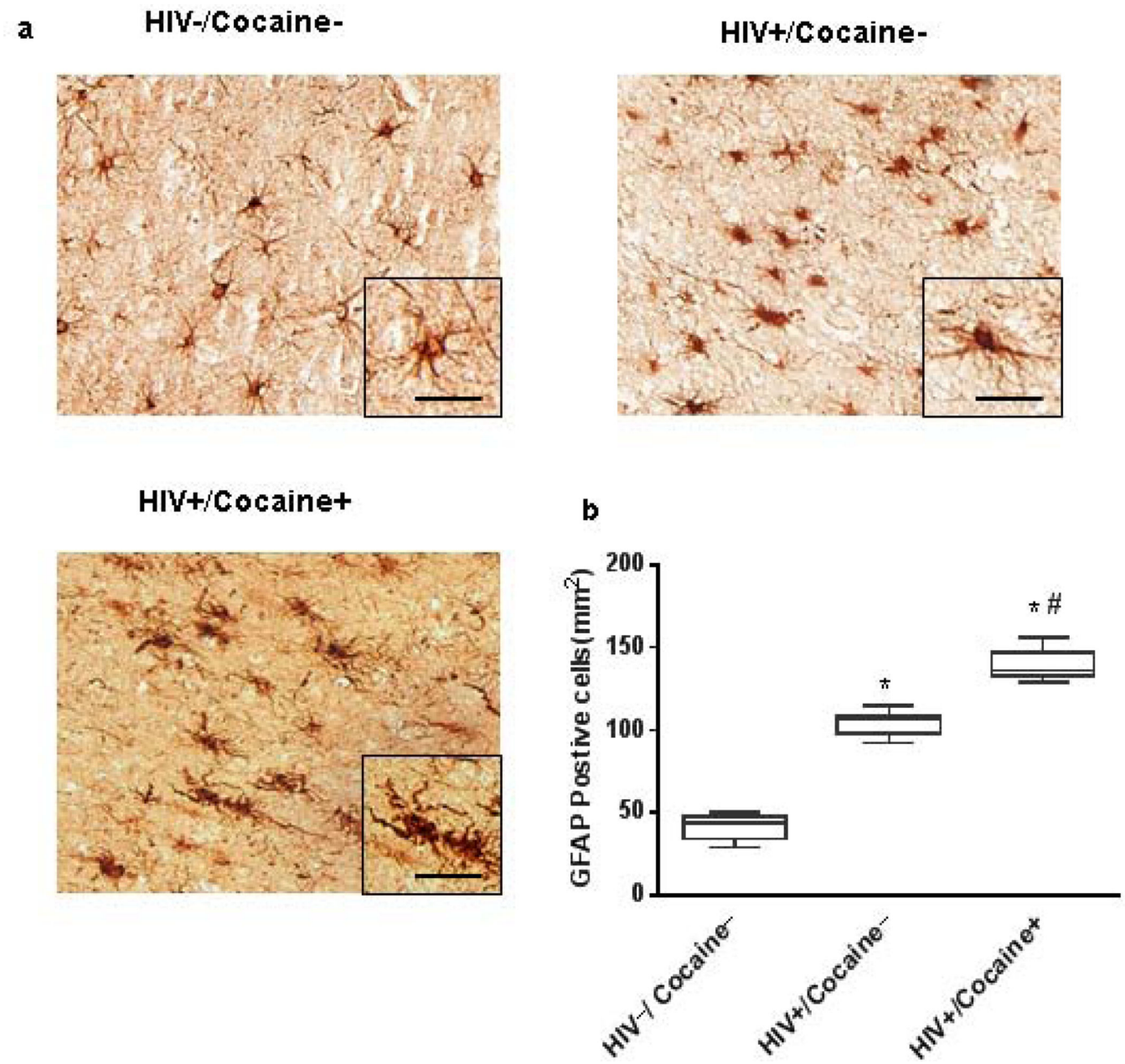

Figure 1. Induction of GFAP expression in astrocytes from HIV-infected drug (cocaine) abusers

(a) Representative images after immunostaining for GFAP showed the increased expression of GFAP positive cells in the cortical sections of brains from HIV+ cocaine abusers patients compared with the HIV+ sections with the latter group demonstrating increased GFAP expression compared to the uninfected controls. Scale bar: 20μm. **(b)** Cell counting using ImageJ indicates that drug (cocaine) abuse resulted in a significant increase of GFAP positive cells per brain section. n=3 sections, threshold=50, * $P<0.05$vs HIV/Cocaine^{-} group, $^{\#}p<0.05$vs HIV^{+}/Cocaine^{-} group.

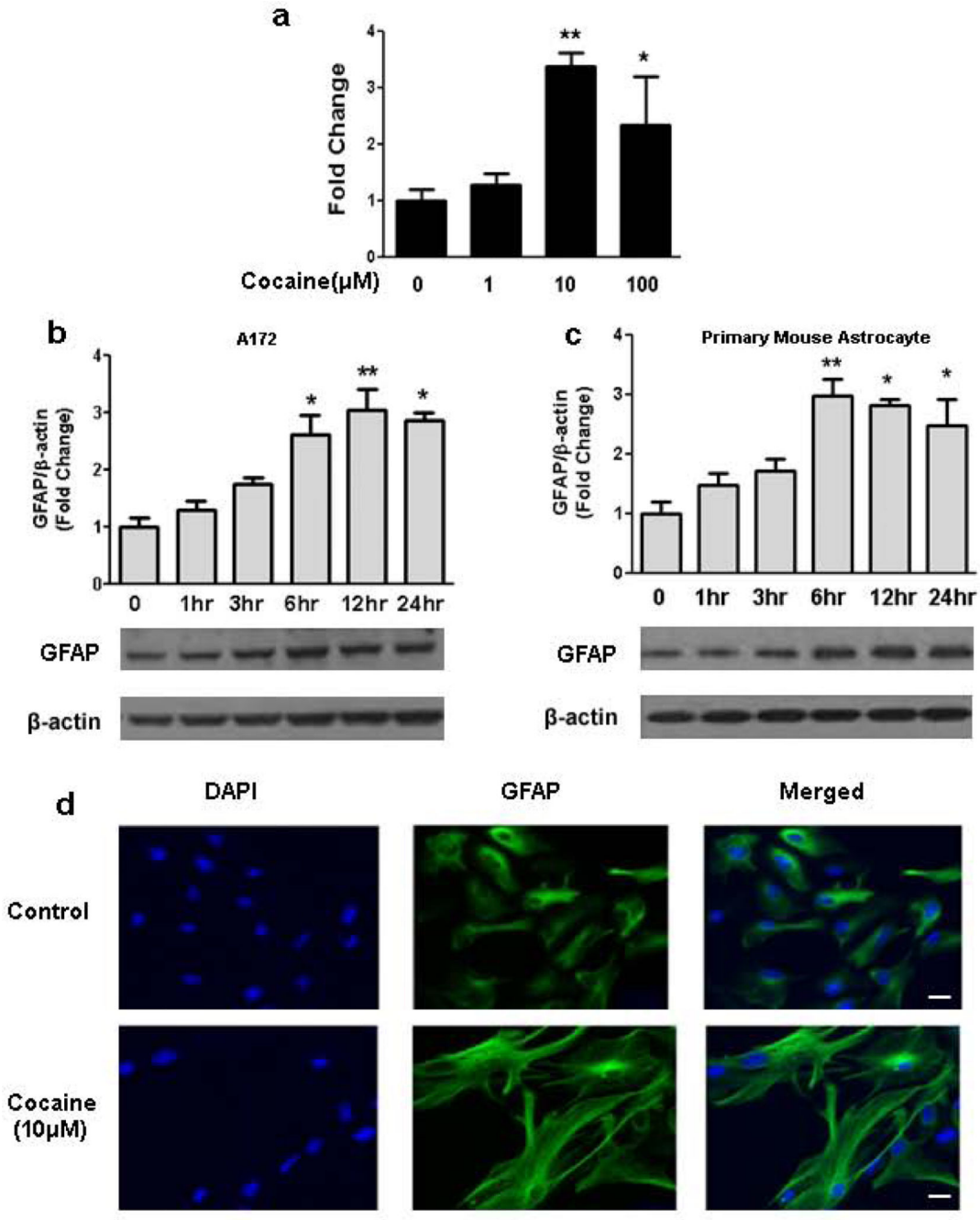

Figure 2. Cocaine induced up-regulation of GFAP expression in human A172 cells & mice primary microglia

(a) Cocaine-mediated induction of GFAP mRNA expression by real-time RT-PCR. Cells were incubated with various concentrations of cocaine (1–100μM) for 12 hr and processed for RNA isolation and real-time PCR. **(b)** Time course of cocaine-mediated induction of GFAP expression in A172 cells. **(c)** Time-course of cocaine-mediated induction of GFAP expression in mouse primary astrocytes. **(d)** Representative image of GFAP staining in

mouse primary astrocytes. Scale bar: 20μm. All data are presented as mean±SEM of 4 individual experiments. *p<0.05, **p<0.01 vs control group.

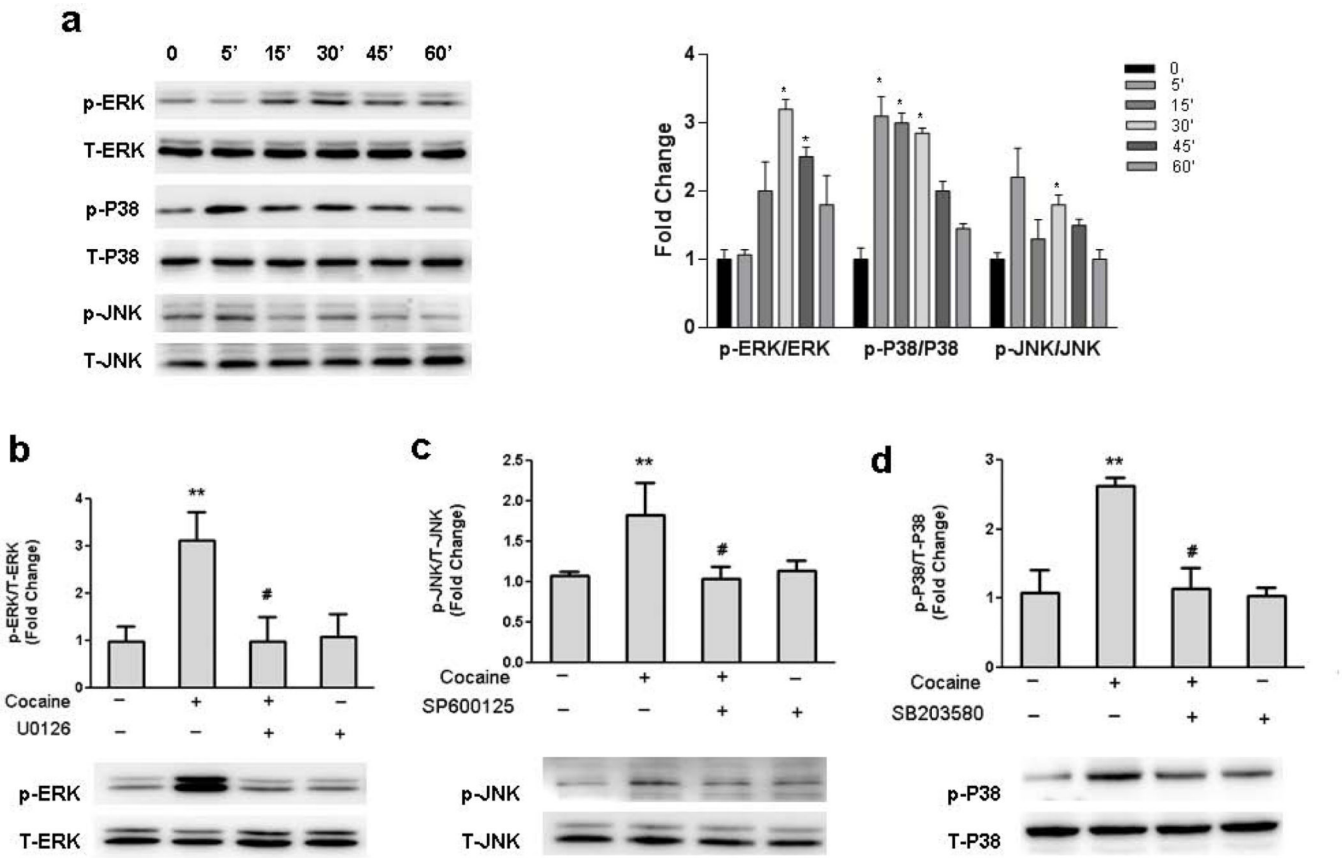

Figure 3. MAPK pathways are activated during cocaine exposure

(a) Treatment of A172 cells with cocaine resulted in increased phosphorylation of ERK, JNK and p38 kinases. **(b)** Pretreatment of cells with ERK pathway inhibitor U0126 (5μM) abrogated cocaine-mediated phosphorylation of ERK. **(c)** Pretreatment of cells with p38 pathway inhibitor SB203680 (10μM) decreased the phosphorylation of p38. **(d)** Pretreatment of cells with JNK pathway inhibitor SP600125 (10μM) abrogated cocaine-mediated phosphorylation of JNK. All data are presented as mean±SEM of 4 individual experiments. **p<0.01 vs control group; #p<0.05 vs cocaine treated group.

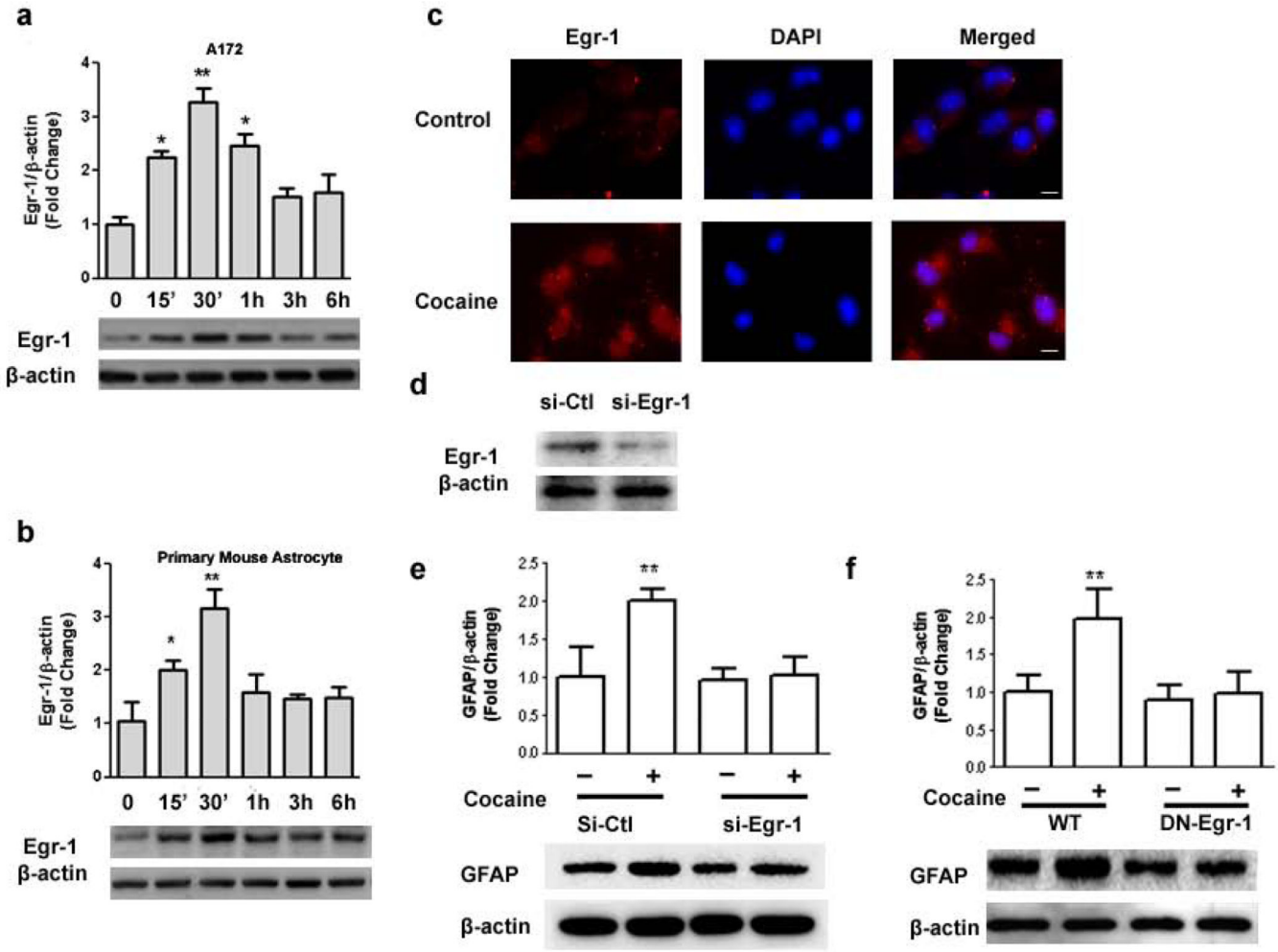

Figure 4. Involvement of Egr-1 in cocaine-mediated up-regulation of GFAP

Cocaine induced time-dependent activation of Egr-1 expression in lysates from **(a)** A172 cells and **(b)** mouse primary astrocytes. **(c)** Representative images of Egr-1 staining in mouse primary astrocytes. Scale bars:20 μm **(d)** Western Blot demonstrating the knockdown efficiency of Egr-1 in A172 cells. **(e)** Egr-1 siRNA but not non-specific siRNA inhibited cocaine-mediated induction of GFAP. **(f)** DN-Egr-1 but not WT-Egr-1 inhibited cocaine-mediated induction of GFAP. All data are presented as mean±SEM of three individual experiments. *p<0.05, **p<0.01 vs control group.

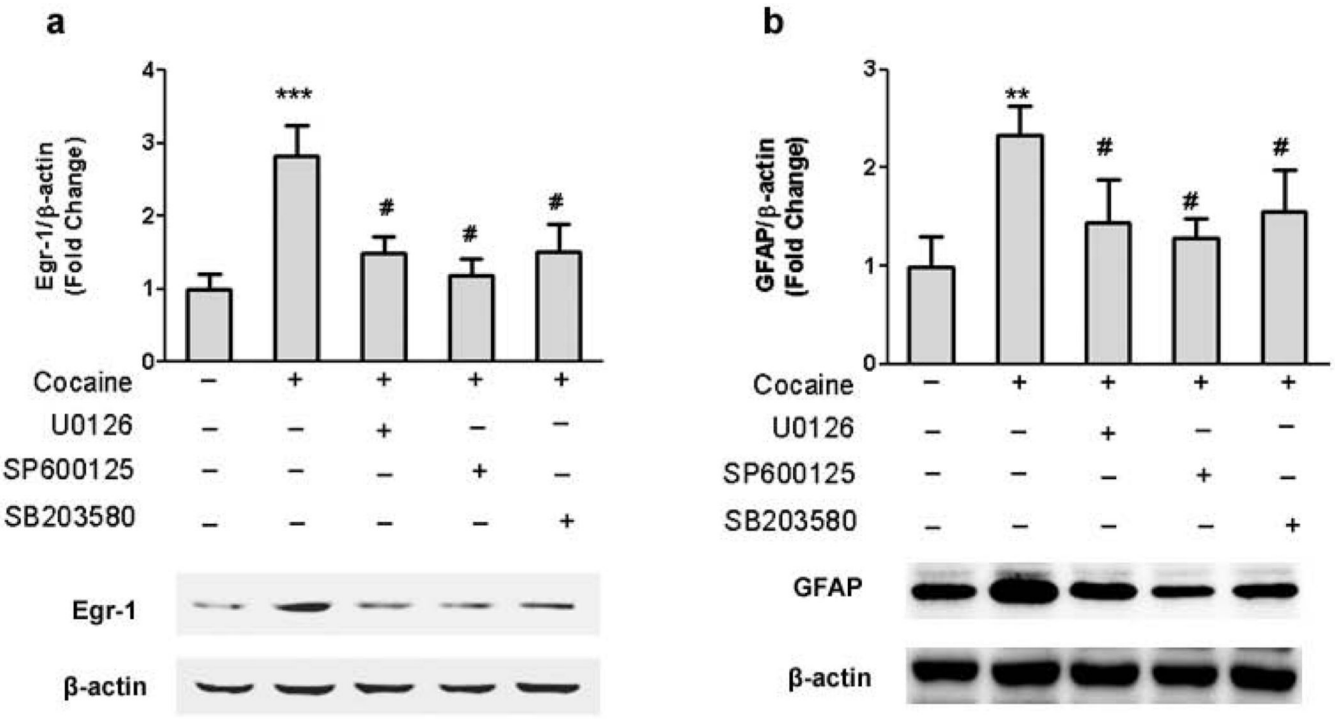

Figure 5. ERK1/2, JNK and p38 pathways are involved in cocaine-mediated in-regulation of GFAP expression in astrocytes

(a) Pharmacological inhibition of ERK1/2 and JNK pathways by MEK (U0126 - 5μM), JNK (SP600125 -10μM) and p38 (SB203580 - 10μM) inhibitors resulted in amelioration of cocaine-mediated induction of Egr-1. **(b)** Pretreatment of cells with MEK (U0126 -5μM), JNK (SP600125 -10μM) and p38 (SB203580 -10μM) inhibitors resulted in amelioration of cocaine-mediated induction of GFAP. All data are presented as mean±SEM of three individual experiments. **p<0.01, ***p<0.001 vs control group; $^{\#}$p<0.05 vs cocaine treated group.

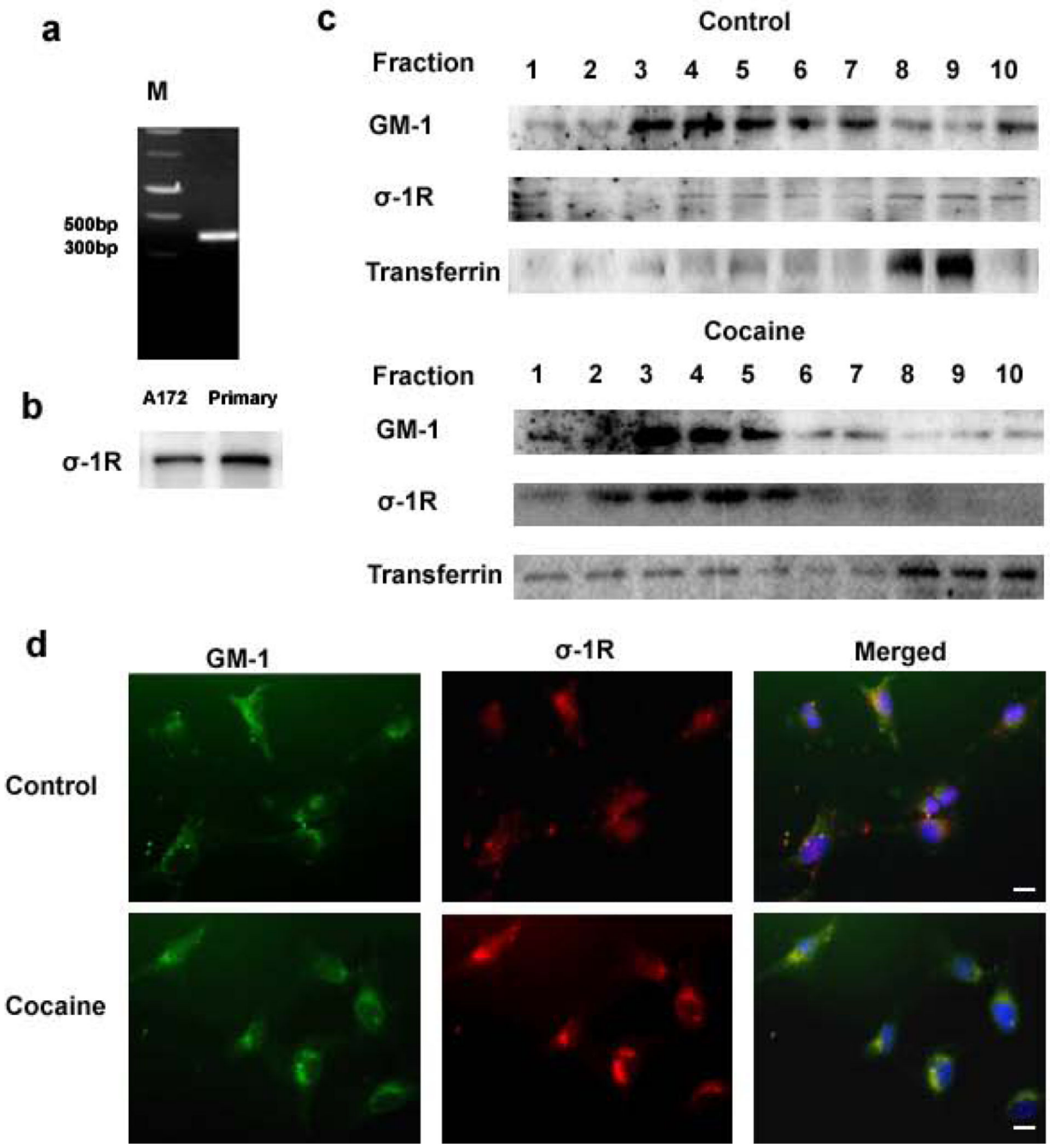

Figure 6. Cocaine exposure results in translocation of σ-1R to lipid rich domains within the astrocytes

(a) RT-PCR and western blotting demonstrating the expression of σ-1R in both human and primary mice astrocytes. **(b)** Detergent-soluble and detergent-resistant membrane fractions were separated using density gradient ultracentrifugation. Western blot analysis of the lysates from two fractions demonstrated translocation of the σ-1R to the lipid raft fractions (GM-1 rich region) compared with lysates from untreated cultures. **(c)** Representative image of immunofluorescence staining demonstrating increased colocalization of the σ-1R with the

lipid raft GM-1 rich microdomains. Untreated cells exhibited a diffuse pattern of σ-1R staining in the cytoplasm. Scale bar: 20μm

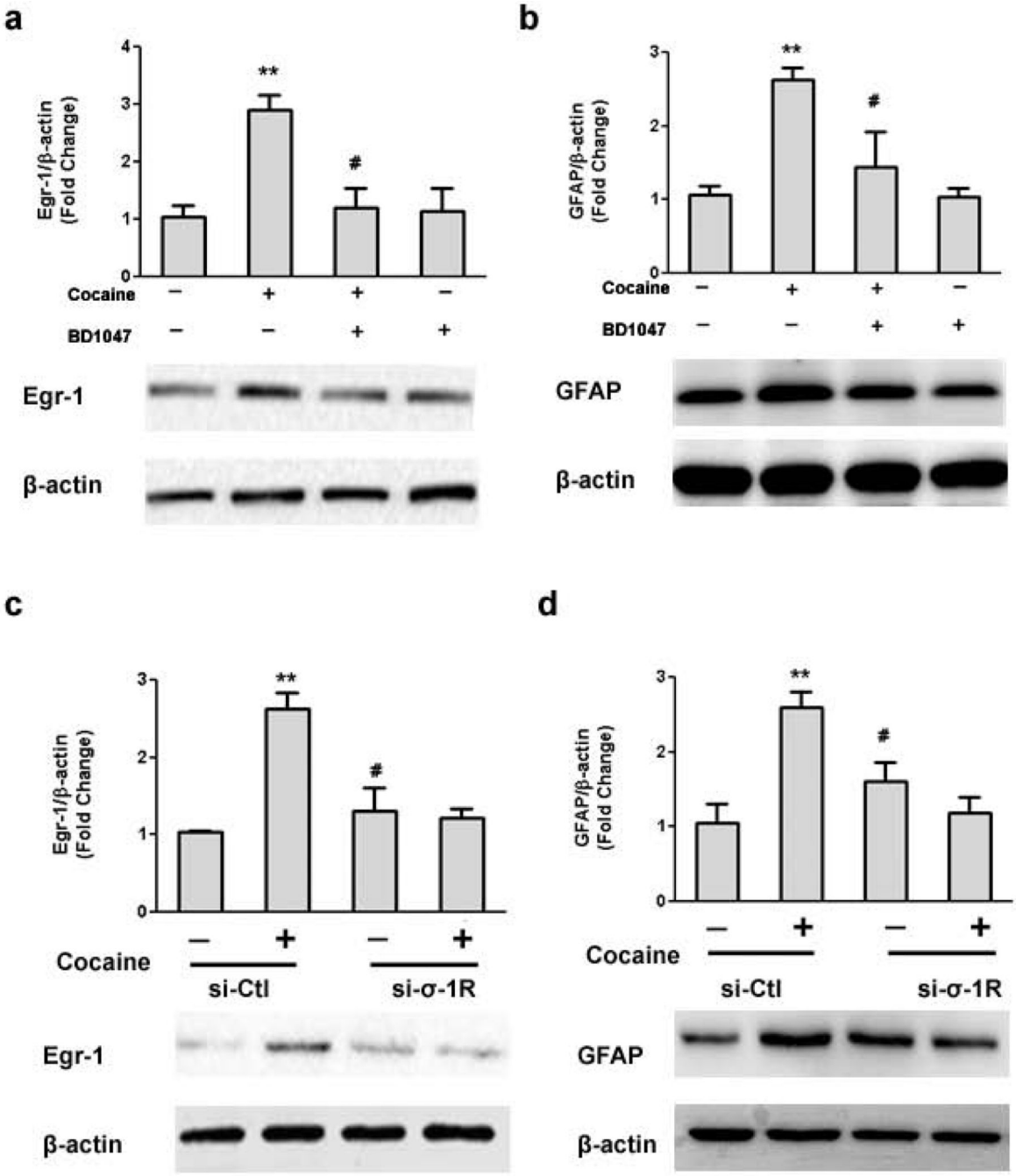

Figure 7. σ-1R is critical for cocaine-induced GFAP expression

Pretreatment of A172 with the σ-1R antagonist BD1047 inhibited cocaine-mediated induction of Egr-1 expression **(a)** and GFAP expression (**b**). σ-1R siRNA but not non-specific siRNA inhibited cocaine-mediated induction of Egr-1 **(c)** and GFAP **(d)** expression. All the data are presented as mean±SEM of four individual experiments. *p<0.05, **p<0.01 vs control group; #p<0.05, ##p<0.01 vs cocaine-treated group.

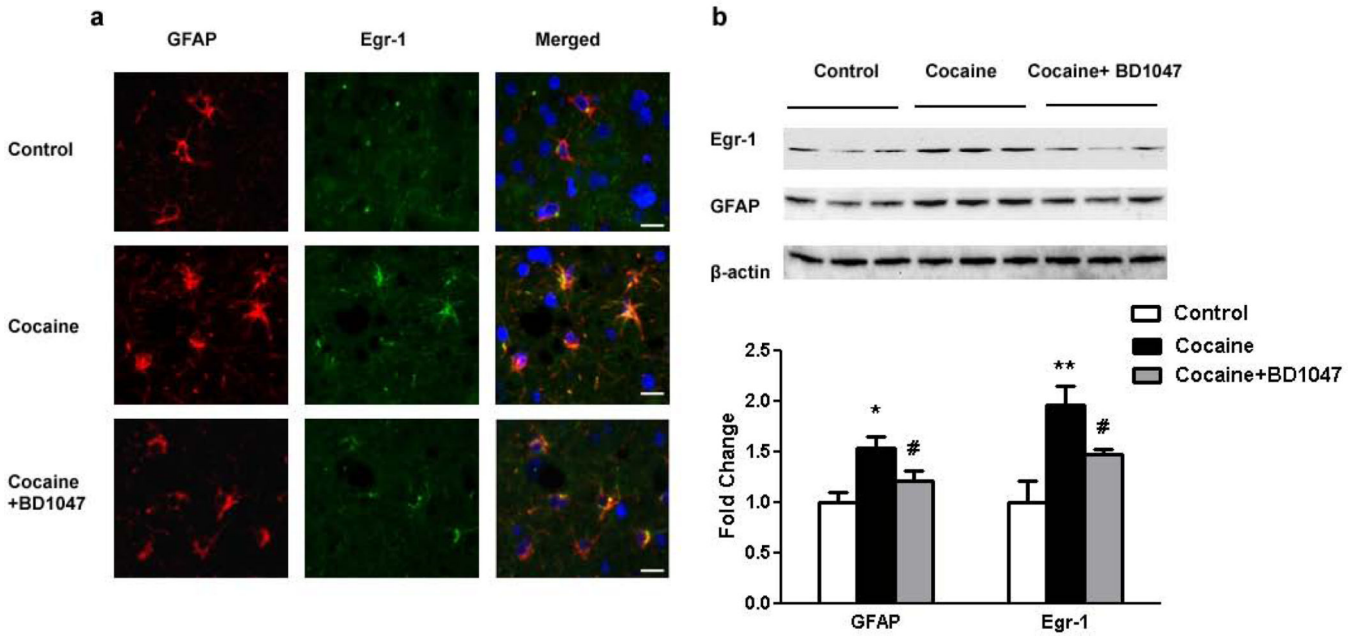

Figure 8. Cocaine-mediated activation of Egr-1 expression and GFAP up-regulation involves σ-1R activation *in vivo*

(a) Cocaine administration resulted in increased expression of GFAP and Egr-1 (middle panels) compared with saline-injected animals (top panels). Pre-treatment of the mice with σ-1R antagonist BD1047 followed by cocaine administration (bottom panels) failed to upregulate both GFAP and Egr-1 expression. Scale bar: 20μm. **(b)** Western blotting demonstrating that cocaine administration to mice resulted in increased expression of Egr-1 and GFAP in brain homogenates, and that this effect of cocaine was ameliorated in mice pretreated with the σ-1R antagonist BD1047. All the data are presented as mean±SEM of four individual experiments. *$p<0.05$, **$p<0.01$ vs control group; #$p<0.05$ vs cocaine-treated group.

Table 1

Clinical data for human brain tissue samples

Case No.	HIV-1 infection	Age (yr)	Gender	Viral load (Plasma)	CD 4 Count	Neurocognitive Disorders	Antiretroviral therapy	Pathological evidence of encephalitis	Drug abuse history
1(3015)	Seronegative control	64	Female	NA	118	Neuropsychological impairment or dementia due to other case	None	HIV−, PML	None
2(ID: 7101958083)	Seronegative control	46	Male	NA	NA	Unable to reliably assign neurocognitive diagnosis	None	Normal	None
3(4084)	HIV+	46	Male	40,519	18	Neuropsychological impairment or dementia due to other case	None	HIV-E, microglial nodule encephalitis	None
4(5007)	HIV+	37	Female	750,000	33	Possible HAD	TFV,dd1,KTA	HIV-E Alzheimer type2, gliosis	None
5(4151)	HIV+	58	Male	4,064	25	Possible HAD	TFV,RTV,DAR, KTA,TZV,ATV, TRU,ATR,ETC	HIV-E	Cocaine, Marijuana, Others NA
6(1004)	HIV+	49	Male	41,124	77	Possible HAD	TFV,dd1,KTA, ATR,ETC	HIV-E	Cocaine, others NA