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RESEARCH ARTICLE

Coronin 1A facilitates calcium mobilization and promotes astrocyte reactivity in HIV-1 neuropathogenesis

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Abstract

Astrocyte reactivity, a phenomenon observed in a variety of neurodegenerative disorders, can have both beneficial and detrimental manifestations which significantly affect neuronal physiology. In neuroAIDS, reactive astrocytes have been observed to severely affect the neuronal population present in their vicinity. Calcium signaling plays a central role in mediating astrocyte reactivity. Coronin 1A, an actin-binding protein, majorly reported in hematopoietic cells, regulates cell activity in a calcium-dependent manner, but its role in astrocyte physiology and reactivity is largely unknown. Using a well-characterized primary culture of human astroglia and neurons, we explored the roles of coronin 1A in astrocyte physiology and its involvement in facilitating astrocyte reactivity. In this study, we report coronin 1A expression in human primary astrocytes and autopsy brain sections, and that it plays activity-dependent roles by facilitating calcium mobilization from the intracellular stores. HIV-1 Tat, a potent neurotoxicant that turns astrocytes reactive, augments coronin 1A expression, apart from affecting GFAP and pro-inflammatory molecules. Also, the autopsy brain tissue of HIV-1 infected individuals has a higher expression of coronin 1A. Downregulation of coronin 1A attenuated the HIV-1 Tat-induced deleterious effects of reactive astrocytes, measured as the upregulated expression of GFAP, pro-inflammatory molecules, and enhanced release of IL-6, and hence reduced astrocyte-mediated neurodegeneration. Our findings also suggest that out of a pool of dysregulated miRNAs studied by us, hsa-miR-92b-5p regulates coronin 1A expression under the effect of HIV-1 Tat. These findings highlight the novel roles of coronin 1A in regulating astrocyte activity in stimulated conditions and astrocyte reactivity observed in HIV-1 neuropathogenesis.

Abbreviations: ACM, astrocyte-conditioned media; AIDS, acquired immunodeficiency syndrome; ATP, adenosine triphosphate; GFAP, glial fibrillary acidic protein; HAND, HIV-1 associated neurocognitive disorder; HIV-1, human immunodeficiency virus -1; ICAM1, intercellular cell adhesion molecule-1; IL-6, interleukin-6; NOS2, nitric oxide synthase-2; NPC, neural precursor cells; PDA, progenitor-derived astrocytes; PDN, Progenitor-derived neurons; PLC, phospholipase C; PTGS2, prostaglandin-endoperoxide synthase-2; Tat, transactivator of transcription; VCAM1, vascular cell adhesion molecule-1.

KEYWORDS

astrocytes, calcium mobilization, coronin 1A, glia-mediated neurodegeneration, reactive astrocytes

1 | INTRODUCTION

Extensive studies on glial cells conducted over the past 30 years, strongly advocate that astrocytes are more than mere supporting cells as they perform many essential functions ranging from neurodevelopment to synaptic plasticity {reviewed in Ref. [1,2]}. Also, growing evidence points towards the fact that astrocytes undergo morphological (including hypertrophy) and molecular changes in various inflammatory or pathological conditions. These changes can further affect the neuronal population. Astrocyte reactivity encompasses morphological and functional changes such as the increase in protein expression of intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, altered calcium signaling, and dysregulated release of numerous factors. The astrocyte reactivity can be beneficial or detrimental for the neurons, based on the context or the neuropathology, and is regulated by several factors.[3,4] Various factors released by the reactive astrocytes can also serve as mediators of the innate immune system.[5]

Astrocytes play a significant role in human immunodeficiency virus (HIV)-1 neuropathogenesis. In fact, in the post-HAART era, the glia-mediated indirect neuronal death is prominent over the direct death of the neurons caused by the virus or the viral proteins.[6–8] Astrocytes contribute significantly to the indirect neuronal death and thus amplify the toxic effects brought about by the activated and/or infected microglia. Up to 19% of astrocytes are found to be positive for HIV-1 DNA in subjects with HIV-associated dementia.[9] The extent of HIV-1 infection in astrocytes is correlated with the severity of the neurological problems associated with AIDS patients. HIV-1 infection in astrocytes is non-productive due to the reduced translation of Gag, Env, and Nef mRNAs, whereas the translation of transactivator of transcription (Tat) mRNA remains unaltered. Studies in the mice model show that HIV-1 Tat is sufficient to induce progressive cognitive abnormalities such as impaired learning and memory deficits.[10-12] Astrocyte reactivity, induced either by HIV-1 infection, the viral proteins, or factors released by other infected cell types, is a key factor in mediating neuronal loss by various mechanisms. Hence, there is an urgent need to better understand the mechanisms that make this supporting cell type reactive and detrimental in the context of neuroAIDS.

Calcium is an important regulator of astrocyte activity. Calcium signaling in astrocytes, once doubted to be significant to bring forth changes in neuronal functions, is now well known to be significantly affecting neuronal physiology.[13] Astroglial $[Ca^{2+}]_i$ flux triggered by neurotransmitters leads to the release of gliotransmitters such as glutamate,[14] D-serine,[15] and adenosine triphosphate (ATP),[16] which after binding to their receptors can cause neuronal excitation.[17] HIV-1 Tat induces Ca^{2+} mobilization in astrocytes from the extracellular milieu or the intracellular stores. These events dysregulate $[Ca^{2+}]_i$ and facilitate altered cell physiology and neuronal death.[18]

We investigated the role of coronin 1A in modulating calcium signaling and astrocyte reactivity in the context of neuroAIDS. Coronin 1A belongs to the coronin family of proteins. The presence of WD repeats in the N-terminal is the hallmark of this family. Its gene is located on chromosome 16 in humans and 7 in mice. This 57 kDa protein regulates several processes such as cytokinesis, cell migration, and phagocytosis. Coronin 1A is expressed majorly by cells of hematopoietic origin; however, some recent studies report its expression in nervous tissue as well.[19–22] It is expressed by adult and embryonic T lymphocytes in mice and accumulates at the F-actin rich protrusions on the membrane of the stimulated T cells. It plays roles in their differentiation, actin cytoskeleton dynamics, and cell activation upon stimulation.[20,23–25] However, the expression and function of coronin 1A in different brain cell types are still underexplored and await detailed molecular investigations.

In this study, we delineated the activity-dependent roles of coronin 1A in cells of non-hematopoietic origin and its involvement in mediating astrocyte reactivity in the presence of neuroinflammatory agents such as HIV-1 Tat. Our experiments were focused to investigate the mechanisms of coronin 1A regulation, its role in astrocyte reactivity, and how it contributes to the indirect neuroinflammation observed in HIV-1 neuropathogenesis.

2 | MATERIALS AND METHODS

2.1 | Human fetal brain-derived neuronal precursor cell culture

Human brain tissues collected from elective abortions with the informed consent of mothers were processed

as per the protocols laid down by the Institutional Human Ethics and Stem Cell Research Committee under strict compliance with the guidelines of ICMR, India. Neural Precursor Cells (NPCs) were derived from the telencephalon region of the aborted fetuses of age 10–14 weeks, as described previously.[26] NPCs were cultured on poly-D-lysine (Sigma-Aldrich, Cat# P7280) coated flasks in Neurobasal Media (Invitrogen, Cat# 21103-049) supplemented with N2 supplement (Invitrogen, USA, Cat# 17505-048), Neural Survival Factor-1 (Lonza, Cat# CC-4323), 20 ng/ml EGF (Peprotech, Cat# AF-100-15-500UG), and 25 ng/ml bFGF (Peprotech, Cat# 100-18B-50UG). NPCs were further assessed for the expression of markers such as Nestin and SOX2, and 99% of the cells were found to be positive. They were also assessed for their ability to form neurospheres and differentiate into astrocytes and neurons.

2.2 | Human primary astrocytes and neuronal culture

NPCs were differentiated into astrocytes by replacing the media with Eagle's Minimal Essential Media (MEM) (Sigma-Aldrich, Cat# M0268-10X1L) containing 10% fetal bovine serum (Gibco, Cat# 10270–106). These cells were cultured for 3 weeks, with half media changes on alternate days, after which they were assessed for the expression of GFAP and vimentin, for which more than 95% of cells were found immunopositive (Figure S1). To differentiate NPCs into neurons, EGF and FGF from NPC media were replaced with 10 ng/ml BDNF (Peprotech, New Jersey, USA, Cat# 450–02-10UG) and 10 ng/ml PDGF-AB (Peprotech, Cat# 100-00AB-10UG). After maintaining them for 3 weeks, with half media changes on alternate days, they were assessed for expression of MAP2, for which more than 95% of cells were immunopositive (Figure S1).

2.3 | Intracellular Ca^{2+} measurement/ live cell imaging

0.2 million cells were seeded in glass-bottom 35-mm dishes (Thermo Fisher, Cat #150682) and incubated with 2.5 μM Fluo-4-AM (Invitrogen, Cat# F14201) in HEPES buffer (130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 20 mM HEPES; pH 7.4) at 37°C for 30 min. After 3 washes, the dish was mounted on the stage for live-cell imaging using a spinning disc confocal microscope (Zeiss AxioObserver Z1, Zeiss). A 10 x objective (Zeiss) was used for the measurement of fluorescent intensity as a measure of the change in intracellular Ca^{2+} levels. The baseline fluorescence intensity was recorded for the initial 1 min; thereafter, 10 μM ATP (Sigma-Aldrich, Cat# A2383-1G) was added to the static bath followed by 5 min of recording (Ex/Em of Fluo-4 Ca^{2+}-bound form: 494/506 nm). ATP remained in the static bath, while the cells were recorded for fluorescence. The change in fluorescence intensity was calculated by subtracting fluorescence at $t = 0$ from all the fluorescence intensity values. The ZEN software (Zeiss) was used for data acquisition and analysis. Areas having a significant number of cells (≥50) were randomly selected for the recordings after which individual cells were marked as the region of interest and the fluorescence intensity was measured across time.

2.4 | Immunocytochemistry

Astrocytes were seeded in a 4-well chamber slide (Nunc, Cat# 177437) at a density of 30,000 cells per well. Cells were fixed after 24 h, using 4% paraformaldehyde, washed thrice with 1X PBS, blocked, and permeabilized using 4% BSA containing 0.4% Triton-X 100. The cells were then incubated with antibodies, mouse anti-coronin 1A (Santa Cruz Biotechnology, 1:100, Cat# sc-100925, RRID:AB_2291951), mouse anti-Ki 67 (Millipore, 1:1000, Cat# MAB4190, RRID:AB_95092), mouse anti-vimentin (Santa Cruz Biotechnology, 1:1000, Cat# sc-6260, RRID:AB_628437), overnight at 4°C, or anti-GFAP (Dako, 1:1000, Cat# Z0334, RRID:AB_10013382) for 1 h at 25°C. The cells were then washed thrice with 1X PBS and incubated with appropriate fluorophore-tagged secondary antibodies (Invitrogen, 1:1000). The wells were washed thrice with 1X PBS and mounted using Hardset mounting media with DAPI (Vector Labs, Cat# H-1500). For each group, a minimum of five images were captured from random fields using the AxioImager Z1 microscope (Zeiss).

2.5 | Immunohistochemistry

Formalin-Fixed Paraffin-Embedded (FFPE) patient brain tissue sections were first deparaffinized and rehydrated. Sections were quenched with 1% H_2O_2 followed by antigen retrieval. Permeabilization was done using 0.1% Triton-X in 1X PBS and later on blocked with 1% BSA, 3% goat or rabbit serum, 0.05% Triton-X in 1X PBS. Sections were incubated overnight with primary antibodies, goat anti-coronin 1A (Abcam, 1:25, Cat# ab14787, RRID:AB_301480), rabbit anti-GFAP (Dako, 1:500, Cat# Z0334, RRID:AB_10013382) in blocking solution. Sections were washed 4 times with 0.02% Tween 20 in

1X PBS (PBST). Secondary antibody incubation was done for 2 h with appropriate biotinylated antibody (Vector labs, 1:500). Washing was done 4 times using PBST, and sections were then treated with a complex of Avidin-Biotinylated HRP (Vector Labs, 1:1, Cat# PK-6100) for 2 h followed by 4 PBST washes. Sections were developed for 1–3 min using the ImmPACT NovaRED kit (Vector Labs, Cat# SK4805) following the manufacturer's protocol, counterstained with hematoxylin, and washed under running water. The sections were dried, treated with xylene, and then mounted using the DPX mountant solution. Each section was imaged thrice randomly using a Leica DMRXA2 (Leica) microscope at 10x and 40x magnifications. Images were then processed and analyzed using the ImageJ software (NIH, RRID:SCR_003070).

2.6 | Small Interfering RNA (siRNA) mediated knockdown of coronin 1A

Coronin 1A was knocked down using siRNA in human primary astrocytes. 10 nM coronin 1A siRNA (Dharmacon, Cat# L-012771-00-0020) or control scrambled siRNA (Dharmacon, USA, Cat# D-001810-10-05) was transfected twice, using lipofectamine RNAimax (Invitrogen, Cat# 133778075) following the manufacturer's protocol. The cells were either harvested after 48 h of transfection to analyze the knockdown efficiency or carried over for further treatments or transfections.

2.7 | ATP stimulations

Coronin 1A was knocked down in astrocytes for 48 h. Cells were serum-starved for 2–3 h and then stimulated with 10 μM ATP (Sigma-Aldrich, Cat# A2383-1G). ATP stimulations were given for either 0, 45, 120, and 300 s or 5, 10, 30, 60, and 120 min, after which the cells were harvested using lysis buffer. The lysates were further processed for protein analysis.

2.8 | HIV-1 Tat transfection

Astrocyte cultures with 80% confluency were used for transfections with expression vector pcDNA3.1 expressing full-length HIV-1 Tat B, which was a kind gift from Prof. Udaykumar Ranga, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), India. Transfections were done using Lipofectamine 3000 (Invitrogen, Cat# L3000008) as per the manufacturer's protocol. Cells were harvested after 24 h as per the experimental requisite.

2.9 | Western blotting

The cells were harvested using lysis buffer, which consisted of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM Sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA (pH 8.0), 2% SDS, and protease inhibitor cocktail (Roche, Cat# 11836170001). Estimation of protein concentration was done using 4% copper sulfate and bicinchoninic acid (Sigma-Aldrich, Cat# B9643-1L). An equal amount of protein samples (25 μg), of different experimental groups, were resolved on 10–13% SDS-PAGE and transferred onto a nitrocellulose membrane (MDI, Ambala, India, Cat# SCNJ8101XXXX101). The blots were blocked using 5% skimmed milk in PBS-Tween 20 (PBST) followed by incubation with primary antibodies, mouse anti-coronin 1A (Santacruz biotech, 1:500, Cat# sc-100925, RRID:AB_2291951), mouse anti-GAPDH (Santacruz biotech, 1:1000, Cat# sc-32233, RRID:AB_627679), rabbit anti-Phospho-p44/42 MAPK (Cell Signaling Technology, 1:2000, Cat# 9101S), rabbit anti-p44/42 MAPK (Cell Signaling Technology, 1:2000, Cat# 9102S), rabbit anti-Phospho-PLCγ1 (Cell signaling, 1:1000, Cat# 2821S, RRID:AB_330855), rabbit anti-PLCγ1 (Cell signaling, 1:1000, Cat# 2822S, RRID:AB_2163702), rabbit anti-GFAP (Dako, 1:20000, Cat# Z0334, RRID:AB_10013382). Blots were washed thrice with TBS-Tween 20 (TBST) and then incubated with appropriate HRP labeled secondary antibodies (Vector Labs, 1:4000) for 2 h at room temperature. The blots were washed thrice with TBST and then developed using Chemiluminescent Reagent (Millipore, USA) and were imaged using Gel Documentation System (Uvitech). Densitometry of the protein bands was done using the ImageJ software (NIH, RRID:SCR_003070).

2.10 | Quantitative real-time PCR

The cells were harvested for RNA isolation using Trizol reagent (Ambion, Cat# 15596018) following the manufacturer's protocol. The purified RNA was utilized for synthesizing cDNA using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Cat# 4368814). qPCR was performed using SYBR Green Master Mix (Applied Biosystems, Cat# 4367659) using the following primers: CORO1A forward 5′-CACCAACATCGTCTACCTCTG-3′ and reverse 5′-ACTCCTTGGAACTGAACATGG-3′, GAPDH forward 5′-CAAGAGCACAAGAGGAAGAGAG-3′ and reverse 5′-CTACATGGCAACTGTGAGGAG-3′, GFAP forward 5′-ACCTGCAGATTCGAGAAACCAG-3′ and reverse 5′-TAATGACCTCTCCATCCCGCATC-3′, vimentin forward 5′- AAGTCCGCACATTCGAGCAA-3′ and

reverse 5′-CTACCAACTTACAGCTGGGC-3′, VCAM1 forward 5′-GGGAAGCCGATCACAGTCAA-3′ and reverse 5′-TCCTGTCTGCATCCTCCAGA-3′, ICAM1 forward 5′-CCGCAGTCATAATGGGCACT-3′ and reverse 5′-GGTTTCATGGGGGTCCCTTT-3′, PTGS2 forward 5′-TGTATGAGTGTGGGATTTGACC-3′ and reverse 5′-TGTGTTTGGAGTGGGTTTCAG-3′, NOS2 forward 5′-CTTTGCCTGTATGCTGATGC-3′ and reverse 5′-GCCTCTGATTTTCCTGTCTCTG-3′.

miRNA along with total RNA was isolated using the miRNEASY mini kit (Qiagen, Cat# 217004) following the manufacturer's protocol. Subsequently, the cDNA was synthesized using the miScript II RT kit (Qiagen, Cat# 218161) as per the manufacturer's protocol. The qPCR assay was done using the miScript SYBR Green PCR kit (Qiagen, Cat# 218073) following the manufacturer's protocol. The qPCR was done in ViiA7^{TM} (Applied Biosystems, RRID:SCR_019582). The specificity of the primers was confirmed via analyzing the melt curve.

2.11 | IL-6-release measurement by flow-cytometry

Astrocyte-conditioned-media (ACM) was collected from astrocytes knocked down for coronin 1A for 48 h and transfected with HIV-1 Tat for 24 h. The conditioned media thus collected was then analyzed for the levels of released pro-inflammatory cytokine interleukin-6 (IL-6) by using flow-cytometry-based cytometric bead array (CBA)-human inflammatory cytokines kit (BD Biosciences, Cat# 551811), and following the manufacturer's protocol.

2.12 | Assessment of neuronal survival by TUNEL Assay

ACM was collected from astrocytes transfected with coronin 1A siRNA or mimic-92b-5p for 48 h and transfected with HIV-1 Tat for 24 h. Neurons at a density of 40,000 cells/well were seeded in poly-D-lysine coated 8 well-chambered slides (Nunc, Cat# 177445). The next day the cells were treated with 50% ACM and neuronal media for 24 h. Cells were then fixed using 4% paraformaldehyde. Washed thrice with 1x PBS, blocked, and permeabilized using 4% BSA containing 0.01% Triton-X 100. Cells undergoing apoptosis were detected using in situ cell death detection kit, TMR red (Roche, Cat# 12156792910), following the manufacturer's protocol. Cells were washed thrice with 1x PBS, and the slide was mounted using hardset mounting media with DAPI (Vector Labs, Cat# H-1500). Four images were captured for each group using the AxioImager Z1 microscope (Zeiss).

2.13 | miRNA target prediction

The miRWalk (http://mirwalk.umm.uni-heidelberg.de/; miRWalk, RRID:SCR_016509) online prediction tool was used to filter miRNAs targeting 3' UTR of CORO1A.

2.14 | miRNA mimic and inhibitor transfection

Transfection was done on astrocytes, in 80% confluent T25 flasks using RNAi MAX (Invitrogen) as per the manufacturer's protocol. 62.5 nM of Syn-hsa-miR-92b-5p miScript miRNA mimic (Qiagen, Cat# MSY0004792) was used for mimic transfection. All-Stars Negative Control siRNA (Qiagen, Cat# SI03650318) was used as mimic control. miRNA inhibition was done using 62.5 nM of the miRCURY LNA miRNA inhibitor against has-miR-92b-5p (Qiagen, Cat# YI04104160-ADAFr), and miRCURY LNA miRNA inhibitor control (Qiagen, Cat# YI00199006-ADA) was used as the inhibitor control. Transfections were done using Opti-MEM media (Gibco, Cat# 31985–070) for 5 h and replaced with complete MEM. Cells were processed after 48 h of transfection for RNA and protein analysis. Effective doses of mimic and inhibitor miRNA were standardized via qPCR and protein studies.

2.15 | Cloning of CORO1A 3' UTR and luciferase assay

The 3′UTR sequence of coronin 1A, being very short in length (111 bp), AGCCCCGCAGGGCCTCCAGCAGGGTCAGC CATTCACACCCATCCACTCACCTCCCATTCCCA GCCACATGGCAGAGAAAAAAATCATAATAAAA TGGCTTTATTTTCTGGT was outsourced in the form of two single-stranded reverse complementary nucleotide chains having restriction sites for SpeI and HindIII, respectively. Both the chains were annealed, digested and were cloned into pMIR-Report plasmid between SpeI and HindIII restriction sites. The cloned plasmid was then sequenced to confirm the successful insertion.

CORO1A 3′ UTR luciferase reporter plasmid was co-transfected with 12.5 nM of mimic-92b-5p into HeLa cells, using Lipofectamine 3000 (Invitrogen, Cat# L3000008) as per the manufacturer's protocol. After 24 h, the samples were harvested for luciferase detection, using the luciferase detection kit (Promega, Cat# E1500) following the manufacturer's protocol, in a Tecan SPARK multiplate reader (Tecan). The readings were normalized using total protein content.

2.16 | Statistical analysis

The experiments were performed independently three to five times; for experiments having only one variable, student's *t*-test was used to assess statistical significance between control and experimental groups, whereas for experiments with two variables, two-way ANOVA followed by Tukey's multiple comparisons test was performed to assess the statistical significance. p values <0.05 were considered statistically significant. * represents $p < 0.05$, ** represents $p < 0.005$, and *** represents $p < 0.0005$.

3 | RESULTS

3.1 | Coronin 1A expression in human astrocytes and brain autopsy tissue

Coronin 1A expression has been reported majorly in cells of hematopoietic origin[20,23,27] and neurons,[22] but its expression in astrocytes was not explored till this study. In this regard, human astrocytes were harvested to assess the protein levels of coronin 1A through western blotting. Jurkat T cells were taken as a positive control for the coronin 1A expression. We observed that human astrocytes, indeed, express coronin 1A (Figure 1A). Additionally, immunocytochemistry was performed, which confirmed coronin 1A (green) localization in the cells along with a marker for astrocytes, GFAP (red) (Figure 1B). Coronin 1A was also found to be expressed in the human brain autopsy sections. Immunohistochemistry performed on the hippocampal sections revealed the coronin 1A expression, as compared to the negative control (NC) (Figure 1C).

3.2 | Coronin 1A downregulation does not affect resting-state human astrocytes

The functional roles of coronin 1A in astrocytes were unexplored; therefore, to study the role of coronin 1A in astrocytes, we carried out a loss of function experiments on human astrocytes. We performed siRNA-mediated knockdown and analyzed the knockdown efficiency after 48 h; the siRNA was found to reduce the mRNA levels by 92% and protein levels by 43% (Figure 2A,B, respectively). To assess if coronin 1A knockdown results in cell death, we performed a live-dead cell assay on coronin 1A siRNA transfected astrocytes. The assay revealed that there was no significant difference in the live-dead cell proportions in the siRNA transfected group compared to the control scrambled siRNA transfected group (Figure S2A). Though mature astrocytes are low-proliferating cells, whatever the rate of proliferation be, was found to be unaffected by coronin 1A knockdown, as assessed through immunocytochemistry using Ki-67 protein as a marker for proliferation (Figure S2B). Further, to investigate the role of coronin 1A in resting-state astrocytes, if any, we assessed the expression of intermediate filament proteins such as GFAP and vimentin. It was found that downregulating coronin 1A neither affected GFAP (Figure 2C,D) nor vimentin expression (Figure 2E,F). These results signify that coronin 1A downregulation does not affect the resting-state astrocytes and hence prompted us to explore whether coronin 1A functions in an activity-dependent manner.

3.3 | Coronin 1A facilitates calcium mobilization and Ca^{2+} dependent pathways in ATP-stimulated human astrocytes

Ca^{2+} mobilization upon stimulation with a ligand is a good measure of astrocyte activity and can be correlated with the effects of astrocyte activity on the neuronal population.[13,28] ATP is a well-known and widely used gliotransmitter that can induce calcium mobilization.[29,30] To investigate the role of coronin 1A in stimulated astrocytes, fluorescence-based live-cell calcium imaging was performed upon stimulation with ATP. An increase in the fluorescence of the calcium-sensitive dye Fluo-4 was taken as a measure of Ca^{2+} flux. An increase in intracellular calcium upon ATP stimulation was recorded in HEPES buffer (+/- $CaCl_2$). Basal fluorescence was recorded for 1 min, and then 10 μM ATP was applied to the bath and recordings were performed for an additional 5 min. ATP stimulation induced the Ca^{2+} flux and fluorescence intensity increased. In the absence of $CaCl_2$ in the HEPES buffer (i.e., the source of Ca^{2+} is just intracellular), astrocytes downregulated for coronin 1A showed a 53.5% decrease in Fluo-4 fluorescence as compared to the control group, suggestive of its role in calcium mobilization in stimulated conditions (Figure 3A). The Ca^{2+} flux defect observed when the HEPES buffer with $CaCl_2$ was used (i.e., the source of Ca^{2+} is both, intracellular and extracellular), was not statistically significant (Figure 3B). This indicated that coronin 1A downregulation affected the calcium mobilization from the intracellular stores in the ATP-stimulated astrocytes.

The reduced Ca^{2+} flux in coronin 1A knocked-down cells could be an outcome of the altered PLCγ1 phosphorylation.[23] To address this possibility, astrocytes transfected with control scrambled siRNA or coronin 1A targeting siRNA were stimulated with 10 μM ATP for varying time points (0, 45, 120, and 300 s) after which the cells were harvested, and the whole cell lysate was analyzed through western blotting for the phosphorylated and the total forms of PLCγ1. It was observed that coronin 1A knockdown resulted in reduced PLCγ1 phosphorylation as compared to the control group

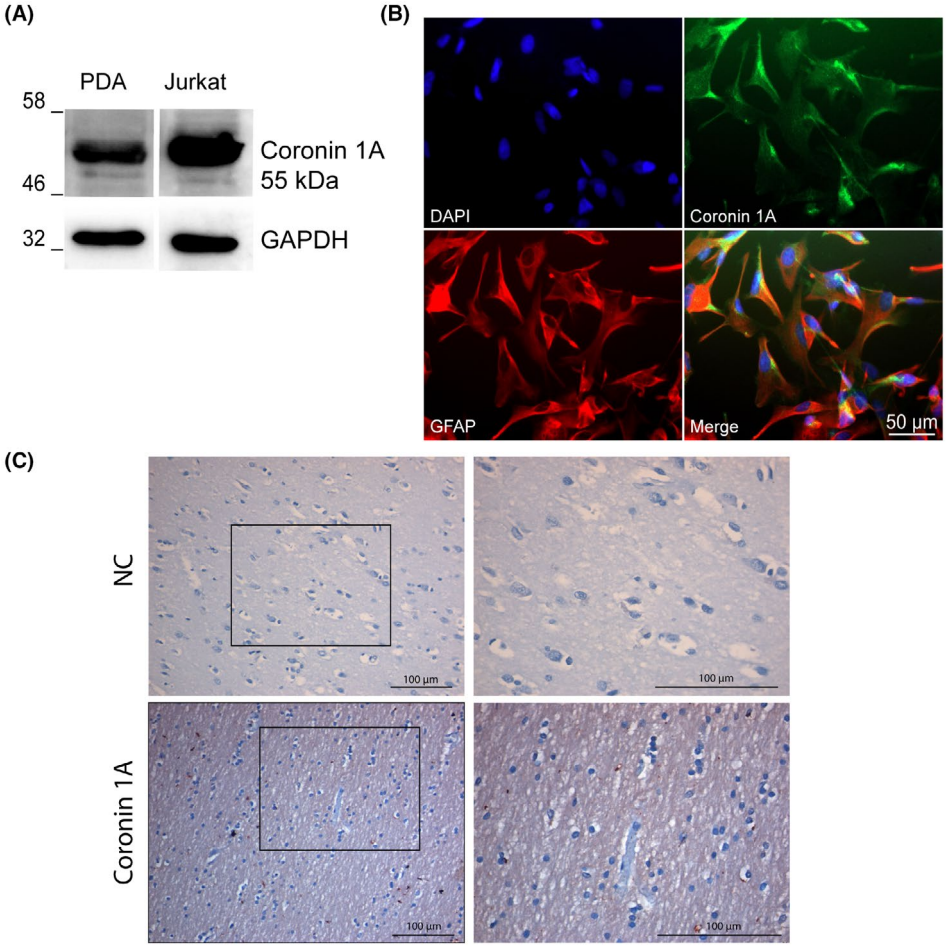

FIGURE 1 Coronin 1A expression in human astrocytes and brain autopsy tissue. (A) Coronin 1A protein expression was assessed in Astrocytes and Jurkat T cells, using western blotting. (B) Immunocytochemistry was performed on astrocytes immunolabeled for Coronin 1A (green), and GFAP (red). Cell nuclei were DAPI stained (blue). Images were acquired using a 20× objective lens and the scale bar represents 50 µm. (C) Immunohistochemistry was performed on human brain autopsy sections of the hippocampal region; the right panel shows the blown-up images of the inset marked in the left panel. Images were acquired using a 20× objective lens and the scale bar represents 100 µm. PDA, progenitor-derived astrocytes; GFAP, glial fibrillary acidic protein; NC, negative control for antibody

(at t = 120 s) (Figure S3A). We also assessed the ERK1/2 phosphorylation pattern after ATP stimulations for varying time-points (0, 5, 10, 30, 60, and 120 min) and observed a slight decrease in ERK1/2 phosphorylation, although not significant (Figure S3B). The above results indicate that, although coronin 1A does not affect the resting-state but plays activity-dependent roles by facilitating calcium mobilization from the intracellular stores upon ATP stimulation.

3.4 | Coronin 1A is upregulated in HIV-1 Tat-induced reactive astrocytes and brain autopsy sections of HIV-1 infected individuals

Neuroinflammatory agent HIV-1 Tat is a well-known neurotoxicant that induces astrocyte reactivity and hence causes astrocyte-mediated neuronal death in HIV-1 neuropathogenesis.[7,31] To further address the possibility of activity-dependent roles of coronin 1A, we assessed whether HIV-1 Tat can modulate the coronin 1A expression in astrocytes. The real-time PCR assay and western blot analysis revealed a 1.4-fold and 1.51-fold increase in the level of coronin 1A mRNA and protein expression, respectively, in astrocytes transfected with the HIV-1 Tat expression vector compared to cells transfected with vehicle control (Figure 4A,B). To validate this finding even further, immunohistochemistry was performed on the human brain autopsy sections. Coronin 1A was found to be upregulated by 1.36-fold in hippocampal sections of HIV-1 positive individuals as compared to their age-matched controls (Figure 4D,E). To confirm the presence of astrocytes in the sections, GFAP staining was performed in the serial sections. Astrocytes were indeed

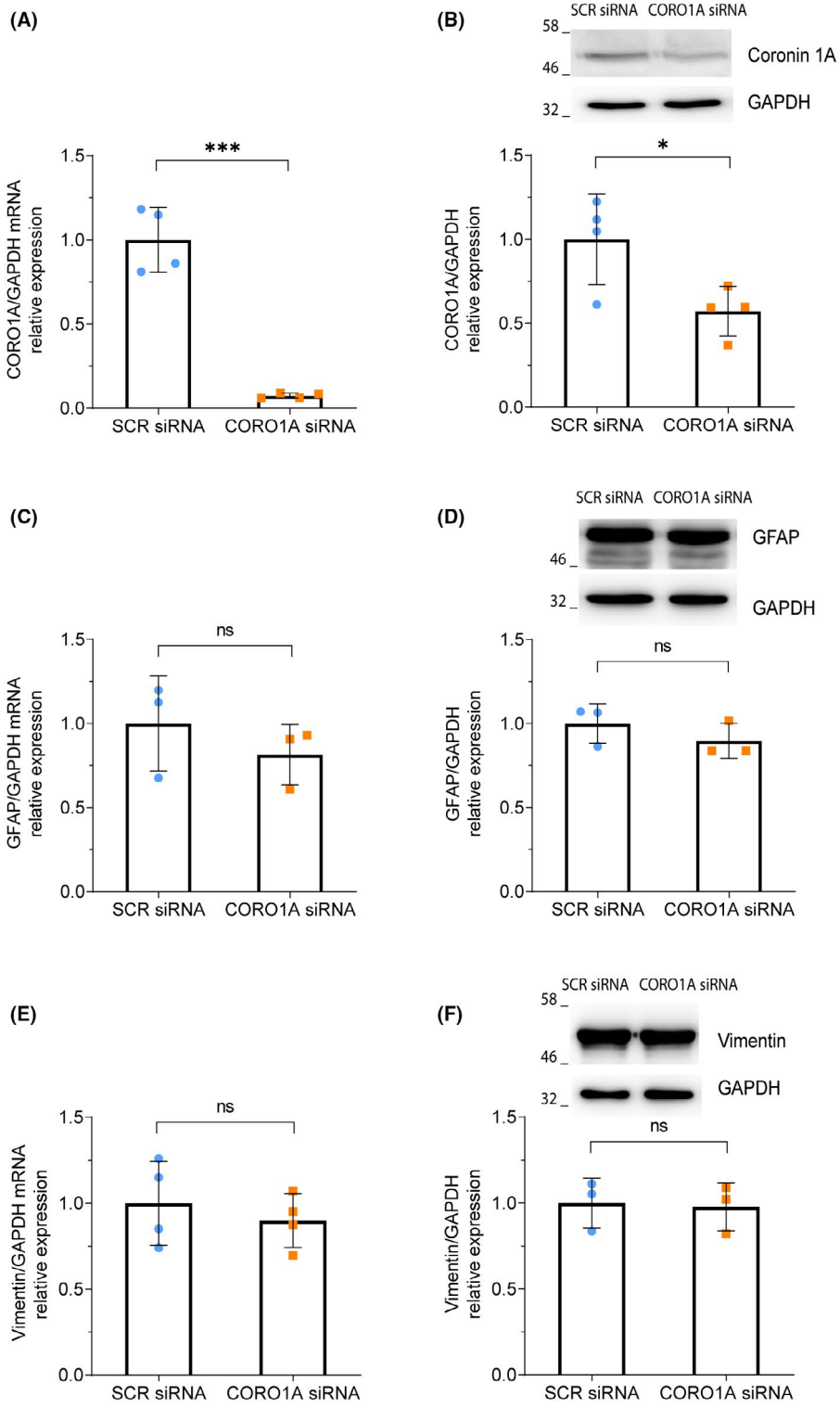

FIGURE 2 Coronin 1A knockdown does not affect the resting-state human astrocytes. Astrocytes transfected with CORO1A siRNA for 48 h, were harvested, and transcript and protein levels were analyzed using real-time qPCR and western blotting, respectively. (A) The bar graph represents the relative expression of coronin 1A mRNA and (B) protein, compared to the control group. Cells downregulated for coronin 1A were assessed for the expression of GFAP (C) mRNA and (D) protein. Vimentin (E) mRNA and (F) protein expression was also assessed in the same experimental group. Blots are representative and the bar graph shows the densitometric analysis of the blots. GAPDH was used as a normalization control for all the analyses. Data represent mean ± SD of three to four independent experiments. * represents $p < 0.05$; *** $p < 0.0005$. SCR, scrambled; CORO1A, coronin 1A; GFAP, glial fibrillary acidic protein

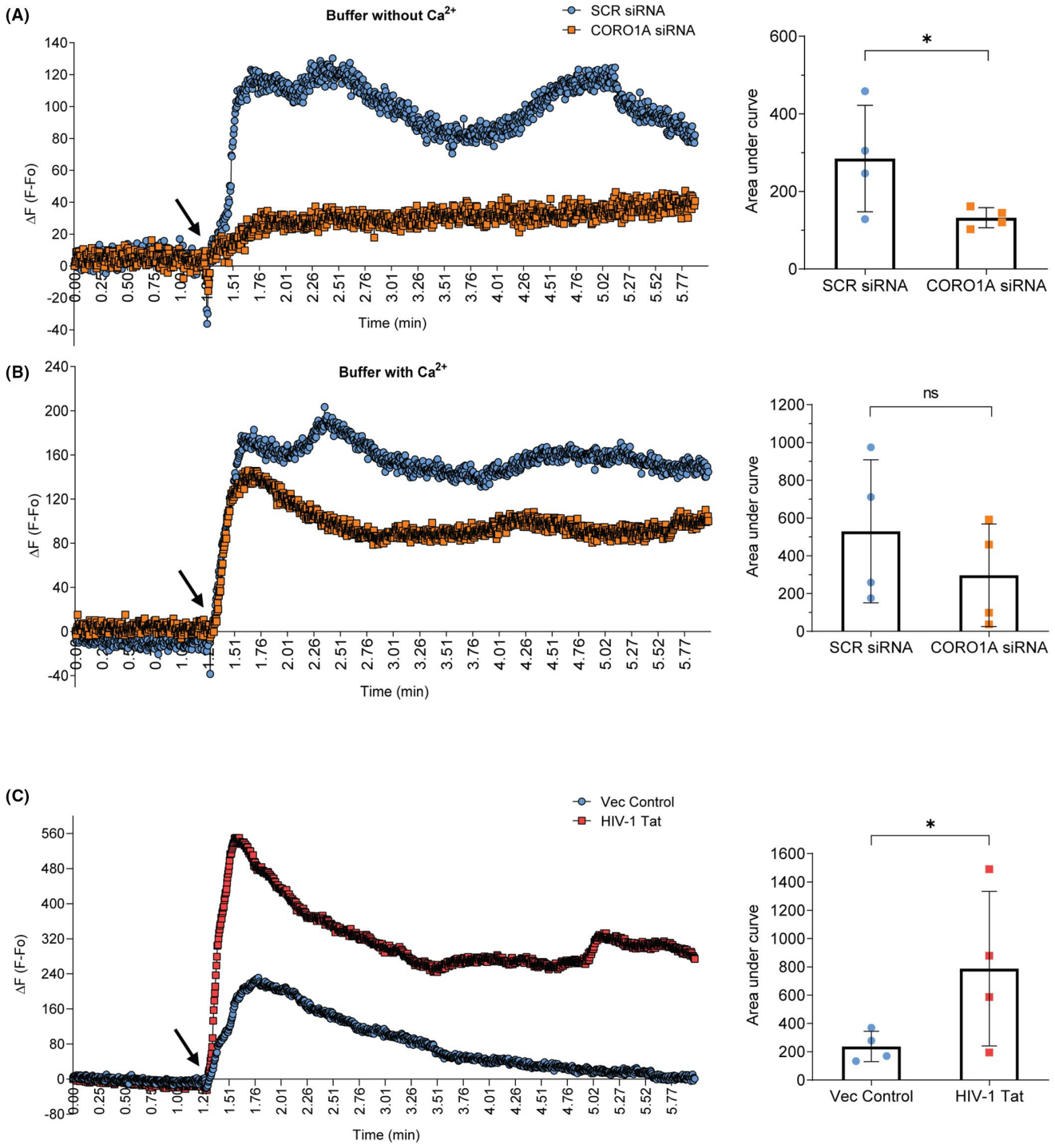

FIGURE 3 ATP-induced Calcium mobilization in Fluo-4 AM labeled human astrocytes. (A, B) Calcium mobilization in cells knocked down for coronin 1A, upon stimulation with 10 μM ATP in (A) HEPES buffer without $CaCl_2$, and (B) HEPES buffer having $CaCl_2$. (C) Calcium mobilization in cells expressing HIV-1 Tat, upon stimulation with 10 μM ATP. Arrow indicates the time of the ATP application in the buffer. The bar graph (right panel) shows the area under the curve (AUC). Data represent mean ± SD of four independent experiments. * represents $p < 0.05$. SCR, scrambled; CORO1A, coronin 1A, ΔF, change in fluorescence intensity

found to be present in the hippocampal section, as clear from the parallel images taken for coronin 1A and GFAP from the similar areas of the serial hippocampal sections (Figure S4).

HIV-1 Tat, indeed, turned the resting state astrocytes reactive as the cells transfected with the HIV-1 Tat expression vector showed enhanced calcium mobilization upon ATP stimulation (3.3-fold) (Figure 3C), enhanced expression

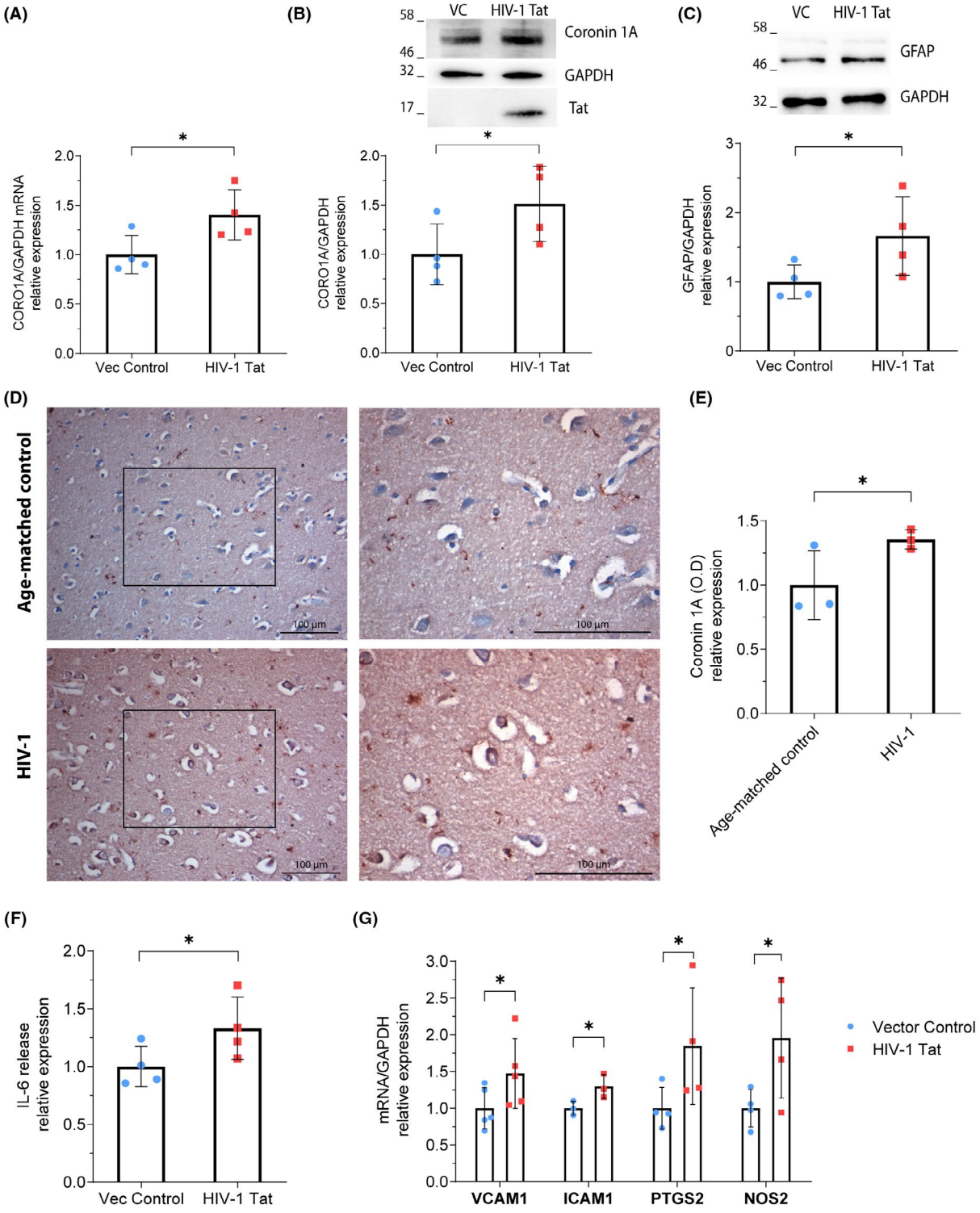

FIGURE 4 HIV-1 Tat augments coronin 1A expression in astrocytes and induces astrocyte reactivity. Astrocytes transfected with HIV-1 Tat expression vector for 24 h were harvested and analyzed for (A) Coronin 1A mRNA levels, using real-time PCR assay, (B) coronin 1A protein, and (C) GFAP protein levels using western blotting; the upper panel shows the representative blots and the lower panel shows the densitometric analysis of the same, compared to cells transfected with vector control. (D) Representative images of immunohistochemistry performed for coronin 1A on human brain autopsy sections of the hippocampal region of HIV-1 patients and their age-matched controls; right panel shows the blown-up images of the inset marked in the left panel and (E) bar graph shows the relative change in coronin 1A optical density in the IHC images. Images were acquired using a 20X objective lens and the scale bar represents 100 µm. The reactive status of the HIV-1 Tat transfected cells was also confirmed by assessing the levels of (F) released IL-6 in the conditioned media and (G) pro-inflammatory genes viz VCAM1, ICAM1, PTGS2, and NOS2, compared to the group transfected with vector control. GAPDH was used as a normalization control for all the mRNA and protein analyses. Data represents mean ± SD of three (for IHC) to five independent experiments. * represents $p < 0.05$. VC, vector control; O.D, optical density

of GFAP (1.66-fold) (Figure 4C), cytokine interleukin-6 (IL-6; 1.33-fold) (Figure 4F), and pro-inflammatory gens such as VCAM1 (1.47-fold), ICAM1 (1.29-fold), PTGS2 (1.84-fold), and NOS2 (1.95-fold) (Figure 4G) as compared to the vector control group. These findings, in conjunction with the previous results, indicate that with the increase in its expression in the inflammatory environment, coronin 1A might be involved in astrocyte reactivity and glia-mediated neurodegeneration.

3.5 | Downregulation of coronin 1A attenuates HIV-1 Tat-induced deleterious effects of reactive astrocytes and supports neuronal survival

Next, we hypothesized that if coronin 1A is important for calcium mobilization and the literature suggests that calcium plays a key role in astrocyte activity and reactivity,[14,32] downregulating coronin 1A might attenuate the detrimental effects of reactive astrocytes observed in various neuroinflammatory contexts, including neuroAIDS. So, to investigate this hypothesis, astrocytes were transfected with siRNA against coronin 1A for 48 h followed by transfection with the HIV-1 Tat expression vector for 24 h. Quantitative PCR and western blotting analyses revealed that the Tat-induced expression of GFAP attenuated in the experimental group where coronin 1A was downregulated beforehand. The scrambled + HIV-1 Tat group showed a 1.51-fold increase in GFAP levels, whereas the coronin 1A siRNA + HIV-1 Tat group showed the GFAP expression similar to the scrambled +vector control group (Figure 5A,B).

Reactive astrocytes release excess cytokines, and/or chemokines which may cause excitotoxicity or neurotoxicity to the neurons present in the vicinity.[5,33-35] In this regard and to validate the findings of the previous experiments, functional assays were performed using the conditioned media derived from astrocytes after coronin 1A knockdown for 48 h followed by HIV-1 Tat transfection

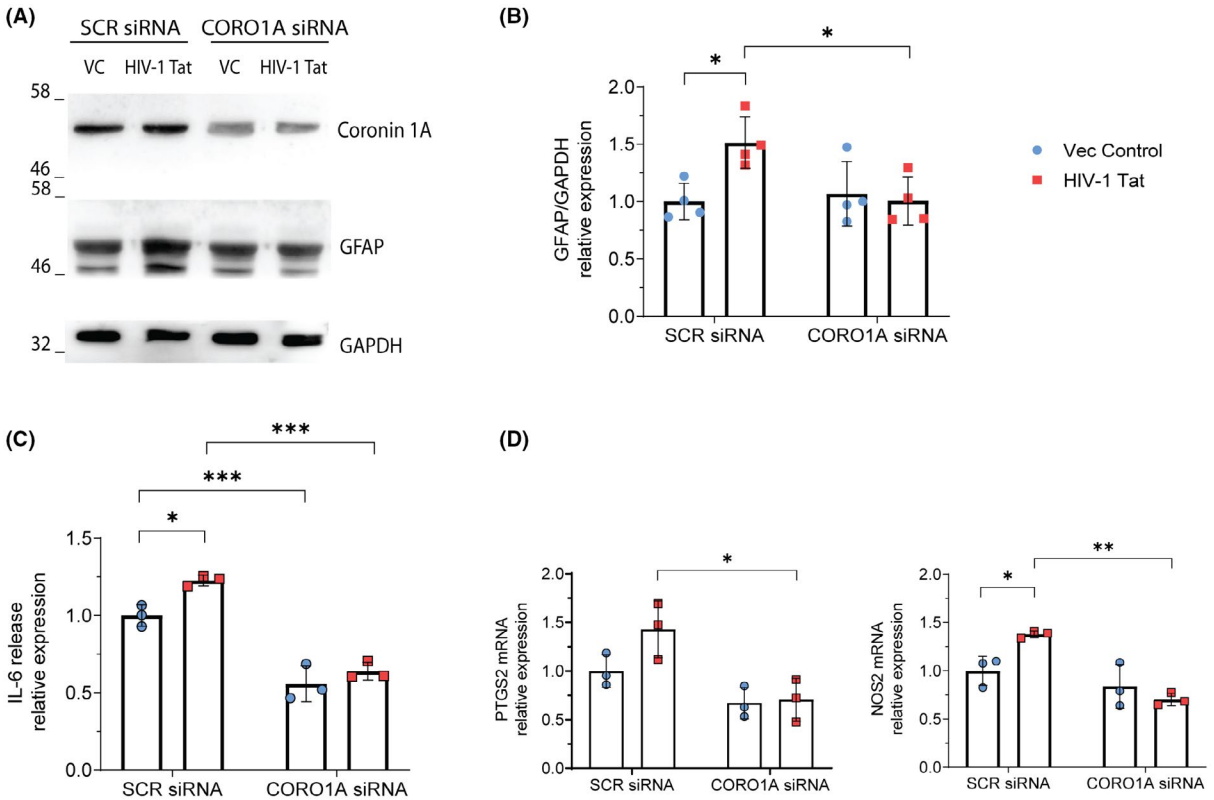

FIGURE 5 Coronin 1A downregulation attenuates expression of HIV-1 Tat-induced GFAP, IL-6, and pro-inflammatory genes in reactive astrocytes. Human astrocytes downregulated for coronin 1A using siRNA for 48 h followed by transfection with HIV-1 Tat for 24 h, were harvested and analyzed for (A) Coronin 1A and GFAP protein expression, using western blotting. (A) representative blots and (B) the densitometric analysis of the GFAP expression. (C) Released IL-6 levels were measured in the conditioned media collected from the above experimental groups, using Flow cytometry and (D) pro-inflammatory genes viz PTGS2 and NOS2, assessed through real-time PCR assay. GAPDH was used as a normalization control for all the mRNA and protein analyses. Data represents mean ± SD of three to four independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. SCR, scrambled; CORO1A, coronin 1A

for 24 h. The freshly collected cell supernatants were analyzed for the levels of released Interleukin-6 (IL-6). The FACS analysis further supported the hypothesis as the enhanced release of the pro-inflammatory cytokine, IL-6, observed under the effect of HIV-1 Tat (1.23-fold) was attenuated when coronin 1A was knocked down (Figure 5C).

Astrocytes are immunocompetent and possess immunological properties.[33] To assess the immune status of the cells, mRNA levels of several pro-inflammatory genes were also assessed in the current setting. Downregulating coronin 1A could attenuate the HIV-1 Tat-induced levels of pro-inflammatory genes such as PTGS2 and NOS2. It was observed that the scrambled + HIV-1 Tat group showed up-regulation in PTGS2 (1.43-fold), NOS2 (1.38-fold) whereas the coronin 1A siRNA + HIV-1 Tat group had PTGS2 and NOS2 levels similar to the scrambled + vector control group (Figure 5D).

The deleterious effects of HIV-1 Tat-induced reactive astrocytes are also manifested in the form of neuronal death. Astrocyte-conditioned media (ACM) collected from astrocytes transfected with HIV-1 Tat, when added to neuronal cultures, increased neuronal death (Figure 6A). So, to further investigate whether HIV-1 Tat-induced reactive astrocyte-mediated neuronal death could be attenuated by downregulating coronin 1A, we transfected astrocytes with coronin 1A siRNA for 48 h followed by transfection with the HIV-1 Tat expression vector for 24 h. ACM was collected from these experimental sets and added to the neuronal cultures to inspect the indirect effects. ACM from the Tat alone group induced 36.2% neuronal death whereas ACM collected from the group which was transfected with coronin 1A siRNA before HIV-1 Tat transfection, showed reduced neuronal death of 18.2% (Figure 6B). These results signified that downregulating coronin 1A is protective as it diminished astrocyte

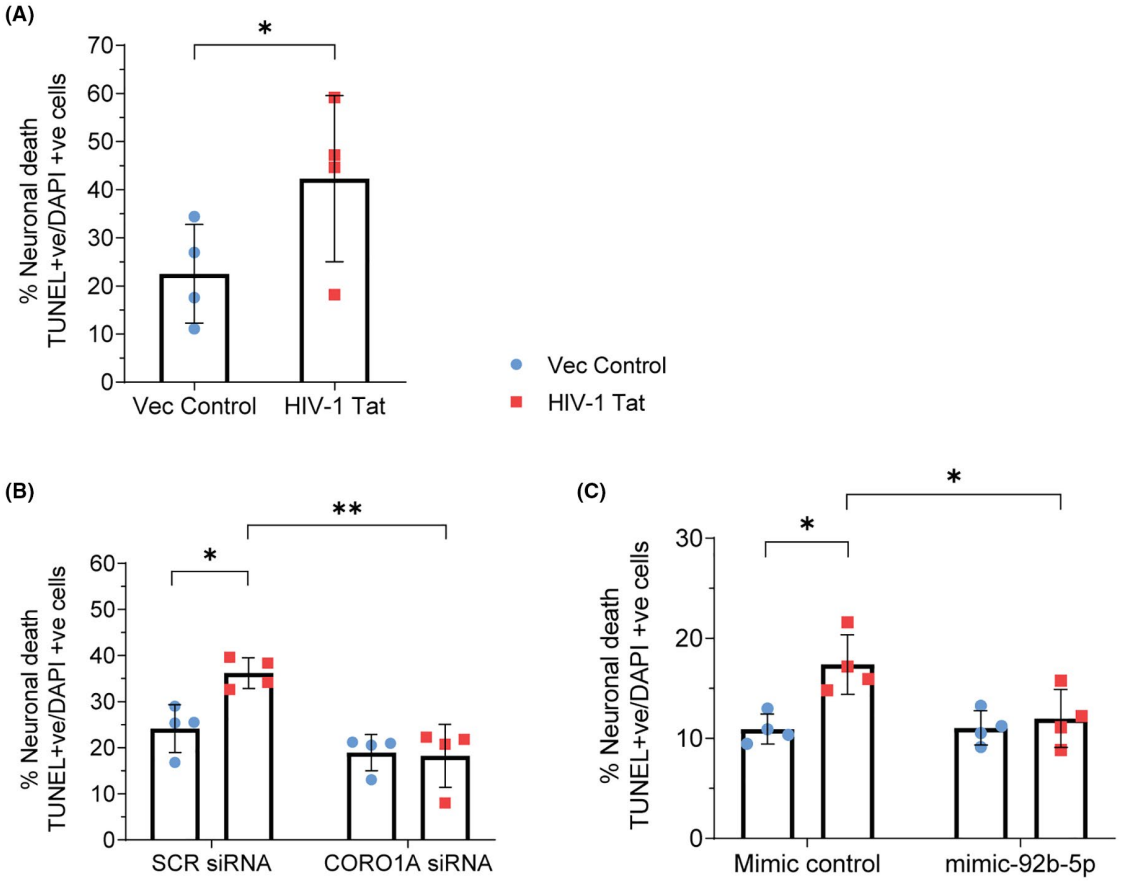

FIGURE 6 Downregulation of Coronin 1A attenuates HIV-1 Tat-induced indirect-neuronal death. Human primary neurons were treated for 24 h with (A) ACM collected from astrocytes transfected with HIV-1 Tat expression vector for 24 h after which neuronal death was assessed by analyzing the TUNEL-positive neurons, (B) ACM collected from astrocytes transfected with CORO1A siRNA for 48 h followed by transfection with HIV-1 Tat expression vector for 24 h and assessed for neuronal death by analyzing the TUNEL-positive neuronal cells, (C) ACM collected from astrocytes transfected with mimic-92b-5p for 48 h followed by transfection with HIV-1 Tat expression vector for 24 h and neurons were then assessed for neuronal death. Data represent the mean ± SD of four independent experiments. *$p < 0.05$, **$p < 0.005$. SCR, scrambled; CORO1A, coronin 1A; ACM, astrocyte conditioned media

reactivity and thus supported our hypothesis that coronin 1A facilitates HIV-1 Tat-induced astrocyte reactivity and subsequent neuronal death.

3.6 | MiR-92b-5p regulates coronin 1A in HIV-1 Tat-induced reactive astrocytes

HIV-infected brains or brain cells also present a dysregulated cellular miRNA profile which might contribute to the virus-induced neurological complications.[36-38] To assess the miRNA regulatory machinery, a small RNA sequencing was outsourced which was performed on astrocytes transfected with HIV-1 Tat expression vector or vector control. A heat map was generated for the miRNAs that were differentially regulated (by ±1.3 to ±2.5-fold) under the effect of HIV-1 Tat. The heatmap function of the display 'R' tool (https://www.displayr.com) was used to generate the heatmap by using fold-change derived from the absolute reads of the sequencing data (Figure S5). Out of all the differentially regulated miRNAs, we narrowed to the downregulated ones, and further, bioinformatics tool miRWalk, was employed to filter down to miRNAs which are predicted to target 3′ UTR of CORO1A (Figure 7A). Individual qPCR assay performed for these miRNAs confirmed their downregulation under the effect of HIV-1 Tat. It was observed that HIV-1 Tat downregulated miR-92b-5p the most (−1.66-fold) (Figure 7B), hence further confirmatory assays were performed only for miR-92b-5p.

To confirm whether miR-92b-5p regulates CORO1A, 111 bp long 3' UTR of CORO1A was cloned in pMIR-REPORT and was used along with mimic-92b-5p for co-transfection into Hela cells. The luciferase assay was performed following the manufacturer's protocol, and relative luciferase units were measured after 24 h of

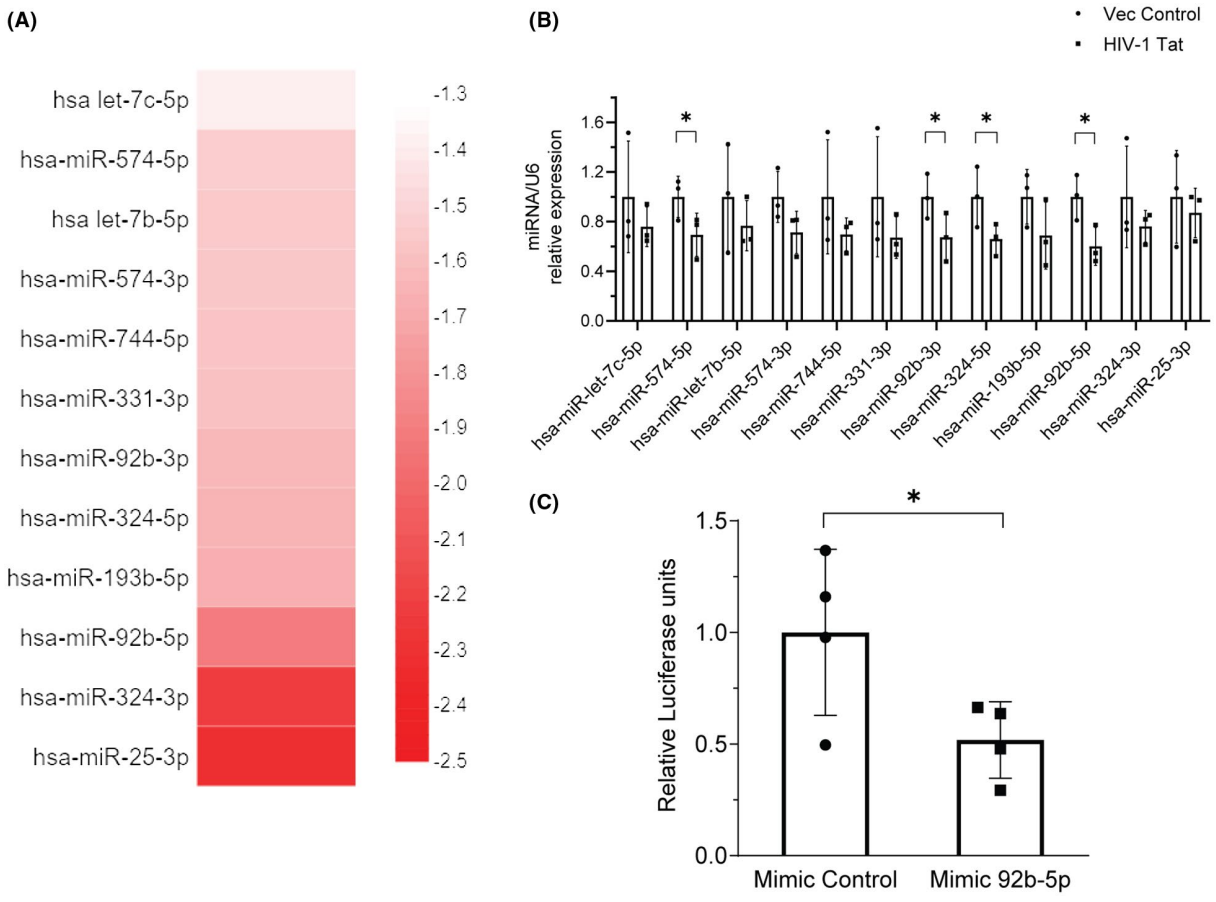

FIGURE 7 MiR-92b-5p is downregulated in response to HIV-1 Tat and targets 3' UTR of CORO1A. Astrocytes were transfected with HIV-1 Tat expression vector for 24 h and were assessed for the expression of miRNAs. (A) Heatmap was prepared using fold-change derived from absolute values of sequencing data, showing miRNAs which are downregulated by −1.3 to −2.5-fold in the presence of HIV-1 Tat, compared to the vector control group, and predicted to target CORO1A. (B) The bar graph represents miRNA levels compared to the vector control group, analyzed using real-time PCR assay; U6 was used for normalizing the data. (C) CORO1A 3' UTR was cloned downstream to the luciferase gene in the pMIR-Report vector and was co-transfected along with mimic control or mimic-92b-5p into Hela cells. Quantitative analysis shows the normalized relative luciferase activity in the indicated groups. The data shown represent three to four independent experiments (mean ± SD). * represents $p < 0.05$

transfection. It was observed that mimic-92b-5p reduced the luminescence by −1.93-fold confirming that it indeed targets 3′ UTR of CORO1A (Figure 7C).

To further validate the above finding, the mimic and inhibitor against miR-92b-5p were employed. Mimic 92b-5p transfection into human astrocytes for 48 h followed by qPCR analysis revealed that miR-92b-5p level increased by 265-fold compared to the cells transfected with mimic control (Figure 8A). The transcript and protein levels of coronin 1A showed a significant decrease by −2.34-fold and −1.38-fold, respectively, because of the mimic-92b-5p transfection, compared to the control transfected group (Figure 8B,C). In contrast, transfection of the inhibitor against miR-92b-5p resulted in significant inhibition of miR-92b-5p levels (−2.34-fold) compared to the control group (Figure 8D). The transcript and the protein levels of coronin 1A showed a significant increase by 1.63-fold and 1.45-fold, respectively, because of the transfection with an inhibitor against miR-92b-5p, compared to the control group (Figure 8E,F).

The effects of miR-92b-5p regulation of the coronin 1A expression under the effect of HIV-1 Tat should also reflect

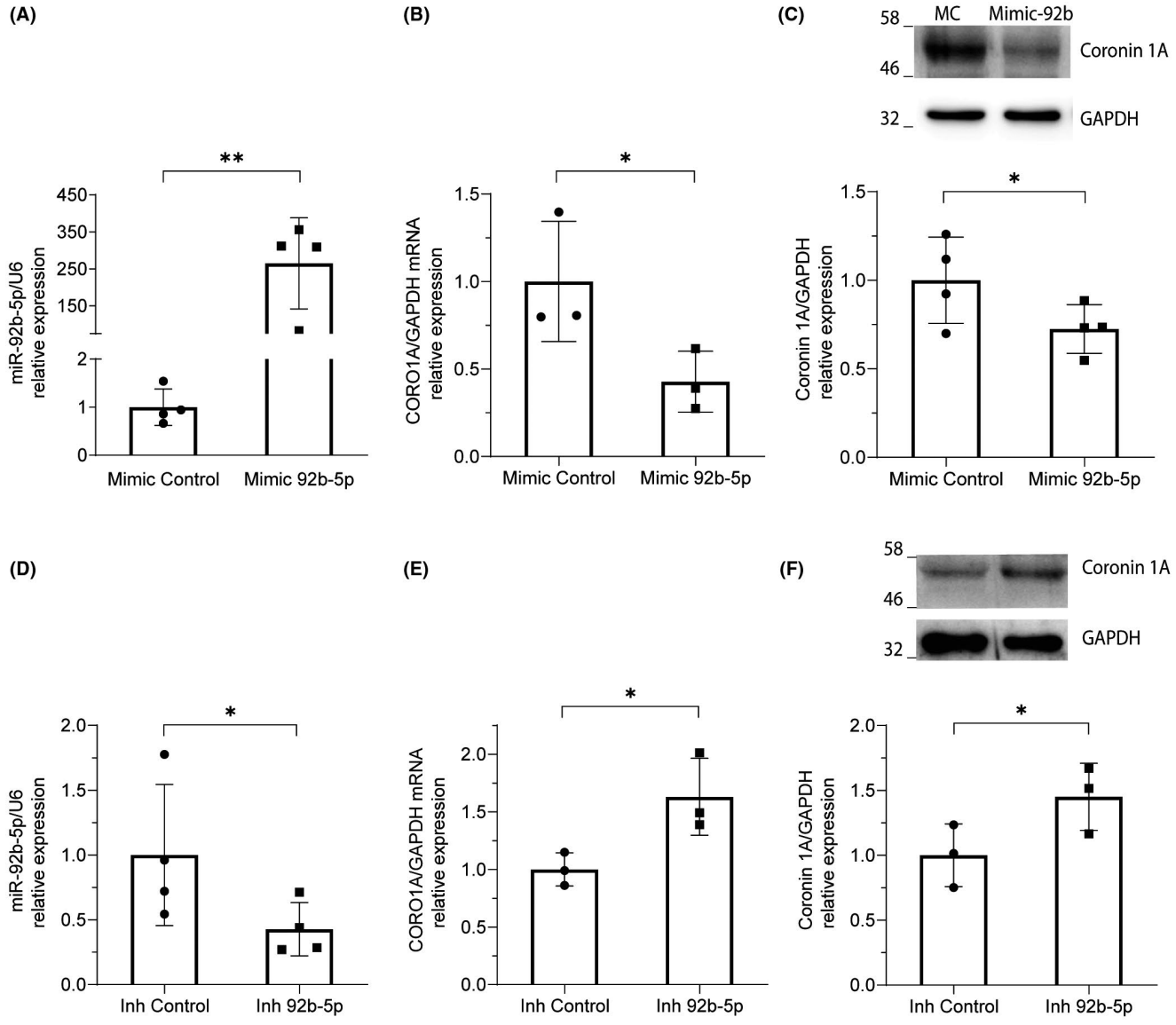

FIGURE 8 MiR-92b-5p mimic downregulates and inhibitor of miR-92b-5p upregulates CORO1A levels. Astrocytes were transfected with mimic-92b-5p for 48 h. Cells were then harvested for miRNA, mRNA, and whole-cell protein. The bar graph shows the effect of mimic-92b-5p on levels of (A) miR-92b-5p, (B) CORO1A transcripts, and (C) coronin 1A protein, compared to their respective levels in the control transfected group. Similarly, astrocytes transfected with an inhibitor against miR-92b-5p were assessed for the effects on levels of (D) miR-92b-5p, (E) CORO1A transcripts, and (F) coronin 1A protein, compared to their respective levels in the control transfected group. miRNA levels were normalized using U6 whereas mRNA and protein levels were normalized with GAPDH mRNA and protein levels, respectively. Data represents mean ± SD of three to four independent experiments. *$p < 0.05$; **$p < 0.005$. Inh, inhibitor

in the neuronal death assay. To explore this idea, we transfected astrocytes with mimic-92b-5p for 48 h followed by HIV-1 Tat overexpression for 24 h. We observed that the group that was transfected with mimic-92b-5p beforehand presented reduced neuronal death of 12% compared to the Tat alone group which presented 17.4% neuronal death (Figure 6C). These observations are congruent with the siRNA-based experimental observations and support our hypothesis even more that HIV-1 Tat, via miR-92b-5p, augments coronin 1A expression which then facilitates astrocyte reactivity and the associated neuronal loss.

4 | DISCUSSION

This study highlights the activity-dependent roles of coronin 1A in astrocytes upon ATP stimulation and its role in promoting astrocyte reactivity under the effect of a neurotoxicant HIV-1 Tat. We demonstrate that coronin 1A facilitates intracellular calcium mobilization in astrocytes stimulated with ATP. It promotes astrocyte reactivity, and its expression is regulated by miR-92b-5p under the effect of HIV-1 Tat. Its role in promoting astrocyte reactivity can have implications in various other neurodegenerative disorders as well, such as Alzheimer's or Parkinson's disease, where the astrocyte reactivity and the associated neurotoxicity are the underlying factors in the disease progression and neuropathogenesis.

Despite being heavily explored, the roles of astrocytes in physiological and pathophysiological contexts are yet to be thoroughly understood. Apart from performing many supportive roles, these cells exhibit varied behavior in pathophysiological conditions. Astrocyte reactivity is a common phenomenon in a variety of neurological disorders.[1,3] The reactive astrocytes may prove to be neuroprotective or neuroinflammatory for the neurons present in the vicinity depending on the pathological context and hence significantly affect the neuronal functions. Reactive astrocyte-mediated neurotoxicity is a leading cause of neuronal loss observed in various neurological disorders including neuroAIDS. NeuroAIDS encompasses the neurological complications associated with HIV-1 infected individuals; around 50% of such individuals manifest some degree of HIV-1 associated neurocognitive disorder (HAND).[39-41]

Calcium excitability of astrocytes is a well-established phenomenon in the field of glia biology. In fact, calcium regulates astroglial secretions including glutamate, cytokines, and/or chemokines.[14,28,32] Hence, it plays a central role in inducing astroglial activity in both physiological and pathophysiological conditions.

Coronin 1A has been found to regulate T cell differentiation, actin cytoskeleton dynamics, and cell activation events.[20] It also regulates the survival as well as proliferation in stimulated naive T cells by mediating calcium mobilization[23] and links cytoskeletal dynamics to T cell receptor (TCR)-induced cell signaling.[21,27] In the brain, it has been found to influence the nerve growth factor (NGF)-tyrosine kinase receptor A (TrkA) endosomal trafficking as well as the signaling events.[42] Coronin 1A also interacts with Gαs, a G protein subunit, upon cell stimulation, to activate the cAMP and PKA signaling pathway. Activated PKA phosphorylates and activates cAMP response element-binding protein (CREB), which facilitates the transcription of genes involved in memory formation and other cognitive functions.[22] Whether the roles of this protein are cell type- and context-dependent is not clear yet and demands extensive exploration. Astrocytes, indeed, play significant roles in regulating neuronal activity, and astrocyte reactivity directly influences neuronal health and functioning in pathological contexts. Whether these cells express coronin 1A, and what role it plays is not known at all. To address this, and to identify the roles of this protein in different cell types and contexts, we investigated the role of coronin 1A in astrocytes in resting and stimulated conditions. We also explored its role in astrocyte reactivity observed in the presence of a potent neurotoxicant HIV-1 Tat B. For the current study, we used a well-characterized model of primary astrocytes and neurons that were differentiated from human fetal brain-derived neural precursor cells.

Coronin 1A has mostly been studied in cells of the hematopoietic origin, as discussed above. So, at first, we assessed the coronin 1A expression in astrocytes; Jurkat T cells were taken as a positive control. Human astrocytes were found to express coronin 1A. Immunocytochemistry performed to confirm the intracellular localization of coronin 1A in astrocytes revealed its expression throughout the cytoplasm of the cell. Coronin 1A expression was also validated on human brain autopsy sections (Figure 1). The astrocytic culture was found to be positive for GFAP and vimentin in more than 95% of the cells (Figure S1).

To explore the functions of this protein in resting-state astrocytes, we deployed a siRNA-mediated loss-of-function approach. The siRNA-mediated coronin 1A downregulation did not alter the GFAP or vimentin expression (Figure 2). Also, the above experimental condition did neither alter the cell viability nor the cell-proliferation (Figure S2). In general, the above results indicate that coronin 1A downregulation does not affect the resting-state astrocytes. Though the scope of this study was limited and focused on human primary astrocytes, generating a cell line lacking coronin 1A, to assess the loss-of-function and/or re-expressing the siRNA-resistant form of coronin 1A, may provide a wider window for manipulation-based studies. Such approaches can be utilized in the future by

researchers of similar interests and are beyond the scope of the current study. The literature about the role of this protein in T cell activation[20,23] provided us the momentum to investigate its involvement, if any, in stimulated conditions and to assess if its roles are activity-dependent.

Astrocytes possess calcium excitability and gliotransmitters such as ATP, glutamate can stimulate astrocytes and cause calcium mobilization followed by downstream calcium signaling.[28-30] To explore whether coronin 1A plays activity-dependent roles, we measured ATP-induced calcium mobilization. Interestingly, we found that coronin 1A knockdown results in impaired calcium mobilization, especially when the extracellular buffer used was devoid of $CaCl_2$, i.e., when the source of calcium was only the intracellular stores (Figure 3A,B). To address the reason behind impaired calcium mobilization, we assessed the phosphorylation pattern of PLCγ1, which is the most common and widely studied enzyme involved in calcium signaling. PLCγ1 phosphorylation was found to be, although similar at $t = 0$ s, but affected at later time-points of ATP stimulation, especially at $t = 120$ s in astrocytes downregulated for coronin 1A. Although it is not very strong evidence, this gave us a hint that this enzymatic pathway might have been affected and can be the cause of impaired calcium mobilization (Figure S3). Such observations, however, demand detailed assessments of several enzymes involved in calcium signaling and are beyond the scope of the current study. We also assessed the phosphorylation of ERK1/2, which is a Ca^{2+} dependent enzyme, and it showed slightly reduced phosphorylation, but the differences were not significant (Figure S3). The impaired Ca^{2+} flux upon ATP-stimulation indicated the activity-dependent roles of coronin 1A in astrocytes and therefore prompted us to look for its involvement in astrocyte reactivity.

HIV-1 Tat is a potent neurotoxicant and is known to induce astrocyte reactivity, which is characterized by an increase in intracellular calcium levels,[18] GFAP expression[7]; it also elevates the expression of cell adhesion molecules such as VCAM-1 and ICAM-1 in astrocytes[43,44] and thus can facilitate the entry of inflammatory cells into the CNS. HIV-1 Tat also induces the expression of inducible nitric oxide synthase (iNOS) and Cyclooxygenase (COX)-2, an enzyme involved in prostanoid synthesis, which further modulates the expression of several inflammatory cytokines and chemokines.[45,46] In agreement with the above-mentioned reports, we report heightened calcium response upon ATP stimulation (Figure 3C) and the enhanced expression of GFAP in human astrocytes transfected with the HIV-1 Tat expression vector, which confirmed the reactive status of these cells. Also, HIV-1 Tat was found to augment the release of IL-6 and the transcript levels of several pro-inflammatory genes such as VCAM-1, ICAM-1, PTGS2, and NOS2. Interestingly, these reactive astrocytes also exhibited the enhanced expression of coronin 1A, which further pointed out its activity-dependent roles. Validating the enhanced expression of coronin 1A on human brain autopsy sections supported our hypothesis even more (Figure 4). Additionally, serial sections stained for GFAP confirmed the presence of astrocytes in the same region of the hippocampal sections (Figure S4).

If coronin 1A has activity-dependent roles, then it might also be involved in the astrocyte reactivity observed in the presence of HIV-1 Tat. Hence, to explore whether it really facilitates astrocyte reactivity, the HIV-1 Tat expression vector was transfected in cells downregulated for coronin 1A, sequentially. It was indeed observed that HIV-1 Tat-induced astrocyte reactivity, assessed by measuring levels of GFAP and some pro-inflammatory genes, PTGS2 and NOS2, diminished when the cells were downregulated for coronin 1A (Figure 5). Coronin 1A downregulation could not diminish the HIV-1 Tat-induced VCAM1 or ICAM1 transcript levels. This could be because the adhesion molecules might not fall in the cascade of coronin 1A-mediated events. The above observations propelled us to also assess the cell functionality by measuring the levels of released factors. Functional assessment performed on the conditioned media revealed that downregulating coronin 1A attenuated the deleterious outcomes of the reactive astrocytes such as the enhanced release of IL-6 (Figure 5). Cumulatively, the reduced inflammatory response further supported primary neurons, as the neuronal survival was found to be improved in the group treated with conditioned media collected from reactive astrocytes downregulated for coronin 1A (Figure 6B). The above findings confirmed our hypothesis of the involvement of coronin 1A in facilitating HIV-1 Tat-induced astrocyte reactivity.

A closer look at the results obtained from the sequential transfection of coronin 1A siRNA followed by transfection of the HIV-1 Tat expression vector points out that downregulating coronin 1A (compare columns with blue dots) suppressed the levels of released IL-6 to a level even lower than what was observed in the scrambled control group (Figure 5C). This observation requires in-depth exploration and has not been addressed in the current study.

Micro-RNAs dysregulated under the effect of HIV-1 often contribute to HIV-1 neuropathogenesis.[36-38] Small RNA sequencing data gave us the pool of HIV-1 Tat-induced dysregulated miRNAs (Figure S5). We further narrowed to miRNAs that are downregulated under the effect of HIV-1 Tat and target the CORO1A 3′ UTR. The expression of short-listed miRNAs was assessed in the cells transfected with the HIV-1 Tat expression vector, using the real-time PCR assay. Out of all the selected miRNAs, miR-92b-5p was found to be affected the most, and hence, it could act as the potential regulator of the

coronin 1A expression in reactive astrocytes. Utilizing the mimic and inhibitor against miR-92b-5p to assess its binding with CORO1A 3' UTR followed by measuring the protein expression further confirmed the involvement of miR-92b-5p in regulating the coronin 1A expression in the HIV-1 Tat-induced reactive astrocytes. Mimic-92b-5p significantly reduced the luciferase activity obtained when CORO1A 3′ UTR was cloned in the pMIR vector having the luciferase gene. The mimic also decreased the transcript and protein levels of coronin 1A. In contrast, inhibitor-92b-5p increased the transcript and the protein levels of coronin 1A (Figures 7 and 8). The above findings illustrate the point that miR-92b-5p mediates coronin 1A regulation in astrocytes under the effect of HIV-1 Tat.

This study concludes that coronin 1A, although does not play an essential role in resting-state astrocytes, plays activity-dependent roles by facilitating calcium mobilization upon ATP stimulation. The coronin 1A expression is elevated in HIV-1 Tat-induced reactive astrocytes and the brain sections of HIV-1 infected individuals, indicating its possible involvement in reactive astrocyte-mediated neuroinflammation. Downregulating coronin 1A attenuates the deleterious effects of reactive astrocytes and hence supports neuronal survival. MiR-92b-5p regulates the coronin 1A expression by targeting its 3′ UTR. By reducing the levels of miR-92b-5p, HIV-1 Tat might augment coronin 1A protein levels, which further promotes astrocyte reactivity and astrocyte-mediated neuroinflammation. Hence, this study reports the non-canonical, activity-dependent roles of coronin 1A in stimulated astrocytes, and its role in promoting HIV-1 Tat-induced astrocyte reactivity.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests related to this article.

AUTHOR'S CONTRIBUTIONS

HSP designed, performed experiments, analyzed, and interpreted the data, and formulated the manuscript. RK performed experiments and helped in analyzing data. Bindu helped in performing experiments. PS helped in designing experiments, analyzing data, and finalizing the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article [and its supplemental information file].

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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