Published in final edited form as:

J Cell Physiol. 2014 August ; 229(8): 1039–1046. doi:10.1002/jcp.24528.

Involvement of IRS-1 interaction with ADAM10 in the regulation of neurite extension

Jin Ying Wang¹, Armine Darbinyan^{1,3}, Martyn K. White¹, Nune Darbinian¹, Krzysztof Reiss⁴, and Shohreh Amini^{1,2,*}

¹Department of Neuroscience, Center for Neurovirology, Temple University School of Medicine, Philadelphia, PA 19140

²Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA 19122

³Department of Pathology, The Mount Sinai Medical Center, New York, NY 10029

⁴Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Abstract

The insulin-like growth factor-1 (IGF-1) signaling pathway plays an important role in neuronal cell differentiation. Recent studies have shown that IGF-1 has the capacity to counteract the retraction of neuronal processes in response to inflammatory cytokines such as TNF- α , which is a known factor for neuronal injury in the CNS. This event is thought to be mediated via interference of TNF- α -induced interaction of β 1-integrin with insulin receptor substrate-1 (IRS-1). Here we demonstrate the interaction of IRS-1 with disintegrin and metalloproteinase ADAM10 through the N-terminal domain of IRS-1 and that this is involved in the regulation of neurite extension and retraction by IGF-1 and TNF- α , respectively. PC12 cells expressing the N-terminal domain show enhanced neurite extension after IGF-1 treatment and reduced neurite depletion relative to control cells after TNF- α treatment. The level of ADAM10 was found to be increased in immunohistochemical studies of HIV encephalitis clinical samples and is present with TNF- α and TNFR1 in both astrocytes and neurons. Altogether, these observations suggest a role for ADAM10 in the mechanism for IGF1/IRS-1 signaling pathway in sustaining the stability of neuronal processes.

Keywords

IRS-1; ADAM10; IGF-1; TNF- α ; neurite extension; signal transduction

*Corresponding Author, Dr. Shohreh Amini, Department of Neuroscience, Center for Neurovirology, Temple University School of Medicine, Room 760 MERB, 3500 N. Broad Street, Philadelphia, PA 19140, Tel: 215-707-4500, Fax: 215-707-4888, shohreh.amini@temple.edu.

INTRODUCTION

A variety of diseases can result in damage and loss of viability and neurite integrity in the central nervous system (CNS) including disorders with neurodegenerative, autoimmune and infectious etiologies. For example, HIV infection of brain causes complex cognitive, behavioral and central motor abnormalities referred as HIV-associated neurocognitive disorder (HAND), which is one of the most common disorders in HIV-infected patients (McArthur et al., 2010) and includes asymptomatic neurocognitive impairment (ANI), HIV-associated mild neurocognitive disorders (MNDs), and HIV-associated dementia (HAD) (Antinori et al., 2007). Neurons are not infected with HIV (Power, 2001) but rather neuronal cell damage is mediated by viral proteins and inflammatory molecules released by other cells in the CNS, e.g., monocyte-derived perivascular macrophages and microglia (González-Scarano and Martín-García, 2005; Kaul et al. 2005). The loss of dendritic arborization and synaptic connections that underlies the pathogenesis of HAND, correlates with neurocognitive decline in AIDS patients and parallels with viral-induced neuroinflammation in the CNS (Masliah et al., 1997). Viral proteins, including gp120 and Tat, and inflammatory cytokines, including tumor-necrosis factor alpha (TNF- α), greatly contribute to synaptic dysfunction through various mechanisms, (Bezzi et al. 2001; Garden et al., 2002; González-Scarano and Martín-García, 2005; Lindl et al., 2010).

In addition to the activation of neurotoxic processes induced by proinflammatory cytokines such as TNF- α , brain injury can also be characterized by the failure of neuroprotective pathways such as insulin-like growth factor-I (IGF-1) signaling. IGF-1 functions primarily via the IGF-1 receptor (IGF-1R), a transmembrane, ligand-activated tyrosine protein kinase highly homologous to the insulin receptor. The mitogen-activated protein kinase and phosphatidylinositol 3' kinase signaling pathways are activated by association of IGF-1 with IGF-1R (Dupont et al., 2003; Vincent and Feldman, 2002). Previous studies have shown that IGF-1 signaling events counteract the retraction of neuronal processes which is mediated by TNF- α (Wang et al., 2006, 2007). Evidently, IGF-1 can interfere with TNF- α -induced interaction of membrane associated β 1-integrin with insulin receptor substrate 1 (IRS-1), which is a key molecule in transmitting signals from the IGF-1R to intracellular pathways (Wang et al., 2006, 2007).

ADAM10 is a member of the ADAM family of disintegrin and metalloprotease transmembrane proteins, which function in integrin-binding, intracellular signaling and cell adhesion activities (Yang et al., 2006). ADAMs play important roles in the development of the nervous system, where they regulate cell migration, proliferation, differentiation and axonal and neurite growth. Studies with Adam10 conditional knock-out mice have shown that ADAM10 plays a crucial role in the developing brain and is essential for the establishment of the brain cortex (Jorissen et al., 2010). Here we show that IGF-1 promotes interaction of IRS-1 with ADAM10 and prevents the destabilizing impact of TNF- α on neuronal processes. This observation provides insight into interaction processes between two signaling pathways with opposing effects on the status of neuronal cells and suggests a new mechanism which can participate in neuronal cell injury in the CNS.

MATERIALS AND METHODS

Cell culture

PC12 rat pheochromocytoma cells (ATCC No. CRL-1721) were cultured as previously described (Wang et al., 2006). To induce neuronal differentiation, PC12 cells were plated on collagen IV (Sigma, St. Louis, MO)-coated dishes and treated with 20 ng/ml nerve growth factor (NGF; Invitrogen, Carlsbad, CA) in serum-free medium (SFM). Neuronal processes began to form within first 24 hr following the treatment and were preserved as differentiated neuronal cultures for 5–10 days. PC12 cells differentiated in the presence of NGF for 5 days were treated with 50 ng/ml TNF- α (BD Biosciences, Palo Alto, CA) or 50 ng/ml IGF-I (Invitrogen).

Mouse cortical neurons were prepared from mouse embryos of 17-day pregnant C57BL6 mice (E17). Cortices were dissected out and placed in Hibernate E medium (BrainBits, Springfield, IL). After careful removal of the meninges, the intact tissue was incubated with Tryple Express enzyme (Invitrogen, Carlsbad, CA) at 37°C for 10 min, followed by three washes with Hibernate E medium. Tissue trituration was performed in culture medium (see below) using a fire-polished glass Pasteur pipette, and single cell suspension was diluted with culturing medium. Finally, cells were plated on 60 mm poly-D-lysine-coated dishes (Sigma) at a density of 2.5×10^6 cells per 60 mm² dishes and cultured in Neurobasal medium containing B27 supplement, 0.25 mM Glutamax and 0.25 mM L-glutamine (all from Invitrogen). Cytosine arabinoside (Ara-C, Sigma, final concentration 1 μ M) was added after 16 hours for two days to reduce glial proliferation (Darbinyan et al., 2013). Treatment of neuronal cultures with Ara-C (48 hours) efficiently depletes proliferating cells. At day 10 or 12 of *in vitro* culturing more than 98% of cells were positive for neuronal marker class III β -tubulin (verified by immunocytochemical analysis). All studies involving the use of animals were approved by the Temple University Animal Care and Use Committee.

Derivation of clonal cell lines from PC12 expressing IRS-1 truncation mutants

PC12/IRS-1 1–300 and PC12/IRS-1 232–529 cell lines stably express IRS-1 mutants 1–300 and 232–529 respectively and were created by transfection of PC12 cells with IRS-1 1–300/pCDNA3.1 or IRS-1 232–529/pCDNA3.1 expression vectors. The IRS-1 1–300aa/pCDNA3.1 and IRS-1 232–529/pCDNA3.1 expression vectors were created by subcloning of the IRS-1/1–300 and IRS-1/232–529 fragments into the *EcoRI*/*Bam*HI sites of pCDNA3.1 vector from the pGEX-5x-1 vector. Geneticin (100 μ g/ml, GIBCO) was used for the selection of stable clones. pCDNA3.1 plasmid was used to make the control cell line. The expression of IRS-1 1–300 and IRS-1 232–529 truncation mutants was confirmed by Western blot assay using anti-IRS-1 (D23G12) rabbit Mab (Cell signaling), which recognizes both endogenous and truncated IRS-1 proteins.

Antibodies

Anti-ADAM10 (A-3, sc-48400), anti-GFAP(C19) goat polyclonal IgG antibodies (sc-6170) were purchased from Santa Cruz Inc. Anti-Cleaved caspase-3 (Asp175), phospho-GSK3 β (Ser9) #9336, and anti-IRS-1 antibody #2382 were obtained from Cell Signaling. Neuronal class III β -Tubulin (TUJ1) monoclonal antibody, Alexa Fluor® Labeled (cat #A488-435L),

was obtained from Covance Inc. Purified mouse anti-Grb2 (#610111) antibody was purchased from BD Biosciences. Anti-Integrin β 1 antibody (EP1041Y)-carboxyterminal end, ab52971 was from Abcam.

Transfection

Mouse cortical neurons were transfected with 50 nM plasmid DNA using Oligofectamine (Invitrogen) according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). In all experiments, Western blot analysis for IRS-1 was performed with protein extracts from control cells and cells transfected with IRS-1⁻specific siRNA to verify IRS-1 knock-down with anti-Grb2 antibody used as a loading control. Cells were treated with either IRS-1 siRNA or non-targeting siRNA using Oligofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). IRS-1 siRNA and nontargeting siRNA were obtained from Santa Cruz (Santa Cruz Biotechnology).

Isolation of membrane raft-enriched fraction

The proteins from membrane rafts and detergent soluble fractions were isolated according to a protocol modified from the method of Tai et al (2003). Briefly, cell pellets were collected from dishes and lysed in ice-cold lysis buffer containing 1% Brij 58, 20 mM Tris (pH 7.5), and 150 mM NaCl with freshly added protease and phosphatase inhibitors. The lysates were centrifuged at 14000 $\times$ g for 25 min at 4°C. The supernatants were saved as detergent soluble fractions. The pellets were detergent-insoluble and contained proteins that bind to the membrane, which were re-suspended in the lysis buffer by the addition of 5% SDS and 1% β -mercaptoethanol. The viscosity was decreased by sonication and the aggregate of denatured nucleic acid was removed by centrifugation.

GST Fusion Proteins and GST pull-down assays

IRS-1 truncation mutants within pGEX-5x-1 vector have been described (Wang et al., 2007). Full-length mouse IRS-1 gene within pGEX 5x-1 vector was generated by inserting full length mouse IRS-1 gene amplified by PCR using pcDNA3.1 IRS-1 into PGEX-5x-1 vector at the NotI site. Cloned pGEX-5x-1IRS-1 vector was verified by sequencing. For preparation of bacterially produced GST fusion proteins, we followed the methods previously described (Gallia et al., 1998). Overnight cultures (100 ml) of *Escherichia coli* DH5 α , were transformed with pGEX-5X1-IRS-1, and pGEX-5X1-IRS1 truncated mutants. GST pull-down assay was performed according to the procedures described previously (Darbinyan et al., 2002). Briefly, cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM NaF, with freshly added protease and phosphatase inhibitor. Five hundred micrograms of protein lysate were incubated with an equal molar amount of purified pGEX-5X1-IRS-1, pGEX-5X1-IRS1 truncated mutants and pGEX-5X1 proteins at 4°C for overnight with rocking, washed thoroughly with lysis buffer, resuspended with reduced Laemmli buffer, boiled and applied to SDS-PAGE followed by Western blotting with anti-ADAM10 (A-3) antibody (sc-48400, Santa Cruz Biotechnology). The ECL enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) was used according to the manufacturer's recommendations.

IP/Western

Immunoprecipitation/western blots were performed as previously described (Wang et al., 2007) using antibody to ADAM10 or IRS-1 for precipitation and antibodies to IRS-1, ADAM10 and β 1-integrin for the Western blot. Precipitation with nonimmune rabbit serum was the negative control. The blots were subsequently washed three times and bound antibody detected either with the ECL kit or with the LI-COR system. For the LI-COR system, blots were incubated with IRDye® 800CW Goat Anti-Rabbit and IRDye® 680RD Goat Anti-Mouse Li-COR dyes and visualized with an Odyssey® CLx Imaging System (LI-COR, Inc., Lincoln, NE) using Odyssey software (LI-COR Biosciences, Lincoln, NE, USA).

Neurite extension and cell growth assays

The measurements of neurite-like processes were taken from series of photographs from selected microscopic fields and reflect an average length of neurite-like processes in a particular microscopic field, as previously described (Wang et al., 2006), using Image J software (NIH). The growth of cells of PC12/IRS-1 1–300 stable cell line was evaluated using Trypan Blue cell exclusion assay at three time points (24hr, 48hrs and 72hrs, respectively). Experiments were performed in triplicate.

Immunocytochemistry

PC12 cells were seeded on Collagen IV-coated glass chamber slides and induced to differentiate with NGF 20 ng/ml. After 5 days NGF was removed and cells were incubated with IGF-1, TNF- α or both. Control cells were kept in serum free medium. After 24 hours cells were fixed with 4% paraformaldehyde, permeabilized for 5 min in 0.25% Triton X100/PBS solution, blocked with 5% normal horse, or goat, or rabbit serum (depending on the type of primary antibody) in 0.1% PBS-BSA for 1 h following incubation with antibody. Control cells were processed without primary antibody. Cells were then washed three times with PBS-0.01% Triton X-100 and incubated with FITC-conjugated anti-mouse, or anti-goat, or anti-rabbit secondary antibody for 1 h. Slides were washed three times with PBS, blocked for a second time with 5% normal horse, or goat, or rabbit serum (depending on the type of primary antibody) for 1 hour followed by incubation with the second set of antibodies. After 16 h, cells were washed, incubated with rhodamine-conjugated secondary antibodies and mounted using mounting medium for fluorescence from Vectashield (cat # H-1400). Nuclear DNA was labeled with DAPI. Slides were examined by fluorescence microscopy.

Clinical brain samples and immunohistochemistry

A total of 6 archival cases of formalin fixed, paraffin embedded HIVE cases (three patients) with their respective controls (three cases) were collected from the NIH National Tissue Consortium at the Manhattan Brain Bank, Mount Sinai School of Medicine, New York, New York. Patients were 41–52 years old males. Immunohistochemistry was performed based on our modified protocol which includes deparaffination in xylene, re-hydration in alcohol up to water, non-enzymatic antigen retrieval in citrate buffer, pH 6.0 at 95°C for 30 minutes. Sections were rinsed with PBS, were blocked with 5% normal horse, or goat, or rabbit serum (depending on the type of primary antibody) in 0.1% PBS-BSA for 1 hour

following incubation with specific antibodies. Primary antibodies utilized included ADAM10, TNF- α , TNFR1, GFAP, and class III β tubulin. Sections were then washed three times with PBS-0.01% Triton X-100 and incubated with FITC conjugated anti-mouse, or anti-goat, or anti-rabbit secondary antibody for 1 hour. Slides were washed three times with PBS, blocked for a second time with 5% normal horse, goat, or rabbit serum (depending on type of second primary antibody) for 1 h following incubation with second set of specific antibodies. After 16 h of incubation, cells were washed, incubated with rhodamine-conjugated secondary antibodies, and mounted using mounting medium for fluorescence from Vectashield. Nuclear DNA was labeled with DAPI. Slides were examined by fluorescence microscopy.

RESULTS

Interaction of ADAM10 with IRS-1 and β 1 integrin in PC12 cells

PC12 cells were untreated or treated with IGF-1, TNF- α or both for 5 days and total cell protein harvested and analyzed by Western blot (Fig. 1A). In all four sets of cells, IRS-1 levels were unchanged but TNF- α induced IRS-1 serine phosphorylation on Ser612 in agreement with earlier studies (Rui et al., 2001; Wang et al., 2007). TNF- α and IGF-1 also induced the expression level of ADAM10, while β 1-integrin expression was largely unchanged. Interestingly, the level of ADAM10 declined in the presence of both TNF- α and IGF-1 and this was unexpected. Membrane raft-enriched cell extract protein fractions were also prepared from the treated and untreated cells and subject to immunoprecipitation (IP) with an anti-ADAM10 antibody. Subsequent analysis by Western blotting using antibodies to IRS-1, phosphor-Ser612-IRS-1, β 1 integrin and ADAM10 is shown in Fig. 1B. ADAM10 bound to IRS-1 in untreated, TNF- α - and IGF-1- treated conditions but the amount of IRS-1 bound to ADAM10 was diminished in the presence of both cytokines. Interestingly, IGF-1 stimulated binding of ADAM10 to phosphorylated IRS-1 and β 1 integrin. Similar results were found for ADAM10 association in the reciprocal IP/Western where α -IRS-1 antibody was used for IP, i.e., ADAM10 association was weakened in the presence of both cytokines (Fig. 1C). To identify the ADAM10 binding region within IRS-1, GST-pull down assays were performed with GST-IRS-1, full-length and truncation mutants (Fig. 1D). Results show the binding region is located in the N-terminal 300 amino acids. This is distinct from the site on IRS-1 to which β -integrin was found to bind as shown in the schematic in the bottom part of Fig. 1C (Wang et al., 2007).

Interaction of ADAM10 with IRS-1 in PC12 cells expressing IRS-1 truncation mutants

To further investigate ADAM10/IRS-1 interaction, we produced a series of clonal PC12 derivatives expressing truncation mutants of IRS-1 as described in Materials and Methods. We performed IP with antibody to ADAM10 and nonimmune mouse serum (NMS) was used as a control. IRS-1 (1–300) was coprecipitated from extracts of the clonal cells that express it (Fig. 2, lane 5) but this was not the case for IRS-1 (232–529), (Fig. 2, lane 6), in agreement with the data from Fig. 1D that this region is involved in IRS-1 binding to ADAM10.

Effect of downregulation of IRS-1

To further investigate the role of IRS-1, mouse primary cortical neurons were transfected with siRNA targeting mouse IRS-1 using Oligofectamine reagent. Untransfected neurons or neurons transfected with the non-targeting siRNA served as controls. After one day, neurons were treated with 50 ng/ml IGF-1, or 50 ng/ml TNF- α , or both for 5 days. Controls were left untreated. Membrane raft-enriched fractions were prepared and Western blot analysis was performed (Fig. 3). Downregulation of IRS-1 by IRS-1 siRNA decreased IRS-1 levels as expected and also the levels of phosphorylated GSK-3 β , which is downstream from IRS-1 in signal transduction. The importance of IRS-1 in apoptotic signaling is indicated by the appearance of activated caspase 3 in all samples where IRS-1 was downregulated, whereas it was not detectable in the absence of IRS-1 siRNA. Importantly, downregulation of IRS-1 decreased the level of ADAM10 associated with the membrane raft-enriched fractions. This experiment was performed three times and these results were reproducible. However note that there is reduced IRS-1 expression in mock transfected cells treated with both TNF- α and IGF-1 (lane 4) that is not seen in NT- siRNA (lane 8) or IRS-1 siRNA treated (lane 12) and this was not reproducible.

Neurite extension and cell growth in PC12 cells expressing IRS-1 truncation mutants

Structural and functional modifications of neuronal processes are essential for formation and maintenance of neuronal connections. We have previously demonstrated that IGF-1 opposes the TNF- α -induced retraction of neuronal processes (Wang et al., 2007). To investigate the impact of TNF- α on neuronal processes and the mechanisms of neuroprotection by IGF-1 signaling, PC12/IRS-1(1–300) and PC12/pCDNA3.1 (Control) cells were plated on collagen IV-coated dishes and induced to differentiate with 20 ng/ml NGF in serum-free medium supplied with 0.1% bovine serum albumin. Neuronal processes start to grow within 24 hours of NGF treatment and the network of fine neuronal processes is formed in 5 days.

Differentiated cells were treated with 50 ng/ml IGF-1, 50 ng/ml TNF- α or both for 5 days and neurite extension measured using the Image J software as described in Materials and Methods. Untreated cells served as a control. IGF-1 exposure enhanced the dense network of neuronal processes and this effect was significantly greater ($p < 0.05$, t-test) in the cells expressing IRS-1(1–300) than the control cells (Fig. 4A, bars 3 and 4). TNF- α treatment lead to decreased density of the PC12 neurites and this was significantly ameliorated ($p < 0.05$, t-test) in the cells expressing IRS-1 1–300 compared to control cells (Fig. 4A, bars 5 and 6).

Treatment with both IGF-1 and TNF- α had an intermediate effect but again the cells expressing IRS-1(1–300) showed significantly more neurite extensions compared to control cells (Fig. 4A, bars 7 and 8). Interestingly, there was observed an opposite effect of IRS-1(1–300) on cell viability with cells expressing IRS-1(1–300) showing a smaller rate of increase in cell number compared to control cells or parental PC12 cells (Fig. 4B).

Presumably, this reflects the occurrence of more effective neuronal differentiation, which is associated with withdrawal from the cell cycle, and a greater expenditure of cell energy on neurite extension rather than survival and/or proliferation in cells expressing IRS-1(1–300). Figure 4C shows a Western blot to verify IRS-1(1–300) expression. Figure 4D shows phase contrast micrographs of PC12 cells after the various treatments. The positive and negative effects of IGF-1 and TNF- α on neurite extension are clearly evident. The clonal derivatives of PC12 appeared qualitatively very similar (data not shown) although the Image J analysis

shows the quantitative differences between them (Fig. 4A). Similarly, the positive and negative effects of IGF-1 and TNF- α on neurite extension were also observed in mouse cortical neurons (data not shown).

Colocalization of ADAM10 with phospho-Ser 612 IRS-1 in PC12 cells

PC12 cells were treated with IGF-1, TNF α or both and analyzed by immunocytochemistry (Fig. 5). Immunofluorescence for phospho-Ser 612 IRS-1 (green) demonstrates predominant expression in neuronal processes. ADAM10 expression (red) increased significantly upon treatment with IGF-1 and TNF α . Increased co-localization of ADAM10 and phospho-Ser 612 IRS-1 (yellow) was observed in neuronal processes upon treatment with IGF-1 or TNF- α but less so with both and this is consistent with the earlier data. Similar results were obtained with rat primary neurons (data not shown).

Detection of ADAM10 and co-expression with TNFR1 and TNF- α in brain tissue samples from patients with HIV

Brain tissue samples from three HIV-positive patients with HIV and three HIV-negative patients with no brain pathology were obtained from the Manhattan Brain Bank and evaluated for expression of ADAM10, TNFR1 and TNF- α by immunohistochemistry (Fig. 6). We examined the expression of ADAM10 in brain tissue samples. Double labeling with GFAP showed increased cytoplasmic expression of ADAM10 in enlarged astrocytes in HIV cases but not in control samples as seen in Fig. 6A (panels on the left). Gliosis in the cerebral cortex from patients with HIV is identified by the presence of glial fibrillary acidic protein (GFAP, green fluorescence), which reveals stellate morphology and fine processes of astrocytes. Also, increased cytoplasmic immunoreactivity for ADAM10 was detected in cortical neurons (red fluorescence) in HIV cases which overlapped with β -tubulin (green fluorescence) as seen in Fig. 6A (panels on the right). ADAM10 was weakly detected in the cytoplasm of neurons in brain samples from the control patients without brain pathology (bottom panel on the right). Increased expression of TNF- α was observed in neurons of HIV cases compared to those from control samples as seen in Fig. 6B (upper panels). Co-localization of ADAM10 with TNF- α was detected in neurons and rarely in perivascular cells in HIV samples. Double labeling with TNFR1 showed that ADAM10 was also co-localized with TNFR1 in neurons and neuronal processes in HIV brain as seen in Fig. 6B (lower panels).

DISCUSSION

The IGF-1/IRS-1 signaling axis is involved in molecular pathways, which regulate structural rearrangements in neuronal processes. Structural and functional modifications of neuronal processes are essential for formation and maintenance of neuronal connections, for synaptic plasticity and ultimately for learning and memory consolidation. We have previously demonstrated that IGF-1 opposes the TNF- α -induced retraction of neuronal processes (Wang et al., 2007). Our previous studies also indicated that interaction of IRS-1 with β -integrin is involved in this effect (Wang et al., 2006, 2007). The cytokine TNF- α has been identified as one of cellular molecules responsible for neuronal injury in CNS seen in a variety of pathological conditions including HAND in HIV-1/AIDS patients (Régulier et al.,

2004). Conversely, brain injury can also be typified by the breakdown of pathways associated with neuroprotection such as those activated by IGF-1/ IGF-1R signaling through its major plasma membrane target IRS-1, e.g., MAPK and PI3K (Dupont et al., 2003; Vincent and Feldman, 2002). Tropea et al (2009) reported that an active peptide fragment of IGF-1 was able to effect a partial reversal of Rett-like Syndrome in MeCP2 mutant mice and Pini et al (2012) have performed a small preliminary clinical study with IGF-1 active peptide to treat Rett Syndrome patients. Landi et al (2011) showed that the dynamic properties of dendritic spines of layer V cortical neurons are deeply affected in a mouse model of Rett syndrome but that 24h after a subcutaneous injection of IGF-1 spine dynamics is restored. In another study, IGF-1 was found to induce cleavage and shedding of the amyloid precursor protein (APP) by activation of the disintegrin and metalloprotease ADAM10 in human neuroblastoma SH-SY5Y cells (Jacobsen et al., 2010). We have now discovered a role for ADAM10 in the interplay between IRS-1 and β 1-integrin, which functions to control neurite extension.

We have found that ADAM10 expression is induced by TNF- α and IGF-1 and that ADAM10 interacts with IRS-1 demonstrated by IP/Western and GST pull-down experiments. This interaction involves the N-terminus of IRS-1, which comprises two functional domains of this docking molecule, pleckstrin homology (PH) and phosphotyrosine binding (PB) (Myers et al., 1994). IRS-1 also binds to β 1-integrin via a central domain within the IRS-1 protein (Wang et al., 2007). TNF- α increased total cellular IRS-1 Serine 612 phosphorylation, yet IGF-1 but not TNF- α increased the association of Serine 612 phosphorylated IRS-1 with ADAM10. This suggests a model whereby an interplay between ADAM10, IRS-1 and β 1-integrin is involved in neurite extension and its regulation by neurotoxic, e.g., TNF- α , and neuroprotective, e.g., IGF-1, cytokines. Hence this interplay is likely a key modulator of neurite damage in various pathological conditions. In support of this, we found that PC12 cells expressing a truncated protein with the IRS-1 N-terminal binding domain was able to enhance the positive effect of IGF-1 on neurite extension and ameliorate the negative effect of TNF- α .

In the light of these findings, we analyzed the status of ADAM10 in clinical samples involving neuronal damage. Immunohistochemistry was used to examine ADAM10 expression in HIVE and a HIV-negative control. We saw an elevation in the levels of ADAM10 in HIVE compared to controls and this was found in neurons and astrocytes as revealed by co-labeling with β III tubulin and GFAP respectively. Since TNF- α has been identified as one of cellular molecules responsible for neuronal injury in CNS seen in HIV-1/AIDS patients (Régulier et al., 2004), we also examined TNF- α levels and TNFR1 in the same samples. Increased cytoplasmic immunoreactivity for ADAM10 was observed in cortical neurons in HIVE cases and increased expression of TNF- α was observed to be associated with neurons, perivascular and parenchymal inflammatory cells compared with control samples.

There are data from other investigators that support our findings. Interestingly, mutant mice deficient in IGF-1 and IGF-1R, as well as those with overexpression of IGF-1 binding proteins that can inhibit IGF-1, exhibit brain growth retardation, decrease in glucose utilization (Cheng et al., 2000; Gualco et al., 2010) and altered lipid and microtubule

metabolism, resulting in impaired growth of neurons and dendritic arborization (Cheng et al., 2003). Also in agreement with our data are recent studies that have shown that IGF-1 can act as an anti-inflammatory cytokine in the brain and potentially inhibits TNF- α (Bluthe et al., 2006). It was also reported that the signaling pathways of IGF-1 and TNF- α may interact during neurodegeneration (Venters et al., 1999) and that overexpression of TNF- α negatively affects brain development in mice via alteration of the expression of IGF-1 system proteins (Ye et al., 2003). In addition, it was demonstrated that IGF-1 is able to rescue TNF- α -induced neuronal cell death (Kenchappa et al., 2004) and reduce neuro-inflammatory response and sensitivity of neurons to the pro-Parkinsonian neurotoxin MPTP (Nadjar et al., 2009). Decreased levels of IGF-1 and growth hormone resistance have been found to be contributing factors in AIDS wasting syndrome (Valcour et al., 2005).

In summary, our studies demonstrate that IGF-1 and TNF- α interplay modulate interactions involving IRS-1, β 1-integrin and ADAM10, which are in turn involved in molecular pathways which regulate structural rearrangements in neuronal processes. Understanding these pathways may offer new therapeutic avenues for the treatment of neuronal cell damage in pathologies of the CNS.

Acknowledgments

We wish to thank past and present members of the Department of Neuroscience and Center for Neurovirology for their continued support, insightful discussion and sharing of reagents and ideas. We also thank Dr. Susan Morgello of the Manhattan Brain Bank for providing samples and to Jernelle C. Miller for help with the neurite extension experiments. This study utilized services offered by the Comprehensive NeuroAIDS Center (CNAC NIMH Grant Number p30MH092177) at Temple University School of Medicine. This work was supported by grants awarded by the NIH to SA.

Contract grant sponsor – NIH, NINDS; Contract grant number – P01 NS43980

LITERATURE CITED

- Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, Cherner M, Clifford DB, Cinque P, Epstein LG, Goodkin K, Gisslen M, Grant I, Heaton RK, Joseph J, Marder K, Marra CM, McArthur JC, Nunn M, Price RW, Pulliam L, Robertson KR, Sacktor N, Valcour V, Wojna VE. Updated research nosology for HIV-associated neurocognitive disorders. *Neurology*. 2007; 69:1789–1799. [PubMed: 17914061]
- Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J, Volterra A. CXCR4-activated astrocyte glutamate release via TNF: amplification by microglia triggers neurotoxicity. *Nature Neurosci*. 2001; 4:702–710. [PubMed: 11426226]
- Bluthe RM, Kelley KW, Dantzer R. Effects of insulin-like growth factor-I on cytokine-induced sickness behavior in mice. *Brain Behav Immun*. 2006; 20:57–63. [PubMed: 16364817]
- Cheng CM, Reinhardt RR, Lee WH, Joncas G, Patel SC, Bondy CA. Insulin-like growth factor 1 regulates developing brain glucose metabolism. *Proc Natl Acad Sci USA*. 2000; 97:10236–10241. [PubMed: 10954733]
- Cheng CM, Mervis RF, Niu SL, Salem N Jr, Witters LA, Tseng V, Reinhardt R, Bondy CA. Insulin-like growth factor 1 is essential for normal dendritic growth. *J Neurosci Res*. 2003; 73:1–9. [PubMed: 12815703]
- Darbinyan A, Darbinian N, Safak M, Radhakrishnan S, Giordano A, Khalili K. Evidence for dysregulation of cell cycle by human polyomavirus, JCIV, late auxiliary protein. *Oncogene*. 2002; 21:5574–5581. [PubMed: 12165856]

- Darbinyan A, Kaminski R, White MK, Darbinian N, Khalili K. Isolation and propagation of primary human and rodent embryonic neural progenitor cells and cortical neurons. *Methods Mol Biol.* 2013; 1078 (In Press).
- Dupont J, Dunn SE, Barrett JC, LeRoith D. Microarray analysis and identification of novel molecules involved in insulin-like growth factor-1 receptor signaling and gene expression. *Recent Prog Horm Res.* 2003; 58:325–342. [PubMed: 12795426]
- Gallia GL, Safak M, Khalili K. Interaction of the single-stranded DNA-binding protein Puralpha with the human polyomavirus JC virus early protein T-antigen. *J Biol Chem.* 1998; 273:32662–32669. [PubMed: 9830007]
- Garden GA, Budd SL, Tsai E, Hanson L, Kaul M, D'Emilia DM, Friedlander RM, Yuan J, Masliah E, Lipton SA. Caspase cascades in human immunodeficiency virus-associated neurodegeneration. *J Neurosci.* 2002; 22:4015–4024. [PubMed: 12019321]
- González-Scarano F, Martín-García J. The neuropathogenesis of AIDS. *Nat Rev Immunol.* 2005; 5:69–81. [PubMed: 15630430]
- Gualco E, Urbanska K, Perez-Liz G, Sweet T, Peruzzi F, Reiss K, Del Valle L. IGF-IR-dependent expression of Survivin is required for T-antigen-mediated protection from apoptosis and proliferation of neural progenitors. *Cell Death Differ.* 2010; 17:439–451. [PubMed: 19834489]
- Jacobsen KT, Adlerz L, Multhaup G, Iverfeldt K. Insulin-like growth factor-1 (IGF-1)-induced processing of amyloid-beta precursor protein (APP) and APP-like protein 2 is mediated by different metalloproteinases. *J Biol Chem.* 2010; 285:10223–10231. [PubMed: 20139073]
- Jorissen E, Prox J, Bernreuther C, Weber S, Schwanbeck R, Serneels L, Snellinx A, Craessaerts K, Thathiah A, Tesseur I, Bartsch U, Weskamp G, Blobel CP, Glatzel M, De Strooper B, Saftig P. The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. *J Neurosci.* 2010; 30:4833–4844. [PubMed: 20371803]
- Kaul M, Zheng J, Okamoto S, Gendelman HE, Lipton SA. HIV-1 infection and AIDS: consequences for the central nervous system. *Cell Death Differ.* 2005; 12(Suppl 1):878–892. [PubMed: 15832177]
- Kenchappa P, Yadav A, Singh G, Nandana S, Banerjee K. Rescue of TNFalpha-inhibited neuronal cells by IGF-1 involves Akt and c-Jun N-terminal kinases. *J Neurosci Res.* 2004; 76:466–474. [PubMed: 15114618]
- Landi S, Putignano E, Boggio EM, Giustetto M, Pizzorusso T, Ratto GM. The short-time structural plasticity of dendritic spines is altered in a model of Rett syndrome. *Sci Rep.* 2011; 1:45. [PubMed: 22355564]
- Lindl KA, Marks DR, Kolson DL, Jordan-Sciutto KL. HIV-associated neurocognitive disorder: pathogenesis and therapeutic opportunities. *J Neuroimmune Pharmacol.* 2010; 5:294–309. [PubMed: 20396973]
- Masliah E, Heaton RK, Marcotte TD, Ellis RJ, Wiley CA, Mallory M, Achim CL, McCutchan JA, Nelson JA, Atkinson JH, Grant I. Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. HNRC Group. HIV Neurobehavioral Research Center. *Ann Neurol.* 1997; 42:963–972. [PubMed: 9403489]
- McArthur JC, Steiner J, Sacktor N, Nath A. Human immunodeficiency virus-associated neurocognitive disorders: mind the gap. *Ann Neurol.* 2010; 67:699–714. [PubMed: 20517932]
- Myers MG Jr, Sun XJ, White MF. The IRS-1 signaling system. *Trends Biochem Sci.* 1994; 19:289–293. [PubMed: 8048169]
- Nadjar A, Berton O, Guo S, Leneuve P, Dovero S, Diguet E, Tison F, Zhao B, Holzenberger M, Bezard E. IGF-1 signaling reduces neuro-inflammatory response and sensitivity of neurons to MPTP. *Neurobiol Aging.* 2009; 30:2021–2030. [PubMed: 18394756]
- Pini G, Scusa MF, Congiu L, Benincasa A, Morescalchi P, Bottiglioni I, Di Marco P, Borelli P, Bonuccelli U, Della-Chiesa A, Prina-Mello A, Tropea D. IGF1 as a Potential Treatment for Rett Syndrome: Safety Assessment in Six Rett Patients. *Autism Res Treat.* 2012; 2012:679801. [PubMed: 22934177]
- Power C. Retroviral diseases of the nervous system: pathogenic host response or viral gene-mediated neurovirulence? *Trends Neurosci.* 2001; 24:162–169. [PubMed: 11182456]

- Régulier EG, Reiss K, Khalili K, Amini S, Zagury JF, Katsikis PD, Rappaport J. T-cell and neuronal apoptosis in HIV infection: implications for therapeutic intervention. *Int Rev Immunol*. 2004; 23:25–59. [PubMed: 14690854]
- Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A, White MF. Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *J Clin Invest*. 2001; 107:181–189. [PubMed: 11160134]
- Tai YT, Podar K, Catley L, Tseng YH, Akiyama M, Shringarpure R, Burger R, Hideshima T, Chauhan D, Mitsiades N, Richardson P, Munshi NC, Kahn CR, Mitsiades C, Anderson KC. Insulin-like growth factor-1 induces adhesion and migration in human multiple myeloma cells via activation of beta1-integrin and phosphatidylinositol 3'-kinase/AKT signaling. *Cancer Res*. 2003; 63:5850–5858. [PubMed: 14522909]
- Tropea D, Giacometti E, Wilson NR, Beard C, McCurry C, Fu DD, Flannery R, Jaenisch R, Sur M. Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. *Proc Natl Acad Sci USA*. 2009; 106:2029–2034. [PubMed: 19208815]
- Valcour VG, Shikuma CM, Shiramizu BT, Williams AE, Watters MR, Poff PW, Grove JS, Selnes OA, Sacktor NC. Diabetes, insulin resistance, and dementia among HIV-1-infected patients. *J Acquir Immune Defic Syndr*. 2005; 38:31–36. [PubMed: 15608521]
- Venters HDQ, Liu TQ, Van Hoy RW, Dantzer R, Kelley KW. A new mechanism of neurodegeneration: a proinflammatory cytokine inhibits receptor signaling by a survival peptide. *Proc Natl Acad Sci, USA*. 1999; 96:9879–9884. [PubMed: 10449788]
- Vincent AM, Feldman EL. Control of cell survival by IGF signaling pathways. *Growth Horm IGF Res*. 2002; 12:193–197. [PubMed: 12175651]
- Wang JY, Grabacka M, Marcinkiewicz C, Staniszevska I, Peruzzi F, Khalili K, Amini S, Reiss K. Involvement of alpha1beta1 integrin in insulin-like growth factor-1-mediated protection of PC12 neuronal processes from tumor necrosis factor-alpha-induced injury. *J Neurosci Res*. 2006; 83:7–18. [PubMed: 16307448]
- Wang JY, Gualco E, Peruzzi F, Sawaya BE, Passiatore G, Marcinkiewicz C, Staniszevska I, Ferrante P, Amini S, Khalili K, Reiss K. Interaction between serine phosphorylated IRS-1 and beta1-integrin affects the stability of neuronal processes. *J Neurosci Res*. 2007; 85:2360–2373. [PubMed: 17593555]
- Yang P, Baker KA, Hagg T. The ADAMs family: coordinators of nervous system development, plasticity and repair. *Prog Neurobiol*. 2006; 79:73–94. [PubMed: 16824663]
- Ye P, Kollias G, D'Ercole AJ. Insulin-like growth factor-I ameliorates demyelination induced by tumor necrosis factor-alpha in transgenic mice. *J Neurosci Res*. 2007; 85:712–722. [PubMed: 17279553]

FIGURE 1. Interaction of ADAM10 with IRS-1 and b1 integrin

The effect of IGF-1 and TNF- α on binding of ADAM10 to IRS-1 and b1 integrin was analyzed. **A**. PC12 cells were treated with 50 ng/ml IGF-1, 50 ng/ml TNF- α or both for 5 days and total cell protein harvested to determine the levels of IRS-1, phospho-ser612-IRS-1, β 1 integrin and ADAM10. Grb2 was the control for protein loading. Each band was quantitated with the Quantity One image analysis software (Bio-Rad, Hercules CA), adjusted to the Grb2 loading control band intensity and normalized to control (Lane 1) except for Caspase 3, which was normalized to Lane 9 because there are no detectable bands

for activated Caspase 3 in Lanes 1–8. Values are printed in italics below each band. **B.** PC12 cells were treated with 50 ng/ml IGF-1, 50 ng/ml TNF- α or both for 5 days and membrane raft protein-enriched whole cell extract was prepared. Seven hundred and fifty micrograms of protein were incubated with anti-ADAM10 antibody and immunocomplexes were immunoprecipitated (IP) with the addition of protein A-Sepharose beads, resolved by SDS-PAGE, and analyzed by Western blotting using an anti-IRS-I, phospho-ser612-IRS-1, anti- β 1 integrin and anti-ADAM10 antibodies. The negative control was performed using nonimmune mouse serum (NMS) for the IP. Each band was quantitated with the Quantity One image analysis software and normalized to control (Lane 1). Values are printed in italics below each band. **C.** As in Panel A but with antibody to IRS-1 used for IP and antibody to ADAM10 used for Western blot. The negative control was performed using nonimmune mouse serum (NMS) for the IP. **D.** GST pull down assays were performed with GST-IRS-1 (full-length and truncation mutants) as described in Materials and Methods, with schematic structure of IRS-1 protein shown below.

FIGURE 2. Interaction of ADAM10 with IRS-1 in PC12 cells expressing IRS-1 truncation mutants

Total cell lysates were prepared from PC12/IRS-1 1–300, PC12/IRS-1 232–528 and PC12/pCDNA3.1. IPs were performed with antibody to ADAM10 and NMS was used as a control. Immunoglobulin G heavy chain – IgG_H; Immunoglobulin G light chain – IgG_L.

FIGURE 3. Effect of downregulation of IRS-1

Western blot analysis of protein lysates prepared from the membrane raft-enriched mouse cortical neurons transfected with siRNA to IRS-1, non-targeting siRNA (NT) or no siRNA as described in Materials and Methods. Each band was quantitated with the Quantity One image analysis software, adjusted to the Grb2 loading control band intensity and normalized to control (Lane 1) except for caspase 3, which was normalized to Lane 9 because there are no detectable bands in Lanes 1–8. Note that inactive procaspase 3 (p32) appears in all lanes while active caspase 3 (p17) appears only in cells treated with IRS-1 siRNA (lanes 9–12) and p12 is not recognized by the antibody. Values are printed in italics below each band.

FIGURE 4. Effect of TNF- α and IGF-1 on the density of the network of neuronal processes
A. PC12/IRS-1 1-300 and PC12/pCDNA3.1.C12 (Control) cells were plated on collagen IV-coated dishes and induced to differentiate in the presence of 20 ng/ml NGF in serum free medium for 5 days, and untreated, treated with 50 ng/ml IGF-1, 50 ng/ml TNF- α or both, and neurite extension measured using the Image J software as described in Materials and Methods. **B.** PC12/IRS-1 1-300, PC12/pCDNA3.1 (Control) and parental PC12 cells were grown in DMEM supplemented with 5% fetal bovine serum and 10% horse serum. Cell numbers were measured by trypan blue exclusion. **C.** Western blot of extracts from PC12/

pCDNA3.1 (Control) and PC12/IRS-1(1–300) cells to verify IRS-1(1–300) expression. Grb2 was used as a loading control. **D.** Differentiated PC12 cells were cultured with serum-free medium (SFM), TNF- α , IGF-1 or both, as indicated and phase contrast images were taken.

FIGURE 5. Immunocytochemistry of ADAM10 with IRS-1

PC12 cells were treated with 50 ng/ml IGF-1, 50 ng/ml TNF α or both for 24 hours, with 4% paraformaldehyde and subjected to immunocytochemical analysis. Immunofluorescence labeling for phospho-Ser 612 IRS-1 (fluorescein, green) and ADAM10 expression (rhodamine, red) was performed as described in Materials and Methods. Nuclei were labeled with DAPI.

FIGURE 6. Immunohistochemical detection of ADAM10, TNF-a and TNFR1 in cases of HIVE
A. Enlarged astrocytes (panels on the left) and cortical neurons (panels on the right) were analyzed by immunohistochemistry for immunoreactivity for ADAM10 (red fluorescence) in HIVE cases. GFAP and β III-tubulin are immunolabeled with green fluorescence. **B.** Expression and colocalization of ADAM10 (green fluorescence) and TNF-a (red fluorescence) were analyzed by immunohistochemistry in HIVE compared with control samples (upper panels). Expression and colocalization of ADAM10 (red fluorescence) and TNFR1 (green fluorescence) is shown in lower panels. High magnification (left) and low

magnification (right) images are shown for HIVE case #3. In all immunohistochemical images, nuclei of cells were stained with DAPI.