JAK/STAT3 Pathway Is Involved in Survival of Neurons in Response to Insulin-like Growth Factor and Negatively Regulated by Suppressor of Cytokine Signaling-3*

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Janus kinases (JAK) and signal transducers and activator of transcription (STAT) proteins are activated in response to many cytokines and growth factors and are well studied in the immune system. This study was conducted to examine the role of the JAK/STAT pathway in neurons in response to tumor necrosis factor-α (TNFα) and insulin-like growth factor-1 (IGF-1), which play a major role during neurodegeneration, and to study their effect on expression of suppressors of cytokine signaling 3 (SOCS-3), belonging to the novel family of feedback regulators of cytokine and growth factor activities. In this report, we showed that TNFα is inhibitory to the survival of primary cortical neurons at higher doses and that IGF-1 can rescue TNFα-stimulated cell death. We showed that the JAK/STAT pathway is involved in this rescue as tyrosphin AG490, a specific inhibitor of JAK/STAT, completely inhibits cell survival in response to IGF-1. STAT3 gets tyrosine-phosphorylated and translocated to the nucleus in response to IGF-1. Northern blot, semi-quantitative reverse transcription-PCR, and real time PCR experiments demonstrated that the JAK/STAT pathway also up-regulated SOCS-3 mainly in response to IGF-1. SOCS-3 associated with the IGF receptor and blocked further STAT3 activation. To our knowledge, this is the first report that demonstrated the importance of the JAK/STAT pathway and the role of SOCS-3 in the survival of neurons in response to IGF-1. We have subsequently shown that SOCS-3 overexpression, on one hand, leads to neuroblastoma cell death and on the other hand leads to primary cell differentiation, indicating the involvement of SOCS-3 in cell survival and differentiation.

Proinflammatory cytokines, especially tumor necrosis factor-α (TNFα), are pleiotropic mediators of a diverse array of physiological and neurological functions and have been implicated in the pathogenesis of several disorders and injuries in the central nervous system. Studies on uninfamed or “normal” brain have generally attributed TNFα to have a neuromodulatory effect (1). In contrast, in inflamed or diseased brain, the abundance of evidence suggests that TNFα has an overall neurotoxic effect (2). On the other hand, IGF-1 exerts anti-apoptotic and neuroprotective effects in primary neuronal cultures and neuronal cell lines (3, 4). In vivo, IGF-1 protects neurons against a variety of brain insults typically associated with overproduction of pro-inflammatory cytokines such as stroke, brain trauma, and multiple sclerosis (3, 4). Cross-talk between the signaling pathways of these two factors has been proposed as a mechanism for regulation of apoptosis and survival (5).

Activation of the Janus kinases (JAK) and signal transducers and activator of transcription (STAT) proteins in response to specific cytokines and growth factors has been investigated primarily in cells of non-neuronal origin. More recently, the JAKs and the STATs have also been found to be active in the developing and mature brain, providing evidence for important roles played by these molecules in the control of neuronal proliferation, survival, and differentiation. Activation of the JAK/STAT pathway has also been demonstrated for nerve regeneration (6), rat cortical injury (7), gangliosides-mediated stimulation of rat and murine primary microglia (8), and in cultured nerve cells (9) and retinal neurons (10) in response to ciliary neurotropic factor. Both STAT3 and STAT1 play an important role in regulating the physiological status of mature neurons (11).

The JAK/STAT signaling pathways are tightly regulated processes. The family of suppressors of cytokine signaling (SOCS) proteins, which includes SOCS-1–7 and cytokine-induced suppressor, has been shown to act as negative regulators of cytokine-induced signaling (12). Although to date studies of the neurobiology of the SOCS family have been limited, we know that many SOCS genes are constitutively expressed in the developing and adult brain, whereas the expression of others, particularly the SOCS-1 and SOCS-3 genes, can be highly regulated (13).

Recently, it has been suggested that SOCS might play a role in negative regulation of receptor tyrosine kinase signaling by insulin and IGF-1 receptors. SOCS-1 and SOCS-3 modulate insulin signaling by targeting insulin receptor substrates 1 and 2 for destruction by proteasome in hepatocytes and liver (14). SOCS-3 may play a role in insulin resistance as it inhibits insulin signaling and is up-regulated in response to TNFα in the adipose tissue of obese mice (15). SOCS-2 has been shown to play an important role in IGF-1 signaling pathway in the brain (16).

In this study we studied the dose-dependent effect of TNFα on primary cortical neurons. With the broad objective of finding...
out various pathways involved in IGF-1-mediated rescue of TNFα-triggered cell death, we examined whether TNFα and IGF-1 mediate their effects through the evolutionarily conserved JAK/STAT signal transduction pathway in neurons and the role of SOCS.

MATERIALS AND METHODS

Reagents—TNFα, IGF-1, and IL-6 were purchased from CytoLab Ltd. (Rehovot, Israel); tyrphostin AG-490, phenylmethylsulfonyl fluoride, and DNase were from Sigma. Antibodies against phospho-STAT3 (sc-8059), STAT3 (sc-483), JAK1(sc-277), JAK2 (sc-278), SOCS-3 (sc-9023), IGFR (sc-713), and actin (sc-7210) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse horseradish peroxidase, anti-rabbit horseradish peroxidase, and anti-rabbit-FITC were from Pierce, and anti-rabbit Texas Red was from The Jackson Laboratories. Dulbecco’s modified Eagle’s medium, basal medium Eagle’s, trypsin, B27, N2 supplements, fetal bovine serum, fetal horse serum, antibiotics, and antimycotics were from Invitrogen. Sepharose A/G beads were from Santa Cruz Biotechnology. Protease inhibitors were from Roche Applied Science.

Animals—Timed mated Sprague-Dawley rats (Charles River Breeding Laboratories, Sulzfeld, Germany) were maintained at a 12-h dark, 12-h light cycle and were allowed to access food and water ad libitum. Pregnant rats were killed by CO2 inhalation, and newborns were physically decapitated. Neocortical tissue was dissected from embryonic day 18 rat brains and collected in PBS. Animal procedures were approved by the Institutional Animal Ethics Committee.

Primary Neuronal Cell Culture—The protocol followed was adapted from a procedure described previously (17). Briefly, the neocortical tissue pieces were incubated for 5 min in PBS containing 1% trypsin and 0.1% DNase. Cells were then transferred to basal medium Eagle’s

FIG. 1. TNFα is inhibitory to neuronal cell survival, whereas IGF-1 can rescue TNFα-inhibited cells. A, in order to find out the effect of TNFα on neuronal cell survival, 3-day-old primary cortical neurons were stimulated with increasing levels of TNFα (1, 10, 50, and 100 pg and 1 and 10 ng per ml) for 48 h. Live cells were counted after trypan blue exclusion. The cell growth is plotted as % growth as compared with control when no TNFα was added. B, primary cortical neurons were stimulated with 10, 20, 50, and 100 ng of IGF-1 and live cells were counted after 48 h. The cell growth is plotted as % growth as compared with control when no IGF-1 was added. C, primary cortical neurons were transfected with EGFP empty vector. After 24 h the cells were either untreated (left) or stimulated with 20 ng/ml IGF-1 (right) and viewed under a fluorescent microscope after 24 h. D, primary cortical neurons were co-stimulated with different concentrations of TNFα (same as mentioned above) and 20 ng/ml IGF-1. Live cells were counted after 48 h. The cell growth is plotted as % growth as compared with control when no factor was added. E, primary cortical neurons were either unstimulated (i) or stimulated with 20 ng/ml IGF-1 (ii), 100 pg/ml TNFα (iii), and 20 ng/ml IGF-1 and 100 pg/ml TNFα (iv) and viewed under light microscope. For all further experiments with primary cortical neurons, IGF-1 and TNFα were used at 20 ng/ml and 100 pg/ml. Bars are mean ± S.E. (n = 3). * indicates the difference between control and sample or samples indicated is significant (i.e. p < 0.05). These are representative data from three sets of independent experiments.
solution containing 0.05% DNase and dissociated by gently triturating through sequentially smaller bore, fire-polished Pasteur pipettes. Cells were centrifuged at 700 × g for 5 min at 4 °C. The pellet was resuspended in basal medium Eagle’s with 10% fetal horse serum and 5% fetal bovine serum. The number of viable cells was counted after trypsin blue exclusion. Cells were seeded in 6-cm dishes, 6-well plates, or Lab-Tek (Nalge Nunc, Naperville, IL) wells that had been coated previously with poly-L-lysine (0.5 mg/ml), at a density of 1000 cells/μl. The culture medium was changed after 1 day to serum-free Dulbecco’s modified Eagle’s medium supplemented with B27 and N2. On the 3rd day AraC (3 μM), from Sigma, was added to inhibit growth of non-neuronal cells. This yielded a 90% pure neuronal population of cells on the 4th day as revealed by neurite-specific neuronal cells. This yielded a 90% pure neuronal population of cells on the 4th day as revealed by neurite-specific neuronal cells. The cells were lysed, and 100 μg of protein was loaded on SDS-polyacrylamide gel and Western-blotted with anti-phospho-Tyr-705 STAT3. Anti-STAT3 antibodies were used as loading control. Data shown are from a single experiment, which is representative of three independent experiments.

**Materials and Methods**

**Measurement of Cell Survival**—10⁶ cells were seeded in Labtek dishes and maintained for 3 days as described before. The cells were then serum-starved for 12–14 h. Subsequently they were stimulated with TNFa and/or IGF-1 in serum-free medium for various time periods. The media were then aspirated, and the cells were washed with ice-cold PBS. Subsequently, the cells were lysed in ice-cold Triton lysis buffer (50 mM Tris, pH 7.5, 1% Triton, 2 mM Na₃VO₄, 2 mM MgCl₂, 10 mM EDTA, 100 mM NaF, 1 mM Na₃VO₄, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, pepstatin A, and leupeptin). The supernatants collected after removing insoluble debris by centrifuging the samples at 12,000 g for 10 min were used in all studies. Proteins concentrations were determined by Pierce BCA assay kit. 100 μg of protein samples along with pre-stained protein molecular weight markers were electrophoresed on denaturing SDS-polyacrylamide gels. The proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), blocked with 5% milk in Tris-buffered saline, and probed with various antibodies. Immunoreactivity was revealed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse (Amersham Biosciences) and enhanced chemiluminescence re-
agents (Amersham Biosciences). The levels of protein were visualized by autoradiography.

Immunoprecipitation—Cells were plated and subsequently treated as described previously and lysed in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 2 mM Na₃VO₄, 100 μM phenylmethylsulfonyl fluoride, and 1 μg/ml of aprotinin, pepstatin A, and leupeptin). 300–500 μg of cellular protein was incubated with 1–2 μg of antibodies. Immunocomplexes were captured with protein A/G-Sepharose beads (Santa Cruz Biotechnology) and blotted as described previously.

Nuclear and Cytoplasmic Protein Extraction—An extraction kit from Geno-technology (St. Louis, MO) was used to extract proteins from the nucleus and cytoplasm as per the manufacturer’s instructions. Briefly, the cells were spun down after treatment, washed with PBS, and resuspended in cytoplasmic extraction buffer. Subsequently, the cells were passed cells through the narrow mouth tip and centrifuged (10,000 × g). The supernatant contained cytoplasmic protein. The pellet was washed several times in the same buffer. Subsequently, nuclear extraction buffer was added and incubated on ice. After centrifugation (30,000 × g), the supernatant contained nuclear extract.

Semi-quantitative RT-PCR—Total RNA from primary cortical neurons and SH-SY5Y cells was isolated using TRIzol reagent (Invitrogen) and was synthesized with first-strand cDNA according to the manufacturer’s instructions. A mock control was carried out to exclude DNA contamination. RT-PCR conditions were 30 min at 50 °C for reverse transcription and 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles. These cycles were preceded by a 15-min incubation at 95 °C for initial heat denaturation and activation of Taq polymerase, followed by a 10-min elongation step at 72 °C. PCR samples were run on agarose gels and detected by ethidium bromide staining. Primers used were the same as those used for cloning rat and human SOCS-3 cDNA. To ensure that the PCR products fell within the linear range, cycle dependence was carried out.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from cells, and 15 μg were resolved by electrophoresis on a 1% agarose/formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N+, Amersham Biosciences) by blotting overnight in 20× SSC and cross-linked under UV light. Prehybridization was carried out overnight at 80 °C in sodium phosphate, SDS, EDTA prehybridization/hybridization solution with 0.5 mg/ml salmon sperm DNA. The membrane was hybridized with a SOCS-3 cDNA probe (Rediprime II; Amersham Biosciences) at 60 °C overnight. The membrane was washed in 0.2× SSC (sodium chloride/sodium citrate), 0.1% SDS twice each at room temperature, and then at 42 °C and then analyzed by Phosphor-Imager (Fuji, Japan). The membrane was subsequently stripped and rehybridized with 32P-radiolabeled GAPDH probe to confirm equal loading of RNA samples.

Real Time PCR—Total RNAs from primary rat cortical neurons were isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). One-step RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit from Qiagen (Hilden, Germany) using 500 ng of total RNA in 25-μl reactions. Each sample was analyzed in triplicate. Condition for the reaction were as follows. Initial incubation for reverse transcription was at 55 °C for 30 min, followed by initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

The primers used were SOCS-3, 5'-ATGGTCACCCACAGCAAGTT; GAPDH, 5'-TCATGACCACAGTC-CGTTCACCACCAGCTGGTAC; 3'-GGATGACCTTGCCCACAGCC.

SYBR Green RT-PCR amplification and real time fluorescence detection were performed using the ABI PRISM 7700 sequence detection system. At the end of the experiments, RT-PCR products were removed from tubes and analyzed by gel electrophoresis to confirm the presence and assess the purity of the amplicons of interest.

Relative gene expression was calculated by the ΔΔCT method. Briefly, the resultant SOCS-3 mRNA was normalized to a calibrator in each case. The calibrator chosen was the unstimulated control sample. Final results were expressed as n-fold difference in gene expression relative to GAPDH RNA and calibrator as follows: n-fold = 2^-ΔΔCt(calibrator-sample), where
significant differences were determined using Student’s t test, with \( p < 0.05 \) considered as significant.

**RESULTS**

**TNFα Inhibits Neuronal Cell Survival and IGF-1 Can Rescue Dying Cells**—TNFα has been shown previously to be able to both enhance and inhibit neuronal cell death (2, 18). Previously, we have shown that TNFα was inhibitory to SH-SY5Y neuroblastoma cell lines, and IGF-1 could rescue these cells from cell death (28). In order to establish its effect on primary cortical neurons, cells were stimulated with increasing concentrations of TNFα. Percentage (%) of live cells was calculated keeping the control untreated cell number as 100%. TNFα was found to promote survival at low concentrations of 1 and 10 pg but inhibited growth at higher concentrations (Fig. 1A). On the other hand, IGF-1 enhanced cell survival by about 40% over unstimulated control at 20 ng/ml concentration, above which it was found to be toxic (Fig. 1B). IGF-1 also increased neuritic outgrowth (Fig. 1C). IGF-1 is usually expressed along with TNFα in degenerating and injured brain (19, 20). It was thus important to find out if IGF-1 could rescue TNFα-mediated cell death. In the presence of both factors, TNFα was found to inhibit IGF-1 actions at lower TNFα concentrations (1 and 10 pg), but IGF-1 could rescue TNFα inhibition at higher concentrations (50 and 100 pg and 1 and 10 ng) (Fig. 1D). IGF-1 was not able to overcome completely the inhibitory effects of TNFα as cell survival never reached levels as with IGF-1 alone. Morphological changes following TNFα and IGF-1 exposure, as shown in Fig. 1E, illustrates cell death following TNFα treatment (Fig. 1E, iii) and rescue of dying cells by IGF-1 (Fig. 1E, iv).

**Involvement of STAT3 in IGF-1-mediated Cell Survival**—Because STAT3 has been shown to be activated in response to other factors like CTNF and fibroblast growth factor in neuronal cells (21), we explored its involvement in IGF-1 and TNFα-mediated neuronal cell survival. Primary cortical neurons were treated with TNFα and IGF-1 for 5 and 15 min. Cell lysates were probed with phospho-STAT3 antibodies. A substantial increase in levels of phospho-STAT3 was observed after 5 min of IGF-1 stimulation with a slight decline after 15 min. Comparatively, TNFα enhanced phospho-STAT3 to a much lower extent (Fig. 2A). In order to find out the role of STAT3 in cell lines of neuronal origin, SH-SY5Y was treated with different concentrations (20, 50, and 100 ng) of TNFα and IGF-1 for 10 min. Higher concentrations of both factors were used as compared with primary cells as our previous experiments demonstrated that the threshold for stimulation was higher for these cells. An increase in phospho-STAT3 levels was observed in response to IGF-1 but not TNFα (Fig. 2B).
JAK1 in both uninduced as well as TNF-associated with IGFR, SY5Y cells were stimulated with IGF-1 and essential for this activation (23). In order to see if STAT3 activated by IGFR upon IGF-1 stimulation, and JAKs are essential for this activation (23). In order to see if STAT3 associated with IGFR, SY5Y cells were stimulated with IGF-1 and TNFα. IGFR was immunoprecipitated, and phospho-STAT3 blot showed that activated STAT3 was associated with IGFR, 5 min post-stimulation in primary cells in response to IGF-1 and not TNFα (Fig. 4A). STAT3 was found to be associated with JAK1 in both uninduced as well as TNFα-induced SY5Y cells (Fig. 4B). Following IGF-1 stimulation, STAT3 dissociated from JAK1 at the early time points of 5 and 15 min possibly due to tyrosine phosphorylation and subsequent processes of STAT activation. Co-stimulation with both factors also led to dissociation of STAT3 from JAK1, although not completely, indicating an inhibitory effect of TNFα on IGF-1 action.

**JAK/STAT Pathway Involved in Survival of Neurons**—In order to explore the role of the JAK/STAT pathway in the survival of neurons, the cells were treated with tyrphostin AG490, an inhibitor frequently used to inhibit this pathway (24). Both SY5Y and primary neuronal cells were treated with increasing doses of the inhibitor. Most interestingly, both cell types were highly susceptible to this inhibitor. At a low concentration of 25 μM, about 80% of SY5Y cells were dead (Fig. 5A), and at 12.5 μM most of the primary cells were dead (data not shown). Most often a dose between 40 and 100 μM is generally used. To re-confirm the role of the JAK-STAT pathway, the SY5Y cells were transfected with a STAT3-EGFP and a dominant negative STAT3-EGFP (plasmids pSV-STAT3 and pSV-STAT3 were kindly provided by Dr. Heinz Baumann, which were further recloned into EGFP vector). After a week of culture, all the dominant negative STAT3-transfected cells (green fluorescent cells) died, whereas the STAT3 cells were healthy (data not shown). Co-stimulation with IGF-1 could not rescue the dying neurons (Fig. 5A), indicating that IGF-1 also uses the JAK/STAT pathway as a major pathway for neuronal survival. Morphological changes following the tyrphostin AG490 treatment, shown in Fig. 5B, reconfirmed cell survival data, where both cell types were highly inhibited following inhibitor treatment and IGF-1 was unable to rescue.

**SOCS-3 Is Up-regulated upon IGF-1 Stimulation**—It has

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**FIG. 5. JAK-STAT pathway is important for neuronal cell survival.** Both primary neuronal cells and SH-SY5Y cells were treated with JAK/STAT inhibitor tyrphostin AG490 (AG) and either left unstimulated or stimulated with IGF-1 for 48 h. A, SY5Y cells were treated with 6.25, 12.5, 25, and 50 μM of AG490, and live SY5Y cells were counted after 48 h after trypan blue exclusion. The cell growth is plotted as % growth as compared with control where neither inhibitor nor IGF-1 was added. An average of four different fields were taken. * indicates the difference between samples indicated is significant (*p < 0.05*) (n = 3). Inhibition by AG490 from 12.5 μM and higher is significant, as compared with uninhibited cells. B, cells viewed under light microscope after 24 h. i and ii are SY5Y and primary cells unstimulated and without inhibitor, respectively. iii and iv are SY5Y and primary cells unstimulated with inhibitor, respectively. v and vi are SY5Y and primary cells IGF-1 stimulated without inhibitor, respectively. vii and viii are SY5Y and primary cells IGF-1 stimulated with inhibitor, respectively. 50 μM AG490 was used for SY5Y and 25 μM primary rat cells. 8–10 fields were viewed, and a representative field is shown. This figure is representative of four independent experiments.

**Nuclear Translocation of STAT3—Tyrosine-phosphorylated STAT3** is known to dimerize and subsequently translocate to the nucleus where it activates various transcription factors (22). Because STAT3 was phosphorylated in response to IGF-1, we wanted to see if it translocated to the nucleus as well. SY5Y cells were stimulated with IGF-1 for 30 min, and cytoplasmic and nuclear proteins were extracted and probed with phospho-STAT3 and FITC-conjugated secondary antibody. Phospho-STAT3 was found to translocate to the nuclear region (Fig. 3i). This observation was confirmed by immunohistochemistry where cortical neurons were stimulated with TNFα and IGF-1 for 30 min and probed with antiphospho-STAT3 and FITC-conjugated secondary antibody. Phospho-STAT3 was found to translocate to the nuclear region of the cell in response to IGF-1 only and not TNFα, which showed a dispersed localization (Fig. 3B).

**STAT3 Is Associated with IGF Receptor and JAK1—**It has been shown previously in other cell types that STATs can be activated by IGFR upon IGF-1 stimulation, and JAKs are essential for this activation (23). In order to see if STAT3 associated with IGFR, SY5Y cells were stimulated with IGF-1 and TNFα. IGFR was immunoprecipitated, and phospho-STAT3 blot showed that activated STAT3 was associated with IGFR, 5 min post-stimulation in primary cells in response to IGF-1 and not TNFα (Fig. 4A). STAT3 was found to be associated with JAK1 in both uninduced as well as TNFα-induced SY5Y cells (Fig. 4B). Following IGF-1 stimulation, STAT3 dissociated from JAK1 at the early time points of 5 and 15 min possibly due to tyrosine phosphorylation and subsequent processes of STAT activation. Co-stimulation with both factors also led to dissociation of STAT3 from JAK1, although not completely, indicating an inhibitory effect of TNFα on IGF-1 action.

**JAK/STAT Is a Major Pathway for Neuronal Cell Survival**—In order to explore the role of the JAK/STAT pathway in the survival of neurons, the cells were treated with tyrphostin AG490, an inhibitor frequently used to inhibit this pathway (24). Both SY5Y and primary neuronal cells were treated with increasing doses of the inhibitor. Most interestingly, both cell types were highly susceptible to this inhibitor. At a low concentration of 25 μM, about 80% of SY5Y cells were dead (Fig. 5A), and at 12.5 μM most of the primary cells were dead (data not shown). Most often a dose between 40 and 100 μM is generally used. To re-confirm the role of the JAK-STAT pathway, the SY5Y cells were transfected with a STAT3-EGFP and a dominant negative STAT3-EGFP (plasmids pSV-STAT3 and pSV-STAT3 were kindly provided by Dr. Heinz Baumann, which were further recloned into EGFP vector). After a week of culture, all the dominant negative STAT3-transfected cells (green fluorescent cells) died, whereas the STAT3 cells were healthy (data not shown). Co-stimulation with IGF-1 could not rescue the dying neurons (Fig. 5A), indicating that IGF-1 also uses the JAK/STAT pathway as a major pathway for neuronal survival. Morphological changes following the tyrphostin AG490 treatment, shown in Fig. 5B, reconfirmed cell survival data, where both cell types were highly inhibited following inhibitor treatment and IGF-1 was unable to rescue.
been suggested that SOCS-3 might play a role in the regulation of receptor tyrosine kinase signaling by insulin receptors (25). SOCS-2 has been shown to associate with IGF-1 receptor (16). Initially screening for SOCS-1, SOCS-2, and SOCS-3 by semi-quantitative RT-PCR revealed that SOCS-3 increased upon IGF-1 stimulation (data not shown). Up-regulation of SOCS-3 RNA was confirmed by stimulating both primary neuronal cells as well as SY5Y cells with IGF-1 and TNF/\alpha. Total RNA was isolated, and a semi-quantitative RT-PCR for SOCS-3 was performed. An increase in levels of SOCS-3 was observed following IGF-1 treatment (Fig. 6A). To confirm these observations, a Northern blot hybridization was performed with 15 μg of total RNA and probed with radiolabeled SOCS-3 DNA probe. RNA isolated from IL-6-stimulated cells was included in this study because IL-6 has been shown previously (26) to up-regulate SOCS-3 in many cell types. More interestingly, levels of SOCS-3 RNA in IGF-1-treated cells were higher than not only TNFα-treated but also IL-6-treated cells (Fig. 6B). In order to define the increase in absolute terms, we performed a one-step real time PCR for SOCS-3 RNA. There was a 2-fold increase in SOCS-3 RNA following IGF-1 stimulation as compared with untreated control and about a 3-fold increase as compared with TNFα-stimulated cells (Fig. 6C). Levels of SOCS-3 RNA in IL-6-treated cells were about 1.5-fold higher than untreated cells. The difference between IL-6 and IGF-1 levels was not statistically significant.

**SOCS-3 Is Associated with IGF Receptor Upon IGF-1 Stimulation**—It has been shown that the SH2 domain of SOCS-3 binds to the Src homology phosphatase-2 binding domains of glycoprotein 130 receptors (27). It is believed that SOCS-3 binds to receptors and inhibits the activity of JAKs by accessing the activation loop of JAKs with its KIR domain. In order to find out whether SOCS-3 associates with IGFR, the receptors from primary cells and SY5Y cells were immunoprecipitated, blotted, and probed with SOCS-3 antibodies following TNFα and IGF-1 stimulation. An increase in the association of SOCS-3 and IGFR corresponded with an increase in SOCS-3 expression (Fig. 7B). The co-localization of IGFR and SOCS-3 protein, upon IGF-1 stimulation, was confirmed by confocal microscopy (Fig. 7C).

**SOCS-3 Inhibits STAT3 Activation following IGF-1 Stimulation**—In order to explore the role of SOCS-3 in IGF-1-mediated JAK/STAT signaling, SOCS-3-EGFP was transiently transfected into SY5Y cells along with an empty vector, and STAT3 activation was observed after 10 and 60 min of IGF-1 stimulation. STAT3 tyrosine phosphorylation was inhibited in SOCS-3-transfected cells but not in stimulated cells transfected with empty vector (Fig. 8A, upper panel). This indicated that SOCS-3 was a negative regulator of IGF-1-mediated JAK/STAT signaling. SOCS-3 expression did not have any effect on STAT3 expression (Fig. 8A, lower panel). In order to check the...
levels of SOCS-3 overexpression following transfection, the unstimulated and 10-min time point lysates were Western-blotted using anti-SOCS-3 antibodies. A SOCS-3 overexpression was seen following transfection (Fig. 8B).

SOCS-3 Inhibits Cell Growth in Response to IGF-1—Because SOCS-3 was found to negatively regulate STAT3, it was thus important to understand the effect of SOCS-3 on cell survival. SY5Y cells were transiently transfected with human SOCS-3-EGFP or empty vector and stimulated for 72 h with TNFα/H9251 or IGF-1, and the surviving fluorescent cells were counted. An 80% decrease in cell survival was observed in SOCS-3-transfected cells following IGF-1 stimulation, as compared with 20% in the case of TNFα (Fig. 9A). Cell death observed in response to TNFα in both mock-transfected and SOCS-3-transfected cells was because of the inhibitory effect of TNFα on SY5Y cells shown before (28). Although unstimulated cells showed no change in cell survival after 72 h, after 1 week of culture most of the cells were dead (data not shown), indicating that the JAK/STAT pathway was important for neuronal cell survival, and SOCS-3 negatively regulated it. Most interestingly, when primary cortical neurons were transiently transfected with rat SOCS-3-EGFP or vector alone, an increase in the length of neurites was observed with SOCS-3 even without simulation (Fig. 9B). This indicated that although SOCS-3 inhibited cell growth in cancerous cells, it probably played a role in primary neuronal cell differentiation. IGF-1 increased the length of neurites both in vector as well as SOCS-3-transfected cells. This reconfirmed our previous observation on the pro-survival role of IGF-1 in primary cells. Stimulation with TNFα inhibited any increase in neuritic length even in the presence of SOCS-3, indicating that TNFα inhibited the ability of IGF-1 to

DISCUSSION

Previously, we have shown that TNFα inhibited SY5Y neuroblastoma cell survival, whereas IGF-1 could rescue these cells from cell death via phosphatidylinositol 3-kinase and c-Jun kinase pathways (28). In this study we looked at the effect of TNFα and IGF-1 on the survival of primary cortical neurons from rats and the involvement of the JAK-STAT pathway in their signaling. We observed that TNFα promoted growth at a low concentration (up to 10 pg/ml) but totally inhibited growth at higher concentrations. TNFα has been previously shown to have protective effect under some conditions of neurological insult (2) or toxic effect (18) depending on the cell type. IGF-1 was found to be neuroprotective at optimal doses (20 ng/ml) and also increased neuritic length (Fig. 1, B and C). Although IGF-1 could rescue the inhibitory effects of TNFα substantially (Fig. 1, D and E), the cell survival never reached levels of IGF-1 alone, indicating that TNFα inhibits the ability of IGF-1 to
promote neuronal survival, an observation similar to Venters et al. (5).

The JAK/STAT pathways, first identified in the interferon systems, are responsive to a wide range of cytokines and growth factors (29). STAT3, a member of the STAT family, plays a central role in mediating cell growth, differentiation, and survival signals. In this study we observed that IGF-1 activated STAT3 both in SY5Y as well as primary cortical neurons (Fig. 2). TNFα also activated STAT3 but to a much lesser extent. The activated STAT3 was found to translocate to the nucleus upon IGF-1 stimulation, indicating its involvement in gene transcription (Fig. 3). To our knowledge this is the first report that demonstrates the involvement of STATs in the IGF-1 pathway in the brain. We also show that activated STAT3 associated with IGFR very early (5 min) upon IGF-1 and not TNFα stimulation (Fig. 4A). Most interestingly, STAT3 was associated with JAK1 under unstimulated conditions as well as with TNFα (Fig. 4B), indicating the fact that STAT3 is constitutively associated with IGFR under basal conditions. STAT binding to receptors that have not bound ligand has been reported earlier (30, 31). It has been shown that STATs are activated through cytokine receptors by two distinct mechanisms, one dependent on receptor tyrosine phosphorylation and the other mediated by the JAK-STAT direct interaction even in an unstimulated state. Upon stimulation with IGF-1, the majority of STAT3 dissociated from JAK1 very early (5 min), presumably because once activated, STAT3 quickly dissociates from JAK, dimerizes, and is transported to the nucleus.

In SH-SY5Y cells, leptin has been shown to induce STAT3 phosphorylation (32). Previous studies have shown that STATs can be activated by a variety of receptor and nonreceptor tyrosine kinases (33, 34). Zong et al. (44) showed that STAT3 is required for the establishment and maintenance of IGFR protein-tyrosine kinase-induced cell transformation. Subsequently, the same group has shown that IGF-1-induced STAT3 activation in the presence of both IGF-IR and Janus kinases in 293T and muscle cell line C2C12 cells (23). IGF1 has been shown to induce proliferation in 1AML6 lens cells, in part through activation of STAT3 and to a lesser extent in STAT1 signal transduction pathways (35). Physiological relevance of STAT pathways in the lens is underscored by inhibition of lens cell proliferation by inhibitors of JAK/STAT pathways (36). The JAK/STAT pathway appears to be an extremely important pathway for cell survival in the brain, as inhibiting it by tyrphostin AG490 decreased cell viability by 90% (Fig. 5). IGF-1 was unable to rescue the dying cells, indicating this was a major pathway involved in IGF-1-mediated survival as well. Our previous data showed that IGF-1 also mediated neuronal survival via c-Jun kinase and phosphatidylinositol 3-kinase, indicating the presence of multiple survival pathways for IGF-1-mediated survival. Although the cross-talk between the key IGF-1 response pathway of insulin receptor substrate proteins (IRS1 and IRS2) Grb2-Sos-MAP kinase and the JAK/STAT pathway needs to be investigated further in the brain, in hematopoietic cells it has been shown that in response to interferon-α the IRS pathway operates separately from the STAT pathway (37). Several possibilities of STAT3 activation by IGF-IR via JAKs exist. First, the activated receptor associates with JAKs, which phosphorylate STAT3. The STAT3-binding site could be generated by activated IGF-IR or JAKs. Second, upon activation by IGF-IR, JAKs could directly phosphorylate STAT3. Third, JAKs could provide STAT3 recruitment sites in the receptor complex. Further experiments could address these probabilities.

The SOCS group of proteins has been implicated in regulation of various cytokine signaling and in a negative cross-talk between distinct signaling pathways. SOCS-1 and SOCS-3 have been shown to bind to activated JAKs and inhibit their catalytic activity, although their precise mechanism is not clear (12). By having shown the importance of the JAK/STAT pathway in IGF-1 signaling in the neuronal cells, we wanted to see if this pathway was negatively regulated by the SOCS proteins in response to TNFα and IGF-1. An increase in SOCS-3 RNA both in rat cortical neurons as well as human SY5Y cells in response to IGF-1 and not in response to TNFα was observed (Fig. 6A). An increase in SOCS-3 RNA upon IGF-1 stimulation was even more than IL-6 stimulation (Fig. 6B), which is known to activate the JAK/STAT pathways in many systems (26). Real time quantitation showed a 2-fold increase in SOCS-3 RNA upon IGF-1 stimulation over control and only 1.5-fold increase upon IL-6 stimulation (Fig. 6C). SOCS-3 was found to associate and co-localize with the IGFR after 1 h of IGF-1 stimulation by confocal microscopy (Fig. 7). This association leads to an inhibition of STAT3 activation, as SY5Y cells transfected with SOCS-3 showed reduced tyrosine phosphorylation of STAT3 (Fig. 8). To our knowledge this is the first report that indicates that SOCS-3 binds to IGF receptor complex upon IGF-1 stimulation and subsequently inhibits STAT3 activation in the brain. SOCS-2 has been shown to bind to the growth hormone receptor and inhibit growth hormone signaling, including attenuation of STAT5 activation in neurons (38). In COS-7 cells, SOCS-3 has been shown to bind to the phosphorylated insulin receptor that prevents STAT5b activation by insulin, indicating that SOCS-3 may be a direct substrate for the receptor tyrosine kinase (25). Also, SOCS-3 decreased insulin-induced insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation.
FIG. 9. **SOCS-3 inhibits cell survival upon IGF-1 stimulation.** SY5Y cells were transiently transfected with human SOCS-3-EGFP construct or empty EGFP vector. After 24 h, the transfected cells were serum-starved and stimulated with TNFα or IGF-1. A, cells were stimulated for 48 h. Green fluorescent cells were counted under a fluorescent microscope, and an average of four different fields were taken. % cell survival was calculated relative to unstimulated vector alone. * indicates the difference between samples indicated is significant (i.e. $p < 0.05$) ($n = 3$). This experiment was repeated three times with similar results. B, cells were stimulated for 24 h and visualized under a fluorescent microscope. Data are representative of two independent experiments.
and its association with p85, a regulatory subunit of phosphatidylinositol 3-kinase (25).

SOCS-3 is expressed in the neuronal cell lines SN56 and IMR32 and negatively regulates leukemia inhibitory factor and ciliary neurotropic factor signal transduction (39). STAT3 induces SOCS-3 expression, during chronic leptin stimulation in nervous system stem cells (38). Influencing SOCS-3 expression and its association with p85, a regulatory subunit of phosphatidylinositol 3-kinase (25). STAT3.

Because SOCS-3 may also be neuroprotective as SOCS-3 and other members of SOCS family increase after focal stroke, cerebral ischemia, traumatic brain injury, and spinal cord injury (7, 43) and antisense knockdown of SOCS-3 expression increases stroke size (43), we explored the role of SOCS-3 in cell survival, in response to TNF-α and IGF-1. An 80% decrease in cell survival was observed in SOCS-3-transfected cells following IGF-1 stimulation, as compared with 20% (statistically insignificant) in the case of TNFα (Fig. 9A). Because we had seen previously that SYSY cells were highly responsive to IGF-1-stimulated conditions. TNF-α there was no difference in cell survival; instead there was an increase in cell survival in degenerative disorders on the other hand. to certain growth factors on the one hand, and to increase cell survival in degenerative disorders on the other hand. to certain growth factors on the one hand, and to increase cell survival in degenerative disorders on the other hand. to increase cell survival in degenerative disorders on the other hand.

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JAK/STAT3 Pathway Is Involved in Survival of Neurons in Response to Insulin-like Growth Factor and Negatively Regulated by Suppressor of Cytokine Signaling-3
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