GFAP Expression and Social Deficits in Transgenic Mice Overexpressing Human sAPPα

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Autistic individuals display impaired social interactions and language, and restricted, stereotyped behaviors. Elevated levels of secreted amyloid precursor protein-alpha (sAPPα), the product of α-secretase cleavage of APP, are found in the plasma of some individuals with autism. The sAPPα protein is neurotrophic and neuroprotective and recently showed a correlation to glial differentiation in human neural stem cells (NSCs) via the IL-6 pathway. Considering evidence of gliosis in postmortem autistic brains, we hypothesized that subsets of patients with autism would exhibit elevations in CNS sAPPα and mice generated to mimic this observation would display markers suggestive of gliosis and autism-like behavior. Elevations in sAPPα levels were observed in brains of autistic patients compared to controls. Transgenic mice engineered to overexpress human sAPPα (TgsAPPα mice) displayed hypoactivity, impaired sociability, increased brain glial fibrillary acidic protein (GFAP) expression, and altered Notch1 and IL-6 levels. NSCs isolated from TgsAPPα mice, and those derived from wild-type mice treated with sAPPα, displayed suppressed β-tubulin III and elevated GFAP expression. These results suggest that elevations in brain sAPPα levels are observed in subsets of individuals with autism and TgsAPPα mice display signs suggestive of gliosis and behavioral impairment.

Key words: sAPPα, astrogliosis, autism, behavior, IL-6, Notch

Introduction

Autism is a heterogeneous neurodevelopmental disorder characterized by impaired communication, social interaction, and restricted, repetitive behaviors and interests (APA, 2000; Steyaert and De la Marche, 2008). Patients with autism exhibit varied patterns of aberrant neuroanatomical and immunological features in addition to behavioral phenotypes (Bailey et al., 1998; Bauman and Kemper, 2005; Casanova et al., 2006; Amaral et al., 2008). Brain overgrowth followed by decelerated development is frequently observed in some autistic patients. This neuropathology is observed early in the developmental course and is thought to involve defects in the normal “pruning” of early neural network arbors. After proliferation and dendritic arbor formation, pruning and programmed cell death are highly regulated multifactorial processes dependent on ordered molecular and cellular interactions involving various players including neural stem cells (NSCs), astrocytes, and microglia.

Typically, glial cells are generated after neurogenesis in the CNS (Sofroniew and Vinters 2010). The full complement of neurons appears during the embryonic period while most gliogenesis occurs within the first month after birth (Jacobson,
secreted APP), and existing in three main isoforms (Selkoe et al., 1988; Turn et al., 2003). APP is processed according to two separate pathways that produce different protein fragments. Protein processing by the amyloidogenic pathway produces, among other fragments, the amyloid-β peptides which constitute the cytotoxic plaques characteristic of Alzheimer’s disease (AD) pathology (Marz et al., 1999; Taga and Fukuda, 2005), led to the upregulation of the astrocyte marker glial fibrillary acidic protein (GFAP) (Bonni et al., 1997; Deverman and Patterson, 2009; Kirsch et al., 2010).

The gp130 pathway cooperates with the Notch1 pathway, which also enhances NSC commitment to glial fate by suppressing neuronal differentiation (Bhattacharya et al., 2008; Sugaya, 2008; Rodriguez-Rivera et al., 2009). There is also evidence of crosstalk between the Notch1 and gp130 pathways as increased Hairy and enhancer of split (Hes), a transcription factor for GFAP (Grandbarbe et al., 2003; Nagao et al., 2007).

Given that astrocytes are important for synaptic pruning during development, promotion of astrocytic differentiation and abnormal glial activation or gliosis appears sufficient to affect neurodevelopment (Oland and Tolbert, 2011; Stephan et al., 2012). Several studies give evidence for gliosis in different brain regions of autistic patients (Ahlsen et al., 1993; Sabaratnam, 2000; Laurence and Fatemi, 2005; Vargas et al., 2005). How abnormal cellular, inflammatory, and neurotropic signals align leading to excessive, aberrant dendritic connectivity via impaired pruning in autistic individuals is still unclear.

The amyloid precursor protein (APP) is a single-pass transmembrane glycoprotein consisting of 695–770 amino acids and existing in three main isoforms (Selkoe et al., 1988; Turner et al., 2003). APP is processed according to two separate pathways that produce different protein fragments. Protein processing by the amyloidogenic pathway produces, among other fragments, the amyloid-β peptides which constitute the cytotoxic plaques characteristic of Alzheimer’s disease (AD) pathology (Mattson, 1997; De Strooper and Annaert, 2000). Conversely, the nonamyloidogenic pathway produces the peptide fragments each have distinct roles in a variety of cellular processes occurring in the brain and other organs (Turner et al., 2003). Numerous reports of sAPPα potentiating neurite outgrowth, preventing neuronal death and aiding in NSC proliferation confirm its neurotrophic and neuroprotective properties (Furukawa and Mattson, 1998; Fu et al., 2002; Copanaki et al., 2010). Further, studies in isolated human NSCs suggest that sAPPα may promote astroglial cell-fate (Kwak et al., 2006a,b) via the IL-6/gp130 signaling pathway (Kwak et al., 2010).
University of South Florida institutional animal care and use committee.

Behavioral Tests

Open Field. Spontaneous locomotor activity and anxiety in mice were assessed in an open field consisting of a 17 inch square arena with plastic walls and floor evenly illuminated by white light. Each mouse was placed in the center of the field and allowed to explore for 10 min. Experiments were video-recorded and total distance traveled as well as time spent in center field was measured using Ethovision behavior analysis software (Noldus Information Technology, Leesburg, VA).

Social Interaction. Test mice were first habituated to the 22 × 15 inch rectangular, three-chambered polycarbonate box with divider walls containing doorways allowing access to each chamber for 10 min. Each of the two side chambers contained an empty wire cage (Galaxy Cup, Spectrum Diversified Designs, Streetsboro, OH) that was inverted and weighed down. After habituation, test mice were enclosed in the center and an unfamiliar mouse of the same strain and gender was enclosed inside one of the wire cages. The location for the “stranger” mouse was alternated between left and right sides of the box. Test mice were allowed 10 min to explore. Experiments were video-recorded and measurements of the time spent in each chamber during both phases of the task were measured and analyzed using Ethovision behavior analysis software (Noldus Information Technology).

Mouse Brain Tissue Isolation and Preparation

Mice were anesthetized using gaseous isoflurane and transcardially perfused with cold 0.01 M PBS (pH 7.4). Brains were rapidly removed and sagitally bisected. Left hemispheres were separated into hippocampus, striatum, cerebellum and cortex regions and each was homogenized in 1X lysis buffer (Cell Signaling Technology) with 1% PMSF (Sigma-Aldrich, St. Louis, MO), centrifuged at 14,000 rpm for 15 min and stored at −80°C. Right hemispheres were fixed overnight with 4% paraformaldehyde and cryoprotected in a graded series of 10, 20, and 30% sucrose solutions, each overnight at 4°C. Right hemispheres were then embedded in Neg50 frozen section medium (Richard-Allan Scientific, Kalamazoo, MI), and sectioned sagitally on a Microm HM 550 cryostat (Thermo Scientific, Richard-Allan Scientific, Kalamazoo, MI) at 25 μm thickness. Free-floating sections were preserved in PBS containing 100 mM sodium azide at 4°C or PBS with 30% glycerol and 30% ethylene glycol at −20°C.

Immunohistochemistry

Sections were washed in PBS, blocked in 5% horse serum in PBS for 1 h at room temperature and incubated overnight at 4°C in blocking solution containing one of the following antibodies: mouse monoclonal anti-Aβ1-17 (6E10, 1:2,000, Covance Research Products, Emeryville, CA); mouse monoclonal anti-GFAP (1:500, Cell Signaling Technology) rabbit polyclonal anti-gp130 (1:4,000, Sigma-Aldrich), rabbit polyclonal anti-α-tubulin (1:1,000, Stem Cell Technologies, Tuikwila, WA); rabbit polyclonal anti-APP C terminus (pAb 396, 1:1,000, kindly provided by S. Gandy and H. Steiner); rabbit polyclonal anti-Notch1 (1:1,000, Epitomics, Burlingame, CA); rabbit polyclonal anti-Notch1 intracellular domain (NICD, activated Notch 1) (1:500, Abcam, Cambridge, MA); rabbit polyclonal anti-α-actin (1:4,000, Sigma-Aldrich). After washing with ddH2O, blots were incubated for 1 h at room temperature with one of the following horseradish peroxidase-conjugated secondary antibodies: horse anti-mouse IgG-HRP linked (1:1,000, Cell Signaling Technology); goat anti-rabbit IgG-HRP linked (1:5,000, Cell Signaling Technology). Blots were developed using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Cell Culture

Cortical primary neurons were isolated from E14 embryos of heterozygous-bred TgAPPz dams. TgAPPz and wild-type (WT) littersmate mouse embryonic brain tissues were mechanically dissociated. Both primary cortical neurons and commercially available murine neurosphere (Stem Cell Technologies, Tuikwila, WA) were cultured in suspension in DMEM/F12 (Invitrogen, Camarillo, CA) containing B27 (Invitrogen), 20 ng/mL human epidermal growth factor (hEGF), and 10 ng/mL fibroblast growth factor (IGF) at 37°C in 5% CO2. Primary neuron cultures from each embryo remained separate and genotypes were identified from embryo tails using real time PCR (Bailey et al., 2012). For differentiation, neurospheres were mechanically dissociated and filtered with a 40 μm cell strainer into single-cell suspensions in DMEM/F12 containing B27, 20 ng/mL hEGF, 10 ng/mL IGF and 10% fetal bovine serum and plated in 24-well plates (Fisher Scientific) at a concentration of 1x105 cells per well and incubated at 37°C in 5% CO2. Human HEK293-expressed human sAPPα (hsAPPα) was generated and purified as previously described (Barger et al., 1995; Furukawa et al., 1996). N2a (murine neuroblastoma) cells (ATCC, Manassas, VA) were grown in complete EMEM supplemented with 10% fetal calf serum. Cells were plated in 24-well collagen coating culture plates at a density of 1x105 cells per well. After overnight incubation, N2a
cells were incubated in neurobasal media supplemented with 3 mM dibutyryl CAMP in preparation for treatment. N2a cells stably over-expressing human sAPP\(\alpha\) (N2a/sAPP\(\alpha\) cells, named 6-1 cell clone) were generated via liposomal delivery using Lipofectamine 2000 (Invitrogen) of a plasmid (pcDNA3.1-sAPP-\(\alpha\)695) containing a human sAPP\(\alpha\) cDNA coding sequence based on the predicted cleavage of the 695 aa isoform of APP (a generous gift from Dr. Steven Barger, University of Arkansas) into N2a cells followed by G418 (400 \(\mu\)g/mL) selection.

**Immunocytochemistry**

After 7 days in culture, neurospheres were gently and mechanically triturated into single-cell suspensions, plated on chamber slides at a concentration of 20,000 cells per well and incubated for 3–7 days at 37°C in 5% CO\(_2\). Cells were fixed with 4% paraformaldehyde (Fisher Scientific) in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min at room temperature, blocked in 5% horse serum for 1 h at room temperature, and incubated overnight at 4°C in 5% horse serum containing one of the following primary antibodies: monoclonal anti-\(\beta\)-tubulin (1:1,000, Stem Cell Technologies), mouse monoclonal anti-GFAP (1:500, Cell Signaling Technology). Cells were then incubated for 30 min at room temperature with fluorescein-conjugated AlexaFluor 488, AlexaFluor 594, and AlexaFluor 555 secondary antibodies (Fluorescein-conjugated IgGs, Invitrogen) at a dilution of 1:200. Images were acquired on an Olympus FV1000 Confocal microscope.

**Statistical Analysis**

Statistical differences between genotype groups were determined using one-way analysis of variance (ANOVA) for multiple comparisons. Other statistical differences were determined using the Student’s \(t\)-test. Statistical analysis for behavioral experiments was performed using GraphPad5 Analysis software. Analyses were performed on Microsoft Excel software.

**Results**

**Elevated sAPP\(\alpha\) and \(\alpha\)-CTF Levels in Brain Tissues From Autistic Children**

Previous studies report elevated levels of sAPP\(\alpha\) in the plasma of children with severe autism (Ray et al., 2011). Since sAPP\(\alpha\) is an established neurotrophic factor (Mucke et al., 1996; Turner et al., 2003), concentrations in autistic patient brains may be affected. A commercially available human sAPP\(\alpha\) ELISA kit was used to measure the sAPP\(\alpha\) concentrations in brain homogenates from autistic patients and controls. Results show that autistic patient brains contain a significantly increased level of sAPP\(\alpha\) compared to the mean level found in the brains of normally developed controls (Fig. 1A). The distributions of individual patient sAPP\(\alpha\) levels around the means show that 4 of the 6 autistic patients had sAPP\(\alpha\) levels greater than the mean sAPP\(\alpha\) levels of the controls (Fig. 1B).

In light of the nonamyloidogenic processing pathway, increased levels of sAPP\(\alpha\) in the brain should convey a corresponding increase in the levels of the APP \(\alpha\)-C-terminal fragment (\(\alpha\)-CTF). Patient brain tissue samples were analyzed by western blot using the polyclonal antibody 396 which binds the APP C-terminal fragment. The results, along with densitometric analysis, show significantly increased expression of the \(\alpha\)-CTF fragment in autistic patient brains compared to normal control brains (Fig. 1C, D). This data suggests that subsets of autistic patients have elevated brain levels of the sAPP\(\alpha\) and \(\alpha\)-CTF fragments of APP, signifying a greater inclination towards the non-amyloidogenic pathway in patient brains and a potential role for the associated fragments in the pathophysiology of autism.

**TgsAPP\(\alpha\) Mice Have Elevated hsAPP\(\alpha\) Levels in the Brain**

To identify the potential role of elevated sAPP\(\alpha\) levels on neurodevelopment, our group has studied a transgenic mouse model that overexpresses human sAPP\(\alpha\) in the brain (Bailey et al., 2012). Brain sections and homogenates from a set of 3-month-old TgsAPP\(\alpha\) littermates were studied by immunohistochemistry and Western blotting techniques using the 6E10 antibody which specifically binds human APP at the A\(\beta\)\(_{1-17}\) region. Homozygous TgsAPP\(\alpha\) (TgsAPP\(\alpha\)+/+ ) mouse brains stained positive for 6E10 in both the cortex and the hippocampus, confirming the expression of the human sAPP\(\alpha\) fragment in these brain regions (Fig. 2A). Western blot analysis and ELISA measurements further corroborated the expression of the protein fragment in TgsAPP\(\alpha\) mice (Fig. 2B–E), demonstrating its absence in WT littermates and genotype-dependent levels of fragment expression in both cortex (Fig. 2B, C) and hippocampus (Fig. 2D, E) regions. These studies confirm the overexpression of hsAPP\(\alpha\) in TgsAPP\(\alpha\) mice.

**Impaired Social Behavior and Hypoactivity in TgsAPP\(\alpha\) Mice**

Social interaction deficits, increased anxiety and hypoactivity are characteristic behaviors of autism patients. To examine social functioning and motor activity in our transgenic mice, we used mouse behavioral tests for open field and social interaction (Crawley 2004).

In the open field task, mice were placed in an open box for 10 min and observed for locomotor activity and time spent in the center of the field. There was no significant difference between groups in time spent in the center (Fig. 3A); however, transgenic mice traveled significantly less distance in the apparatus than WT littermate controls (Fig. 3B), suggesting hypoactivity.

For the social interaction experiment, test mice were observed for 10 min in a 3-chambered apparatus containing an empty wire cage on one end and a wire cage enclosing a...
stranger mouse on the other end. Whereas WT mice \((n=5)\) spent significantly more time in the chamber containing the stranger mouse than in the chamber with the empty wire cage, there was no significant difference in the periods of time that TgsAPP\(\alpha\) mice \((n=4)\) spent in each of these chambers (Fig. 3C,D). Ratios of time spent with stranger/time spent with empty wire cage showed a decrease in time spent with stranger mice by TgsAPP\(\alpha\) mice compared to WT littermates. This finding demonstrates a decreased preference for sociability in TgsAPP\(\alpha\) mice compared to WT controls.

Increased GFAP Expression in Brains of Adult TgsAPP\(\alpha\) Mice

An association between APP and increased GFAP expression in the brain has been firmly established in studies on patients suffering from AD and Down Syndrome (DS) (Jorgensen et al., 1990; Sugaya et al., 2007). To find out whether the overexpression of sAPP\(\alpha\) was associated with an increase in this astrocyte marker, brain sections from 3-month-old TgsAPP\(\alpha\) mice and WT controls were subjected to immunohistochemical staining with GFAP (Fig. 4A). Other brain sections from these mice were double stained with GFAP and Nissl, which identifies cell bodies and delineates morphology in brain tissues (Fig. 4B). Image analysis and quantification of GFAP staining show that there are significantly more GFAP-positive cells in the hippocampus and entorhinal cortex of TgsAPP\(\alpha\) mice compared to WT controls (Fig. 4C). Immunohistochemistry findings were verified by Western blot analysis on cortical and hippocampal brain homogenates and, as expected, significantly increased GFAP expression was found in TgsAPP\(\alpha\) mice in both regions compared to WT controls (Fig. 4D). This GFAP increase is also seen in 1-month-old and 6-month-old TgsAPP\(\alpha\) mice compared to WT controls (data not shown).

Increased Glial Differentiation in TgsAPP\(\alpha\) Derived Murine Neuronal Stem Cells

Murine NSCs derived from TgsAPP\(\alpha\) and WT E14 embryos were cultured under differentiating conditions for 3 days. Using immunofluorescence techniques, these differentiated stem cells were fixed and stained with antibodies against neuronal marker \(\beta\)-tubulin III and GFAP. Compared to murine NSCs from WT embryos, there was less \(\beta\)-tubulin III expression and greater GFAP expression in murine NSCs derived from TgsAPP\(\alpha\) embryos (Fig. 5A). Immunofluorescence findings were verified by Western blot analysis on cell lysates prepared from mouse NSCs (Fig. 5B). As expected, significantly increased GFAP expression was found in TgsAPP\(\alpha\) mouse-
derived NSCs (Fig. 5C). The levels of human sAPP\(_a\) (hsAPP\(_a\)) secreted into the media by the murine NSCs from both groups of mice were measured using a commercially available ELISA kit. The ELISA results confirmed that the hsAPP\(_a\) transgene is expressed in murine NSCs from TgsAPP\(_a\) embryos and that the protein fragment is secreted from these murine NSCs (Fig. 5D). Overall, these results support the association between sAPP\(_a\) and increased GFAP expression in vivo.

**Increased Expression of IL-6, NICD, and gp130 Correlated With Elevated sAPP\(_a\) Levels**

Standard mechanisms of action by which sAPP\(_a\) achieves its effects in the brain are still yet to be determined. With these experiments, we aimed to identify possible signaling pathways involved in sAPP\(_a\) function. Previous studies implicate the IL-6/gp130 pathway and the activity of Notch1 intracellular domain (NICD, activated Notch1) in glial differentiation of NSCs (Rodriguez-Rivera et al., 2009; Kwak et al., 2010). IL-6 concentrations in cortical brain homogenates from 3-month-old WT (\(n = 4\)) and TgsAPP\(_a\) (\(n = 4\)) mice were measured by ELISA. TgsAPP\(_a\) mice demonstrated a significantly increased mean concentration of IL-6 per milligram of protein than WT littermates (Fig. 7A). Brain homogenates were also subjected to Western blot analysis using antibodies against human sAPP\(_a\), gp130, NICD, and \(\beta\)-actin. The blots exhibited greater protein expression of gp130 and NICD in the TgsAPP\(_a\) mice, which verifiably express hsAPP\(_a\), compared to WT littermates (Fig. 7B).

To determine whether this effect is specifically due to the presence of hsAPP\(_a\) we transfected N2a neuroblastoma cells with the hsAPP\(_a\) gene to create a cell line, named clone 6-1, which overexpresses the hsAPP\(_a\) protein fragment. Conditioned media from triplicate cultures of each of these two cell lines were collected 18 h after plating (without treatment)
and subjected to IL-6 ELISA. The 6-1 cells secreted a significantly higher level of IL-6 than N2a cells under normal culture conditions (Fig. 7C). Lysates from each cell line were analyzed by Western blot using antibodies against hsAPPα, gp130, activated Notch1, and β-actin. Results confirm the successful transfection of the hsAPPα fragment in the 6-1 line and show increased expression of gp130 and NICD in this cell line compared to the WT neuroblastoma line (Fig. 7D).

Finally, N2a cells and murine NSCs were treated with 0, 0.5, 1, and 2 nM doses of recombinant hsAPPα (rhsAPPα) for 24 h. IL-6 concentrations secreted into the media were measured by ELISA and the cell lysates were subjected to Western blot analysis using antibodies against gp130, activated Notch1, and β-actin. Although the murine NSCs secreted notably less IL-6 than the N2a cells, treatment with rhsAPPα induced both types of cells to secrete increased levels of IL-6 in a dose-dependent manner (Fig. 7E). Dose-dependent increases in gp130 and activated Notch1 were also evident in cell lysates after treatment with rhsAPPα (Fig. 7F).

In addition, we observed the cortical expression of gp130 in TgsAPPα mice and WT controls by immunohistochemistry. TgsAPPα mice exhibit increased gp130-positive staining compared to WT littermates (Fig. 8A,B). As further support, mouse brain homogenates were prepared from dissected cortical tissues and subjected to Western blot analysis. Notably, these results confirmed the increased expression of gp130 in the cortical region (Fig. 8C).

**Discussion**

This work reports the discovery of elevated levels of sAPPα and γ-CTF in the insular cortex of autism patients and suggests increased GFAP expression-associated hypoactivity and social deficits in mice designed to over-express hsAPP in brain tissues. The work further reveals that the observed GFAP upregulation is correlated with elevations in IL-6, gp130, and Notch1. These observations support our hypothesis that subsets of patients with autism exhibit elevations in CNS sAPPα and mice generated to mimic this observation display markers suggestive of gliosis and autism-like behavior.

Since the neurotrophic and neuroprotective functions of the sAPPα fragment are generally accepted, previous studies revealing the presence of sAPPα in autism patient plasma raise questions about the association between the APP fragment and autism pathophysiology (Sokol et al., 2006; Bailey et al., 2012). We discovered, in this study, that autism patients exhibit elevated levels of sAPPα as well as notable...
increases in α-CTF compared to controls in a portion of the insular cortex (Fig. 1) which is part of the gray matter in the CNS. The insular cortex was selected because it is part of the neocortex that shows neurobiological abnormalities in some autistic populations and appears to be involved in language and attention (Bailey et al., 1998; Binstock, 2001). The initial study proposing this unconventional association mentioned that the presence of the sAPPα fragment could be evidence of heightened α-secretase processing (Sokol et al., 2006); and our findings, particularly the dramatic increase in α-CTF levels, support this inference.

APP is in fact upregulated in the brain in response to neural injury, but, as previously mentioned, sAPPα plays a neuroprotective role (Mattson et al., 1993a). As Table 1 shows, several of the samples from both control and autistic cases involve causes of death that could impact brain APP levels. Some of the individual causes of death occur in both groups of cases, thereby alleviating a potential confounding variable. For example, a comparison of the levels of α-CTF in control cases of drowning with the α-CTF level in the autism case of drowning shows increases in α-CTF expression in the control cases of drowning compared to control cases with other causes of death. However, there is an even greater increase in α-CTF expression within the brain sample from the autistic case of drowning compared to the control cases.

Our data confirms that the TgsAPPα mouse we generated exhibits greater expression of hsAPPα in the cortex and hippocampus regions of the brain compared to WT littermate controls. In light of our observations of the autistic insular cortex,
the TgsAPPα mice mimic the human condition of elevated cortical sAPPα levels. We have previously reported that these mice also demonstrate high sAPPα levels in the plasma (Bailey et al., 2012), which also mimics the original finding in autism patient plasma (Sokol et al., 2006). It is worth noting that hsAPPα in heterozygous TgsAPPα mice is expressed at over 10 times the sAPPα levels we observed in the human condition, and homozygous mice have over 20 times greater hsAPPα expression compared to patients studied here. This exaggeration of sAPPα overexpression in the model represents a significant limitation in the translation of the results to the human condition.

Additionally, TgsAPPα mice demonstrate behavioral impairments that simulate autistic behaviors in humans. In addition to impaired social interaction, which is a cardinal autistic behavioral phenotype, patients have also demonstrated hypoactivity (Gillberg and Billstedt, 2000; Maestro et al., 2005; Receveur et al., 2005). TgsAPPα mice exhibit reduced preference for social interaction, indicating impaired sociability (Fig. 3A). Further, in the open field task, TgsAPPα mice demonstrated hypoactive exploratory behavior (Fig. 3C,D). Altogether, the neuropathological and behavioral features of TgsAPPα mice are suggestive of components of an autism phenotype, however further studies are required to substantiate sAPPα’s direct causation.

Evidence of increased GFAP and gliosis in autism patients has been demonstrated in several studies (Bailey et al., 1998; Sabaratnam, 2000; Casanova, 2006). One study shows that the level of GFAP in the CSF of autism patients was at almost three times the level normally developed patients (Ahlsen et al., 1993). Vargas et al. and Fatemi et al. report increased GFAP reactions in different regions of autistic patient postmortem brains, including the middle frontal gyrus and the superior frontal cortex (Laurence and Fatemi, 2005; Vargas et al., 2005). These observations in autism patients may be related to the longstanding theory that astrocytes participate in the brain's immune response and indicate tissue damage (Casanova, 2006). Adult TgsAPPα mice demonstrate increased GFAP
expression in the entorhinal cortex and hippocampus regions compared to WT littermate controls (Fig. 4). These results suggest a possible role for the elevated levels of sAPPα and increased GFAP expression observed in postmortem autism brains.

There is minimal emphasis in the literature on sAPPα function in other brain cells besides neurons. Its trophic and protective effects on neurons have been well-documented (Malliah et al., 1992; Milward et al., 1992; Mattson et al., 1993b; Roch et al., 1994; Smith-Swintosky et al., 1994; Luo et al., 2001). Evidence exists supporting sAPPα activity on microglial cells, stimulating the release of interleukin-1 (Li et al., 2000), glutamate and markers of inflammation (Barger and Harmon, 1997; Barger and Basile, 2001). The cellular function of sAPPα in astrocytes was introduced with reports that treatment of human NSCs with recombinant sAPPα caused increased astrogliogenesis in vitro (Kwak et al., 2006a). More recently, sAPPα has been proven to increase subgranular zone (SGZ)-derived neural progenitor cell (NPC) proliferation in culture, and treatment of these cells with sAPPα increased NPC differentiation into astroglial cells (Baratchi et al., 2011).

We observed increased GFAP expression and decreased β-tubulin III expression by primary murine NSCs derived from TgsAPPα mice (Fig. 5). We also detected dose-dependent increases in GFAP-positive cells differentiated from WT murine NSCs treated with rhsAPPα (Fig. 6), suggesting that in cell cultures sAPPα could promote glial cell fate. Despite these findings it is likely that upregulation of α-CTF itself (Fig. 1) has effects on APP and its binding partners beyond those of sAPPα. Interestingly, it is not known whether α-CTF and sAPPα can bind together and form a heterodimeric receptor as in the case of Notch1 and other similar receptors (Blaumueller et al., 1997; Furukawa et al., 1996; Van Nostrand et al., 2002; Shaked et al., 2006; Chen et al., 2006; Kedikian et al., 2010;
FIGURE 7: Increased expression of IL-6, NICD, and gp130 correlated with elevated sAPPα levels. A: Cortical brain homogenates were prepared from 3-month-old TgsAPPα mice and WT littermates (n = 4) and subjected to IL-6 ELISA (mean ± sd of pg of IL-6/mg total protein). IL-6 production was enhanced in cortical tissues from TgsAPPα mice. B: WB analysis of these tissues for sAPPα (6E10), gp130, NICD, and β-actin. C: Conditioned media collected after 18 h from N2a cells overexpressing human sAPPα (6-1 cell clone) show enhanced IL-6 production by ELISA compared to N2a cells. Data presented as mean ± SE (pg of IL-6/mg protein) from three independent experiments, with three replicates per group. D: WB analysis of these cells for sAPPα, gp130, NICD, and β-actin. WT N2a cells (E, top panel) or murine NSCs (E, bottom panel) were treated with sAPPα at doses indicated for 24 h and subjected to IL-6 ELISA revealing enhanced levels of IL-6 in sAPPα treatment groups. IL-1β, IL-4, TNFα, and IFN-γ were undetectable (data not shown). F: In parallel, the cell lysates were subjected to WB analysis using gp130 and NICD antibodies. Data presented as mean ± SD (pg of IL-6 per mg total protein from three independent experiments, three replicates per group). **P<0.01; ***P<0.001.
Isbert et al., 2012). Hence the properties of α-CTF in subsets of individuals with autism remain elusive.

Several binding partners for sAPPα have been identified, such as apolipoprotein E (Barger and Harmon, 1997), the class A scavenger receptor (Santiago-Garcia et al., 2001), and others (Kounnas et al., 1995; Knauer et al., 1996; Das et al., 2002), providing a number of candidates that may be responsible for its cellular activities. However, none of these proteins or receptors is involved in glial differentiation. IL-6 family proteins promote the differentiation of NSCs into astrocytes and inhibit neurogenesis through activation of the JAK-STAT pathway (Bonni et al., 1997). The binding of leukemia inhibitory factor (LIF) ligand to its receptor recruits membrane gp130 to form a complex that triggers the JAK-STAT pathway (Taga and Fukuda, 2005). Additionally, Notch1 inhibits neuronal cell fate and promotes glial differentiation of NSCs via its intracellular domain (Wang and Barres, 2000; Gaiano and Fishell, 2002). In particular, Notch1 guides cells that were already destined to become glia towards an astrocytic rather than oligodendrocytic cell fate (Grandbarbe et al., 2003; Lasky and Wu, 2005). Sugaya et al. showed that sAPPα induces glial differentiation of human NSCs by activating the IL-6/gp130 pathway (Kwak et al., 2010). This group also reported that Notch1 signaling is involved in sAPPα-induced glial differentiation of NSCs (Kwak et al., 2011). Our findings confirm these sAPPα mechanisms of action in murine NSCs and N2A cells treated with conditioned medium from NSCs obtained from TgsAPPα mouse NSC culture. Moreover, our observation of increased NICD expression after these experiments suggests that sAPPα operates through the Notch1 pathway (Fig. 7). The sAPPα fragment may not be directly binding to the receptors that trigger either the IL-6 or Notch1 pathways; however, these findings imply that other sAPPα binding partners exist. Velasco et al. reported that stimulation of glial differentiation in rat NSCs via the Notch1 pathway is more potent than stimulation via the IL-6/gp130 activated pathway (Rodriguez-Rivera et al., 2009). Our results suggest that sAPPα can promote glial cell fate of murine neural cells.

In addition to its well-established function in glial differentiation during development, gp130 participates in the regulation of serotonergic gene expression in the mouse brain (Kulikov et al., 2010) and, along with other constituents of the IL-6 pathway, is activated in the astrocytic response to traumatic brain injury (Oliva et al., 2012). Kirsch et al. previously reported that the IL-6/gp130 pathway regulates the astrocytic response and axonal sprouting of neurons after entorhinal cortex lesion in adult rats (Xia et al., 2002). Here, we report that adult TgsAPPα mice demonstrated marked increases of gp130 in the entorhinal cortex by immunohistochemistry (Fig. 8). This suggests that the overexpression of sAPPα in the brain is associated with continually increased presence of gp130, even after development. In light of the aforementioned results (Fig. 4), this gp130 increase may correlate with the GFAP increase we observed in TgsAPPα mice;
however direct causation is lacking. Whether excess sAPPα may potentially be impacting gliosis and abnormal brain development through upregulation of gp130 and consequently gp130-related pathways such as Notch1 and LIF in some individuals with autism remains to be determined.

In summary, we have shown that subsets of patients with autism exhibit elevations in sAPPα and α-CTF in the insular cortex compared to normally developed children. Mice generated to mimic this observation show abnormal social behavior and hypertrophy, increased GFAP expression, and decreased β-tubulin III with associated elevations in IL-6, gp130, and Notch1. NSCs derived from sAPPα overexpressing mice, as well as N2a cells treated with rhsAPPα displayed increased markers suggestive of glial cell fate. Gliosis during critical windows of development may contribute to dysregulated synaptic pruning, maintenance or function, leading to aberrant synaptic connections and components of the autistic phenotype.

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