ASTROCYTE-INDUCED SYNAPTOGENESIS IS MEDIATED BY TRANSFORMING GROWTH FACTOR BETA SIGNALING THROUGH MODULATION OF D-SERINE LEVELS IN CEREBRAL CORTEX NEURONS


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Background: Synapse formation and function is modulated by intrinsic and extrinsic non-autonomous factors.

Results: Astrocytes induce synapse formation through TGF-β1 pathway. TGF-β1 synaptogenic property is dependent on D-serine signaling.

Conclusion: TGF-β induces excitatory glutamatergic synapses in vertebrates.

Significance: This is a novel molecular mechanism that might impact synaptic function and shed light on new potential therapeutic targets for synaptic-deficit diseases.

Assembly of synapses requires proper coordination between pre- and postsynaptic elements. Identification of cellular and molecular events in synapse formation and maintenance is a key step to understand human perception, learning, memory, and cognition. A key role for astrocytes in synapse formation and function has been proposed. Here, we show that transforming growth factor beta (TGF-β) signaling is a novel synaptogenic pathway for cortical neurons induced by murine and human astrocytes. By combining gain and loss of function approaches, we show that TGF-β1 induces the formation of functional synapses in mice. Further, TGF-β1-induced synaptogenesis involves neuronal activity and secretion of the co-agonist of the NMDA receptor, D-serine. Manipulation of D-serine signaling, by either genetic or pharmacological inhibition, prevented the TGF-β1 synaptogenic effect. Our data show a novel molecular mechanism that might impact synaptic function and emphasize the evolutionary aspect of the synaptogenic property of astrocytes, thus shedding light on new potential therapeutic targets for synaptic-deficit diseases.

Human cognitive ability is associated with hugely complex synaptic connections (1).
Conversely, synaptic dysfunctions may lead to several human neurodevelopmental disorders, including autism, schizophrenia, epilepsy and others. Thus, understanding the mechanism by which synapses are formed, specified and maintained is a key step, not only in understanding human cognitive advantages, but also in designing therapeutic approaches to repair the injured human nervous system. Despite compelling advances in the identification of cellular and molecular events in synapse formation, a fundamental question remains unresolved: is the number of synapses an intrinsic property of neurons, or is it controlled by extrinsic signals or non-neuronal determinants?

The TGF-β (transforming growth factor beta) superfamily is constituted by multifunctional polypeptide members, which perform critical functions in nervous system development and repair (2-9). Disturbances in TGF-β pathways have been associated with a broad spectrum of behavioral abnormalities, including cognitive impairment, affective disorders and deficits in sensorimotor gating (10-13).

Members of the TGF-β superfamily have been strongly implicated in synapse formation in invertebrates (14-18), and more recently, in vertebrates (19-22).

Glia cells are key determinants in the formation, stabilization and elimination of synapses, in either the peripheral or central nervous system (CNS) (23-32). It remains unclear whether glia-driven synaptogenesis is a general principle that applies to all neuronal cell types and brain regions, as well as being a conserved feature for all mammals, including humans.

The fact that TGF-β1 is secreted by central and peripheral glia (2,3,6), and is expressed in the embryonic and postnatal brain during the synaptic period (33), led us to investigate the hypothesis of TGF-β1 as a synaptogenic molecule for vertebrate neurons.

Here, we provide evidence that TGF-β signaling pathway is involved in murine and human astrocytes synaptogenic property. Further, we unveil a mechanism underlying TGF-β1-induced synaptogenesis involving D-serine, a co-agonist of the N-methyl D-aspartate (NMDA) subtype of glutamate receptor. Our results not only contribute to understanding the mechanism by which astrocytes and growth factors modulate synapse development, but may potentially lead to the development of novel therapeutic approaches to neurological disorders, based on the pharmacological manipulation of the levels of excitatory amino acids.

**EXPERIMENTAL PROCEDURES**

**Animals:** For neuronal cultures, embryonic (E14; 14-day embryonic) mice were used and for astrocyte cultures, newborn (P0) Swiss mice were used. All animal-use protocols were approved by the Animal Use Ethics Committee of the Federal University of Rio de Janeiro.

**Astrocyte cultures:** Primary astrocyte cultures were prepared from the cerebral cortex of newborn Swiss mice as previously described. Briefly, the mice were decapitated, the brain structures were removed and the meninges were carefully stripped off. Tissues were washed in phosphate-buffered saline (PBS) with 0.6% glucose (Sigma Chemical Co., St. Louis, MO, USA) and dissociated into single cells in a medium consisting of Dulbecco's minimum essential medium (DMEM) and nutrient mixture F12 (DMEM/F12, Invitrogen, Carlsbad, CA, USA), enriched with glucose (3.3x10^-2 M), glutamine (2x10^-3 M) and sodium bicarbonate (0.3x10^-2 M). Dissociated cells were plated onto glass coverslips previously coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA), on a 24-well plate (Corning Incorporated, Corning, NY, USA), in DMEM/F12 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cultures were incubated at 37°C in a humidified 5% CO₂, 95% air chamber for 7-10 days until reaching confluence. After growing to confluence, cells were subjected to passages to generate pure astrocytic cultures. This protocol yields an astrocyte-enriched culture constituted by more than...
95% of GFAP (glial fibrillary acidic protein)-positive cells.

Astrocyte-conditioned medium: Astrocyte-conditioned medium (ACM) was prepared as previously described (34). After reaching confluence, secondary astrocyte cultures were thoroughly washed to eliminate residual serum, and the cultures were maintained in a medium consisting of Dulbecco's minimum essential medium (DMEM) and nutrient mixture F12 without fetal bovine serum (DMEM/F12, Invitrogen, Carlsbad, CA, USA), for one day. The medium was then collected and centrifuged to remove cellular debris and used immediately. To analyze the effect of astrocyte conditioned medium in neuronal synapse formation, neuronal cultures were grown in Neurobasal for 12 days in vitro. After this period, the neuronal culture was pre-incubated with vehicle or the TGF-β pathway inhibitor, SB-431542, for 30 min. Then, the neuronal cultures were maintained simultaneously in the presence of ACM and the inhibitor for additional 3 h, followed by fixation and immunostaining of synaptic proteins. SB-431542 (10 μM) (Sigma Chemical Co., St. Louis, MO, USA) was diluted in dimethyl sulfoxide (C₂H₆OS, DMSO, Sigma Chemical Co., St. Louis, MO, USA). Cells were exposed to a maximum concentration of 0.4 % DMSO. Addition of DMSO to ACM had no effect on synapse formation (data not shown).

Neuronal cultures: Dissociated primary neuronal cultures were prepared from the cerebral cortex of E14 Swiss mice and maintained in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with B-27, penicillin, streptomycin, L-glutamine, fungizone, and Ara C (0.65 μM, Sigma Chemical Co., St. Louis, MO, USA). Neurons were plated at a density of 50,000-150,000 per well of 13 mm diameter and 800,000 cells per well of 35 mm diameter, previously coated with poly-lysine (10 μg/mL, Sigma Chemical Co., St. Louis, MO, USA). One-third of the medium was changed for fresh medium every three days. This protocol produces a neuronal-enriched culture (98% of neurons and 2% of astrocytes).

Treatment of neuronal cultures: Neurons were treated with TGF-β1 (10 ng/mL, R&D Systems, Buckinghamshire, UK) or D-serine (0.4 mM, Sigma Chemical Co., St. Louis, MO, USA) for different times (zero, three, six and nine days in vitro – DIV). For pharmacological inhibition assays, neuronal cultures were plated and incubated in the presence or absence of specific inhibitors for three days in vitro (10 μM of 5,7-dichlorokynurenic acid; 10 μM MK-801 (Sigma Chemical Co., St. Louis, MO, USA). To analyze the effect of D-serine degradation on synapse formation, neuronal cultures were simultaneously treated with TGF-β1 and the enzyme D-aminoacid oxidase for three days in vitro (DAO; 20 μg/mL, MP Biomedicals LLC, Illkirch, France). For assurance of DAO activity specificity, cultures were also treated with heat inactivated DAO, boiled for 5 minutes. Removal of hydrogen peroxide derived from the degradation of D-serine was assayed by addition of catalase (10 U/mL, Sigma Chemical Co., St. Louis, MO, USA).

Human astrocyte cultures: Adult primary human astrocytes were isolated from surgically resected anterior temporal lobe tissue, from patients selected for surgical treatment of temporal-lobe epilepsy associated with hippocampus sclerosis (TLE-HS). The selected patients were evaluated by video-electroencephalography monitoring with a 132-channel Nihon-Kohden® apparatus, and the ictal onset zone was concordant with neuroimaging and semiology data. The pathological tissue targeted in surgery for these cases is the gliotic hippocampus, and the anterior temporal lobe resection is used merely as a surgical pathway to the diseased area. As previously described (35), only healthy cortical tissue was used to produce astrocyte cultures. All patients gave written consent to the study, and the procedures were in agreement with the Brazilian Ministry of Health Ethics Committee (CONEP No. 2340).
Experimental protocols were performed as previously described (36). Briefly, tissues were washed in DMEM medium, mechanically dissociated, chopped into small (<2 mm³) pieces with a sterile scalpel, and incubated in 10 mL of 0.25% trypsin solution at 37°C for 10 min. After centrifugation for 10 min, the cell pellet was resuspended in DMEM/F12 growth medium supplemented with 10% FCS, and plated onto tissue culture plates in a humidified 5% CO2, 95% air atmosphere at 37°C for 2 h in order to achieve adherence of microglial cells. The nonadherent astrocytes were transferred into other culture plates, previously coated with poly-L-lysine. Adherent astrocytes were allowed to grow by replacing the medium once a week. New passages of cells were generated by harvesting confluent astrocyte cultures using trypsin-EDTA solution (0.25% trypsin with EDTA, Invitrogen, Carlsbad, CA, USA). Human astrocytes from up to the third passage were used in the study.

Presynaptic activity analysis: Cells were washed with extracellular solution (150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4). After that, the cells were incubated with a high-potassium solution (97 mM NaCl, 57 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) containing FM1-43 (Molecular Probes, Paisley, UK; 5 μM) at room temperature for 5 min. Cells were washed twice with the extracellular solution, then fixed, and observed by means of a TE2000 Nikon microscope.

D-Serine level analysis: The media of astrocyte or neuronal cultures were supplemented with 10 mM L-serine (Sigma Chemical Co., St. Louis, MO, USA) to maximize D-serine formation, in the presence or absence of 10 ng/mL TGF-β1 (R&D Systems, Buckinghamshire, UK). After 24 h, the conditioned medium was removed and treated with trichloroacetic acid (5% final concentration) to precipitate proteins and extract the free amino acids. Samples were centrifuged (20,000 g for 5 min), the supernatants were extracted three times with water-saturated diethyl ether to remove trichloroacetic acid, and the amino acids were measured by HPLC, as described (37).

Transfection of neuronal cultures with shRNA: Sequences complementary to the rat homolog of the serine racemase gene were designed and cloned into the pENTR/U6 plasmid (Invitrogen, Carlsbad, CA, USA). This vector induces the expression of short hairpin RNAs (shRNAs) in mammalian cells, under the control of the U6 promoter and RNA polymerase III. Two sequences targeting the serine racemase gene and one control sequence targeting the exogenous LacZ gene were chemically synthesized and cloned into the pENTR/U6 plasmid to generate the shRNA pENTR/U6 vectors. SR597-pENTR/U6 (Top strand: CACCGCTCTCACCCTATGCTGCTAAACGAAATTTAGCAGCATAGGTGAGAGC); SR1063-pENTR/U6 (Top strand: CACCGCTAAATACCTGGCCTATTA CGAATAATAGCCAGGTATTTAAGC); LacZ-pENTR/U6 (Top strand: CACCGCTACAAATACGCGATTTCGA AAAATCGCTGATTTGTGTAG). For transfection, neuronal cultures were incubated for 4 h with 0.5 mL of Opti-MEM (Invitrogen, Carlsbad, CA, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), in the presence of 1 μg of SR597-pENTR/U6 plus 1 μg of SR1063-pENTR/U6, or 1 μg of LacZ-pENTR/U6. After transfection, cells were maintained for an additional 48 h in the regular media followed by treatment with 10 ng/mL TGF-β1 (R&D Systems, Buckinghamshire, UK) for 3 days and subsequent immunocytochemistry analysis.

ELISA of neuronal and astrocyte conditioned medium: Concentrations of murine TGF-β1 were measured in cell-free conditioned media derived from astrocyte and neuron cultures. A standardized sandwich ELISA protocol with matched antibody pairs from PeproTech and R&D Systems was carried out according to the manufacturers’ instructions. Results are expressed as picograms per milliliter. The
experimental groups consisted of triplicate samples from three independent experiments.

**Immunocytochemistry:** After fixation with 4% paraformaldehyde for 15 min, the cultures were permeabilized with 0.2% Triton X-100 for 5 min at room temperature, and unspecific sites were blocked with 10% bovine serum albumin (Sigma, St. Louis, MO, USA) for 1 h before immunoreactions with the following antibodies: rabbit anti-glial fibrillary acidic protein (GFAP, DAKO Cytomation, Glostrup, Denmark; 1:500); mouse anti-class III βTubulin (Promega Corporation, Madison, WI, USA; 1:1,000); mouse anti-α-synaptophysin (Chemicon International, Billerica, MA, USA; 1:200); rabbit anti-PSD-95 (Cell Signaling, Beverly, MA, USA; 1:100); rabbit anti-serine racemase (Abcam, Inc., Cambridge, UK; 1:500); rabbit anti-TGFβ RII (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200); guinea pig anti-glutamate-aspartate transporter (α-GLAST; Chemicon International, Billerica, MA, USA; 1:100); mouse anti-human leukocyte antigen-ABC (α-HLA-ABC; BD Bioscience, NJ, USA; 1:100). After primary antibody incubation, the cells were thoroughly washed with PBS and incubated with secondary antibodies for 2 h, at room temperature. Secondary antibodies were Alexa Fluor 546 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes, Paisley, UK; 1:1,000) or with Alexa Fluor 488 (goat anti-rabbit IgG, goat anti-guinea pig IgG, goat anti-mouse IgG; Molecular Probes, Paisley, UK; 1:300). Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole, dilactate; Sigma, St. Louis, MO, USA). To analyze excitatory synapse formation, only DAPI-labelled neurons away from a distance equivalent of at least two diameters of the cell body were selected. After immunostaining, cells were observed with the aid of a TE2000 Nikon microscope and a Leica Confocal microscope. Colocalization of synaptic proteins was analyzed as described in Puncta analysis section.

**Puncta analysis:** Neurons with at least two diameters away from the neighboring neuron were randomly selected and identified using the DAPI stain. After capturing 15 to 20 images per experimental condition, the green and red channels were aligned and neurites of the same sizes (10 μm) were selected and quantified using the Puncta Analyzer plug-in in NIH Image-J as previously described (24). The neurites were chosen randomly, following only one criterion: they had to be located at a distance of one cell body from the neuronal cell soma. In each image, 8-12 neurites were analyzed, totaling at least 1000 μm of neurite length analyzed per experimental condition. In each experiment at least 30-40 neurons were analyzed per experimental condition. Experiments were done in duplicate and each result represented the mean of at least three independent neuronal cultures.

**Neuronal morphology and quantitative analysis:** Cell density, neuron number and neurite outgrowth were analyzed. Neuronal cultures were immunostained with the antibody against the neuronal marker βTubulin III (Promega Corporation, Madison, WI, USA; 1:1,000) and observed and counted using a TE2000 Nikon microscope and a Leica Confocal microscope. Neurite length was measured as the summed distance from one cell body to the end of all its neurites. At least ten fields were analyzed per well. In all cases, at least 100 randomly chosen neurons were observed per well. The experiments were done in duplicate, and each result represents the mean of at least three independent experiments.

**Statistical analysis:** Graphpad software, version 5.0 (GraphPad Software, La Jolla, CA, USA), was used for statistical analysis of the quantitative data. As all statistical tests involved multiple conditions, the Anova test was applied in all comparisons, followed by a non-directional Tukey’s post-test when statistical significance was achieved. A confidence interval of 95% was used, and a P-value < 0.05 was considered statistically significant. Densitometry of blotted gels was performed using the program Un-Scan-It gel version 6.1 (Silk Scientific, Inc., Orem, UT,
USA). Data are reported as mean ± SEM and error bars in the graphs represent SEM.

**Electron microscopy:** Cortical neurons were fixed on coverslips for 4 h in 2.5% glutaraldehyde (Sigma, St. Louis, MO, USA) in 0.1 M cacodylate buffer (pH 7.3). After fixation, the cells were post-fixed for 20 min in a solution containing 2% OsO$_4$ and 0.8% potassium ferrocyanide in 0.3 M cacodylate buffer, washed in the same buffer, dehydrated, and flat-embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss 900 electron microscope. Images were recorded in three independent experiments, and the number of synapses was counted by a investigator blinded to the origin of the images. The images were morphometrically analyzed using ImageJ software. Only excitatory synapses recognized by high post-synaptic density regions were evaluated.

**Immunoblotting assays:** Protein concentration in cell extracts was measured by the BCA™ Protein Assay Kit (Cole-Parmer Canada Inc., Montreal, QC, Canada). Forty micrograms of protein per lane was electrophoretically separated in 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After separation, the proteins were electrically transferred to a Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 1.5 h. Unspecific sites were blocked by membrane incubation in Tris-buffered saline-Tween 20 (TBS-T; Merck, Darmstadt, Germany) containing 5% milk for 1 h. Primary antibodies were incubated in block solution overnight, followed by 2 h-incubation with peroxidase-conjugated secondary antibodies. Proteins were observed using the enhancing chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; Pierce Biotechnology, Milwaukee, WI, USA), and PVDF membranes were exposed to autoradiographic films. Primary antibodies were mouse anti-α-synaptophysin (Chemicon International, Billerica, MA, USA; 1:500); rabbit anti-PSD-95 (Cell Signaling Technology, Beverly, MA, USA; 1:1,000) and rabbit anti-serine racemase (Abcam, Inc., Cambridge, UK; 1:1,000). Secondary peroxidase-conjugated antibodies were goat anti-rabbit IgG and goat anti-mouse IgG (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK; 1:3,000).

**Electrophysiology:** Whole-cell membrane currents were recorded at a membrane potential of −70 mV with an EPC-7 (List, Darmstadt, Germany) patch-clamp system. Currents were low-pass filtered at 3 kHz and digitized with a LabMaster interface under the control of pClamp software (Axon Instruments, Foster City, CA, USA). The standard extracellular solution was 165 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM D-glucose, 5 mM HEPES, and ~2 mM NaOH, pH 7.3. This solution replaced the culture medium approximately 20 min before the recordings and was continuously perfused at a rate of ~1 mL/min throughout the experiments. Patch micropipettes were made from borosilicate glass capillaries (WPI, Sarasota, FL, USA) in a P-97 horizontal puller (Sutter Instruments, Novato, CA, USA). The intracellular solution was 80 mM CsCl, 80 mM CsF, 10 mM EGTA, 10 mM HEPES, and ~26 mM CsOH, pH 7.3. Reported membrane potentials are corrected for a −7 mV liquid junction potential measured between solutions. The filled patch microelectrodes had resistances of 2 to 5 MΩ in the bath; the access resistance (28 ± 12 MΩ) was left uncompensated. Experiments were performed in 6-10 neurons from treated and untreated paired cultures, prepared from the same cell suspension, either at 3 or 12 days in vitro. Membrane capacitance was estimated from the analog compensation of the transient response to a hyperpolarizing test pulse. Approximately 2 min after achieving the whole-cell configuration, voltage sensitive sodium currents were evoked by depolarizing square pulses to 0 mV (50 ms) from a holding potential of −70 mV. Leak currents were subtracted by a fractional (P/4) method. Immediately after this protocol, recordings of the spontaneous activity were made during 3
min for each neuron. Only cells that responded to the depolarizing pulse with a fast-inactivating inward current were considered neurons and were used for the spontaneous activity experiments. Recordings were made at room temperature (~23°C). Current traces were analyzed with pClamp software. Synaptic currents larger than four times the baseline S.D. (noise) were detected by automatic template matching followed by manual removal of spurious events.

**RESULTS**

Astrocytes from rodents and humans induce synapse formation between cerebral cortex neurons through TGF-β pathway. It remains unclear if the synaptogenic property of astrocytes also applies to humans. Here, we tested the involvement of TGF-β family members as synaptogenic factors derived from both human and murine astrocytes. Members of the TGF-β family elicit their bioactivity via a heteromeric receptor complex, consisting of TGF-β receptor (TGFβR) types I and II. TGFβRII is required for the transmembrane signaling of TGF-βs, whereas phosphorylation of TGFβ RI by TGFβ RII is required for activating Smad proteins and propagating the downstream cellular signal. To directly test the involvement of TGF-β family members as synaptogenic factors derived from astrocytes, we analyzed the effect of SB-431542, a TGF-β pathway inhibitor, in astrocyte-induced synaptogenesis. Neuronal cultures were grown in Neurobasal for 12 days in vitro, after this period, the neuronal culture was pre-incubated with vehicle or the TGF-β pathway inhibitor, SB-431542, for 30 min. Then, the neuronal cultures were maintained simultaneously in the presence of astrocyte conditioned medium derived from either murine (MACM) or human astrocytes (HACM), and the inhibitor for additional 3 hours. Human astrocytes were obtained from normal subcortical white matter (NSWM) of the anterior left temporal lobe (Fig. 1A). Those cells highly express astrocyte markers such as GFAP (Fig. 1B) and GLAST (Fig. 1C) and the human leukocyte antigen (HLA) (Fig. 1D), attesting their human and astrocytic nature. The NSWM was obtained from patients with drug-resistant temporal lobe epilepsy associated with hippocampal sclerosis (TLE-HS) who underwent surgery (Fig. 1A). As expected, murine conditioned medium increased the number of synapses, by 185%, as revealed by quantification of double-immunolabeled puncta for the pre- and post-synaptic proteins, synaptophysin and PSD-95, respectively (Fig. 2A-B, 2F). Addition of SB-431542 partially prevented this event, showing that TGF-β signaling is involved in the synaptogenic effect of the murine astrocyte-conditioned medium (Fig. 2A-C, 2F). Interestingly, human conditioned medium increased by 260% the number of synapses of murine cerebral cortex neurons, whereas addition of SB-431542 completely prevented this event, suggesting that synaptogenic effect of human astrocyte-conditioned medium also involves TGF-β signaling (Fig. 2D-F). In order to measure synaptic functionality in the presence of HACM, pre-synaptic activity was assayed by the fluorochrome FM1-43, which labels active presynaptic vesicles (Fig. 2G-I). Addition of SB-431542 to non-conditioned medium had no effect in synapse number (data not shown).

These data not only highlight the evolutionary aspect of the synaptogenic property of astrocytes, but point to TGF-β signaling as a conserved synaptogenic glial-induced pathway present in invertebrates and vertebrates, including humans.

**TGF-β1 induces synapse formation between cerebral cortex neurons.** Among TGF-β family, TGF-β1 has been strongly implicated in synapse formation in invertebrates (Chin et al., 2002) and in vertebrate peripheral nervous system (Feng and Ko, 2008). We then investigated the possibility of TGF-β1 as a synaptogenic molecule for glutamatergic vertebrate synapses. The demonstration of high levels of TGF-β1 in astrocyte-conditioned medium analyzed by ELISA (Fig. 3A) and the presence of TGFβ RII protein in neuronal cultures support this hypothesis (Fig. 3B-D).
To test whether TGF-β1 alone could reproduce the effects of astrocyte-conditioned medium, we cultured cerebral cortex neurons in the presence of TGF-β1 for 12 days and analyzed synapse formation by immunocytochemistry, western blotting, electron microscopy and electrophysiological assays. The presence of synapses in cerebral cortex cultures was first morphologically assessed by immunostaining for synaptophysin and PSD-95. After 3 days in culture (data not shown), the untreated neurons contained strikingly few sites of colocalized immunoreactivity, which progressively increased in number during 12 days in culture (Fig. 4A-C). TGF-β1 enhanced the number of synaptic puncta after 3 (data not shown) and 12 days in culture (Fig. 4A-C and Fig. 5A-E). Levels of pre- and post-synaptic proteins were also increased by this treatment (Fig. 4D).

Synapses induced by TGF-β1 were ultrastructurally identical to those from control cultures (Fig. 5A-D, F). Pre- and post-synaptic specializations could be easily detected in both cultures. There was no difference in the average length of the post-synaptic densities (PSD), but their number increased by 150% (Fig. 5E, F).

To ascertain if the increase in colocalized puncta reflected the formation of functional synapses, cortical neurons with 3 and 12 days in culture were treated with TGF-β1 since day 1, and used in whole-cell patch-clamp experiments. Spontaneous activity, Na\textsuperscript{+} currents and membrane capacitance were recorded. Spontaneous post-synaptic currents in 12-day treated neurons (n=19) showed significantly higher frequencies (2.5 ± 0.3 s\textsuperscript{−1} versus 1.6 ± 0.3 s\textsuperscript{−1} in control; p<0.05, Student’s t-test) and amplitudes (130 ± 14 pA versus 75 ± 5 pA; p<0.001, Student’s t-test) than control neurons (n=18). The distributions of inter-event intervals and amplitudes were also significantly different (Fig. 5G, H). Treated and untreated cortical neurons cultured for 3 days showed either very few or (more often) no spontaneous currents. The mean voltage-activated Na\textsuperscript{+} currents (VAC) in neurons treated with TGF-β1 (3395 ± 233 pA, n=7) were significantly greater than those of untreated neurons (1374 ± 255 pA, n=8; p<0.001, two-way ANOVA) in 12-day cultures (Fig. 5I) but not in 3-day cultures (data not shown). TGF-β1 also increased neuronal membrane capacitance, reflecting an increased membrane area. Measured capacitances were 15 ± 2.0 pF for untreated (n=8) and 25 ± 2.7 pF for 12-day treated neurons (n=7; p<0.001, two-way ANOVA). Again, no differences were observed in 3-day cultures. Together, these data demonstrate that cortical neurons are targets for TGF-β1, which induces formation of morphologically organized and functional synapses in vitro.

Several mechanisms are proposed to underlie the astrocyte effect on synapse number and efficacy, including increased dendritic maturation and neuronal arborization, enhanced neuronal survival, modulation of synapse elimination or stabilization, and secretion of gliotransmitters or neuromodulators (38-40). To determine whether the effect of TGF-β1 in synaptogenesis was due to an increase in dendritic arborization or neuronal density, the number of neurons and their arborization were evaluated. TGF-β1 did not induce significant changes in the number of total or β-Tubulin-immunoreactive cells or in dendritic morphology, indicating that TGF-β1 increases synaptogenesis independently of dendrite outgrowth or neuronal survival (Fig. 6).

D-serine also induces synapse formation between cerebral cortex neurons. What mechanism underlies the synaptogenesis induced by TGF-β1? It is known that synaptic transmission mediated by the N-methyl D-aspartate (NMDA) type of glutamate receptor participates in structural plasticity of synaptic networks (41,42). To investigate if TGF-β1 synaptogenic property was dependent on NMDA receptor activity, we blocked the receptors with MK-801. Blockade of NMDA receptors completely abolished the effect of TGF-β1 on synaptogenesis (Fig. 7A).

D-serine is the main endogenous ligand of the co-agonist site at the NMDA receptors, and, together with glutamate, is
required for NMDA receptor function in synaptic transmission and plasticity (43-46). We investigated whether D-serine mediates the effect of TGF-β1 on synaptogenesis.

TGF-β1 markedly increased the levels of extracellular D-serine from both neuronal and astrocyte cultures (Fig. 7B). Levels of the neurotransmitter glutamate were not altered by TGF-β1 in neither cell types (Fig. 7B). D-serine formation is mediated by the enzyme serine racemase, which converts L-serine to D-serine. TGF-β1 did not modulate serine racemase levels or distribution in either neurons or astrocytes (Fig. 8).

In order to address the effects of D-serine on synaptogenesis, cerebral cortex neurons were treated with D-serine, followed by synapse evaluation as previously described. As shown for TGF-β1, D-serine increased the PSD/synaptophysin puncta by 218% (Fig. 9A-C) and nearly doubled the levels of these pre- and post-synaptic proteins (Fig. 9D). D-serine induced-synaptogenesis was mediated by the co-activation of NMDA receptors, since the specific antagonist of the co-agonist site 5,7-dichlorokynurenic acid (DCK) and the NMDA receptor activity inhibitor, MK-801, completely blocked the effect (Fig. 9C). Cell viability assays revealed no drug toxicity at the concentrations used (data not shown). MK and DCK alone had no effect on synapse formation (data not shown).

Synapses induced by D-serine were ultrastructurally identical to those from the control and TGF-β1-treated cultures (Fig. 9E-H, J). There was no difference in PSD length, although synapse number increased by 133% (Fig. 9I, J). As shown for TGF-β1 and other synaptogenic molecules such as thrombospondin (24), D-serine also did not induce significant changes in dendritic morphology or neuronal survival (Fig. 10). As seen with TGF-β1, treatment with D-serine for 12 days also significantly changed the distributions in both amplitude and inter-event intervals of spontaneous synaptic currents (Fig. 9K, L). However, this treatment did not significantly change voltage-activated currents or membrane capacitance (Fig. 9M).

**TGF-β1-induced cerebral cortex synapse depends on D-serine.** To determine whether the increase in synaptogenesis induced by TGF-β1 resulted from increased D-serine release, the effects of TGF-β1 on synaptogenesis were evaluated after inducing degradation or impairing formation of D-serine. We reduced D-serine content by adding exogenous D-amino acid oxidase (DAO), an enzyme that specifically degrades D-amino acids, and by down-expressing serine racemase by a shRNA approach. As previously shown, treatment of cortical neurons with TGF-β1 increased PSD/synaptophysin puncta by 90%, whereas pre-treatment with DAO 20 μg/mL completely prevented this effect (Fig. 11A-F). Treatment with heat inactivated DAO (hiDAO) had no effect in TGF-β synaptogenic property, attesting the specificity of the enzyme (Fig. 11D, F). Further, DAO or hiDAO alone, in the absence of TGF-β, had no effect on synapse number (Fig. 11F).

Loss of function with serine racemase shRNA completely abolished the TGF-β1 synaptogenic effect (Fig. 11H). Knocking down of serine racemase by shRNA decreased the levels of serine racemase by approximately 50% (Fig. 11G). Measurement of D-serine levels by HPLC revealed that either DAO or shRNA treatment decreased the levels of extracellular D-serine (decrease of 18.8% and 41.4%, respectively). Further, addition of D-serine completely rescued DAO (Fig. 11E, F) and serine racemase knocking down (Fig. 11H) effect in TGF-β synapse induction, thus supporting the hypothesis that this effect depends on D-serine synthesis. Interestingly, neurons transfected with serine racemase shRNA had a tendency to exhibit lower synaptogenic levels compared to LacZ control (although not statistically relevant), uncovering the basal role of D-serine in synapse formation (Fig. 11H). Together these results demonstrate that the effect of TGF-β1 in synaptogenesis is dependent on D-serine levels.
DISCUSSION
Identification of signaling pathways involved in synapse formation and function provides basic knowledge that may help in manipulating neural circuitry during development and adulthood. In the present study, we described a novel non-cell-autonomous mechanism by which a growth factor controls synapse formation in vertebrates: by increasing the extracellular levels of the NMDA receptor co-agonist, D-serine, TGF-β1 raises the number of functional cerebral cortex neuronal synapses. Further, the data presented here address an attractive and unknown issue, the evolutionary conservation of astrocyte and TGF-β1 synaptogenic properties, thus providing new insights into the role of glial cells in neurodevelopmental disorders.

Members of the TGF-β superfamily have been implicated in synapse formation, especially in invertebrates (14-18). Recently, in vivo and in vitro assays using animals deficient for members of the TGF-β family revealed impaired synaptic development and cognition deficit (12,19-22,47,48).

TGF-βs and their receptors are widely expressed in the embryonic and adult brain (33,49). TGF-β1 expression was identified in proliferative zones and distributed over the entire rodent cortical plate from E16 to P30, including during the synaptogenic period. Both astrocytes and neurons are sources of TGF-β1 and express their receptors (2,3,50). This molecular arrangement supports the possibility that TGF-β1 is a local regulator of synapse formation in the cerebral cortex. Our data corroborate the recent identification of TGF-β1 as a Schwann cell-derived synaptogenic factor in the Xenopus and rat peripheral nervous systems (51), and suggest that TGF-β members might also represent glia-derived synaptogenic factors for the vertebrate CNS in addition to others already described (24,52-55).

Here we found that synapses induced by TGF-β1 are both pre- and post-synaptically active, as revealed by the increased incidence and mean amplitude of spontaneous synaptic currents. This is a significant observation, since several astrocyte-derived synaptogenic molecules identified so far induce pre-synaptic differentiation, although post-synaptically inactive synapse (24,52).

We found that TGF-β1 increased voltage-activated sodium currents, which may reflect a general effect of TGF-β1 on neuronal maturation, akin to the transient up-regulation of potassium channels in cerebellar granule neurons (56). These data, together with the fact that TGF-β1 is not synaptogenic for retinal ganglion cells (24) in spite of being synaptogenic for neuromuscular junction (51) and cerebral cortical neurons (shown here), support the concept that the TGF-β1 effect is cell-specific.

Interestingly, inhibition of TGF-β1 receptor activity only partly impaired the synaptogenic effect of astrocyte-conditioned medium on cortical neurons. The remaining activity is likely to be due to other astrocyte-derived factors already described, including thrombospondin, cholesterol, hervin, and D-serine, the latter described here (24,53-55,57-60). However, like D-serine and unlike most of these molecules, TGF-β1 induces ultrastructurally normal and post-synaptically active synapses. The present study may suggest that TGF-β1 itself is an instructive molecule for excitatory synapse formation, although not the only molecule secreted by astrocytes.

Our data are in apparent contradiction to those generated from forebrain-specific Smad4 knockout mice, which show an enhancement of paired-pulse facilitation in excitatory synaptic transmission and stronger paired-pulse depression of GABA_A currents in the hippocampus (12). It is also possible that TGF-β1-triggered Smad4 signaling promotes synaptogenesis in developing neurons but later down-modulates the short-term plasticity of mature synapses, which we did not examine. Furthermore, astrocytes have been reported to enhance both excitatory and inhibitory synapses, suggesting that through activation of different signaling pathways, astrocytes balance the number of excitatory and inhibitory inputs (54,61). In fact, we cannot completely rule out an effect
of TGF-β1 on inhibitory synapses between the cortical neurons in our study.

Several mechanisms are proposed to mediate synapse formation, including enhancement in dendritic arborization, neuronal survival, modulation of stabilization and elimination of dendritic spines (38-40). In contrast to our observations on cortical neurons, TGF-β1 has been shown to induce neurite outgrowth and dendritic spine formation in hippocampal neurons in vitro (62). However, as we observed, thrombospondin also does not affect glutamatergic axon length or branching, either in hippocampal neurons or in retinal ganglion cells (RGCs) (24,58,63). In contrast, glia-derived cholesterol induces synapse from RGCs through increasing neurite outgrowth and dendritic differentiation (30). Additionally, astrocytes have been shown to increase inhibitory synapses by enhancing GABAergic axon length and branching (58), raising the possibility that astrocytes might promote inhibitory and excitatory synaptogenesis through different pathways and molecules.

Our results suggest that the TGF-β1-induced number of synapses is not attributable to an increase in the number or density of neurons or the result of non-specific trophic effects on cell death, even though TGF-β members are potent neuronal survival factors. A possibility is that trophic stimulation in our culture system might have elicited the maximum effect for neuronal survival and neurite outgrowth. A similar explanation has been proposed for the TGF-β1 effect at the neuron-muscular junction (51).

Although our data have shown that most TGF-β1 activity in excitatory synapse formation is mediated by D-serine, we cannot rule out the possibility that TGF-β1 might itself have a residual synaptogenic activity. TGF-β1 binds to several extracellular matrix proteins and their receptors, leading to the possibility that binding to adhesion molecules facilitates the recruitment of pre- and postsynaptic molecules at new contact sites. In fact, additional pathways have been proposed to explain TGF-β1-modulation of synapse efficacy, such as induction of synapsin phosphorylation and redistribution in invertebrates (15), or binding to the nerve-derived heparin sulfate proteoglycan, agrin (51). Further, TGF-β1 has been reported to increase the levels of thrombospondin in astrocytes (64). The fact that D-serine increased synaptic currents similarly to TGF-β1, but failed to increase inward voltage-gated currents, also suggests that not all effects of TGF-β1 in the cortical neurons were mediated by D-serine. Although this might occur, the loss of function and pharmacological inhibition assays for serine racemase and D-serine, respectively, suggest that most TGF-β1 synaptogenic activity depends on D-serine. Increased D-serine levels induced by TGF-β1 were not explained by changes in total serine racemase protein levels in both neurons and astrocytes. On the other hand, several regulatory mechanisms and protein interactions have been proposed to play a role in regulating serine racemase activity and D-serine availability. For example, the scaffolding molecule, protein interacting with C-kinase 1 (PICK1) has been shown to bind to serine racemase (65) leading to increase in D-serine formation (66). Interactions between PICK1 and PKC have been shown to reduce serine racemase activity by phosphorylation of serine amino acid residues (67). Recent findings revealed the PICK1 as an important regulator of TGF-β signaling (68). It remains to be investigated whether the increase in D-serine levels yielded by TGF-β1 pathway is related to modulation of serine racemase regulators function.

D-serine is the dominant endogenous co-agonist of NMDA receptors in several brain regions and its production and secretion are involved in synaptic plasticity in the hippocampus, pre-frontal cortex and hypothalamus (69). Here we identified a previously unknown activity of D-serine, as a synaptogenic molecule. Inhibition of the NMDA receptor activity by the uncompetitive antagonist MK-801 suggests that the synaptogenic effect of D-serine relies on its co-activation of post-synaptic currents. Interestingly, D-serine levels in the rodent...
brain peak around postnatal day 7, a period of intense synaptogenesis. Consistent with a role of D-serine in synapse formation, recent evidence demonstrated that serine racemase knockout mice show memory deficit and impairment in cortical dendrite morphology (70).

Indirect evidence is provided by the observation that TGF-β1 knockout mice with the NIH genetic background (which rescues TGF-β1 mice from embryonic lethality) show reduced synaptic density, although this phenomenon might be attributed to the extensive neuronal death found in these animals (7). Further in vivo support for a correlation between TGF-β1 and glutamatergic responses is provided by the recent demonstration of increased levels of NR1 and NR2A/B receptor subunits in the hippocampus of TGF-β1 overexpressing mice (62) and association between deficits in TGF-β pathways and neuropsychiatric disorders (10,12,13).

It remains unclear whether the glia-synaptogenic property, initially described for rodents, is a general principle that applies to other mammals, including primates. Previously, murine astrocytes have been shown to induce neuronal maturation and synapse formation from human embryonic stem cells (71). It is not clear, however, if this is an autonomous characteristic of human neurons, since synaptogenic properties of human astrocytes have not been shown so far. This hypothesis was recently strengthened by the observation that astrocytes derived from Down’s Syndrome fetuses are directly involved in the development of spine malformations and reduced synaptic density (72). However, this is indirect evidence, and direct proof of the involvement of healthy human astrocytes in synapse development is still lacking. We showed here that human astrocytes increase the number of functional synapses between murine cerebral cortex neurons revealed by puncta analysis and FM1-43 labeled vesicular secretion.

It is of note that the pharmacological inhibitor of TGFβR partially impaired the synaptogenic activity of murine astrocytes, but fully prevented that of human astrocytes. This suggests that TGF-β pathway might represent a more important role for human astrocyte-induced synapses than for mice, thus implying that the synaptogenic property of glial cells may be a conserved aspect of synaptic biology.

Our data suggest that the synaptogenic effect of TGF-β1 involves the activation of a cascade, which includes an increase in the release of D-serine and the activation of the co-agonist site of NMDA-glutamate receptors. Exogenous administration of D-serine enhances mechanisms related to synaptic plasticity, both in vitro and in vivo (37,67,73). Based on our present findings, we speculate that increased synaptogenesis can participate in the cognitive improvement induced by D-serine administration. Our work raises the question of whether manipulation of TGF pathways and/or D-serine levels might represent a potential tool to restore synapses in the normal brain or enhance the regeneration of new synapses in aged, degenerating and acutely injured CNS.
REFERENCES


ABBREVIATIONS
ACM, astrocyte-conditioned medium; NCM, neuronal-conditioned medium; CNS, central nervous system; DMEM/F12, Dulbecco’s Modified Eagle Medium supplemented with F12 nutrient mixture; DMSO, dimethyl sulfoxide; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; GLAST, glutamate-aspartate transporter; PBS, phosphate-buffered saline; TGF-β1, transforming growth factor beta 1; DCK, 5,7- dichlorokynurenic acid.; MK, MK-801; SB, SB-431542; DAO, D-aminooacid oxidase enzyme; D-Ser, D-serine; shRNAs, short hairpin RNAs; DIV, days in vitro; HLA, human leukocyte antigen-ABC; GLAST, glutamate-aspartate transporter; Ser Rac, serine racemase; TGFβ RI, transforming growth factor beta 1 receptor type I; TGFβ RII, transforming growth factor beta 1 receptor type II.
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do Rio de Janeiro (FAPERJ).
FIGURE LEGENDS:

FIGURE 1. Human astrocyte cultures express typical markers. Adult primary astrocytes were isolated from human temporal-lobe tissue derived from epileptic patients submitted to surgical procedures. Magnetic resonance image shows the brain region used for astrocyte isolation (A), arrow represents the healthy subcortical area of the temporal lobe, from where tissue was obtained for astrocyte cultures. Arrowhead shows the atrophied hippocampal tissue affected by epileptic disorder. Human astrocytes were grown in DMEM/F12 supplemented with 10% FCS, and the distribution of typical astrocytic proteins was analyzed by immunocytochemistry. Human astrocytes express the typical astrocyte marker GFAP (B), GLAST in a typical punctate distribution pattern in their membranes (C), and the HLA in a spread distribution pattern over the monolayer (D). Scale bars, 50 µm.

FIGURE 2. Astrocyte-induced synapse formation is mediated by TGF-β pathway. Cerebral cortex neurons were maintained in the presence of DMEM/F12 (Control; A, F), murine (MACM) and human (HACM) astrocyte-conditioned medium alone (CM; B, D), simultaneously with the TGF-β pathway inhibitor, SB-431542 (CM+SB; C, E). Number of Synaptophysin/PSD-95 puncta was quantified after 12 days (F). Presynaptic activity for HACM assays was evaluated by FM1-43 incubation followed by puncta quantification (G-I). Scale bars, 20 µm. *p<0.001, ** p<0.0001.

FIGURE 3. Levels of TGF-β1 secreted by astrocytes and TGFβRII distribution in cerebral cortex neurons. Levels of TGF-β1 present in neuronal (NCM) and astrocyte (ACM) conditioned media were measured by ELISA assays (A). Astrocytes secrete higher amounts of TGF-β1 than neurons in vitro. β-TubulinIII-positive neurons cultured for 12 days express the TGF-β type II receptor (TGFβRII) in their membranes (B-D). Scale bar, 20 µm. ** p<0.0001.

FIGURE 4. TGF-β1 induces synaptogenesis in cerebral cortex neuronal cultures. Cerebral cortex neurons were cultured for 12 days in the presence of Neurobasal medium alone or supplemented with 10 ng/mL of TGF-β1. Number of Synaptophysin/PSD-95 puncta was quantified after 12 days (A-C). Levels of synaptic proteins were evaluated by Western Blotting assays (D). Scale bar, 20 µm. ** p<0.0001.

FIGURE 5. TGF-β1 induces ultrastructurally normal and functional synapses in vitro. Cerebral cortex neurons were cultured for 12 days in the presence of Neurobasal medium alone or supplemented with 10 ng/mL of TGF-β1. Synapse formation was evaluated by electron microscopy quantification of number of synapses (A, C, E) and length of PSD density (B, D, F), and electrophysiological assays (G-I). Arrowheads (A, C) and brackets (B, D) indicate postsynaptic density. In both cases, asymmetric synapses (presumably excitatory) structurally normal are observed. TGF-β1 increased the number of synapses, although their ultrastructure seemed to be indistinguishable from control synapses. Scale bars, 0.5 µm for A and C; 0.2 µm for B and D. **p<0.0001. Electrophysiological effects of TGF-β1 treatment (G-I). Representative raw current traces illustrate differences in spontaneous activity recorded in the control and TGF-β1-treated group (G). The distributions of the amplitude and interval between events of the two groups were significantly different (p<0.001; Kolmogorov-Smirnov test; n = 5,177 and 8,580 events, from 19 and 18 neurons, respectively) (H). Averaged voltage-activated Na⁺ current traces obtained from five randomly chosen neurons in each condition illustrate the effect of TGF-β1 (p<0.001 vs. control) (I).

FIGURE 6. TGF-β1 does not affect neuronal survival and neurite arborization. Cerebral cortex neurons were cultured for 12 and 3 days in control medium (Control; A, E) or in the presence of 10 ng/mL of TGF-β1 (TGF-β1; B, F). Numbers of β-Tubulin III cells (C), total cells (D), total neurite...
FIGURE 7. **TGF-β1 synaptogenic property is dependent on NMDA receptor activity and involves increase in the extracellular levels of the aminoacid, D-serine.** Cerebral cortex neurons were cultured in the presence of Neurobasal medium alone, with 10 ng/mL of TGF-β1, and simultaneously with TGF-β1 and the NMDA receptor inhibitor MK-801 (TGF-β1+MK). After 3 days, the number of Synaptophysin/PSD-95 puncta was quantified (A). *TGF-β1 increases the extracellular levels of D-serine in neuronal and astrocyte cultures.* Extracellular levels of the neurotransmitter glutamate and the amino acid D-serine (B) were quantified by HPLC chromatography assays of neuronal and astrocyte conditioned medium. TGF-β1 raised the level of extracellular D-serine in either neuronal and astrocyte cultures. *p<0.001.

FIGURE 8. **TGF-β1 does not affect Serine racemase distribution and levels.** Cerebral cortex astrocytes and neurons were cultured in control medium (A-C and H-J) or in the presence of 10ng/mL of TGF-β1 (D-F and K-M). Serine racemase distribution and labeling intensity were evaluated by immunocytochemistry assays (B,E and I,L), and protein levels were analyzed by Western Blotting assays (G and N). There were no statistical differences in distribution, labeling intensity and protein levels of Serine racemase in astrocytes and neurons treated with TGF-β1 compared with control condition. Scale bar: 30µm.

FIGURE 9. **D-serine induces ultrastructurally organized and functional synapses.** Cerebral cortex neurons were cultured in the presence of Neurobasal medium alone, with 0.4 mM of D-serine alone, and simultaneously with D-serine and NMDA receptor inhibitors MK-801 and DCK (D-ser + MK; D-ser + DCK). After 12 days, the number of Synaptophysin/PSD-95 puncta was quantified (A-C) and levels of synaptic proteins were evaluated by Western Blotting assays (D). Synapse formation was also evaluated by quantitative electron microscopy (E-J) and electrophysiological assays (K-M). Number of synapses (I) and length of PSD density (J) were evaluated by electron microscopy. Arrowheads (E, G) and brackets (F, H) indicate postsynaptic density. In both cases, structurally normal synapses are observed. D-serine increased the number of synapses, although their ultrastructure seemed to be indistinguishable from control synapses. Scale bars, 20 µm for A, B; 0.5 µm for E, G; 0.2 µm for F and H; *p<0.001; **p<0.0001. **Electrophysiological effects of D-serine treatment (K-M).** Representative raw current traces illustrate differences in spontaneous activity recorded in the control and D-serine-treated group (K). The distributions of the amplitude and interval between events of the two groups were significantly different (*p<0.001; Kolmogorov-Smirnov test; n=5,644 and 6,127 events, from 25 and 21 neurons, respectively) (L). Averaged voltage-activated Na⁺ current traces obtained from five randomly chosen neurons in each condition illustrate the effect of TGF-β1 (*p<0.001 vs. control) (M).

FIGURE 10. **D-serine does not affect neuronal survival and neurite arborization.** Cerebral cortex neurons were cultured for 12 and 3 days in control medium (Control; A, E) or in the presence of 0.4 mM of D-serine (D-ser; B, F). Numbers of β-Tubulin III cells (C), total cells (D), total neurite length (G, H) or neurites (I) were obtained using the Image J software. There was no statistical difference between the groups. Scale bars, 50 µm (A); 30 µm (E).

FIGURE 11. **Synaptogenesis induced by TGF-β1 is mediated by D-serine.** Cerebral cortex neurons were cultured for 3 days in the presence of control medium (Control, A, F), 10 ng/mL of TGF-β1 (TGF-β1, B, F), simultaneously with TGF-β1 and the D-serine degradation enzyme, DAO (TGF-β1+DAO, C, F). For addressing DAO specificity, neurons were also simultaneously treated with...
TGF-β1 and heat-inactivated DAO (TGF-β1+hiDAO, D, F). Neurons were also transfected with plasmidial vectors containing two different shRNA constructions against Serine Racemase enzyme mRNA (shSR1 and shSR2), or with control constructions (LacZ) (H). Rescue assays were addressed by adding 0.4 mM of D-Serine to either DAO- or shSR-treated TGF-β neuronal cultures (F, H). Neurons were also cultured in the presence of DAO, hiDAO and shSR1 and shSR2 alone (F, H). DAO Synapses were evaluated by immunolabeling for Synaptophysin and PSD-95 followed by quantification (A-F, H). Serine Racemase knockdown levels were evaluated by Western blotting assays (G). Scale bar, 5 µm. *p<0.001; ***p<0.0001.
Figure 1
Figure 2
Figure 3

(A) Bar graph showing TGF-β1 levels (pg/mL) for NCM and ACM. The graph indicates a significant difference (**).

(B) Image of βTubulinIII/TGFβRII/DAPI immunostaining.

(C) Higher magnification of βTubulinIII/TGFβRII/DAPI staining in a neuron.

(D) Another view of βTubulinIII/TGFβRII/DAPI staining.
Figure 4
Figure 6

(A) Control  (B) TGF-β1

12 DIV

(C) T tubulin cells (% of total)

(D) DAPI

(E) Control  (F) TGF-β1

3 DIV

(G) Average of neurite length (μm)

(H) Average of the longest neurite (μm)

(I) Number of Neurons (% of total)

Control  TGF-β1

Bars 1, 2, 3, 4, 5 or +
Figure 7
### Figure 8

<table>
<thead>
<tr>
<th>Astrocyte</th>
<th>Neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><strong>TGF-β1</strong></td>
<td><strong>TGF-β1</strong></td>
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</tbody>
</table>

**GFAP**
- A: Control
- D: TGF-β1

**Ser Rac**
- B: Control
- E: TGF-β1

**merge**
- C: Control
- F: TGF-β1

**βTubulin**
- H: Control
- K: TGF-β1

**merge**
- J: Control
- M: TGF-β1

**Ser Rac**
- I: Control
- L: TGF-β1

**αTubulin**
- N: Control
- O: TGF-β1

**Ser Rac levels (relative to αTubulin)**
- Bar graph showing Ser Rac levels in control and TGF-β1 conditions.

**αTubulin**
- Bar graph showing αTubulin levels in control and TGF-β1 conditions.
Figure 9
Figure 10
Figure 11
Astrocyte-induced synaptogenesis is mediated by transforming growth factor beta signaling through modulation of D-serine levels in cerebral cortex neurons


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