A Critical Role for System A Amino Acid Transport in the Regulation of Dendritic Development by Brain-derived Neurotrophic Factor (BDNF)*

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Dendritic development is essential for the establishment of a functional nervous system. Among factors that control dendritic development, brain-derived neurotrophic factor (BDNF) has been shown to regulate dendritic length and complexity of cortical neurons. However, the cellular and molecular mechanisms that underlie these effects remain poorly understood. In this study, we examined the role of amino acid transport in mediating the effects of BDNF on dendritic development. We show that BDNF increases System A amino acid transport in cortical neurons by selective up-regulation of the sodium-coupled neutral amino acid transporter (SNAT)1. Up-regulation of SNAT1 expression and System A activity is required for the effects of BDNF on dendritic growth and branching of cortical neurons. Further analysis revealed that induction of SNAT1 expression and System A activity by BDNF is necessary in particular to enhance synthesis of tissue-type plasminogen activator, a protein that we demonstrate to be essential for the effects of BDNF on cortical dendritic morphology. Together, these data reveal that stimulation of neuronal differentiation by BDNF requires the up-regulation of SNAT1 expression and System A amino acid transport to meet the increased metabolic demand associated with the enhancement of dendritic growth and branching.

Development of the nervous system proceeds through a sequence of complex ontogenetic processes that includes cell proliferation, migration, neurite outgrowth, axon guidance, and synapse formation (1). Neuronal development is determined by both intrinsic and extrinsic factors. Among the latter, neurotrophic factors play a key role. In particular, BDNF, a member of the neurotrophin family, controls the survival and differentiation of specific neuronal populations in the peripheral and central nervous system (2). In the developing visual cortex, BDNF has been shown to regulate the dendritic growth and complexity of pyramidal neurons (3, 4). Furthermore, over-expression of BDNF in pyramidal neurons of the visual cortex leads to sprouting of basal dendrites and regression of dendritic spines (5). Because dendritic morphology determines the number, pattern, and types of synapses received by a neuron, regulation of cortical dendritic growth and branching by BDNF is likely to play a major role for the proper functioning of the brain and especially the cerebral cortex.

Although BDNF regulates cortical dendritic development, little is known about the cellular and molecular mechanisms underlying these effects. In particular, the role of amino acid transport in mediating the effects of BDNF on dendritic growth and branching remains unknown.

System A is a ubiquitous amino acid transport system that mediates the Na+-dependent transport of short-chain neutral amino acids such as alanine, serine, and glutamine (6). In addition, System A is the major amino acid transport system subject to regulation by environmental conditions, proliferative stimuli, developmental changes, hormones, and growth factors (6). During gestation, inhibition of System A-mediated amino acid transport is associated with intrauterine growth retardation (7). Recently, three isoforms of the System A family of transporters have been cloned and termed sodium-coupled neutral amino acid transporter (SNAT)1, SNAT2, and SNAT4 (8). SNAT1 is found primarily in the brain, SNAT2 is expressed ubiquitously in mammalian tissues, and SNAT4 is restricted almost exclusively to the liver (8). A role has been proposed for SNAT1 in supplying glutamatergic and GABAergic neurons with glutamine, the preferred precursor for the synthesis of the neurotransmitters glutamate and GABA (9, 10). However, recent data have revealed that SNAT1 is not observed in axon terminals but is localized almost exclusively to the somatodendritic compartment of glutamatergic and GABAergic neurons (11), indicating that the physiological role of SNAT1 in the brain remains controversial.

In the present study, we examined the role of System A and SNAT1 in the regulation of cortical dendritic development by BDNF. We show that stimulation of cortical neurons by BDNF up-regulates SNAT1 expression and System A amino acid transport. Induction of SNAT1 expression and System A activity is required for the increase in dendritic length and branching.
of cortical neurons in response to BDNF. We also provide evidence that up-regulation of SNAT1 expression and System A activity by BDNF is necessary to enhance levels of tissue-type plasminogen activator (tPA), a protease whose secretion is essential for the effects of BDNF on dendritic morphology.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All experiments were performed in accordance with the European Communities Council Directive regarding the care and use of animals for experimental procedures. Primary cultures of cerebral cortical neurons were prepared from 17-day-old Swiss mouse embryos as described previously (12). If not otherwise indicated, cells were plated at a density of 55,000/cm² on culture plates or glass coverslips in Neurobasal medium supplemented with B27 (Invitrogen, Basel, Switzerland) and 500 µM glutamine. Human embryonic kidney cells (HEK293; Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 25 mM glucose, 0.1 mM nonessential amino acids (Invitrogen), 50 units/ml penicillin, 50 µg/ml streptomycin and kept at 37 °C in a humidified atmosphere at 95% air, 5% CO₂.

**Dendritic Analysis**—Cortical neurons were grown at a density of 1400 cells/cm² to allow morphological analysis of single neurons. After 3 days in culture, cortical neurons were exposed to 10 ng/ml BDNF with or without 20 mM MeAIB or 2 µM tPA stop and immunostained for MAP2. Neurons were traced in a blind manner using a microscope equipped with ×63 and ×100 objectives, epifluorescence, a Lucid camera system (MicroBrightField Inc., Williston, VT), and the Neurolucida software (MicroBrightField Inc.). As Sholl analysis of preliminary studies revealed that the effects of BDNF on dendritic length and branching were maximal within a radius of 80 µm from the cell body (not shown), data presented in this study include analysis of dendritic morphology within this radius. When the effects of SNAT1 siRNA and control (Ctrl) siRNA were tested, cortical neurons were cotransfected 2 days after plating with the enhanced green fluorescent protein (EGFP) expression vector pEGFP (0.1 µg/well; Clontech, Basel, Switzerland) together with SNAT1 siRNA or Ctrl siRNA (0.5 µg/well). One day after transfection, neurons were treated for 24 h with 10 ng/ml BDNF in Neurobasal/B27 medium and fixed with 4% paraformaldehyde. EGFP-positive neurons were traced and analyzed using Neurolucida software.

**RT-PCR and DNA Constructs**—4 µg of total RNA extracted from cultured cortical neurons using TRIzol reagent (Invitrogen) were reverse-transcribed with oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. SNAT1, SNAT2, and SNAT4 cDNA fragments were PCR-amplified using the following primers: SNAT1 forward 5'-CACGCTATGGGAAAGAGG-3' and reverse 5'-GGTCTTCAAGAGACACGC-3'; SNAT2 forward 5'-CGAACATGTTGGGACATAAGG-3' and reverse 5'-CTCTGACGCTTATCACTCC-3'; SNAT4 forward 5'-GGGGTACCCATTACGG-3' and reverse 5'-ACACAAAATAGACGAGG-3'. The SNAT1 and SNAT2 PCR products were T/A-cloned into pGEM-T Easy vector (Promega, Wallisellen, Switzerland) and used to produce riboprobes for Northern blots. To generate hemagglutinin (HA)-epitope tagged SNAT1 (HA-SNAT1) plasmid, the complete coding sequence of SNAT1 was amplified by RT-PCR using the following primers (SNAT1 forward 5'-GGGGTACCCATTACGG-3' and reverse 5'-GGGAATTCTCAGTGCCCTTCGTTGCCTCCAA-3') and then cloned into the KpnI and EcoRI sites of pcDNA3 (Invitrogen) containing a 5' HA tag.

**Small Interfering (si) RNAs**—SNAT1 and control (no significant sequence homology to any known mammalian gene) 21-nucleotide duplexes were designed and synthesized by Qiagen (Basel, Switzerland). The target sequence for SNAT1 siRNA was 5'-AACGAACTTTCTGAGCATATA-3', corresponding to nucleotides 511–531 of the mouse SNAT1 cDNA sequence.

**Transfection**—Transfection of plasmid as well as cotransfection of plasmid with siRNA were performed using the Transfectin reagent (1 µl/well in 24 well plates; Bio-Rad, Glattbrugg, Switzerland) according to the manufacturer's guidelines. When siRNA duplexes (2.5 µg/35 × 10-mm plate) were transfected alone, GeneSilencer reagent (Gene Therapy Systems, Axon Lab, Baden-Dättwil, Switzerland) was used following the manufacturer's manual.

**Immunocytochemistry**—Immunostaining was performed as described previously (13). Cortical neurons were fixed for 20 min in 4% paraformaldehyde except for double-labeling experiments with GABA and MAP2 antibodies where cortical neurons were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde. Mouse anti-MAP2 antibody (Sigma, Basel, Switzerland), rabbit anti-SNAT1 antibody (J. D. Erickson), rabbit anti-GABA (Sigma), and CY3-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, La Roche, Switzerland) were used at a final dilution of 1/100, 1/2000, 1/15,000, and 1/500, respectively. Glass coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

[^14C]-(Methylamino)isobutyric Acid ([14C]MeAIB) and[^3H]Glutamine Uptake—After 3–6 days in vitro, cortical neurons grown on 35 × 10-mm plates were treated with 10 ng/ml BDNF in HEPES buffer (in mM: 10 HEPES, pH 7.0; 140 NaCl; 7.5 NaHCO₃; 2 KCl; 2 CaCl₂; 3 MgSO₄; 2 KH₂PO₄; 5 glucose). At the end of the stimulation, uptake was performed for 20 or 3 min at 37 °C in the same medium containing either 0.5 µCi/ml [14C]MeAIB (specific activity, 51.5 mCi/mmol; PerkinElmer Life Sciences, Geneva, Switzerland) or 2 µCi/ml [3H]glutamine (specific activity, 49 Ci/mmol; GE Healthcare, Otelfingen, Switzerland), respectively. For [3H]glutamine uptake, the HEPES buffer also included 50 µM unlabeled glutamine and 20 mM 2-aminoisobutyric acid (2, 2, 1-heptane-2-carboxylic acid) to inhibit glutamine uptake mediated by System L. Indeed, [3H]glutamine uptake by cortical neurons was primarily mediated by System L (66.3 ± 1.1% of total [3H]glutamine uptake, n = 6), whereas contribution by System A (MeAIB-sensitive uptake) was less than 30% (27.2 ± 1.1% of total [3H]glutamine uptake, n = 6). The uptake was terminated by three washes with ice-cold phosphate-buffered saline, and cells were lysed in 2 ml of 10 mM NaOH; 0.1% Triton X-100. Aliquots of 500 µl were assayed for radioactivity by liquid scintillation counting, and protein con-
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tent was determined by the BCA protein assay kit (Pierce, Lausanne, Switzerland). For kinetic analysis, [14C]MeAIB uptake was measured in HEPES buffer containing increasing concentrations of unlabeled MeAIB. For each MeAIB concentration, [14C]MeAIB was adjusted to keep the [14C]MeAIB/MeAIB concentration ratio constant. Nonspecific tracer binding was determined by measuring cell-associated radioactivity in the presence of an excess of unlabeled MeAIB (20 μM) or glutamine (10 μM). Nonspecific binding to cells accounted for 8.47 ± 1% of total [14C]MeAIB uptake (n = 15 from five independent experiments) and for 12 ± 1.2% of total [3H]glutamine uptake (n = 10 from three independent experiments). Nonspecific uptake was subtracted from total uptake to yield specific uptake values.

**LDH Release**—LDH release into the culture supernatant was quantified using a sensitive detection kit (CytoTox Detection kitPLUS (LDH), Roche Diagnostics, Mannheim, Germany).

**MTT Reduction**—Measurement of cellular MTT reduction was performed as described (14).

**Protein Synthesis**—Analysis of protein synthesis was performed as described previously (13).

**Zymography**—tPA activity was assayed by zymography as described previously (12). Bands of caseinolysis at ~68 kDa corresponding to the molecular weight of tPA were analyzed using ScionImage Software, version Beta 4.0.2 (Scion Corp., Frederick, MD).

**Northern Blotting**—Northern blots were carried out using a previously described procedure (12). 32P antisense SNAT1 and SNAT2 riboprobes were generated by *in vitro* transcription of a linearized pGEM-T Easy vector containing the corresponding cDNA fragment (see “RT-PCR and DNA Constructs”).

**Western Blotting**—Western blots were performed as described previously (13). For immunodetection of SNAT1, SNAT2, hemagglutinin, and β-tubulin, blots were incubated overnight at 4 °C with an antibody against SNAT1 (1/2000; J. D. Erickson), SNAT2 (1/2000; J. D. Erickson), hemagglutinin (1/1000; Cell Signaling Technology, Bioconcept, Allschwil, Switzerland), or β-tubulin (1/1000; Sigma), respectively.

**Statistical Analysis**—Data were analyzed for statistical significance by using one-way analysis of variance followed by Bonferroni *post hoc* test except for time course analysis of MeAIB uptake where one-way analysis of variance was followed by Dunnett *post hoc* test.

**RESULTS**

**Regulation of System A Amino Acid Transport by BDNF**—System A can be distinguished from the other neutral amino acid transport systems by its ability to transport N-methylated amino acids such as the nonmetabolizable amino acid analog MeAIB (15). Regulation of System A amino acid transport activity by BDNF was examined by measuring MeAIB uptake. We found that treatment of cortical neurons with BDNF caused a marked increase in MeAIB uptake (Fig. 1). Time course analysis revealed that MeAIB uptake enhanced significantly after 20 min and reached maximal levels (179.4 ± 7.6% of control) after 5 h of stimulation by BDNF (Fig. 1A). These data indicate that BDNF increases System A activity in cortical neurons. In neurons, System A plays an important role in the Na+/H+ exchange-reciprocal plots of the results presented in C. transport of glutamine, the primary precursor for the synthesis of the neurotransmitters glutamate and GABA (16). This led us to examine the effect of BDNF on glutamine transport, and we found that BDNF also markedly increased glutamine uptake by cortical neurons (Fig. 1B). Regulation of System A activity by BDNF was further characterized by performing kinetic analysis of MeAIB uptake in control and BDNF-treated cortical neurons. The rates of MeAIB uptake in control and BDNF-treated cultures exhibited Michaelis-Menten kinetics (Fig. 1C). Double-reciprocal plots of MeAIB uptake rate versus extracellular MeAIB concentration revealed that the Vmax of MeAIB uptake increased significantly from 10.5 ± 0.9 to 18.3 ± 2.5 pmol/mg of protein/min when cortical neurons were exposed to BDNF (Fig. 1D). In contrast, no significant effect was observed on the Km value as Km was 447.4 ± 14.2 μM in control neurons and 410.5 ± 65.6 μM in BDNF-treated neurons (Fig. 1D). The increased Vmax value of MeAIB uptake in response to BDNF provides evidence that BDNF enhances the number of functional System A transporters in cortical neurons. Because we have previously shown that treatment of cortical neurons with...
BDNF increases overall protein synthesis (13), we examined the role of System A amino acid transport in BDNF-induced protein synthesis. Treatment of cortical neurons with an excess of MeAIB to competitively inhibit System A amino acid transport caused a complete suppression of BDNF-induced protein synthesis (Ctrl = 100 ± 2%, BDNF = 129.8 ± 2.1%, BDNF + MeAIB = 92.45 ± 1.99%, n = 12). These data indicate that up-regulation of System A amino acid transport is necessary for the increased protein synthesis by BDNF.

Up-regulation of System A Amino Acid Transport Is Required for the Regulation of Dendritic Morphology by BDNF—Because BDNF regulates dendritic development of cortical neurons (3, 4, 17), we investigated whether up-regulation of System A transport contributes to the effects of BDNF on the dendritic morphology of cortical neurons. To address this issue, cortical neurons were stimulated by BDNF in the presence of the amino acid analog MeAIB, and the dendritic morphology was analyzed. When compared with untreated cultures, neurons exposed to BDNF exhibited an increased number of branch points (Fig. 2, A and B). In marked contrast, treatment of cortical neurons with MeAIB completely suppressed the effect of BDNF on dendritic branching (Fig. 2, A and B), indicating that stimulation of System A amino acid transport is required for the regulation of cortical dendritic morphology by BDNF. Suppression of BDNF-induced dendritic development by MeAIB did not result from a decrease in neuronal viability, as determined by LDH release and MTT reduction (Fig. 3, A and B). Further analysis of the regulation of dendritic branching by BDNF showed that BDNF increased the number of branch points in both GABAergic (21.5% of the total neuronal population) and non-GABAergic neurons, although the effect of BDNF was more pronounced in non-GABAergic cells (Table 1). In addition, inhibition of System A amino acid transport by MeAIB suppressed BDNF-induced dendritic branching in both populations of cortical neurons (Table 1).

**BDNF Increases SNAT1 Expression**—To investigate further the role of System A in the regulation of neuronal differentiation by BDNF, we examined the System A amino acid transporter isoform involved in the effects of BDNF on the dendritic development of cortical neurons. In agreement with the tissue distribution of SNAT isoforms (8), RT-PCR analysis revealed that SNAT1 and SNAT2 mRNAs were expressed in cultured cortical neurons, whereas SNAT4 mRNA was not detected (Fig. 4A). On the basis of these findings, we tested the effect of BDNF on the expression of SNAT1 and SNAT2. Treatment of cortical neurons with BDNF caused an up-regulation of SNAT1 mRNA expression, whereas SNAT2 mRNA levels remained unchanged (Fig. 4, B and C). Consistent with increases in SNAT1 mRNA levels, we found that BDNF enhanced SNAT1...
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**FIGURE 4.** BDNF increases SNAT1 mRNA and protein expression. A, RT-PCR analysis of SNAT1, SNAT2, and SNAT4 mRNAs in cultured cortical neurons. The same experiment was repeated in the absence of reverse transcriptase (−RT). B, Northern blot analysis of SNAT1 and SNAT2 mRNAs following stimulation of cortical neurons with 10 ng/ml BDNF for various periods of time. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, Quantitative analysis. Data are the mean ± S.E. percentages of Ctrl values from at least five independent experiments. D, Western blot analysis of SNAT1 and SNAT2 protein expression following stimulation of cortical neurons by 10 ng/ml BDNF for 7 h. E, Quantitative analysis. Densitometric values for SNAT1 and SNAT2 proteins were normalized to corresponding β-tubulin values and expressed as the mean ± S.E. percentages of Ctrl values from at least six independent experiments.

**FIGURE 5.** Inhibition of SNAT1 expression by siRNA. A, Western blot analysis of HEK293 cells cotransfected with HA-SNAT1 plasmid together with Ctrl siRNA or SNAT1 siRNA. B, Western blot analysis of SNAT1 protein levels in cortical neurons transfected with Ctrl siRNA or SNAT1 siRNA and stimulated or not with 10 ng/ml BDNF for 7 h. C, Quantitative analysis. Densitometric values for SNAT1 protein were normalized to corresponding β-tubulin values and expressed as the mean ± S.E. percentages of Ctrl values from five independent experiments.

Among proteins whose expression is induced by BDNF in developing cortical neurons, we have shown previously that BDNF increases the expression and secretion of tPA (12), a serine protease that regulates neurite outgrowth, neuronal migration, and synaptic plasticity (18–20). Because there is evidence that tPA can regulate neurite outgrowth and sprouting, we examined the role of BDNF-induced tPA secretion in the regulation of dendritic morphology. To address this issue, we tested the effect of 2,7-bis-(4-amidinobenzylidoxy)-cycloheptan-1-one (tPA stop), a bis-benzamidine derivative that
inhibits single- and two-chain forms of tPA (21), on the increased dendritic length and branching elicited by BDNF. Treatment of cortical neurons with tPA stop suppressed BDNF-induced dendritic length and branching (Fig. 7), indicating that secretion of tPA is required for the regulation of cortical dendritic development by BDNF.

Role of System A and SNAT1 in the Up-regulation of tPA Levels by BDNF—On the basis of these observations, we determined whether stimulation of System A activity by BDNF was necessary for the elevation of tPA levels by BDNF. Zymographic analysis revealed that treatment of cortical neurons with MeAIB markedly reduced (65% inhibition) increases in tPA activity levels by BDNF (Fig. 8, A and B). These results indicate that up-regulation of System A amino acid transport plays a critical role in the enhancement of tPA levels by BDNF. We next examined whether induction of SNAT1 expression contributes to increases in tPA levels by BDNF. In agreement with the marked reduction of BDNF-induced tPA levels by MeAIB, treatment of cortical neurons with SNAT1 siRNA strongly reduced (69% inhibition) the increased tPA activity by BDNF (Fig. 8, C and D), providing evidence that induction of SNAT1 expression is essential for the enhancement of tPA levels by BDNF.

DISCUSSION

Dendritic development is guided by an intrinsic growth program that is capable of generating a basic dendritic arborization as well as by several extracellular factors such as neuronal activity, growth factors, and guidance molecules that are essential for sculpting dendrites to their final form (22–24). Of these extracellular factors, BDNF plays an important role in regulating the growth and branching of cortical dendrites (3, 4). Regulation of dendritic growth by BDNF is thought to require the activation of a transcriptional program and new protein synthesis (22, 24). Consistent with this hypothesis, BDNF increases both overall and local protein synthesis in cortical neurons (13, 25, 26). In this study, we examined the role of amino acid transport in the regulation of cortical dendritic development by BDNF. We show that BDNF increases amino acid transport mediated by System A (Fig. 1A), the major system responsible for the transport of short-chain neutral amino acids. Kinetic analysis of MeAIB uptake revealed that stimulation of System A...
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Among proteins underlying dendritic development, there is evidence that the serine protease tPA is secreted from growing neurites and promotes neurite outgrowth (18, 20). With regard to the regulation of tPA, we have shown previously that BDNF enhances the expression and release of tPA in developing cortical neurons (12). Here, we provide strong evidence that increases in tPA levels by BDNF require up-regulation of SNAT1 expression and System A amino acid transport. In support of this observation, the enhancement of tPA levels by BDNF is strongly reduced by suppressing BDNF-induced SNAT1 expression or System A activity (Fig. 8). Our results also demonstrate that tPA stop blocks the increased dendritic length and branching of cortical neurons by BDNF (Fig. 7), indicating that secretion of tPA by BDNF is essential for the effects of BDNF on dendritic development. Together, these data provide an example of a protein whose induction by BDNF requires the up-regulation of SNAT1 expression and System A activity and whose secretion plays a critical role in the regulation of cortical dendritic development by BDNF.

In conclusion, our data indicate that promotion of neuronal differentiation by BDNF requires the up-regulation of SNAT1 expression and System A amino acid transport to ensure an adequate substrate supply for the increased synthesis of proteins necessary for the enhancement of dendritic growth and branching. These results underscore the importance of the regulation of amino acid transport in the control of dendritic development.
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