Differential Cellular Expression of Neurotrophins in Cortical Tubers of the Tuberous Sclerosis Complex

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Neurotrophins and their receptors modulate cerebral cortical development. Tubers in the tuberous sclerosis complex (TSC) are characterized histologically by disorganized cortical cytoarchitecture and thus, we hypothesized that expression of neurotrophin mRNAs and proteins might be altered in tubers. Using *in situ* transcription and mRNA amplification to probe cDNA arrays, we found that neurotrophin-3 (NT3) and trkB mRNA expression were reduced whereas neurotrophin-4 (NT4) and trkC mRNA expression were increased in whole tuber sections. Alterations in mRNA abundance were defined in single microdissected dysplastic neurons (DNs) and giant cells (GCs). NT3 mRNA expression was reduced in GCs and trkB mRNA expression was reduced in DNs. NT4 mRNA expression was increased in DNs and trkC mRNA expression was increased in both DNs and GCs. In three patients, *TSC2* locus mutations were confirmed and the mean tuberin mRNA expression levels was reduced across all nine cases. Consistent with these observations, NT3 mRNA expression was reduced but trkC mRNA expression was increased *in vitro* in human NTera2 neurons (NT2N) transfected with a tuberin antisense construct that reduced tuberin expression. Western analysis of tuber homogenates and computer-assisted densitometry of immunolabeled sections confirmed the neurotrophin mRNA expression data in whole sections and single neurotrophin immunoreactive cells. We conclude that alterations in NT4/trkB and NT3/trkC expression may contribute to tuber formation during brain development as downstream effects of the hamartin and tuberin pathway in TSC. (*Am J Pathol* 2001, 159:1541–1554)

Tubers in the tuberous sclerosis complex (TSC) are developmental abnormalities of cerebral cortical cytoarchitecture that are associated clinically with epilepsy.^{1–3} Electrocorticography has shown that tubers are epileptogenic and seizures in TSC patients are often medically intractable despite anticonvulsant polytherapy.^{4–6} TSC is an autosomal-dominant disorder resulting from mutations in one of two genes, *TSC1* or *TSC2*.^{7,8} although the mechanism by which mutations in either TSC gene leads to tuber formation is unknown. Disorganized cortical lamination and aberrant cellular morphology are important histological features of tubers. Large dysplastic neurons (DNs) and giant cells (GCs) are prominent cell types in tubers.⁹ DNs and GCs share select morphological features including cytomegaly, the extension of aberrant processes often of unclear identity, ie, axons *versus* dendrites, and the expression of neural protein markers such as neurofilament and α -internexin.^{1,9,10}

The *TSC2* knockout mouse¹¹ and the Eker rat strain¹² do not fully model human brain pathology in TSC, and thus, analysis of human tuber specimens provides the only direct avenue to study the mechanisms of tuber formation. One strategy to investigate the molecular pathogenesis of cytoarchitectural disorganization in tubers is to evaluate the expression of candidate genes and proteins in human tuber specimens that are relevant to cortical development.¹⁰ Neurotrophins and their cognate receptors comprise a family of proteins that mediate proliferation, differentiation, migration, and process outgrowth during cortical development,^{13,14} and thus, are ideal candidate molecules to investigate in tubers. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) exert their effects on neurons by binding selectively to a family of neuronal cell membrane receptors, trks A to C.^{13,14} NGF signals through trkA, BDNF and NT4 through trkB, and NT3 through trkC. These neurotrophins and their receptors are expressed throughout cortical development and likely contribute to the organized formation of cortical laminae.¹⁵ An additional protein, ciliary neurotrophic factor (CNTF), is enriched primarily in the peripheral

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Table 1. Patient Characteristics

Age/sex	Seizure type	Tuber location	EEG/EcoG	Surgical outcome
Surgical TSC cases				
7/F	cps, gtc	Rt DLF cortex	Rt DLF spikes	Reduced seizures 1 year after surgery
20/F	cps, gtc	Lt Temp cortex	Lt Temp spikes	Seizure free at 2 years after surgery
17/M	cps	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 2 years after surgery
4/M	gtc	Rt DLF cortex	Rt DLF spikes	Reduced seizures at 6.5 years after surgery
1/M	cps	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 4 years after surgery
18/F	gtc	Rt Temp cortex	Rt Temp spikes	Reduced seizures at 3 years after surgery
3/M	gtc, cps	Rt DLF cortex	Rt DLF spikes	Reduced seizures at 4 years after surgery
Post-Mortem TSC cases				
24/F	cps	DLF		
46/F	cps	DLF		

Abbreviations: cps, complex partial seizure; gtc, generalized tonic-clonic seizure; DLF, dorsolateral frontal; Temp, temporal; Rt, right; Lt, left; M, male; F, female; EcoG, electrocorticography.

nervous system and binds selectively to the CNTF receptor (CNTFR).¹⁶ BDNF and NT3 regulate neurogenesis and contribute to differentiation of neuronal progenitor cells within the telencephalic ventricular zone via interaction with trkB and trkC. NT3 and NT4 are critical for dendritic arborization¹⁷ and for axonal pathfinding during corticogenesis. Exposure of developing cortex to excess neurotrophins disrupts cortical lamination *in vitro*¹⁸ and recent evidence suggests that several of these proteins may contribute to epileptogenesis.^{19,20}

Only one study to date has reported expression of trkA and trkB proteins in human cortical dysplasia not associated with TSC²¹ and there has been no investigation of these mRNAs or proteins in TSC. In view of the disorganized cytoarchitecture observed in tubers, we hypothesized that expression of select neurotrophins would be altered in tubers and that these changes may be defined in select cell types. We determined the abundance BDNF, CNTF, CNTFR, NT3, NT4, NGF, trkA, trkB, and trkC mRNAs as well as the chemoattractants netrin1 and netrin2 mRNAs in whole tuber sections resected from TSC patients with medically intractable epilepsy. Netrin1 and netrin2, although not members of neurotrophin family per se, play important roles in axon growth and cell migration, and thus, are also relevant to cortical development.²² We then determined the expression of these mRNAs in single DNs and GCs microdissected from tubers in an attempt to define the cellular specificity of these mRNA changes. Although the phenotypic distinctions between these cells remain a source of debate, we attempted to devise a strategy to classify these cells based on size and morphology. Neurotrophin mRNA expression was also evaluated in human NTera2 neurons (NT2N),²³ a human neuronal cell line that expresses p75NGFR, trkA, trkB, and trkC mRNAs.²⁴ These neurons were examined in their native state and after stable transfection with a tuberin antisense construct that reduced tuberin expression. We were specifically interested in

tuberin because overall TSC severity, tuber formation, and epilepsy are likely more severe in TSC2- than TSC1-associated cases.²⁵ Finally, we used Western blotting and quantitative immunohistochemistry with computer-assisted image analysis to demonstrate that observed alterations in neurotrophin mRNA expression predicted changes in protein expression in tubers.

Materials and Methods

Patient Selection and Tissue Samples

Tubers were obtained intraoperatively from seven patients with clinically and radiographically defined TSC²⁶ and medically intractable complex partial epilepsy (Table 1). Tubers were resected from the dorsolateral prefrontal cortex in three patients and the temporal neocortex in four patients. Tubers were identified on preoperative brain MRI and were targeted for surgical resection based on scalp electroencephalography or intracranial electrocorticography that revealed epileptiform discharges such as spikes, sharp waves, or seizures. The histopathological diagnosis was confirmed in these specimens by a neuropathologist (Figure 1). Genotype analysis on four patients was obtained retrospectively and revealed that one case resulted from a mutation in the TSC1 locus whereas three cases resulted from mutations in the TSC2 locus.

Two tubers and adjacent nontuberal dorsolateral frontal cortex was obtained at postmortem examination from two female patients (ages 24 and 46 years) with TSC.²⁶ The postmortem interval to autopsy was 14 ± 4 hours. Both patients had a history of epilepsy. The causes of death were respiratory failure from pulmonary lymphangiomyomatosis and myocardial infarction. Histologically, GCs and DNs were not present in the nontuberal

Figure 1. Tuber specimen immunolabeled with NeuN antibody. Note disorganized lamination. GCs (**large arrows**) exhibit cytomegaly, a laterally displaced nucleus, and short processes of unclear identity. DN's (**small arrows**) also exhibit a dysmorphic cell soma and disorganized orientation with respect to the pial surface (**top**). Scale bar, 100 μ m.

cortex and the lamination pattern was morphologically similar to control cortex.

Control temporal neocortex was obtained postmortem from four age-matched patients (two females, two males; age, 6 to 36 years; mean age, 22 years). All died of nonneurological causes. Their average postmortem interval to autopsy was 10 ± 3 hours. Seizures were not among the terminal events in these patients and none had a history of epilepsy or TSC. Histological evaluation of these specimens showed no abnormality.

All patients or appropriate family members consented to the use of resected or necropsy material in accordance with the University of Pennsylvania Institutional Review Board and Committee on Human Research.

Tissue Processing and Immunohistochemistry

Brain samples were either fixed by immersion in ice-cold 70% ethanol/150 mmol/L NaCl or 4% paraformaldehyde because previous work²⁷ suggested that rapid cold fixation of human brain specimens yields consistent immunohistochemical results with neurotrophin antibodies. Postmortem specimens were flash-frozen on dry ice at -70°C . Fixed specimens were embedded in paraffin, sectioned at 7 μ m, and mounted on coated slides.

Fixed sections were rehydrated through xylenes and graded ethanols. Sections were immersed in a 150-ml methanol/30 ml H_2O_2 (30%) solution for 30 minutes and then rinsed in cold tap water for 10 minutes. Sections were washed in a 0.1 mol/L Tris (pH 7.4) solution for 5 minutes and then in a Tris/2% fetal bovine serum solution for another 5 minutes. All tuber and control sections were probed with one of several antibodies. In the first experiments, sections were labeled with a mouse monoclonal antibody that recognizes the DNA binding protein NeuN (1:500 dilution; Chemicon, Temecula, CA), a pan-neuronal marker²⁸ that permits reliable identification of neurons in fixed, paraffin-embedded sections before microdissection (see below). In additional experiments, rabbit polyclonal antibodies recognizing BDNF (1:100 dilution),

NT3 (1:100), NT4 (1:100; Santa Cruz Biotechnology, Burlingame, CA), trkB (1:50; Chemicon), or trkC (1:50; Oncogene, Cambridge, MA) were used to probe tuber and control sections. Primary antibody labeling for all antibodies was performed overnight at 4°C . Immunolabeling was visualized using the avidin-biotin conjugation method (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine. NeuN-labeled sections were stored in $2\times$ standard saline citrate buffer awaiting single cell microdissection while the neurotrophin-labeled sections were mounted with Permount (Fisher Scientific, Pittsburgh, PA) for morphometric and densitometric analysis.

In Situ Transcription

The synthesis of a radiolabeled mRNA to probe cDNA arrays begins with generation of cDNA directly on the fixed tissue section by *in situ* (reverse) transcription.²⁹ After NeuN immunolabeling, tissue sections selected for mRNA analysis were treated with Proteinase K (50 $\mu\text{g}/\text{ml}$) at 37°C for 30 minutes and then washed in diethyl pyrocarbonate-treated water. To initiate *in situ* transcription, an oligo-dT₂₄ primer coupled to a T7 RNA polymerase promoter was annealed to cellular poly(A) mRNA directly on the tissue section overnight at room temperature. Sections were then washed in $2\times$ standard saline citrate buffer and cDNA was synthesized on the section in *in situ* transcription reaction buffer (10 mmol/L HEPES buffer, pH 7.4, 120 mmol/L KCl, 1 mmol/L MgCl_2 , 250 $\mu\text{mol}/\text{L}$ dATP, dCTP, dGTP, TTP) with avian myeloblastosis reverse transcriptase (0.5 U/ μl ; Seikagaku America, Falmouth, MA). cDNA generated from cellular poly(A) mRNA was extracted from sections with NaOH, 0.1% sodium dodecyl sulfate, and 5 mol/L KAc followed by ethanol precipitation. cDNA from the whole sections was later processed for mRNA amplification and synthesis of a radiolabeled RNA probe.²⁹

NT2N Neuronal Cultures

The NTera 2 cell line is available through the American Type Culture Collection (NTera 2 C1.D1, ATCC CRL-1973; Rockville, MD). NTera 2 human teratocarcinoma cells were differentiated into postmitotic neurons (NT2N) as previously described.²³ Differentiation was induced by culturing cells (3.2×10^3 cells/ cm^2) in the presence of 10 $\mu\text{mol}/\text{L}$ all-trans retinoic acid for 5 weeks followed by replating at 1:6 density without retinoic acid in the presence of mitotic inhibitors (10 $\mu\text{mol}/\text{L}$ 5-fluoro-2'-deoxyuridine, 10 $\mu\text{mol}/\text{L}$ uridine, 1 $\mu\text{mol}/\text{L}$ cytosine- β -D-arabinose furanoside). One week later, a final replating was done using culture vessels coated with Matrigel (Collaborative Biomedical Products, Bedford, MA), and cells were maintained in the presence of mitotic inhibitors for an additional 2 to 3 weeks. The differentiated cultures contain >95% postmitotic neurons, with <5% remaining undifferentiated cells as previously demonstrated by immunofluorescent labeling with anti-MAP-2 antibodies.²³

Generation of Antisense Constructs and Establishment of Transfected NT2N Lines

Three distinct tuberin constructs were generated and subcloned in either the sense (S) or antisense (AS) direction into pcDNA3.1 plasmid vector following a CMV promoter. One construct (5') contains nucleotides 1 to 606 of the full-length tuberin molecule whereas the M and G (GAP domain) constructs contain nucleotides 1902 to 2592 and 4351 to 5347, respectively. The efficacy of the antisense constructs in reducing tuberin expression was first assayed in C6 rat glioma cells 48 hours after lipofectamine-mediated transfection by Western analysis (see below) using rabbit polyclonal tuberin (1:1000 dilution, C-20; Santa Cruz Biotechnology) or mouse monoclonal tubulin (1:5000 dilution, DM1-A; Sigma Chemical Co., St. Louis, MO).

Undifferentiated NT2 cells (4×10^5 cells) were transfected (Calcium Phosphate Transfection Kit; 5 prime->3 prime, Inc., Boulder, CO) with 10 μ g of S- or AS-tuberin expression plasmids for 16 hours, followed by incubation of cultures in medium containing 300 μ g/ml active G418 (Life Technologies, Inc., Gaithersburg, MD). After 10 days, control cells transfected with an irrelevant plasmid had no surviving cells, whereas transfected cell wells showed resistant colonies. NT2N were kept under selection for at least 6 weeks before utilization, to ensure establishment of stable transfectants. These cells were then differentiated into postmitotic neurons (see above).

Microdissection and Aspiration of Single Neurons from Human Specimens and NT2N

The phenotypic distinctions between GCs and DNPs is a source of debate and indeed, they may reflect a continuum of cell types rather than clearly separate neurobiological cell categories because there are no well-characterized markers to identify either cell type. However, because GCs and DNPs do exhibit morphological and pharmacological differences,^{1,2,9,10} we attempted to more fully evaluate neurotrophin gene expression changes in these cells. For the purposes of single-cell gene and protein expression analysis, GCs and DNPs were defined using morphometric parameters (cell diameter, length, and width) to divide them into groups for analysis. Specifically, using computer-assisted image analysis, we determined the area of >20,000 individual labeled cell bodies in nine tuber specimens. The diameter of the GCs exceeded 80 μ m and GCs exhibited little polarization into axonal or dendritic segments. DNPs were smaller (40 to 70 μ m) and exhibited dendrites and axons that were characteristic of neurons. DNPs also exhibited disorganized orientation with respect to the pial surface. Astrocytes were small (<30 μ m in size) and were excluded from the groups. Cellular morphology was visually corroborated with somatic area and parceled cells into three broad groups. Cells whose area was among the largest 20% of recorded size and that exhibited the most aberrant cell

morphology were deemed GCs. The next largest cell types (the second 20% in cell area size) exhibited features more consistent with neurons and were deemed DNPs. These strict criteria may have actually excluded specific cell types, for example, excessively large DNPs or more diminutive GCs, yet they provided a point of reference to initiate these types of analyses. Before final assignment into GC or DP groups, each 4mm² region of interest (ROI) was visually inspected and any cellular elements erroneously included in the computerized analysis were deleted. In the control sections, all neurotrophin immunoreactive neurons within the targeted 4-mm² ROIs were selected for density analysis. Axons, dendrites, or blood vessels in tuber and control specimens were excluded using maximum-minimum length, width, and area parameters.

Single NeuN-immunolabeled GCs, DNPs, or control pyramidal neurons selected from cerebral cortical layer V were microdissected ($n = 30$ cells in each group) from the sections under light microscopy using a glass Femtotip (Eppendorf) and joystick micromanipulator. Dissected cells were then aspirated into a second glass microelectrode filled with *in situ* transcription reaction buffer and avian myeloblastosis reverse transcriptase. NT2N were fixed briefly in 4% paraformaldehyde before aspiration. Single nontransfected control, S- or AS-transfected NT2N were visualized under phase contrast microscopy and were directly aspirated into glass electrodes filled with *in situ* transcription reaction buffer and avian myeloblastosis reverse transcriptase ($n = 20$ cells in each group). The aspirated cell and reaction buffer were transferred to a microfuge tube and incubated at 40°C for 90 minutes to ensure cDNA synthesis in the single dissected cell.

mRNA Amplification: Tissue Sections, Single Immunolabeled Cells, and Single NT2N

Amplification of mRNA from whole sections and fixed neurons has been described previously in detail.^{10,29,30} cDNA extracted from whole sections or generated from single cells served as a template for synthesis of double stranded template cDNA with T4 DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN). mRNA was amplified (aRNA) from the double-stranded cDNA template with T7 RNA polymerase (Epicentre Technologies). aRNA served as a template for a second round of cDNA synthesis with avian myeloblastosis reverse transcriptase, dNTPs, and N-6 random hexamers (Boehringer-Mannheim). cDNA generated from aRNA was made double-stranded and served as template for a second aRNA amplification incorporating ³²PCTP. The size range of radiolabeled aRNA from whole sections or single GCs and DNPs was assayed on a 1% agarose denaturing gel (not shown). Radiolabeled aRNA from whole tissue sections or single cells was used to probe candidate cDNA arrays.

cDNA Array Analysis

Linearized plasmid cDNAs including BDNF, CNTF, CNTFR (courtesy S. Scherer), NGF, netrin1, netrin2 (courtesy M. Tessier-Lavigne), NT3, NT4, trkA, trkB, and trkC (courtesy R. Madison, Duke University) were adhered to nylon membranes via UV crosslinking to generate a slot-blot array. β -actin, GFAP, and α -internexin cDNAs were included to serve as housekeeping genes to confirm the blot hybridization efficacy. A cDNA encoding tuberin (courtesy J. Sampson, University of Wales, College of Medicine) was also included on the blot so that tuberin mRNA expression could be quantified in each case. Plasmid pBluescript cDNA (pBS) served to define background, nonspecific hybridization of the aRNA probe to the cDNA blot. Blots were hybridized with the radiolabeled aRNA probe for 48 hours in 6 $\times$ SSPE buffer, 5 $\times$ Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate, and 200 μ g/ml salmon sperm DNA at 42°C. Slot blots were washed in 1 $\times$ standard saline citrate and were exposed to a phosphorimager cassette screen for 24 to 48 hours.

Western Analysis

BDNF, NT3, NT4, trkB, and trkC expression in human brain tissue samples and tuberin expression in NT2N were assessed by Western analysis. Frozen tuber and nontuber samples were dissected on a freezing table and homogenized. Tissue homogenates or NT2N were lysed in the RIPA buffer containing 1 $\times$ phosphate-buffered saline, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10% protease inhibitor cocktail (Sigma Chemical Co.). The total cell lysates were centrifuged at 10,000 $\times g$ for 15 minutes and the protein concentration estimated in the supernatant using the bicinchoninic acid protein assay (Bio-Rad, Richmond, CA). Cell lysate (100 to 200 μ g) was electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membrane. Western blots were probed with primary antibody (NT3, 1:500 dilution; NT4, 1:500 dilution; trkC, 1:80 dilution; rabbit antituberin, C-20, 1:100 dilution) overnight at 4°C, then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution; Amersham, Piscataway, NJ) for 1 hour at room temperature. Antibody labeling was visualized by enhanced chemiluminescence.

Quantitative Immunohistochemical Analysis

Three representative contiguous digital photos were obtained (magnification, $\times 20$) from each tissue section using image acquisition and analysis software (Phase 3 Imaging System integrated with Image Pro Plus; Media Cybernetics, Silver Spring, MD) and a Spot RT CCD camera (Diagnostic Instruments Inc.).

The relative optical density ratio (ODR) of GCs, DNPs, and control neurons immunolabeled with BDNF, NT3, NT4, trkB, and trkC antibodies was calculated using the IPP software. Because fixed brain tissue specimens can

bind antibodies with varying avidity, the ODR obviates these differences by determining the intensity of cell immunoreactivity when compared with the noncellular background. The ODR for the selected cell groups served as an index of immunolabeling intensity in single cells and whole sections. The absolute pixel staining density of selected single cells and the noncellular background (the cells were digitally subtracted from the image) was determined in the ROIs from each case and was assigned a numeric value by IPP ranging from the darkest (0) to the whitest (63,535) pixel. A mean optical density value for single GCs or DNPs in tubers and pyramidal neurons in control cortex was calculated and expressed as a ratio (ODR) of the mean optical density of the background. The total mean ODR for all cells in nine tubers (GCs and DNPs combined) was tabulated and compared with control neurons for each neurotrophin antibody. The total mean ODR ($\pm$ SE) of GCs and DNPs separately *versus* control neurons was also compared so that selective changes in individual cell types could be assessed. Statistical comparison of ODR in these groups was accomplished with a one-way analysis of variance and Fisher's post hoc test accepting $P < 0.001$.

Quantitative mRNA Expression Analysis

The relative abundance of neurotrophin and tuberin mRNAs in whole sections, single GCs and DNPs, and single NT2N was determined by analyzing the aRNA-cDNA hybridization intensities of the cDNA array phosphorimage (ImageQuant 5.0 software). Nonspecific hybridization to pBS plasmid cDNA was subtracted from hybridization intensity of each aRNA-cDNA. Relative hybridization intensity were determined by first averaging the autoradiographic density of all of the aRNA-cDNA hybrids on the blot and then expressing each as a percentage of the average hybridization intensity of the entire blot.³¹ mRNAs whose hybridization intensity was $>125\%$ of the mean blot hybridization intensity were designated as very high abundance, those $>75\%$ of the mean blot hybridization intensity were high abundance, those between 25 to 75% were medium abundance, and those that were $<25\%$ were of low abundance. Differences in relative abundance were determined using a one-way analysis of variance comparing each cDNA against section or cell type. To control for multiple experimental comparisons, a Bonferroni correction factor was used and significance was confirmed with a Fisher's post hoc test ($P < 0.05$).

Results

Fidelity of mRNA Amplification

aRNA amplification using T7 RNA polymerase proceeds by linear kinetics and therefore aRNA-cDNA hybridization intensity reflects relative abundance of cellular poly(A) mRNAs. The fidelity of aRNA amplification procedure from sections and single cells was verified using several approaches. First, only those whole sections and single cell samples with aRNA attaining 2 kb or more in length

Figure 2. Mean relative hybridization intensity ($\pm$ SE bar) of tuberin mRNA from tuber ($n = 18$) and control ($n = 8$) sections (mRNA amplified from two sections per case). Note overall reduction in tuberin mRNA expression in whole tuber sections compared with control cortex ($P < 0.05$).

as evidenced by a denaturing gel (not shown) were used to probe the arrays. Thus, differences in mRNA abundance on the cDNA arrays did not merely reflect differences in the length of amplified mRNAs from sections and individual cells. Second, the incorporation of ^{32}P -CTP into aRNA probes was similar in each cell type ($\sim 10^6$ to 10^7 counts incorporated) and thus, differential hybridization of aRNA to the cDNA blots did not reflect variable incorporation of ^{32}P -CTP into the aRNA probe. Third, aRNA probes from whole sections hybridized to the β -actin, GFAP, and α -internexin cDNAs (not shown) and validated the hybridization conditions. Finally, aRNA probes from sections and single cells exhibited minimal hybridization ($<5\%$ total average blot hybridization intensity) with pBS plasmid cDNA.

Genotype Analysis and Tuberin Expression

In an attempt to define the relationship of neurotrophin gene expression alterations to reductions in tuberin, the abundance of tuberin mRNA was determined in the nine TSC cases. Radiolabeled aRNA from whole tuber sections was used to probe reverse Northern blots containing the tuberin cDNA and hybridization intensity was determined as a measure of mRNA expression. Three cases resulted from mutations at the *TSC2* locus and one case from a *TSC1* locus mutation while genotype information on the five remaining cases was unavailable. Although sample numbers were too few to make appropriate statistical distinctions between cases, by visual inspection, tuberin mRNA expression was similar to con-

trol cortex in the one case associated with a *TSC1* locus mutation (and three other nongenotyped cases). In the three cases associated with a *TSC2* mutation (and the two remaining nongenotyped cases), tuberin mRNA expression was reduced. Indeed, mean tuberin mRNA expression was modestly but significantly reduced (two-fold) across nine all tuber specimens when compared with control cortex (Figure 2).

Differential Expression of Neurotrophin Genes in Whole Tuber Sections

Radiolabeled aRNA probes amplified from tuber, nontuber TSC cortex, and control cortex sections exhibited differential hybridization to the neurotrophin cDNAs. The abundance of neurotrophin mRNAs in control cortical sections was similar to previous reports in normal cerebral cortex as demonstrated by *in situ* hybridization.^{15,32,33} Netrin1 and trkB were very high abundance mRNAs; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, netrin2, NGF, NGFR, NT4, trkA, and trkC were low abundance mRNAs in control cortex. The relative expression of these mRNAs did not differ between nontuberal cortex and control cortex (percent hybridization when compared to mean hybridization on the blot; Table 2). The assessment of mRNA levels in nontuberal cortex compared with control cortex provided a valuable internal control for potentially confounding patient variables in TSC patients such as the effects of longstanding epilepsy, the use of psychotropic and anti-epileptic medications, and the single *TSC* gene mutation (heterozygote state) presumed present in all nontuber neurons of TSC patients. The similarity in neurotrophin gene expression in nontuberal cortex also highlighted the specificity of altered neurotrophin expression within tubers.

When compared with control and nontuber cortex sections, the relative abundance of NT3 and trkB mRNAs was reduced in tuber sections (Table 2). In contrast, the relative abundance of NT4 and trkC mRNAs was increased in tuber sections. The relative abundance of the chemoattractant netrin1 mRNA was diminished in tuber sections ($301 \pm 26\%$ in controls *versus* $150 \pm 19\%$ in tubers, $P < 0.05$) but netrin2 mRNA was unchanged. The relative abundance of the remaining neurotrophin

Table 2. mRNA Expression and Optical Density Ratios in Tuber and Control Sections

	mRNA expression			ODR section		ODR single cells	
	Control	Tuber	Nontuber	Control	Tuber	GC	DN
BDNF	49 $\pm$ 7%	44 $\pm$ 6%	40 $\pm$ 6%	1.27 $\pm$ 0.01	1.30 $\pm$ 0.02	1.31 $\pm$ 0.02	1.29 $\pm$ 0.02
NT3	70 $\pm$ 8%	33 $\pm$ 7%*	81 $\pm$ 11%	1.38 $\pm$ 0.02	1.27 $\pm$ 0.02*	1.32 $\pm$ 0.01	1.26 $\pm$ 0.03*
NT4	20 $\pm$ 6%	110 $\pm$ 13%*	33 $\pm$ 11%	1.29 $\pm$ 0.02	1.42 $\pm$ 0.01*	1.30 $\pm$ 0.02	1.38 $\pm$ 0.03*
TrkB	187 $\pm$ 30%	103 $\pm$ 12%*	166 $\pm$ 22%	1.35 $\pm$ 0.01	1.29 $\pm$ 0.01*	1.33 $\pm$ 0.03	1.27 $\pm$ 0.02*
TrkC	5 $\pm$ 2%	35 $\pm$ 6%*	7 $\pm$ 1%	1.29 $\pm$ 0.01	1.37 $\pm$ 0.03*	1.49 $\pm$ 0.05*	1.35 $\pm$ 0.03

Left, expression of neurotrophin mRNAs from $n = 12$ control sections (three sections per case), $n = 18$ tuber sections (two sections per case), and $n = 6$ nontuber TSC sections (three sections per case). Numbers reflect summed mean percent hybridization intensity of aRNA amplified from whole tuber sections to neurotrophin cDNA arrays. The hybridization intensity is expressed as a percentage of total blot intensity (*, $P < 0.05$). Middle, relative ODR of neurotrophin-immunolabeled sections reflecting GCs and DNs in tubers and all neurons in control sections (*, $P < 0.01$). Right, relative ODR of GC and DN compared with control neurons in middle panel (*, $P < 0.01$).

Figure 3. Relative abundance of BDNF, NT3, NT4, and netrin1 mRNAs in single control neurons, DNs, and GCs ($n = 30$ cells in each group; mean percent aRNA-cDNA hybridization intensity, $\pm$ SE bar; *, $P < 0.05$). BDNF mRNA expression was similar in all cell types. Note selective changes in mRNA expression within individual cell types (reduced NT3 mRNA in GCs, increased NT4 mRNA in DNs, and reduced netrin 1 mRNA in both cell types).

mRNAs (NGF, trkA, CNTF, CNTFR) did not differ between tuber, nontuber, and control specimens. Hybridization of aRNA from the entire section to GFAP, α -internexin, and β -actin cDNAs confirmed that representative cellular elements ie, neuronal, glial, endothelial cell types within the tuber contributed to the mRNA expression profile.¹⁰ In an effort to more accurately define alterations in neurotrophin expression solely in DNs and GCs, we assayed mRNA expression in single microdissected cells.

Differential Expression of Neurotrophin mRNAs in GCs and DNs

With the exception of GFAP, all of the assayed mRNAs were detected in control neurons. Netrin1 and trkB were very high abundance mRNAs; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, netrin2, NT4, NGF, trkA, and trkC were low abundance mRNAs. Because there were no observed differences in mRNA abundance in nontuber cortex, single cell analysis in these samples was not performed. The detection of α -internexin but not GFAP in the control neurons as well as the DNs and GCs provided strong evidence for the neural phenotype of these cells. The absence of GFAP mRNA in single DNs and GCs allowed us to conclude that amplification of glial mRNAs did not contaminate the cell expression profile data.

The expression of neurotrophin genes was distinct in DNs and GCs when compared with control neurons and highlighted the cellular specificity of the changes in mRNA expression determined in whole sections (Figures 3 and 4). NT4 mRNA expression was increased and trkB mRNA expression was reduced in DNs compared with GCs and control neurons. NT3 mRNA levels were reduced in GCs when compared to DNs and control neurons. In contrast, trkC mRNA abundance was increased and netrin 1 mRNA levels were decreased in both DNs and GCs when compared with control neurons. Expression of BDNF, CNTF, CNTFR, NGF, trkA, and netrin2 mRNAs did not differ across the cell groups.

mRNA Expression in NT2N Transfected with Tuberin Antisense Constructs

Two of the AS constructs (5' and GAP domain) effected a 70% reduction in tuberin expression in transfected C6 rat glioma cells compared to S-transfected controls. Reduced tuberin expression in NT2N by Western analysis was most pronounced (~60%) in the NT2N transfected with the 5' AS-construct when compared with nontransfected and S-transfected controls (Figure 5) and thus these cells were assayed for changes in neurotrophin mRNA expression. The expression of control proteins, tubulin, and β -actin (not shown), did not change in the 5'

Figure 4. Relative abundance trkA, trkB, and trkC mRNAs in single control neurons, DNs, and GCs ($n = 30$ cells in each group; mean percent aRNA-cDNA hybridization intensity, $\pm$ SE bar; *, $P < 0.05$). Expression of trkA mRNA was similar in all cell types. Expression of trkB mRNA was reduced in DNs whereas trkC expression was increased in both DNs and GCs.

AS-transfected C6 glioma cells or the NT2N. Tuberin mRNA levels in the control NT2N and S-transfected NT2N were similar, whereas tuberin mRNA expression was significantly decreased (fivefold reduction) in the AS-transfected NT2N ($n = 20$ cells in each group; Figure 5). No differences in hybridization intensity of α -internexin and β -actin cDNA was observed between the control, S-, and AS-transfected NT2N and GFAP mRNA (an exclusively glial mRNA) was not detected in NT2N. There were no morphological changes observed in the AS-transfected NT2N. Cells extended normal-appearing processes (axons and dendrites) and did not display cytomegaly.

Among the neurotrophin mRNAs analyzed, trkB was a very high abundance mRNA; NT3 was a high abundance mRNA; BDNF was a medium abundance mRNA; and CNTF, CNTFR, NT4, NGF, trkA, and trkC were low abundance mRNAs. The expression of trkC and NT4 mRNAs

Figure 5. The effect of tuberin antisense constructs on tuberin expression. **A:** Western blot depicting reduced tuberin expression in AS-transfected C6 glioma cells using three distinct AS constructs (5'-, M-, and G-constructs, see Materials and Methods for nucleotide sequence) compared with S-transfected control cells. No change in control protein tubulin expression in S- or AS-transfected cells. **B:** Relative hybridization abundance of tuberin mRNA in NT2N transfected with 5'-tuberin antisense construct ($n = 20$ NT2N in each group, $P < 0.001$). Note reduction (nearly fivefold) in tuberin mRNA levels in antisense-transfected cells compared with sense-transfected and untransfected control cells. **C:** Western blot depicting reduced tuberin protein expression in NT2N transfected with 5'-tuberin antisense (AS) construct compared with sense (S)-transfected and untransfected control (C) NT2N. **Arrow** depicts marker size of ~200 kd.

was increased in AS-transfected NT2N compared with nontransfected and S-transfected NT2N (Figure 6). The levels of other neurotrophin mRNAs including trkB and NT3 that were altered in tubers, did not differ between control, S-, and AS-transfected NT2N. Netrin1 mRNA was a high abundance mRNA in control and S-transfected NT2N and netrin2 was a medium abundance mRNA. Netrin1 mRNA abundance was reduced in AS-transfected NT2N while netrin2 mRNA levels did not change.

Neurotrophin Proteins in Tubers: Western Analysis and Immunohistochemistry

The expression of select neurotrophin proteins (BDNF, NT3, NT4, trkB, and trkC) was determined by Western analysis in homogenates of frozen tubers and control cor-

Figure 6. The effect of tuberin antisense constructs on neurotrophin and netrin mRNA expression in NT2N ($n = 20$ NT2N in each group; mean percent aRNA-cDNA hybridization intensity, $\pm$ SE bar; *, $P < 0.05$). **A:** Reduced netrin1 mRNA expression in antisense-transfected NT2N and increased NT4 (**B**) and trkC (**C**) mRNA expression in antisense-transfected NT2N compared with sense-transfected NT2N.

tex. Changes in the abundance of neurotrophin mRNAs identified in whole tuber sections in part predicted altered levels of these proteins in tubers (Figure 7). For example, in two tubers assayed NT4 and trkC protein levels were higher, on visual inspection, than in control cortex. NT3 protein levels were reduced in these two tuber specimens compared with control cortex. BDNF and trkB protein levels did not differ on visual inspection between tubers and control cortex.

Changes in neurotrophin protein expression in whole sections in part reflected altered expression of mRNAs in whole tissue sections. Because differential mRNA abundance in whole tuber sections reflected cell-specific changes in gene transcription as determined by single cell mRNA analysis, we defined the expression of BDNF, NT3, NT4, trkB, and trkC proteins in single GCs and DNs by computer-assisted image quantification of immunohis-

Figure 7. Western analysis depicting altered neurotrophin protein levels (NT3, **top**; NT4, **middle**; trkC, **bottom**) in tuber homogenates. Note reduction in NT3, but increase in NT4 and trkC expression in tubers (**lanes 2 and 3**) compared with control cortex (**lane 1**).

tochemically labeled sections. Immunolabeling of nontuber and control cortex with neurotrophin antibodies revealed specific staining patterns consistent with previous reports.^{32,33} For example, robust BDNF and modest NT3 and NT4 labeling was noted in the somatodendritic domains of pyramidal neurons in layers III, V, and VI. BDNF staining was prominent in the soma and apical dendritic segments of pyramidal neurons. In contrast, trkB staining was modest in cells within all cortical layers and faint trkC immunoreactivity was observed in cell bodies and axons of passage throughout the cortical laminae. The patterns of neurotrophin staining in nontuber and control cortex did not differ.

All tuber specimens probed with BDNF, NT3, NT4, trkB, and trkC antibodies exhibited immunolabeling (Figures 8 and 9). Using our morphological criteria for DNs and GCs, the number of neurotrophin immunolabeled GCs and DNs in tubers was determined. A total of 20,053 neurotrophin-immunolabeled cells (GCs and DNs combined for all antibodies) were identified in the nine representative 4-mm ROIs across the nine tuber specimens whereas 6637 neurons were identified in the four control-section ROIs. Within the nine tuber ROIs, 3525 neurotrophin-labeled cells met morphological criteria for GCs whereas 16,528 cells met criteria for DNs demonstrating that the number of DNs far exceeded that of GCs.

The ODR of GCs and DNs combined in tubers and the mean ODR of individual GCs and DNs was determined for each neurotrophin antibody and compared with con-

Figure 8. Representative tuber sections probed with neurotrophin antibodies. **A:** trkB-immunolabeled tuber sections. Diminished trkB immunoreactivity in DNs (small arrows) compared with GCs (large arrows). **B:** BDNF immunolabeling. Note approximately equal staining density of DNs (small arrows) and GCs (large arrows). Scale bar = 150 μ m.

trol neurons (Table 2). For example, the NT3 ODR for DNs and GCs combined was reduced in tubers compared to control sections and was reduced selectively in DNs compared with GCs or control neurons. The NT4 ODR in tubers was increased compared with control sections and the NT4 ODR of DNs was selectively increased compared with GCs and control neurons. The trkB ODR was reduced in tubers compared to control cortex and was decreased in DNs compared with GCs. The total trkC ODR was increased in tubers *versus* control sections and was selectively increased in DNs and GCs compared to control neurons. BDNF ODR did not differ in tubers *versus* control cortex or between GCs, DNs, and control neurons.

Discussion

We show that expression of select neurotrophin and neurotrophin receptor mRNAs and proteins is altered in tubers compared with nontuber cortex in TSC and control cortex. The changes in neurotrophin mRNA expression determined in whole sections was corroborated in part by Western analysis of neurotrophin protein levels in tuber

homogenates or by densitometry of entire immunohistochemically labeled sections. Thus, the expression of NT4 and trkC mRNA and protein was increased whereas the expression of NT3, trkB, and netrin1 was reduced in tubers compared with control cortex. These results argue that enhanced or diminished transcription of neurotrophin genes in tubers is reflected in expression of the cognate proteins. We generated an *in vitro* system using an anti-sense tuberin construct to assay the effects of reduced tuberin on neurotrophin expression in a human neuronal cell line that corroborated in part the changes in neurotrophin mRNA and protein expression observed in tubers. Finally, quantitative optical densitometric analysis of immunolabeled sections in part corroborated mRNA expression differences in single DNs and GCs. These studies are the first to define altered neurotrophin gene and protein expression in TSC.

mRNA Analysis in Tissue Sections and Single Cells in Neurological Disease

mRNA analysis in archival pathological material provides an experimental strategy to assay alterations in mRNA abundance across select brain lesions such as tubers and to define cell-specific changes in gene expression in individual cell types. Some mRNA degradation occurs within archival pathological material yet numerous investigators have confirmed the fidelity of single-cell mRNA amplification from fixed tissue.^{10,29,30,34–37} These reports have demonstrated that the relative abundance, complexity, and size (often attaining 3 kb in size) of mRNA transcripts amplified from fixed tissue is consistent and can be reliably used for cDNA array analysis.

Single-cell mRNA analysis was designed to surmount the fact that interpretation of gene expression data derived from analysis of whole brain regions may be complicated by the presence of multiple distinct cell types within a region that make differential contributions to the observed profile of mRNA expression.^{10,34,35} Single cell mRNA analysis should be viewed as a strategy to screen

Figure 9. Tuber section probed with trkC antibodies. Note immunoreactivity in both DNs (small arrows) and GCs (large arrows).

large numbers of candidate genes that may have significance at the protein expression level. Comparative analysis of gene expression in single cells provides a unique opportunity to identify genes that are differentially expressed within select cell populations. In the present study, single cell mRNA analysis demonstrated that the alterations in gene expression detected in whole sections was explained by differential expression of these mRNAs in distinct cell populations. An important theoretical caveat is that alterations in mRNA levels predict but do not necessarily define alterations in functional protein expression. Additional protein assays such as Western analysis or immunohistochemistry provide corroborative data. For example, altered expression of single GABA_A or NMDA receptor subunit mRNAs was shown to modify neurotransmitter receptor function as defined by patch-clamp recording³⁶ or receptor-ligand binding in whole tissue homogenates.³⁷

Changes in neurotrophin mRNA expression assayed in whole sections and single cells was highly concordant with changes observed in protein expression in tuber homogenates by Western analysis or by densitometric analysis of immunolabeled sections with two exceptions. NT3 mRNA was reduced in whole sections and NT3 protein expression was reduced as determined by Western analysis and densitometry of entire tuber sections. However, in single cells NT3 mRNA was reduced in GCs whereas by single cell immunodensitometry, reduced NT3 protein levels were noted in DN. One explanation is that translational control mechanisms regulate protein expression. Thus, reduced NT3 mRNA in GCs is compensated for by enhanced protein expression and a post-transcriptional modification reduced NT3 protein expression in DN. Similarly, reduced *trkB* mRNA and protein in whole sections and single DN was not detected by Western analysis. The reductions in *trkB* were modest and potentially not detected by Western assay.

Neurotrophin and Tuberin Expression

We wished to specifically investigate the effects of altered tuberin on neurotrophin expression because overall TSC severity, tuber number, and consequently, epilepsy is more severe in *TSC2*- than *TSC1*-associated cases.²⁵ We generated an *in vitro* system using an tuberin AS-construct in human NT2 neurons to reduce tuberin expression because NT2N are a human cell line known to express both hamartin and tuberin.³⁸ The tuberin antisense experiments were designed to model the effects of reduced tuberin, as may occur in gene deletions, as one of many potential mechanisms by which tuberin function may be compromised in patients. Thus, we hypothesized that any observed effects of reduced tuberin expression on neurotrophins in NT2N might corroborate these changes in human neurons *in vivo*. Indeed, NT2N express a variety of neurotrophins²⁴ and the relative abundance of neurotrophin and netrin mRNAs in S-transfected and control NT2N was similar to control cortex. In addition, previous studies in human neuroblastoma cell lines have demonstrated that tuberin AS-constructs reduce tuberin

expression and alter cell cycle dynamics.³⁹ The AS-constructs did not alter cellular morphology in the NT2N, yet tuberin mRNA and protein expression was diminished by >60% and a similar reduction in tuberin expression observed in the C6 glioma cell line supported the validity of the AS-construct effects on tuberin expression. The apparently normal morphology of AS-transfected NT2N cells may have reflected the presence of enough functional tuberin to maintain cellular cytoarchitecture or may require more rigorous evaluation of subtle structural alterations. Increased NT4 and *trkC* mRNA and reduced netrin1 mRNA expression detected in AS-transfected NT2N was similar to that identified in tubers and appeared to be a direct consequence of reduced tuberin levels.

Tuberin mRNA levels were determined in our tissue samples. In five specimens, three of which were obtained from individuals with an identified *TSC2* locus mutation, there was reduced tuberin mRNA levels. In four specimens, one from a patient with a known *TSC1* locus mutation, tuberin mRNA expression was similar to control tissue. Reduced tuberin mRNA levels in two nongenotyped cases supports, but does not prove that these were *TSC2* mutations. In contrast, unchanged tuberin mRNA levels in three of five nongenotyped cases may reflect a *TSC1* mutation but may also support a *TSC2* mutation without quantitative reductions in tuberin gene transcription. In fact, in some TSC tuber specimens, tuberin is robustly expressed in similar populations of cells even in the presence of *TSC2* germline mutations.^{38,40} In our samples, alterations in neurotrophin expression were consistent across all cases and thus we propose that changes in neurotrophin genes and proteins in tubers may result from reduced tuberin function but more likely reflect a common downstream effect of the hamartin-tuberin pathway. There is recent evidence, based on identification of functional protein-protein interactions between hamartin and tuberin, to suggest that hamartin and tuberin comprise a cellular pathway that contributes to cell cycle passage, cell-cell interactions, and possibly cell migration.^{41,42} Taken together, these reports imply that mutations in either the *TSC1* or *TSC2* gene likely results in a downstream cascade of common cellular events that includes changes in neurotrophin expression.

Additional factors may modulate neurotrophin expression in tubers because not all changes in neurotrophin mRNA expression observed in tubers were identified in tuberin AS-treated NT2N. For example, because tuberin reduction in the NT2N was only ~60%, a greater reduction in tuberin may be required to elicit all changes in neurotrophin mRNA expression observed in tuber specimens. Second, tubers consist of a heterogeneous population of cell types whereas the NT2N are a clonal human cell line. Additional effects of astrocytes, GCs, or oligodendrocytes present in human specimens on neurotrophin expression are absent from the NT2N system. Finally, some of the changes in neurotrophin expression may reflect more longstanding developmental effects of *TSC1* or *TSC2* mutations on cells in tubers. Clearly, further studies to define a mechanistic relationship between *TSC1* and *TSC2* gene mutations and changes in neuro-

trophin mRNA expression may prove crucial in understanding tuber formation in TSC.

Altered Neurotrophin Expression and Aberrant Cytoarchitecture in Tubers

NT3/trkC and NT4/trkB constitute two neurotrophin receptor-ligand pathways affected in TSC. If alterations in neurotrophin gene and protein expression persist from embryogenesis, then the downstream effects of these gene expression changes may account for abnormal cytoarchitecture in tubers. The observed changes in gene expression, e.g., enhanced expression of NT4 and diminished expression of trkB, may occur as a direct effect of *TSC1* or *TSC2* gene mutations or imply that changes in the expression of receptor might feedback to diminish expression of its ligand. Recent studies have suggested a role for neurotrophins in the growth and refinement of neural connections,⁴³ dendritic arborization,¹⁷ programmed cell death,⁴⁴ cortical lamination,^{18,45} synaptogenesis,⁴⁶ and in activity-dependent synaptic plasticity.^{47,48} Altered expression of NT3, NT4, trkB, and trkC as well as netrin 1 may have numerous potentially deleterious effects on cortical lamination during development. For example, an intriguing study demonstrated that administration of exogenous NT4 to organotypic cortical slice cultures results in dyslamination and neuronal heterotopia within cortical layers I to II.¹⁷ Thus, enhanced expression NT4 could contribute to the altered laminar cytoarchitecture characteristic of tubers. Reductions in both neuronal and glial cell populations have been observed in NT3 knockout mice.⁴⁹ Overexpression of trkC inhibits the growth of intracerebral xenografts of a medulloblastoma cell line in nude mice.⁴⁴ TrkB knockout mice have decreased densities of axonal varicosities, lower densities of synaptic contacts, and decreased density of synaptic vesicles,⁴⁶ a finding that has been suggested in tubers and non-TSC cortical dysplasia.⁵⁰ Cell culture experiments revealed reduced survival by trkB^{-/-} cortical neurons, a quantitatively significant defect in the formation of dendrites, and a significant reduction in neurite outgrowth by surviving trkB^{-/-} neurons.⁴⁸ Reduced levels of netrin1 are associated with impaired axon pathfinding and aberrant neuronal migration.⁵¹

Neurotrophin Expression, Epilepsy, and Epileptogenesis

Tubers are highly correlated with epilepsy in TSC patients⁵² and presurgical evaluations of TSC patients with medically intractable seizures have demonstrated that tubers are epileptic foci.⁶ Changes in neurotrophin expression in tubers may have relevance to the epileptogenic properties of these lesions. An important theoretical consideration is whether the observed changes in neurotrophin gene and protein expression are secondary to recurrent seizures emanating from the tubers because these specimens were resected for the direct purpose of seizure control. For example, variable increases in NGF,

BDNF, trkB, and trkC mRNA, but decreases in NT3 mRNA expression have been reported after seizures in experimental animals induced by pilocarpine, kainic acid, quinolinic acid, or hippocampal stimulation. Thus, reduced NT3 and enhanced trkC mRNA expression in tubers was consistent with previous experimental seizure paradigms and could in part reflect effects of recurrent seizures on gene transcription. However, NGF and BDNF mRNA expression was not altered in tubers and a decrease (rather than an increase) in trkB mRNA was observed. Thus, a generalized effect of seizures on gene and protein expression in tubers cannot be invoked. Additionally, neurotrophin gene changes in animals are transient and related to early phases of epileptogenesis whereas resected tubers are more chronic epileptic foci. The response of GCs and DNs to seizures may be distinct from normal hippocampal or cortical pyramidal neurons, and thus these neurotrophin gene changes might be unique to tubers.

Altered expression of neurotrophins and trks may contribute to epileptogenesis through a variety of mechanisms. For example, BDNF and NT3 promote the morphological differentiation of a subpopulation of GABAergic neurons in cortex⁵³ and a recent study showed markedly diminished numbers of GABAergic neurons in tubers as evidenced by GAD65 immunolabeling.³⁷ Similarly, NT3 and trkB have a role in the promotion of activity-dependent inhibitory synaptogenesis^{48,49} and thus, reduced NT3 and trkB expression may alter the formation of inhibitory synapses in tubers. Reduced expression of several GABA_A receptor subunits has been demonstrated in tubers and thus, a reduction in GABAergic neurons coupled with diminished GABA-mediated receptor inhibition may foster epileptogenesis. In contrast, a recent study demonstrated that BDNF and NT4 could act as potent excitatory modulators in the hippocampus, cortex, and cerebellum that exerted an effect as rapidly as the neurotransmitter glutamate.²⁰ Thus increased expression of NT4 in tubers could enhance excitatory synaptic activity in these lesions and foster epileptogenesis.

The differential expression of select neurotrophin and chemoattractant genes in tubers suggests pathways that could interfere with appropriate cortical development and promote tuber formation in TSC patients. Further investigation of the functional relationship between these genes and mutations in the TSC genes may shed light on the pathogenesis of tuber formation and epilepsy in TSC patients.

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