Neurotrophins in Mesial Temporal Lobe Epilepsy With and Without Psychiatric Comorbidities

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Abstract

Despite the strong association between epilepsy and psychiatric comorbidities, data on clinicopathologic correlations are scant. We previously reported differential mossy fiber sprouting (MFS) in mesial temporal lobe epilepsy (MTLE) patients with psychosis (MTLE + P) and major depression (MTLE + D). Because neurotrophins (NTs) can promote MFS, here, we investigated MFS, neuronal density and immunoreactivity for NGF and BDNF in specific subfields versus those not taking haloperidol. There were no differences in neuronal density and immunoreactivity for NGF and BDNF in specific subfields versus those not taking haloperidol. There were no differences in NT3 immunoreactivity among the groups. These findings support a close association between MFS and NT expression in the hippocampi of MTLE patients and suggest that distinct structural and neurochemical milieu may contribute to the genesis or maintenance of psychiatric comorbidities in MTLE.

Key Words: Brain-derived neurotrophic factor, Depression, Nerve growth factor, Neurotrophin, Psychiatric comorbidity, Psychosis, Temporal lobe epilepsy.

INTRODUCTION

Temporal lobe epilepsy (TLE) is the most common cause of intractable epilepsy in adults. The mesial subtype (MTLE) usually shows hippocampal sclerosis (HS), neuronal loss, gliosis, and mossy fiber sprouting (MFS) (1–5). Psychiatric comorbidities are very frequent in TLE patients (6–11), although the precise nature of this association is still a matter of debate (12, 13). We have recently shown neuropathologic findings suggesting that there is a structural basis for psychiatric manifestations in MTLE patients, such as increased MFS in patients with a history of major depression and decreased MFS in interictal psychosis (4).

Neurotrophins (NTs) are low-molecular-weight growth factors that act as extracellular ligands and affect differentiation, maintenance, and survival of cells. Among these endogenous proteins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3) were shown to promote MFS in different animal and in vitro models of TLE (14–16). Neurotrophins are upregulated in the dentate gyrus of TLE patients (17), and in animal models, seizure activity results in a transient increase of NGF and BDNF in hippocampal and neocortical neurons (14, 18–21). Although unaltered in the kindling model (15), NT3 expression is increased in the subchronic phase of the kainate model (22). Moreover, prolonged NT3 intracerebral infusion triggers sprouting and might play an antiepileptogenic role (15). In major depression and schizophrenia, decreased NT expression has been documented (23–27), whereas antidepressant and antipsychotic treatment might restore NT plasma levels (23, 28, 29). Interestingly, chronic treatment with antidepressants, antipsychotics, and benzodiazepine in naive rats does not alter BDNF or NGF hippocampal levels (30, 31).

Although growth factor involvement in TLE and neuro-psychiatric disorders seems unequivocal, the detailed expression patterns and functions of NTs within the human hippocampal formation remain largely unknown. Here, we characterized NT expression in the hippocampi of MTLE patients.
immunoreactivity in subfields of the mesial temporal structures, including the hippocampus, subiculum complex, and entorhinal cortex in a new cohort of MTLE patients with and without co-morbid major depression and psychosis. We also investigated the possible correlation between NT immunoreactivity and MFS and further clinical characteristics.

MATERIALS AND METHODS

Patients

We analyzed the hippocampal formation from MTLE specimens freshly collected in the operating room and non-epileptic controls from necropy, collected between 3.5 and 6 hours after death. A less than 24-hour postmortem time limit allows comparison of necropsy tissue with freshly collected surgical specimens for their protein levels, cell morphology, and tissue integrity (32, 33). Tissue collection and processing were conducted according to protocol approved by our institution’s research ethics board.

Mesial temporal lobe epilepsy specimens were derived from 40 MTLE patients who underwent a standard en bloc anterior temporal resection (including 3-4 cm of the hippocampus) for medically intractable seizures. All had clinical neuropathologic confirmation of HS. They were divided into 3 groups: 14 MTLE patients without any history of psychiatric disorder (MTLE group); 13 MTLE patients with interictal psychosis (MTLE + P group); and 13 MTLE patients with a diagnosis of major depression (MTLE + D group). According to Blümcke HS categories (34), 17 patients had severe HS, 16 classical HS, 5 CA1 HS, and 2 CA4 HS. No differences were seen among MTLE groups with respect to HS categories (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A508). For comparison, 10 nonepileptic control hippocampi from necropsies were processed and analyzed in the same manner as the surgical cases. Underlying diseases causing death were cardiomyopathy, pulmonary infarct, or renal-hepatic failure, with no history of hypoxic episodes before death, seizures, or neurologic diseases. Furthermore, there was no evidence of pathologic abnormalities of the mesial temporal structures on postmortem examination. Mesial temporal lobe epilepsy and control specimens were collected between 1996 and 2006. Clinical characteristics of all groups are shown in the Table.

Clinical Features of MTLE Patients

All patients were referred for presurgical assessment because of drug-resistant seizures, as defined by Berg (35). Patients
were evaluated at the Ribeirão Preto Epilepsy Surgery Program using standardized protocols approved by the institution’s ethics committee, and written consent was obtained from each patient. Presurgical investigation at the Epilepsy Monitoring Unit included detailed clinical history, neurologic examination, interictal and ictal scalp/sphenoidal electroencephalography, neuropsychology evaluation, and intracarotid amobarbital memory and language procedure whenever deemed clinically necessary.

Mesial temporal lobe epilepsy was defined following Engel criteria (36): 1) seizure semiology consistent with MTLE, usually with epigastric/autonomic/psychic auras, followed by complex partial seizures (CPSs); 2) presurgical investigation confirming seizure onset zone in the temporal lobe; 3) anterior and mesial temporal interictal spikes on electroencephalography; 4) no lesions other than unilateral or bilateral hippocampal atrophy on high-resolution magnetic resonance imaging scans (reduced hippocampal dimensions and increased T2 signal); 5) clinical histopathologic examination compatible with HS; and 6) no evidence of dual pathology identifiable by any of the assessment methods described (clinical, electrophysiology, neuroimaging, and histopathology). Exclusion criteria were focal neurologic abnormalities on physical examination, generalized or extratemporal electroencephalography spikes, and marked cognitive impairment indicating dysfunction beyond the temporal regions.

Information regarding antecedent of an initial precipitant injury, febrile seizures, seizure types, drug regimen, and estimated monthly frequency (within the 2 years before surgery) were retrospectively collected from medical records for each patient. Psychiatric evaluations were conducted in all MTLE patients. Each diagnosis of major depression was independently established during the presurgical evaluation by 2 psychiatrists with experience in psychiatric disorders associated with epilepsy using the guidelines of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Once a consensus on the classification of psychotic syndromes associated with epilepsy is lacking, and neither Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, nor International Classification of Disease, 10th Revision, has addressed this issue specifically, the diagnosis of psychosis associated with MTLE was established according to Sachdev (37), that is, patients with interictal psychosis did not experience the following: psychotic disorder temporally associated with seizures, changes in antiepileptic medications, epileptic status, delirium, and psychosis for paradoxical normalization. This group was defined by a prolonged psychotic state that was not related to the epileptic seizures. Typically, the psychotic states closely resemble schizophrenia, with paranoid ideas that might become systematized, ideas of influence, and auditory hallucinations often of a menacing quality. The points of difference are common religious coloring of the paranoid ideas, tendency of the affect to remain warm and appropriate, and no typical deterioration to the hebephrenic state (9). Patients had no history of psychiatric disorders (before seizure onset) or of substance dependence at any time. Global IQ was calculated after neuropsychologic tests (complete WAIS-III or WAIS-R protocol).

Tissue Collection and Immunohistochemical Processing

Specimens were segmented into 1-cm blocks transversely oriented to the hippocampal long axis. Blocks were placed in neo-Timm fixative solution (0.1% sodium sulfide [Sigma, St. Louis, MO] in Millonig buffered glutaraldehyde [Vetc, Rio de Janeiro, Brazil]) or buffered paraformaldehyde (Sigma). After 48 to 96 hours, the specimens were processed for neo-Timm histochemistry and hematoxylin and eosin (Laborclin, Pinhais, Brazil) staining or paraffin-embedded for immunohistochemistry.

Cryostat sections were mounted on chrome-alum gelatinoated slides and air-dried for neo-Timm staining (2). Slides were processed in batches containing at least 1 MTLE and 2 control slides. Slides were immersed in a physical developer maintained at 26°C in a darkroom. Developer consisted of 10 mL of a 50% gum arabic (Sigma) solution, 30 mL of an aqueous solution of 1.3 mol/L citric acid (Merck, Darmstadt, Germany), 0.9 mol/L sodium citrate (Merck), 90 mL of an aqueous solution of 0.5 mol/L hydroquinone (Merck), and 1.5 mL of a 17% silver nitrate solution (Merck). Light sections were developed for 40 minutes and dark sections for 50 minutes. Slides were washed in distilled water for 5 minutes and running tap water for 10 minutes. They were subsequently air-dried, ethanol dehydrated, xylene cleared, and coverslipped.

Immunohistochemistry was performed with antibodies that identified immunoreactivity for NeuN, a nuclear protein found in the nuclei of mature neurons (1:1000 dilution; Chemicon-Millipore, Billerica, MA) and for the NTs: neurotrophic growth factor (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), BDNF (1:30 dilution; Chemicon-Millipore), and NT3 (1:30 dilution; Chemicon-Millipore). According to the manufacturers and selected references, these antibodies show no cross-reactivity with other NTs and/or are capable of neutralizing the biologic activity of its own isoform only (38–40). Their Western blot profile in human tissue is shown in Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A509. Briefly, paraffin-embedded MTLE and control hippocampi were processed together for each antibody as described in Kandratavicius et al (5), with overnight incubation at room temperature and developed simultaneously for 10 minutes in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL) and 0.01% H2O2 (Merck). After sufficient colorization, reaction was halted by washing in several rinses of distilled water, dehydrated through graded ethanol to xylene (Merck), and coverslipped with Krystalon (EM Science, Gibbstown, NJ). Adjacent sections were hematoxylin and eosin stained and examined for tissue integrity. Control sections without the primary antisera did not reveal staining (data not shown).

Cell Count and Neo-Timm Quantification

Mesial temporal lobe epilepsy and control hippocampi were compared for neuronal density and neo-Timm staining. Neuronal counting was performed based on Lorente de No classification (41), including fascia dentata granular and subgranular cells, polymorphic hilar neurons (limited to a region between stratum granulosum and CA4 pyramidal cells, being at least 50 μm from the stratum granulosum and 100 μm...
from CA4), as well as pyramidal cells in CA4 (the portion of Ammon horn that permeates the inner part of the dentate gyrus), CA3, CA2, CA1, prosubiculum, subiculum, parasubiculum, and entorhinal cortex layer III. Cell densities (neurons per cubic millimeter) were estimated in 8-μm NeuN-stained slices at 400× magnification with a morphometric grid methodology using Abercrombie correction (42), as previously described and well established in the literature for surgical hippocampal fragments (1, 3–5, 43–47). We also performed measurements of granular layer dispersion using NeuN-stained sections and the straight line tool of ImageJ analysis system (National Institutes of Health, USA, public

![Image](http://jnen.oxfordjournals.org/)

**FIGURE 1.** Neuronal density in human hippocampal formation subfields. (A, B) NeuN-stained hippocampal formation in a mesial temporal lobe epilepsy (MTLE) patient (A) and a control (B). There is marked neuronal loss in the MTLE dentate gyrus, Ammon, horn and Sommer sector, as well as granular layer (GL) dispersion. (C) Neuronal density values from MTLE (black bars), MTLE + major depression (MTLE + D) (gray bars), MTLE + psychosis (MTLE + P) (light gray bars), and from nonepileptic controls (white bars) are indicated as mean ± SD. **Significant difference between epileptics and the control group (p < 0.001). Neuronal loss was observed in GL, hilus, CA4, CA1, prosubiculum, and entorhinal cortex. There is also a statistical trend (tr: 0.05 ≥ p ≤ 0.07) to decreased neuronal density in MTLE + P CA2 versus control. ENT, entorhinal cortex; HIL, hilus; PAR, parasubiculum; PRO, prosubiculum; SUB, subiculum. Scale bar = (A, B) 2 mm.
Mossy fiber sprouting was evaluated in neocortex-stained sections in the hilus, granular layer, and inner molecular layer, always in the superior blade of the dentate gyrus. Each subfield of each specimen had 3 different samples measured and averaged for statistical analysis. Measurements of gray value and length were estimated using ImageJ, as previously described (4). In brief, length measurements of sprouting consisted of the distance from the outer border of the granular layer to the visible limit of the sprouted mossy fibers within the molecular layer, including the sprouted mossy fibers that eventually invaded the outer molecular layer (measurement referred as visible sprouting in the molecular layer). Images were collected and digitized with a high-resolution CCD monochrome camera attached to an Olympus microscope. This method was used to obtain digitized images of neocortex and anti-NeuN, -NGF, -BDNF, and -NT3-stained slides. Uniform luminance was maintained and checked every 10 measurements using an optical density standard and a gray value scale ranging from 0 (white) to 255 (black).

Semiquantitative Analysis of Immunohistochemistry

Slides adjacent to those examined for neuronal density were analyzed for NT immunoreactivity. In brief, all digitized images taken in 20× magnification were analyzed with ImageJ software, following the same criteria: 1) the software identifies the gray value distribution of a subfield’s digital image (total area for each subfield analyzed = 313.7 × 235.3 μm, which corresponds, e.g., to approximately one-third of an Ammon horn subfield; 2) the immunoreactive area, that is, positively stained pixels, is selected, limited to a threshold range; and 3) the threshold range is presettled based on control group sections to exclude the low-intensity gray value of background staining from the analysis. A similar approach was used by our group elsewhere (48). Results for granular layer included granular cell layer per se and proximal molecular layer. Analyses were conducted by one investigator (Ludmyla Kandratavicius), blinded to hippocampal pathology and group classification.

Data Analysis

Data were analyzed using the statistical program PAWS (version 18.0) and SigmaPlot (version 11.0). Groups were compared using analysis of variance (ANOVA one-way, with Bonferroni post hoc test) or unpaired t test for variables with normal distribution and Kruskal-Wallis one-way ANOVA on ranks (with Dunn post hoc test) or Mann-Whitney U rank sum test for variables without normal distribution. Fisher Exact test was applied for comparison of relative frequencies of clinical variables between groups. Other statistical tests included Pearson correlation analyses and analysis of covariance. Statistical significance was set at p < 0.05, and values were presented as mean ± SD.

RESULTS

Clinical Profiles

The 4 patients groups did not show significant differences in sex, age, or collected side (Table). Clinical variables such as the presence of an initial precipitant injury and febrile seizures, age of first seizure and seizure onset, seizure frequency and epilepsy duration, HS side, handedness, IQ, years at school, and performance in verbal memory tests were homogeneously distributed among MTLE groups. The MTLE + D patients exhibited a trend to increased proportion of patients with secondarily generalized seizures versus patients without psychiatric comorbidities (0.05 ≥ p ≤ 0.07), and MTLE + P patients exhibited a trend to worse performance in nonverbal memory tasks.

All epileptic patients were on antiepileptic drugs (carbamazepine, oxcarbazepine, phenobarbital, and/or phenytoin). In addition, patients were also taking benzodiazepines (MTLE...
group, 8 of 14; MTLE + D, 10 of 13; MTLE + P group, 10 of 13), fluoxetine (MTLE + D, 4 of 13), and haloperidol (MTLE + P group, 10 of 13). No differences in neuronal density, MFS, or NT immunoreactivity were seen between patients taking or not taking benzodiazepines. No differences in neuropsychologic tests between patients taking or not taking benzodiazepines, fluoxetine, or haloperidol were seen.

Neuropathologic Characterization: Neuronal Density and MFS

Evaluation of epileptogenic and control hippocampal formation (Fig. 1A, B) showed reduced neuron density in the granular layer, hilus, CA4, CA1, and prosubiculum of all MTLE groups when compared with control (Fig. 1C). In addition, there was a significant neuron density reduction in the entorhinal cortex of MTLE patients with major depression and interictal psychosis versus controls and a trend to decreased neuronal density in CA2 of MTLE + P specimens. The MTLE groups exhibited increased granular layer width when compared with that in controls (ANOVA F3,47 = 8.31, p < 0.0001), with no statistically significant differences between MTLE groups (ANOVA F2,37 = 0.20, p = 0.817) (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A508). The MFS gray value and length were increased in MTLE groups versus...
those in control (Fig. 2). Moreover, MTLE + P specimens exhibited decreased MFS gray value in the molecular layer when compared with those in MTLE and MTLE + D groups. Sprouting length was also decreased in the MTLE + P group. Specimens from the MTLE + D group showed increased MFS length versus those in other MTLE groups (Fig. 2F).

Interestingly, neuronal density correlated inversely with seizure frequency in CA4 (r = −0.41, p = 0.045), CA2 (r = −0.50, p = 0.021), prosubiculum (r = −0.38, p = 0.043), subiculum (r = −0.37, p = 0.039), and parasubiculum (r = −0.45, p = 0.015), whereas molecular layer gray value of MFS correlated positively with seizure frequency (r = +0.46, p = 0.014). Also, granular cell dispersion in the superior blade of the dentate gyrus correlated inversely with CA4 neuronal density (r = −0.49, p = 0.023), suggesting that dispersion could be related to loss of granular cells original targets. Within the MTLE + D group, no differences in MFS or neuronal density were observed between patients taking fluoxetine or not. The MTLE + P patients taking haloperidol had decreased neuronal density when compared with those not taking haloperidol in CA3 (9.2 ± 2.9 × 10^3 neurons/mm^3 vs 26.0 ± 2.7 × 10^3 neurons/mm^3, t8 = −7.54, p = 0.002) and subiculum (12.1 ± 7.5 × 10^3 neurons/mm^3 vs 31.0 ± 7.1 × 10^3 neurons/mm^3, t11 = −3.22, p = 0.012).

**NT Immunoreactivity**

Strong NGF immunoreactivity was detected in all hippocampal formation subfields (Fig. 3). In MTLE specimens, increased NGF expression was detected in hypertrophic hilar cells and pyramidal neurons in the Ammon horn, subicular complex, and entorhinal cortex. Glia-like cells were also evident in the MTLE Sommer sector (CA1 and prosubiculum; Fig. 3K, M). Nerve growth factor immunoreactivity in the granular layer (Fig. 4A–D) was increased in MTLE and MTLE + D groups versus the control group (Fig. 4F). Among epileptic groups, specimens from the MTLE + D group showed greater NGF immunoreactivity, and specimens from the MTLE + P group had less immunoreactivity (Fig. 4A–C). Other hippocampal subfields with decreased NGF immunoreactivity in the MTLE + P group were the hilus, CA2, CA1, prosubiculum, subiculum, and parasubiculum (Fig. 4F).

Nerve growth factor immunoreactivity in CA3 of epileptic patients correlated positively with granular cell dispersion in the superior (r = +0.47, p = 0.027) and inferior blade (r = +0.45, p = 0.036), indicating that the increased trophic tone from CA3 to the dentate gyrus might support the reverberatory excitatory activity between CA3, mossy cells, and granule cells, and possibly reinforced by MFS (49). Moreover, NGF immunoreactivity in the granular layer correlated with MFS gray value (r = +0.54, p = 0.008) and length (r = +0.51, p = 0.014) and with seizure frequency (r = +0.40, p = 0.016). Nerve growth factor immunoreactivity correlated positively with neuronal density in CA1 (r = +0.39, p = 0.038) and prosubiculum (r = +0.42, p = 0.042). In addition, NGF immunoreactivity in the granular layer (r = +0.44, p = 0.008), subiculum (r = +0.34, p = 0.047), and parasubiculum (r = +0.47, p = 0.009) correlated positively with global IQ and with neuropsychologic nonverbal scores in the same regions (granular layer NGF: r = +0.44, p = 0.011; subiculum NGF: r = +0.42, p = 0.017; parasubiculum NGF: r = +0.58, p = 0.001). No differences in NGF immunoreactivity were seen in MTLE + D patients taking fluoxetine versus those not taking fluoxetine. The MTLE + P patients taking haloperidol showed less NGF immunoreactivity in parasubiculum (2,022.31 ± 410.6 × 10^2 μm^2 vs 3,303.65 ± 138.2 × 10^2 μm^2, t10 = −2.85, p = 0.019). Moreover, when we analyzed NGF expression considering seizure type, we found that patients with secondarily generalized seizures exhibited decreased values versus those with CPS in the hilus (1,640.12 ± 693.4 × 10^2 μm^2 vs 2,249.85 ± 816.2 × 10^2 μm^2, t17 = 2.20, p = 0.03), CA2 (2,482.40 ± 747.9 × 10^2 μm^2 vs 3,316.06 ± 1,194.1 × 10^2 μm^2, t14 = 2.17, p = 0.04), and subiculum (2,571.59 ± 813.10 vs 3,950.24 ± 846.25, t16 = 2.71, p = 0.013).

**FIGURE 4.** Nerve growth factor (NGF) expression in mesial temporal lobe epilepsy (MTLE) specimens with and without psychiatric comorbidities and in nonepileptic controls. (A–D) MTLE + major depression (MTLE + D) granular layer (B) exhibited greater NGF expression versus MTLE (A), MTLE + psychosis (MTLE + P) (C), and control (D) groups. (E) NGF-immunoreactive area values were higher in epileptic groups versus controls (** p < 0.001; * p < 0.05), and there were differences among MTLE groups (## p < 0.001; # p < 0.05). In CA2 and CA1, there was a trend (tr: 0.05 ≥ p ≤ 0.07) to decreased NGF expression in MTLE + D versus MTLE. Values from MTLE (black bars), MTLE + D (gray bars), MTLE + P (light gray bars), and from nonepileptic controls (white bars) are indicated as mean ± SD. Scale bar = (A–D) 50 μm.
Brain-derived neurotrophic factor immunoreactivity in neurons was detected in all subfields of the hippocampal formation (Fig. 5). Glia-like cells were also immunostained, particularly in MTLE dentate gyrus and Ammon horn (Fig. 5A–K). Comparing all groups, the granular layer was the subfield with major differences among them (Fig. 6A–D). The MTLE and MTLE + D showed increased BDNF-immunoreactive area versus that of the control group (Fig. 6E); and MTLE + P granular layer BDNF immunoreactivity was less than that of MTLE (Fig. 6E). In the hilus, BDNF in MTLE + P was less than that of controls, and in CA1, greater BDNF immunoreactivity area was seen for MTLE and MTLE + D (Fig. 6E). Brain-derived neurotrophic factor immunoreactivity correlated positively with neuronal density of epileptic patients in the hilus ($r = +0.65$, $p = 0.001$), CA3 ($r = +0.56$, $p = 0.045$), and subiculum ($r = +0.41$, $p = 0.035$), but not with MFS or with dispersion in the granular layer. Brain-derived neurotrophic factor immunoreactivity in the granular layer ($r = +0.36$, $p = 0.037$) and subiculum ($r = +0.42$, $p = 0.022$) also correlated positively with global IQ, but not

FIGURE 5. Brain-derived neurotrophic factor (BDNF) expression in the human hippocampal formation. (A–T) There is greater immunoreactivity in mesial temporal lobe epilepsy (MTLE) granular cells and in cells with glial profiles, for example, in dentate gyrus and Ammon horn (arrows, A, C, K, M). ENT, entorhinal cortex; GL, granular layer; HIL, hilus; PAR, parasubiculum; PRO, prosubiculum; SUB, subiculum. Scale bar = (A–T) 50 µm.
with other neuropsychologic scores. Brain-derived neurotrophic factor immunoreactivity correlated inversely with age of seizure onset ($r = -0.40$, $p = 0.032$) and CA3 ($r = -0.51$, $p = 0.020$). Regarding MTLE + D patients taking fluoxetine, greater BDNF immunoreactivity in CA1 was seen when compared with those not taking fluoxetine ($3,920.77 \pm 161.8 \times 10^2 \mu m^2$ vs $2,529.46 \pm 451.4 \times 10^2 \mu m^2$, $t_{11} = +4.11$, $p = 0.009$). In contrast, MTLE + P patients taking haloperidol showed decreased BDNF immunoreactivity in the hilus ($1,038.60 \pm 440.6 \times 10^2 \mu m^2$ vs $1,840.75 \pm 207.3 \times 10^2 \mu m^2$, $t_{11} = -2.96$, $p = 0.016$) and CA4 ($1,451.76 \pm 638.9 \times 10^2 \mu m^2$ vs $3,100.54 \pm 284.0 \times 10^2 \mu m^2$, $t_{11} = -4.22$, $p = 0.002$) versus those not taking haloperidol.

Neurotrophin 3 expression was detected in neurons and glia, as well as in blood vessels through all hippocampal formation subfields (Fig. 7A–T). No differences in NT3-immunoreactive area (Fig. 7U) or in the qualitative assessment of how strong NT3 staining was in blood vessels (data not shown) were found among groups in any subfield. Neurotrophin 3 and MFS correlated inversely in CA4 ($r = -0.64$, $p = 0.003$), CA1 ($r = -0.58$, $p = 0.005$), subiculum ($r = -0.52$, $p = 0.008$), and entorhinal cortex ($r = -0.59$, $p = 0.013$). No differences in NT3 expression between patients taking or not fluoxetine or haloperidol were seen.

Given the differences in neuronal densities (Fig. 1), an important question was whether the statistical differences in NT immunoreactivity for the 4 groups could be accounted for by changes in neuronal densities. Therefore, we performed an analysis of covariance comparing groups and neuronal densities with NGF, BDNF, and NT3-immunoreactive area. Differential BDNF expression in the hilus, where a positive correlation was seen between neuronal density and BDNF expression, lost its statistical significance after neuron count correction. Differences between groups in other subfields and for other NTs remained significant after cell count correction, indicating that the 4 patient categories showed significant differences in NT expression levels that were not influenced by changes in neuronal densities.

**DISCUSSION**

An extensive number of neuropathologic studies have been done on hippocampi from MTLE patients, whereas fewer have examined extrahippocampal tissue (50). Postmortem studies with specimens from patients with schizophrenia and major depression have also shown several abnormalities in the hippocampal formation (12, 13). Despite the strong association between epilepsy and psychiatric comorbidities, neuropathologic data are scant. In another series of patients, we previously reported decreased MFS in MTLE patients with psychosis and increased MFS in MTLE patients with major depression (4); this result was confirmed in the present series. Another important replicated finding was decreased neuronal density in the entorhinal cortex of MTLE patients with psychiatric comorbidities (4). More severe neuron loss in the entorhinal cortex would contribute to disrupted communication between the hippocampal formation and neocortical and limbic sites, which are known to have a profound effect in modulation of the psychopathologic state (12).

**NTs, Epilepsy, and Psychopathologic States**

Neurotrophins are part of a set of molecules that can influence MFS development. Indeed, a positive correlation between MFS progression and increased NGF concentration, but not increased BDNF concentration, was demonstrated in the kainate model of epilepsy (22), which is similar to the correlations we found in this MTLE series. In particular, a study on transgenic mice suggests that BDNF is not essential to MFS because hippocampal slices of a BDNF-deficient (-/-) mouse display MFS as well as the wild type (51). With respect to NT3, a negative correlation with MFS was found, in
contrast to animal model results (22), and measurements of NT3 mRNA in the granular layer in epileptic patients (52). It has been suggested that NT3 can trigger sprouting and inhibit epileptogenesis (15), and we hypothesize that, in human MTLE, where a seizure-prone circuit is already established, decreased NT3 input to the dentate gyrus (e.g. from the entorhinal cortex) would contribute in the chronic phase to sustain hyperexcitability and to halt further progression of sprouting. Indeed, we found a positive correlation between seizure frequency, MFS, and granular layer NGF, in agreement with earlier work in animal models (53, 54). Neurotrophin 3 expression in non-neuronal tissue such as blood vessels has been verified by others, and one of the proposed mechanisms would result in increased nitric oxide production via endothelial nitric oxide synthase (55). Although increased neuronal nitric oxide production in MTLE may be inferred (56), we did not identify qualitative differences in NT3 expression in hippocampal blood vessels between control and epileptic patients, suggesting that NT3-mediated endothelial nitric oxide production is unaltered.

The association between seizure facilitation and increased NGF expression has been shown in animal models of epilepsy (14, 53) and is in agreement with our result of positive correlation between seizure frequency and NGF expression in the granular layer. On the other hand, we found that patients with secondarily generalized seizures exhibited decreased NGF expression in the hilus, CA2, and subiculum versus patients with CPS. In a recent study, Alapirtti et al (57) compared blood samples of 3 TLE patients with secondarily generalized seizures and 11 patients with CPS, suggesting that the more severe the seizure type (i.e. secondarily generalized seizures), the stronger the inflammatory response after an acute seizure. In our series, a molecule related to seizure facilitation was found downregulated in patients with more severe seizures; however, when NGF is not able to interact with their receptors, seizure development is halted (58). Inasmuch as we found distinct hippocampal subfields related to seizure frequency and seizure type, differential modulation and action of NGF are feasible. Future studies with NTs receptors will be able to clarify this finding.

Despite NGF upregulation found in most hippocampal formation subfields of patients with epilepsy, specimens with MTLE and psychosis showed decreased NGF immunoreactivity in the granular layer, hilus, CA2, CA1, and all subicular complex when compared with MTLE without psychiatric comorbidities. An equivalent result was seen for BDNF immunoreactivity in the granular layer. There is a significant and stable association between schizophrenia and memory impairment (59), although its basis is unknown (60). Most studies comprise verbal memory deficits, but poor performance of auditory memory tasks and visuospatial delayed recognition have also been reported in patients with schizophrenia and their relatives (61) and in healthy subjects injected with ketamine (62). Diminished hippocampal NGF and BDNF immunoreactivity might be related to the lower cognitive performance trend seen in the MTLE + P group because NGF and BDNF, but not NT3 (63), are required for optimal cholinergic neurotransmission (64). In turn, low cholinergic function results in poor memory performance, as seen in schizophrenia and Alzheimer disease (65, 66). In accordance with our findings of positive correlations between NGF immunoreactivity and IQ and nonverbal memory tasks, it has been shown in rodents that increases in hippocampal and neocortical NGF reverse deficits in learning and memory in spatial navigation and object recognition tasks (67). More importantly, in conditions of glutamatergic hypofunction (as it occurs in schizophrenia and animal models of schizophrenia based on N-methyl-d-aspartate antagonism [68–70]), there is a decrease in BDNF expression (71), as we found in the MTLE + P group versus the MTLE group. Likewise, rats injected with ketamine show decreased MFS induced by electroconvulsive seizures and decreased BDNF expression (72). Low BDNF levels have been observed in the plasma of schizophrenic patients (25, 73, 74) and seem independent of medication despite the high variability between the studies (75). Other studies have also shown association between BDNF polymorphisms and schizophrenia and cognitive deficits (76–79), but not in cases of febrile seizures (80) or TLE (81). Decreased BDNF expression has been described in postmortem hippocampus of schizophrenic patients (24, 77), and, in contrast, there are also reports of increased BDNF expression (82, 83). The 2 latter studies speculate that the results would be related to a deficient BDNF secretion leading to intraneuronal BDNF accumulation. In view of reciprocal BDNF/glutamate modulation (84), normalization of BDNF levels in psychosis would counteract glutamatergic hypofunction. Based on the increased BDNF expression found in epilepsy, a comorbid psychotic state would display milder symptoms than in schizophrenia, as is indeed depicted in interictal psychosis (9).

**Drugs and NT Expression**

Dose-dependent fluoxetine-induced neuroprotection has been described in animal models of epilepsy, ischemia, and Parkinson disease (85–87). In our series, we did not observe differences in neuronal density but found increased BDNF immunoreactivity in CA1, the same region reported as protected, and with increased BDNF levels in fluoxetine-treated ischemic gerbils (85). A similar result has also been found in the human dentate gyrus (88). Interestingly, the CA1 region is one of the most affected subfields by neuronal loss in MTLE. In our series, most BDNF immunoreactivity detected in CA1 was in glial cells, and it has been shown that astrocytes are particularly affected by fluoxetine and paroxetine (but not tricyclics) treatment and respond with BDNF upregulation (89).

**FIGURE 7.** Neurotrophin 3 (NT3) expression in mesial temporal lobe epilepsy (MTLE) specimens with and without psychiatric comorbidities and in nonepileptic controls. (A–T) There was faint to moderate NT3 expression in neuronal and glial cells and faint to strong staining in blood vessels (arrows) in all groups and subfields of the hippocampal formation. (U) No differences in NT3-immunoreactive area were found among groups. ENT, entorhinal cortex; GL, granular layer; HIL, hilus; PAR, parasubiculum; PRO, prosubiculum; SUB, subiculum. Scale bar = (A–T) 50 μm.
The effects of typical and atypical antipsychotics on cell proliferation and apoptosis have been investigated repeatedly, with controversial results. Haloperidol treatment in animal models has been shown to increase hippocampal neurogenesis (90), or to have no effect (91), to promote survival of hippocampal stem cells (92) and to induce apoptosis in cortical neurons (93). In our series, significant neuronal loss was seen in CA3 and subiculum of MTLE + P patients taking haloperidol versus those not taking haloperidol in the same group, in agreement with evidence that antipsychotics can reduce brain tissue volume (94). Our results also showed decreased BDNF and NGF hippocampal expression in MTLE + P patients taking haloperidol, in accordance with animal model studies showing decreased BDNF hippocampal levels (95) and NGF cortical levels (96).

CONCLUSIONS

It is important to acknowledge some limitations inherent to our findings in this study. Even with a relatively small sample size, the present results suggest that further studies exploring MTLE and related comorbidities are worthwhile. Although we could not perform stereologic counts because of limited tissue source from surgery, our neuron density numbers are in agreement with recent hippocampal stereologic counts performed in MTLE specimens (97). In fact, because all MTLE surgical specimens were freshly collected and submitted to identical processing, differences among them are particularly relevant.

In summary, the present results provide the first demonstration of differential NT expression in human MTLE hippocampal formation with and without psychiatric comorbidities, supporting the close association between MFS and NTs. It also indicates that the use of haloperidol in MTLE might relate to increased neuronal loss and decreased NT expression. Our results are in agreement with most studies done with postmortem major depression, schizophrenia specimens, and animal models that have independently provided important pathophysiological hallmarks. The relatively high prevalence of psychiatric symptoms in MTLE patients suggests mechanisms and/or substrates shared in these conditions. Clearly, different psychopathological states in MTLE rely on distinct structural and neurochemical milieu. In the static concept of chronic MTLE, we are not able to define which exact variable might contribute to the genesis or to the maintenance of a particular psychiatric comorbidity, but it is hoped that future research on the morphologic and biochemical abnormalities in this scenario will delineate the molecules that may become targets for new treatments.

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