

## Human Temporal Lobe Epilepsy Analyses by Tissue Proteomics

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**ABSTRACT:** Although there are many types of epilepsy, temporal lobe epilepsy (TLE) is probably in humans the most common and most often studied. TLE represents 40% of the total epilepsy form of the disease and is difficult to treat. Despite a wealth of descriptive data obtained from the disease history of patients, the EEG recording, imaging techniques, and histological studies, the epileptogenic process remains poorly understood. However, it is unlikely that a single factor or a single mechanism can cause many changes associated with this neuropathological phenomenon. MALDI mass spectrometry imaging (MSI) coupled to protein identification, because of its ability to study a wide range of molecules, appears to be suitable for the preparation of molecular profiles in TLE. Seven neuropeptides have been identified in Dentel gyrus regions of the hippocampus in relation with TLE pathology. Shot-gun studies taking into account gender influence have been performed. Tissue microextraction from control (10) toward 10 TLE patients have been analyzed after trypsin digestion followed by separation on nanoLC coupled to LTQ orbitrap. From the shot-gun analyses, results confirmed the presence of specific neuropeptides precursors and receptors in TLE patients

as well as proteins involved in axons regeneration including neurotrophins, ECM proteins, cell surface proteins, membrane proteins, G-proteins, cytoskeleton proteins and tumor suppressors. Among the tumor suppressors identified, the Leucine-rich glioma inactivated 1 (LGI1) protein was found. LGI1 gene recently been demonstrated being implicated in heritability of TLE. We have also demonstrate the presence a complete profile of tumor suppressors in TLE patients, 7 have been identified. Refining this analysis taken into account the gender influence in both control and in TLE reflected the presence of specific proteins between male and female and thus mechanisms in pathology development could be completely different. © 2014 Wiley Periodicals, Inc.

**KEY WORDS:** neuroproteomic; epilepsy; mass spectrometry imaging; neuropeptide; sexome

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## INTRODUCTION

Epilepsy is characterized by an overactive brain and can occur at any age; 50,000,000 cases of epilepsy have been identified worldwide. Epilepsy is a chronic neurological disorder characterized by recurrent seizures. It is the expression of abnormal operation and acute and transient electrical activities of a brain region, defined clinically by the occurrence of at least two crises (Fischer, 2005). Although there are many types of epilepsy, temporal lobe epilepsy (TLE) is probably in humans the most common and most often studied. TLE represents 40% of the forms of the disease and is difficult to treat. In most cases, TLE is refractory to drug treatment and it is possible to resort to surgery (Benbadis et al., 1996; Benbadis and Luders, 1996a, b). Surgical treatments remove 80 to 90% of seizures in patients with TLE in cases of surgical perspective is available. TLE is often associated with hippocampal sclerosis characterized by the loss of pyramidal neurons and gliosis in regions 1 and 3 of the Ammon's horn. The hippocampal sclerosis associated TLE may be due to a brain injury, tumor, meningitis, encephalitis, or a crisis in childhood (Engel et al., 1989; Mathern et al., 1995a, 1995b; Engel, 1996; Cendes, 2004; Wieser and Hane, 2004; Wieser, 2004; Fischer, 2005; Lewis, 2005). Thus, despite the wealth of descriptive data obtained from the disease history of patients, EEG recordings,

imaging techniques and histological studies, the epileptogenic process remains poorly understood. However, it is unlikely that a single factor or a single mechanism can cause all these changes associated with this neuropathy. Epilepsy results in neuronal cell death, loss of neurons, or neurogenesis by changing the propagation of neural circuits (Jacobs et al., 2009). Recently, Childhood absence epilepsy, a prototypic form of generalized non-convulsive epilepsy, has been investigated by MALDI MSI using BS/Orl and BR/Orl mouse lines. Six potential markers including Myelin basic protein, Neurogranin, Full length Purkinje cell protein 4 with one acetylation, ubiquitin, a fragment of Synapsin-I, and the short isoform of Thyrosin  $\beta$ -4 with one acetylation have been identified by this MALDI MSI coupled to a Top-Down approach (Lagarrigue et al., 2012). In this report, we demonstrate using a MALDI MSI approach in human TLE tissues the exact anatomical localization of seven neuropeptides (NPY (1-30), somatostatin 14, neurokinin B, galanin, corstitatin, chromogranin B fragment, cocaine- and amphetamine-regulated transcript (CART) peptide fragment) within the hippocampal formation of these patients. Shot-gun studies were performed from tissue sections obtained from male and female patients. Data revealed differences by taking into account the gender differences. In males, proteins are involved related to inflammation and neurogenesis whereas in females proteins are involved related to axon guidance and polarization, odorant receptors and opioids. These data indicated that mechanisms implicated in male and female epilepsy are different. This confirms that epilepsy is a different disease for women than men.

## EXPERIMENTAL PROCEDURES

### Chemicals

All chemicals were of the highest purity obtainable. Water, formic acid (FA), trifluoroacetic acid (TFA), acetonitrile (ACN), and methanol (MeOH) were purchased from Biosolve B.V. (Valkenswaard, the Netherlands). Ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), ethanol (EtOH),  $\alpha$  cyano-4-hydroxycinnamic acid (HCCA), aniline (ANI), DL-dithiothreitol (DTT), iodoacetamide (IAA), and reagents for Haematoxylin-eosin-safran (HES) staining were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Tetramethylethylenediamine (TEMED), ammonium persulfate (APS) and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad (Marnes La Cocquette, France). Sequencing grade, modified porcine trypsin was purchased from Promega (Charbonnières, France).

### Tissue Collection

The epileptic hippocampal tissue was dissected for therapeutic purposes from patients with medial temporal lobe epilepsy who had hippocampal sclerosis (Table 1). All patients submitted informed consent. During surgery, the head and body of the hip-

poampus was dissected. After trimming down of the head and end of the body, about 6 mm of the hippocampus was immediately frozen using isopentane floated liquid nitrogen, and then kept in liquid nitrogen alone. Tissue acquisition and storage procedures were conform to the guidelines of the Institutional Review Board of Seoul National University Hospital (H-0507-509-153) for hippocampi from epilepsy patients. Twenty TLE hippocampi have been collected and used in this study.

### Tissue Sample Preparation

Collected human epileptic hippocampi were frozen in isopentane cooled at  $-50^\circ\text{C}$ . Samples were then stored at  $-80^\circ\text{C}$  until processing. Ten  $\mu\text{m}$  human brain tissue sections were cut using a cryostat CM1510S (Leica Microsystems, Nanterre, France) and applied onto indium-tin oxide (ITO)-coated conductive glass slides (Bruker Daltonics, Bremen, Germany). The tissue sections were vacuum-dried in a desiccator during 10 min then soaked subsequently in 70% EtOH, 95% EtOH, and chloroform for 30 s each with concomitant drying under vacuum for 5 min. The sections were then dried and stored under vacuum until further use. For some sections, after MALDI analysis, the matrix was removed by washing the slides with 70% ethanol and the sections were stained using the Hematoxylin, Eosin, Safran (HES) staining.

### MALDI Mass Spectrometry Imaging


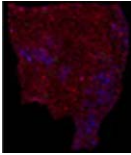
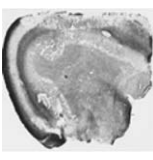
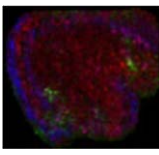
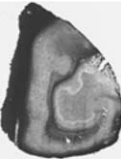
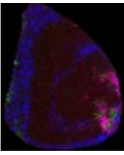

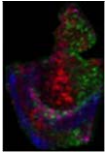
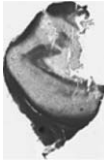
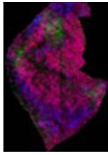

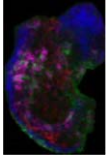
HCCA/ANI as solid ionic matrix (10mg/mL of HCCA, dissolved in 7:3 AcN/0.1%TFA, v/v containing 1.5 molar equivalent of ANI) was applied on the tissue surface using the ImagePrep device (Bruker Daltonics, GmbH). MALDI MSI experiments were performed on an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, GmbH) equipped with a smartbeam laser (Nd:YAG, 355nm). Peptide mass spectra were acquired in linear positive ion mode at a mass range of  $m/z$  500 to 10,000. The distance between raster points was set to 50  $\mu\text{m}$  and a total of 300 laser shots were accumulated at 200 Hz repetition rate for each pixel. Spectra were processed by baseline correction and smoothing using the FlexAnalysis 3.2 software (Bruker Daltonics, GmbH). Image analysis and data visualization were performed with the FlexImaging 2.1 software (Bruker Daltonics, GmbH). For statistical analysis, the data set obtained from FlexImaging was loaded into the ClinProTools 2.2 software (Bruker Daltonics, GmbH) to conduct hierarchical clustering. Unsupervised clustering was selected with Euclidean as the distance method and ward as the linkage method. Results of the hierarchical clustering were exported to FlexImaging 2.1 software to reconstruct the areas with different profiles.

### SwePep Databank

SwePep consists of a dynamic web interface, a relational database, and a business tier, which uses the client input from the web interface to construct and execute queries to the database. SwePep is specifically designed for endogenous peptides. Every peptide in the database is connected to the following

TABLE 1.

Description of the patients gender, pathology, diagnosis, surgery details, seizure, medicine taking period, tissues sections of the hippocampus stained by HES or used for MALDI MSI analyses [Color table can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Gender	Age	Surgery Date	Diagnosis	invasive study	Surgery details	seizure	medicine-taking period	H&S	MALDI MSI
Male	30	01/11/2010	Medial temporal lobe epilepsy	N	Temporal Lobectomy [ATL and amygdalohippocampectomy]	Y	Since 2008		
Female	35	2010-12-20 2010-12-27	Temporal lobe epilepsy	Y	Invasive monitoring ant-tem-poral lobectomy	Y	Since 2006		
Male	37	2010-12-22 2010-12-29	Temporal lobe epilepsy	Y	Invasive monitoring craniotomy and lesionectomy	Y	Since 2009		
Female	50	18/04/2011	Temporal lobe epilepsy With intractable epilepsy	N	Selective Amygdalohippocampectomy (trans-sylvian)	Y	28 years		
Female	19	2011-04-13 2011-04-20	Temporal lobe epilepsy With intractable epilepsy	Y	Invasive monitoring Operation of epilepsy, Temporal Lobectomy [Anterior	Y	10 years		
Male	35	11/05/2011	Temporal lobe epilepsy With intractable epilepsy	N	Operation of epilepsy, Temporal Lobectomy [Ant. temporal lobectomy and amygdalohiDDOcamDecto	Y	10 years		

information: name, sequence, and precursor protein, position in precursor sequence, modifications, location, organisms, reference, mass, and pI. SwePep consists of 4,180 unique endogenous peptides, and many of these are post-translationally modified. The neuropeptides in SwePep have been derived from 1,643 precursor proteins from 394 different species. All peptides have searchable descriptors such as mass (monoisotopic and average), modifications, precursor information, and organism affiliation. Because the experimental data contain peptides and proteins in the mass range up to 10 kDa, the SwePep database also contains 25,047 small proteins with sequence length less than or equal to 120 amino acids (Falth et al., 2006, 2007).

## Tissue Proteomic

### Tissue protein extraction

Twenty micrometers hippocampal tissue sections were mounted on a parafilm covered glass slide and the tissue was microdissected manually using a binocular. The pieces were extracted by incubating in 20  $\mu$ l of 50 mM bicarbonate buffer containing 50 mM DTT and 1% SDS at 55°C for 15 min. The extracts were then loaded on 12% polyacrylamide gel and separated at 70 V for 15 min and then 120 V until the dye front reaches the other end of the gel. After migration, the gel was incubated in the gel fixative solution for 30 min and stained with colloidal Coomassie brilliant blue overnight. The stain was removed by washing the gel four times with distilled deionized water.

### In gel digestion

The gel was cut into 10 pieces. Pieces were washed with 300  $\mu$ l of distilled deionized water for 15 min, 300  $\mu$ l of ACN for 15 min, and 300  $\mu$ l of  $\text{NH}_4\text{HCO}_3$  100 mM (pH 8) for 15 min. Then a mix of 300  $\mu$ l of  $\text{NH}_4\text{HCO}_3$ /ACN (1:1, v/v) for 15 min and 300  $\mu$ l of ACN for 5 min. Band pieces were dried in a Speedvac for 5 min. The reduction of cystine residues was made with 50  $\mu$ l of 10 mM of DTT in  $\text{NH}_4\text{HCO}_3$  100 mM (pH 8). Pieces were incubated at 56°C for 1 h. Alkylation of cysteines was made with 50  $\mu$ l of 50 mM of IAA in  $\text{NH}_4\text{HCO}_3$  100 mM (pH 8). Pieces were incubated at room temperature in the dark for 30 min. Band pieces were washed a second time with 300  $\mu$ l of  $\text{NH}_4\text{HCO}_3$  100 mM (pH 8) for 15 min. Then a mix of 300  $\mu$ l of  $\text{NH}_4\text{HCO}_3$ /ACN (1:1, v/v) for 15 min and 300  $\mu$ l of ACN for 5 min. Band pieces were dried in a Speedvac for 5 min. A digestion of band pieces was made with trypsin (12.5  $\mu$ g/ml) in  $\text{NH}_4\text{HCO}_3$  20 mM (pH 8), enough to cover pieces. Pieces were incubated at 37°C overnight. Peptides were extracted on shaking platform with 50  $\mu$ l of FA 1% two times for 20 min, then 150  $\mu$ l of ACN for 10 min. The supernatant was transferred in new tube and dried with Speedvac.

### NanoLC-HR-MSMS

Samples were resuspended in 20  $\mu$ l of TFA0.1%, then they were desalted on a C-18 Ziptip (Millipore, Saint-Quentin-en-Yvelines, France), dried under vacuum and then resuspended in

AcN/0.1% FA, 2:8, v/v). The samples were separated by online reversed-phase chromatography using a Thermo Scientific Proxeon Easy-nLC system equipped with a Proxeon trap column (100  $\mu$ m ID  $\times$  2 cm, Thermo Scientific) and C18 packed tip column (75  $\mu$ m ID  $\times$  10 cm, Thermo Scientific). Elution was carried out using an increasing gradient of AcN (5% to 30% over 120 min) and a flow rate of 300 nl/min. A voltage of 2 kV was applied via the liquid junction of the nanospray source. The chromatography system was coupled to a Thermo Scientific LTQ-Orbitrap XL mass spectrometer programmed to acquire in a data dependent mode. The survey scans were acquired in the Orbitrap mass analyzer operated at 60,000 (FWHM) resolving power. A mass range of 300 to 1600  $m/z$  and a target of 1E6 ions were used for the survey scans. Precursor ions observed with an intensity over 500 counts were selected "on the fly" for ion trap collision-induced dissociation (CID) fragmentation with an isolation window of 4 a.m.u. and a normalized collision energy of 35%. A target of 5,000 ions and a maximum injection time of 200 ms were used for MS<sup>2</sup> spectra. The method was set to analyze the top 10 most intense ions from the survey scan and a dynamic exclusion was enabled for 60 s.

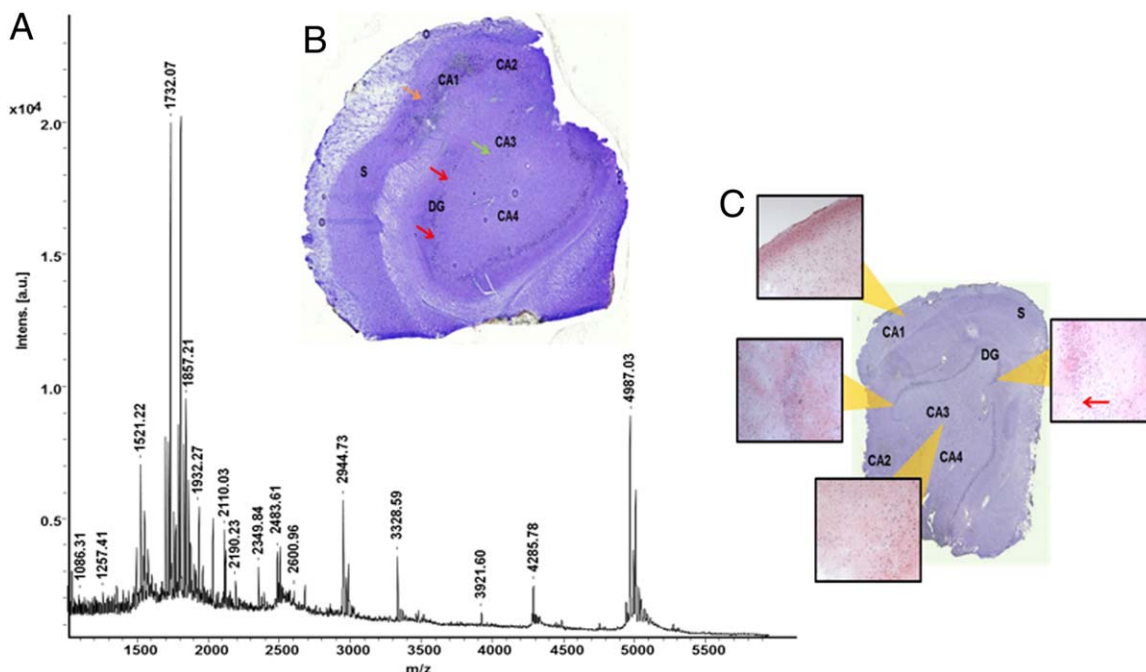
## Analysis

Tandem mass spectra were processed using the Thermo Scientific Proteome Discoverer software version 1.4. Resultant spectra were matched against the Swiss-Prot® Human database (version January 2013) using the SEQUEST® algorithm (Thermo Fisher Scientific, San Jose, CA; version 1.3.0.339). All MS/MS samples were analyzed using Sequest. Sequest was set up to search Human\_uniprot\_fullproteome.fasta.fasta (unknown version, 71,956 entries). N-terminal acetylation; phosphorylation of tyrosine, serine, and cysteine carbamidomethylation; methionine oxidation; and arginine deamidation were set as variable modifications. The search was performed by selecting trypsin as the enzyme with two missed cleavages allowed. The precursor mass tolerance was 10 ppm, and the fragment mass tolerance was 0.5 Da. Peptide validation was performed using the Percolator algorithm. The peptides were filtered based on a  $q$ -value of 0.01, which corresponds to a 1% false discovery rate (FDR). Only proteins with a score of over 5, which represents the proteins identified with two or more unique peptides, were kept for analysis. The relative protein expression was calculated based on the protein score, which was shown to be an adequate relative indicator of the relative differential expression (Colinge et al., 2005). We compared the acquired results with an analysis using the Scaffold 4.1.1. software (Searle, 2010). We considered this method to be quite accurate because it gave similar results when a quantitative comparison of the different tissues' proteins relied on spectral counting.

## Label Free Quantification

Scaffold (version Scaffold\_4.1.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and





**FIGURE 1.** (A) MALDI mass spectrum recorded from the section (c) of an epileptic hippocampus. Inset is the optical image of a section of annotated epileptic hippocampus stained with toluidine blue: Horn of Ammon (CA), dentate gyrus (DG) and subiculum (S). (B) Optical image of a section of annotated TLE hippocampus stained with toluidine blue: Horn of Ammon (CA), dentate gyrus (DG), and subiculum (S). (C) Optical image of a

section of annotated control hippocampus stained with toluidine blue: Horn of Ammon (CA), dentate gyrus (DG), and subiculum (S). Insets consist of microscopic images of four regions of interest obtained from a serially cut section followed by HES coloration. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

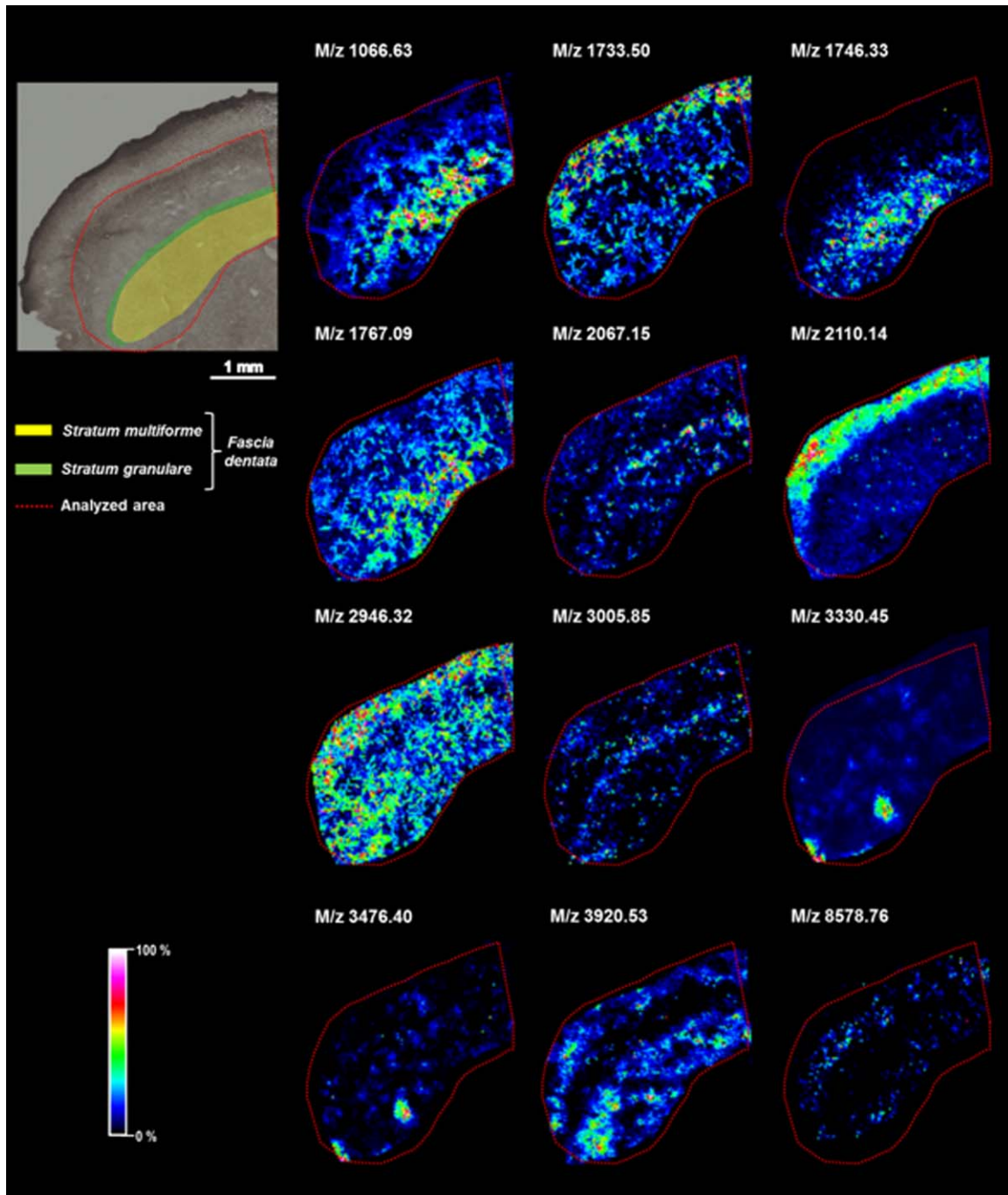
protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction (Higdon et al., 2007; Zhang et al., 2009). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Choi et al., 2008). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Normalization was done on top three total ion current (TIC) in addition to spectral counting.

## RESULTS

### Endogenous Peptide Analysis of Human TLE by MALDI MSI

Histological observation of the hippocampi of epileptic patients shows the different structures found in a typical hippocampus (Table 1), but with considerable differences (Figs. 1B,C). One can observe a significant neuronal loss in the CA3 and CA4 fields and at the level of the subiculum. This massive loss of neurons in the subiculum makes it difficult to define

this structure. Localized loss of neurons is evident in the CA1 region and in the granular layer of the DG (Fig. 1B). The distinction between the CA4 and the hilar layer is not feasible because of the almost total loss of neurons in the CA4 field (Fig. 1A). A study at the molecular level has been conducted subsequently to correlate the histological and molecular changes (Fig. 1A). Many peptides were detected in the mass range  $m/z$  1,000 to 5,000 (Fig. 1A). However, peptides weakly present in the hippocampus are highly localized or may not even be highlighted on the average spectrum from the acquisition of spectra in different regions of the cut. In addition, various studies have shown that the DG has an important role in the balance of excitatory/inhibitory limbic system. Granule cells are glutamatergic excitatory neurons located at the entrance of the hippocampus and thus act as intermediaries between the entorhinal cortex and the pyramidal cells of the CA1 and CA3 fields. The DG is believed to act as a gate controlling nerve impulses entering the hippocampus (Hsu, 2007). Indeed, while many mechanisms exist in the brain for regulation between excitation and inhibition of nerve signals, the ability of the DG to control the excitation is very strong and robust. Disruption of this filter function has a disproportionate impact on the excitation/inhibition balance of the limbic system. For this reason, a large number of studies on TLE focused on the DG (Dudek and Sutula, 2007). These observations led us to perform MALDI molecular images in the region bound by the



**FIGURE 2.** Distribution by MALDI MSI of peptides from different layers of the dentate gyrus of epileptic patients: polymorphic layer (Stratum. Multiforme, S. mu), granular layer (Stratum granulare, S.gr) and molecular layer (Stratum moleculare, S.mo). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

DG molecular layer including the polymorphic layer and the granular layer (Fig. 2). To highlight the peptidome in thin structures, including the granular layer, the spatial resolution chosen for this MALDI imaging experiment was 25 microns (Fig. 2). The measured peptide at  $m/z$  1066.5 (Fig. 2) is located in the hilar layer (stratum multiforme) like the one at  $m/z$  1,746.33 where the mossy fibers are positioned so that the pep-

tide  $m/z$  2110.1 (Fig. 2) is found in the molecular layer. The ions  $m/z$  3,330.4 and 3,376.4 are specifically detected in a group of hilar cell layer of the DG (Fig. 2). Ions at  $m/z$  1,767.09 and 2,946.32 are highly expressed in the three layers of the DG whereas the ones at  $m/z$  2,067.15, 3,005.85, 3,920.53, and 8,578.76 are less expressed but are also present in the whole of the DG. Other peptides of particular location

TABLE 2.

List of the Neuropeptides Identified in the Different Structure of the Hippocampus [Color table can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Layers <i>m/z</i>	Neuropeptides	Sequences	Stratum moleculare	Stratum granulare	Stratum multiforme
1,066.5	CART (28-36)	pQEDAELQPRA			
1,210.4	Neurokinin B	DMHDFVGLM			
1,637.5	Somatostatin-14	AGCKNFFWKTFSTC			
1,746	Cortistatin 14	PCKNFFWKTFSSCK			
1,763	Cortistatin 14 Na <sup>+</sup>	PCKNFFWKTFSSCK(Na <sup>+</sup> )			
2,110.1	Oxidized Chromogranin B (357-374) + phosphate	GLQYR(O)GRG-phosphoSEEDRAPRPR			
3,155.1	Galanin	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS			
3,454.4	Neuropeptide Y (1-30)	YPSKPDNOGEDAPAEDMARYYSALRHYINL			
4,938.6	Thymosin Beta 4	SDKPDM(O)AEIEKFDKSKLKKTTETQEKNP PSKETIEQEKQAGES			

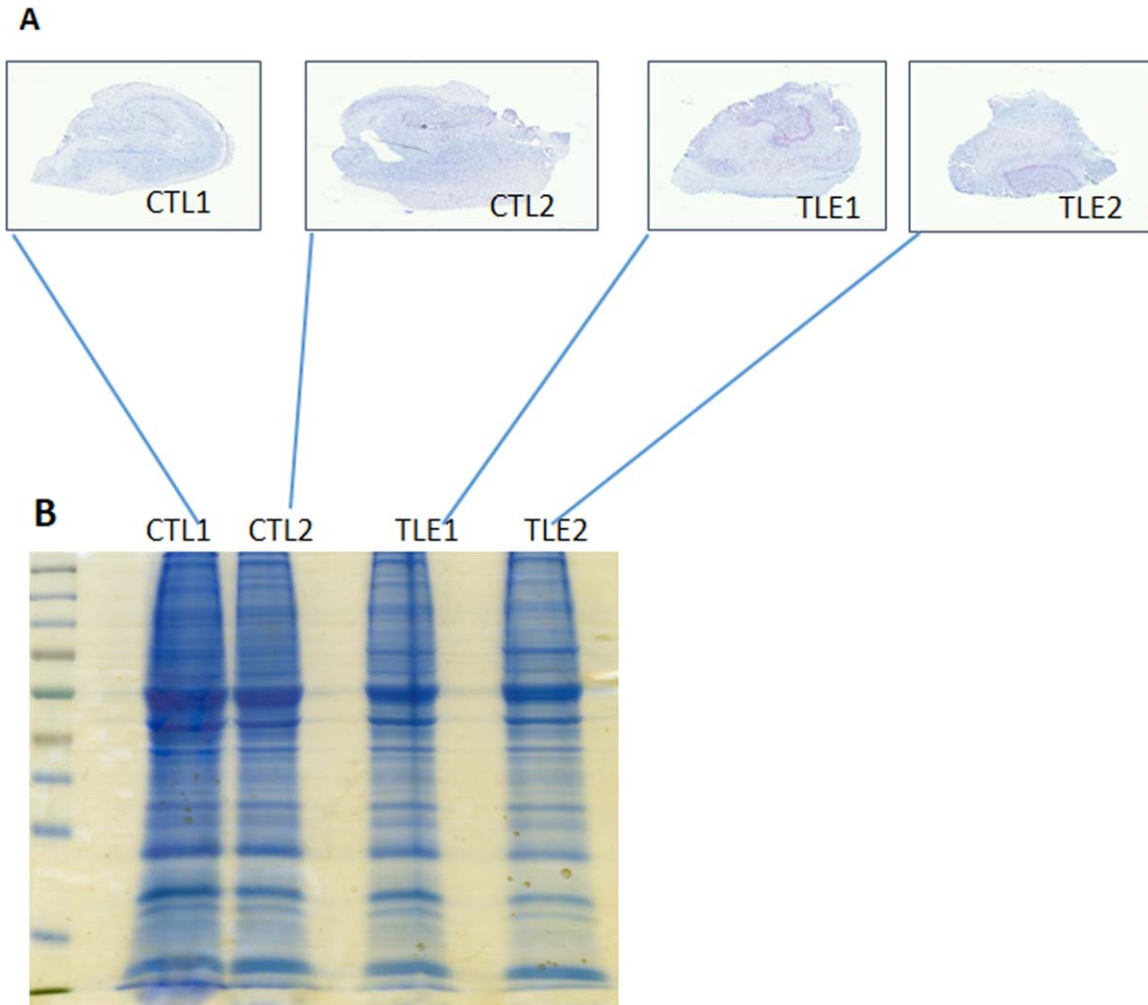
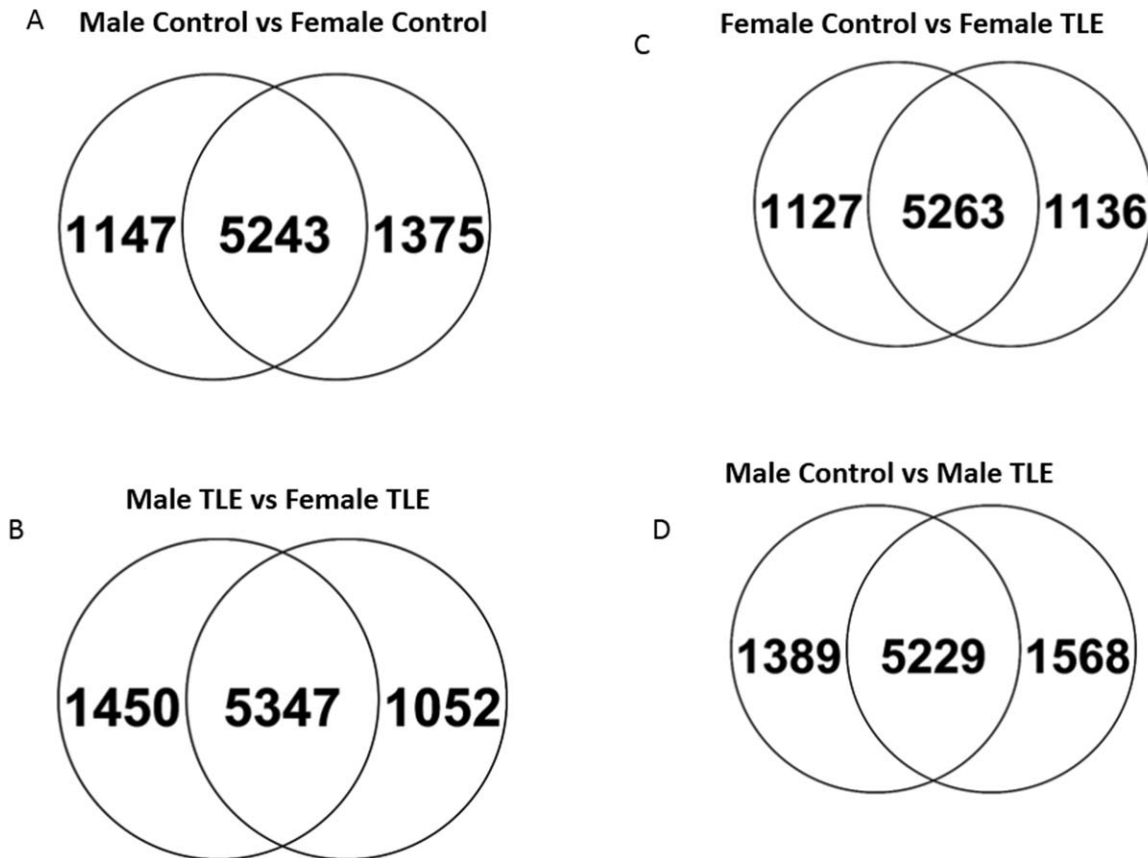


FIGURE 3. Schematic representation of the tissue proteomic procedure used to characterize proteins in control and TLE patients. (A) Sections of control and TLE sections colored with toluidine blue. (B) SDS-PAGE of the protein extracted from the tissue section used in (A). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



**FIGURE 4.** Venn diagrams obtained after Scaffold analysis taking into account the gender of control and TLE patients. Each experiments were performed three times per sample. Specific proteins to male or female TLE have been identified (Supporting Information data 1). (A) Comparison between control male and female control. (B) Comparison between male and female TLE. (C) Comparison between female control and female TLE. (D) Comparison between male control and male TLE.

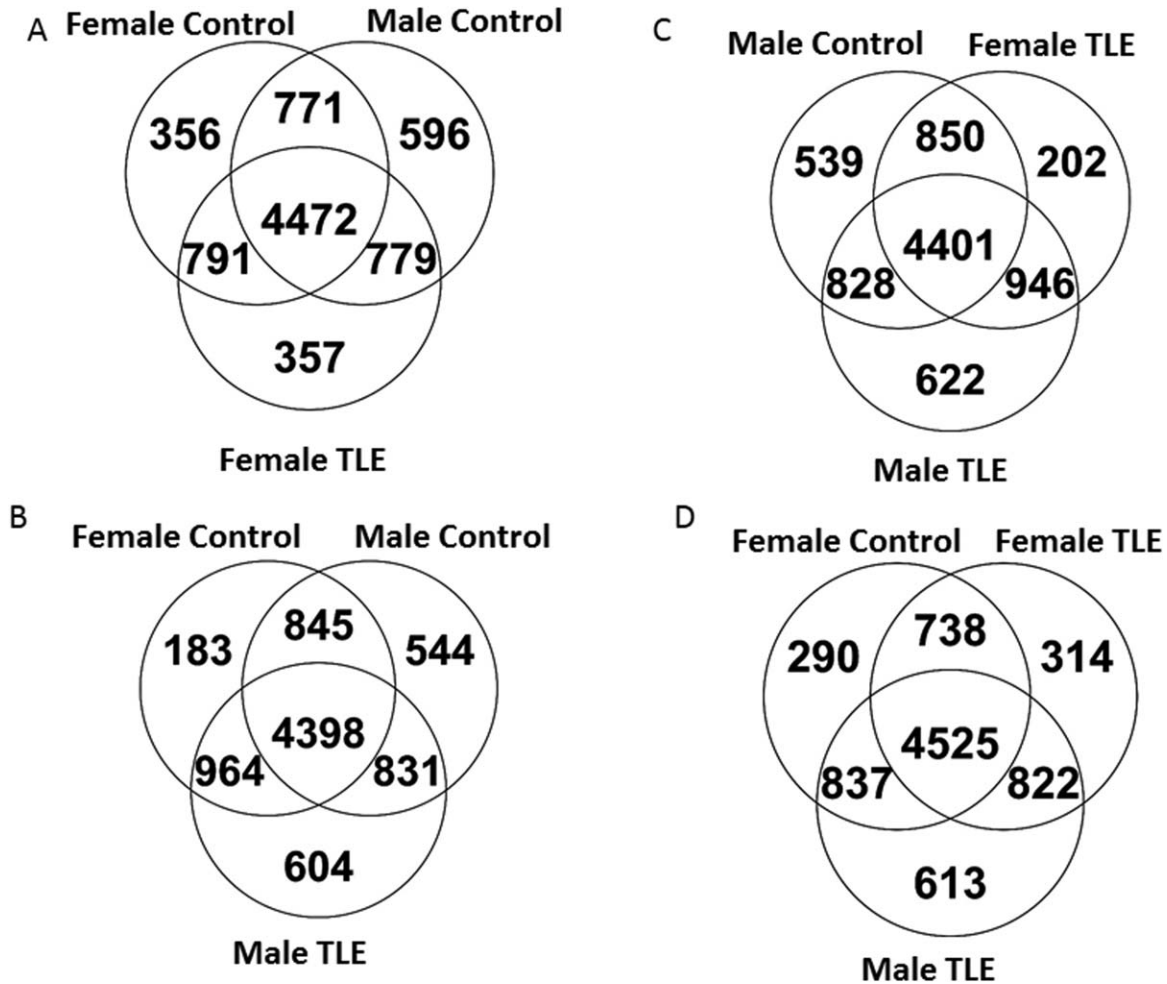
in DG have also been detected and are reported in Table 2. Identification of the peptides was performed firstly by querying a database of peptides such as Swepep (Falth et al., 2006, 2007, 2008) or then by MS/MS (Table 2). Comparison of the  $m/z$  values with those in the Swepep databank data suggests that the peptide measured at  $m/z$  3,155.1 located in the granular layer of the DG is galanin. Similarly, the measured peptide at  $m/z$  1,210.4 with the same distribution corresponds to neurokinin B as was also mentioned by (Schwarzer et al., 1995; Shughrue et al., 1996; Magloczky et al., 2000). Two signals,  $m/z$  of 3,454.4 and  $m/z$  1,637.5, corresponded to the C-terminally truncated fragment NPY (1-30) and somatostin 14, respectively. They were specifically detected respectively in the hilar layer and molecular layers of the DG which is also in line with reports from (de Lanerolle et al., 2010).

### Global Proteomic Analyses of TLE Patients Versus Controls

In order to identify, the protein pattern present in tissue sections from patients (Table 2), tissue proteomic extraction was performed. Proteins extracted from tissues (Fig. 3a) were then

separated in 1 SDS-PAGE. Gels were then cut in eight pieces before subjected to trypsin digestion followed by nanoLC separation and identification by LTQ-orbitrap mass spectrometer. This procedure was performed three times per patient (Fig. 3b). Data were then analyzed in proteome scape software 1.4. The reproducibility in term of number and identification of protein identified is >98% from the three samples per patient analyzed. By taking into account the gender origin of control and TLE a complete study performed in scaffold 4.1.1. (Fig. 4). Proteins (8,435) have been identified with a protein threshold of 99% and at least two peptides minimum based on 96,115 spectra at 95% minimum and 19.5% decoy FDR. Venn diagrams (Fig. 4) showed that male TLE contains similarly the same number of proteins that in controls (1,450 vs. 1,375, respectively) and same conclusions can be drawn between female TLE and control (1,052 vs. 1,147, respectively). The specific proteins detected in TLE patients correspond to 27.1% in male versus 19.6% for the female. Comparison between control and TLE specific identified proteins in function of the gender, showed that male TLE contain 29.9% specific proteins whereas female TLE contain less specific proteins (21.5%). Three hundred fifty seven specific





**FIGURE 5.** Venn diagrams obtained after Scaffold analysis based on label free quantification taking into account the gender of control and TLE patients. Each experiments were performed three times per sample. Specific proteins to male or female TLE have been identified (Supporting Information data 1). (A) Comparison between male control, female control and female TLE. (B) Comparison between male control, female control and male TLE. (C) Comparison between male and female TLE and male control. (D) Comparison between male and female TLE and female control.

proteins have been detected in female TLE versus 596 in male control and 356 in female control (Fig. 5A) versus 604 in male TLE versus controls (Fig. 5B) whereas comparison between male control, male TLE and female TLE gave 622 specific for male TLE and 202 for female TLE (Fig. 5C). Similarly, comparison between female control, female TLE and male TLE gave 613 specific for male TLE and 314 for female TLE (Fig. 5D). These data confirm the fact that male TLE expressed more specific TLE proteins than female TLE ( $613 \pm 6$  vs.  $291 \pm 21$ , respectively).

Table 3 represents a list of selected proteins identified from the 10 patients (male and female mixed) selected according to their score in label free quantification (Fig. 6). The complete protein list identification can be found in Supporting Information Data 1. As showed in Table 3, proteins identified are involved in neurites outgrowth, neuronal differentiation,

tubules polarization, cell migration, cytoskeleton network, intracellular contacts, growth factors, cellular signaling and tumor suppressors. Among the tumor suppressors known to be implicated in TLE, the leucine rich glioma inactivated 1 gene (LGI1) has been identified (Kalachikov et al., 2002). These data clearly show that specific proteins in hippocampus are related to the gender female or male (Fig. 6). In fact only in male, tumor suppressors (TS) have been identified including DMBT1, TUSC2, MGEA5; GBAS, CNBP2 and LIG1. These TS proteins present a specific signature in male TLE (Table 3). This signature is completed with the presence of specific hormones the Follistatin related protein 4 and BPI fold coninating family B member 1, neurites outgrowth (slit-robo rho GTPase activating protein 3, G protein regulated inducer of neurite outgrowth), and proteins know to be implicated in Alzheimer (amyloid beta A4). This signature is

TABLE 3.

List of selected proteins identified from 10 male and 10 female patients showing their molecular functions and their representation following the gender. [Color table can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Protein reference	Protein name	Function	Male	Female
LG11_HUMAN	Leucine-rich glioma inactivated 1	Tumor suppressor		
TUSC2_HUMAN	Tumor suppressor candidate 2	Tumor suppressor		
DMBT1_HUMAN	Deleted in malignant brain tumors 1	Tumor suppressor		
CNDP2_HUMAN	Metallopeptidase 20	Tumor suppressor		
GBAS_HUMAN	Glioblastoma amplified sequence	Tumor suppressor.		
MGEA5_HUMAN	meningioma expressed antigen 5	Tumor suppressor		
PVRL1_HUMAN	Poliovirus receptor-related 1	Heterophilic interactions have been detected between PVRL1/nectin-1 and PVRL3/nectin-3 and between PVRL1/nectin-1 and PVRL4/nectin-4		
FSTL4_HUMAN	Follistatin related protein 4	Inhibits the anterior pituitary's secretion of follicle-stimulating hormone		
GRIN1_HUMAN	G protein regulated inducer of neurite outgrowth 1	Neurites outgrowth		
BPIB1_HUMAN	BPI fold-containing family B member 1	May play a role in innate immunity in mouth, nose and lungs.		
SRGP2_HUMAN	SLIT-ROBO Rho GTPase activating protein 3	Attenuate RAC1 signaling in neurons		
APBA1_HUMAN	Amyloid beta (A4) precursor protein-binding	Modulate processing of the beta- amyloid precursor protein (APP) and hence formation of beta-APP		
REEP2_HUMAN	Odorant receptor accessory protein 1	Enhance the cell surface expression of odorant receptors		
OPTN_HUMAN	Optineurin	Part of TNF alpha signaling pathway		
OPALIN_HUMAN	Oligodendrocytic myelin paranodal and iner loop protein	cell proliferation and differentiation		
AP2S1_HUMAN	Adaptor-related protein complex 2	Adaptor protein complexes function in protein Transport via Transport vesicles in different membrane traffic pathways.		
SRC8_HUMAN	Cortactin	Cellular growth regulation and transformation		
SESN2_HUMAN	Sestrin 2	Involved in the reduction of peroxiredoxin		
CTCF_HUMAN	Transcriptional repressor CTCF	Acts as transcriptional repressor binding to promoters of vertebrate MYC gene and BAG1 gene		
ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	Involved in protein-protein interactions that result in protein complexes, receptor-ligand binding or cell adhesion.		
LIN7C_HUMAN	Lin-7 homolog C	Ensures the proper localization of GRIN2B (subunit 2B of the NMDA receptor) to neuronal postsynaptic density		
ABI1_HUMAN	Abl-interactor 1	Plays a role in regulation of EGF-induced Erk pathway activation		
BMR1B_HUMAN	Bone morphogenetic protein receptor type-1B	Receptor for BMP7/OP-1 and GDF5		
SFXN5_HUMAN	Sideroflexin-5	Potential iron transporter		
IRF2_HUMAN	Interferon regulatory factor 2	Antagonizes IRF1 transcriptional activation		

TABLE 3. (continued).

Protein reference	Protein name	Function	Male	Female
NRP2_HUMAN	Neuropilin 2	High affinity receptor for semaphorins		
CNTFR_HUMAN	Ciliary neurotrophic factor receptor	Binds to CNTF		
CADM1_HUMAN	Cell adhesion molecule 1	Mediates homophilic cell-cell adhesion in a Ca(2+)- independent manner		
CLAP1_HUMAN	Cytoplasmic linker associated protein 1	Required for the polarization of the cytoplasmic microtubule arrays in migrating cells		
AIDA_HUMAN	Axin interactor, dorsalization associated	Antagonizes a Wnt/beta-catenin-independent dorsalization pathway activated by AXIN/JNK-signaling		
MAP7_HUMAN	Microtubule-associated protein 7	Plays a role in the formation of intercellular contacts		
RHOC_HUMAN	Ras homolog gene family, member C: RHOC	Regulates a signal transduction pathway		
NTRI_HUMAN	Opioid binding protein/cell adhesion molecule-like	Neural cell adhesion molecule		
CAPR1_HUMAN	Cell cycle associated protein 1	Regulate the transport and translation of mRNAs of proteins involved in synaptic plasticity in neurons and cell proliferation		
KIT_HUMAN	KIT - v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	Receptor for stem cell factor (mast cell growth factor).		
ARHG7_HUMAN	Rho guanine nucleotide exchange factor (GEF) 7	Functions in cell migration		
PKP2_HUMAN	Cell adhesion molecule 1	Mediates homophilic cell-cell adhesion		
DESP_HUMAN	Desmoplakin	Major high molecular weight protein of desmosomes		
SPTA1_HUMAN	Spectrin, alpha	Spectrin is the major constituent of the cytoskeletal network		
LIG1_HUMAN	Leucine rich repeat and Ig domain containing 1	Functional component of the Nogo receptor signaling complex (RTN4R/NGFR)		

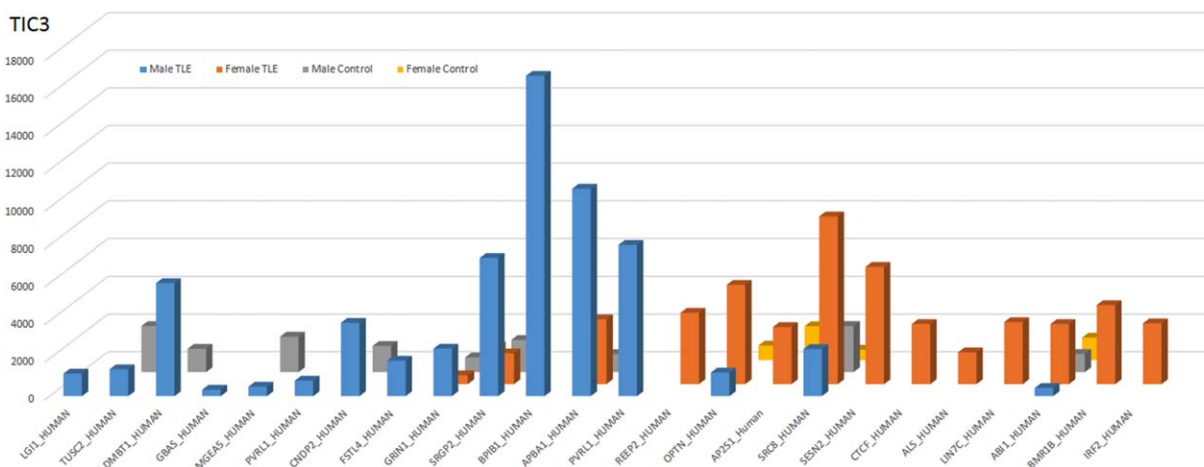
common to the five male TLE samples. In fact individual variations between the five male patients ranged between 1% and 3.8% from the 2,506 common proteins identified based on scaffold analysis.

In female, the signature is based on proteins involved in brain synaptic plasticity (optineurin, opalin, Lin7 homolog C), odorant receptor (odorant receptor accessory protein 1), growth factor (sestrin, insulin-like growth factor, bone morphogenic protein receptor type 1, interferon regulatory factor 2, Abelson interactor 1), and actin-associated cytoskeleton proteins (cortactin, APS2). These data reflect that mechanisms implicated in male and female epilepsy seems really different to each other's.

## TLE and Genders

In male TLE, from the TS identified, the LGI1 protein (Fig. 7) is interesting due to the fact that recent investigations by genetics showed mutations in its gene in approximately 50% of families with autosomal dominant partial epilepsy with auditory features (ADPEAF), and have a penetrance of about 67%

(Rosanoff and Ottman, 2008). LGI1 is expressed normally in the developing and adult central nervous system especially in the neocortex, granule cells of the dentate gyrus and CA3–CA1 region of the hippocampal pyramidal cell layer (Kusuzawa et al., 2012). In our data, LGI1 has been detected in 66.6% of male TLE patients. Moreover, label free quantification performed with Scaffold 4.1.1 based on a protein threshold of 99% and at least two peptides minimum confirmed the presence of neuropeptide precursors as well as their specific receptors in TLE patients (Fig. 8). Secretogranin-1, secretogranin-2, galanin, and appetite regulated hormone isoform 2, angiotensinogen and neuroendocrine convertase 2 have been detected in a high level in male TLE whereas pro-enkephalin B and growth hormone 2 have been specifically detected in female TLE. Regarding their receptors, male TLE specifically contained NPY type 2 receptor, vasopressin as well as the mu opioid slice variant receptors. Female TLE highly expressed VIP and leptin receptors. These results confirmed the data obtained in MSI (Fig. 2). Moreover, these data also revealed that endocrine hormones and their receptors are highly involved in TLE. Male



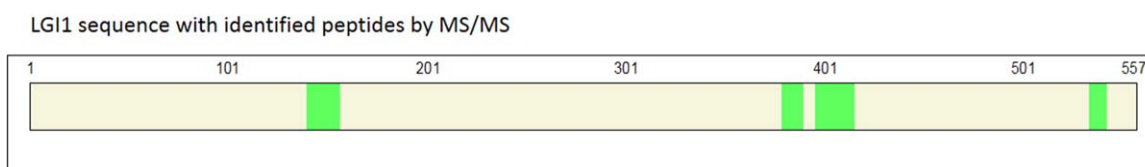
**FIGURE 6.** Label Free quantification of identified proteins specifically in male or female. The normalization has been performed based on the Total Ion Current (TIC) of the three major proteins. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

TLE highly expressed steroid hormone receptor and FSH receptors while Female TLE have GH2 and LHRH receptors. These data showed that endocrine and hormones are involved in TLE and seems to be gender dependent.

## DISCUSSION

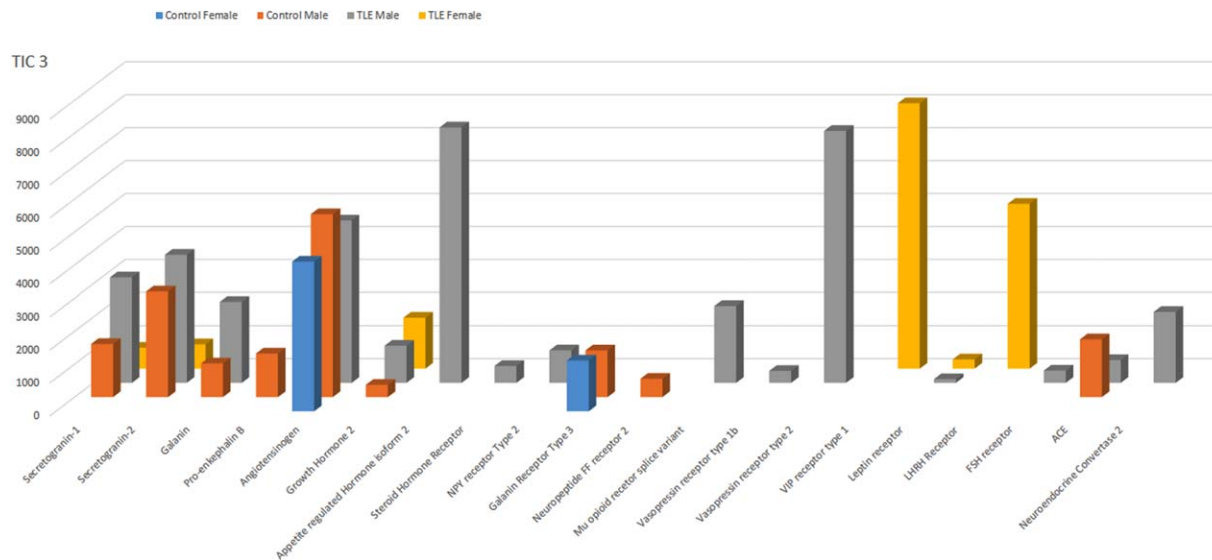
This study clearly establishes that specific neuropeptides are involved in TLE and is in line with previous work based on immunocytochemistry or in situ hybridization. In fact, the presence of galanin in the DG region is in accordance with previous reports by other groups (Melander et al., 1986; Harrison and Henderson, 1999; Mazarati et al., 2000; Petersson et al., 2000; Schott et al., 2000). Neurokinin B has been previously mentioned by (Schwarzer et al., 1995; Shughrue et al., 1996; Magloczky et al., 2000). For C-terminally truncated fragment NPY(1–30) and somatostatin 14 which have been specifically detected in the hilar layer and molecular layers of the DG respectively, these data are in line with reports from (de Lanerolle et al., 2010). Processing of NPY resulting in the formation of the C-terminally truncated fragment NPY (1-30) has previously been demonstrated in the hippocampus by the group of Silberring (Schwarzer et al., 1995; Stenfors et al., 1997). NPY is normally contained in GABAergic interneurons in DG, many

of which also contains somatostatin and cortistatin and give rise to the dense NPY innervation of the dentate outer molecular layer (Decressac et al., 2011). In epilepsy, there is an up-regulation of NPY and NPY receptor expression in the DG after seizures and this may be important in seizure-induced neurogenesis (Furtinger et al., 2001, 2002, 2003; Kokaia, 2011; Gotzsche et al., 2012). NPY seems to be intimately associated with diseases that affect the hippocampus, including a possible role in modulating neurogenesis in some pathological conditions. The neuropeptide somatostatin is expressed in a discrete population of interneurons in the DG. These interneurons have their soma in the hilus and project to the outer molecular layer onto dendrites of DG cells, as can be seen in the MALDI MSI images (Fig. 2). Somatostatin-containing interneurons are very sensitive to excitotoxicity, and thus are vulnerable to a variety of neurological diseases and insults, including epilepsy. SST plays an important role in cognition by modulating the response of neurons to synaptic input. In the dentate, somatostatin and the related peptide cortistatin reduce the likelihood of generating long-term potentiation, a cellular process involved in learning and memory (Qiu et al., 2007; Tallent, 2007; Tallent and Qiu, 2008). Somatostatin and cortistatin are important neuromodulators in the DG, and disruption of this signaling system has a major impact on hippocampal function. For the CART peptide, recent studies in immunohistochemistry have shown that mossy cells express this peptide (Seress et al., 2009). In most cases the



**FIGURE 7.** Sequence of the LGI1 tumor suppressor protein. In green are presented the peptides characterized by MS/MS. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]





**FIGURE 8.** Label Free quantification of neuropeptides precursors, receptors, enzymes and hormones from control and TLE patients taking into account their gender. The normalization has been performed based on the Total Ion Current (TIC) of the three major proteins. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

number of mossy cells was reduced in the epileptic hippocampi than in controls; however, there was a significant loss of pyramidal cells and a partial loss of granule cells in the same epileptic hippocampi in which mossy cell loss was apparent. The loss of mossy cells could be correlated with the extent of hippocampal sclerosis, patient age at seizure onset, duration of epilepsy, and frequency of seizures (Seress et al., 2009). Taken together, the localization of the identified neuropeptides in the different layers of DG by MALDI MSI reinforced the data obtained by immunohistochemistry and confirmed their involvement in TLE. This is important as they can serve as potential targets for drug treatments. Moreover, the shot-gun study confirms the presence of the neuropeptides precursor consolidating the MALDI MSI data. The comparison between TLE patients and controls taking into account the gender of the patients established the presence of specific proteins in TLE. Among the specific proteins several tumor suppressors (TS) have been detected. The presence of the LGI1 is interesting since genetic linkage studies placed LGI1 mutation has one of the cause of the hereditary form of autosomal dominant partial epilepsy with auditory features (ADPEAF) (Fukata et al., 2006). Recent work has shown that the DEP (Dishevelled containing protein) domain containing 5 (DEPDC5) TS is also implicated in TLE heredity (Ishida et al., 2013). We have demonstrated in this study that five other TS have been discovered and especially in male TLE patients. Some have been detected in schizophrenia like the meningioma expressed antigen 5 (MGEA5) (Moon et al., 2006) or in epilepsy associated to brain tumors like the deleted in malignant brain tumors 1 (DMBT1), CD66a, glioblastoma amplified sequence (GBAS), Tumor suppressor candidate 2 (TUSC2) (Stefan and Theodore, 2012). In male the presence of follistatin related protein (FSRP) is in line with previous works showing link between sex steroid hormone levels,

the hypothalamic–pituitary axis, and testicular function (Montouris and Morris, 2005). Moreover, some proteins identified in this study have previously been detected in autism or schizophrenia pathologies like proteins involved in neurites outgrowth (SRGP2 or the LSAMP, GRIN1), neuroligins, beta amyloid protein (Martucci et al., 2003; Bailey et al., 2008; Gandal et al., 2012). Concerning the data obtained in female TLE patients, these are more related to NMDA receptors or signaling (LIN7C). In female, hormones seem to have a major impact in TLE especially through NMDA receptors. In fact, estradiol level increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input. Estrogen also dynamically alters synaptic connectivity which is in line with the proteins we identified (NCS1, Synaptopodin, AP2S1, contactin associated protein like 2, and cortactin). The net effect of these steroid actions is to alter neuronal excitability over physiological cycles (Morrell, 1999).

Taken together, considering the protein profiles obtained in male of in female TLE, a sexome can be observed. This sexome is linked to TLE pathological process which is different between male and female. Previous data have shown that a gender susceptibility to the development of specific epilepsy subtypes. In fact, cryptogenic localization-related epilepsies seem more frequent in women and localization-related symptomatic epilepsies in men (Christensen et al., 2005). Our data confirm this assumption.

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