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Morphological Aberrations in Therapy-Resistant Partial Epilepsy (TRPE)

Confocal Laser Scanning and 3D Reconstructions of Lucifer Yellow Injected Atypical Pyramidal Neurons in Epileptic Human Cortex

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Abstract

Epileptic temporal and parietal cortices, removed from 6 patients with therapy-resistant (intractable) partial epilepsy (TRPE) during neurosurgery, were studied. Neurons (40–50 in each slice) in laminae I–VI and white matter were injected with Lucifer Yellow (LY). Samples were examined in a confocal laser scanning microscope (BioRad [Richmond, CA] MRC 600), and individual cells were scanned at 0.1–2 μm incremental levels. 2D maximal linear projection was used for overview. Frames (50–60) of scanned neurons were transformed into 3D volumes, using VoxelView software on a Silicone Graphics workstation, and rotated. All samples contained pyramidal neurons with duplicated apical dendrites, additional basal dendrites, or were misplaced in a horizontal position in the white matter. Rarely were such cells observed in normal cases. The relation between the observations and the disease is discussed.

The attempt to simultaneously apply immunofluorescence was successful concerning synaptic vesicle antigens. This approach will be used for a detailed study of the synaptology of this disease.

Index Entries: Epilepsy; human cortex; Lucifer Yellow; intracellular injections; pyramidal neurons; confocal laser scanning; 3D reconstructions; morphology; immunofluorescence.

Introduction

The Jacksonian type of partial epilepsy affects the motor cortex of the contralateral frontal lobe. However, most of the cases of partial epilepsy originate in the temporal lobes, either in the limbic structures or the temporal neocortex. A substantial number of these cases have proven to be resistant to anticonvulsive

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medical treatment, and this condition is now classified as *therapy resistant (intractable) partial epilepsy* (TRPE). The cause of TRPE is so far unknown, but specific aberrations in the architectonic arrangement of the neuronal elements are suggested to form the basis of this condition (*see*, for example, refs. *1,2*). Many of the cases are now suitable for surgical removal of the cortical region demonstrated to be the epileptogenic zone using a number of clinical and electrophysiological tests. The cortical samples, removed from such patients, form the basis of the present investigation.

Neuropathological findings from such cases include, in about 20% of the cases, the diagnosis of "mild cortical dysplasia" (=microdysgenesis), which is defined by:

1. Ectopic nerve cells in the molecular layer (lamina I);
2. Ectopic nerve cells in the subcortical white matter;
3. Clustering of neurons;
4. Indistinct lamination; and
5. Increased columnation of the neocortex *(3)*.

For a diagnosis of "microdysgenesis" at least 2 of the above criteria must be fulfilled *(3,4)*.

Until now, all studies on surgically removed epileptic cortices have been performed at the 2D light microscopic level, and have focused on populations of nerve cells, not individual neurons. Since it is important to study individual neurons that are 3D, their position, form, and details of the connections between single nerve cells, the present study was undertaken. We have used the recently developed Lucifer Yellow (LY) intracellular staining technique *(5,6)* together with confocal laser scanning *(7,8)* and have made an attempt to combine this approach with immunofluorescence of, i.e., synaptic constitutents. This would enable detailed investigations on transmission related antigens in relation to the LY-labeled cells.

Materials and Methods

Patients

Six cases of TRPE were investigated at the time of the conference. (Since then 5 more cases have been investigated, and these will be described in a separate publication)

Male Born 1973

Seizures since the age of 10 yr. The left temporal lobe was resected September 1990, and we reoperated with extended temporal lobe resection September 1991. PAD showed supernumerary nerve cells in the white matter and indistinct border between layers I and II owing to increased number of nerve cells in lamina I. *Diagnosis: Microdysgenesis, gliosis.*

Female Born 1963

Seizures since the age of 22 yr, starting after encephalitis. The left temporal lobe was resected October 1991. PAD showed diffuse border between gray and white matter, with many neurons observed in lamina I. Marked gliosis in both white and gray matter. *Diagnosis: Microdysgenesis, gliosis.*

Female Born 1966

Seizures since the age of 7 yr. The posterior part of the left parietal lobe was resected October 1991. PAD showed focal presence of very large neurons in cortex and white matter. Bizarre giant astrocytes and giant neurons in white matter. *Diagnosis: Focal cortical dysplasia.*

Male Born 1945

Seizures since the age of 19 yr. The anterior right temporal lobe was resected September 1992. PAD showed gliosis, but no other neuronal aberrations observed light microscopically. *Diagnosis: Gliosis.*

Male Born 1967

Seizures since the age of 15 yr. The anterior right temporal lobe was resected November 1992. PAD showed gliosis, but no other neuronal aberrations observed light microscopically. *Diagnosis: Gliosis.*

Female Born 1987

Seizures since the age of 1.5 yr. The left parietal lobe was resected November 1992. PAD showed many ectopic neurons in the white matter, and in laminar I, and gliosis. *Diagnosis: Microdysgenesis, gliosis.*

Control Material

The opportunities to collect normal brain specimens are scarce. We chose not to use material removed during neurosurgery operations from patients with brain tumors or traumatic cases, since the validity of these as "normal control" tissues may be debated. Instead we have collected autopsy material from three neurologically healthy cases, where the postmortem time was kept within 6 h. Areas from the temporal and parietal cortex areas were used.

Intracellular Injections

Fresh brain samples, removed from the six TRPE patients at neurosurgery (or autopsy material, *see above*) were immersion fixed in 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). A small block of the cortex (2 × 1 × 1 mm) was excised from the tissue, immersed in the same cold fixative for 1 h, and then rinsed in PB. The tissue was serially sectioned perpendicular to the pial surface with an Oxford vibratome at 150 μm thickness and stored at 4°C. At injection, slices were floated onto a glass plate, and held down by a Millipore (Bedford, MA) filter with a slightly smaller window than the underlying tissue. The preparation was immersed in a Petri dish with PB and this injection chamber was transferred to a fixed-stage microscope (Zeiss ASM) positioned on a vibration isolation table. A glass microelectrode was filled with 6% aqueous solution of LY (Molecular Probes, Eugene, OR), which has a strong greenish-yellow fluorescence and a quantum yield of 0.2–0.4, which does not change with pH *(7)*.

A number of pyramidal and nonpyramidal neurons in a few sequential slices were iontophoretically injected. Cells selected for injection were visualized by the presence of autofluorescent lipopigment (lipofuscin and/or ceroid) particles under the visual control by fluorescent microscopy. After penetration of the microelectrode into the cell body, LY was iontophoretically injected by a negative constant current of 2–5 nA for 5–10 min until all fine dendritic branches and collaterals of axons appeared brightly fluorescent *(6,7)*. Up to 40–50 neurons were injected in each slide. The preparations were mounted on a microscope slide and coverslipped with glycerol.

3D Reconstruction

A confocal laser scanning instrument (Bio-Rad MRC 600, Lasersharp UK), coupled to a NIKON Optiphot FX microscope) and the VoxelView/GT program (Vital Images, London, UK) running on a Silicone Graphics work station, were used for analysis. The selected neurons were scanned at 0.1–2 μm increments, and 10 scans (N) at each incremental level were collected in the Kalman filtering mode. Incremental levels were collected through the thickness of each studied neuron, usually 50–70 focal levels. The stack of 2D files was then transferred to the Silicon Graphics workstation, and interpreted into 3D volumes *(9)* using the VoxelView software from Vital Images. This volume could then be rotated in various directions to enable a detailed study of individual dendrites and their branchings. Photographs were taken off screen with an external camera, using Kodak Ectachrome 200 ASA diapositive film. The 2D reconstructions, created by maximal linear projection of the frames in the stacks, were photographed from the Bio-Rad monitor, using Kodak 400 ASA Tri-X film.

Immunofluorescence

We tested the possibility to combine the LY injections with immunoincubations, and chose to incubate with antisera against various synaptic vesicle components (antisynapsin I or anti-p38; *10*). The incubations were performed after as well as before the LY injections, using free floating slices. Triton X-100 inclusion in the incubation media was found to be deleterious for the LY fluorescence, which leaked out from the injected cells and blurred the picture. The best results were achieved when the immunoincubations were performed, without detergents, for an extended (doubled) period of time, after the LY injections were performed. Only Texas Red-labeled secondary antisera could be used in the dual channel scanning system of the Bio-Rad 600 CLSM. This fluorophore gives a fluorescence wavelength that has a good separation in the "red channel" from the LY-induced fluorescence, which is recorded in the "green channel" of the instrument *(11)*.

Results

LY-Injected Neurons

Patient #1

LY-injected nerve cells showed to be pyramidal neurons in laminar III and V with atypically branching apical and basal dendrites (Fig. 1). Also inverted pyramidal neurons, in addition to many subcortical pyramidal neurons, many with horizontally oriented dendrites, were observed (Fig. 1A).

Patient #2

LY-injected nerve cells were pyramidal neurons in laminar III with two or three apical dendrites (Fig. 1B) and inverted pyramidal neurons in laminar V, with one apical dendrite, were present. Some large neurons, probably atypical pyramidal neurons, were present in the white matter (Fig. 1A).

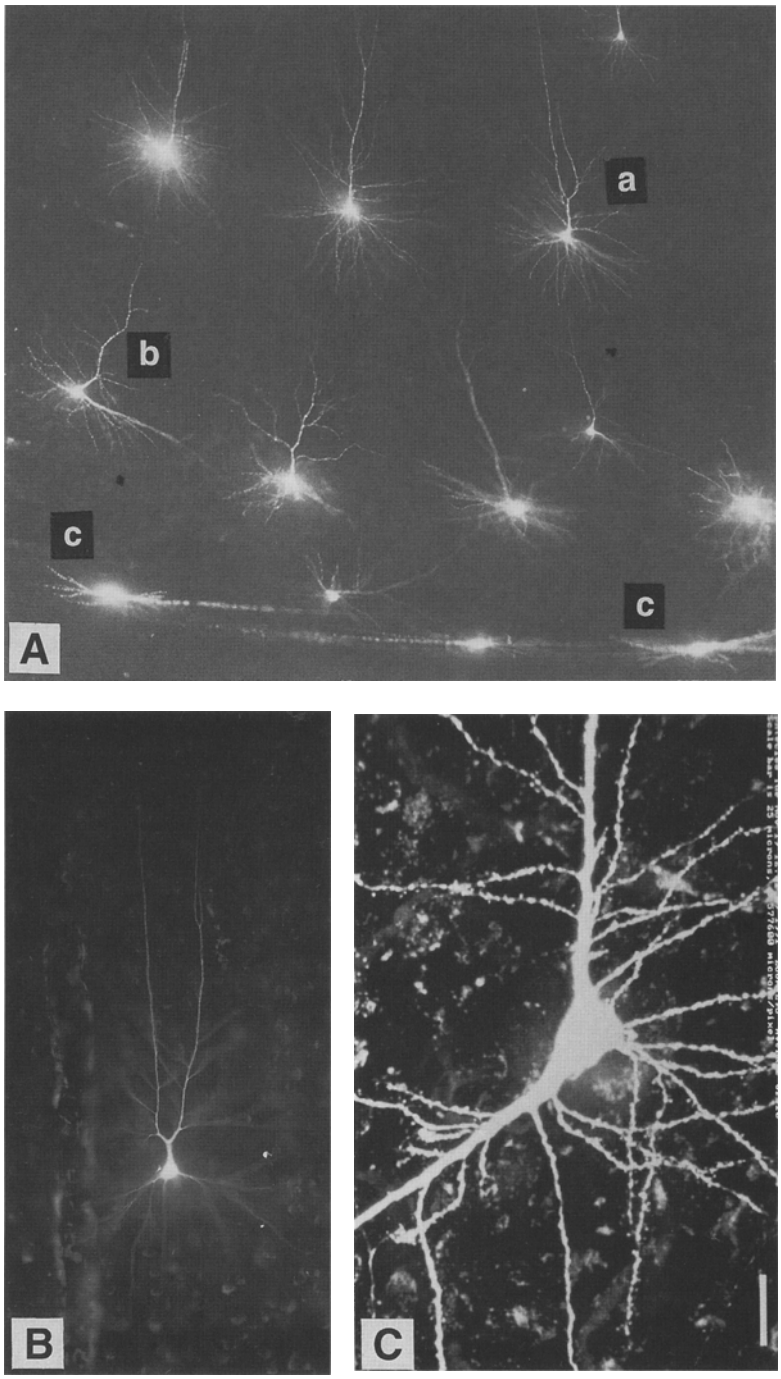

Fig. 1. **(A)** Different types of neurons in the temporal cortex removed at surgery from patient (#1) with intractable partial epilepsy (IPE), visualized after intracellular injection with Lucifer Yellow. Nine neurons were injected. Some have a normal appearance, but the one marked (a) has a forched apical dendrite, the one marked (b) has a thick basal dendrite, and (c) are aberrant pyramidal neurons residing in the white matter. Fluorescence microphotograph. Bar indicates 20 μm. **(B)** Neuron with a bifurcated apical dendrite, photographed in the epifluorescence microscope. A blood vessel is seen to the left. Bar is 20 μm. **(C)** Neuron with one apical and one basally projecting thick dendrite (a "dinosaur" neuron). Confocal laser scanning picture of a 2D reconstruction of 30 incremental levels collected at 2-μm intervals, and projected using the maximal projection command. The details of the various dendritic branches of this LY-injected neuron is very clear, and single spines can be identified. Autofluorescent pigment in surrounding nerve cells are indicated by arrows. Bar is 25 μm.

Patient #3

In specimens from this patient, LY-injected pyramidal neurons in laminar III and V had aberrant apical dendrites, and a basal large dendrite ("dinosaur cell," Fig. 1C). Also, horizontally oriented neurons in the white matter were observed.

Patients #4 and 5

Many pyramidal neurons in laminar II had bifurcate apical dendrites. A few neurons were observed in the white substance, but the number was clearly less than in patients 1–3.

Patient #6

LY-injected pyramidal neurons in laminar III and V had aberrant apical dendrites, and a basal large dendrite ("dinosaur cell"). From the basal dendrites, branches were often seen to turn toward the pial surface (Fig. 2). Also, horizontally oriented neurons in the white matter were frequent.

In the control and autopsy specimens the lamination was distinct, and only occasionally could we observe neurons in the lamina I. A few atypical pyramidal cells with bifurcated apical dendrites and a thick basal dendrite ("dinosaur" cell) were detected. No neurons could be seen in the white matter (Fig. 3).

The injected nerve cells were registered and the occurrence of the various morphological variants and their locations were marked in a large drawing of the whole slice. A summary of the observations is given in Table 1.

Immunofluorescence

The penetration of antibodies could be decided with accuracy in the CLSM. Whereas the LY-injected cells were distributed through the thickness of the slice, immunofluorescence could be observed to extend up to 15 μm from each surface (free floating sections incubated). This enabled the observation of immunoreactive material in relation to LY-labeled dendrites within a limited thickness, but still this combination of methods (the LY injection method and immunofluorescence) is of potential great value, since, e.g., transmitter related substances in structures near labeled normal or atypical neuronal dendrites can be studied.

The immunofluorescent picture after incubation with synaptic vesicle-directed antibodies is shown in Fig. 2. A large number of fluorescent spots, probably representing synaptic boutons, could be seen, often in direct closeness to LY-labeled dendritic ramifications.

Discussion

The intracellular injection technique using highly fluorescent naphtalimide tracers, e.g., LY, was originally developed by Stewart *(12,13)*. The technique was first described for fixed tissues in 1984 *(14)* and later demonstrated in slice preparations of fixed tissue in 1986 *(15,16)*. It was further developed and described in detail in 1989 by Buhl and Lübke *(5)* and Buhl and Schlote *(17)*. The first application of the technique on human autopsy material was described by Einstein in 1988 *(18)*. We have now used this LY injection method *(6)* for human brain tissue and combined it with CLSM, which has enhanced considerably the usefulness of the technique *(7,8)*.

The morphological features of individual neurons constitute a basis of our understanding of both normal and abnormal functions of the nervous system. Since neurons are inherently 3D, the traditional morphological characterization in two dimensions is insufficient. Using this technique to fill single neurons and their arborizations with fluorescent dyes (which can be photooxidized to electron dense products), in combination with confocal laser scanning microscopy *(7,8)*, coupled to powerful computer hard- and software (Bio-Rad MRC 600 and Silicon Graphics IRIS work station with VoxelView 3-D imaging program) we have undertaken a study of the architecture of individual neurons in neocortex specimens, surgically removed from patients with TRPE. A major advantage of confocal laser scanning is the rejection of out-of-focus plane (for a description of the use of CLSM in biological tissues, *see* refs. *19,20*), allowing true optical sections to be recorded in digital format, and facilitating computer manipulation and image reconstruction *(9)*.

The preliminary results described in the present article show different types of interesting atypical morphologies of single neurons in temporal and parietal cortices in laminar III, laminar V, and subcortical white matter in patients suffering from TRPE. Such large atypical architectonic variations were not observed in earlier investigations, using LY injections, on autopsy material from non-epileptic cortices *(6)*. However, in normal cortices a large variation among neurons in dendritic morphology has been described in Golgi impregnated material (e.g., *21*). Also, the presence of neurons in the white subcortical matter has been observed *(22)*. Therefore, the relation between the observed irregularities and the disease requires a careful statistical analysis, which is presently being performed

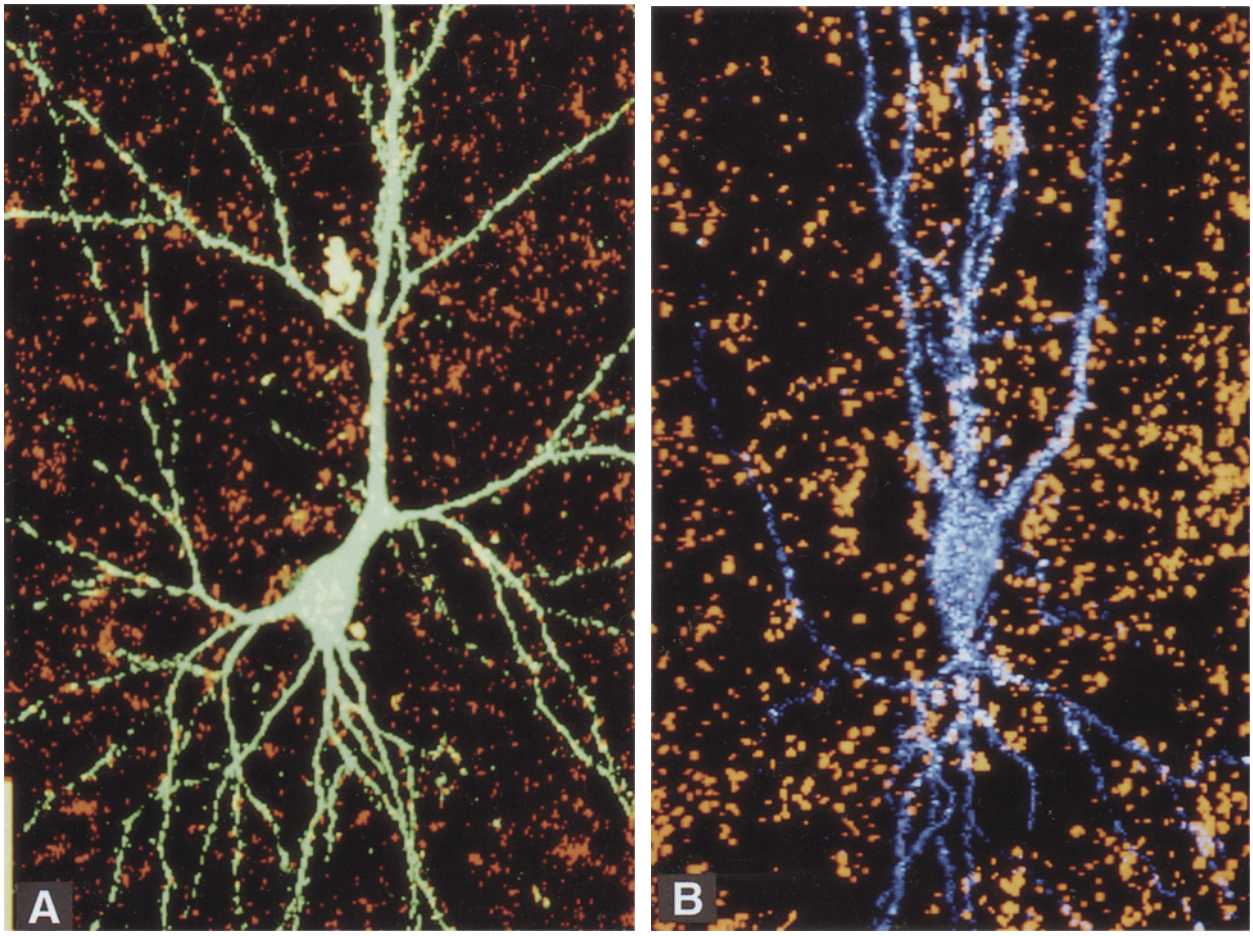

Fig. 2. Confocal laser scanning pictures of two pyramidal neurons injected with LY, and incubated with antisera against synaptic vesicle antigens. The slices were scanned in the Bio-Rad 600 instrument using dual channel registration mode after activation of fluorescence with the krypton/argon laser. For the neurons 50 frames at 1-μm increments were collected and projected linearly using the maximal projection command. The red immunofluorescence was scanned at 1-μm increments, and 12 frames were projected with the maximal linear projection command. The two sets of data were then merged together using the VolumeMath software from the VoxelView program. **(A)** A pyramidal neuron from lamina III located in the posterior parietal cortex, removed from patient (#3) with TRPE. After injection of LY into the neurons this slice was immunoincubated with rabbit antiserum against the synaptic vesicle protein p38 (synaptophysin) in the first incubation, followed by biotinylated goat-antirabbit-IgG, and Texas Red-labeled streptavidin. LY is seen in green, whereas the Texas Red-labeled immunoreactive elements (dots presumably representing synaptic boutons) are seen in red (pseudocolors). An abnormally deviating dendritic branch, emanating from the basal dendrite is indicated (arrow). **(B)** This slice was, after injection of the LY, immunoincubated with rabbit-antisynapsin I, followed by the secondary incubations as described in A. The neuron is pseudocolored in blue. This neuron has many apical dendrites (arrows) and one deviating basal dendritic branch (arrowhead), projecting toward the pial surface. The bars represent 25 μm. Direction of pial surface to the top.

including the five additional cases of TRPE that were processed since the time of the meeting.

However, it is likely that TRPE is triggered by multiple causes, one of which may be the architectonic aberrations observed to be more frequent in TRPE tissues than in control material (Table 1). In some of the patients described, the onset of seizures was connected to trauma or infection, which may

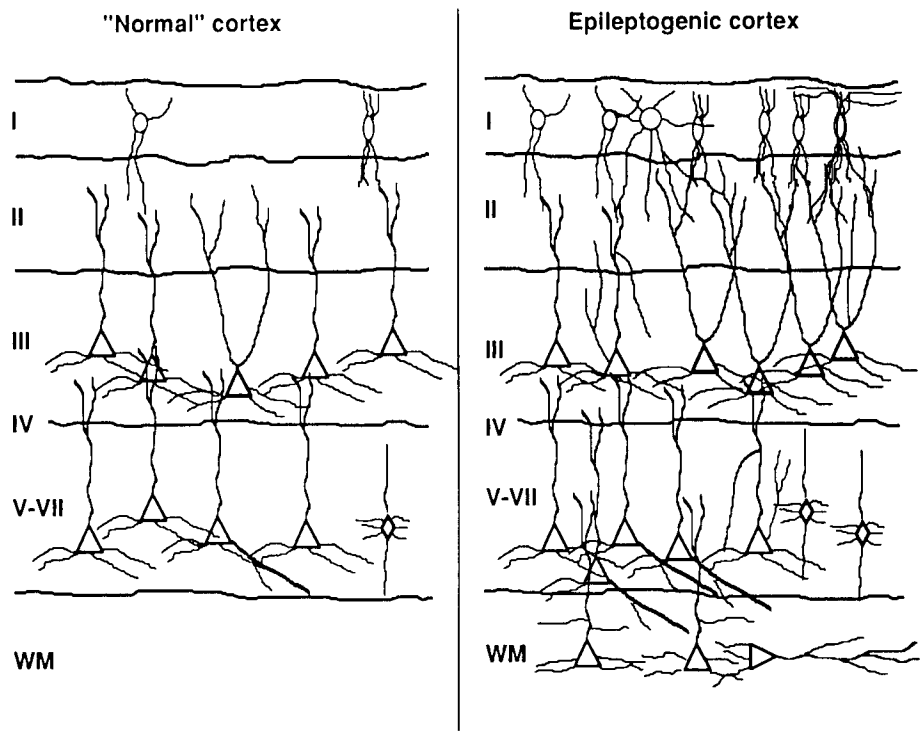

Fig. 3. White matter of control autopsy specimens. Note the lack of neurons.

Table 1
Characterization of LY-Injected Pyramidal Nerve Cells in Temporal Cortices

	"Normal" appearance	"Dinosaur" cells	Inverted pyramids	Strange morphology	White matter
Control cortex	50%	45%	5%	0%	0%
TRPE cortex	25%	45%	10%	10%	10%

The approximate percent of the distribution of counted cells in control and TRPE cortical specimens.

have played the role of "the drop overflowing the goblet" in a situation when a buffering capacity of the brain was needed to balance/compensate for an abnormal neuronal wiring.

Conclusions

Epilepsy is still poorly understood. The intracellular injection method, combined with confocal laser scanning and 3D reconstructions, was used in the present study to investigate the morphology of single neurons in cortex specimens from patients with TRPE. In this study we have observed some clear differences in cortical architectonics and morphology between cortices from patients with TRPE and normal cases:

1. Lamination was poor;
2. Many nerve cells were present in laminar I;
3. Many nerve cells were also located in the white matter;
4. Inverted pyramidal cells were frequent;
5. Pyramidal cells with two or three thick apical dendrites, or with a thick basal dendrite ("dinosaur cells") were frequent;
6. Bi- or trifurcated apical dendrites were often observed; and
7. There appeared to be a selective loss of spines over areas of primary and secondary dendrites.

We will continue our studies, as outlined above, in a larger number of disease cases and in normal control material, in order to establish any significant correlation between the occurrence of neurons

with strange morphology, as described in the present article, and TRPE. Other diseases will also be studied, and the combination with immunofluorescent localization of transmitter-related antigens in close proximity to the LY-labeled neurons, demonstrated to be a successful strategy in this article, will be pursued.

Acknowledgments

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