Vigabatrin, the GABA-Transaminase Inhibitor, Damages Cone Photoreceptors in Rats

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Epileptic patients experienced an irreversible loss of their peripheral visual field upon treatment with vigabatrin (gamma-vinyl GABA), an inhibitor of the GABA degrading enzyme, GABA transaminase. Subsequently, central visual function was reported to also be irreversibly altered. This visual loss is associated with a decrease in the electroretinogram measurement localizing the deficit to the retina. To investigate its cellular origin, we treated rats daily with vigabatrin for 45 days. Two days after arresting this treatment, rats exhibited an irreversible decrease in the photopic electroretinogram, the flicker response, and the oscillatory potentials. These functional alterations were associated with a peripheral disorganization of the outer retina. However, photoreceptor damage was not limited to these disorganized areas, but cone inner and outer segments were severely injured in more central areas and their numbers were irreversibly decreased by 17 to 20%. Ultrastructural examination of the retina confirmed the presence of major photoreceptor damages, which were further supported by terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) and caspase-3 activation both indicative of photoreceptor apoptosis. This study suggests that the visual field loss in vigabatrin-treated epileptic patients may result from a sequence of events starting from cone cell injury to a more severe disorganization of the photoreceptor layer.


In epilepsy, enhancing GABA inhibitory neurotransmission has become a major therapeutic strategy to limit the occurrence of seizures. In this context, vigabatrin, gamma-vinyl GABA (VGB), used to be a prevalent drug in the treatment of partial epilepsy, pharmacoresistant epilepsy, and infantile spasms, because it blocks the GABA transaminase, the GABA degrading enzyme. With VGB treatment, the seizure frequency was reduced by at least 50% and many patients were rendered seizure-free. Unfortunately, long-term VGB administration resulted in irreversible visual field constriction in approximately 40% of patients that appeared asymptomatic. Central vision dysfunction were subsequently reported.

In visual electrophysiology, the main change was an amplitude decrease in the photopic electroretinogram (ERG), which can be interpreted as a dysfunction of the cone photoreceptor pathway. This alteration was sometimes associated with a reduction in the scotopic ERG b-wave, suggesting additional perturbations in the rod pathway. Moreover, the reversible EOG amplitude decrease indicated that the cellular interactions between photoreceptors and the retinal pigment epithelium were affected. Inner retinal function, as measured by oscillatory potentials, was occasionally reported to be decreased.

In animals, VGB was found to cross the blood–retinal barrier and to accumulate in the retina at a concentration fivefold higher than in the brain. As a consequence, the GABA concentration also was found to increase sixfold in the retina for control conditions. Immunohistochemical observations showed GABA accumulation in Müller glial cells, whereas GABA is normally located in amacrine cells and often in horizontal cells depending on animal species. Although the VGB treatment was reported to disturb the retinal architecture in the peripheral retina of rats, the cellular origin of the visual constriction was not elucidated.

To further understand the VGB-induced retinal toxicity, we treated rats for 45 days with VGB daily injections. Electroretinogram measurements and histological observations of the retina point to cone photoreceptor damage as an early event in VGB retinal toxicity.

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Materials and Methods

Vigabatrin Treatments

Wistar rats Rj Wi IOPS Han were purchased from Janvier (Le Genest-St-Isle, France) at 7 weeks and acclimatized for 2 weeks. VGB dissolved in 0.9% NaCl at 125mg/ml was daily and intraperitoneally injected at a final concentration of 250mg/kg as described for animal treatment of epilepsy.17 This dose was slightly higher than that prescribed to adult patients (1–6gm/day), children (100mg/kg/day), or infants (150mg/kg/day).18 Control rats received vehicle solution.

Electroretinogram Recordings

ERG were recorded as described previously19 at either 2 days (Group I: 13 rats) or 43 days (Group II: 7 rats) after the last VGB injection. Control animals (n = 12) from the two groups were pooled because they were not statistically different. Anesthesia was induced by an intraperitoneal injection (1–1.5ml/kg) of a solution containing ketamine (40mg/ml) and xylazine (4mg/ml Rompun, Bayer Vital, Leverkusen, Germany). Five recordings were averaged with an interstimulus interval 10 to 20 seconds for the lower level intensities or 40 seconds for higher intensities. Oscillatory potentials (OPs) obtained at 5cdsm⁻² with an interstimulus interval of 30 seconds were isolated by further filtering the ERG responses (100–300Hz). Flicker responses were obtained with 50 light flashes of 2.5cdsm⁻² intensity.

Tissue Preparation for Immunohistochemistry

Eye cups were fixed overnight at 4°C in 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS) 0.01M, pH 7.4. After cryoprotection in PBS containing successively 10, 20, and 30% sucrose at 4°C, the tissue was embedded in tissue. After cryoprotection in PBS containing successively 10, 20, and 30% sucrose at 4°C, the tissue was embedded in OCT (Labonord, Villeeneuve d’Ascq, France). Vertical sections (8–10μm thickness) were permeabilized for 5 minutes in PBS containing 0.1% Triton X-100 (Sigma, St. Louis, MO), rinsed, and incubated in PBS containing 1% bovine serum albumin (Eurobio, Les-Ulis, France), 0.1% Tween 20 (Sigma), and 0.1% sodium azide (Merck, Fontenay-Ss-Bois) for 2 hours at room temperature. The primary antibody added to the solution was incubated for 2 hours at room temperature. Antibodies were anti–GFAP rabbit polyclonal antibody (1:400; DAKO), and antiactive caspase-3 rabbit polyclonal antibody (1:100; Cell Signaling Technology, Beverly, MA). After rinses, sections were incubated with the secondary antibody, goat anti–rabbit IgG conjugated to either Alexa TM 594 or Alexa TM 488 at 1 to 500 (Molecular Probes, Eugene, OR) for 2 hours. Cone photoreceptors were stained with peanut agglutinin lectin coupled to Texas Red (PNA, 1:40; Sigma). DNA fragmentation was demonstrated with the In Situ End Labeling/terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (ISEL/TUNEL) method (Roche Molecular Biochemicals, Penzburg, Germany). The dye, diamidophenyl-indole (DAPI), was added to any incubation solution. Sections were mounted with Fluorsave reagent (Calbiochem, San Diego, CA). PNA-stained cone inner/outer segments were counted on retinal sections in eight consecutive 300μm-wide optical windows on each side of the optic nerve under the ×40 objective. Areas with a disorganized outer nuclear layer were specifically excluded from this count. Vertical sections were stained with hematoxylin and eosin.

Tissue Preparation for Electron Microscopy

Eye cups were fixed overnight at 4°C in cacodylate 0.1M (Merck) containing 2% paraformaldehyde, 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and CaCl₂, 2.5mM at pH 7.4. The tissue was rinsed in cacodylate 0.15M and postfixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 hour. After dehydratation in ethanol, and propylene oxide, the tissue was embedded in epon. Semithin sections were stained with methylene blue and ultrathin sections (80nm) contrasted with lead citrate and uranyl acetate.

Statistical Analysis

Statistical analysis of the results was performed by a two-way analysis of variance with unequal numbers. A probability (p < 0.05) was taken as a criterion for statistical significance.

Results

Functional Evaluation of the Retina

After the VGB treatment, ERG responses to a 1cdsm⁻² flash appeared slightly smaller in treated an-
imals, but the difference was not statistically significant (Fig 1A,B) (a wave, control group: 225 ± 17μV, SEM, n = 12, and treated Group I: 215 ± 20μV, SEM n = 13, b wave, control group: 780 ± 42μV, treated Group I: 628 ± 59μV). Implicit times of the a- and b-waves were not different in treated and control animals (data not shown). These ERG measurements suggested that the rod pathway was not affected significantly by the VGB treatment.

To assess cone function, we measured photopic ERGs in the presence of a continuous background light (25cdm⁻²) saturating rods (see Fig 1C). Photopic ERG amplitudes decreased by 40% in VGB-treated animals (127 ± 17μV, SEM, n = 13) for control rats (211 ± 13μV, SEM, n = 12) at the maximum light intensity (25cdsm⁻² F²₈ = 17.10; p < 0.001; n = 32). The difference was statistically significant at all light intensities (see Fig 1D).

Consistent with the photopic ERG modification, flicker responses were decreased by 37% at 5Hz in VGB

![Figure 1](image-url)
Group I treated animals (107 ± 11 μV, SEM, n = 13) when compared with control animals (169 ± 11 μV, SEM, n = 12) (F^1_{27} = 5.48 p < 0.05, n = 31). The same decrease was observed at 10 and 15 Hz with F^2_{27} = 19.93 p < 0.001 and F^1_{27} = 25.38 p < 0.001, respectively (see Fig 1E, F). Implicit times were not changed.

To determine whether animals could recover from these functional alterations in the cone pathway, we treated rats for 45 days and withheld treatment during an additional 43-day recovery period. In these recovering VGB-treated animals (Group II), scotopic ERGs were still insignificantly decreased (see Fig 1B). In contrast, photopic ERGs (see Fig 1D) and flicker responses (see Fig 1F) remained significantly reduced. These amplitudes were similar and not significantly different from those described above in Group I, but they were significantly different from the values obtained with the control group. These observations indicated that the VGB treatment produced irreversible modifications in the cone pathway.

When inner retinal function was assessed by measuring OPs (see Fig 1G), all individual wavelets (OP1-OP2-OP3-OP4) showed a decrease in amplitude in VGB-treated animals as compared with control responses. OP1 response amplitude was decreased by 17% in the Group I (115 ± 11 μV; n = 13) and 31% in the Group II (96 ± 10 μV; n = 7) for the control group (139 ± 7 μV; n = 12) (F^1_{28} = 6.17; p < 0.05; n = 32). The decrease in OP2 amplitude was similar to that observed for OP1 (see Fig 1G, H). OP3 and OP4 waves appeared more affected by the treatment, and a partial recovery appeared to be observed between Group I and Group II. OP3 wave was decreased by 50% for Group I and 40% for Group II (F^1_{28} = 19.2; p < 0.001; n = 32), whereas the OP4 wave was decreased by 71% for Group I and 48% for Group II (F^1_{28} = 45.64 p < 0.001; n = 32; see Fig 1H). These observations suggested that the VGB treatment can induce irreversible alterations in inner retinal function at this observed recovery time.

Retinal Morphology
To investigate the cellular origin of the decrease in the photopic ERG response, we examined retinal sections of VGB-treated animals shortly after the treatment (Group I, n = 21) and after the longer recovery period (Group II). Most Group I animals (16/21) presented a severely disorganized peripheral outer retina with photoreceptor nuclei found in apposition to retinal pigment epithelium (Fig 2). Photoreceptor inner and outer segments had disappeared in some peripheral areas or appeared included in rosette-like structures. These disorganized outer retina extended on varying lengths in each animal from 78 to 2,695 μm (872 ± 186 μm, n = 16, SEM). Although such retinal disorganizations appeared more extended in VGB-treated animals with greater reduc-

ions in the ERG response amplitude, a strict correlation was not observed. When the VGB treatment was followed by a 43-day recovery period, 57% of the Group II animals (4/7) exhibited a disorganized peripheral retina. These areas with distorted outer nuclear layer (ONL) were reduced in size (some nuclei to 292 μm) despite similar ERG amplitudes as in Group I.

To locate the extent of VGB-induced retinal damage, we immunolabeled retinal sections with glial filament acidic protein (GFAP) antibody, because retinal damage is typically associated with GFAP expression in Müller glial cells around the damaged area.20 In control animals, GFAP expression was confined to glial cell processes located at the inner limiting membrane (presumed astrocytes) and to some processes extending vertically in the retina or horizontally in the outer plexiform layer (Fig 3A). In contrast, GFAP expression was greatly increased in glial cells extending vertically from the inner to the outer limiting membrane (OLM) in all disorganized and more central retinal areas (see Fig 3B). In highly disorganized retinal areas, the OLM became discontinuous and GFAP-immunopositive processes extended beyond the ONL into the layers of photoreceptor outer and inner segments (see Fig 3E). Also, in more central and normal-appearing regions, glial cell processes also were occasionally observed to cross the OLM (see Fig 3D, arrow). These observations indicated that the VGB treatment induced retinal gliosis in the entire retina.

Because ERG recordings located the visual dysfunction to the cone pathway, cone photoreceptor inner/outer segments and axon terminals were stained with peanut agglutinin lectin (PNA). In VGB-treated animals, PNA-stained cone outer and inner segments often appeared disorganized or truncated. Such damaged cone photoreceptors were not restricted to the highly altered peripheral retina but extended to more central areas with normally structured retina (see Fig 3D, G). In these apparently normal retinal areas, some GFAP-positive processes were found to break through the OLM and surround PNA staining (see Fig 3D). In disorganized peripheral retinal areas, the PNA staining was more fragmented, suggesting a more advanced degradation of cone inner and outer segments (see Fig 3E). These observations suggested that cone photoreceptors were directly affected by the VGB treatment.

To further determine whether cone photoreceptors were damaged by the treatment, we estimated the cone density on retinal sections by counting their PNA-stained inner segments. Cone density decreased by 17.7% in VGB-treated rats from Group I (11.86 ± 0.98 cells/300 μm, SEM, n = 7) when compared with control rats (14.41 ± 0.58 cells/300 μm, SEM, n = 6). After the 43-day recovery period (Group II), a further 21.1% cone cell density decrease was observed in VGB-treated animals (11.37 ± 0.80 cells/300 μm, SEM, n = 7).
Fig 2. Histological changes in the peripheral retina of vigabatrin-treated rats. (A–D) Vertical section of the retina from a vigabatrin-treated animal (Group I) (A) with disorganized peripheral areas (B, D, between arrowheads in A) and an apparently normal central area (C). The peripheral disorganization was not symmetrical because it was limited to the area seen in D on the opposite side to that illustrated in A. (E, F) Semithin section of the peripheral retina in control (E) and vigabatrin-treated animals (Group I) (F). In the retina of the vigabatrin-treated rat, displaced photoreceptor nuclei are found in contact to the retinal pigment epithelium (arrows in B, D, F) or in the outer plexiform layer (OPL) (arrowheads in F). Layers of photoreceptor inner and outer segments are also distorted in the area of ONL disorganization. Scale bars in A, B, E = 100\( \mu \text{m} \) in A and B–D and 30\( \mu \text{m} \) in E, F, respectively.
This cone cell quantification indicated that the decrease in the photopic ERG response amplitude may result from a cone cell loss in VGB-treated rats or from the loss of their outer and inner segments.

To examine whether photoreceptor cells actually were degenerating in VGB-treated rats, we labeled retinal sections for DNA fragmentation using TUNEL (Fig 5). Control retina did not show any TUNEL-positive cells in any nuclear layers. In VGB-treated an-
imals, the retina presented some TUNEL-positive cells in the ONL in both disorganized peripheral retinal areas and in apparently normal retinal areas (see Fig 5A–C,E). To analyze further the apoptotic pathway of photoreceptors in VGB-treated animals, we immunolabeled retinal sections with an antibody directed against the active form of caspase-3. Active caspase-3–immunopositive cells were again observed in disorganized and apparently normal ONL areas of VGB-treated animals (see Fig 5D,E,G,H,J,K). When the caspase-3 immunolabeling was combined with PNA staining of the cones, some PNA-labeled structures were found in apposition to caspase-3–immunopositive cell nuclei (see Fig 5F,G,H,I,J,K). These observations indicated that the VGB treatment induced apoptosis with caspase-3 activation in photoreceptors.

When the retina was examined at the ultrastructural level, photoreceptor inner and outer segments often appeared disorganized in peripheral and apparently normal retinal areas of VGB-treated rats (Fig 6B–D) in contrast with the normal retina (see Fig 6A). These anomalies included vacuolization of the inner segment, disruption of the disc stacks, dilation of the discs, and formation of membranous bodies in the outer segments. Also, more numerous phagosomes also were observed in the retinal pigment epithelium facing the photoreceptors (see Fig 6E, F). These observations indicated that the VGB treatment caused structural modifications of photoreceptors consistent with their degeneration.

Discussion
VGB-treated rats were found to exhibit a significant and irreversible decrease in the photopic ERG response that originates in the cone pathway. Histological examination showed a profound alteration of the retinal architecture in the peripheral retina with a severely disorganized outer retina. In more central areas, although the general retinal architecture appeared preserved, cone photoreceptor inner and outer segments were greatly distorted and their numbers significantly decreased. Furthermore, photoreceptor apoptosis was attested in these areas by TUNEL and caspase-3 activation. Cell damage in these more central regions was finally confirmed by the glial reaction indicated by increased GFAP protein expression and the extension of glial cell processes beyond the OLM. These histological and functional observations indicated that photoreceptors, especially cones, were irreversibly injured by the VGB treatment in rats.

Retinal Cell Damage
In humans, VGB was found to induce an irreversible constriction of the visual field. Visual deficits were associated with decreases in amplitude of the photopic ERG, flicker ERG, and oscillatory potentials. These electrophysiological data suggested that the cone pathway was more severely affected than the rod pathway because the scotopic ERG appeared less altered in these studies. Alteration in the cone pathway was confirmed by analysis of color vision, visual acuity, and other visual tasks depending on cones.

In animals, VGB treatments were first reported to induce a disorganization of the photoreceptor layer in the peripheral retina. This observation was confirmed in our study, and we showed further cone dysfunction correlated to cone photoreceptor injury and photoreceptor apoptosis in more central areas. These data were obtained from Wistar albino rats that showed longer dark adaptation to scotopic illumination and higher susceptibility to photoreceptor light damage than pigmented rats. Furthermore, in contrast with humans, rats have a nocturnal life and therefore a rod-dominated retina. Cone photoreceptor damage in VGB-treated epileptic patients would be consistent with the reported electrophysiological measurements, the constriction of the visual field, the altered color vision and reduced visual acuity. Note that, as in humans, the VGB-treatment produced different degrees of damage in the rat retina. Oscillatory potentials also were reduced in amplitude in
VGB-treated rats as previously reported in humans, but it remains unclear whether this effect reflected cone photoreceptor dysfunction as reported in cone dystrophy or whether amacrine cells generating these electrical signals were specifically damaged. Increasing the external GABA concentration is also known to partially suppress oscillatory potentials. These comparisons of our observations with the human data suggest that the VGB-treated rats may provide an adequate animal model to study the physiopathological mechanisms of VGB retinal toxicity in epileptic patients.

Cellular Mechanisms of Photoreceptor Injury
The graduation in retinal damage from the periphery to the center may provide a spatial representation of the sequence of events occurring at the periphery. Cone photoreceptor alteration therefore would be one of the earliest events occurring in retinal VGB toxicity.
If it appears that the entire retina is affected by VGB toxicity because increased GFAP labeling is classically restricted to lesioned areas, it is still unclear, however, whether the glial reaction preceded or followed cone photoreceptor damage. Similar features with glial cell processes extending beyond the OLM along cone inner/outer segments also were described after a few days of retinal detachment. Furthermore, a complete disorganization of the ONL as described here was observed after long retinal detachment periods in which...
cone photoreceptor injury was attributed to hypoxia.29 Retinal hypoxia also could occur in VGB treatment as indicated by the decrease in ocular perfusion11 and the EOG alteration10 representative of the relationship between the retinal pigment epithelium and photoreceptors. In this context, note that an epileptic patient (Case 3) treated with VGB and carbamazepine for 75 months had a retinal detachment before the age of 28 years, although it is unclear whether it was related to the VGB treatment.9 VGB treatment therefore may offer similar similarities with retinal detachment.

In the retina, VGB distribution was not homogeneous but with a peak in the peripheral retina which may explain its higher peripheral toxicity (Pow DV, 2000, 38th ISCEV symposium, Sydney, Australia). VGB is known to suppress the GABA-T activity without affecting GABA synthesis by glutamic acid decarboxylase (GAD).30 As a consequence, the level of GABA was reported to increase sixfold in the retina of treated rats.15 GABA, which is normally located in horizontal and amacrine cells, was found to accumulate in glial Müller cells15 where it is taken up by GABA transporters but cannot be degraded by the VGB-inhibited GABA-T. Inhibiting GABA-T, a mitochondrial enzyme, which provides 8 to 10% of substrates to the tricarboxylic cycle via the GABA shunt, could generate a metabolic dysfunction at the origin of the VGB damage. However, the implication of GABA itself in the VGB-elicited retinal damage is supported by the observation of similar disorganization of the outer nuclear layer in mice with a normal GABA-T but overexpressing GAD under an α-cristalline promoter (Szabo and colleagues 2002, 32th meeting of The Society for Neuroscience, Orlando, FL). GABA can become excitatory and potentially excitotoxic in adult neurons after trauma or excessive stimulation of GABA receptors.31,32 Therefore, all retinal neurons, except rod photoreceptors that do not express GABA receptors, could be directly affected by the abnormal increase in GABA. In the retina, GABA is known to activate different GABA receptors including GABA<sub>C</sub> receptors that exhibit a higher affinity for the neurotransmitter and do not desensitize as GABA<sub>A</sub> receptors do.33 Therefore, similarly to GABA<sub>A</sub> receptors, GABA<sub>C</sub> receptor overactivation is very likely to induce neuronal cell death. In the mammalian retina, GABA<sub>C</sub> receptors are located in cone photoreceptors and bipolar cells, whereas GABA<sub>A</sub> receptors are more generally distributed in all neurons except for rod photoreceptors. In cones, GABA receptor activation not only contributes to retinal information processing, but also provides a trophic signal at least during development.34 Further studies beyond the scope of this article therefore are needed to investigate whether GABA receptor activation is the cause of cone photoreceptor alteration.

GABA also can act on different membrane GABA transporters expressed in Müller cells and retinal pigment epithelium cells.35,36 However, glial alteration rather may result from the gliotoxin and glutamate analogue, amino adipic acid (AAA). Its concentration was indeed reported to increase significantly in rat cortical tissue after VGB administration.37 Furthermore, AAA levels were reported to increase in the plasma and urine of epileptic patients under VGB therapy.38 AAA is long known to be a gliotoxin that affects Müller glial cells,39 leading to an upregulation of GFAP and secondarily to photoreceptor dysmorphogenesis.40 The involvement of AAA gliotoxicity in VGB-induced visual field constriction is further supported by the histological similarities between AAA-treated developing retina39 and retina of VGB-treated animals.

Conclusions

This study indicates that VGB which increases GABA and AAA concentration in the retina damages cone photoreceptors. Retinal GABA increase appears as a common feature in different animal models of retinal pathologies including retinitis pigmentosa42 and diabetic retinopathy.43 Further understanding the molecular mechanisms of the VGB toxicity therefore may generate interesting outcomes for the validation of new therapeutic agents in epilepsy but also for the treatment of retinal pathologies.

References


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