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mTOR Inhibition Mitigates Molecular and Biochemical Alterations of Vigabatrin-Induced Visual Field Toxicity in Mice

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Abstract

BACKGROUND—Gamma-vinyl- γ -aminobutyric acid (GABA) (vigabatrin) is an antiepileptic drug and irreversible GABA transaminase inhibitor associated with visual field impairment, which limits its clinical utility. We sought to relate altered visual evoked potentials associated with vigabatrin intake to transcriptional changes in the mechanistic target of rapamycin (mTOR) pathway and GABA receptors to expose further mechanisms of vigabatrin-induced visual field loss.

METHODS—Vigabatrin was administered to mice via an osmotic pump for two weeks to increase GABA levels. Visual evoked potentials were examined, eye samples were collected, and gene expression was measured by quantitative reverse transcription-polymerase chain reaction. Similarly, human retinal pigment epithelial cells (ARPE19) were exposed to vigabatrin and treated with mTOR inhibitors for mTOR pathway analysis and to assess alterations in organelle accumulation by microscopy.

RESULTS—Dysregulated expression of transcripts in the mTOR pathway, GABA_{A/B} receptors, metabotropic glutamate (Glu) receptors 1/6, and GABA/glutamate transporters in the eye were found in association with visual evoked potential changes during vigabatrin administration. *Rrag* genes were upregulated in both mouse eye and ARPE19 cells. Immunoblot of whole eye revealed greater than three fold upregulation of a 200 kDa band when immunoblotted for ras-related guanosine triphosphate binding D. Microscopy of ARPE19 cells revealed selective reversal of vigabatrin-induced organelle accumulation by autophagy-inducing drugs, notably Torin 2. Changes in the mTOR pathway gene expression, including *Rrag* genes, were corrected by Torin 2 in ARPE19 cells.

CONCLUSIONS—Our studies, indicating GABA-associated augmentation of RRAG and mTOR signaling, support further preclinical evaluation of mTOR inhibitors as a therapeutic strategy to potentially mitigate vigabatrin-induced ocular toxicity.

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Keywords

vigabatrin; GABA; mTOR; gene expression; visual evoked potentials; visual field loss; ARPE19 cells; epilepsy

Introduction

Gamma-vinyl-GABA (vigabatrin; VGB) is a unique antiepileptic drug, approved by Food and Drug Administration for infantile spasms and refractory epilepsy. It irreversibly inactivates GABA transaminase, preventing the metabolism of GABA and effectively increasing central nervous system GABA levels. The therapeutic utility of VGB is limited by its potential to cause peripheral visual field loss. As many as 40% of patients taking VGB, in controlled studies, have experienced irreversible visual field loss as measured by amplitude decreases of both the photopic electroretinogram (ERG) β -wave and the 30 Hz flicker response.^{1–3} Additional clinical pathologies include abnormal macular reflexes, surface wrinkling retinopathy, and narrowed retinal arteries.¹

The mechanism by which VGB induces visual field loss has been investigated in preclinical models, but the root cause remains unclear. Butler et al.⁴ observed lesions to the retinal pigment epithelium when VGB was administered to Sprague Dawley rats (300 mg/kg). Elevated GABA levels, resulting from VGB administration, has been postulated to induce perturbations in cellular homeostasis and recycling processes in eye, including apoptosis and autophagy.^{5–7} VGB administered to mice (35 or 250 mg/kg/day, intraperitoneally, once daily for seven days) increased GABA levels threefold to sixfold in the brain and 3.7- and 5.1-fold in the eye, respectively,⁷ and resulted in a significant increase in mitochondria number in the retina, brain, and liver. These findings indicated that mitophagy (selective autophagy of mitochondria) is dysfunctional under hyperGABAergic conditions in the eye.

Mechanistic target of rapamycin (mTOR) is the catalytic subunit of two functionally distinct protein complexes that influence autophagy, cell growth, and proliferation. Dysregulation of mTOR signaling occurs in numerous neurodevelopmental and neuropsychiatric disorders: epilepsy, autism, schizophrenia, depression, drug addiction, neurodegenerative disorders, congenital disorders of autophagy, and Down syndrome.^{8,9} Increased levels of GABA inhibit mitophagy and pexophagy in yeast cultures by activating TOR1.⁶ Furthermore, rapamycin (an inhibitor of TOR1 and mTOR) over-rode GABA-induced defects in both yeast and mammalian cells, as well as in murine model of the rare defect of GABA metabolism, the so-called aldehyde dehydrogenase 5a1^{-/-} (*aldh5a1*^{-/-}) mice, a model of heritable succinic semialdehyde dehydrogenase deficiency. In the latter, mitochondria number and aberrant antioxidant levels induced by elevated GABA were also normalized by rapamycin.⁶ Furthermore, activation of mTOR via phosphorylation in liver homogenates of VGB-treated mice suggested a strong likelihood of similar GABA-induced mTOR activation in eye.⁷ These findings formed the rationale for the present study.

Here we report the *in vivo* effects of VGB on visual evoked potentials (VEPs) and the association of these alterations with transcriptional changes in signaling molecules known to regulate mTOR activity and GABA and glutamate transporters and receptors in the eye (Fig

1), using both *in vivo* (mouse) and *in vitro* evaluations (ARPE19 cells). Our unifying hypothesis in this study was that exposure to VGB and increased GABA levels in the eye would alter the expression of GABA receptors and mTOR pathway-related genes.

Materials and Methods

Chemicals

VGB (Sabril) was obtained from Tocris Biosciences (Bristol, United Kingdom). Rapamycin, Torin 1 (Tor1), and Torin 2 (Tor2) were purchased from Cayman Chemical (Ann Arbor, MI) and trehalose from Sigma Aldrich (St. Louis, MO). Cell culture grade dimethyl sulfoxide (DMSO) was purchased from Thermo Fisher Scientific (Waltham, MA).

Animal studies

All animal studies were approved by the Washington State University Institutional Animal Care and Use Committee (protocols ASAF 4232/4276), and complied with the guidelines published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals).

Osmotic pump implantation

C57/B6 mice, aged 8 weeks, were bred in-house and administered VGB (50 mg/mL, $n = 3$) or vehicle (phosphate-buffered saline, $n = 3$) via subcutaneously implanted osmotic pump model 2002 (Alzet, Cupertino, CA) at a flow rate of 0.5 μ L/hour for 2 weeks. Mouse retinal development occurs between E11.5 and postnatal day P8; therefore these studies tested ocular function in the mature retina.

Electrode implantation

Young-adult mice (aged six to eight weeks) underwent stereotaxic electrode implantation into the primary visual cortex under isoflurane anesthesia (5% induction and 3% maintenance). Two stainless-steel polyimide-insulated electroencephalographic (EEG) electrodes (Plastics, Roanoke, VA part #E363/1/SPC; diameter, 0.280 mm) were implanted in V1 visual cortex at -3.0 mm A/P, and ± 2.5 mm lateral relative to bregma and at 0.5 mm depth from the skull, then soldered to a plastic connector affixed to the skull (Pinnacle Technologies, part # 8201-SS). Reference and ground electrodes were implanted in the frontal cortex (A/P, $+2.5$; Lat, -1.5 ; A/P, $+2.5$; Lat, $+1.5$). Presoldered stainless steel electromyographic (EMG) electrodes were implanted and sutured into the trapezius muscles to record muscular inputs. Mice were implanted 4 days before recording and received a daily subcutaneous bolus of buprenorphine (0.1 mg/kg) for three days as post-surgical analgesic.

Light-adapted recordings were performed 4 hours into the light phase of the 12-hour light and 12-hour dark schedule. Dark-adapted recordings commenced in the dark phase (1900 hours), after at least 30 minutes of dark adaptation in their individually housed home cages. Dark adaption potentiates visual evoked potential (VEP) amplitude relative to light.¹⁰ After a 10-minute baseline EEG recording, light emitting diode light stimulation (10 milliseconds pulses) was delivered from a fiber optic probe positioned between the eyes. A total of 780 stimuli were delivered at 0.1 Hz ($n = 60$ stimuli over a 10-minute interval), 0.2 Hz ($n = 120$

stimuli over a 10-minute interval), and 1 Hz ($n = 600$ stimuli over a 10-minute interval) sequentially during a 30-minute interval. EEG and electromyographic signals were recorded using Pinnacle Technologies Sirenia Acquisition software. Data shown in Fig 2 are from the 1 Hz stimulus condition. VEP was measured as the electrical potential at 500 Hz from 0.5 seconds before the onset of the visual stimulus to 0.5 seconds after the onset of the stimulus.^{11,12} These values were summed at each time point across all 600 stimuli to yield an averaged VEP curve for each animal.

Animal tissue harvest for RNA isolation and quantitative reverse transcription-polymerase chain reaction

After dark-adapted electrophysiological recordings, animals were sacrificed and tissues rapidly excised, snap-frozen in liquid nitrogen, and stored at -80°C prior until further analysis. RNA was prepared from one whole eye per subject ($n = 3$ per treatment group), then pooled for each group. Isolation of RNA used standard chloroform and isopropanol isolation. iScript mastermix (BioRad, Hercules, CA) was used to synthesize cDNA in an Eppendorf Master cycler as per manual instructions. Sybr green (10 μL , BioRad) reactions were loaded with 50 ng of cDNA per well into prearrayed and validated pathway analysis plates (PrimePCR mTOR signaling plate). A BioRad CFX384 real-time PCR instrument operated with BioRad CFX manager v3.0 software was used for data acquisition, normalization, expression, and statistical analysis with $P < 0.05$ significant on the basis of two replicates per group. A list of genes examined and their full names are shown in Supplementary Table.

ARPE19 cell line validation, culture, and fluorescent microscopy

A T175 flask of ARPE19 cells was collected at passage 9 and pelleted for cell line verification using the CellCheck 9 (9 marker STR profile and interspecies contamination test performed at Idexx Bioresearch; Columbia, MO). ARPE19 cells were purchased from American Type Culture Collection and expanded with Dulbecco's modified Eagle's medium (DMEM/F12). For fluorescent microscopy, cells were counted and 24-well plates were seeded with 3.0×10^5 cells and cultured in the presence of vehicle (dimethyl sulfoxide (DMSO, $<0.01\%$), VGB (5 μM), VGB plus rapalog (rapamycin 100 nM, Tor1, or Tor2 10 nM), or the mTOR-independent autophagy-inducing alpha-linked disaccharide trehalose (100 nM) for three days. VGB concentrations were selected based on the reported brain levels measured using microdialysis in traumatic brain injury patients (5.22 [range 4.2 to 7.1] μM^{13}). Live cells were imaged on a Leica DMI8 inverted fluorescent microscope (4 wells per group and three images per well) averaged for a constant area and normalized to NucBlue fluorescence after 0 (peroxisome) and 6 hours (lysosome and mitochondria) of starvation in media (DMEM/F12 devoid of fetal bovine serum). Organelle-specific probes (NuclearBlue; MitoTracker green, LysoTracker red) were obtained from Molecular Probes (Invitrogen, Thermo Fisher Scientific) and incubated under the manufacturer's instructions. Statistical analysis included one-way ANOVA computed with a significance threshold of $P < 0.05$.

Preparation of ARPE19 cell pellets for transmission electron microscopy

Media was removed from six-well plates of ARPE19 cells after 3 days of VGB (5 μ M) or DMSO (0.15%) treatment (similar approach as fluorescent microscopy) and media was replaced with fetal bovine serum-depleted DMEM/F12 for 6 hours. After isolation and washing of cells, the latter were prepared for electron microscopy as previously described using paraformaldehyde, glutaraldehyde and osmium tetroxide,⁷ using an FEI Tecnai G2 T20 microscope.

Expression analysis of ARPE19 cells

ARPE19 cells were cultured under similar treatment and starvation conditions, with $n = 6$ replicate wells for DMSO, VGB, and VGB plus Tor2 groups. After six hours in replete conditions the media was removed and Trizol immediately added to wells. The wells were scraped for cell collection and then processed in an identical fashion to that used for whole lysate expression analysis described previously.

Immunoblotting of mouse whole eye

One whole eye from each mouse was mechanically homogenized with a tapered dowel for immunoblot analysis following standard protocols employing nitrocellulose and alkaline phosphatase-conjugated primary antibody. Protein bands were detected with chemiluminescent CDP-Star Substrate with Nitro-Block-II Enhancer (Novex, Wadsworth, OH) and normalized to beta-actin (loading control).

Results

Application of VGB alters VEPs in the mouse

To gauge the effect of VGB exposure on neurophysiological function, eight week-old mice received VGB (50 mg/mL) or vehicle (phosphate-buffered saline) at 0.5 μ L/hour flow rate via an osmotic pump implanted subcutaneously for 2 weeks. Averaged peristimulus histograms from a total of 600 stimuli delivered at 1 Hz over a ten-minute interval indicated profound disruption of the VEP by VGB exposure (Fig 2). Repeated measures analysis of variance on VEP amplitude (defined by the maximum negative deflection within the peristimulus histogram; Fig 2B) indicated significant main effects for dark versus light ($P = 0.008$) and pharmacology ($P = 0.015$) and their interaction ($P = 0.021$). VEP amplitude was significantly attenuated in VGB-exposed mice relative to saline controls in both the light-adapted (by 59%) and dark-adapted (by 76%) conditions.

Application of VGB associates with dysregulation of gene expression in mouse eye

Gene expression profiling of mTOR and GABA/glu receptors was undertaken in extracts of eye derived from mice that had undergone chronic VGB application. Upregulation of *Rragb* in the eye of VGB-treated mice ($\uparrow 8.2$ -fold; Fig 3A) was observed, although this failed to achieve statistical significance. Furthermore, VGB exposure resulted in an increased expression of *Akt1s1* (also known as *Pras40*, $\uparrow 4.1$ -fold) and *Mlst8* ($\uparrow 6.3$ -fold) and the decreased expression of *Fkbp1a* ($\downarrow 3.6$ -fold*) and *Prkag2* in the eye ($\downarrow 5.7$ -fold*). In addition, decreased expression of *Ilk* ($\downarrow 4.8$ -fold*) and *Prkcb* ($\downarrow 5.1$ -fold) and an increase in *Vegfa*

($\uparrow$ 5.0-fold) and *Tbp* ($\uparrow$ 5.0-fold; Fig 3A) were observed in the eye. Asterisked values represent significant changes. See Supplementary Table for descriptions of genes and associated abbreviations that were evaluated in our expression studies.

VGB mediates changes in GABA and glutamatergic gene expression profiles in the eye

Changes in the differential gene expression of eye tissue receptor subunits included *Grm1/6*, *Gabra6* (not significant), *Gabrd*, *Gabre* (not significant), *Gabbr1*, and *Gabbr2* and transporters *Slc17a7*, *Slc1a6*, *Slc6a13*, and *Slc17a6* (Fig 3B,C). *Grm1* was highly upregulated in the eye ($\uparrow$ 70.1-fold; Fig 3B), although this failed to reach significance. See Supplementary Table for descriptions of genes and abbreviations evaluated.

VGB induces an increase in RagD/mTOR complex formation in mouse eye

To further assess the hypothesis that increased GABA may act similarly to certain amino acids such as leucine, ras-related G protein D (RAGD) was examined using Western blot in whole eye extracts of mice. Immunoblot bands corresponding to RAGD (~50 kDa) showed similar intensity between treatment and control wells; however, a significant increase of 3.25-fold was observed at the molecular weight ~200 kDa (Fig 4). This larger molecular weight band is consistent with the RAGD–regulatory-associated protein of mTOR (RAPTOR) complex¹⁴ and provided corroboration of molecular expression studies at the protein level for RAGD.

Rapalogs attenuate VGB-induced effects on organelle abundance in ARPE19

To extend *in vivo* studies, we undertook fluorescent microscopy studies of human retinal pigment epithelial cells ARPE19. Our rationale here for *in vitro* studies was that examination of organelles would be considerably more facile for organelle evaluation in the setting of tissue culture. We observed that ARPE19 cells cultured in VGB show enhanced organelle-specific fluorescence (Fig 5). The left side of the figure depicts the cumulative data for treatments and organelle fluorescence, while the right side depicts representative fluorescent staining for each intervention and organelle. For the latter, upper panels depict individual organelle stain, while bottom panels show the merge of each stain with 4',6-diamidino-2-phenylindole (NucBlu). An asterisk indicates significantly different from vehicle (DMSO) treatment, whereas the absence of an asterisk indicates normalization of fluorescence (not significantly different from DMSO treatment). In the case of peroxisomes, this enhanced fluorescence occurred without serum starvation, but only after six hours of starvation for lysosomes and mitochondria. Enhancement of fluorescence by VGB was normalized for all three organelles by Tor2 (10 nM) and trehalose (100 nM) (Fig 5). The disaccharide trehalose has a mechanism of autophagy induction that is distinct from that of Tor1 and Tor2. Trehalose inhibits glucose transport members of solute carrier 2A (also known as GLUT, for glucose transporter), thereby inhibiting glucose transport to enhance adenosine monophosphate–activated kinase (AMPK)-dependent autophagy (see Fig 1).¹⁵ Rapamycin (also known as sirolimus, 100 nM) blocked VGB enhancement of fluorescence in peroxisomes and lysosomes (Fig 5). The dual mTORC1/2 inhibitor Tor1 (10 nM) blocked VGB enhancement of fluorescence only in peroxisomes (Fig 5). In addition, VGB increased mitochondrial abundance in transmission electron micrographs, an observation compatible with a reduced rate of mitophagy (Fig 6) and consistent with previous studies in *aldh5a1*^{-/-}

mice in which supraphysiological GABA associated with increased mitochondrial number in brain and liver.¹⁶

Tor2 attenuates VGB-induced effects on mTOR pathway expression changes in ARPE19 cells

Treatment of ARPE19 cells with VGB significantly altered the expression of 34 genes (*Tsc2* $p = ns$) related to the mTOR signaling pathway (Fig 7). Of these, VGB resulted in a 3.0-fold increase in 24 genes. Of these, coculture with Tor2 returned expression levels to within threefold of control values of 20 genes (including *Akt1s1*, *Akt3*, *Eif4b*, *Eif4bebp1*, *Fkbp8*, *Gsk3b*, *Igfbp3*, *Mapk*, *Pik3ca*, *Prkg2*, *Prkca*, *Rictor*, *Rsp6*, *Rps6kb1*, *Raptor*, *Tbp*, *Tsc1*, *Ulk2*, *Vegfb*, and *Vegfc*). Although not fully corrected (less than threefold control levels), Tor2 reduced the overexpression of *Rhoa* (63.7- to 3.7-fold), *Rragc* (192.8- to 24.2-fold), *Ragd* (17.4- to 5.5-fold), *Vegfb* (8.2- to 3.0-fold), and *Ywhaq* (72.0- to 5.1-fold). Modest downregulation (less than fourfold) associated with VGB treatment occurred for eight genes (*Ilk*, *Prkag1*, *Prkag3*, *Pten*, *Rraga*, *Tsc2*, *Ulk1*, and *Vegfa*). Several of these genes corroborated findings from *in vivo* studies in the eye, including upregulation of *Akt1s1*, *Tbp*, *Vegf*, and *Rrag* complex genes and downregulation of *Ilk*. Supplementary Table provides a more comprehensive description of gene expression studies.

Discussion

In an earlier report, we demonstrated that mice treated with VGB undergo changes in mitochondria that are consistent with perturbations in autophagy.⁷ In the present work, we have extended this evaluation, by confirming that VGB alters VEPs *in vivo*, which associates with dysregulated mTOR-related gene expression profiles, as well as a functional increase in RAPTOR–RAGD complex formation. We extended the *in vivo* analyses through *in vitro* studies, which demonstrated the capacity of an mTOR inhibitor, Tor2, to correct VGB-induced dysregulation of gene expression profiling in ARPE19 cells. As a functional readout of the latter, we also evaluated the fluorescent microscopy of these ARPE19 in the presence and absence of various mTOR inhibitors and functionally evaluated mitochondrial number using transmission electron microscopy.

The VEP was examined in mice administered VGB or vehicle for two weeks via subcutaneously implanted osmotic minipumps. Our studies demonstrate that the effects of VGB on network properties can be gauged with a functionally relevant signal via VEP. Our prediction in the use of VEP was that VGB, via blockade of GABA transaminase, would reduce VEP amplitude. As predicted, VGB attenuated VEP amplitude and dark adaptation potentiated it. These data confirm the feasibility of measuring VEP as a quantitative gauge of the effect of VGB on visual field function. A complimentary approach to quantify the effects of VGB *in vivo* would include the use of electroretinography. Electroretinography could provide specific information on retinal toxicity through the evaluation of detailed analysis of dark-adapted (rod) versus light-adapted (cone) testing. Moreover, looking at the response amplitudes and latencies (time from stimulus flash to peak of response) could be very informative as to whether the insult(s) associated with VGB resides at the

photoreceptors versus inner retinal cells. These studies are being developed in our laboratory.

In the present study, we focused our attention on Tor2, because it represents an improved mTOR inhibitor with better bioavailability and metabolic stability,⁶ and characterized trehalose as an mTOR-independent measure of autophagy. Our results are the first cell-based studies indicating a global effect of VGB on organelle proliferation and suggesting that VGB disrupts pexophagy, lysophagy, and mitophagy. In the retinal pigment epithelia, autophagy, and mitophagy (and most likely pexophagy and lysophagy) are cytoprotective functions linked mechanistically to protection against all-*trans*-retinal- and light-induced damage and photoreceptor cell death.^{17,18} Results with trehalose, a nonreducing α -disaccharide, are particularly interesting because trehalose works in an mTOR-independent mechanism.^{19,20} If regulation of autophagy is the primary mechanism through which rapalogs exert their efficacy through mTOR, trehalose could possess synergistic properties with mTOR inhibitors, including organelle stabilization and improved antioxidant status, while simultaneously reversing organelle proliferation.

The mechanism of autophagy induction by trehalose has recently been described. Trehalose, a weevil-produced disaccharide, allows for AMPK-driven autophagy through blockade of glucose transport.¹⁵ This mechanism indicates that autophagy suppression is the likely mechanism behind the observed organelle accumulation that we identified. Collective evidence of *Rrag* gene expression and correction by Tor2 suggests that mTOR inhibits autophagy in response to amino acid signaling cues initiated by elevated GABA levels. RAGA/B-RAGC/D complexing is required for amino acid-mediated activation of mTOR. Correction of VGB-induced organellular changes by rapalogs (rapamycin, Tor1, and Tor2) highlights the role of mTOR under hyperGABAergic conditions in an ocular-tissue derived cell line. The functional role of ARPE19 cells in the pathology of VGB-induced visual field loss likely represents only one of the important cell types of the retina that are impacted with VGB. These cells were selected based on their relative ease of culture, commercial availability, and the important pathologies observed in the retinal pigment epithelium.⁴

In terms of cellular bioenergetics, AMPK (Fig 1) becomes active under low nutrient conditions, favoring processes, which increase adenosine triphosphate levels such as fatty acid oxidation and autophagy,²¹ while inhibiting mTOR. AMP binding to the gamma regulatory subunit of AMPK (encoded by the *Prkag1/2* transcripts) leads to allosteric activation. As a nonprotein amino acid, GABA may act similarly to leucine, which triggers mTOR-mediated inhibition of autophagy in a Rag complex-dependent manner.²² Leucine acts through the vacuolar ATPase in the lysosomal membrane to activate the Rag complex (Fig 1). The active Rag complex (composed of guanosine triphosphate [GTP]-bound RagA/B and guanosine diphosphate [GDP]-bound RagC/D) binds to Raptor of mTORC1 and recruits mTOR to the lysosome, where it binds to active GTP-bound Rheb²³ to inhibit autophagy. Collective evidence for GABA-induced activation of RAG-signaling to mTOR in this report includes the upregulation of *Rragb* in the whole eye of VGB-treated mice ($\uparrow$ 8.2-fold; Fig 3A) and increased *Raptor*, *Rictor*, *Rragc*, and *Rragd* gene expressions in ARPE19 by VGB, which was partially corrected by Tor2 treatment (Fig 7). The RAGD GTPase has a molecular weight of ~50 kDa and is unstable when unbound.^{14,24} The detection of a band at

200 kDa in our immunoblotting analysis likely corresponds to that of the RAGD–RAPTOR complex, with 3.25-fold increased intensity in VGB-treated mice. The 200 kDa band represents a direct interaction between Rag proteins and mTORC1.^{14,25} Further support for this hypothesis in ARPE19 gene expression profiling is found with upregulated glycogen synthase kinase-3 (*Gsk3*), which also acts as a positive mediator of RAPTOR/mTORC1 amino acid sensing through phosphorylation of S⁸⁵⁹RAPTOR²⁶ (Fig 7). Finally, culture of ARPE19 cells with VGB resulted in altered gene expression for additional mTOR complex–affiliated genes in the eye, including *Akt1s1* (also known as PRAS40, ↑4.1-fold; Fig 3A) and *Mlst8* (↑6.3-fold; Fig 3A), a subunit of mTORC1 and mTORC2 with unclear function,⁸ which were upregulated, as well as *Fkbp1a* (↓3.6-fold*; Fig 3A), a target for the inhibitory effect of rapamycin on mTOR, which was downregulated. As a functional readout of these dysregulated gene expression profiles associated with VGB treatment, we found correction of numerous mTOR-affiliated genes with Tor2 (Fig 7).

In support of our gene expression studies looking at GABAergic and glutamatergic signaling, a growing body of literature relates GABA and glu levels and receptor activity to mTOR signaling²⁷ (Fig 1). Electrophysiological studies have shown that hyperactive mTOR increases evoked synaptic responses in both glutamatergic and GABAergic neurons, with attenuation of these abnormalities and a decrease of glutamatergic (but not GABAergic) synaptic transmission mitigated by rapamycin.²⁷ Neurotransmitter transporter functionality is also critical to the retinal microenvironment,²⁸ which provided a component of our rationale to evaluate these transporters in our expression studies. In addition to changes in mTOR pathway gene expression, expression changes in eye tissue were found for GABA and Glu receptors, including *Grlm1/6*, *Gabra6*, *Gabrd*, *Gabre*, *Gabbr1*, and *Gabbr2* and neurotransmitter transporters *Slc17a7*, *Slc1a6*, *Slc6a13*, and *Slc17a6* (Fig 3B,C). Dysregulated expression profiles for GABA and glu receptors and transporters are consistent with *in vitro* neurotransmission studies linking GABA and glu receptor function to mTOR.

In a study of school age children who had received VGB as infants, Riikonen et al.²⁹ detected peripheral visual field deficits (pVFDs) in one third, and the rate of pVFDs increased from 9% to 63% as VGB treatment duration increased.²⁹ To track the retinal toxicity associated with VGB, pediatric neurologists currently rely on serial fundoscopic examinations (which are insensitive) and occasionally ERGs, as well as ocular coherence tomography (investigational). There is no consensus as to what testing is sensitive enough to detect early onset pVFDs, and even ERGs are debatable, since the normal findings change with development, and the findings in infants remain questionable. Child neurologists tend to use VGB for as short a time as possible, indicating an urgent unmet clinical need for improving the interventional utility of this unique drug. Along these lines, ocular administration of rapamycin has improved the engraftment of corneal allografts.^{30,31} Rapalog administration directly to the eye (either intravitreally or subretinally, the latter requiring specialized equipment) could be advantageous in bypassing the potential for systemic toxicity associated with rapalogs. Pharmaceuticals that eliminate the retinal toxicity of VGB would provide a major advance in epilepsy treatment, and our studies suggest that mTOR inhibitors should receive further preclinical attention as a therapeutic modality to potentially mitigate VGB-induced visual field pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

The mTOR signaling pathway with potential contributions from GABA and Glu receptors. The mTOR pathway receives cues from many bioenergetics processes, encompassing hormones (insulin, AVP), tyrosine kinase receptors, PI3K, Ras/Mapk, AMPK (low energy), Akt, and amino acids, which signal to mTOR via Rag GTPases. The two distinct protein complexes mTORC1 (includes Raptor) and mTORC2 (includes Rictor) perform distinct effector roles for catalytic mTOR. These functions include suppression of autophagy, which has been linked to GABA elevations in numerous experimental model systems. Impaired autophagy can result in changes to cellular homeostasis and oxidative damage, as well as organelle and protein aggregate accumulation. VGB irreversibly inhibits the ABAT enzyme (also known as GABA-T) in the GABA degradation pathway leading to GABA accumulation. VGB application and resulting increase in GABA changes the neurotransmitter receptor landscape, including GABA and glu ionotropic and metabotropic receptors. VGB, vigabatrin; SSA, succinic semialdehyde; GABA, 4-aminobutyric acid; Glu, glutamate; Gln, glutamine; vGAT, vesicular GABA transporter; EAAT, excitatory amino acid transporter; mGluR, metabotropic glutamate receptor; NMDAR, glutamatergic *N*-methyl-D-aspartate receptor; GABA_AR, ionotropic GABA_A receptor; GABA_BR, metabotropic GABA_B receptor; GAT1, GABA transporter 1; AVP, arginine vasopressin; TBP, TATA binding protein; Vegfa, vascular endothelial growth factor A; IGFBP3, insulin growth factor binding protein 3; IGF1, insulin growth factor 1; S6K, ribosomal S6 kinase; PI3K, phosphoinositide 3-kinase; PTEN, phosphate and tensin homolog; PDK1, pyruvate dehydrogenase kinase 1; PIP₂ and PIP₃, phosphatidyl inositol bisphosphate and phosphatidyl inositol (3,4,5) triphosphate, respectively; mTORC2, mechanistic target of rapamycin complex 2; RAS, rat sarcoma GTPase; Raf, rapidly accelerated fibrosarcoma kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; Snca, synuclein alpha; Akt, protein kinase B; C-SK3β, cytosolic serine kinase β3 subunit; BCL-2, B-cell lymphoma 2 apoptosis regulator; FOXO, forkhead box protein; αPKC, protein kinase C α subunit; HIF-1, hypoxia inducible factor 1;

c-fos, protooncogene c-fos; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1 α ; TSC1 and 2, tuberous sclerosis proteins 1 and 2, respectively; mTORC1, mechanistic target of rapamycin complex 1; Adora2a, adenosine A2a receptor; AC, adenylate cyclase; GPP or GDP, guanosine pyrophosphate or diphosphate; GTP, guanosine triphosphate; Rag A–D, ras-related GTP binding proteins A–D; Rheb, ras homolog enriched in brain; FKBP, FK506 binding protein; ULK1, Unc-51-like autophagy activating kinase 1; α , β , γ -subunits of the trimeric G-coupled protein; ATG13, autophagy-related 13 protein; FIP200, focal adhesion kinase family interacting protein (200 kDa); PRAS40, proline-rich Akt substrate (40 kDa); Prod1, proline dehydrogenase 1 protein; AMPK, adenosine monophosphate-activated kinase; PP2A, protein phosphatase 2a; LKB1, liver kinase B1 tumor suppressor protein; CAB39, calcium binding protein 39. (The color version of this figure is available in the online edition.)

FIGURE 2.

Effects of VGB on VEPs. (A) Representative peristimulus histograms (from 0.5 second before the onset of the 10 milliseconds visual stimulus to 0.5 seconds after the onset of the stimulus). Curves are averages from a total of 600 stimuli delivered to mice treated with either vehicle (left) or VGB (right). Data are from light-adapted (upper) or dark-adapted (lower) conditions. (B) Maximum negative deflection within the peristimulus histogram in the two treatment groups across the two lighting conditions. *Significantly attenuated relative to vehicle in the same lighting condition. Statistical analyses performed with Fisher's least significant difference (LSD) method. EEG, electroencephalographic; VEP, visual evoked potential; VGB, vigabatrin.

FIGURE 3. Gene and protein expression changes in eye. Gene expression changes in the mTOR pathway (A), Glu receptors and transport (B), and GABA receptors/transport pathways (C) in the eye of VGB-treated mice relative to control mice. * $P < 0.05$, ** $P < 0.01$, # $P = 0.0520$ by one-way analysis of variance with Dunnett’s *post hoc* analysis for multiplicity adjusted P values. See Supplementary Table for further information on genes evaluated. mTOR, mechanistic target of rapamycin; VGB, vigabatrin. (The color version of this figure is available in the online edition.)

FIGURE 4.

Relative RAGD protein levels (requisite for amino acid-mediated activation of mTOR) in whole eye homogenates of mice administered PBS or VGB. Bar graph denotes the mean $\pm$ S.D. of RAGD intensity at 200 kDa normalized to β -actin. * $P < 0.05$, by one-way ANOVA with Dunnett's *post hoc* analysis for multiplicity adjusted P values. mTOR, mechanistic target of rapamycin; PBS, phosphate-buffered saline; VGB, vigabatrin.

FIGURE 5.

Alterations of organelle number in cultured human retinal pigment epithelial (ARPE19). ARPE19 cell cultures and live cell fluorescent imaging as a measure of peroxisome, lysosome, and mitochondrial content. In the case of peroxisomes, this enhanced fluorescence occurred without serum starvation, but only after 6 hours of starvation for lysosomes and mitochondria. The right side of the figure depicts representative fluorescence staining for each organelle (top panel), and the overlay with NucBlu (bottom panel for each organelle). Boxes indicate the mean and error for standard deviation; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Dunnett's *post hoc* analysis for multiplicity adjusted P values. DMSO, dimethyl sulfoxide; VGB, vigabatrin. (The color version of this figure is available in the online edition.)

FIGURE 6.

Transmission electron microscopy of ARPE19 cells for mitochondrial number. Average mitochondrial counts per micrograph with error bars reflecting the standard deviation (A) and micrographs for DMSO vehicle (B) and VGB-treated (5 μ M) ARPE19 cells (C) were imaged at $\times 5000$ magnification. Gross alterations of morphology (overall shape and number of cristae) were not observed. Statistical analysis used a two-tailed *t* test, $^{**}P < 0.01$. DMSO, dimethyl sulfoxide; VGB, vigabatrin. (The color version of this figure is available in the online edition.)

FIGURE 7.

Gene expression analysis in cultured ARPE19 cells ($n = 6$ wells per group). Cells were collected after 6 hours of serum starvation and 3 days of exposure to vehicle control, 5 μM VGB (blue; normalized to a control group with DMSO) or 5 μM VGB + 10 nM Tor2 overlaid (pink; also normalized to a control group with DMSO). All replicate comparisons are statistically significant ($P < 0.05$) between groups except Tsc2 ($P = 0.2$). Thirty-five expression changes occur including eight downregulations in the pathway for VGB and 27 upregulations. See Supplementary Table for further information on genes evaluated. DMSO, dimethyl sulfoxide; VGB, vigabatrin. (The color version of this figure is available in the online edition.)