Published in final edited form as:

Epilepsy Res. 2020 October ; 166: 106395. doi:10.1016/j.epilepsyres.2020.106395.

Transcriptome Analysis in Mice Treated with Vigabatrin Identifies Dysregulation of Genes Associated with Retinal Signaling Circuitry

Dana Walters^{a,*}, Kara R. Vogel^{b,*}, Madalyn Brown^a, Xutong Shi^a, Jean-Baptiste Rouillet^a, K. Michael Gibson^{a,**}

^aDepartment of Pharmacotherapy, Washington State University College of Pharmacy and Pharmaceutical Sciences, Spokane, Washington, USA

^bDepartment of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA

Abstract

Vigabatrin (VGB; γ -vinyl-GABA) is an antiepileptic drug that elevates CNS GABA via irreversible inactivation of the GABA catabolic enzyme GABA-transaminase. VGB's clinical utility, however, can be curtailed by peripheral visual field constriction (pVFC) and thinning of the retinal nerve layer fiber (RNFL). Earlier studies from our laboratory revealed disruptions of autophagy by VGB. Here, we tested the hypothesis that VGB administration to animals would reveal alterations of gene expression in VGB-treated retina that associated with autophagy. VGB (140 mg/kg/d; subcutaneous minipump) was continuously administered to mice (n=6 each VGB/vehicle) for 12 days, after which animals were euthanized. Retina was isolated for transcriptome (RNAseq) analysis and further validation using qRT-PCR and immunohistochemistry (IHC). For 112 differentially expressed retinal genes (RNAseq), two databases (Gene Ontology; Kyoto Encyclopedia of Genes and Genomes) were used to identify genes associated with visual function. Twenty four genes were subjected to qRT-PCR validation, and five (*Gb5*, *Bdnf*, *Cplx9*, *Crh*, *Sox9*) revealed significant dysregulation. IHC of fixed retinas verified significant down-regulation of *Gb5* in photoreceptor cells. All of these genes have been previously shown to play a role in retinal function/circuitry signaling. Minimal impact of VGB on retinal autophagic gene expression was observed. This is the first transcriptome analysis of retinal gene expression associated with VGB intake, highlighting potential novel molecular targets potentially related to VGB's well known ocular toxicity.

^{**}Correspondence: Department of Pharmacotherapy, Health Sciences Building Room 210C, Washington State University, College of Pharmacy and Pharmaceutical Sciences, 412 E. Spokane Falls Boulevard, Spokane, WA 99203-2131; mike.gibson@wsu.edu; 509 358 7954; fax 509 358 7744.

^{*}equal contributors

Conflicts of interest: All authors declare that they have no conflicts of interest in this study.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Vigabatrin; retina; gene expression; transcriptome; visual field constriction; RNA sequencing (RNAseq)

1. Introduction

In 2015, the United States Center for Disease Control (CDC) estimated that roughly 3.5 million adults and children were affected with epilepsy, representing > 1% of the population (<https://www.cdc.gov/epilepsy/data>). Currently, twenty-eight antiepileptic drugs (AEDs) are currently available, and most act via modulation of ion channels (Na^+ , Ca^{+2}) activity (Vossler et al, 2018; Nicholas et al, 2012). Of these, only vigabatrin (VGB; γ -vinyl GABA) directly alters activity of the GABA metabolic pathway via irreversible inhibition of GABA-transaminase resulting in the elevation of inhibitory GABA (Fig. 1; Ricci et al, 2006; Sulaiman et al, 2003). Therapeutic indications for VGB include infantile spasms (West Syndrome), focal-onset seizures, and an inherited disorder of GABA metabolism, succinic semialdehyde dehydrogenase deficiency (SSADHD) (Scheffer et al 2017; Messer et al, 2020; van der Poest et al, 2020; Osborne et al, 2019; Gaily et al, 2012; Carmant et al, 2011; Gibson et al, 1995).

The antiepileptic action of VGB primarily derives from elevation of GABA, the major inhibitory neurotransmitter in the adult central nervous system (CNS; Fig. 1), and the resulting offset of excitatory glutamatergic signaling (Bak et al, 2006). Although GABA is abundant in mammalian retina, its signaling activity in retina is not as clearly defined as its role in the CNS. Selected studies indicate roles for GABA in retinal ganglion function (McCall et al, 2002; Popova et al, 2015), axonal protection, and maintenance of ganglia cell integrity (Ishikawa et al, 2018). Disruptions in CNS GABA signaling associated with elevated GABA have been suggested to induce neurodegeneration (Bittigau et al, 2002). Since retina is an extension of the CNS, it is reasonable to assume that an hyperGABAergic state associated with VGB may also induce retinal neurodegeneration.

Approved for use in Europe in 1989, VGB did not gain United States Food and Drug Administration (FDA) approval until 2009 due to evidence of intramyelinic edema in animals (Shields et al, 2011). VGB carries a black box warning (<https://www.sabril.net/>) indicating a significant risk of permanent bilateral peripheral visual field constriction (pVFC) (Hawker et al, 2008). In addition to pVFC and thinning of the RNFL, additional toxic side effects with VGB have been reported, which may be grouped as follows: 1) *structural* (damage to rod/cone photoreceptors, reduced ocular/cerebral blood flow); 2) *imaging* (white matter spongiosis, restricted globus pallidus diffusion); 3) *metabolic* (amino acid (taurine, ornithine) anomalies, reduced glutamine synthetase and serum transaminase activities, oxidative stress); and 4) *genomic alterations* (dysregulation of genes active in autophagy, damage to DNA) (Wild et al, 2019; Pearl et al, 2018; Froger et al, 2014; Sorri et al, 2010; Hisama et al, 2001). Light exposure may also have a role, suggesting a link to the phototransduction cascade, the process by which a photon of light is translated into an electrical signal (Yang et al, 2012; Tao et al, 2016).

The mechanism(s) of VGB's ocular toxicity remain unclear, thwarting the development of therapeutic strategies for mitigation that could expand its clinical utility. Earlier studies from our laboratory provided evidence that inhibition of the mammalian target of rapamycin (mTOR), a central signaling component of autophagy, had potential to mitigate the ocular toxicity in VGB-treated animals (Vogel et al, 2015; 2017a; 2017b). Here we have tested the hypothesis that VGB administration to animals would induce dysregulation of autophagic gene expression in the murine retina. The overriding objective was to provide insight into the potential of pharmacotherapeutics that influence autophagy as an approach to mitigating VGB retinal toxicity, with concomitant extension of its clinical utility.

2. Materials and Methods

2.1. Animals and drug administration

All procedures were approved by the Washington State University Institutional Animal Care and Use Committee (Protocols ASAF 4232–42 and 6134), and consistent with the National Institutes of Health guide for the care and use of laboratory animals. C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were bred in-house to 8–10 weeks of age (20.8–26.1 g in weight). Adult animals were selected to determine VGB's effect on the developed retina. Cohorts consisted of six male animals per group. Males were chosen to initially control for gender-related differences in gene expression and visual function (van Alphen et al, 2009). Animals were maintained under a 12 h: 12 h light-dark cycle with access to food and water *ab libitum*. Osmotic minipumps, model 2002 (Alzet, Cupertino, CA) were prepared prior to surgery with a VGB dose calculated as 140 mg/kg/day; PBS (phosphate buffered saline; pH 7.4) served as vehicle. The minipumps delivered VGB at a constant flow rate of 0.5 μ L/h for up to 14 days. The VGB dosing paradigm was specifically chosen to mimic that employed in humans (Walters et al, 2019). Animals were randomly assigned to vehicle or drug cohorts and were euthanized after 12 days of VGB exposure. Retinas were rapidly excised and flash frozen in liquid nitrogen and stored at -80°C until RNA isolation was performed. For immunohistochemistry, tissues were collected as described below. All dissections were performed between 12 PM – 3 PM to minimize circadian variability (Tosini et al, 2008).

2.2. RNA isolation, library preparation, and RNAseq

Total ribonucleic acid (RNA) was isolated from mouse retinas using miRNAeasy Kit (QIAGEN, cat no.: 217004. Hilden, Germany) according to manufacturer's protocol. RNA quality and quantification were assessed using Nanodrop, agarose gel electrophoresis, and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) for RNA integrity (RIN). The RIN score for all samples was between 9 and 9.6, and the 260/280 nm ratios for all samples was less than 2.1 indicating acceptable suitability for sequencing. RNA library construction, sequencing and data processing was performed by Novogene, Inc (Sacramento, CA). RNA Libraries were sequenced using an Illumina HiSeq 4000 (Illumina Inc, San Diego, CA)

2.3. Data processing

Raw reads were filtered to remove low quality reads and reads with adaptors, the latter representing linkers that were ligated to each single DNA molecule during library

preparation. Reads were then directly mapped to a reference genome downloaded from the genome website browser (Mus_musculus_mm10, NCBI/UCSC/Ensembl) using the STAR software for mapping. Reference genome indexes were built also using STAR and the paired-end clean reads were aligned to the reference genome using STAR (v2.5) (Dobin et al, 2013) which used the maximal mappable prefix (MMP) to produce precise mapping results for junction reads. Visualization of mapping results took place in the Integrative Genomics Viewer (IGV), a high-performance visualization tool for interactive exploration of large, integrated genomic datasets (Thorvaldsdóttir et al, 2013).

2.4. Data analysis and statistics

Data analysis was performed using a combination of programs, including STAR, HTseq, Cufflink and Novogene's propriety wrapped scripts. Alignments were parsed using Tophat program and differential expression was determined through DESeq2/edgeR (Robinson et al, 2010). DESeq2 provided statistical routines for determining the differential expression in digital gene expression using a model for negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach (Zhang et al, 2019) to control for false discovery rate (FDR). Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were implemented for pathway analysis using the clusterProfiler R package (Shen et al, 2012). The threshold for determining significant differential gene expression was a corrected pvalue (padj, or p adjusted) of less than 0.05 and an absolute fold change of 1.3 (see Fig. 2).

2.5. qPCR validation of RNA-seq results

GO and KEGG analysis guided the selection of genes related to visual function for further validation using quantitative polymerase chain reaction (qPCR) and custom RT² Profiler PCR Arrays (QIAGEN, Hilden, Germany). RNA (150 ng) from each sample was converted to cDNA using the RT² First Strand Kit. The cDNA was then aliquoted into a 384-well custom RT² Profiler array and the reaction was performed on a CFX 384 (Bio-Rad Laboratories, Hercules, CA). All genes were normalized to the geometric mean of the housekeeping genes *Pgk1* and *Sdha* (Wang et al, 2019).

2.6. Immunohistochemistry (IHC)

In order to further validate both RNAseq and qRT-PCR findings, we selected *Gb5* (guanine nucleotide binding protein (G protein), beta 5) for evaluation at the protein level using IHC. C57/Bl6 mice (n=6 each VGB, vehicle) were dosed once daily (intraperitoneally) with VGB (12.5 mg/kg/day) for 10 days from day of life 10 to 20. Higher doses of VGB in adolescent animals resulted in toxicity (Levav-Rabkin et al, 2010). The use of adolescent mice provided insight into whether the alterations of gene expression found in the developed retina of adult mice would also be associated with the developmental period, which is clinically relevant to dosing in patients where VGB is administered from infancy to adulthood. At 20 days, animals were sedated with ketamine and xylazine prior to cardiac perfusion with saline and 4% paraformaldehyde (PFA) in PBS. Following perfusion, ocular tissues were rapidly excised into 0.05% glutaraldehyde, 4% paraformaldehyde, 2% sucrose (in 0.1 M phosphate buffer) solution and incubated overnight at 4°C; retinas were subsequently embedded in agarose (6%) and sectioned (100 micron sections) with a vibratome. Retina slices were

incubated with primary antibody 1:500–1000 overnight at +4°C in 1% bovine serum albumin (BSA). Primary antibody (anti-*Gb5* (C-6), Santa Cruz Biotechnology, Inc (sc-515379) was used to detect *Gb5*. After rinsing with PBS, samples were treated with Alexa Fluor (488 and 568, 1:5000) secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI; 1:5000) for nuclear staining prior to mounting and imaging with a Zeiss Z2 (4 color). Images (3 images per retina section) were acquired at 40x magnification and oil immersion. Background fluorescence for each region was subtracted from the *no primary* antibody, the latter serving as negative control. Three regions of interest were averaged per image for both photoreceptors and ganglion cell layers and no outliers were eliminated after intensity measurement in Zen. Relative quantification was normalized to DAPI intensity and statistics were analyzed using a Welch's *t*-test in GraphPad Prism.

3. Results

3.1 Differentially expressed genes identified by RNAseq

112 differentially expressed genes were identified with a $\text{padj} < 0.05$ in animals receiving 140 mg/kg/day VGB as compared to untreated animals (58 down-regulated and 54 up-regulated genes (Supplementary Tables S1; Fig. 2). Combinatorial application of GO and KEGG identified a total of 26 of these 112 genes that were associated with visual system in relation to phototransduction, plasticity, signaling and apoptosis. In addition to genes related directly to the visual system, GO analysis indicated dysregulation in the modulation of chemical synaptic transmission ($n=11$), regulation of GABAergic synaptic transmission ($n=4$), learning and memory ($n=8$), negative regulation of ion transport ($n=6$), regulation of epithelial cell proliferation ($n=4$) and the positive regulation of neuron projection development ($n=8$) (Supplementary Table S2).

3.2 qRT-PCR validation of genes identified by RNAseq

qRT-PCR is an accepted method by which to validate RNAseq results. For the 26 genes associated with the visual system, 24 were selected for validation by qRT-PCR using custom RT² profiler arrays (Qiagen, Hilden, Germany). A limitation of the RT² profiler array was that only 24 genes could be analyzed per analysis, and thus we selected the most extensively dysregulated genes from the original initial 26 for further confirmation via qRT-PCR. The RT² profiler array revealed that 11 gene were up-regulated and 13 down-regulated (Table 1). Only five demonstrated a significant p (<0.05) value, including *Gb5*, *Bdnf*, *Crh*, *Sox9* and *Cplx3*.

3.3. Immunohistochemistry (IHC)

IHC was chosen to further validate RNAseq and qRT-PCR results. We opted to perform IHC on *Gb5* (Table 1) as a representative example of significantly dysregulated genes. Our rationale was that the fold-regulation for *Gb5* was intermediate to that of *Bdnf* and *Crh* (Table 1). Moreover, determining a gain-of-signal for up-regulated genes (*Sox 9*, *Cplx3*) was considered challenging with IHC. Sections of retina derived from VGB-treated animals demonstrated decreased immunofluorescence for *Gb5* that was restricted to photoreceptors and not the ganglion cell layer (Figs. 3 & 4; $p<0.05$, compared to vehicle). These results provided additional verification of RNAseq and qRT-PCR data.

4. Discussion

Continuous administration of VGB to mice resulted in significant dysregulation of five genes: *Gb5* (guanine nucleotide binding protein (G protein), beta 5), *Crh* (corticotropin releasing hormone), *Bdnf* (brain derived neurotrophic factor), *Sox 9* (SRY-box containing gene 9), and *Cplx3* (complexin 3). We previously demonstrated that 140 mg/kg/d VGB, administered to adult mice resulted in GABA accumulation in eye exceeding that of brain (Walters et al 2019; Chan et al, 2020). The relationship of these five genes with GABA, GABAergic receptors, or intracellular signaling has been previously established (Colmers et al 2018; Puller et al 2014; Maqsood et al 2016). Whether elevated GABA alone, or other processes associated with VGB administration, is responsible for the dysregulation of these genes remains to be determined.

Gb5 is responsible for termination of the light response in retinal rods, which requires hydrolysis of guanosine triphosphate (GTP) catalyzed by a complex of *Rgs9-1* and *Gb5* involved in phototransduction. This complex inactivates the transducin complex, which is responsible for the exchange of GTP to GDP (guanosine diphosphate; Chen et al, 2015). Studies in *Rgs9-1^{-/-}* and *Gb5^{-/-}* mice found that loss of either leads to inhibition of transduction deactivation (Morgans et al 2007). As well, characterization of dark/light adapted *Gb5^{-/-}* rod cells confirmed a profound effect on phototransduction inactivation and disrupted light adaptation. Taken together, our results of combined RNAseq, qRT-PCR and IHC for *Gb5* provide evidence for a detrimental effect of VGB on the inactivation of the phototransduction cascade.

Crh is convulsant in the brain of developing animals (Hollrigel et al, 1998), and VGB treatment leads to down-regulation of *Crh* in the immature rat brain (Tran et al, 1999), consistent with our results in adult murine retina. Certain *Crh* positive amacrine cells form GABAergic synapses with alpha ganglion cells, maintaining and balancing GABA release during fast hyperpolarization (Park et al, 2018). Thus, in the presence of excessive GABA linked to VGB intervention, it is not unreasonable to see down-regulation of retinal *Crh*. It is also noteworthy that *Crh* is released from the hypothalamus and stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary. In addition to VGB, ACTH represents a targeted therapeutic in patients with West Syndrome (infantile spasms). It would appear paradoxical, however, that *Crh* is proconvulsive in selected situations, yet its downstream hormonal target (ACTH) has anticonvulsive properties in certain instances (infantile spasms). These offsetting convulsive/anticonvulsive actions may, however, represent a homeostatic balancing mechanism for *Crh*.

Patients exposed to VGB may manifest retinal nerve fiber layer (RNFL) thinning (Origlieri et al 2016). Individuals with permanent hemianopia (partial to total vision impairment in half of the visual field) manifest RNFL thinning that correlates with thickening of the photoreceptor layer (de Araujo et al, 2017). These authors concluded that axonal injury to the inner retina led to secondary damage in the outer retina in their patients. Brain derived neurotrophic factor (BDNF; a member of the neurotrophin growth factor family) plays a significant role in axonal protection, retinal ganglia cell integrity, and stabilization of growing retinal axons via BDNF/nitric oxide signaling (Ernst et al 2000). Dysregulation of

Bdnf levels are consistent with the significant extension of dendrites into the retinal outer nuclear layer that we recently reported (Chan et al, 2020), but more detailed studies are needed to examine the potential link(s) between *Bdnf* and RNFL.

Sox 9 plays an important role in the development of the vertebrate eye (Huang et al, 2013). Wang and colleagues (2018) found that knockout of *Sox 9* resulted in a loss of glial fibrillary acidic protein (GFAP) in retinal glial (Müller) cells. Hyperstimulation of Müller cells is damaging to retinal tissue, with GFAP representing a reliable biomarker of Müller cell activation. In addition to GFAP suppression, the loss of *Sox 9* also attenuated the migration speed of Müller cells to areas of retinal wound (Wang et al 2018). Upregulation of *Sox 9* (Table 1) may represent a protective response to VGB intervention in retinal Müller cell. Moreover, *Sox 9* upregulation may have correlated with extensive rod and cone bipolar, and horizontal cell remodeling, that we observed with VGB administration (Chan et al, 2020), hypotheses which require further investigation.

SNARE (SNAP receptors) represent protein complexes that mediate vesicle fusion in mammals. *Complexins* (*Cplx*s, *synaphins*) are SNARE complex regulators controlling the speed and Ca^{2+} sensitivity of SNARE-mediated synaptic vesicle fusion and exocytosis (Yoon et al, 2018). *Cplx3* regulates signaling in retinal circuits by altering exocytosis at ribbon synapses, the latter structures that maintain the polarity necessary for protein trafficking involving vesicle fusion and exocytosis (Mohrman et al 2015). Deletion of *Cplx3* results in abnormal function/structure of retinal ribbon synapses. As noted by Roth and Draguhn (2012), the release of GABA by interneurons must be responsive to cellular needs in terms of quantity and spatiotemporal function. We hypothesize that significantly elevated retinal GABA associated with VGB correlates with the significant up-regulation of *Cplx3* (> 12-fold; Table 1), a process that requires further study, and most importantly developmental characterization.

In view of its unique mechanism of action, and its broad clinical utility in multiple epileptic syndromes, understanding and mitigating the ocular toxicity of VGB remains a serious unmet clinical challenge. Our study (as well as many reports of VGB-associated toxicity) were performed in adult animals, and the genes we identified as responsive at the transcriptional level of VGB (*Gb5*, *Crh*, *Bdnf*, *Sox 9*, *Cplx3*) all have roles in retinal development, morphogenesis, and signaling (Fig. 5) (Grabowska et al 2008; Wagle et al 2011; Forouzanfar et al 2019; Ahmed et al 2004; Zanazzi and Mathews, 2010). A limitation of our study was the limited analysis of only retina, and it will be of interest to examine the transcriptomics of other CNS compartments (e.g., hippocampus, pons, cortex, etc) to determine if we observe similar outcomes as for retina. Thus, transcriptome analyses from early development through adulthood with VGB administration would be a reasonable next step in our investigations. Further, since the retina consists of different cell populations (photoreceptors, cones, rods, amacrine and bipolar cells, ganglion cells, etc) it would be valuable to examine transcriptomics in specific cell populations in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health, National Eye Institute, NIH R01EY027476 (KMG). The authors gratefully acknowledge the assistance of Novogene Inc. in performance of RNAseq studies. We also acknowledge the assistance of Dr. Steven Fliesler for providing expertise on retinal dissection.

References

- Ahmed F, Torrado M, Zinovieva RD, et al., 2004 Gene Expression Profile of the Rat Eye Iridocorneal Angle: NEIBank Expressed Sequence Tag Analysis. *Invest. Ophthalmol. Vis. Sci* 45, 3081–90. 10.1167/iov.04-0302. [PubMed: 15326124]
- Bak LK, Schousboe A, and Waagepetersen HS, 2006 The Glutamate/GABA-Glutamine Cycle: Aspects of Transport, Neurotransmitter Homeostasis and Ammonia Transfer. *J. Neurochem* 98, 641–53. 10.1111/j.1471-4159.2006.03913.x. [PubMed: 16787421]
- Bittigau P, Sifringer M, Genz K, et al., 2002 Antiepileptic Drugs and Apoptotic Neurodegeneration in the Developing Brain. *Proc. Natl. Acad. Sci. USA* 99, 15089–94. 10.1073/pnas.222550499. [PubMed: 12417760]
- Carmant L, 2011 Vigabatrin Therapy for Infantile Spasms: Review of Major Trials in Europe, Canada, and the United States; and Recommendations for Dosing. *Acta Neurol. Scand. Suppl* 192, 36–47. 10.1111/j.1600-0404.2011.01599.x.
- Chan K, Hoon M, Pattnaik BR, et al., 2020 Vigabatrin-Induced Retinal Functional Alterations and Second-Order Neuron Plasticity in C57BL/6J Mice. *Invest. Ophthalmol. Vis. Sci* 61, 17 10.1167/iov.61.2.17.
- Chen C-K, Woodruff ML, Fain GL, 2015 Rhodopsin Kinase and Recoverin Modulate Phosphodiesterase during Mouse Photoreceptor Light Adaptation. *J. Gen. Physiol* 145, 213–24. 10.1085/jgp.201411273. [PubMed: 25667411]
- Colmers PLW, Bains JS, 2018 Balancing Tonic and Phasic Inhibition in Hypothalamic Corticotropin-Releasing Hormone Neurons. *J. Physiol* 596, 1919–29. 10.1113/JP275588. [PubMed: 29419884]
- Dobin A, Davis CA, Schlesinger F, et al., 2013 STAR: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* 29, 15–21. 10.1093/bioinformatics/bts635. [PubMed: 23104886]
- De Araújo RB, Oyamada MK, Zacharias LC, et al. 2017 Morphological and Functional Inner and Outer Retinal Layer Abnormalities in Eyes with Permanent Temporal Hemianopia from Chiasmal Compression. *Frontiers Neurol.* 8, 619 10.3389/fneur.2017.00619.
- Ernst AF, Gallo G, Letourneau PC, et al., 2000 Stabilization of Growing Retinal Axons by the Combined Signaling of Nitric Oxide and Brain-Derived Neurotrophic Factor. *J. Neurosci* 20, 1458–69. [PubMed: 10662836]
- Forouzanfar F, Shojapour M, Aghili ZS, et al., 2019 Growth Factors as Tools in Photoreceptor Cell Regeneration and Vision Recovery. *Curr Drug Targets* 11 20 10.2174/1389450120666191121103831.
- Froger N, Moutsimilli L, Cadetti L, et al., 2014 Taurine: The Comeback of a Neutraceutical in the Prevention of Retinal Degenerations. *Progr. Retinal Eye Res* 41, 44–63. 10.1016/j.preteyeres.2014.03.001.
- Gaily E, 2012 Vigabatrin Monotherapy for Infantile Spasms. *Expert Rev. Neurotherap* 12, 275–86. 10.1586/ern.12.3.
- Gibson KM, Jakobs C, Ogier H, et al., 1995 Vigabatrin Therapy in Six Patients with Succinic Semialdehyde Dehydrogenase Deficiency. *J. Inherit. Metab. Dis* 18, 143–46. 10.1007/bf00711750. [PubMed: 7564230]
- Grabowska D, Jayaraman M, Kaltenbronn KM, et al., 2008 Postnatal Induction and Localization of R7BP, a Membrane-Anchoring Protein for Regulator of G Protein Signaling 7 Family-Gbeta5 Complexes in Brain. *Neurosci.* 151, 969–82. 10.1016/j.neuroscience.2007.11.045.
- Hawker MJ, Astbury NJ, 2008 The Ocular Side Effects of Vigabatrin (Sabril): Information and Guidance for Screening.” *Eye* 22, 1097–98. 10.1038/eye.2008.139. [PubMed: 18497834]

- Hisama FM, Mattson RH, Lee HH, et al., 2001 "GABA and the Ornithine Delta-Aminotransferase Gene in Vigabatrin-Associated Visual Field Defects. *Seizure* 10, 505–7. 10.1053/seiz.2001.0524. [PubMed: 11749107]
- Hollrigel GS, Chen K, Baram TZ, et al., 1998 The Pro-Convulsant Actions of Corticotropin-Releasing Hormone in the Hippocampus of Infant Rats. *Neurosci.* 84, 71–79. 10.1016/s0306-4522(97)00499-5.
- Huang X-Q, Huang Z-X, Li Z-L et al., 2013 C-Jun Terminal Kinases Play an Important Role in Regulating Embryonic Survival and Eye Development in Vertebrates. *Curr. Molec. Med* 13, 228–37. [PubMed: 23116265]
- Ishikawa M, Yoshitomi T, Covey DF, et al., 2018 Additive neuroprotective effects of 24(S)-hydroxycholesterol and allopregnanolone in an ex vivo rat glaucoma model. *Sci Rep.* 8:12851. doi: 10.1038/s41598-018-31239-2. [PubMed: 30150786]
- Levav-Rabkin T, Melamed O, Clarke G, et al., 2010 A Sensitive Period of Mice Inhibitory System to Neonatal GABA Enhancement by Vigabatrin Is Brain Region Dependent. *Neuropsychopharmacol.* 35, 1138–54. 10.1038/npp.2009.219.
- Maqsood R, Stone TW, 2016 The Gut-Brain Axis, BDNF, NMDA and CNS Disorders. *Neurochem. Res* 41, 2819–35. 10.1007/s11064-016-2039-1. [PubMed: 27553784]
- McCall MA, Lukasiewicz PD, Gregg RG, et al., 2002 Elimination of the Rho1 Subunit Abolishes GABA(C) Receptor Expression and Alters Visual Processing in the Mouse Retina. *J. Neurosci.* 22, 4163–74. <https://doi.org/20026386>. [PubMed: 12019334]
- Messer R, Knupp KG, 2020 Infantile Spasms: Opportunities to Improve Care. *Semin. Neurol.* 3 6 10.1055/s-0040-1705121.
- Morgans CW, Liu W, Wensel TG, et al., 2007 Gbeta5-RGS Complexes Co-Localize with MGluR6 in Retinal ON-Bipolar Cells. *Eur. J. Neurosci* 26, 2899–2905. 10.1111/j.1460-9568.2007.05867.x. [PubMed: 18001285]
- Mohrmann R, Dhara M, Bruns D, 2015 Complexins: Small but Capable." *Cell. Molec. Life Sci* 72, 4221–35. 10.1007/s00018-015-1998-8. [PubMed: 26245303]
- Nicholas JM, Ridsdale L, Richardson MP, et al. 2012 Trends in Antiepileptic Drug Utilisation in UK Primary Care 1993–2008: Cohort Study Using the General Practice Research Database. *Seizure* 21, 466–70. 10.1016/j.seizure.2012.04.014. [PubMed: 22608976]
- Origlieri C, Geddie B, Karwoski B, et al., 2016 Optical Coherence Tomography to Monitor Vigabatrin Toxicity in Children. *J. Amer. Assoc. Pediatr. Ophthalmol. Strabismus* 20, 136–40. 10.1016/j.jaapos.2015.10.020.
- Osborne JP, Edwards SW, Alber FD, et al., 2019 The Underlying Etiology of Infantile Spasms (West Syndrome): Information from the International Collaborative Infantile Spasms Study (ICISS). *Epilepsia* 60, 1861–69. 10.1111/epi.16305. [PubMed: 31418851]
- Park SJH, Pottackal J, Ke J-B, et al., 2018. Convergence and Divergence of CRH Amacrine Cells in Mouse Retinal Circuitry. *J. Neurosci* 38, 3753–66. 10.1523/JNEUROSCI.2518-17.2018. [PubMed: 29572434]
- Pearl PL, Poduri A, Prabhu SP, et al., 2018. White Matter Spongiosis with Vigabatrin Therapy for Infantile Spasms. *Epilepsia* 59, e40–44. 10.1111/epi.14032. [PubMed: 29473152]
- Popova E, 2015 GABAergic Neurotransmission and Retinal Ganglion Cell Function. *J. Comp. Physiol. A (Neuroethology, Sensory, Neural, and Behavioral Physiology)* 201, 261–83. 10.1007/s00359-015-0981-z.
- Puller C, Haverkamp S, Neitz M, et al., 2014 Synaptic Elements for GABAergic FeedForward Signaling between HII Horizontal Cells and Blue Cone Bipolar Cells Are Enriched beneath Primate S-Cones. *PloS One* 9, e88963 10.1371/journal.pone.0088963. [PubMed: 24586460]
- Ricci L, Frosini M, Gaggelli N, et al., 2006 Inhibition of Rabbit Brain 4-Aminobutyrate Transaminase by Some Taurine Analogues: A Kinetic Analysis. *Biochem. Pharmacol* 71, 1510–19. 10.1016/j.bcp.2006.02.007. [PubMed: 16540097]
- Robinson MD, McCarthy DJ, Smyth GK, 2010 edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 26(1), 139–140. doi:10.1093/bioinformatics/btp616. [PubMed: 19910308]

- Roth FC, Draguhn A, 2012 GABA Metabolism and Transport: Effects on Synaptic Efficacy. *Neural Plasticity*, 805830 10.1155/2012/805830.
- Shen S, Park JW, Lu ZX., et al., 2014 rMATS: Robust and Flexible Detection of Differential Alternative Splicing from Replicate RNA-Seq Data. *PNAS*. (rMATS), 10.1073/pnas.1419161111,
- Yu G, Wang L, Han Y and He Q*, 2012 clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology*. 16, 284–287. doi: 10.1089/omi.2011.0118. [PubMed: 22455463]
- Shields WD, Pellock JM, 2011 Vigabatrin 35 Years Later - from Mechanism of Action to Benefit-Risk Considerations. *Acta Neurol. Scandin. Suppl* 192, 1–4. 10.1111/j.1600-0404.2011.01606.x.
- Sorri I, Brigell MG, Mályusz M, et al., 2010 Is Reduced Ornithine-δ-Aminotransferase Activity the Cause of Vigabatrin-Associated Visual Field Defects? *Epilepsy Res.* 92, 48–53. 10.1016/j.epilepsyres.2010.08.006. [PubMed: 20850272]
- Sulaiman SAJ, Suliman FEO, Barghouthi S, 2003 Kinetic Studies on the Inhibition of GABA-T by Gamma-Vinyl GABA and Taurine. *J. Enzyme Inhib. Med. Chem* 18, 297–301. 10.1080/1475636031000118428. [PubMed: 14567543]
- Tao Y, Yang J, Ma Z, et al., 2016 The Vigabatrin Induced Retinal Toxicity Is Associated with Photopic Exposure and Taurine Deficiency: An In Vivo Study. *Cell. Physiol. Biochem* 40, 831–46. 10.1159/000453143. [PubMed: 27941319]
- Thorvaldsdóttir H, Robinson JT, Mesirov JP, 2013 Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform.* 14, 178–192. doi:10.1093/bib/bbs017. [PubMed: 22517427]
- Tosini G, Pozdeyev N, Sakamoto K, et al., 2008 The Circadian Clock System in the Mammalian Retina. *BioEssays* 30, 624–33. 10.1002/bies.20777. [PubMed: 18536031]
- Tran V, Hatalski CG, Yan XX, et al., 1999 Effects of Blocking GABA Degradation on Corticotropin-Releasing Hormone Gene Expression in Selected Brain Regions. *Epilepsia* 40, 1190–97. 10.1111/j.1528-1157.1999.tb00847.x. [PubMed: 10487181]
- van Alphen B, Winkelman BHJ, Frens MA Age- and Sex-Related Differences in Contrast Sensitivity in C57BL/6 Mice. *Invest. Ophthalmol. Vis. Sci* 50, 2451–58. 10.1167/iovs.08-2594.
- van der Poest Clement E, Jansen FE, Braun KPJ, et al., 2020 Update on Drug Management of Refractory Epilepsy in Tuberous Sclerosis Complex.” *Paediatr Drugs* 22, 73–84. 10.1007/s40272-019-00376-0. [PubMed: 31912454]
- Vogel KR, Ainslie GR, Jansen EEW, et al., 2015 Torin 1 Partially Corrects Vigabatrin-Induced Mitochondrial Increase in Mouse. *Ann. Clin. Translational Neurol* 2, 699–706. 10.1002/acn3.200.
- Vogel KR, Ainslie GR, Schmidt MA, et al., 2017a mTOR Inhibition Mitigates Molecular and Biochemical Alterations of Vigabatrin-Induced Visual Field Toxicity in Mice. *Pediatr. Neurol* 66, 44–52.e1. 10.1016/j.pediatrneurol.2016.09.016. [PubMed: 27816307]
- Vogel KR, Ainslie GR, Pearl PL, et al., 2017b Aberrant mTOR Signaling and Disrupted Autophagy: The Missing Link in Potential Vigabatrin-Associated Ocular Toxicity? *Clin. Pharmacol. Therap* 101, 458–61. 10.1002/cpt.581. [PubMed: 27864823]
- Vossler DG, Weingarten M, Gidal BE, and American Epilepsy Society Treatments Committee. Summary of Antiepileptic Drugs Available in the United States of America: WORKING TOWARD A WORLD WITHOUT EPILEPSY. *Epilepsy Curr.* 18, 1–26. 10.5698/1535-7597.18.4s1.1.
- Wagle M, Mathur P, Guo S, 2011 Corticotropin-Releasing Factor Critical for Zebrafish Camouflage Behavior Is Regulated by Light and Sensitive to Ethanol. *J. Neurosci* 31, 214–24. 10.1523/JNEUROSCI.3339-10.2011. [PubMed: 21209207]
- Walters DC, Jansen EEW, Ainslie GR, et al., 2019 Preclinical Tissue Distribution and Metabolic Correlations of Vigabatrin, an Antiepileptic Drug Associated with Potential Use-Limiting Visual Field Defects. *Pharmacol. Res. Perspect* 7, e00456 10.1002/prp2.456. [PubMed: 30631446]
- Wang X, Shu Q, Ni Y, et al., 2018 CRISPR-Mediated SOX9 Knockout Inhibits GFAP Expression in Retinal Glial (Müller) Cells. *Neuroreport* 29, 504–8. 10.1097/WNR.0000000000001143. [PubMed: 29538098]

- Wild JM, Aljarudi S, Smith PEM, et al., 2019 The Topographical Relationship between Visual Field Loss and Peripapillary Retinal Nerve Fibre Layer Thinning Arising from Long-Term Exposure to Vigabatrin.” *CNS Drugs* 33, 161–73. 10.1007/s40263-018-0583-8. [PubMed: 30637668]
- Yang J, Naumann MC, Tsai Y-T, et al., 2012 Vigabatrin-Induced Retinal Toxicity Is Partially Mediated by Signaling in Rod and Cone Photoreceptors. *PloS One* 7, e43889 10.1371/journal.pone.0043889. [PubMed: 22970106]
- Yoon T-Y, Munson M, 2018 SNARE Complex Assembly and Disassembly. *Curr. Biol.* 28, R397–401. 10.1016/j.cub.2018.01.005. [PubMed: 29689222]
- Zanazzi G, Matthews G, 2010 Enrichment and Differential Targeting of Complexins 3 and 4 in Ribbon-Containing Sensory Neurons during Zebrafish Development. *Neural Develop.* 5, 24 10.1186/1749-8104-5-24.
- Zhang MJ, Xia F, Zou J Fast and Covariate-Adaptive Method Amplifies Detection Power in Large-Scale Multiple Hypothesis Testing. *Nature Commun.* 10, 3433 10.1038/s41467-019-11247-0.

Highlights

- RNA sequencing/validation was performed on vigabatrin (VGB)-treated mouse retinas
- 5 genes linked to structure/function/signaling were significantly dysregulated
- 3 genes (*Gb5*, *Bdnf*, *Crh*) were down-regulated; 2 (*Sox9*, *Cplx3*) up-regulated
- This is the first transcriptome analysis of VGB effects on retinal gene expression
- The results may highlight novel gene targets correlated to VGB's ocular toxicity

Fig 1. GABA metabolism and the site of action of vigabatrin.

Abbreviations: GAD, glutamic acid decarboxylase; GABA-T, GABA transaminase (also aminobutyrate aminotransferase); SSADH, succinic semialdehyde dehydrogenase. Succinic acid ultimately converts the carbon skeleton of GABA into a substrate for the Krebs cycle in the form of succinic acid. Note that the obligate nitrogen acceptor in the GABA-T reaction is 2-oxoglutarate, which generates a mole of glutamic acid for each mole of GABA metabolized. The latter cyclical process is referred to as the “GABA shunt”.

Fig 2. Volcano plot of differential gene expression associated with vigabatrin (140 mg/kg/day) administration.

The horizontal axis displays the gene fold change ($\log_2(\text{fold change})$), while the vertical axis represents statistically significant degrees of changes in gene expression levels ($-\log_{10}(\text{padj})$). Red points indicate up-regulated genes (54/112) and green points indicated down-regulated genes (58/112).

Fig. 3. Representative immunofluorescence micrograph of retinal layers stained for DAPI and *Gb5*.

Shown are DAPI (blue) and *Gb5* (green), as well as capture with brightfield and merged (left to right; 100X magnification). Below the upper two panels is a zoom of photoreceptors focused on *Gb5* staining (green). Each individual region of interest (ROI) was placed in a blinded manner across the image for analysis. Sections included one/animal, 3 regions of retina, with 3 ROIs for each region imaged, n=4–5 animals for one vibratome slice per animal. Abbreviations employed: RPE, retinal pigment epithelium; PR, photoreceptors; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL,

inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; Veh, vehicle; VGB, vigabatrin (12.5 mg/kg/d for 10 days). Scale for 100X and zoom is shown for 50 μ m segments. Abbreviations: DAPI, 2-[4-(aminoiminomethyl)phenyl]-1H-Indole-6-carboximidamide hydrochloride (a stain for DNA); *Gb5*, guanine nucleotide-binding protein subunit beta-5.

Fig 4. Quantitation of *Gb5* expression in photoreceptor and ganglion cell layers.

The absolute immunofluorescence of *Gb5* was corrected for DAPI immunofluorescence in the same tissue. Animals were treated for 10 days, age of life 10–20 days, with VGB at 12.5 mg/kg/d, or vehicle (n=6 per cohort). Each individual data point represents three averaged ROIs (regions of interest) from one individual scanned region, with 3 regions/animal and n=4–5 eyes per group. Data presented as mean + SEM. Statistical analysis employed a two-tailed *t* test.

Fig. 5. Schematic diagram of the dysregulated genes identified in the current study.

The roles of these genes in retinal circuitry, signaling or function is shown below the gene abbreviation (*italics*), along with their potential impact on retinal components in the center. Directionality of arrows indicate either down-regulation or up-regulation (orange). Note that the up-regulation of *Cplx3* (12-fold) was considerably larger in magnitude than any of the other genes shown.

Table 1:

Validation of RNA-SEQ Hits using qRT-PCR

Gene ID	Gene Name	Fold Regulation	P Value
Rem2	Rad and gem related GTP binding protein 2	-1.6149	0.064004
Gb5	Guanine nucleotide binding protein (G protein), beta 5	-1.5228	0.012241
Ptpn5	Protein tyrosine phosphatase, non-receptor type 5	-1.2928	0.437776
Vgf	VGF nerve growth factor inducible	-2.4344	0.070605
Bdnf	Brain derived neurotrophic factor	-1.3205	0.000487
Crh	Corticotropin releasing hormone	-2.3208	0.036148
Grk1	G protein-coupled receptor kinase 1	-1.121	0.597184
Gcap1	Guanylate cyclase activator 1a (retina)	-1.127	0.198999
Aipl1	Aryl hydrocarbon receptor-interacting protein-like 1	-1.1622	0.175192
BC027072	CDNA sequence BC027072	-1.1869	0.338464
Notch1	Notch gene homolog 1 (Drosophila)	-1.2042	0.375271
Sox9	SRY-box containing gene 9	1.3214	0.026125
Syt7	Synaptotagmin VII	1.0844	0.469485
Hrh3	Histamine receptor H3	1.0346	0.793852
Nlgn1	Neurologin 1	1.2214	0.16884
Cnr1	Cannabinoid receptor 1 (brain)	1.2974	0.163387
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	1.0903	0.780956
Serpine2	Serine (or cysteine) peptidase inhibitor, clade E, member 2	1.1261	0.605115
Nlgn3	Neurologin 3	-1.1403	0.484625
Calcr1	Calcitonin receptor-like	1.0237	0.749487
Slc25a13	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13	1.0944	0.646481
Cplx3	Complexin 3	12.2884	0.039817
Slc13a3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	1.1296	0.848099
Arl6ip1	ADP-ribosylation factor-like 6 interacting protein 1	-1.0689	0.439723

RNA for this study was identical to those samples employed for RNA-Seq studies. The p values are calculated based on a Student's t-test of the replicate $2^{(-\Delta\Delta CT)}$ values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in red. These data reflect relative quantification whereas the RNA-seq data is absolute quantification.