Very long-chain fatty acids support synaptic structure and function in the mammalian retina

Blake R. Hopiavuori1,2,*, Lea D. Bennett5, Richard S. Brush2,4, Matthew J. Van Hook6, Wallace B. Thoreson6,7 and Robert E. Anderson1–4

1 Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, 608 S. L. Young Boulevard, Oklahoma City, OK 73104 Oklahoma, USA
2 Dean McGee Eye Institute, Oklahoma City, Oklahoma, USA
3 Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA
4 Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA
5 Retina Foundation of the Southwest, Dallas, Texas, USA
6 Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, Nebraska, USA
7 Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska, USA

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Abstract – Elongation of Very Long chain fatty acids-like 4 (ELOVL4) is a fatty acid elongase responsible for the biosynthesis of very long chain (VLC; ≥ C26) fatty acids in the retina, brain, skin, Meibomian gland, and testes. Heterozygous inheritance of mutant ELOVL4 causes juvenile macular degeneration in autosomal dominant Stargardt-like macular dystrophy (STGD3). Retinal photoreceptors are enriched with VLC polyunsaturated fatty acids (VLC-PUFAs), which have been shown by our group and others to be necessary for the survival of rod photoreceptors. Our group performed a series of studies using mice conditionally depleted of retinal Elov4 (KO) aimed at understanding the role of VLC-PUFAs in long-term retinal health and function, focusing on the role of these fatty acids in mediating synaptic function between the photoreceptors and the rest of the neural retina. The absence of VLC-PUFA from the retina of KO mice resulted in a marked decrease in retinal b-wave responses of the electroretinogram as well as a decrease in the amplitude of the oscillatory potentials mediated by the neural retina. Although there were no measurable differences between KO and wild type (WT) mice in either pre-synaptic rod calcium channel function or post-synaptic bipolar cell glutamate receptor responses, ultrastructural analysis revealed a marked decrease in the diameter of synaptic vesicles in rod terminals. Recent quantification suggests that this decrease in synaptic vesicle size due to the absence of VLC-PUFAs in KO mice, and the consequent decrease in glutamate content, could account for the decrease in b-wave response amplitudes that were previously measured in these animals.

Keywords: ELOVL4 / very long chain fatty acids / very long chain polyunsaturated fatty acids / Stargardt-like macular dystrophy / Lipids in retinal structure and function

Résumé – Les acides gras à très longue chaîne participent à la structure et à la fonction synaptique dans la rétine des mammifères. L’ELOVL4 (elongation of very long chain fatty acids-like 4) est une élongase d’acide gras impliquée spécifiquement dans la voie de biosynthèse d’acides gras à très longue chaîne (VLC de longueur de chaîne ≥ 26 atomes de carbone) dans la rétine, le cerveau, la peau, la glande de Meibomius (glande sébacée située dans l’épiderme des paupières) et les testicules. La transmission de la mutation hétérozygote ELOVL4 provoque une dégénérescence maculaire juvénile dans la dystrophie maculaire autosomique dominante de type Stargardt (STGD3). Les photorécepteurs rétinien sont enrichis en acides gras polyinsaturés à très longue chaîne (AGPI-VLC), qui s’avèrent nécessaires à la survie des photorécepteurs en bâtonnet. Afin de mieux comprendre le rôle spécifique de ces acides gras dans le fonctionnement rétinien et à plus long terme la santé, notre groupe de recherche a effectué une série d’études utilisant des souris génétiquement dépourvues (KO) d’ELOVL4 rétinienne, en se concentrant sur la médiation de la fonction synaptique entre les photorécepteurs et le reste de la rétine neurale. L’absence d’AGPI-VLC dans la rétine de la souris KO pour ELOVL4 conduit à une diminution marquée de la réponse électrique de la rétine, à savoir ondes b de l’électrorétinogramme et amplitude des potentiels oscillatoires véhiculés par la rétine neurale.

* Correspondence: blake-hopiavuori@ouhsc.edu

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Bien qu’il n’y ait pas de différences mesurables entre les souris KO et sauvage au niveau des fonctions présynaptique des cellules en bâtonnet (canaux calciques) et postsynaptique des cellules bipolaires (récepteurs au glutamate), une analyse ultrastructurale a révélé une diminution marquée du diamètre des vésicules synaptiques au niveau terminal (bâtonnets). Une donnée récente suggère que cette diminution de la taille des vésicules synaptiques chez la souris KO pour ELOVL4 déficiente en AGPI-VLC, et la diminution consécutive du contenu du glutamate, pourrait expliquer la diminution des amplitudes de réponse des ondes b qui ont été précédemment mesurée chez ces animaux.

**Mots clés :** Acides gras à très longue chaîne / acides gras polyinsaturés à très longue chaîne / dystrophie maculaire de type Stargardt / lipides dans la structure et la fonction rétinienne

1 Introduction

Elongation of Very Long chain fatty acids-like 4 (ELOVL4) is a fatty acid elongase responsible for the biosynthesis of very long chain (VLC; ≥ C26) fatty acids that are found as components of more complex lipid molecules such as sphingolipids and phospholipids in the retina, brain, skin, Meibomian gland, and testes (Agbaga et al., 2008; Aveldano, 1987; Brush et al., 2010; Poulos et al., 1987; Vasireddy et al., 2007). In the retina the predominant VLC fatty acids are polyunsaturated fatty acids (VLC-PUFA) which are typically esterified within a phosphatidylcholine molecule alongside DHA (22:6 n3) (Agbaga et al., 2010) (Fig. 1). VLC saturated fatty acids (VLC-SFA) are primarily found in sphingolipids (Brush et al., 2010; Poulos, 1995).

Autosomal dominant Stargardt-like macular dystrophy (STGD3) is a juvenile form of progressive macular degeneration that begins with onset of vision loss as early as nine years of age and is characterized by loss of the macula and subsequent formation of a central scotoma. STGD3 is caused by a five base pair deletion and frameshift mutation in exon six of the ELOVL4 gene (Donoso et al., 2001; Edwards et al., 2001; Griesinger et al., 2000; K尼亚泽va et al., 1999; Zhang et al., 2001). The frameshift mutation induces a pre-mature stop codon and causes a premature termination of the transcript, resulting in a truncated ELOVL4 protein devoid of its ER-retention motif. Since the ELOVL4 protein must be retained in the ER to perform its enzymatic function (Agbaga et al., 2008; Barabas et al., 2013; Harkewicz et al., 2012; Logan et al., 2014), loss of the ER-retention motif causes the ELOVL4 protein to be mislocalized within the cytosol (Agbaga et al., 2014). The mutant protein does not have any enzymatic activity of its own (Logan et al., 2013). However, using *in vitro* cell-based and cell-free microsomal assays, we found that co-expression of different forms of both wild-type and mutant ELOVL4 resulted in a significant dominant-negative effect of the mutant protein on both localization and enzymatic activity of the wild-type protein (Logan et al., 2013). This suggests that the retina phenotype observed in STGD3 results from a loss of VLC-PUFA products due to the dominant negative effect of an enzymatically inactive mutant protein.

It is known that VLC-PUFA are incorporated into phosphatidylcholine and are densely packed into photoreceptor outer segment membranes, but their presence and function in retinal synapses was relatively unknown until recently. To determine the role of VLC-PUFA in the structure and function of retinal synapses, we conditionally deleted *Elovl4* from rod and cone photoreceptors in mice and evaluated inner retinal function, synaptic architecture, and the ultrastructure of VLC-PUFA-depleted photoreceptor terminals (Bennett et al., 2014).

2 Retinal synapses contain VLC-PUFA

Ribbon and conventional synapses were prepared from fresh bovine retinas by sucrose gradient centrifugation.
3 Loss of VLC-PUFA from retinal synapses results in a decrease in both synaptic vesicle diameter and number

Transmission electron microscopy (TEM) was used to evaluate ultrastructural changes within the retinal synapses of Elov14 KO and control mice. A randomized blind study revealed that synaptic vesicle diameter was significantly reduced in KO mice at 12 months of age. WT mice had an average vesicle diameter of 29.5 ± 0.93 nm, whereas KO mice had an average vesicle diameter of 24.5 ± 0.62 nm, with the majority measuring less than 29.0 nm (Figs. 2C and 2D). In addition,}

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<th>Table 1. Phosphatidylcholine molecular species are different in retinal synaptosomes compared to photoreceptor outer segments.</th>
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<td>PC34:01, 16:0/18:1†‡</td>
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<td>PC38:06, 16:0/22:6†‡</td>
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<td>PC40:06, 18:0/22:6†‡</td>
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<td>Σ VLC-PUFA†</td>
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PC number of carbons; number of double bonds Σ VLC-PUFA, sum of very long chain polyunsaturated fatty acids; ROS, rod outer segments. † RS versus ROS, p < 0.05. ‡ CS versus ROS, p < 0.05. † RS versus CS, p < 0.05. (This research was originally published in Investigative Ophthalmology and Visual Science. Bennett et al., 2014. © Association for Research in Vision and Ophthalmology.)
the number of tethered presynaptic vesicles (within 40 nm of the synaptic ribbon) was significantly reduced in the KO mice with an average of 3.6 ± 0.2 vesicles/μm of the presynaptic ribbon, while WT mice had an average of 4.7 ± 0.3 vesicles/μm of the presynaptic ribbon (Figs. 2E and 2F).

4 Loss of VLC-PUFA results in synaptic reorganization

Immunohistochemistry performed on WT and KO retinas revealed a notable change in synaptic organization in Elovl4 KO mice (Figs. 3A and 3B). The rod glutamatergic terminals (marked by VGLUT1 staining) make synaptic connection with bipolar cells (marked by PKC-α staining) within the outer plexiform layer (OPL). In the case of the KO mice, the rod photoreceptor terminals (green) appear to withdraw their terminals from the OPL where they should be making connections with the bipolar cell dendrites (orange). This results in a loss of VGLUT1 positive terminals in the OPL of KO mice and an increase in PKC-α staining within the outer nuclear layer (ONL) as the bipolar cell dendrites appear to extend downward in an attempt to re-connect with the withdrawn presynaptic terminals. Therefore it is likely that the decreased presynaptic vesicle size and number resulted in decreased synaptic efficiency and drove the subsequent reorganization of the rod terminals and the bipolar dendrites within the OPL.

5 Loss of VLC-PUFA results in rod-mediated functional deficits within the neural retina

Electroretinography (ERG) provides a non-invasive means to assess the electrophysiological responses of the photoreceptor outer segments (a-wave) and the neural retina (b-wave) in response to varying intensities of photostimulation. ERG was performed as described (Bennett et al., 2014) in order to analyze the various stages of the retinal response to light. These
studies revealed that the outer segment-mediated a-wave was significantly reduced in the KO mice compared to WT mice. This loss of a-wave response could be explained by the loss of rod photoreceptor cells. In addition, the b-wave response induced by the photoreceptor pre-synaptic terminals and mediated by the neural retina was also significantly reduced in KO mice compared to WT (Figs. 4A and 4B). This reduction in b-wave amplitude, however, was greater than predicted from the loss or rod photoreceptor cells and represented specific changes in the retinal synapses in the KO mice. Using a Butterworth filter (30 and 80 Hz) to remove a- and b-wave contamination allowed for the isolation of oscillatory potentials (OPs), which are mediated by the synaptic feedback responses of amacrine, horizontal, and bipolar cells to the initial rod photoreceptor response (Wachtmeister, 1998). OP amplitudes were significantly decreased in KO mice compared to WT mice, suggesting a decrease in synaptic efficiency within the neural retina following depletion of VLC-PUFA (Figs. 4C and 4D).

6 Deficits in synaptic transmission due to the absence of vlc-pufa are not due to deficits in pre-synaptic calcium currents or post-synaptic glutamatergic currents

Whole-cell patch clamp recordings were used to evaluate the amplitude and voltage dependence of pre-synaptic rod photoreceptor inward calcium currents ($I_{Ca}$) as well as glutamate-mediated post-synaptic currents in retinal slices from both WT and KO mice. Recordings were performed under standard conditions as described previously (Bennett et al., 2014; Van Hook and Thoreson, 2013). There was no significant difference in $I_{Ca}$ between WT and KO mice, indicating that loss of pre-synaptic $I_{Ca}$ are not responsible for any decreases in synaptic transmission in KO mice (Figs. 5A–5C). Post-synaptic glutamatergic currents mediated by mGluR6 were evaluated by measuring responses to the mGluR6 antagonist CPPG applied in the presence of the metabotropic glutamate receptor group 3-selective agonist L-2-amino-4-phosphonobutyric acid (L-AP4). Rod bipolar cells were voltage clamped at $-60$ mV and responses were evoked by transient and localized CPPG application. Inward rod bipolar cell mediated currents were measured and no significant differences were detected between WT and KO mice, indicating that changes in the b-wave were not mediated by dysregulation of post-synaptic glutamate receptor currents (Figs. 5D–5F). A lack of significant differences between WT and KO mice in both pre-synaptic $I_{Ca}$ and post-synaptic rod bipolar cell glutamate receptor currents indicates that the decrease in synaptic transmission is most likely due to deficits in pre-synaptic release downstream of $I_{Ca}$, but upstream of post-synaptic metabotropic glutamate receptor responses. This is consistent with the possibility that the deficit in the b-wave arises from decreases in presynaptic release of glutamate, perhaps due to a small number of pre-synaptic vesicles or a decrease in vesicle size.

7 Decreases in B-wave neural retina responses correlate with decreases in pre-synaptic release of glutamate

The diameters of synaptic vesicles from KO mice were significantly smaller than those from WT mice, 24.5 nm vs.
29.5 nm (Figs. 2C and 2D). Because volume scales with the cube of radius, this relatively small change in diameter would result in a reduction in volume (and thus glutamate content) of ~57% (43% of control). This reduction in glutamate content could explain the roughly 50% decrease in b-wave amplitude in KO mice.

8 Discussion

Our studies demonstrate a clear role for ELOVL4 in supporting the structural and functional integrity of neuronal synapses within the mammalian retina. VLC-PUFA are not exclusively expressed in photoreceptor outer segments as was previously thought, but rather are present within both the large ribbon synapses made up of the photoreceptor and bipolar cells as well as the smaller conventional synapses utilized by the rest of the neural retina. The depletion of these very long chain fatty acids clearly provoked changes in the membrane structure of presynaptic photoreceptor terminals, causing them to withdraw into the ONL away from the bipolar cell dendrites, creating a larger distance for glutamate to diffuse before reaching its post-synaptic target. This retraction of pre-synaptic terminals from their post-synaptic targets, combined with a decrease in synaptic vesicle diameter and number, results in less glutamate being released and translates into a gross dysregulation of synaptic efficiency, which can be measured directly by decreases in the electrophysiological responses of the scotopic system. This dysregulation cannot be accounted for by changes in inward pre-synaptic \( I_{\text{Ca}} \), which are necessary for synaptic vesicle docking and release (DeLorenzo and Freedman, 1978; Katz and Miledi, 1967) or by changes in post-synaptic glutamate receptor-mediated currents. This further supports the idea that the changes measured within the scotopic system are mediated by the decrease in pre-synaptic vesicle diameter and number, translating to a smaller pool of releasable vesicles as well as a decrease in the quantal size of individual vesicles. The reduction in vesicle diameter results in an average volume reduction of 57%, which correlates with a decrease in scotopic b-wave responses of ~50% at higher stimulus intensities. It is important to note that these studies were conducted on 12-month-old mice and that this is an age-dependent phenotype where over time the reduction of these VLC-products results in retinal degeneration, synaptic remodeling, and dysregulation of synaptic function.

In an earlier publication from our group, (Brush et al., 2010), we found very long chain saturated fatty acids (sum of 26:0 + 28:0 + 30:0) in the neutral sphingolipids of the rat and bovine retina, as well as in bovine ROS. Since ELOVL4 is responsible for the synthesis of all very-long chain fatty acids (≥ C26) regardless of their degree of unsaturation, another possible scenario is that these very long chain saturated fatty acids, which exist as components of sphingolipid molecules,
are providing a significant level of structural support for synaptic membrane size, while the very-long chain polyunsaturated fatty acids, which exist as components of phosphatidylcholine molecules, are providing a significant level of structural support for synaptic membrane morphology and fluidity. The longer the fatty acid chain, the more dynamic its influence can be on the structural and biophysical properties of a membrane. The biophysical properties of these two types of VLC-FA are very different, so it is possible that their ratios must be carefully balanced to achieve the proper size and curvature of a synaptic vesicle membrane. The loss of the VLC-PUFA could explain the significant changes in synaptic vesicle morphology, such as the lack of curvature, while the loss of the VLC-SFA could explain the significant reduction in synaptic vesicle size, as both ultrastructural changes were found in Elolv4 KO mice (Bennett et al., 2014).

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References


