Excitatory amino acid transporter EAAT5 improves temporal resolution in the retina

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Abstract

Excitatory amino acid transporters (EAATs) remove glutamate from the synaptic cleft. In the retina, EAAT1 and EAAT2 are considered the major glutamate transporters. However, it has not yet been possible to determine how EAAT5 shapes the retinal light responses because of the lack of a selective EAAT5 blocker or EAAT5 knock-out animal model. In this study, EAAT5 was found to be expressed in a punctate manner close to release sites of glutamatergic synapses in the mouse retina. Light responses from retinae of wild-type and of a newly generated model with a targeted deletion of EAAT5 (EAAT5\(^{-/-}\)) were recorded in vitro using multi-electrode arrays. Flicker resolution was considerably lower in EAAT5\(^{-/-}\) retinae than in wild-type retinae. The close proximity to the glutamate release site makes EAAT5 an ideal tool to improve temporal information processing in the retina by controlling information transfer at glutamatergic synapses.

Significance statement

Neurons communicate with other neurons at synaptic connections by release of neurotransmitters acting at postsynaptic receptors. Neurotransmitters are removed from the synaptic cleft by transporters. Using the mouse retina as a model for the central nervous system, the role of EAAT5 that functions as glutamate transporter and as glutamate-gated ion channel was investigated in retinal information processing. EAAT5 was found highly localized to the glutamate release site at retinal synapses, suggesting a role in shaping of synaptic responses. In a mouse model devoid of EAAT5, temporal resolution of the retina was severely compromised. The results demonstrate that glutamate transporters like EAAT5 can exert a tremendous effect on information processing in neuronal networks.
Introduction

Excitatory amino acid transporters (EAATs) are secondary active transporters that belong to solute carrier family 1 (SLC1). EAATs rapidly remove glutamate from the synaptic cleft after its release from presynaptic nerve terminals (Vandenberg and Ryan, 2013; Rose et al., 2018) to terminate glutamatergic synaptic transmission and prevent glutamate excitotoxicity. EAATs function as both glutamate transporters and glutamate-gated anion channels (Machtens et al., 2015; Fahlke et al., 2016). To date, five different EAAT isoforms have been described in mammals (EAAT1 - EAAT5). These differ significantly in their glutamate transport rates (Gameiro et al., 2011; Mim et al., 2005), but not in their unitary anion current amplitudes (Schneider et al., 2014; Torres-Salazar and Fahlke, 2007). Whereas EAATs 1–3 are effective glutamate transporters (with glutamate uptake currents that exceed their anion currents), the transport rates of EAAT4 and EAAT5 are smaller; therefore, they are assumed to predominantly function as glutamate-gated anion channels.

Glutamate is the major excitatory neurotransmitter in the mammalian retina, where it is used by photoreceptors in the outer plexiform layer (OPL) and bipolar cells in the inner plexiform layer (IPL). EAAT1 (also called GLAST in the mouse) is expressed at high levels in Müller cells and seems to be responsible for most retinal glutamate reuptake (Izumi et al., 2002; Sarthy et al., 2005; Derouiche and Rauen, 1995; Rauen et al., 1996; Rauen et al., 1998; Lehre et al., 1997; Pow and Barnett, 1999; Kugler and Beyer, 2003; Fyk-Kolodziej et al., 2004; Rauen et al., 2004). EAAT2 (GLT-1) is expressed in photoreceptors and bipolar cells (Rauen et al., 1996; Rauen et al., 1998; Rauen and Wiessner, 2000; Harada et al., 1998) (both glutamatergic cell types), suggesting a role for EAAT2 in glutamate reuptake and recycling. The retinal distribution and function of EAAT5 are less clear owing to lack of a selective EAAT5 blocker or an EAAT5 knock-out animal model. EAAT5 has been localized immunohistochemically to both synaptic layers (Wersinger et al., 2006; Tse et al., 2014; Fyk-
Kolodziej et al., 2004), but also to the somata of some bipolar, amacrine, and ganglion cells, as well as to photoreceptors, including their inner segments (Wersinger et al., 2006; Pow and Barnett, 2000; Lee et al., 2013). Electrophysiological measurements have also suggested the presence of EAAT5 on the terminals of photoreceptors and rod bipolar cells (Wersinger et al., 2006; Veruki et al., 2006).

In this study, the first genetically modified mouse model (EAAT5\(^{-/-}\)) with a targeted deletion of the \textit{Slc1a7} gene, which encodes EAAT5 was generated and analyzed. The EAAT5 expression pattern was defined in normal mouse retinae using a newly generated monoclonal anti-EAAT5 antibody. EAAT5 was strongly expressed in a punctate manner close to glutamate release sites in both synaptic layers of the mouse retina. This signal was completely lost in the EAAT5\(^{-/-}\) retina, confirming the specificity of the antibody. The EAAT5\(^{-/-}\) retina displays normal anatomical and synaptic organization, and features robust light responses in the form of the local field potential or ganglion cell spiking under all light regimes. However, flicker resolution was considerably compromised in EAAT5\(^{-/-}\) retina, suggesting that an important role of EAAT5 is to improve temporal resolution in the retina.

**Materials and methods**

**Animals**

Wild-type C57BL/6J and C57Bl/6N mice were obtained from Charles River. C57Bl/6N-EAAT5\(^{-/-}\) mice were generated by microinjection of the transcription activator-like effector nuclease (TALEN) in fertilized eggs (Cyagen Biosciences, Santa Clara, US). Breeding animals delivered by the company displayed irregular retinal morphology that was observed in the +/+, +/-, and -/- genotypes. Mice were back-crossed into the C57Bl/6J background for nine generations. The resulting mice had normal retinal morphology, and immunohistochemical staining with a variety of retinal markers yielded results identical to...
wild-type strains. C57Bl/6J-EAAT5<sup>-/-</sup> and C57Bl/6J-EAAT5<sup>+/+</sup> mice from heterozygous breeding were used for electrophysiological measurements and immunohistochemistry studies. All animals were kept on a 12-h light/dark cycle with food and water ad libitum. Zeitgeber time (ZT) of the experiments was 3 to 8 (ZT0 = 7 a.m.) All experiments were performed in accordance with the German Law for the Protection of Animals and following approval by the regulatory authorities.

**Generation of the anti-EAAT5 antibody, 20F12**

A monoclonal antibody against murine EAAT5 was generated using a glutathione S-transferase (GST) fusion protein. An intracellular epitope (C-terminal amino acids 441–560) present in all postulated splice-variants of rat EAAT5 (Lee et al., 2012) was chosen to ensure the detection of all potential murine variants. The amino acid sequence was: tddinliavwaldrfrtminvlgdalaagimahicrdfaqdmtekllpcetkpvltqceivaqqngcvksvaeasegtlgptcp hhipvqveqdedpaasldhctieisetnv.

Rats (strain: Lou/C) were immunized with the GST-fusion construct using standard procedures (R. Feederle, Antibody Core Facility, Helmholtz Zentrum München) and spleen cells were fused to a mouse myeloma cell line (P3X63-Ag8.653) to generate hybridoma cells. The primary supernatant was tested by ELISA and then immunochemically. Hybridoma cells were subcloned via single cell distribution until all reacted positively. The final clone was expanded and used to generate supernatant containing the monoclonal antibody (20F12; subtype IgG₂).

**Immunohistochemistry**

**Sections.** For immunohistochemistry, animals were deeply anesthetized with isoflurane and killed by decapitation. The eyes were then enucleated and opened by an encircling cut at the limbus. Eyecups with the retinas were immersion-fixed for 30 min in 4%
paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) at room temperature and then
washed in PB several times. The tissue was incubated in 10% sucrose in PB for 1 h, and then
in 30% sucrose in PB overnight. Retinae were then isolated from the eyecups, flat embedded
and frozen in optimal cutting temperature compound (NEG-50, Richard Allen Scientific,
Thermo Fisher Scientific, Germany). Vertical sections (i.e. perpendicular to the retinal layers,
20-μm thick) were cut on a cryostat (HM 560 CryoStat, Microm, Walldorf, Germany),
collected on SuperFrost Plus slides (Menzel, Braunschweig, Germany), and stored at -20°C.

**Dissociated cells.** Eyes were prepared as described above. Retinae of one animal were
removed from the eyecups, washed in 1 mL warm calcium/magnesium-free Hank’s Balanced
Salt Solution (CMF-HBSS; Sigma Aldrich) for 5 min at 37°C, and incubated for 20 min at
37°C in 1 mL warm papain (Worthington) solution (20 units of papain in 1 mL CMF-HBSS).
Papain action was stopped by washing the retinae twice in warm HBSS. The tissue was gently
dissociated in 1 mL fresh HBSS by pipetting 15–20 times using a cut and fire-polished
1000 μL plastic tip (Nerbe plus, Germany). After dissociation, cells were seeded and settled
for 30 min on poly-L-lysine-coated (Sigma) coverslips at room temperature (RT) in a
humidified chamber before fixation (5 min at RT with 4 % paraformaldehyde). The fixative
was then removed and cells were washed in PB (twice for 10 min each).

**Antibody staining.** For anti-EAAT5 antibody (20F12) staining, an antigen retrieval step
was performed: 5-min pretreatment with 1% sodium dodecyl sulfate (AppliChem) in
phosphate-buffered saline, followed by three 5-min washes in PB at RT. Sections were
incubated overnight at RT with primary antibody diluted in 5% Chemiblocker (Chemicon,
Hofheim, Germany), 0.5% Triton-X100, and 0.05% NaN₃ in PB. Sections were washed in
PB, incubated for 1 h at RT with fluorescently labeled secondary antibody diluted in PB
containing 5% Chemiblocker and 0.5% Triton-X100, and then washed in PB. The 20F12
antibody (rat IgG₂ subtype) was detected with secondary mouse anti-rat-IgG2 antibody (gift
of R. Feederle, Helmholtz Zentrum München; 1:100 in CTA; 1 h at RT) and visualized with
donkey-anti-mouse Cy3 antibody. Retinal sections were then mounted on coverslips using Aqua-Poly/Mount (Polysciences, Eppelheim, Germany). Sections and cells were examined using a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Heidelberg, Germany) with a 63×/1.4 oil immersion lens. Images were processed using Adobe Photoshop.

Primary antibodies are listed in Table 1. Secondary antibodies are listed in Table 2. Streptavidin Alexa647 (Molecular Probes), at 1:100 dilution, was used to visualize biotinylated peanut agglutinin.

**Multi-electrode arrays and data recording**

Multi-electrode arrays (MEAs; Multi Channel Systems MCS GmbH, Reutlingen, Germany) had 60 active electrodes in an 8 × 8 matrix layout with electrode diameters (d) of 30 μm and inter-electrode distances of 200 μm. Electrodes were coated with porous titanium nitride with impedance levels of 50 kΩ at 1 kHz. MEAs were pre-treated in a plasma cleaner (Diener Electronic GmbH + Co. KG, Germany) and coated with poly-D-lysine hydrobromide (Sigma, Germany).

The MEA60 data acquisition system (MC_Card, Multichannel system, Reutlingen, Germany) consisted of an RS-232 interface, an integrated preamplifier and MEA 1060 bandpass filter (amplification gain: 1200), and a personal computer. The waveforms were recorded with a sampling frequency rate of 25 kHz/channel. Data were later converted to ASCII files by MC_Data for further analysis with OriginPro8 and custom made MATLAB scripts.

Retinae of adult wild-type and EAAT5/− mice were prepared for MEA recordings. Briefly, mice were dark-adapted overnight. Retina preparation and recording were performed in dim red light. Mice were deeply anesthetized with isoflurane and killed by decapitation. The eyeballs were enucleated and retinae were isolated in carbonate-buffered Ames' solution
(Sigma), bubbled with 95% O$_2$ + 5% CO$_2$ at a pH of ~7.4 (AMES). Retinae were cut in half and stored in AMES in the dark at RT. For the experiment, one half retina was transferred with the ganglion cell side up onto a nitrocellulose filter (pore size 0.8 μm, MF-Millipore Membrane Filters, Millipore, Germany, size 5 mm × 5 mm with a central hole of 2 mm diameter). The filter/retina sandwich was transferred onto an inverted petri dish, excess buffer was removed with filter paper, and the sandwich was mounted with the ganglion cells toward the electrode side of the MEA. During recording, the retina was continuously perfused with AMES at a flow rate of 3 mL/min at RT.

Light stimulation and analysis. Retinae were stimulated in full-field mode using a white LED (ANSI white, 3465K, 185 lm at 700 mA, rise and fall time of about 100 μs) positioned below the MEA. Stimuli were generated by an external stimulator (STG 4002, Multi Channel Systems MCS GmbH) controlled by MC-Stimulus software. Each light flash was 20 ms in length. Flicker stimuli were either applied with increasing frequency at a single light intensity or at a single frequency with rising intensity. Intensities were calculated as rhodopsin isomerizations per rod and flash (rho*/rod/flash), according to Wyszecki and Stiles (2000) and Lyubarski et al. (2004). Scotopic and mesopic stimuli were applied without background light. For photopic stimulation, the retina was light-adapted on the MEA by a background light of 10,000 rho*/rod/s for 10 min (Seeliger et al., 2011) and photopic stimuli were applied on that background. Recordings of both LFP and action potentials were subjected to Fast Fourier Analysis using scripts in Neuroexplorer or Matlab. The response was considered correct when the dominant peak in the FFT matched the stimulus frequency. The fraction of correct responses (normalized to the response at 2 Hz) were plotted against stimulus frequencies.
**Results**

**Targeted EAAT5 deletion does not affect retinal anatomy or synaptic organization**

Mice carrying a homozygous deletion of *Slc1a7* developed normally and were viable. They were fertile with normal mating efficiency and no apparent behavioral deficit or disorder. No anatomical differences were observed between wild-type (Fig 1, WT, left) and EAAT5\(^{−/−}\) retinae (KO, right) in retinal thickness or layering (Fig 1A). In addition, immunohistochemical staining patterns were similar between genotypes for markers (see Fig. 1 legend for details) of photoreceptors (Fig 1B), glia cells (Fig 1C), neurons of the inner retinal network (Fig 1D), and pre- and postsynaptic elements of photoreceptor synapses (Fig 1E, F).

**EAAT5 is expressed in a punctate manner at glutamatergic synapses**

Although no EAAT5 splice variants have been reported in the mouse, several have been described in the rat (Lee et al., 2013). Therefore, a monoclonal antibody was raised against mouse EAAT5 using a C-terminal epitope homologous to a region common to all rat EAAT5 splice variants to ensure broad specificity for all potential EAAT5 splice forms in the mouse. EAAT5 was highly localized in wild-type retinae, often to small puncta in the IPL and OPL (Fig 2A, arrows). In the inner nuclear layer, some weakly labeled bipolar cell somata (arrowhead) were occasionally visible, with their processes projecting into the IPL. In Fig 2A, asterisks mark blood vessels that were nonspecifically labeled by secondary antibodies (the signal was also present in negative controls; data not shown). Importantly, punctate staining was only observed in wild-type but not in EAAT5\(^{−/−}\) retinae, indicating the high specificity of the antibody (Fig 2A, right).

Genetic ablation of EAAT5 did not change the expression patterns of EAAT1 (GLAST; Fig 2B) or EAAT2 (GLT-1; Fig 2C). Consistent with previous studies in the rat (Jungblut et
strong EAAT1 staining was found in Müller cell somata and their processes spanning the retina vertically. EAAT2 staining was localized to the somata and processes of photoreceptors (mostly cones) and bipolar cells (Rauen et al., 1996; Rauen et al., 1998; Rauen and Wiessner, 2000; Harada et al., 1998).

Fig 2D–J shows the close association of EAAT5-positive puncta with the glutamate release site on photoreceptors. Photoreceptor terminals comprise one (rod spherule) or several (cone pedicle) invaginations, each with a ribbon structure marking the glutamate release site and three to four postsynaptic processes formed by the dendrites of ON-bipolar and horizontal cells (Dowling and Boycott, 1966). Triple labeling was performed of EAAT5, mGluR6 (the glutamate receptor expressed postsynaptically in ON-bipolar cell dendrites) (Nomura et al., 1994), and the presynaptic protein piccolo. In the OPL, the anti-piccolo antibody labeled the ribbons of rods and cones (Regus-Leidig et al., 2013). In Fig 2D, several rod spherules (arrowheads) and two cone pedicles (arrow) could be distinguished. Fig 2E–J show the terminals at higher magnification. Depending on the viewing angle, the rod ribbon (blue) appeared as a line, a horseshoe shape, or an intermediate shape. The number of EAAT5 puncta per spherule seemed to depend on the viewing angle, with one punctum (Fig 2E) or two puncta (Fig 2F) between the mGluR6 and piccolo labels (indicated by arrows in Fig 2G and J) or with an additional third punctum between the two mGluR6 puncta on the rod bipolar cell dendrites; these sometimes overlapped slightly but never clearly co-localized (arrowheads in Fig 2G and J).

Multiple EAAT5 puncta might indicate EAAT5 expression by different cell types. First, the puncta might represent the tips of horizontal cells (Dowling and Boycott, 1966). However, in double labeling experiments with antibodies against the CabP28K calcium-binding protein (a good marker for horizontal cell dendritic tips) (Haverkamp and Wässle, 2000), no co-localization with EAAT5 was observed (data not shown). Second, EAAT5 might localize to a more distal site than mGluR6 on ON-bipolar cell dendrites; however, co-localization with
typical rod bipolar cell markers such as PKCα or Gα0 was not observed. Finally, EAAT5 might be expressed presynaptically in the photoreceptor terminal, with its localization restricted to parts of the plasma membrane within the invagination. Evidence for EAAT5 expression in photoreceptors comes from other studies (Wersinger et al., 2006; Pow and Barnett, 2000; Lee et al., 2013; Eliasof and Werblin, 1993; Hasegawa et al., 2006; Picaud et al., 1995). Cone pedicles harbor several invaginations: Fig 2H and I show cone pedicles in the side (2H) and top (2I) views. Similar to in rod spherules, EAAT5 was found closely associated (but never co-localized) with the ribbons and mGluR6.

Several EAAT5-positive bands could be observed in the IPL (Fig 2A). At higher magnification (Fig 3A), triple labeling showed the fine detail of EAAT5 (red) localization relative to vGlut1 (green; vesicular glutamate transporter 1; labels all bipolar cell terminals) (Johnson et al., 2003; Haverkamp et al., 2003) and ChAT (blue; choline acetyl transferase; labels bands of cholinergic processes that subdivide the IPL in a characteristic manner) (Haverkamp and Wässle, 2000). Decoration of bipolar cell terminals (green) with numerous EAAT5-positive puncta (white ellipses) was commonly seen in sublaminae 4 and 5 (SL4, SL5) but rarer in the other sublaminae. Most terminals in sublaminae 4 and 5 originate from rod bipolar cells. Therefore, retinal sections were triple labeled for piccolo (green), EAAT5 (red), and PKCα (blue, protein kinase Cα; to label rod bipolar cell terminals; Fig 3B, C) (Negishi et al., 1988; Greferath et al., 1990). Note that in the IPL, piccolo is also present in conventional synapses and, therefore, is seen outside bipolar cell terminals (Regus-Leidig et al., 2013). Nevertheless, ribbons of rod bipolar terminals can be easily identified by their close association with the PKC signal. As seen in photoreceptor invaginations, EAAT5 puncta were in close proximity to the glutamate release site of rod bipolar terminals marked by piccolo-positive ribbons (blue; Fig 3C).
Owing to the resolution limits of light microscopy, it is difficult to unequivocally distinguish pre- and postsynaptic sites at a synapse in retinal sections. Therefore, cells from mouse retinae were isolated by enzymatic digestion. In the cell preparation, EAAT5 puncta were observed on the axon terminals of acutely dissociated rod bipolar cells, again in close proximity to the ribbons (Fig 3D, E). Isolated rod and cone terminals were also found with similar EAAT5 and ribbon labeling to that seen in intact sections (compare the top right inset in Fig 3E with Fig 2F and Fig 3F to Fig 2I). These staining patterns suggest that EAAT5 is expressed on rod bipolar cell terminals and photoreceptor terminals, in agreement with previous studies (Wersinger et al., 2006; Tse et al., 2014; Fyk-Kolodziej et al., 2004; Veruki et al., 2006).

These results demonstrate that EAAT5 is closely associated with glutamatergic synapses in both synaptic layers of the retina, where it is perfectly located to mediate glutamate-driven negative feedback. Other than loss of the EAAT5 signal, no differences in marker staining were seen between wild-type and EAAT5−/− retinae. Hence, targeted EAAT5 deletion does not produce gross pathological changes in the retina. In conclusion, the EAAT5−/− mouse proved a good model to study the function of EAAT5 in generating and shaping light responses in the intact retina.

**EAAT5 improves temporal resolution in the retina**

Next, light responses were recorded from wild-type and EAAT5−/− retinae using multi-electrode arrays under three light regimes: scotopic (dark-adapted, low-intensity stimuli, rods only), mesopic (dark-adapted, medium-intensity stimuli, rods and cones active), and photopic (light-adapted with bright background, high-intensity stimuli, cone dominated). Flicker stimuli were applied with frequencies of between 2 Hz and 30 Hz. Depending on the light regime, responses to flicker stimuli start to fuse at different frequencies (i.e. the flicker fusion
frequency, which in mouse in all lighting regimes was found to be below 30 Hz). Fig 4A compares representative recordings of the local field potential (LFP) of dark-adapted retinas from wild-type (left) and EAAT5\(^{-/-}\) (right) animals when stimulated in the high mesopic range. The LFP recorded \textit{in vitro} roughly corresponds to the electroretinogram (ERG) that can be recorded \textit{in vivo} (Fujii et al., 2016). At light onset, a negative deflection was observed that corresponds to the a-wave of the ERG (mostly originating from the closure of cyclic nucleotide-gated ion channels in photoreceptors during the photoreceptor), followed by a positive deflection corresponding to the b-wave (which reflects the activity of ON-bipolar cells). In both wild-type and EAAT5\(^{-/-}\) retinas at 2 Hz, each successive light flash of the flicker stimulus initiated additional deflections superimposed on the overall waveform triggered by the first flash. At 24 Hz, retinas of neither genotype could resolve the flicker stimulus: both responses were consistent with the application of a continuous light stimulus. The wild-type retina could resolve the 10-Hz flicker stimulus, with individual responses on top of the large deflection in LFP throughout the stimulus duration, albeit at a lower amplitude than at 2 Hz. In contrast, these individual deflections could not be observed in the EAAT5\(^{-/-}\) retina (i.e. this retina could not resolve the 10-Hz flicker stimulus). For both wild-type and EAAT5\(^{-/-}\) retinas, the fraction of correct responses was determined and plotted over the stimulus frequency (Fig 4B). In EAAT5\(^{-/-}\) retina (gray curve), temporal resolution was significantly compromised: half-maximal resolution was at 10 Hz, compared with 14 Hz in the wild-type retina (black curve).

To test whether this difference also affects retinal output to the brain, the spiking behavior of ganglion cells was recorded. Up to 30 different types of ganglion cells are postulated in the mouse retina (Baden et al., 2016), each with a characteristic response behavior. Fig 5A shows one type of response that was routinely recorded in this study both in wild-type (left) and EAAT5\(^{-/-}\) (right) retina. In this ON-ganglion cell type, a 2-Hz flicker
stimulus led to relatively long bursts triggered by the first two flashes in the first part of the response, which tended to merge (“ON-merge cell”); in the last two-thirds of the response, well-separated bursts were triggered by every flash. Flicker stimuli of 2 Hz and 8 Hz could be resolved in both genotypes. At 16 Hz, the stimulus was well resolved in the wild-type cell (note the gaps separating the short bursts in the second half of the recording and the correct peak at 16 Hz in the power spectral density analysis shown below the trace) but not in the EAAT5−/− cell (very few gaps and a peak at 24 Hz instead of 16 Hz). Plotting the fraction of correctly responding cells over the flicker frequency (Fig 5B) revealed that temporal resolution by this cell type is significantly worse in EAAT5−/− (gray curve) than wild-type (black curve) retina.

The impact of EAAT5 on temporal resolution depends on stimulus intensity

The bright light stimuli employed in these experiments (Figs 4–5) resulted in large changes in the membrane potential of photoreceptors and bipolar cells concomitant with large changes in the amount of glutamate released at the terminals of these cells. It is reasonable to assume that the impact of EAAT5 on the shaping of retinal light responses depends on the extent to which glutamate release is changed by the stimulus and, hence, on the stimulus intensity. Using a flicker frequency of 12 Hz (at which large differences were observed between responses of wild-type and EAAT5−/− retina), retinæ of both genotypes were stimulated at stimulus intensities from the scotopic to the high mesopic range. Fig 6 shows the fraction of correctly responding cells plotted against stimulus intensity for different ganglion cell types. For ON-merge cells (Fig 6A), other ON-ganglion cell types (Fig 6B, pooled ON-ganglion cells except for ON-merge cells), and OFF-ganglion cells (Fig 6C) at low stimulus intensities, these values were small and differences between the two mouse genotypes were not significant. At the highest intensity, the fraction of correctly responding cells was larger in wild-type retina than in EAAT5−/− retina. The difference was statistically significant for ON-
but not for OFF-ganglion cells. With higher stimulus intensity, OFF-ganglion cells failed to respond to each flash of a flicker series, probably because they received more inhibition mediated by the rod bipolar/AII pathway (Smith et al., 1986; Wässle et al., 1986; Müller et al., 1988). The failure to respond to each flash reduced their apparent flicker resolution.

Next, it was tested whether temporal resolution in the EAAT5^{−/−} was also compromised under photopic conditions. After light adaptation in vitro for 10 min to suppress the rod contribution and isolate cone-driven responses (Seeliger et al., 2011), the LFP waveform changed to resemble the characteristic ERG for light-adapted retinae (Fig 7A). The large a-wave-like deflections were strongly reduced and the b-waves became shorter (compare Fig 4A with Fig 7A).

In the light-adapted state, differences in temporal resolution of the two genotypes were much smaller than under mesopic conditions. As shown in Fig. 7A, the retinae of both genotypes could resolve the 2-Hz flicker stimulus equally well but not the 30-Hz flicker stimulus. For the wild-type, amplitudes of the initial and successive b-waves were very similar for 2-Hz and 10-Hz flicker stimuli; at 18 Hz, the initial b-wave was twice as large as its successor. In the EAAT5^{−/−} retina at 10 Hz, amplitudes of the second and following b-waves were reduced by two-thirds compared with the initial b-wave; at 18 Hz, the flicker stimulus was barely resolved. Recordings were performed at stimulus intensities of 1,000 (Fig. 7B) and 19,000 (Fig. 7C) isomerizations per rod and flash. While there was a tendency for lower resolution in EAAT5^{−/−} retina (gray curves) compared to wild-type retina (black curves), differences did not reach statistical significance under photopic conditions.
Discussion

This study introduces a new mouse model that enabled studying the impact of EAAT5 on retinal light responses in the intact retina for the first time. The major outcome of the study is: EAAT5 is highly localized in close proximity to the glutamate release sites at photoreceptor and bipolar cell synapses. Genetic ablation of EAAT5 severely affected retinal output by compromising temporal resolution.

EAAT5 expression. In rod bipolar cells, fluorescence staining showed bright puncta of EAAT5 at the axon terminal in retinal sections, as well as in dissociated cells. In the OPL, the situation is less clear. EAAT5 puncta in the invagination co-localized with neither the CabP28K marker of horizontal cell dendritic tips (Haverkamp and Wässle, 2000) nor with mGluR6 (on ON-bipolar cells). In the rat retina, mGluR6 localizes not to the extreme tip of ON-bipolar cell dendrites but more proximally, at 200–600 nm from the active zone of the invagination (Vardi et al., 2000). Thus, EAAT5 might localize more distally than mGluR6 on ON-bipolar cell dendrites. Unfortunately, rod bipolar cell markers (PKCα and Gα0) did not label dendrites distal to mGluR6, so co-localization with EAAT5 could not be tested (data not shown). Finally, EAAT5 might be expressed presynaptically in the photoreceptor membrane within the invagination. EAAT5 expression in photoreceptors was suggested previously (Wersinger et al., 2006; Pow and Barnett, 2000; Lee et al., 2013; Eliasof and Werblin, 1993; Hasegawa et al., 2006; Picaud et al., 1995). Tse et al. (2014) also reported punctate EAAT5 staining (using a commercial antibody) in the OPL of mouse retina, but did not associate these puncta with the glutamate release site. Using the same commercial antibody in double labeling experiments, 100% co-localization with the newly raised anti-EAAT5 antibody was found (data not shown). Other studies on EAAT5 have described a more diffuse signal with no clear association with the glutamate release site (e.g. labeling of the entire terminal,
somata, and even the inner segments of photoreceptors) (Pow and Barnett, 2000; Wersinger et al., 2006). Of course, the antibodies used in these experiments have not yet been tested on EAAT5−/− retina.

**Function of EAAT5.** So far, the absence of an EAAT5-specific blocker has made it impossible to distinguish the functions of this transporter from those of other EAAT isoforms in the intact retina, in particular in shaping light responses. For example, in all previous studies EAAT-mediated currents in the retina were identified using EAAT blocker DL-threo-β-Benzylxoyaspartic acid (TBOA), which does not discriminate between EAAT isoforms. The kinetics of TBOA-blockable currents in bipolar cells (Veruki et al., 2006) were not identical to those in cells with heterologously expressed EAAT5 (Schneider et al., 2014; Gameiro et al., 2011). These differences may depend on the recording conditions or reflect differences between the recorded cell types; however, the possibility that the results of previous studies may not reflect the function of EAAT5 alone, but may also include those of other EAAT isoforms (e.g. EAAT2 in bipolar cells (Rauen et al., 1996; Rauen et al., 1998; Rauen and Wiessner, 2000; Harada et al., 1998)) cannot be excluded.

In this study, by establishing an EAAT5 knock-out mouse model, functions could be specifically attributed to this EAAT isoform. EAAT5 deletion specifically affected retinal output by compromising temporal resolution. EAAT5 might improve temporal resolution via different mechanisms: 1. Glutamate-gated chloride currents mediated by EAAT5 could trigger feedback inhibition; 2. By buffering glutamate or clearing glutamate from the synaptic cleft, EAAT5 would reduce the action of glutamate at postsynaptic as well as on presynaptic glutamate receptors.

In neuronal networks, diverse feedback mechanisms adjust the gain control to limit the response amplitude (thereby preventing saturation of cellular and network activity) and increase temporal resolution by shortening the responses. In the retina, presynaptic and postsynaptic feedback mechanisms control every step of signal processing. An example of
presynaptic feedback is activation of HCN1 channels (hyperpolarization-activated and cyclic
nucleotide-gated ion channel 1) during the hyperpolarizing light response of photoreceptors,
which effectively curtails the light response (Fain, 1978; Knop et al., 2008; Seeliger et al.,
2011). Targeted deletion of HCN1 reduced flicker resolution and led to saturation of the
retinal network by rod activity (Seeliger et al., 2011). EAAT5 might mediate another
presynaptic feedback mechanism at retinal glutamatergic synapses that strongly improves
temporal resolution. EAAT5 was found strategically localized for this function.

**EAAT5 in rod bipolar cells.** The effect of EAAT5 on temporal resolution was particularly
pronounced under mesopic light conditions when both rods and cones are active but rods
contribute a substantial fraction to the light response. Upon EAAT5 deletion, ON-ganglion
cell spiking as well as b-waves of the LFP were strongly affected, both of which depend on
rod bipolar cell activity. Characteristics of the b-wave can be affected by changing
photoreceptor input to rod bipolar cells. For example, application of APB acting at mGluR6 at
rod bipolar cell dendrites can abolish the b-wave (Slaughter and Miller, 1981; Massey et al.,
1983). Changing signal processing within the rod bipolar cells also affects the b-wave. For
example, deletion of PKCα, which is highly expressed in rod bipolar cells, affects proper
activation and termination of the rod bipolar cell responses and, hence, the characteristics of
the b-wave (Ruether et al., 2010; Xiong et al., 2015).

The effect of EAAT5 deletion is in perfect agreement with the postulated role of EAAT5
at rod bipolar cell terminals. First, EAAT5 was unequivocally localized in form of brightly
fluorescent puncta to the output synapses in both sections and isolated rod bipolar cells.
Second, EAAT5 was shown to act as glutamate-gated chloride channel in rod bipolar cells
(Veruki et al., 2006; Wersinger et al., 2006; Bligard et al., 2020). Upon depolarization of the
rod bipolar cell, glutamate release at the output synapse would activate not only the
postsynaptic glutamate receptors but also presynaptic EAAT5, leading to chloride influx,
hyperpolarization of the cell, and, consequently, reduced bipolar cell output. Indeed, chloride
currents could be elicited by depolarization and were inhibited by TBOA, providing support for a postulated EAAT5-mediated feedback mechanism. Bligard et al. (2020) postulated that EAAT-mediated inhibition is important for gain control at the rod bipolar cell output synapse. Owing to the time delay needed for activation, EAAT5-mediated inhibition may preferentially affect the later stage of depolarization and, therefore, shorten rod bipolar cell output. Shorter signals would enable the transmission of higher frequencies at this synapse. Finally, flicker resolution is higher in the cone system than in the rod system. By reducing rod bipolar cell output under mesopic conditions, EAAT5-mediated feedback might shift the balance toward the cone system to enable higher flicker resolution. However, the improvement of temporal resolution might also depend on functions of EAAT5 apart from its function as a chloride channel. EAAT5 might improve glutamate buffering and reuptake in the synaptic cleft. This glutamate clearance would help to sharpen the action of glutamate at postsynaptic cells, thereby increasing temporal resolution in wild-type retina. In EAAT5<sup>-/-</sup> retina, at low stimulus frequencies used (e.g. at 2 Hz), glutamate might diffuse from the synaptic cleft to be removed by EAAT1 and local glutamate concentration could return to baseline between individual light flashes even in the absence of EAAT5. At higher flicker frequencies, however, synaptic events might be considerably prolonged, thereby “blurring” synaptic activity and reducing temporal resolution.

Genetic ablation of EAAT5 only slightly lowered flicker resolution under photopic conditions (where the cone system dominates the light response). In electrophysiological experiments, no EAAT-like currents have been identified in mouse cone bipolar cells (Wersinger et al., 2006; Bligard et al., 2020). However, since there are at least 12 types of cone bipolar cells (for review: Euler et al., 2014) and bipolar cells can differ substantially in their inventory of ion channels (Ivanova and Müller, 2006), the small samples of recorded cells in these studies might not have included all bipolar cell types. Some EAAT5-immunoreactive puncta were associated with cone bipolar cell terminals (Fig 3A). Based on...
their stratification level in the IPL, some of these terminals might correspond to type 5 cone bipolar cells, which are ON-cone bipolar cells. EAAT5-like immunoreactivity in some cone bipolar cells was also reported by Pow and Barnet (2000) and Fyk-Kolodziej et al. (2004). Thus, while the evidence of EAAT5 expression is less compelling for cone bipolar axon terminals and EAAT5 seems to be of lesser importance for photopic vision, immunohistochemical data suggest that EAAT5 might play a role in controlling synaptic output at least in some cone bipolar cell types.

**EAAT5 in the outer retina.** There is also evidence for EAAT5 expression in photoreceptor terminals (this study; see also Wersinger et al., 2006; Pow and Barnett, 2000; Lee et al., 2013; Eliasof and Werblin, 1993; Hasegawa et al., 2006; Picaud et al., 1995). Photoreceptor synapses show a complex organization and multiple feedback mechanisms at these synapses have been described. Therefore, the physiological role of EAAT5 and the effect of EAAT5 deletion in the outer retina are more difficult to interpret than in the inner retina. Although EAAT5 is considered a glutamate transporter with a low transport rate (Gameiro et al., 2011; Schneider et al., 2014), Hasegawa et al. (2006) reported that glutamate clearance at the photoreceptor synapse mediated by EAAT5 is important for shaping of light responses at the rod–rod bipolar cell synapse in mice. Knock-out of EAAT5 in photoreceptors might lead to elevated levels of glutamate in the synaptic cleft, triggering a variety of possible effects. For example, a metabotropic glutamate receptor was reported on cone terminals that might become activated and could affect the rate of glutamate release (Van Hook et al., 2017). Depolarization of horizontal cells by increased glutamate levels would affect photoreceptor output in a complex manner. For example, feedback by horizontal cells does not only affect photoreceptor membrane potential but also causes changes in peak amplitude as well as a shift in the voltage-dependence of cone calcium channels (Verweij et al., 1996; for review see Wen and Thoreson, 2019 and Thoreson and Mangel, 2012). Finally, EAAT5 might affect photoreceptor output via its function as chloride channel. Measurements of the chloride
reversal potential in salamander (Thoreson and Bryson, 2004) and mammalian cones (Szmajda and DeVries, 2011) indicate a relatively positive chloride Nernst potential, which would argue against a negative feedback. Moreover, in a substantial body of work on salamander photoreceptors, Thoreson et al. speculated that chloride dynamics in the photoreceptor terminal affect the activation properties of voltage-activated calcium channels (Li et al., 2008; Thoreson et al., 2000; Thoreson et al., 2003; Thoreson and Bryson, 2004).

Activation of EAAT5 triggers a chloride conductance that might affect the chloride concentration in the small terminal, and hence, photoreceptor output. This delicate interaction would be suited to fine tune photoreceptor output and must be determined in future studies.

In summary, the mechanisms by which EAAT5 could affect information transfer in outer retinal synapses are quite diverse and more experiments, in particular involving single cell recordings or paired recordings of photoreceptors, horizontal cells, and bipolar cells are required to address this question. This is well beyond the scope of the present study that focused on the introduction and characterization of a new mouse model to study EAAT5 function, thereby overcoming the lack of an EAAT5-specific inhibitor. The mouse model enabled to demonstrate for the first time the role of EAAT5 in the intact retina. EAAT5 considerably increases temporal resolution of the retina. EAAT5 was found highly localized to glutamatergic ribbon synapses in both synaptic layers, consistent with a role in shaping the output of glutamatergic cells in the retina. Interestingly, EAAT5 is also expressed at the ribbon synapses of vestibular hair cells (Dalet et al., 2012) but not at the calyx of Held (Palmer et al., 2003), a well-studied conventional glutamatergic synapse. Synaptic activity in ribbon synapses is based on graded potentials of the presynaptic cell and is typically associated with much higher, sustained vesicular release compared with conventional more transient synapses. It is, therefore, tempting to speculate that EAAT5-mediated feedback triggered by glutamate release might be a common mechanism to regulate synaptic output at ribbon synapses.
References


Dalet A, Bonsaquet J, Gaboyard-Niay S, Calin-Jageman I, Chidavaenzi RL, Venteo S, Desmadryl G, Goldberg JM, Chabbert C. Glutamate transporters EAAT4 and EAAT5 are expressed in vestibular hair cells and calyx endings. *PLOSone* 2012; 7(9)e46261.


Vandenberg RJ, Ryan RM (2013) Mechanisms of glutamate transport. *Physiol Rev*


Figure legends

**Fig. 1. The EAAT5<sup>−/−</sup> retina appears histologically normal.** (A–F) Left: wild-type retina (WT), right: EAAT5<sup>−/−</sup> retina (KO). (A) Nissl staining reveals retinal thickness and retinal layering. (B) Photoreceptors, recoverin (rec, green, photoreceptor somata and inner segments), cone cyclic nucleotide-gated (CNG) channel (red, cone outer segments), rhodopsin (rhod, blue, rod outer segments). (C) Glial cells, glutamine synthetase (GS, red, Müller cells), glial fibrillary acidic protein (GFAP) (green, astrocytes). (D) Inner retinal cells, protein kinase C (PKC)<sub>α</sub> (green, rod bipolar cells, arrowheads), calcium binding protein (CabP, red, horizontal cells, arrows), calretinin (Cal, blue, several types of amacrine cells). (E) OPL, metabotropic glutamate receptor 6 (mGluR6, green, glutamate receptor on ON-bipolar cell dendrites), PKCα (red, rod bipolar cell dendrites), piccolo (Pic, blue, photoreceptor presynaptic ribbon). (F) OPL, piccolo (Pic, green, photoreceptor presynaptic ribbon), pan α1 calcium channel subunit (red, calcium channel in rod photoreceptors), postsynaptic density protein 95 (PSD95, blue, plasma membrane marker for rod terminals). GCL: ganglion cell layer, INL: inner nuclear layer, IPL: inner plexiform layer, IS: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, OS: outer segments. Scale bar in (A) also applies to (B) and (C). Wild-type and EAAT5<sup>−/−</sup>: n = 6 animals.

**Fig 2. Localization of EAATs in mouse retinal sections.** (A) EAAT5 expression. In wild-type (WT) retina (left), arrows indicate brightly labeled puncta or short processes in the OPL and IPL, the arrowhead indicates bipolar cell soma in the INL, and asterisks indicate...
nonspecific staining of blood vessels by the secondary antibody (n = 10 animals). In EAAT5\(^{-/-}\) (KO) retina (right), puncta are absent; only nonspecific staining in blood vessels is seen (n = 5 animals). (B) EAAT1 (GLAST) was strongly expressed in Müller cells that span almost all retinal layers. No difference in expression pattern or level was observed in the EAAT5\(^{-/-}\) retina (right) (n = 3 animals for wild-type and for EAAT5\(^{-/-}\)). (C) EAAT2 (GLT1) was mainly expressed in photoreceptors and bipolar cells, with no differences in expression between wild-type (left) and EAAT5\(^{-/-}\) (right) retinae (n = 3 animals for wild-type and for EAAT5\(^{-/-}\)). (D–J) Triple staining with antibodies against mGluR6, EAAT5, and piccolo (Pic) in a wild-type retina (n = 5 animals). (D) In the OPL, EAAT5 (red) is closely associated with both the piccolo-positive ribbon (blue) of photoreceptors, and the mGluR6 label on ON-bipolar cell dendrites (green). Arrow: cone terminal, arrowheads: rod terminals. (E–G, J) Higher magnification of rod spherules at different viewing angles, showing EAAT5-positive puncta (red) marked with arrows between the ribbon (blue) and mGluR6 (green) and marked with arrowheads between mGluR6 puncta. (H, I) Single cone pedicles, showing close association of EAAT5-positive puncta with both ribbons and mGluR6. GCL: ganglion cell layer, INL: inner nuclear layer, IPL: inner plexiform layer, IS: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, OS: outer segments. The scale bar in (C) also applies to (A) and (B); the scale bar in (G) also applies to (E,F,H–J).

**Fig 3. Localization of EAAT5 on bipolar cell terminals in the mouse retina.** (A) Triple staining. EAAT5-positive puncta (red) were mostly found on vGluT1-positive (green) bipolar cell terminals in sublaminae (SL) 4 and 5. Choline acetyl transferase (ChAT)-positive processes (blue) subdivide the IPL in a characteristic way (n = 3 animals). (B, C) Triple staining. Close association of EAAT5-positive puncta (red, arrowheads) and ribbons (piccolo (Pic), green, arrows) on rod bipolar terminals (PKCa, blue) (n = 6 animals). (D–F) Acutely dissociated rod bipolar cells and photoreceptor terminals (3 experiments, two retinae of one
animal/experiment). (D) Dissociated rod bipolar cell (PKCα, blue) with an axon terminal decorated with EAAT5 (red) puncta and ribbons (CTBP2, green). (E) Axon terminal of the same cell. Insets are at higher magnification. Top right corner: isolated rod terminal with horseshoe-shaped ribbon (CTBP2, green) and EAAT5 puncta (red). (F) Isolated cone terminal (PEA (peanut agglutinin), blue) with closely associated ribbons (CTBP2 green) and EAAT5 puncta (red).

**Fig 4.** Temporal resolution of the EAAT5−/− retina is significantly impaired in the mesopic range. Flicker stimuli were applied for a series of frequencies from 2 Hz to 30 Hz. (A) The LFP of the EAAT5−/− retina (right) showed impaired resolution for the 10-Hz flicker stimulus compared with the wild-type retina (left). In neither genotype could the 24-Hz flicker stimulus be resolved. Bars: duration of flicker stimulus. (B) When the fraction of correct responses was plotted against the stimulus frequency, a significant reduction in temporal resolution was apparent for EAAT5−/− (gray curve, mean ± SEM of 30 retinal pieces from 13 animals) compared with the wild-type (black curve, mean ± SEM of 33 retinal pieces from 13 animals). Two-way ANOVA with Bonferroni multiple comparisons. Flicker stimulus: 610 activated rhodopsin molecules (rho*) per rod and flash (rho*/rod/flash); total stimulus duration: 3 s; individual flash duration: 20 ms.

**Fig 5.** Temporal resolution of ON-ganglion cells is significantly impaired in the EAAT5−/− retina in the mesopic range. (A) Typical response of one type of ON-ganglion cell (“ON-merge cell”) in the wild-type (left) and EAAT5−/− retina (right). Bars: duration of flicker stimulus. (B) The fraction of correctly responding cells was significantly reduced in EAAT5−/− (gray curve, mean ± SEM of 32 cells of 10 animals) compared with wild-type (black curve, mean ± SEM of 40 cells of 10 animals) cells at frequencies of 10–20 Hz. Two-way ANOVA with Bonferroni multiple comparisons. Flicker stimulus: 610 activated rhodopsin molecules...
(rho*) per rod and flash (rho*/rod/flash) (mesopic conditions); total stimulus duration: 3 s; individual flash duration: 20 ms.

**Fig 6.** The impact of EAAT5 on temporal resolution in ganglion cells increases with stimulus intensity under mesopic conditions. Black curves: wild-type; gray curves: EAAT5\(^{-/-}\). (A) ON-merge cells (wild-type, 32 cells; EAAT5\(^{-/-}\) 15 cells). (B) ON-cells (except for ON-merge; wild-type, 92 cells; EAAT5\(^{-/-}\) 96 cells). (C) OFF-cells (wild-type, 39 cells; EAAT5\(^{-/-}\), 20 cells). Two-way ANOVA with Bonferroni multiple comparisons. Flicker stimulus: 12 Hz, total stimulus duration: 3 s; individual flash duration: 20 ms. Stimulus intensities: 17, 56, 183, 330, 610 activated rhodopsin molecules (rho*) per rod and flash (rho*/rod/flash).

**Fig 7.** Temporal resolution of the EAAT5\(^{-/-}\) retina is not significantly impaired in the photopic range. Flicker stimuli were applied for a series of frequencies from 2 Hz to 30 Hz. (A) For the 2-Hz flicker stimulus, both genotypes showed a clear response. The LFP of EAAT5\(^{-/-}\) retina (right) showed slightly impaired temporal resolution for 10-Hz and 18-Hz flicker stimuli compared with the wild-type retina (left). Neither wild-type nor EAAT5\(^{-/-}\) retinas could resolve the 30-Hz flicker stimulus. Bars: stimulus duration. (B) The fraction of correct responses plotted against the stimulus frequency for stimulus intensity of 1,000 activated rhodopsin molecules (rho*/rod/flash) shows no significant reduction for EAAT5\(^{-/-}\) (gray curve, mean ± SEM of eight retinal pieces from six animals) compared with the wild-type (black curve, mean ± SEM of 10 retinal pieces from six animals). Two-way ANOVA with Bonferroni multiple comparisons. (C) The fraction of correct responses plotted against the stimulus frequency for stimulus intensity of 19,000 activated rhodopsin molecules (rho*/rod/flash) shows a mild but not significant reduction for EAAT5\(^{-/-}\) (gray curve, mean ± SEM of eight retinal pieces from six animals) compared with the wild-type (black curve,
mean ± SEM of 10 retinal pieces from six animals). Two-way ANOVA with Bonferroni multiple comparisons. Total stimulus duration: 3 s; individual flash duration: 20 ms; background light: 10,000 rho*/rod/s.
Table 1: Primary antibodies and markers

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