Regulation of Rapid Signaling at the Cone Ribbon Synapse via Distinct Pre- and Postsynaptic Mechanisms

by

Matthew JM Rowan

A Dissertation Submitted to the Faculty of

The Charles E. Schmidt College of Science

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Florida Atlantic University

Boca Raton, Florida

August 2011

Regulation of Rapid Signaling at the Cone Ribbon Synapse via Distinct Pre- and Postsynaptic Mechanisms

by

Matthew JM Rowan

This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Wen Shen, College of Medicine, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

SUPERVISORY COMMITTEE:

Wen Shen, Ph.D.
Dissertation Advisor

Howard Prentice, Ph.D.

Janet Blanks, Ph.D.

Xupei Huang, Ph.D.

John Wu, Ph.D.

Jason Christie, PhD.

Rodney K. Murphey, Ph.D.
Chair, Department of Biological Sciences

Gary W. Perry, Ph.D.
Dean, The Charles E. Schmidt College of Science

Barry T. Rosson, Ph.D.
Dean, Graduate College

3/29/2011
Date

Acknowledgements

I would like to acknowledge all my mentors, past and present, that helped me achieve my personal goals. Most notably of this group, I acknowledge my advisor Dr. Wen Shen for all the support and guidance she provided. I would also like to thank my dissertation committee, and the Department of Chemistry at FAU for supporting my work and helping me grow as a scientist and educator, and to NIH and NSF for monetary support. Lastly, but certainly not least, I acknowledge my family and children, who help drive me to be the best I can be every day.

Abstract

Author:	Matthew JM Rowan
Title:	Regulation of Rapid Signaling at the Cone Ribbon Synapse via Distinct Pre- and Postsynaptic Mechanisms
Institution:	Florida Atlantic University
Dissertation Advisor:	Dr. Wen Shen
Degree:	Doctor of Philosophy
Year:	2011

Background: Light-adaptation is a multifaceted process in the retina that helps adjust the visual system to changing illumination levels. Many studies are focused on the photochemical mechanism of light-adaptation. Neural network adaptation mechanisms at the photoreceptor synapse are largely unknown. We find that large, spontaneous Excitatory Amino Acid Transporter (EAATs) activity in cone terminals may contribute to cone synaptic adaptation, specifically with respect to how these signals change in differing conditions of light. EAATs in neurons quickly transport glutamate from the synaptic cleft, and also elicit large thermodynamically uncoupled Cl^- currents when activated. We recorded synaptic EAAT currents from cones to study glutamate-uptake events elicited by glutamate release from the local cone, and from adjacent photoreceptors. We find that cones are synaptically connected via EAATs in dark; this synaptic connection is diminished in light-adapted cones.

Methods: Whole-cell patch-clamp was performed on dark- and transiently light-adapted tiger salamander cones. Endogenous EAAT currents were recorded in cones with a short depolarization to -10mV/2ms, while spontaneous transporter currents from network cones were observed while a local cone holding at -70mV constantly. DHKA, a specific transporter inhibitor, was used to identify EAAT2 currents in the cone terminals, while TBOA identified other EAAT subtypes. GABAergic and glycinergic network inputs were always blocked with picrotoxin and

strychnine. Results:

Spontaneous EAAT currents were observed in cones held constantly at -70mV in dark, indicating that the cones received glutamate inputs from adjacent photoreceptors. These spontaneous EAAT currents disappeared in presence of a strong light, possibly because the light suppressed glutamate releases from the adjacent photoreceptors. The spontaneous EAAT currents were blocked with TBOA, but not DHKA, an inhibitor for EAAT2 subtype, suggesting that a non-EAAT2 subtype may reside in a basal or perisynaptic area of cones, with a specialized ability to bind exocytosed glutamate from adjacent cones in dark. Furthermore, these results could be artificially replicated by dual-electrode recordings from two adjacent cones. When glutamate release was elicited from one cone, the TBOA-sensitive EAAT currents were observed from the other cone.

Conclusions: Cones appear to act like a meshwork, synaptically connected via glutamate transporters. Light attenuates glutamate release and diminishes the cone-cone synaptic connections. This process may act as an important network mechanism for cone light adaptation.

Regulation of Rapid Signaling at the Cone Ribbon Synapse via Distinct Pre- and Postsynaptic Mechanisms

List of Figures

Abbreviations/Chemical Names

(S)-AMPA	(*S*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BPC	Bipolar Cell
CaCl2	Calcium chloride
CdCl2	Cadmium chloride
CCD	Charged Coupled Device
CGP	55845 (2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride
CNS	Central Nervous System
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CPPG	((RS)-alpha-cyclopropyl-4-phosphonophenylglycine ((RS)-)
CoCl2	Cobalt chloride
EAAT	Excitatory Amino Acid Transporter
EGTA	Ethylene glycol tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPL	Inner Plexiform Layer
KCl	Potassium chloride
MCPG	Methyl-4-carboxyphenylglycine
MDL	11939 α-Phenyl-1-(2-phenylethyl)-4-piperidinemethanol

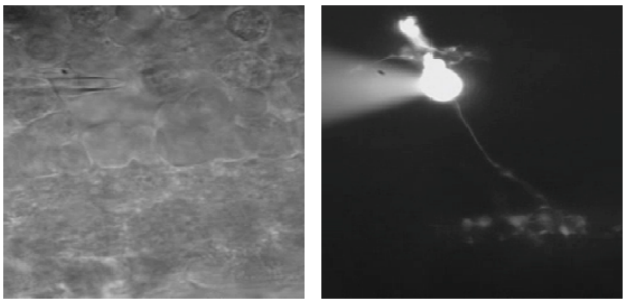
MgCl2	Magnesium chloride
NaCl	Sodium chloride
NMDA	*N*-Methyl-D-aspartic acid
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PKI	Protein kinase inhibitor-(14-22)-amide, myristoylated
TBOA	DL-*threo*-β-Benzyloxyaspartic acid
TEA	Tetraethyl ammonium chloride
VGKC	Voltage Gated Potassium Channel

Chapter 1- Introduction

1.1 Introduction to the Retina; Factors Contributing to the Modulation of the Cone Synapse

The retina is a thin layer of neural tissue attached to the back of the eye. It contains light-sensitive sensory photoreceptors and the neural circuits which organize and process visual information. The retina is considered an extension of the brain, as its cells do not functionally differ from those found in the CNS, at least with respect to the general aspects of neurons. The retina is an ideal system to study basic neurophysiology, as its neuronal anatomy is very well characterized. Furthermore, the functional aspects of each synaptic layer are generally well known physiologically; therefore further insight into more complex, novel mechanisms of signaling can continue to be elucidated by utilizing this important model. In addition to the broad insights into the nervous system gained by studying the retina, there are important questions still unanswered on how visual information is processed. This information is vital to future clinical endeavors such as retinal implants, or optogenetic signal control in patients with neurodegenerative retinal disease. The outer or proximal retina contains the first visual synapse, made between light sensitive photoreceptors and 2nd order neurons called bipolar cells (BPCs) (see Fig. 1), and horizontal cells that provide lateral integration at the Outer Plexiform Layer (OPL).

A1 A2



B

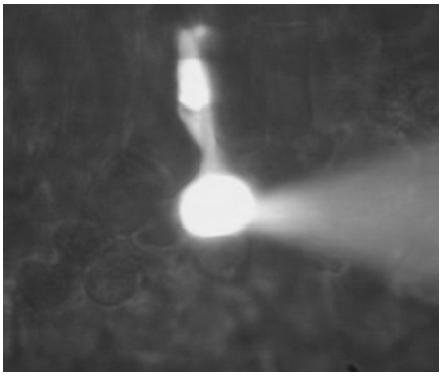


Figure 1. Lucifer Yellow fluorescence of a cone and BPC in salamander retinal slices in Whole-cell recording configulation.

A1, Phase contrast image of retinal slice with a whole-cell recording electrode, taken with a CCD camera. *A2*, In the same slice, fluorescence image of a bipolar cell dialyzed with Lucifer Yellow through the whole-cell recording electrode. *B*, Fluorescence image of a cone photoreceptor with internal dialysis of Lucifer Yellow in a retinal slice.

Information is passed from BPCs to the inner retina, where they synapse with excitatory, spiking ganglion cells. This photoreceptor-BPC-ganglion cell pathway is known as the vertical processing pathway, and is well conserved among the higher vertebrate species. Photoreceptors and BPCs are completely glutamatergic, meaning all excitatory information in the vertical pathway is passed chemically via glutamate. The bipolar cells can be described as analog signal generators, the summation of which lead to digital output from ganglion cells. Generally, two subtypes of photoreceptor and BPC are described- rod and cone photoreceptors, and ON and OFF bipolar cells, respectively. Both cone and rod photoreceptors hyperpolarize with a flash of light, as do the sign-conserving OFF-BPCs. Conversely, ON-BPCs respond with a depolarization as known as sign-inverting (Dowling and Werblin 1969). This ON/OFF segregation of light signals is the most fundamental feature of visual processing within the retina. The synaptic inputs of BPCs within the salamander have been more recently defined as rod-dominant, cone-dominant, as well as mixed input of rods and cones (Pang et al., 2004). Mammalian bipolar cell inputs are similarly segregated.

1.1.1 Functional Organization of the Retina

In the salamander, photoreceptors make synaptic contacts with multiple types of BPCs, deemed cone-dominant, rod-dominant or mixed BPCs. (Yang & Wu, 1996; Yang & Wu, 1997; Zhang, Zhang, & Wu, 2006). The information is later organized within different layers of the inner retina (IPL, see Figure 2). It is this parallel processing which allows for the separation of cone and rod information in BPCs and therefore downstream at the ganglion cell layer. Because of this segregation, we may focus specifically on the contributions of cones or rods by selecting cone-dominant or rod dominant BPCs for study. Cone

photoreceptors receive lateral inhibition from horizontal cells in the outer retina, the mechanism most recently described is through a regulation of presynaptic Ca^{2+} channels via a proton feedback phenomenon. (Cadetti 2006). Information from the vertical signaling pathway is also integrated in the inner retinal synapse (inner plexiform layer; IPL) by amacrine cells, through a number of mechanisms including electrical and inhibitory chemical synapses.

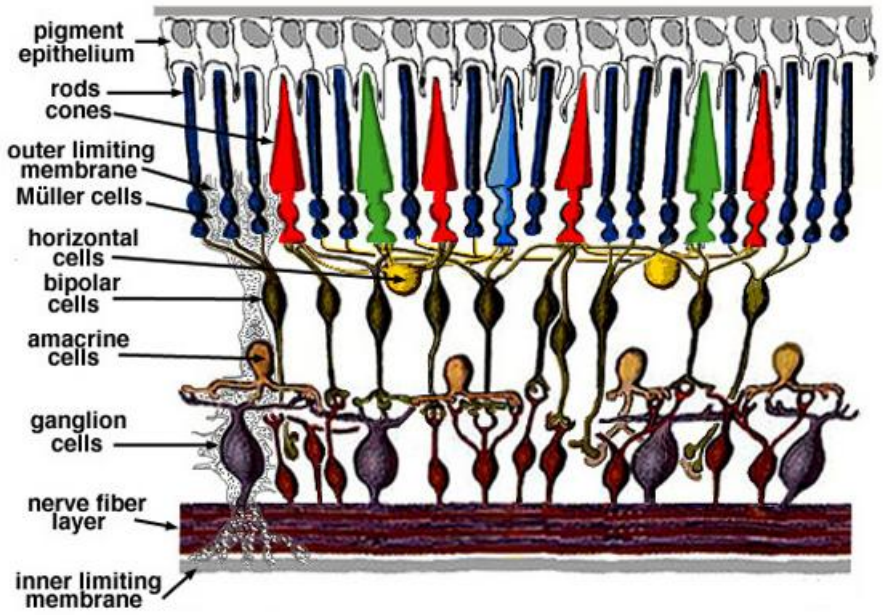


Figure 2. Neural Organization of the Retina.

In general, five types of retinal neurons consist of a vertical and lateral neural organization. Photoreceptors-BPCs-ganglion cells pass glutamatergic signals via vertical pathway; whereas horizontal cells and amacrine cells provide lateral inputs to regulate the vertical signals respectively in the OPL and IPL, the two synaptic zones in the retina. Ganglion cells are output neurons in the retina, sending primary visual information to the brain through optic nerve fibers. The Muller cells are glial cells that support the neural structure and function within the retina.

1.1.2 Glutamatergic synapses in the OPL

The chemical synapse between photoreceptors and bipolar cells exists at the Outer Plexiform Flayer (OPL) of the retina. Photoreceptors are thought to be exclusively glutamatergic neurons, which exhibit high rates of exocytosis, sustained by specialized active zones which contain ribbon-like structures. The ribbon is thought to promote single multi-vesicular release events, as well as continuous or 'tonic' vesicular release, by tethering vesicles close to the synaptic cleft (Sterling & Matthews, 2005). Another form of ribbon signaling has been deemed phasic release from cones, and occurs directly following periods of hyperpolarization where many vesicles are tethered and primed for release on the ribbon end.

Recently, the mechanisms by which the cone ribbon adapts to different signaling paradigms, namely tonic and phasic signals, have been elucidated in some detail (Jackman et al 2009). Interestingly, in comparison with vesicle delivery by free diffusion through the cytoplasm, the ribbon actually will slow delivery, thereby restraining release in a controlled manner. Their results indicate that the rate-limiting step controlling cone vesicle fusion switches from vesicle resupply during tonic (or continuous) transmission, due its slow rate, to Ca^{2+}-dependent fusion during phasic transmission, as vesicles in this paradigm are primed at the ribbon and ready to be released.

Tonic release may have been adapted to encode slower changes in background light, as control of the release rate by vesicle resupply can separate tonic transmission from noisy signals which can be generated by stochastic openings of Ca^{2+} channels in darkness. Phasic release improves the encoding of temporal and intensity light information with even greater precision, as these signals are rapid and can vary in amplitude in direct proportion to the

length and/or intensity of light proceeding. Exactly how these signals are modulated or influenced by other synaptic factors is of great interest and high relevance with regards to this study.

Glutamate exerts its signaling role in neuronal synapses, including those found in retina, by acting on glutamate receptors both pre- and post-synaptically. Glutamate concentrations in the surrounding extracellular fluid determine the extent of receptor stimulation, and therefore visual processing. Simple diffusion appears to be an important mechanism for glutamate removal from the synaptic cleft, at least at synapses with small diameters of vesicles, in many neurons. Nevertheless, diffusion only works quickly over very short distances (hundreds of nanometers) and is only an efficient synaptic removal mechanism as long as the glutamate concentration in the extracellular fluid outside synapses is kept low (Danbolt 2001). Synapses in the OPL are purposefully tight, with bipolar dendrites contacting an invagination area within the photoreceptor terminal. Glutamate concentrations are also relatively high at this synapse, as glutamate-containing vesicles are constantly released in the dark. Therefore, other mechanisms to attenuate signals in the OPL, such as glutamate uptake or re-uptake, are vitally important in preventing glutamate toxicity, for maintaining internal glutamate levels, and most importantly for this study- in maintaining and regulating visual transduction.

1.1.3 Light responses in photoreceptors and BPCs and relevant postsynaptic receptors involved in glutamatergic synapses

Signal transduction begins in the outer retina, where photoreceptors respond directly to light stimulation. Phototransduction is now well understood at the molecular level (see Burns and Arshavsky, 2005; Lamb and Pugh, 2006; Fu and Yau, 2007 for review). In dark conditions both rod and cone photoreceptors are relatively depolarized, therefore they are constantly releasing glutamate. Photo-stimulation causes a hyperpolarization in photoreceptors, which leads to the closure of voltage-dependent Ca^{2+} channels in the terminals of photoreceptors, and ultimately reduces glutamate release in the OPL. As stated, the physiological response of BPCs is dependent on their respective subtype, as ON-BPCs depolarize while OFF-BPCs hyperpolarize in response to light. Both responses are dependent on the amount of glutamate in the synapse, as the ON-BPC light response is characterized via mGluR6-type metabotropic glutamate receptors (Nakajima et al., 1993; Nomura et al., 1994) and OFF-bipolar cells are dependent on AMPA-type ionotropic glutamate receptors (Gilbertson et al., 1991; DeVries, 2000). Cones are also regulated by their own glutamate release in the OPL, as metabotrophic glutamate receptors are expressed presynaptically (Brandstatter and Hack, 2001; Koulen et al. 1999). Cone photoreceptors can also produce transient depolarization responses following light flashes. These voltage overshoots are thought to been generated through a Ca^{2+} spiking mechanism in rod photoreceptors (Slaughter 2005); cone photoreceptors (Deschenes 1992), conveyed by gap junction channels. The importance of glutamate transporters in the OPL cannot be overstated from a regulatory standpoint.

1.1.4 Glutamate and glutamate transporter in regulation of voltatge-dependent Ca^{2+} channels in photoreceptors:

The release of glutamate from presynaptic photoreceptors in the OPL is contingent upon Ca^{2+} influx from channels which remain open in the dark, therefore leading to the depolarization of the cell and subsequent glutamate release. Therefore, the regulation of voltage-dependent Ca^{2+} channels in photoreceptors will modify all downstream signaling in the retina and visual cortex. The ability to regulate glutamate levels at this synapse has been shown to have an effect on the regulation of Ca^{2+} currents in photoreceptors, and therefore, downstream bipolar cells. Ca^{2+} currents appear to be regulated both directly through the activation of excitatory amino acid transporters (EAATs), and indirectly via external glutamate. Thoreson et al. has described an inhibition in L-type Ca^{2+} currents of approximately 20% after EAAT activation in cones, while blocking metabotrophic glutamate responses. Hosoi et al. have noted that activation of metabotrophic glutamate receptor mGluR6 in cone photoreceptors causes a negative feedback mechanism. L-type voltage-dependent Ca^{2+} channels become inhibited with mGluR6 activation, which in turn lead to a reduction in glutamate release. Taken together, any changes in EAAT2 activity within the OPL may in turn have important regulatory implications for signal processing, as it is likely required to keep glutamate at precise physiological levels in the synaptic cleft in the OPL.

1.2 Excitatory Amino Acid Transporters (EAATs)

Five subtypes of EAATs have been cloned from human, with a representative homologue for each subtype found present in the salamander retina. The five EAAT subtypes comprise the group of transporters known as the high-affinity glutamate transporters, with K_m values varying from 1-100μM. Human brain expresses the transporter

subtypes EAAT1, EAAT2, and EAAT3 (Arriza et al., 1994) as well as EAAT4 (Fairman et al., 1995). EAAT2 is the most prevalent of the transporters within the central brain. EAAT1, EAAT2, and EAAT5 have been shown to be present within the mammalian retina, and are similarly expressed within salamander retina. EAAT1 appears to be expressed solely in glial cells of the salamander retina, whereas EAAT2 and EAAT5 are found within glial cells and neuronal cells in the salamander retina (Amara 1998). The molecular structures of EAATs are well conserved in mammalian and non-mammalian neurons and glial cells, and are expressed in the photoreceptors and bipolar cells of primate (Hanna & Calkins, 2007), mouse (Rauen et al., 2004) and salamander (Eliasof et al., 1998a). These transporters display two separate conductance when activated; a coupled $Na^+/H^+/K^+$ dependent conductance which is necessary for the transporter to bind and translocate glutamate, and a stoichiometrically uncoupled Cl^- conductance (Danbolt, 2001). The anionic conductance is indicative of EAAT uptake and has been used to assess EAAT activity (Arriza et al., 1997; Otis & Jahr, 1998). An EAAT-mediated Cl^- conductance has been well documented within photoreceptors (Picaud et al., 1995; Grant and Werblin, 1996; Gaal et al., 1998). This study focuses upon the importance of the excitatory amino acid transporter (EAAT) EAAT2, otherwise known as GLT-1, in the outer retina, where the first visual synapse occurs between light-sensitive photoreceptors and bipolar cells (Figure 2). Within the outer portion of the retina, different EAATs are expressed, including EAAT2.

One of the goals of my dissertation study is to explain the contribution of neuronal EAATs to visual processing, and to uncover any insights into the modulation of its activity or expression. As EAATs are responsible for removing synaptically released glutamate from the extracellular space, the failure of EAAT to carry out this role can lead to excessive stimulation of glutamatergic receptors, causing excitotoxicity and cell death. In the outer

retina, glutamate is constantly released from photoreceptors in the dark, and can also be released in larger, shorter bouts of intense strengths. All of these processes are likely to be controlled, to some extent, by neuronal EAATs.

1.2.1 EAAT2 expression patterns in the outer retina

The general nature of EAAT expression in salamander has been studied, but very little is known about the detailed spatial arrangement of EAATs in the OPL synapse. (Hasegawa 2006). The salamander homologue of human EAAT2 has been shown to share the highest percentage of sequence similarity of any in the 5 described subtypes within the EAAT family, making it an attractive model for study. In salamander, two specific isoforms of EAAT2 have been characterized by molecular cloning, which have been deemed sEAAT2A and 2B. Their expression is prominent in the OPL in salamander, with previous staining patterns which localize the expression of sEAAT2B to OFF-BPCs. sEAAT2A may be expressed neuronally as well, with staining patterns suggesting presynaptic expression in cones (Amara 1998a). Staining in OFF-BPCs of both rat and macaque are also EAAT2 positive (Grunert et al., 1994; Rauen and Kanner, 1994). My preliminary data show that sEAAT2B is not only isolated to OFF -BPCs in the OPL, but also within the dendrites of ON -BPCs as well. Evidence from the experimental data (Fig. 4) suggests that sEAAT2B is completely postsynaptic, which when combined with physiological data (Fig. 5) provides a strong case for specific sEAAT2A expression at pre-synaptic sites (photoreceptors) in the OPL.

1.2.2 EAAT2 activity, contribution to visual processing, and regulation

The glutamate transporter EAAT2 is the most prominent glutamate transporter in the mammalian central nervous system (Danbolt 2001). EAATs in the CNS maintains extracellular glutamate, support re-uptake of glutamate into the presynaptic terminal for the re-cycling of glutamate, reduces synaptic release through negative feedback, and prevents neurotransmitter spill over (Wong 2005). EAAT2 co-transports glutamate into the cell along with three Na^+ and one H^+, followed by the counter transport of a K^+. In addition, the binding of glutamate and Na^+ activates an uncoupled Cl^- conductance that is universally observed in EAATs, which again may be of interest as a regulatory factor in the outer retina.

EAATs in the retina have been shown to play a major role in visual transduction. The majority of cone dominated bipolar cell signals were shown to be mediated by EAATs in telosts (Wong et al. 2004); but neither the mechanism behind its action, nor the contributions of specific EAAT subtypes were determined. Presynaptic EAATs in rod photoreceptors were proven as essential tools for proper signal transduction to bipolar cells (Hasegawa 2006) as well, as fast glutamatergic cleanup is necessary for high resolution signaling. Experimental data presented in this study suggests a strong connection to the telost study in salamander, as cone dominated BPC light responses are seen be to highly regulated via EAAT2 activity (Fig. 7). The data also suggests an important role for EAAT2 in both rod and cone dominant BPCs (Fig. 6), as it is necessary to maintain a steady dark membrane potential at these synapses.

1.2.3 Other EAAT subtypes within photoreceptors

In this study, EAAT2 is described as a major contributor in glutamate regulation in the outer retina. Another subtype of EAAT, namely EAAT5, has been proposed to be of significant importance photoreceptor cells, among other retinal neurons (see Amara 1998, Wersinger et al 2006). Both subtypes of EAAT2A and EAAT5 have been experimentally isolated within salamander cone photoreceptors (Rowan 2010; Amara 1998a/b). It is likely that each isoform plays a distinct role in visual signal processing, and may be isolated to distinct physical locals in the cone. As will be described in the following chapters, these hypotheses were confirmed.

1.3 Metabotropic Actions of Taurine and Glycine in the Retina

1.3.1 Glycine and Taurine in the Retina

Glycine is traditionally known to act as an inhibitory neurotransmitter. In the OPL, glycinergic input is from a group of interplexiform cells that are located in the inner retina and have synaptic axonal processes terminating in the OPL. Glycine is known to activate Cl^- permeable channels, and the expression of ionotropic glycine receptors has been described in amphibian cones in bullfrog (Gea et al 2007), mouse cones (Havercamp 2003), and BPC dendrites and axon terminals. These receptors are also expressed in 3rd order neurons of the retina. Recently, the evidence (Hou et al. 2008) has suggested that novel, metabotrophic action of glycine might exist. The aforementioned paper suggests a metabotrophic glycine regulation of Ca^{2+} channels within retinal cells. This metabotrophic action was confirmed in this study within BPCs, as a specific modulation of voltage gated K^+ channels occurs via glycine through a metabotropic site.

Taurine, is a sulfur containing amino acid, is found at high concentrations in mammalian plasma and cells. Quite similar in structure to neurotransmitters glycine and GABA, taurine is the second most abundant free amino acid in the CNS, after glutamate (Sturman, 1988). Taurine has also been implicated in neurotransmission, although its direct targets remain elusive. (Kuriyama et al., 1982), neuromodulation (Muramatsu et al., 1978),

Taurine is prevalent within the outer retina, especially within photoreceptors (Militante JD, Lombardini JB 2002), although the exact roles it may play in neuronal processing in the OPL is not clear. A strong link has been observed between taurine deficiency and visual dysfunction, a problem that can be reversed through dietary supplementation (Lombardini,1991). Although taurine can activate ionotropic receptors, such as glycinergic receptors, it does so only at non-physiologically relevant concentrations (S. Bulley, personal communication). Therefore, any active site for taurine still needs to be elucidated- an endeavor undertaken in the current studies. Indeed, I hypothesized that taurine might act through a similar metabotropic site as glycine. Through the course of these studies, it was discovered that taurine does act metabotrophically, at a distinct receptor site and at physiological concentrations. Interestingly, the metabotropic taurine action leads to a specific modulation of voltage gated K^+ channels, as with glycine, but in an opposite manner.

1.3.2 Voltage gated K+ channels within BPCs

As a consequence of this study, it was determined that both taurine and glycine act as upstream modulators of voltage gated K^+ channels. Voltage-gated K^+ channels repolarize neurons, allowing K^+ efflux and consequently reduce excitability (Hille, 2001). They are involved in a number of neuronal functions including regulating neuronal excitability, synaptic plasticity, learning and memory (Kandel and Schwartz,1982; Kaczmarek and Levitan, 1987.). Therefore, any changes in the conductance of these channels will have widespread consequences.

The vast majority of results support a modulation of delayed rectifier channels via glycine and taurine. The delayed rectifier channels (including Kv1.1, Kv1.2, Kv1.3, Kv2.1 and Kv3.1) have a higher activation threshold compared to the A-type K^+ channels and also display much slower inactivation rates. A number of modulatory pathways of these channels is possible in retinal BPCs, including a number of protein kinase pathways. It has been proposed that metabotropic glycine receptor modulation of Ca^{2+} channels occurs through a PKA dependent pathway (Hou et al 2008), and therefore a similar kinase-dependent pathway may be in play regarding the regulation of voltage gated K^+ channels via glycine and taurine.

Chapter 2- Methods

2.1 Retinal Slice Preparation

Larval tiger salamanders (Ambystoma tigrinum),purchased from Kons Scientific (Germantown,WI, USA) and Charles Sullivan (Nashville, TN, USA), were used in this study. The animals were kept in aquaria at 13◦C under a 12 h dark–light cycle with continuous filtration. The retinas were collected from animals kept at least 6 h in the dark. Briefly, the animals were decapitated and double-pithed and the eyes were enucleated. All procedures were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University's Animal Care Committee. The retinal slices were prepared in a dark room, under a dissecting microscope equipped with powered night-vision scopes (BEMeyer Co., Redmond,WA, USA), an infrared illuminator (850 nm), an infrared camera and a video monitor. Briefly, the retina was removed from an eyecup in Ringer solution and mounted on a piece of microfilter paper (Millipore, Billerica,MA, USA), with the ganglion cell layer downward. The filter paper with retina was vertically cut into 250 nm slices using a tissue slicer (Stoelting Co.,Wood Dale, IL, USA).

2.2 Whole-Cell Patch Clamp Recording

A recording chamber was placed on an Olympus BX51WI microscope equipped with a CCD camera linked to a monitor. Whole-cell patch-clamp recordings were performed on photoreceptors and bipolar cells in dark-adapted retinal slices, using anEPC-10 amplifier and Pulse software (HEKA Instruments Inc., Bellmore, NY, USA). Patch electrodes (5–8MΩ) were pulled with an MF-97 microelectrode puller (Sutter Instrument Co., Novato, CA, USA). All recordings were performed under 850 nm infrared illumination to avoid exposure to visible light. Photic stimulation was performed for differing lengths or intensities with red LED illumination(660 nm peak emission) focused directly upon the retinal slice, controlled with a HEKA amplifier output.

For exocytotic capacitance recordings, the phase angle setting was made with Pulse software based on the EPC-10+ amplifier circuitry, and verified using a model cell. A gravity-driven perfusion system was used to superfuse all external solutions. The perfusion tube was placed 3mm away from the retinal slice and was manually controlled for delivering drugs during the experiments. All of the chemicals used in this study were purchased from Sigma and Tocris Bioscience (Ellisville, MO, USA).

2.3 Extracellular and Intracellular Solutions

A single retinal slice was mounted in a recording chamber and superfused with oxygenated Ringer solution, consisting of (mM): NaCl (111), KCl (2.5), $CaCl_2$ (1.8), $MgCl_2$ (1.0), Hepes (5.0) and dextrose (10), pH7.7. For recording Ca^{2+} channel currents from cones, 10mM $BaCl_2$ and 40mM TEA were substituted for an equimolar amount of Na^{+}. For

recordings of capacitance and endogenous EAAT currents in cones and bipolar cells, 20mM TEA was substituted for an equimolar concentration of Na^+.

For recording transporter currents in response to exogenous glutamate perfusion, electrodes were filled with (mM): KCl (40), potassium gluconate (60), $MgCl^2$(1), EGTA (5.0), Hepes (10), and E_{Cl}=−20 mV in photoreceptors or E_{Cl} =0 mV within OFF-BPCs. For experiments on BPC dark membrane currents and light responses, 5mM KCl and 95 mM potassium gluconate were substituted for to calibrate the at $E_{(Cl)}$= −60 mV. To record current–voltage relationship curves from photoreceptors and bipolar cells, KCl and potassium gluconate were replaced with CsCl and CsF, respectively, to block large K^+ conductances. For capacitance recordings from cones, the internal solution included (mM): CsF (94), TEA (9.4), $MgCl_2$ (1.9), MgATP (9.4), EGTA (5.0), Hepes (32.9). For recordings of fast endogenous EAAT currents, CsF was replaced with $CsNO_3$ in order to enhance EAAT currents; the other solutions remained the same as for capacitance measurements. In all recordings, inhibitory inputs from glycine and GABA were blocked with 10 μM strychnine and 100μM picrotoxin, respectively.

2.4 Intracellular Fluorescence dye Staining

Cell morphology was visualized in living retinal slices through the use of Lucifer Yellow fluorescence. Images will be acquired by using a x40 water immersion objective (n.a.=0.9), the 460nm excitation and 505nm emission filter, along with a mercury burner light source. Photoreceptors and rod- or cone-dominant BPCs were identified by their morphology with Lucifer Yellow dialysis through whole-cell recording electrodes, as well as with their light response patterns.

2.5 Immunohistochemistry

Freshly enucleated eyes were fixed in 4% paraformaldehyde in Ringer solution for 30 min followed by dissection of the retina from the eyecup and then rinsed extensively in Ringer solution. The eyecups were dehydrated in graded sucrose solutions (10%, 15%, 20% and 30%), and following overnight immersion in 30% sucrose, they were embedded in OCT compound (Ted Pella, Redding, CA, USA), frozen, and sectioned at 14 μm thicknesses. Frozen sections were collected on slides, air dried, and stored at 80°C.

Immunohistochemistry was performed on retinal sections and flat-mount retinas that were removed from eyecups after fixation. Retinal sections and flat-mounts were rinsed with PBS containing 0.1% Tween and 0.3% Triton X-100 (PBST-T), and then treated with a blocking solution consisting of 10% normal goat or donkey serum and 0.1% sodium azide in PBST-T. Retinal sections and flat mounts were then incubated with the primary antibody mixture, either with 3% goat or donkey serum and 0.1% sodium azide in PBST-T for 2 h at room temperature or for 5 days at 4°C, respectively. Controls lacking the primary antibody were unstained. After numerous washes in PBS with 0.1% Tween (PBST) containing 0.1% sodium azide, immunoreactivity was tested after incubation with immunofluorescent secondary antibodies for 40 min at room temperature for retinal sections and overnight at 4°C for flat mounts in PBST-T containing 0.1% sodium azide. The retinas were subsequently rinsed with PBST, mounted with Vectorshield (Vector Laboratories, Burlingame, CA, USA) and viewed with a confocal laser scanning microscope (Zeiss, LMS 700). Images were acquired via 40× and 60× oil-immersion objectives, and then processed with Zen software (Trumbull, CT, USA).

2.6 Data Analysis

Single- and dual-whole-cell voltage and current clamp data were analyzed and plotted using Pulse (HEKA) and Igor (WaveMetrics, Inc., Lake Oswego, OR, USA) and Microsoft Excel software. The Junctional conductance (G_j) was estimated by calculating the slope of the linear regression curve used to fit the original data. The rate of current and voltage decay in figure ?? and ?? was obtained by measuring the time constant (τ) for the current decay of 90% of the peak current, determined by curve fitting with a single exponential function: $y=y_0+Aexp[(x-x_0)/\tau]$, where y_0 is an offset, A is the amplitude, and x_0 is a constant start fitting time. The statistics data were generated with two-tailed T-test and expressed as mean $\pm$ SE. The data fitting and statistics were performed with Igor and Excel software. The statistic data were obtained from average of numbers of cells with standard deviations.

2.7 Light stimuli

A white light-emitting diode (LED) was placed under the glass bottom of the recording chamber, very close to retinal slices in the chamber. Light responses were detected from IP cells and amacrine cells in dark-adapted retinal slices. To reduce bleaching of photoreceptors, a dim light stimulus was used to activate most rods and some cones. In some experiments a bright light stimulus was also used to activate most of the cones in comparison with a dim light-evoked response. The duration of a light stimulus was .2-4 seconds.

Chapter 3- Results

The salamander retina is an ideal system in which to examine the role of EAAT2 in photoreceptor transmission, as salamander photoreceptors are readily accessible for electrophysiological study. Two forms of EAAT2 have been isolated and cloned in the salamander retina, designated sEAAT2A and sEAAT2B. sEAAT2A has been localized immunohistochemically to photoreceptor terminals and Müller cells within the OPL, while sEAAT2B is thought to be localized specifically in Off-bipolar cells. Importantly, both sEAAT2A and sEAAT2B have similar pharmacological properties, as both transporters are similarly inhibited with DHKA, with no significant difference in sensitivity within the micromolar range (Eliasof et al., 1998b).

In the following results I used whole-cell patch-clamp recording combined with fluorescence dye dialysis and antibody labeling methods to examine in detail the function of both EAAT2 isoforms at the glutamatergic synapse between photoreceptors and bipolar cells, both in the dark and in response to photic stimulation. I find that sEAAT2A and 2B are involved in maintaining a low level of tonic glutamate input to bipolar cells in darkness, and that fast presynaptic EAAT2 uptake acts to limit the light offset signal from cones. A similar mechanism might be of general importance within glutamatergic synapses in retinas of other species.

3.1 Physiological Roles of Neuronal EAAT2 in the OPL

3.1.1 sEAAT2A in photoreceptor terminals

Antibody-labeling has shown that sEAAT2A is present in photoreceptor terminals (Eliasof et al, 1998b). DHKA, a non-transported and EAAT2-selective antagonist, was used to separate sEAAT2A currents from total glutamate transporter currents in both rods and cones. DHKA is a reliable antagonist with Ki values between 24μM and 79 μM for EAAT2, and has no significant effect on other EAATs near this concentration (Eliasof et al., 1998a). Experiments were performed on dark-adapted retinal slices with an inhibitory cocktail consisting of 100μM picrotoxin and 10μM strychnine to block the effects of GABAergic and glycinergic inputs from the network. A high Cl^- intracellular solution was used for photoreceptors to mimic natural conditions, in which the rod E_{Cl} is around -20mV (Thoreson et al., 2002). Because glutamate uptake occurs most efficiently at lower voltages (Levy et al., 1998), photoreceptors in dark-adapted retinal slices were held at -50mV, a potential negative to the E_{Cl} in the experiments. 1mM glutamate was applied to activate glutamate transporters, which elicited large, sustained steady state inward currents in both rods and cones (Fig.3A).

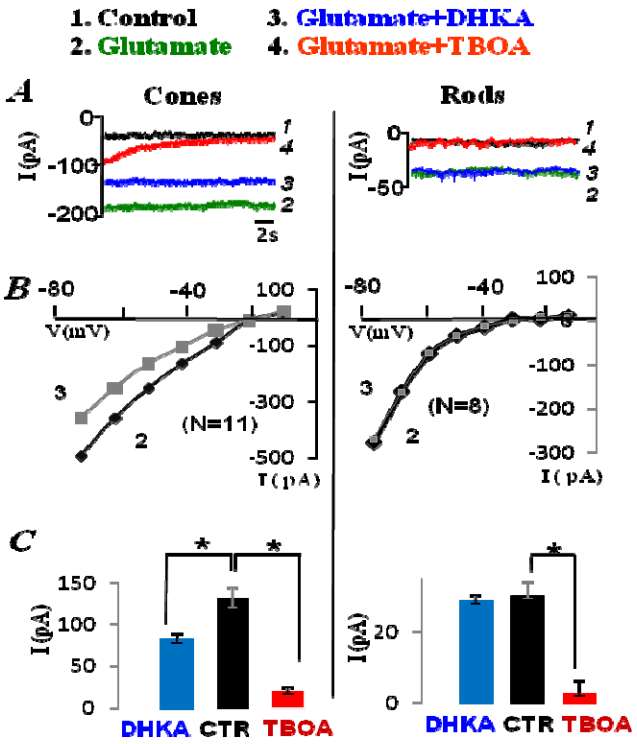


Figure 3. 1mM glutamate-elicited EAAT currents in photoreceptors at holding voltage -50mV, $E_{(Cl)}$ = -20mV.

A, Steady state glutamate currents in cones (*left)* and rods (*right*) in control and with either 100µM DHKA or 3µM TBOA. *B*, Typical Current-Voltage relationship of glutamate-elicited currents in cones (n=11) and rods (n=8). Cones are inhibited by DHKA (*grey trace*) over a wide voltage range, while rods were unaffected. *C*, Average peak steady state glutamate currents (*black bars*) in cones and rods. Cones are significantly inhibited by DHKA (36.97% ± 5.65; $p < .0001$; n=7), whereas rods are unaffected. Both cones and rods are fully inhibited by TBOA (90.10% ± 6.57; $p < .0005$; n=7 and 95.83% ± 3.39; $p < .01$; n=7), respectively. Asterisk denotes statistically significant differences.

These inward currents are EAAT specific Cl^- currents, as identified in previous studies (Grant and Werblin, 1996; Gaal et al., 1998). With the cells held at -50 mV, the amplitude of the glutamate-elicited currents at -50mV were significantly larger in cones (131.21pA ± 12.56; mean ± SEM; n=7), as compared with -30.35pA ± 3.73 (n=7) in rods. 100µM DHKA reduced the inward currents in cones but had no effect on rod inward currents; residual DHKA-insensitive currents in cones and rods were completely blocked by 3µM TBOA (Fig. **3A**). Plots of the current-voltage relationship of glutamate transporter currents from cones and rods (Fig. **3**B), showed a characteristically linear relationship at potentials negative to the E_{Cl}. Currents reversed near the E_{Cl}, -20mV. In cones, 100µM DHKA reduced the amplitude of glutamate-elicited currents over a broad voltage spectrum, but had no effect on the E_{Cl} reversal potential (Fig. **3**B, left panel, n=11).

In cones, DHKA inhibited a significant portion (36.97% ± 5.65) of the control glutamate currents at -50mV (Fig.1C left panel, $p < .0001$, n=7), whereas 90.10% ± 6.57 of the remaining transporter current in cones was blocked with TBOA (Fig. 3C left panel, $p < .0005$, n=7). The TBOA-sensitive glutamate current is probably mediated by at least one other subtype of sEAAT, most likely sEAAT5, previously detected in salamander photoreceptors (Eliasof et al., 1998b). Our results agree with those of a previous study (Eliasof 1993) of isolated cones. Although rods were unaffected by 100µM DHKA under the same conditions (Fig. 3A & 3B, right panels), the vast majority of the overall glutamate-induced currents in rods were blocked by TBOA, which suppressed 95.83% ± 3.39 of the current recorded in the control group ($p < .01$, n=8, Fig.3C right panel). The data from rods is consistent with a previous study in rat, in which rod EAAT currents were completely inhibited with TBOA, but were unaffected by 200µM DHKA (Hasegawa et al, 2006).

Therefore, presynaptic sEAAT2A clearly plays a unique role within cone photoreceptors in the OPL of tiger salamander retina.

3.1.2 Evidence of sEAAT2B in OFF-bipolar cells

The presence of EAAT2 in bipolar cells has been reported in primate, rat and salamander retina (Eliasof et al. 1998a ; Rauen et al., 2004; Hanna and Calkins, 2007). A previous study depicted sEAAT2B as a neuronal-specific glutamate transporter in bipolar cells of the salamander retina (Eliasof et al., 1998b). To determine the specific locale of sEAAT2B, we used a salamander-specific antibody against sEAAT2B, developed by Dr. Susan Amara. Confocal imaging of a retinal section labeled with sEAAT2B with and without background staining of Acridine Orange (AO), a cell nuclei marker, indicates that sEAAT2B is present in both bipolar cell dendrites and axon terminals within the distal layer of the IPL (Fig. 4). sEAAT2B-labeling was present in displaced Off-bipolar cells, which have somas located in the outer nuclear layer (ONL) (Fig. 4A). Displaced Off-bipolar cells in salamander has been shown to receive predominant input from cones (Maple et al., 2005). Localization of sEAAT2B within the OPL was detected by single-plate confocal scanning at the photoreceptor terminal with sEAAT2B and SV2 double-labeling, a synaptic vesicle protein II (SV2) which labels photoreceptor terminals, in the flat-mounted retina. Figure 2B displays an example of sEAAT2B (red) and SV2 (green) labeling at the distal boundary of the OPL, and a superimposed image of both stains.

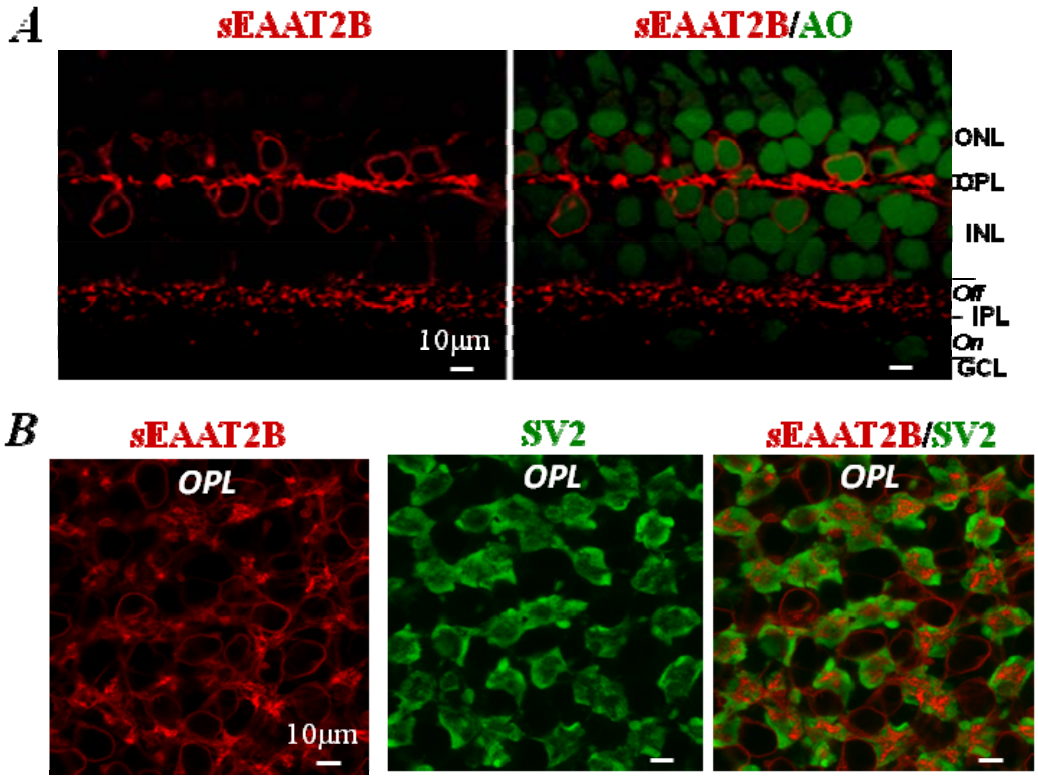
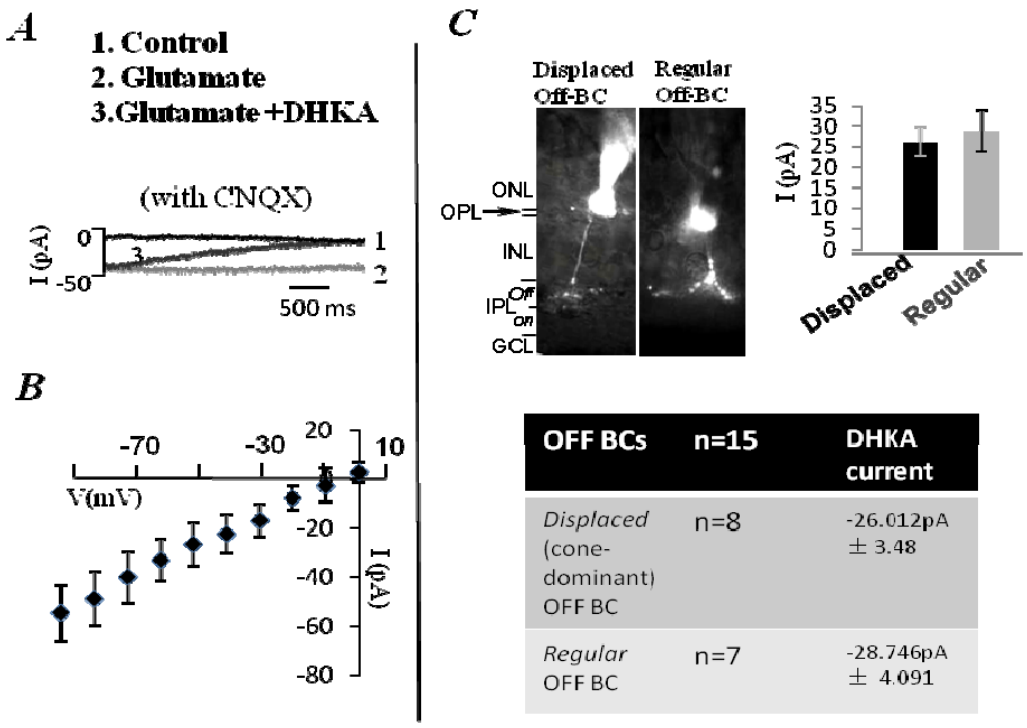


Figure 4. Confocal imaging of sEAAT2B labeling in retina.

A, sEAAT2B labeling (*red*) with and without background acridine orange (AO) (*green)* nuclear stain in a retinal section. sEAAT2B labels both regular and displaced Off-bipolar cells located in the INL and ONL, respectively. *B*, Single scanning imaging from a double-labeled flat-mounted retina, in which SV2 and sEAAT2B are separately located at photoreceptor terminals and the postsynaptic dendrites, respectively, in the distal OPL.

Double-labeling indicated that anti-sEAAT2B and anti-SV2 labeled both bipolar cell dendrites and photoreceptor terminals, with no co-localization detected at the terminals of rods or cones (Fig. 4B, right panel). sEAAT2A and sEAAT2B are therefore separately expressed at the pre- and post-synaptic sites of the OPL, offering an opportunity to study the role of two EAAT2 variants at specific pre- and post-synaptic sites within the retina.

sEAAT2B-mediated currents were investigated in Off-bipolar cells in dark-adapted retinal slices in whole-cell voltage-clamp mode. Lucifer Yellow was added to the electrode solution to depict the cell morphology after recording. Recordings were made with E_{Cl} = 0mV. In the control, glutamate receptors in Off-bipolar cells were blocked by 100μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a specific AMPA receptor inhibitor. 100μM PTX and 10μM strychnine were applied to block inhibitory inputs. 1mM glutamate with CNQX elicited an inward current in cells held at -60mV. The CNQX-insensitive currents were blocked with 100μM DHKA, indicating an EAAT2-specific transporter current (Fig. 5A). The current-voltage relationship of the DHKA-sensitive currents was linear and had a reversal potential near the E_{Cl}. (Fig 5B, n=6). Similar results were obtained in displaced Off-bipolar cells and non-displaced Off-bipolars (Fig. 5C).



OFF BCs	n=15	DHKA current
Displaced (cone-dominant) OFF BC	n=8	-26.012pA ± 3.48
Regular OFF BC	n=7	-28.746pA ± 4.091

Figure 5. sEAAT2B-mediated currents in Off-bipolar cells.

A, With CNQX added to block AMPA receptors in Off-bipolar dendrites, glutamate elicits a steady inward current that is blocked by DHKA, indicating an sEAAT2B-mediated current. *B*, The current-voltage relationship curve of the DHKA-sensitive currents in Off-bipolar cells. *C*, Morphology of displaced and regular (non-displaced) Off-bipolar cells, following Lucifer Yellow dialysis during whole-cell recoding, with statistical analysis of the sEAAT2B-mediated currents from each morphological type. EAAT2B currents were not significantly different among the subtypes of Off-bipolar cells.

Displaced Off-bipolar cells displayed the morphological features of cone-dominated bipolar cells, as previously described (Maple et al., 2005).

Non-displaced Off-bipolar cells normally had the morphology of rod-cone mixed cells, with the axon terminals located throughout the Off sublamina, as described previously (Pang et al. 2004). We analyzed DHKA-sensitive currents from Off-bipolar cells with somas located in the ONL and INL, and found that the average currents were 26.01$\pm$3.48 pA (n=8) and 28.74$\pm$4.09 pA (n=7), respectively (Fig. 5C). This indicates that sEAAT2B-mediated currents in Off-bipolar cells were significantly smaller than sEAAT2A-mediated currents in cones.

Glutamate transporter currents were investigated from On-bipolar cells in the presence of specific antagonists, CPPG ((RS)-alpha-cyclopropyl-4-phosphonophenylglycine ((RS)-) and MCPG ((+)-Methyl-4-carboxyphenylglycine) to block metabotropic glutamate receptors in On-bipolar dendrites. 1mM glutamate elicited glutamate transporter currents in On-bipolar cells that were DHKA-insensitive, but were TBOA-sensitive (data not shown), indicating that On-bipolar cells lack sEAAT2B, but are likely to express other EAATs. Our data agrees with immuno-antibody labeling (Eliasof et al.1998a) that sEAAT2B is Off-bipolar cell specific in the salamander.

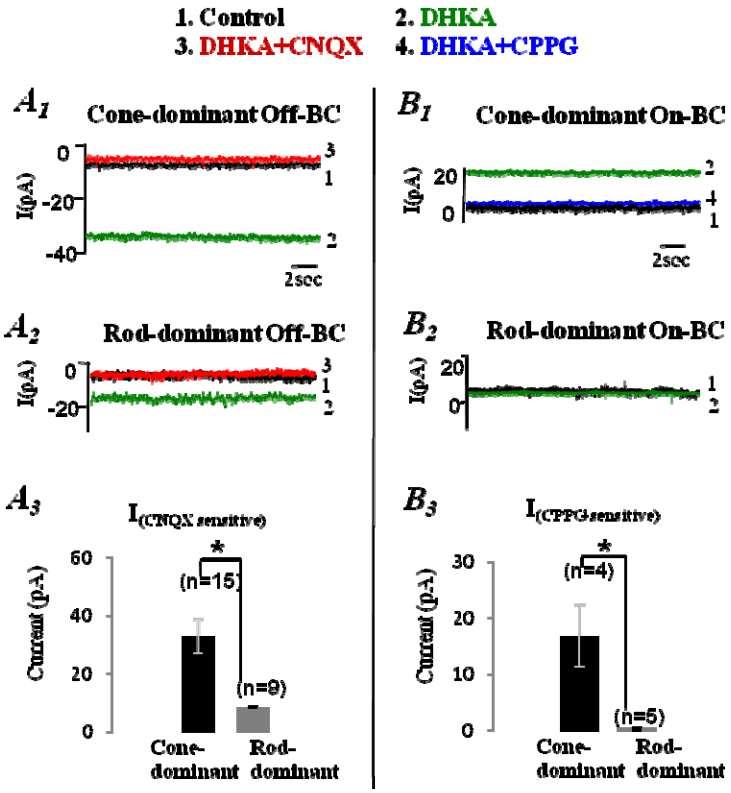


Figure 6. DHKA-elicited currents in rod- and cone-dominated bipolar cells in dark-adapted retinal slices at holding voltage -60mV, $E_{(Cl)}$= -60mV. *A_1*, DHKA elicits an inward current, which can be blocked by CNQX, in cone-dominated Off-bipolar cells (n=15). *A_2*, DHKA elicits a CNQX sensitive inward current in rod-dominant Off-bipolar cells (n=9). *A_3*, Comparison of the average absolute DHKA-elicited currents in cone- and rod-dominant Off-bipolar cells. DHKA elicited a significantly larger current in cone-dominant Off-bipolar cells (*cone-dominant:* -32.76pA ± 6.00*; rod dominant:* -8.373pA ± 0.4; $p < .01$*).* *B_1*, DHKA elicits an outward current, which can be blocked by CPPG, in cone-dominant On-bipolar cells (*n= 4*). *B_2*, DHKA

3.1.3 sEAAT2 maintains synaptic glutamate levels in dark

Rod and cone photoreceptors are constantly depolarized in dark, which allows for a continuous release of glutamate. Therefore, sEAAT-specific glutamate uptake most failed to elicit a current in all rod-dominant On-bipolar cells ($n=5$). B_3) Comparison of the effects of DHKA likely takes place at the photoreceptor terminal to prevent glutamate depletion at the pre-synaptic release site (Hasegawa et al., 2006). Accordingly, blocking sEAAT2-mediated glutamate reuptake in cones would cause an increase in glutamate levels in the OPL. Cone- and rod-dominant On-bipolar cells (*cone-dominant:* 16.79 ± 5.62; *rod-dominant:* .035pA ± .18; $p < .05$). Asterisk denotes statistically significant differences.

In general, bipolar cells in salamander retina receive mixed inputs from both rods and cones, although some bipolar cells receive a majority of input from either rods or cones, which are designated rod-dominant and cone-dominant bipolar cells, respectively (Pang et al., 2004). Rod- or cone-dominant neurons can be distinguished by the location of their axon terminals. In addition, rod-dominant bipolar cells display a slow recovery at light offset, resembling the light response of rods (Schnapf and Copenhagen, 1982), while cone-dominant bipolar cells inherit the features of the cone light response, characterized by a fast response at the offset of a light stimulus. These criteria were used to characterize the effect of DHKA on the different subtypes of bipolar cells. Cell morphology was revealed with Lucifer Yellow dialysis. 100μM picrotoxin and 10μM strychnine blocked inhibitory inputs, and 100μM cyclothiazide (CTZ) was included to prevent AMPA receptor desensitization in bipolar cells. Dark currents were recorded from Off-bipolar cells that were voltage-clamped at -60mV, near the E_{Cl}, to minimize EAAT currents. 100μM DHKA was then applied, which generated a steady inward current in Off-bipolar cells (Fig. $6A_1$ & $6A_2$). Since DHKA inhibits

sEAAT2 reuptake of glutamate at the synapse, the observed inward current in Off-bipolar cells was most likely glutamatergic, caused by glutamate accumulation in the synaptic cleft and subsequent activation of postsynaptic AMPA receptors. CNQX, which specifically blocks AMPA receptors located in Off-bipolar cell dendrites, was used to verify that the DHKA-elicited current was a consequence of increased synaptic glutamate in the OPL. Indeed, these inward currents were fully blocked by 100□M CNQX (Fig. $6A_1$ & $6A_2$). The average DHKA-induced AMPA currents were significantly larger in cone-dominant Off-bipolar cells (-32.76pA ± 6.00, n=15), when compared with rod-dominant Off-bipolar cells (-8.373pA ± 0.4, n=9) as illustrated in Fig.6 A_3 ($p < .01$, n=9). As DHKA blocks both sEAAT2A and sEAAT2B, the larger DHKA-induced current in cone-dominated Off-bipolar cells (Fig. $6A_1$) is likely due to the fact that both sEAAT2A and sEAAT2B uptake was blocked at this synapse, while at rod-dominated Off-bipolar cell synapses (Fig. $6A_2$), only EAAT2B uptake is blocked. This finding also suggests that presynaptic uptake via sEAAT2A is significantly stronger at the cone to bipolar cell synapse.

The effect of DHKA was also investigated in On-bipolar cells, which utilize metabotropic glutamate receptors on their dendrites. Activation of these metabotropic receptors closes cation permeable channels in the cells; therefore On-bipolar cells are hyperpolarized in dark by constant glutamate release from photoreceptors. DHKA-induced glutamatergic responses were recorded from cone- and rod-dominant On-bipolar cells held at -60mV. 100μM DHKA increased glutamatergic inputs to cone-dominant On-bipolar cells, resulting in an outward current in the neurons, but did not affect rod-dominant On-bipolar cells (Fig. $6B_1$ & $6B_2$). The outward currents in cone-dominant On-bipolar cells were blocked by 100μM CPPG, a specific antagonist for metabotropic glutamate receptors in On-bipolar dendrites (Fig. 6B, n=4). The average DHKA-induced metabotrophic currents were

significantly larger in cone-dominant On-bipolar cells (16.79 ± 5.62pA, n=5) when compared with rod-dominant On-bipolar cells (0.035 ± .18pA, n=5), as shown in Fig. $6B_3$ ($p < .01$, n=5). Results from On-bipolar cells suggest that presynaptic sEAAT2A plays an important role at the synapses of cone-dominant On-Bipolar cells. These findings appear in accordance with the results in figures 1, namely, that sEAAT2 is a cone-pathway specific transporter.

3.1.4 Fast uptake via sEAAT2A shapes light-evoked cone signals to bipolar cells

As presynaptic sEAAT2A uptake was found to maintain lower tonic glutamate levels from cones in dark, we postulated that the transporter might also play a role in shaping light-evoked, transient glutamate release from cones to bipolar cells. Bipolar cells receiving cone inputs receive fast, transient potentials when presynaptic cones are depolarized (Cadetti et al., 2005, 2008), a phenomenon that naturally occurs at light offset. The "off-overshoot" response is a cone-specific signal that encodes the intensity and duration of a light stimulus, and perhaps the motion of light across the visual field (Wu, 1988). The effect of sEAAT2A on the light-evoked, glutamatergic "off-overshoot" was studied in bipolar cells with application of DHKA. Intracellular and extracellular solutions were kept as in figure 3, with E_{Cl}=-60mV; controls included 100µM picrotoxin, 10µM strychnine and 100µM CTZ. A two-second light stimulus was used to evoke current responses in the neurons. The effect of 100µM DHKA on light-evoked responses was tested on both cone- and rod-dominant bipolar

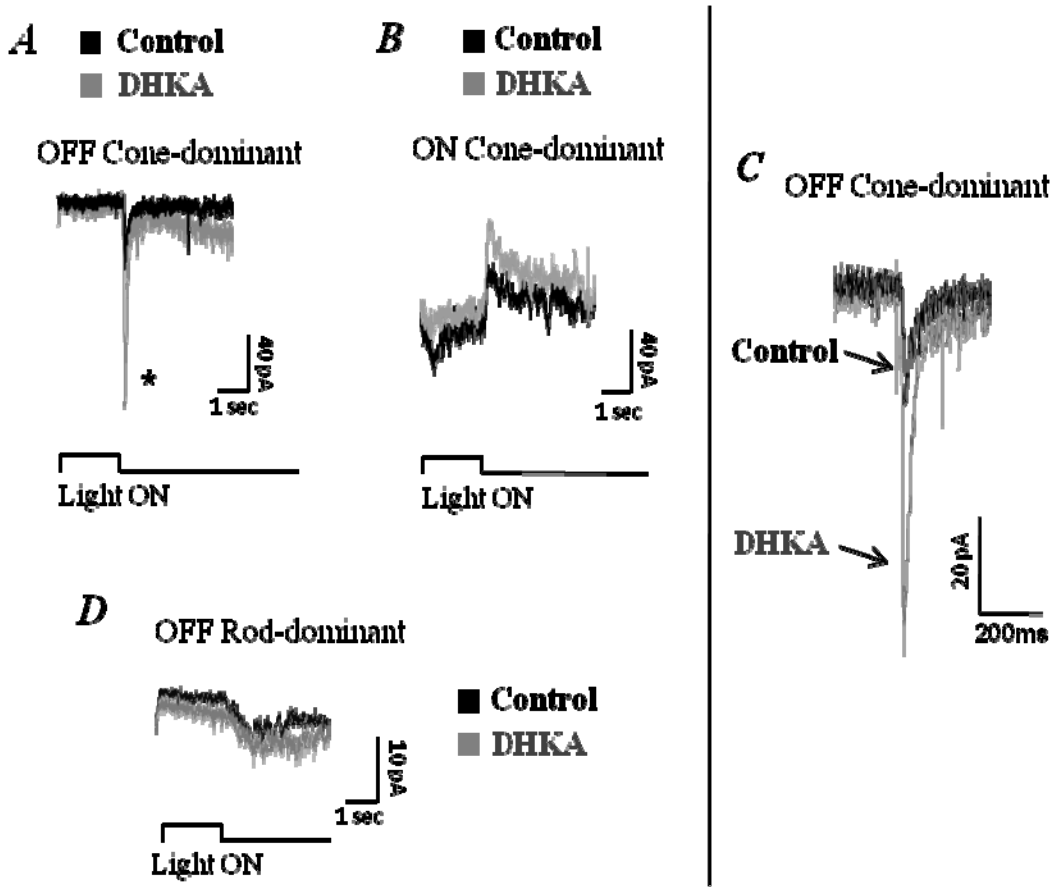


Figure 7. The contribution of sEAAT2A to light responses in rod- and cone-dominated bipolar cells.

A, Cone-dominated Off-bipolar cells display a transient inward current at the offset of a 2 second light-stimulus. DHKA causes a large enhancement in the light-offset current. *B*, Cone-dominated On-bipolar cells display a transient outward current at the offset of a 2 second light-stimulus. DHKA enhances the light-offset response. *C*, Enlarged light-offset currents from a cone-dominated Off-bipolar cell. *D*, Rod-dominated Off-bipolar cells display a slow light-offset response; DHKA did not alter the light-offset response in the neurons.

cells. Typical light-evoked current responses in Off- and On-bipolar cells receiving inputs predominately from cones are depicted in Fig. **7** (black traces). Light stimulation produced an outward current in Off-bipolar cells and an inward current in On-bipolar cells. Cones rapidly release a store of vesicles at the offset of a light stimulus which in turn evokes a transient glutamatergic current in cone-dominated bipolar cells, as shown by the black traces in Figs. 7A & 7B. DHKA significantly increased the amplitude of the transient current at the light offset in both cone-dominant Off- and On-bipolar cells, although the increase was much larger in Off-bipolar cells (gray traces, Fig. 7A & 7B). In cone-dominant Off-bipolar cells, the overshoot was enhanced by DHKA from an average control amplitude of -12.88pA ± 2.11 to -59.94pA ± 5.15 ($p < .01$ n=6, Fig. 7C). The average enhancement of the overshoot in On-bipolar cells was far less, i.e., from 14.07pA ± 3.52 to 24.05pA ± 5.89 with DHKA ($p < .05$, n=5, Fig. 7B). In contrast, DHKA had no apparent influence on the light offset current response in cells receiving a majority of inputs from rods (Fig. 7D). Our results suggest that sEAAT2 rapidly binds extracellular glutamate, therefore limiting the amplitude of the "off-overshoot" signal from cones to bipolar cells, most significantly within Off-bipolar cells.

3.1.5 Inhibition of sEAAT2A did not affect vesicular release from cones

Light-evoked offset responses in second-order neurons originate from pre-synaptic cones (Wu, 1988). In order to isolate a mechanism underlying the observed EAAT2-specific suppression of cone signals, it is necessary to determine whether EAAT2A inhibition directly increases the magnitude of glutamate release from cones. To test this, we recorded the effects of DHKA on the cone light response, the voltage-dependent Ca^{2+} channel currents, and cell capacitance changes following depolarization. Controls included 100μM PTX and 10μM strychnine to block inhibitory synaptic inputs.

The effect of 100µM DHKA on light-evoked responses was recorded from cones in dark-adapted retinal slices, with the same internal solution used for Fig. 3. Cones were voltage-clamped at -50mV, slightly negative to the dark membrane potential of the neurons. A light stimulus closed cGMP gated channels in the cones, ceasing cation influx in dark, and causing an outward current shift. At the termination of a light stimulus, a large inward current can be elicited in cones (Fig. 8A). 100 µM DHKA had no effect on light-evoked responses in cones when a low Cl^- intracellular solution (K^+-gluconate) was used (n=5), indicating that the inhibition of sEAAT2A had no direct effect on the light-evoked cone response. DHKA also had no effect on the cone dark membrane potential, in agreement with a previous study in salamander (Yang 1997).

A shift in voltage-gated Ca^{2+} channel currents following EAAT2A inhibition might alter the amount of vesicular release from cones. To test if an EAAT2A-directed modulation of cone voltage-gated Ca^{2+} channels occurs, 100µM DHKA was applied while recording Ca^{2+} channel currents in cones. Ba^{2+} and TEA were added to the external solution, with TEA and CsCl-containing electrode solution to isolate Ca^{2+} channel currents. Maximal sustained inward Ca^{2+} channel currents were elicited from cones by a single voltage-step from the holding potential to -10mV, and were recorded for 25ms (Fig. 8B). DHKA had an insignificant effect on the voltage gated Ca^{2+} channel

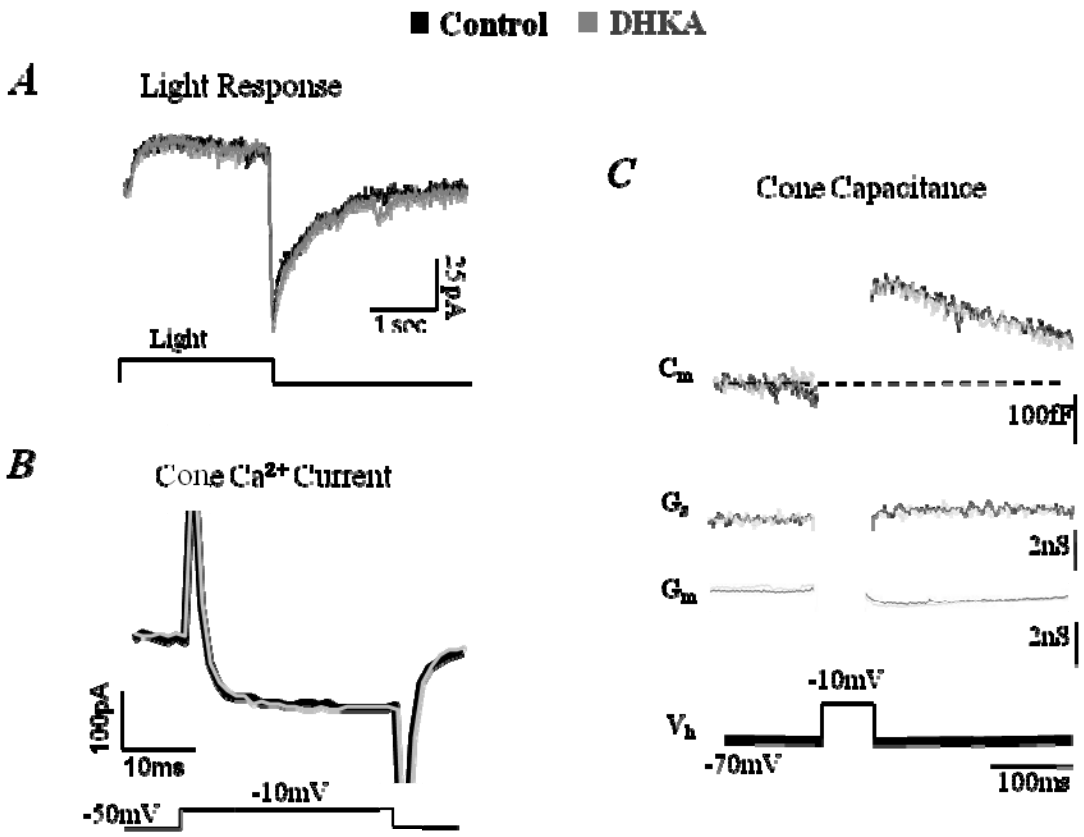


Figure 8. EAAT2A inhibition does not alter cone light responses or exocytosis.

A, Cones display a characteristic outward current during a light response. DHKA has no significant effect on the light-evoked responses of cones, or on currents following the light response (*n=5*). *B*, Voltage gated Ca^{2+} currents were isolated in cones with external Ba^{2+} with a 25ms voltage step to -10mV. DHKA does not alter voltage-dependent Ca^{2+} channel currents in cones (*n=7*). *C*, Cellular capacitance measurements (C_m) were performed prior to and following a 100ms depolarizing step from -70mV to -10mV to measure exocytosis. DHKA did not affect the depolarization-evoked capacitance changes (*n=5*). Gs: access conductance, Gm: membrane conductance.

currents in cones (n=7), suggesting that the Ca^{2+}-dependent glutamate release in cones is unaltered after EAAT2A inhibition.

To provide further evidence that EAAT2A activity did not modulate vesicular glutamate release, we recorded capacitance (C_m) changes following a depolarizing step. Transient increases in cell capacitance are correlated with the binding of vesicles at the plasma membrane. If EAAT2A inhibition changes any exocytotic mechanism, or somehow modifies the readily releasable pool of vesicles, a change in C_m following a depolarization would be apparent. Cells were voltage-clamped at -70 mV, and were subjected to a 600-Hz sine wave of ±30 mV was applied around the holding potential. Heka software 'Lock-in' mode was utilized to measure the capacitance (C_m), G_s (access conductance), and G_m (membrane conductance) for each sine wave during the recordings. G_s and G_m did not significantly change during C_m recordings, indicating a stable control. 30-second intervals were permitted between depolarizing steps, which allows cone intracellular Ca^{2+} levels to return to prestimulus levels between pulses (Innocenti 2008). Cones had a total cell capacitance ranging from 18.3 - 24.8 pF in dark conditions. 75ms depolarizing steps from -60mV to -10mV elicited a 202.4 fF ± 24.3 (n=5) peak capacitance increase, within the range of values previously described in salamander cones in the slice preparation (Rabl et al 2005). 100µM DHKA did not affect the depolarization-evoked capacitance changes (Fig. 8C, n=5). Taken together, the light-offset enhancement observed in bipolar cells after EAAT2A inhibition is probably not due to an enhanced presynaptic glutamate release, and EAAT2A activity therefore does not modulate the output of cones.

3.1.6 Comparison of EAAT2-mediated uptake at presynaptic cones and postsynaptic bipolar dendrites following fast glutamate release

Our results show that sEAAT2 plays a significant role in the encoding of fast cone signals in the OPL. Uncovering the distinct actions of the cone EAAT2A and bipolar cell EAAT2B is necessary, and offers a unique perspective into the physiological importance of presynaptic and postsynaptic transporters at the same synapse. Two separate experiments were devised to study the natural activity of EAAT2 in cones and bipolar cells following fast glutamate exocytosis. Both experiments utilized intracellular $CsNO_3^-$ in the pipette solution (Figs. 9A, 9B) to amplify transporter kinetics following vesicular release from cones, as the NO_3^- ion is more readily conducted by the transporter after binding glutamate. TEA was included in the bath to reduce membrane conductance as in capacitance measurements, and the majority of cellular capacitance was compensated for during these experiments. 100μM Picrotoxin and 10μM strychnine were perfused constantly. All experiments were performed in dark conditions.

Cones were held at -70mV and subjected to a short, 2ms depolarizing step to -10mV in order to measure evoked EAAT currents following vesicular release, as previously demonstrated in retinal bipolar cells of various species (Palmer et al 2003, Wersinger et al. 2006). A 15 second interval was allowed following each depolarization. Large, transient inward currents were immediately evoked following the short depolarizing step (Fig. 9A). This inward tail current was partially inhibited with 100μM DHKA, accounting for 35.3% ± 7.8 of the observed peak current following vesicular release (n=7). This DHKA-sensitive transporter current indicated that cone specific EAAT2A rapidly binds extracellular glutamate following release. In addition, the DHKA-insensitive inward current following

depolarization was completely eliminated with 50μM TBOA (data not shown). TBOA likely also inhibits the EAAT5 subtype in cones, as suggested earlier. Our results suggest that the presynaptic cone EAAT2A plays a significant role in limiting light-offset signals, through an immediate buffering of synaptic glutamate following release. The contribution of both transporters together is considered in our discussion.

The activity of postsynaptic EAAT2B in Off-bipolar cells was studied in cells with truncated axons, to remove any possible transporter-associated currents that likely exist within the terminal. Only cone-dominant, displaced Off-bipolar cells were used in this experiment, which were readily accessible within the ONL. Immediately after breaking in, cells held at -60mV were subjected to a 2 second light stimulus. Cells receiving strong cone inputs were selected (Fig. 9B, black trace) and 100μM CNQX was then applied to inhibit AMPA inputs. The NO_3^- containing intracellular solution was allowed to diffuse for 1 minute before resuming light responses. If EAAT2B was significantly activated following light offset, an observable DHKA-sensitive inward current should exist after the light cessation. We did not observe a significant DHKA sensitive current throughout the experiments (n=4), indicating that dendritic sEAAT2B uptake following large glutamate release is significantly less effective as compared with presynaptic uptake via sEAAT2A. However, baseline currents were shifted inward slightly after the application of DHKA, suggesting that dendritic EAAT2B might play a lesser role in controlling the concentration of glutamate in the dark.

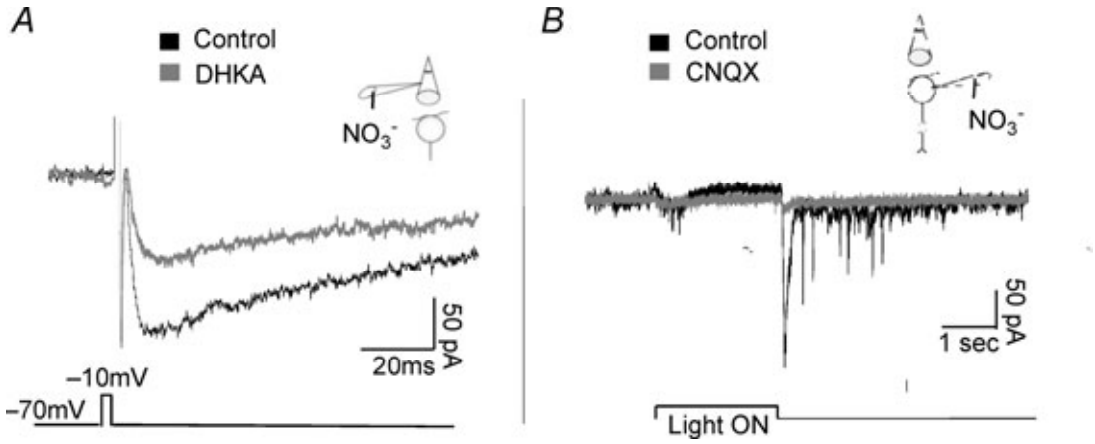


Figure 9. Presynaptic EAAT2A rapidly removes glutamate released from cones.

A, with intracellular NO3− added in the pipette to enhance EAAT currents, an inward current was elicited in cones following a 2 ms depolarizing step. A significant portion of the current was sensitive to DHKA. *B*, displaced Off-bipolar cells with truncated axons were chosen to study the EAAT2B activation following fast exocytosis in cones at the light offset. With intracellular NO3−, light responses were recorded from the Off-bipolar cells. Light offset responses were almost completely blocked with CNQX, indicating that transient glutamate release from cones has a negligible effect on EAAT2B in the dendrites of the Off-bipolar cells.

3.1.7 *The function of EAAT2A at the cone to bipolar cell synapse*

The function of EAAT2A in the regulation of cone to bipolar cell signaling was studied by recording light-evoked glutamate currents, as in Figure 7, in bipolar cells in response to photic stimuli of differing duration. Since glutamate vesicles are readily replenished at cone terminals when light hyperpolarizes the cones, the longer the hyperpolarization, the more vesicles are accumulate in the terminals. Therefore, an increase in light duration would be expected to increase glutamate vesicle release in cones at the end of the light stimulation. Using stimuli of 400ms, 700ms, 1s and 4s durations, we were able to demonstrate this variation in glutamate release from cones (Fig. 10) in the postsynaptic current recordings from cone-dominated Off-bipolar cells. Figure 10A (dark trace) shows typical current responses as the duration of light stimulation was increased. With application of 100μM DHKA to block EAAT2A, the control current amplitudes and duration were enhanced. The amplitude of enhancement was directly correlated to the duration of light, as the longer 1sec and 4sec light steps are much more pronounced when compared to the 400ms light stimulus (Fig. 8A, grey traces). These results suggest that glutamate uptake via EAAT2A is maximally facilitated in response to longer light exposures which produce a greater glutamate release at the termination of the photic stimulus.

To confirm that glutamate uptake in cones is facilitated following strong glutamate release, we recorded glutamate transporter currents using a similar protocol as described in figure 9A. Cones were held at -35mV to mimic the dark membrane potential, and were hyperpolarized to -70mV before triggering a short 2ms depolarization pulse to -10mV. The duration of the hyperpolarizing steps was varied from 400ms to 4s to match the time course of light stimulation used in figure 10A. The patch pipette again delivered NO_3 intracellularly

to enhance transport currents, so that inward transporter currents were readily observed at the end of the depolarizing pulse (Fig. 10 B). As predicted, transporter currents increased with increasing hyperpolarizing steps. These depolarization evoked transporter currents were again DHKA sensitive (data not shown).

3.1.8 Model of EAAT2s role in shaping large synaptic signals from cones

Figure 11C depicts a possible mechanism for sEAAT2 in modulating the transient glutamatergic synapses of cones-to-bipolar cells. In this model, sEAAT2A-mediated uptake into cone terminals rapidly buffers glutamate levels in the OPL, thus limiting glutamate diffusion after transient glutamate release in the dark-adapted retina. Blockage of the presynaptic transporter allows for glutamate spillover to adjacent areas in the synaptic cleft, resulting in a larger synaptic input to bipolar cells after light offset-evoked transient glutamate release. In addition, sEAAT2B may serve as a postsynaptic transporter in Off-bipolar cell dendrites, thereby contributing to glutamate uptake in the cone-to-Off-bipolar cell synapse. Our model also suggests that sEAAT2A might be in closer proximity to glutamate release sites in cone terminals than previously suggested.

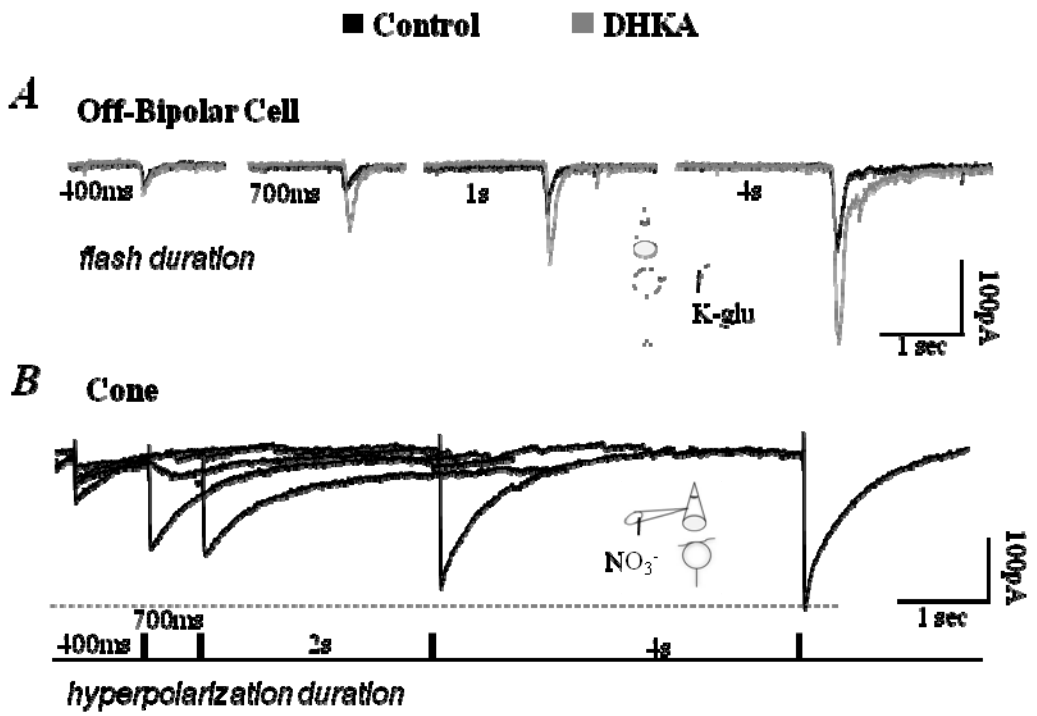


Figure 10. The role of EAAT2A in modulating glutamate release.

A, light responses recorded from an Off-bipolar cell stimulated with flash durations of 400 ms, 700 ms, 1 s and 4 s. DHKA enhances the amplitudes and durations of light responses when the flash duration exceeded 400 ms. *B*, glutamate transporter currents were recorded from a cone that was held at the resting dark potential of −35 mV, and were hyperpolarized to −70 mV for durations of 400 ms, 700 ms, 2 s and 4 s. This was followed by a brief depolarization to −10 mV at each duration to evoke glutamate release. The glutamate uptake currents were elicited by each depolarization pulse and were increased progressively with longer hyperpolarization times.

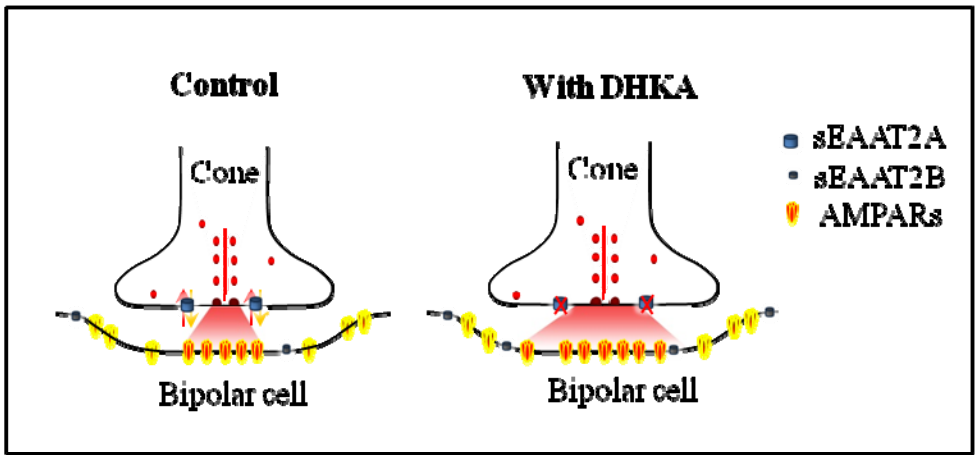


Figure 11. Hypothetical model depicting the role of sEAAT2 at the cone to Off-bipolar cell synapse of the dark-adapted retina.

In control conditions, glutamate released stimulates AMPA receptors on Off-bipolar cells, and excess glutamate is taken up into the terminals by the sEAAT2A transporter. Blocking transporter activity with DHKA produces accumulation of glutamate in the synaptic cleft, which binds to more AMPA receptors and greatly enhances light offset response.

3.2 Diverse Roles of EAAT subtypes within cones

3.2.1 The EAAT2A isoform is isolated near release sites within cones

As previously stated, at least two types of presynaptic sEAATs, sEAAT2A and sEAAT5, have been localized in salamander cone terminals. In addition their ability to functionally regulate glutamate uptake, these EAATs also cause thermodynamically uncoupled anions to cross the membrane; therefore EAATs can quickly transfer a synaptic glutamate signal directly into an electrical signal within cone terminals. To indicate that the cone terminal specific EAATs perform like autoreceptors in this glutamatergic synapse, I directly measured anionic transporter currents from the cone terminals following an elicited vesicular release of varying degrees, triggered by an excitatory voltage pulse. Experiments were performed in dark adapted cones in tiger salamander retinal slices with continuous bath perfusion of an inhibitory cocktail consisting of 100μM PTX and 10μM strychnine to block the network effects of GABAergic and glycinergic inputs that converge upon the distal retina. Again, $CsNO_3$ intracellular solution was used to amplify the transporter anion current following vesicular release from cones, as the NO_3^- ion is more readily conducted by the transporter after binding glutamate. Cones were held at -70mV, below the reversal potential for anions in the neurons; also glutamate uptake occurs most efficiently at the lower voltages (Levy et al., 1998). Cones were applied to a brief, 2ms depolarizing pulse to -10mV in order to measure evoked sEAAT currents following vesicular release, as previously demonstrated in cones and bipolar cells of various species (Rowan et al., 2010; Palmer et al 2003, Wersinger et al. 2006).

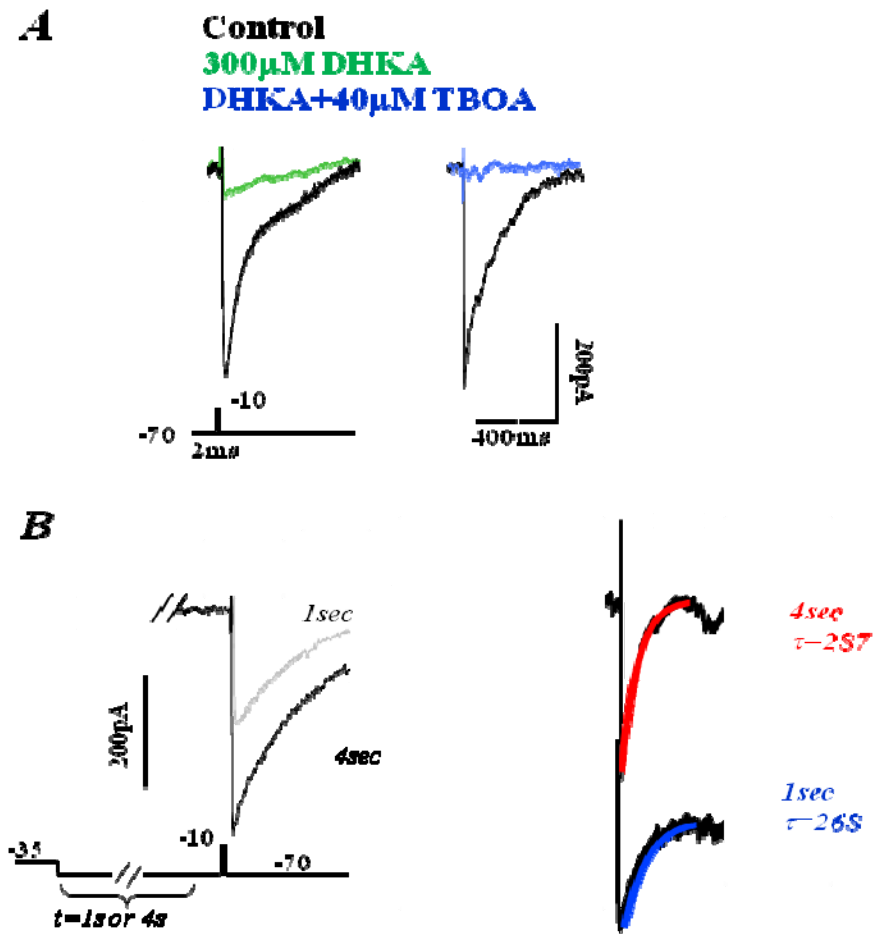


Figure 12. EAAT2 is located close to the release site

A, Elicited EAAT activation via short 2ms depolarization in cones is completely inhibited via 300µM DHKA (*left*) as well as 40M TBOA (*right*). B, Alternating the concentration of glutamate release from cones with varying hyperpolarization time course (*left*). Kinetics with low, 1sec hyperpolarization did not differ from that following strong, 4 sec release (*right*).

A 40 second interval was allowed for the recovery following each depolarization, as duration described as necessary to prevent vesicular rundown. A large, transient inward current was immediately evoked following the brief depolarization in the cone (Fig. 12A, left). This vast majority of this inward tail current was inhibited with 300μM DHKA, accounting for over 90% (n=5) of the observed peak current following vesicular release. DHKA selectively inhibits EAAT2 within a concentration range from .1-1mM (Eliasof 1998a/b). 300μM DHKA had a greater inhibition on the sEAAT2A current in the cones compared with 100μM DHKA used in the previous section. This is likely due to the competitive nature of DHKA with respect to the exocytosed glutamate. This DHKA-sensitive current reached its peak within the 2ms depolarizing stimulation, indicating that EAAT2A in cone terminals rapidly binds synaptic glutamate upon the vesicles released from a nearby site. The small remaining DHKA-insensitive current was completely eliminated by 40μM TBOA (Fig.12A, right), a highly potent and non-competitive EAAT antagonist. EAATs expressed in cones have been shown to shift membrane potential following exogenous glutamate application, and been suggested to impose an inhibitory feedback mechanism during tonic vesicular release within the dark. These results suggest that synaptic EAAT2 currents may therefore cause large, albeit transient, membrane potential changes in cone terminals.

To further study the sensitivity of the EAATs to synaptic glutamate levels in cone synapses, we again directly altered the amount of vesicular glutamate release in cones. Glutamate release can naturally be ceased by a light stimulus, because cones are hyperpolarized during the light step. The cessation of release during light causes vesicle accumulation, and subsequently an increase in the releasable poor in cone terminals. The

amount of vesicular release that rapidly occurs after a light (phasic release) depends on the amount of vesicles accumulated during this hyperpolarization (Jackman et at., 2009).

We again adopted this time-dependent vesicle accumulation protocol to manipulate synaptic glutamate levels. The sensitivity of EAATs to various synaptic glutamate levels was studied by measuring synaptic EAAT currents in the cones. Cones in retinal slices were held at the dark membrane potential -35mV, and dialyzed with a NO_3^- containing electrode solution. EAAT currents in cones were evoked by a 2ms depolarization pulse with a pre-pulse applied 1 or 4 second hyperpolarization step (-70mV) to allow glutamate vesicles to accumulate on the synaptic ribbon. A 1 second hyperpolarization only allows for approximately one third of the maximum glutamate vesicle accumulation; after a 4 second hyperpolarization cone ribbons are nearly saturated (Jackman et at., 2009). Figure 12B shows that the amplitude of the synaptic sEAAT currents was significantly increased with a 4 second pre-pulse at -70mV, indicating that EAAT activation is highly coupled to the synaptic glutamate level in cone synapses.

To estimate the kinetics of sEAAT currents, measured the rise times and decay constants of these two elicited EAAT currents. The time to peak for each trial did not differ, reaching a peak within 1-2ms. The decay time for these two currents can easily be fit with a single exponential curve with an average time constant (τ) of 268ms and 287ms for 1s and 4s pre-pulse, respectively (Fig. 12B, right). By comparing these currents, we found that the size of the synaptic glutamate transient did not affect the decay rate of the transporter currents, suggesting that the kinetics of glutamate uptake by EAATs are not significantly shifted by changes in glutamate in the synaptic zone.

3.2.2 Dark adapted cones receive large, non-EAAT2 unitary transporter events

EAATs in cone terminals are readily available for reuptake of glutamate spontaneously released in dark. The reuptake via EAAT2 seen following during tonic glutamate release and following phasic release (see figure 12) appear to occur at local regions, which are very close to the release site (likely the synaptic ribbon). It is unclear if any spontaneous glutamate signals, large or small, can influence surrounding cones to form a network connection. This signaling paradigm has been proposed, but not experimentally determined. Again utilizing $CsNO_3$ intracellular solution to largely amplify EAAT currents, we observed large, slowly decaying transients when the cells were continuously hyperpolarized to -70mV. (Figure 13A). These spontaneously occurring events displayed completely distinct kinetics with respect to elicited EAAT currents following a depolarization, decaying over a much longer period of time, normally around 1 second. Furthermore, these currents were completely different pharmacologically (Figure 13A & B). High concentrations of DHKA did not inhibit the size or decay of these transients. Conversely, TBOA completely eliminated them, suggesting a unique role for non-EAAT2 glutamate transporter. The fact that these currents were DHKA-insensitive, coupled with the knowledge that EAAT2 serves to quickly bind glutamate following release from a cone, suggests that these events may reflect the activity of peripheral transporters which encode spontaneous glutamate inputs from surrounding cones. The following data of section 3.2 will address this hypothesis.

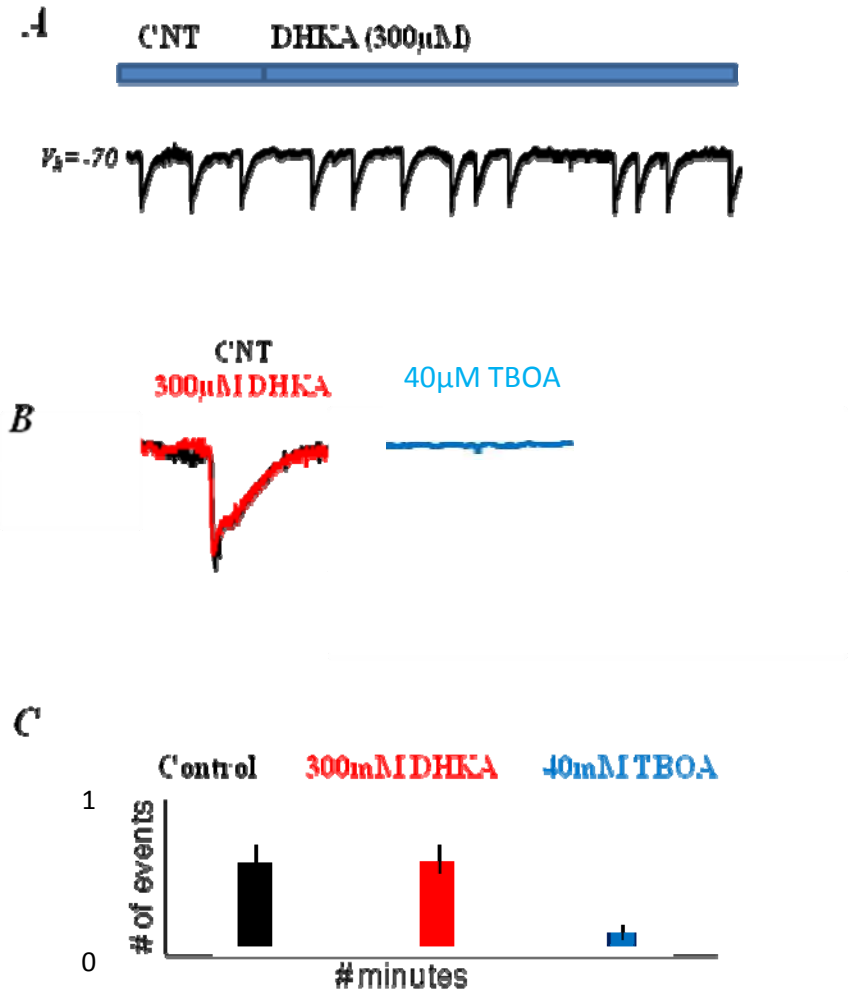


Figure 13. Spontaneous EAAT activity in dark adapted cones

A, Spontaneous currents arising in dark adapted cones, which are DHKA-insensitive. B, DHKA does not affect the kinetics of these unitary currents, although they are completely blocked via TBOA. C, Summary of pharmacological data.

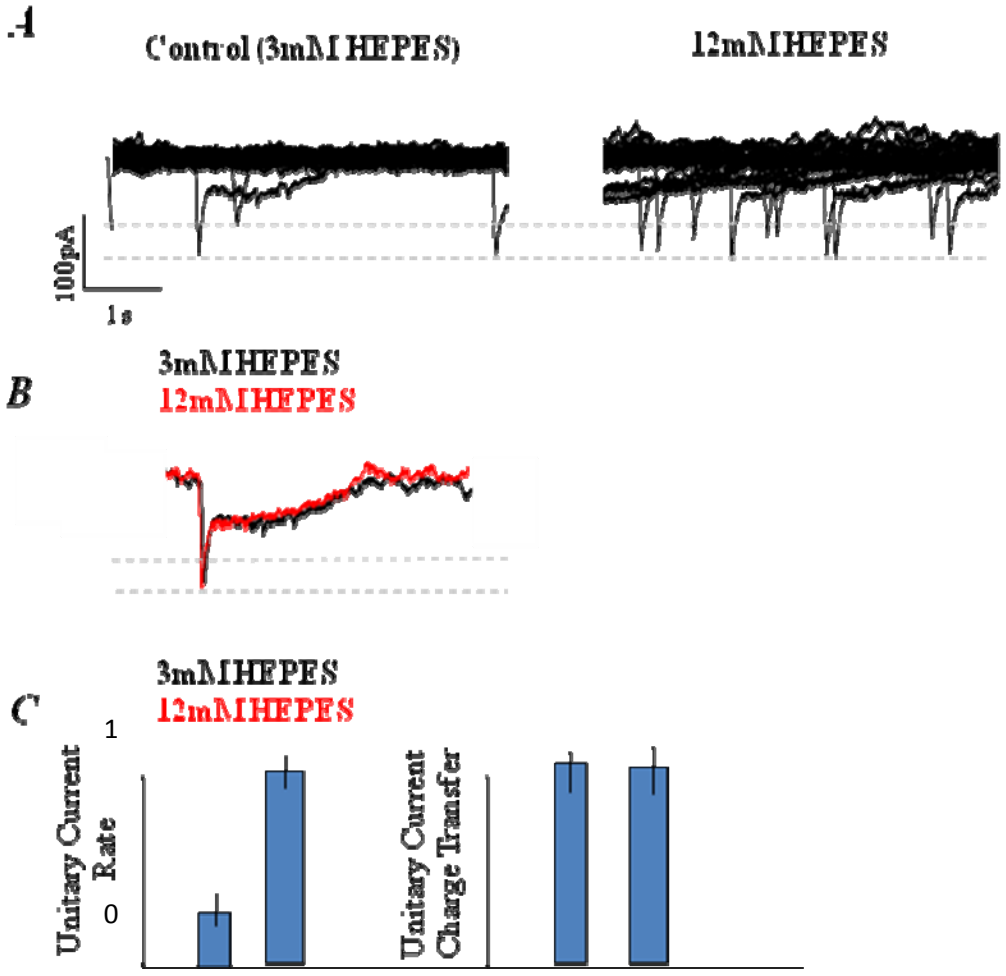


Figure 14. Induced increase in spontaneous EAAT activity from surround

A, Spontaneous EAAT currents in cones under 3mM and 12mM HEPES. B, Differing HEPES concentrations does not affect unitary kinetics. C, Summary of data.

3.2.3 Increasing surrounding photoreceptor release probability by buffering proton levels increases the rate of unitary transporter events in dark adapted cones

To address the hypothesis of direct, glutamatergic communication of adjacent photoreceptors to a 'central' cone, an experiment was devised to change the release probability from the surrounding photoreceptors, while maintaining a relatively stable, and low, release probability in the recorded central cone. In photoreceptors, as in other tonically releasing ribbon synapses such as rod bipolar cells, the continuous release of vesicles is largely determined by the Ca^{2+} conductance through VGCCs- a higher tonically releasing ribbon synapses such as rod bipolar cells, the continuous release of vesicles is largely determined by the Ca^{2+} conductance through VGCCs- a higher conductance will consequently lead to an increase in the rate of release. The most commonly understood factor involved is the increase in Ca^{2+} conductance with cell depolarization. Other factors also determine Ca^{2+} driving force through VGCCs, notably external protons, which act to inhibit this conductance (Devries 2000). Therefore, one feasible method to control the vesicular release of surrounding photoreceptors is to shift the concentration of the proton buffer HEPES. The recorded cone would still be subject to HEPES in the bath, although keeping it voltage clamped at -70mV will avoid any changes in Ca^{2+} through major cone VGCCs, which are normally inactive at voltages lower than -40mV.

A cone was voltage clamped at -70mV in dark adapted retinal slices to prohibit glutamate release from the cell. In the control Ringer's solution, 3mM HEPES was used to buffer local pH in the distal retina. Few spontaneous currents appeared in the cone under these conditions, appearing at a rate of ~10/min (Fig. 14A *left*). The observed rate of these currents largely increased when the bath Ringer's solution was switched to 12mM HEPES

buffer Ringer's solution, at around ~30/min (Fig. 14A, *right*). As higher HEPES strongly buffers local synaptic protons, the 12mM condition favors Ca^{2+}-dependent glutamate release in cone terminals. Although an increase in the rate of events was observed, the overall kinetics of the events was not changed, as shown in Figure 14B, and summarized in 14C. These findings should strengthen the hypothesis that the unitary EAAT currents in cones arise from chemical crosstalk between a central cone and surrounding photoreceptors.

3.2.4 Direct evidence of cone-to-cone crosstalk via extrasynaptic EAATs

To address whether two transporter currents are mixed at the same synaptic sites or at separated locations of the basal and invaginating areas which the data suggests, double-electrode patch-clamp experiments was performed on paired cones in dark adapted retinal slices. In one cone, arbitrarily denoted cone 1, a 100ms depolarizing step was applied to elicit fast EAAT currents in one cone. The other cone (cone 2), changes in membrane current were observed. Both cones were held at -70mV to avoid spontaneous glutamate vesicle release. The brief depolarization pulse applied to cone 1 (Figure 15A), elicited a large transporter current at the end of the pulse, as earlier described. Of interest was the small, transient inward current observed in cone 2. This current could be blocked with 40μM TBOA, but was left unchanged with 300μM DHKA (Figure 15A'), indicating a non-EAAT2 specific EAAT current. This result strongly suggests that glutamate released from the depolarized cone quickly diffuses to activate EAATs on the adjacent cone. The DHKA insensitive nature of the current is further evidence that surrounding photoreceptors, specifically cones in this case, can activate glutamate transporters at the basal area of the cone pedicle, which are accessible for uptake of diffused glutamate in the parasynaptic regions.

The results from paired cone recording experiments suggest that a distinct EAAT subtypes, possibly EAAT5, exists in the basal areas of the cone pedicles and receives glutamate signals from adjacent cones. This glutamate-directed feedback regulation in cones most likely occurs via EAATs localized to basal regions of the presynaptic pedicle.

As indicated in Figure 14, the activity of EAATs in cones was highly coupled to the spontaneous vesicle release rate in dark, as the unitary transporter currents increased with an increase in external HEPES. It is well understood that spontaneous glutamate vesicle release in cones is strongly suppressed with bright light. Therefore, if the observed EAAT–specific signal is coupled to vesicle release from surrounding cones, these unitary currents should be suppressed with bright background light.

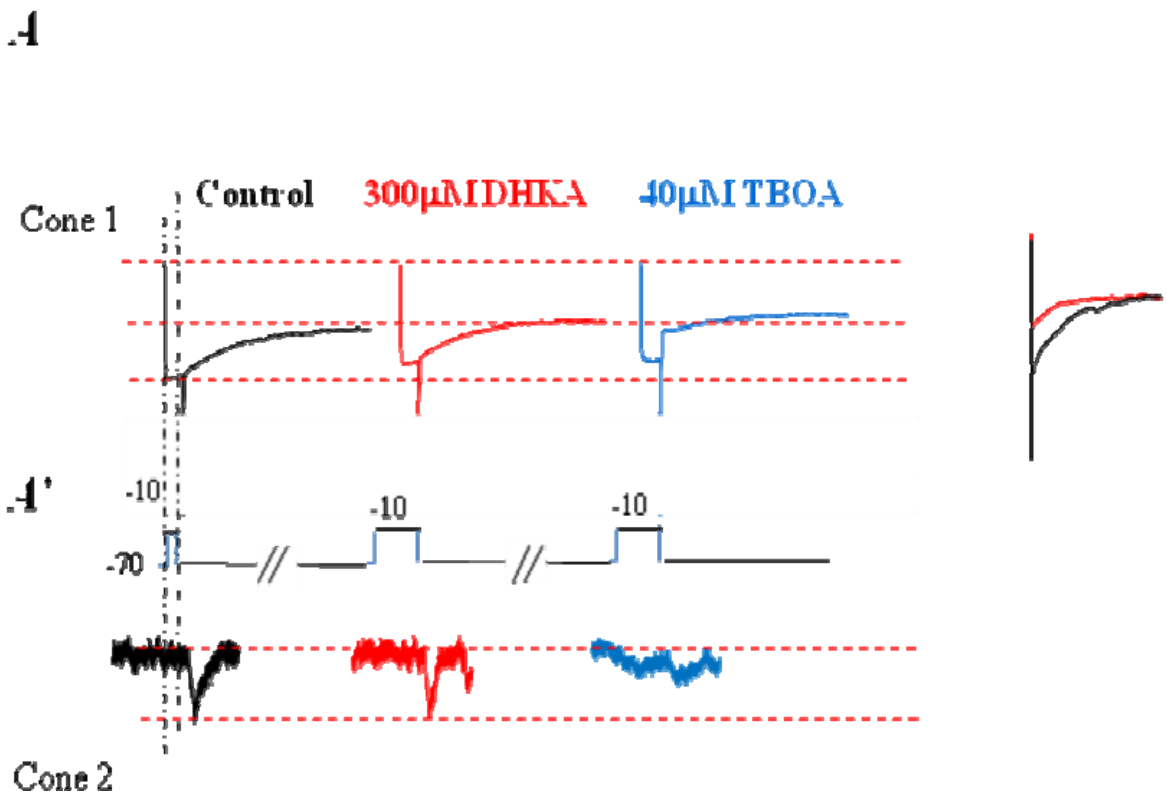


Figure 15. Dual recordings from adjacent cone describe EAAT connection

A, Simultaneous recorded cone (1) with elicited 100ms depolarization; *Black* control, *Red* 300µM DHKA, *Blue* 40µM TBOA.. *Inset*- Same cone (1) under a 2 ms depolarization to show DHKA sensitivity clearly. A', Simultaneous recorded cone (2), held at -70mV.

3.2.5 Natural elicitation of unitary transporter events during and following light stimulation

In order to test this hypothesis, unitary transporter events were observed in a dark adapted cone, as previously discussed, and subjected to bright red light for multiple seconds, repeating throughout a recording. Figure 16A shows that the spontaneous EAAT currents were indeed light sensitive. In dark the spontaneous EAATs currents appeared at -70mv with CsNO3 in the electrodes. The currents were completed diminished (did not occur) with a bright background light to ceased glutamate release from surrounding photoreceptors. Normally, recorded cones display an inward current at -70mV in dark conditions. This is due to the holding membrane potential was negative to the cones dark membrane potential, usually around -35mV to -40mV. Therefore, cations flowing through cyclic nucleotide gated channels open in dark receive a large driving force at the hyperpolarized -70mV. A light stimulus nearly reversed these inward currents, as these light sensitive channels were closed. The significant factor in this experiment is that surrounding cones, which were depolarized in the dark are now hyperpolarized, closing VGCCs in their terminals. This light-driven suppression of the surround tonic glutamate release completely ceased EAAT-mediated synaptic communication between cones, as expected.

It may be further hypothesized that surrounding cones could activate EAATs in a central cone following phasic glutamate release- the state directly following light stimulation. Recorded cones held steadily at -70mV did indeed receive a bursting signal at the offset of a light stimulus from surrounding photoreceptors. Figure 16B shows a set of recordings from a cone in a dark-adapted retinal slice. A large and transient inward current at the offset of light

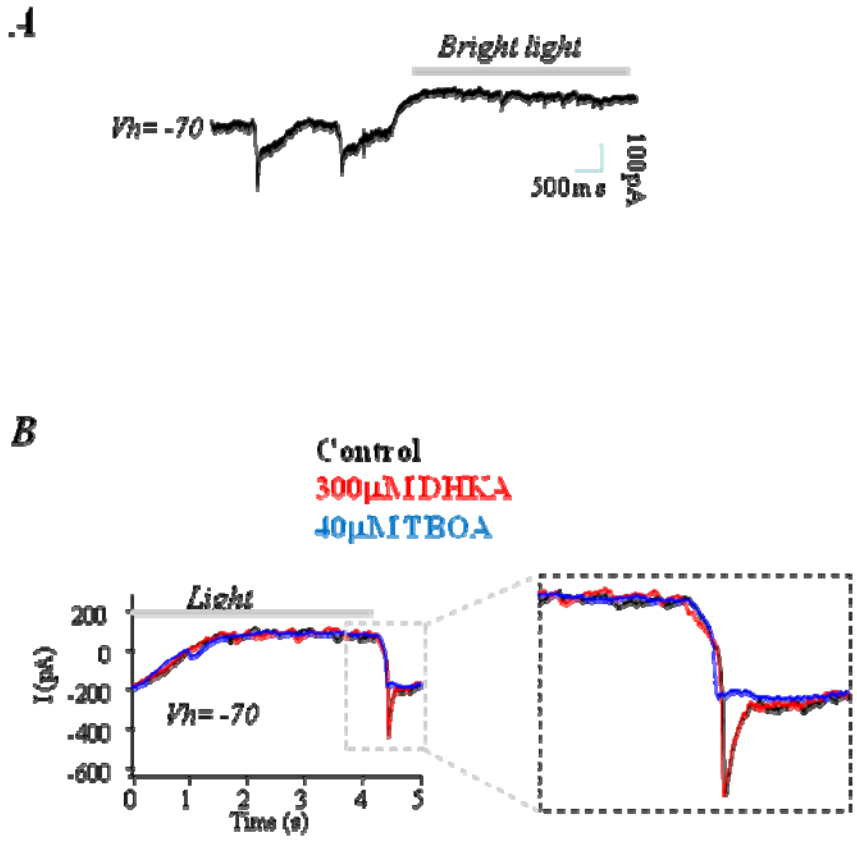


Figure 16. Natural light evoked EAAT5 current from adjacent cone release

A, Natural light stimuli suppressed all unitary EAAT currents seen in darkness. B, Naturally evo3ked EAAT current from adjacent photoreceptors at light offset. Response is blocked with 40µM TBOA, but not effected by 300µM DHKA.

stimulation was recorded from the cone. The offset response in the cone was blocked by TBOA, but was DHKA-insensitive (see Fig. 16B, *dotted box*). These light offset currents should again be elicited by glutamate inputs from surrounding cones, because 1) the local cone was hyperpolarized at -70mV and 2) rods in salamander retinas usually have a slow voltage depolarization at the light offset, without offset overshot response.

3.2.6 A Plausible Model for the distribution of Glutamate Transporters in cone terminals

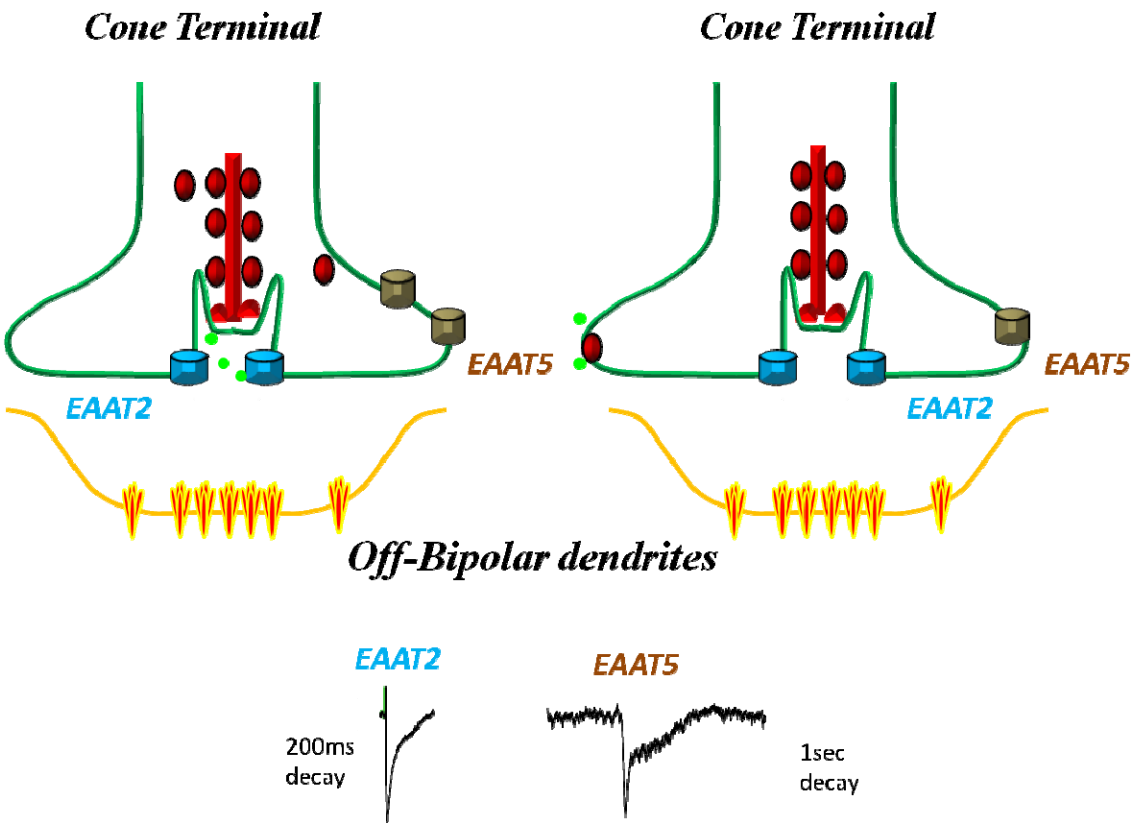


Figure 17. Proposed Model for EAAT2 and EAAT5s distinct roles

3.3 Novel Regulation of Synaptic EAAT2 Following Short Periods of Light

3.3.1 EAAT2s ability to limit the phasic glutamate signal is inversely correlated with the intensity of light proceeding the event

Earlier, (see section 3.1.4) EAAT2s ability to shape large, postsynaptic glutamate signals was discussed in detail. Importantly, it was realized that EAAT2 limits postsynaptic AMPA signals with great efficacy. These results reflect a natural, light-evoked control of phasic glutamate signals in the outer retina. One question that arises concerning this transporter phenomenon has to do with the intensity of light preceding the phasic cone signal. It has been noted that the more intense a light flash is, the larger the phasic cone signal encoded in postsynaptic neurons (Jackman et al. 2009). What has not been described is a mechanism underlying this phasic signal gain control. If EAAT2, as it is intimately coupled with the phasic signal, is directly or indirectly affected by shifts in light intensity, could provide some insight.

To examine this possibility, postsynaptic off bipolar cells receiving cone inputs were chosen for recordings; specifically monitoring the phasic AMPA responses after light flashes of constant time but with varying degrees of intensity. Figure 18 A demonstrates the effect of the shifting intensity (see left panel). As light intensity is increased, the phasic signal also rises in amplitude. Of interest was the effect of EAAT2 inhibition following each intensity change (Figure18 A, *right panel*). It was noted that EAAT2 has a shifting – dependent on the intensity of light proceeding the phasic signal. Blocking EAAT2 with 100μM DHKA led to an increase in the phasic signal amplitude, as seen earlier, but with one caveat in this experiment. After the light signal reached a certain intensity, blocking EAAT2 led to no further enhancement of the signal. This finding may be interpreted in two ways- that

increasing light intensity eventually modulates EAAT2s ability to limit the phasic signal, or simply that at some point, EAAT2 becomes overly saturated by the size of the release, and cannot overcome.

To ensure that the apparent transporter modulation with changing light intensities was not due to a saturating effect, the following experiments were conducted in an individual off bipolar cell (Figure 18B). The effect of DHKA on 2 sec light steps were first observed following low and high light intensities. 2 second steps were chosen, as they will not lead to a saturated phasic response (see Jackman et al 2009, Rowan et al. 2010). Even in this condition, DHKA only led to an enhancement in the in the low intensity paradigm (Figure 18B, *see overlaid traces*). To further confirm that the high light intensity did not saturate the response at 2 seconds, the same cell was subjected to a 4 second stimulus of high intensity. The larger size of this response with respect to the 2 second stimuli confirmed the apparent EAAT2 modulation via intense light. The ability of EAAT2 to naturally limit phasic signals in postsynaptic neurons is therefore controlled by the background intensity itself. This phenomenon could contribute to how signal output of cones is adapted in varying conditions of light.

3.3.2 Evidence that strong background light directly influences EAAT2 uptake

To truly confirm the hypothesized EAAT2 modulation via strong light, it was necessary directly observe EAAT2 currents within the cone during these varying light conditions. To address this, a familiar protocol was developed for the cone, which were dialyzed with NO3- and held at Vh of -70mV. Large transporter transients were observed following 2ms steps to -10mV (Figure 19A, *black trace*). These signals are EAAT2 specific (see section 3.2.1). When the retinal slice was subjected to a 4 second light stimuli

proceeding the depolarization, the resulting EAAT2 current was significantly reduced (red trace), indicating the possibility that less glutamate was bound with background light. As a precautionary measure, the light step was always continued throughout the entire trace as to eliminate the influence of surrounding photoreceptor activity. When both traces are scaled, they do not appear kinetically different- only changes in amplitude are observed. This likely rules out a mechanism involving internal ion shifting, as shifts in internal K+ are necessary to complete the transporter co-localization, and would therefore vary the signal kinetics of the EAAT signal as previously observed with shifts in internal K+ (Wadiche et al. 2006). In addition, internal Cs^+ was always included in the pipette, further negated a mechanism involving internal K^+ ions shifting.

If EAAT2 is truly inhibited with an increasing intensity of light, then these currents may be concurrently reduced in a step-wise manner. Indeed, this result was seen Figure 19B). With respect to currents in the dark, the elicited EAAT2 current displayed nearly 2-fold decrease in the displayed cell with high intensity light, and was significantly reduced on average.

One simple, although seemingly paradoxical explanation for the reduced EAAT2 current with background light is that glutamate release is reduced or changed because of it, and therefore the EAAT2 current is shifted. This is unlikely, but worth checking to be thorough. Figure 19D shows the exact protocol as in Figure 19A and B, but this time checking for changes in exocytotic capacitance (see methods), to observe any possible flaws in how glutamate release is being controlled in light and dark. As expected, there was no significant change in the capacitance increases following a 2ms depolarization, either in fully dark conditions or with a 4 second background of high intensity light. The 50fF jump in

capacitance is expected for saturated ribbons, as described by Jackman et al 2009. The lack of changes in vesicular release under these conditions is likely also due to the strong depolarization step to -10mV, a voltage at which shifts in Ca^{2+} conductance that can shift via network feedback (see Cadetti and Thoreson 2006) from horizontal cells is largely avoided. In short, the inhibited EAAT2 current with background illumination is not due to an indirect change in phasic glutamate levels.

These data provide part of a feasible mechanism for the cones spectacular ability to encode light intensity via phasic signals. Undoubtedly, other factors are involved in low light intensity shapes this signal, such as Ca^{2+} driving force or vesicular buildup, or surround influences from lateral integration.

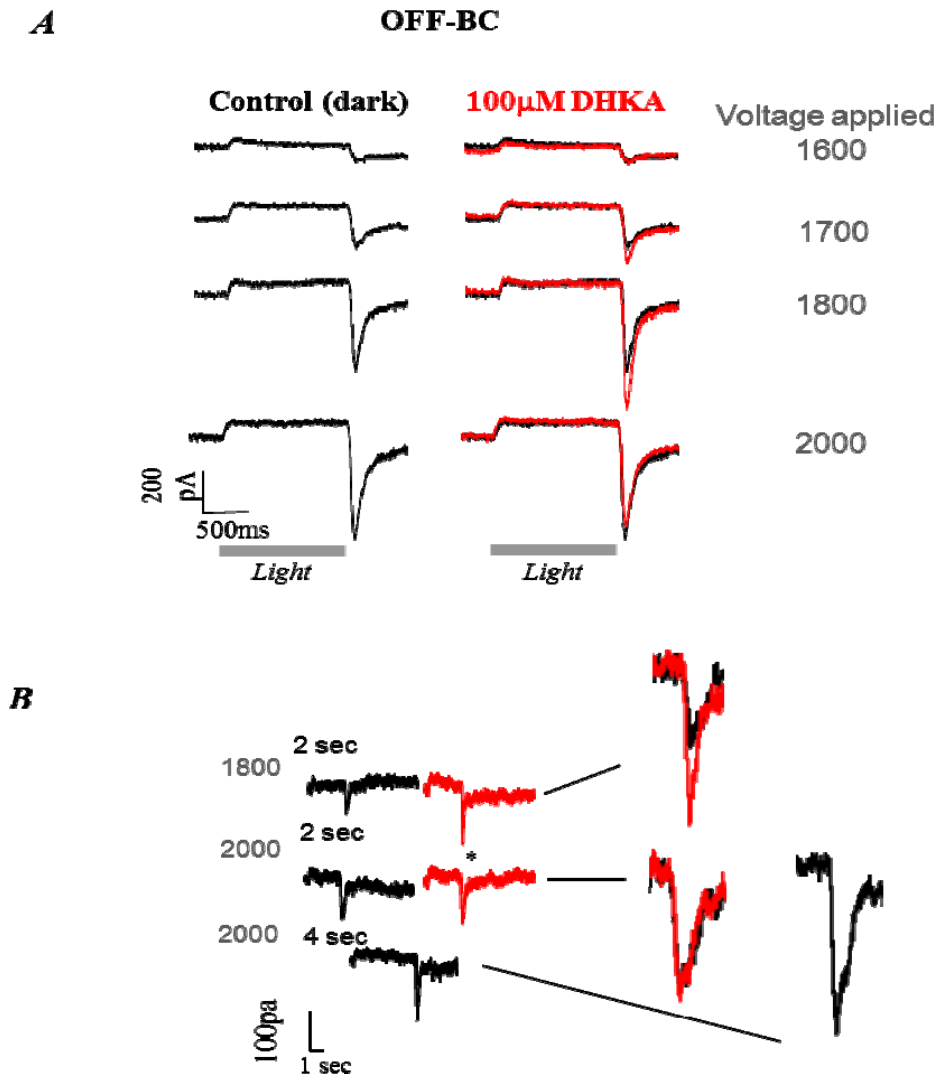


Figure 18. Effects of differing light intensities on EAAT2 in the OPL

A, DHKA enhancement of postsynaptic light offset signals (cone phasic release) under differing conditions of light intensity. B, High light intensity suppression of DHKA enhacement is not due to saturation of signal.

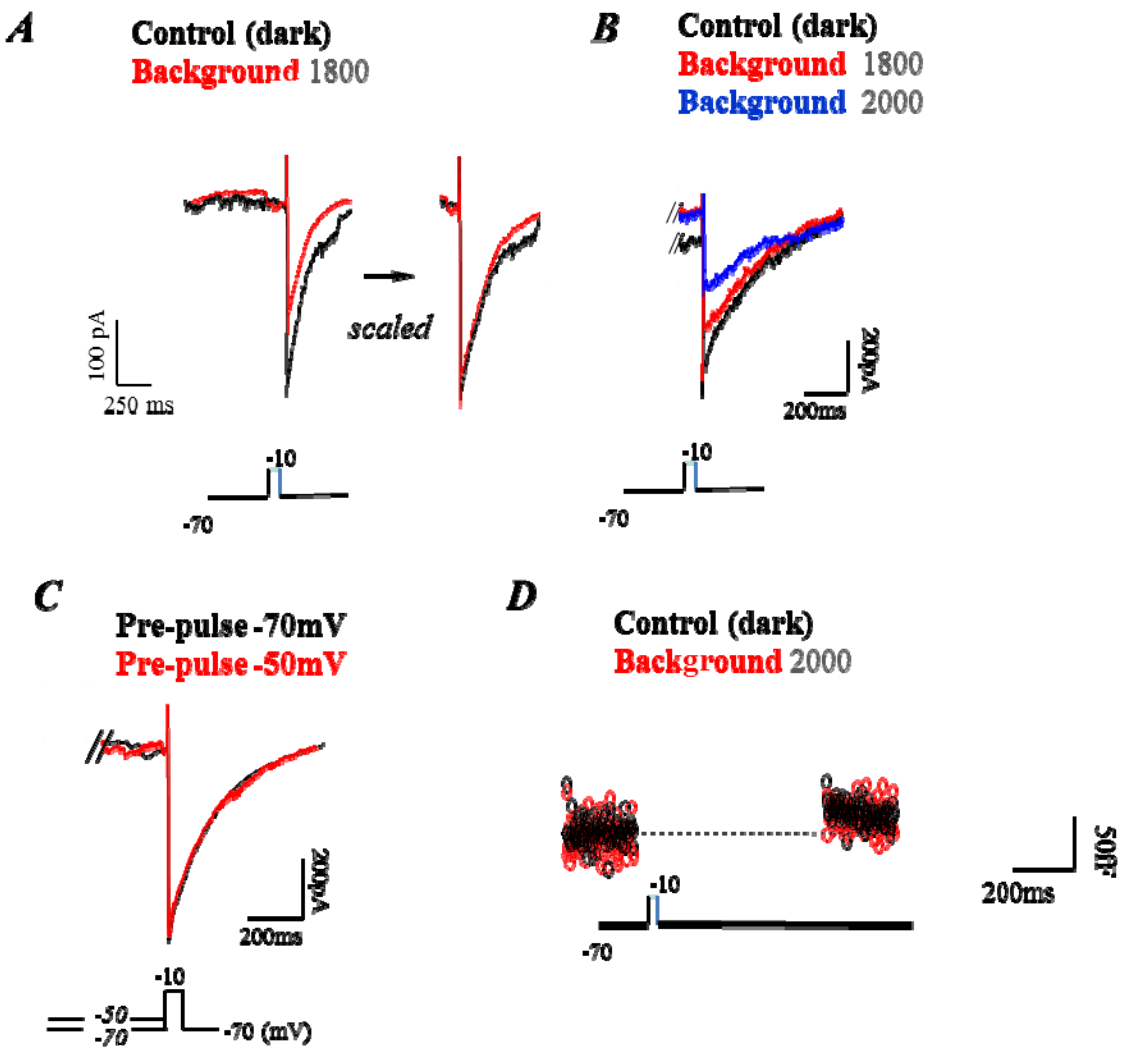


Figure 19. Intense light directly modulates EAAT2 activity

A, Comparison of elicited EAAT2 currents in darkness and following 4 seconds of light stimuli. B, Increases light intensity is in direct relation to elicited EAAT current inhibition. C, Pre-pulse voltage does not effect EAAT transient. D, Background light does not inhibit glutamate release from cones, confirmed via capacitance recordings.

3.3.3 Shifting proton levels offer a mechanism for the light-specific ability of EAAT2 regulation

As an internal mechanism that involves the shifting of K+ ions is unlikely, another plausible mechanism for this light-adaptive phenomenon was tested. One variable, specific to the binding activity of EAATs (Larsson et al 2004) and which is known to shift in concentration at the OPL during light adaptations (Dmitriev 2001) is the proton. Indeed, pH increases (increasing alkalinity) somewhat considerable as a whole in the synaptic areas, due to the acidic nature of glutamatergic vesicles constantly being exocytosed in darkness (see Devries 2001), and likely due to other mechanisms. It was therefore hypothesized, especially because of the possibility that protons appear to lock in the Na+ ion necessary for glutamate binding of EAATs, that a natural reduction in synaptic protons with light might affect the binding efficacy of EAAT2 in cone photoreceptors. Figure 20 shows direct evidence for this hypothesis. Light more readily inhibits EAAT2 currents in conditions of high proton buffering, (Figure 20B) compared with lower proton buffering (Figure 20A). Our results also agree with Palmer et al. 2003, who described proton buffers as synaptic EAAT inhibitors in bipolar cells terminals.

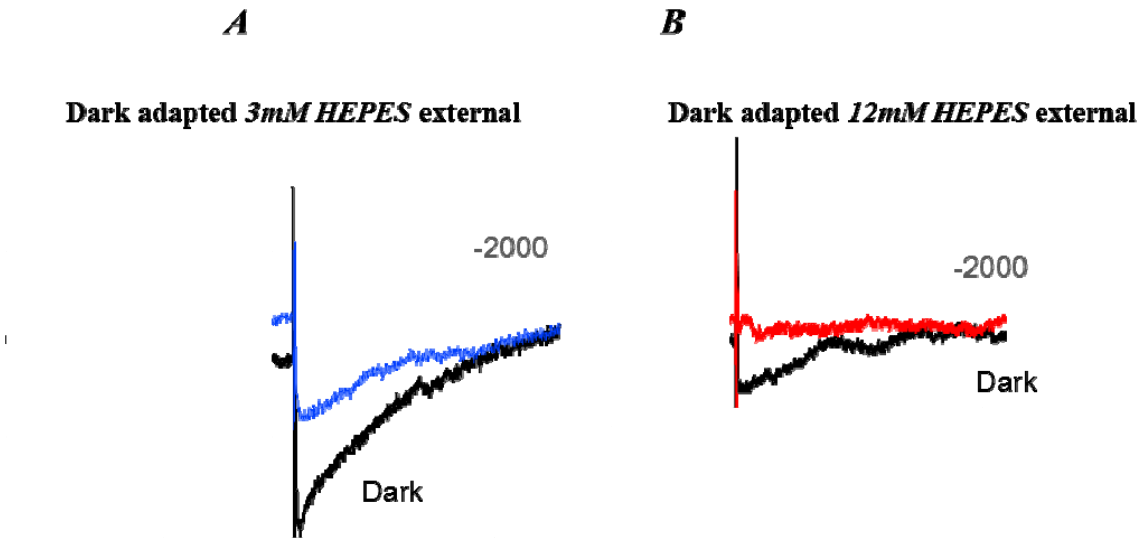


Figure 20. Proton-specific mechanism of light-induced EAAT2 modulation

A, Trace of elicited EAAT current in 3mM HEPES before (*black*) light stimuli and after 4 second high intensity stimuli (*red*). B, Trace in 12mM before (*black*) HEPES and after (*red*) 4 second high intensity stimuli.

3.3.4 Possible Model for the action of extracellular protons in regulating EAAT2 binding

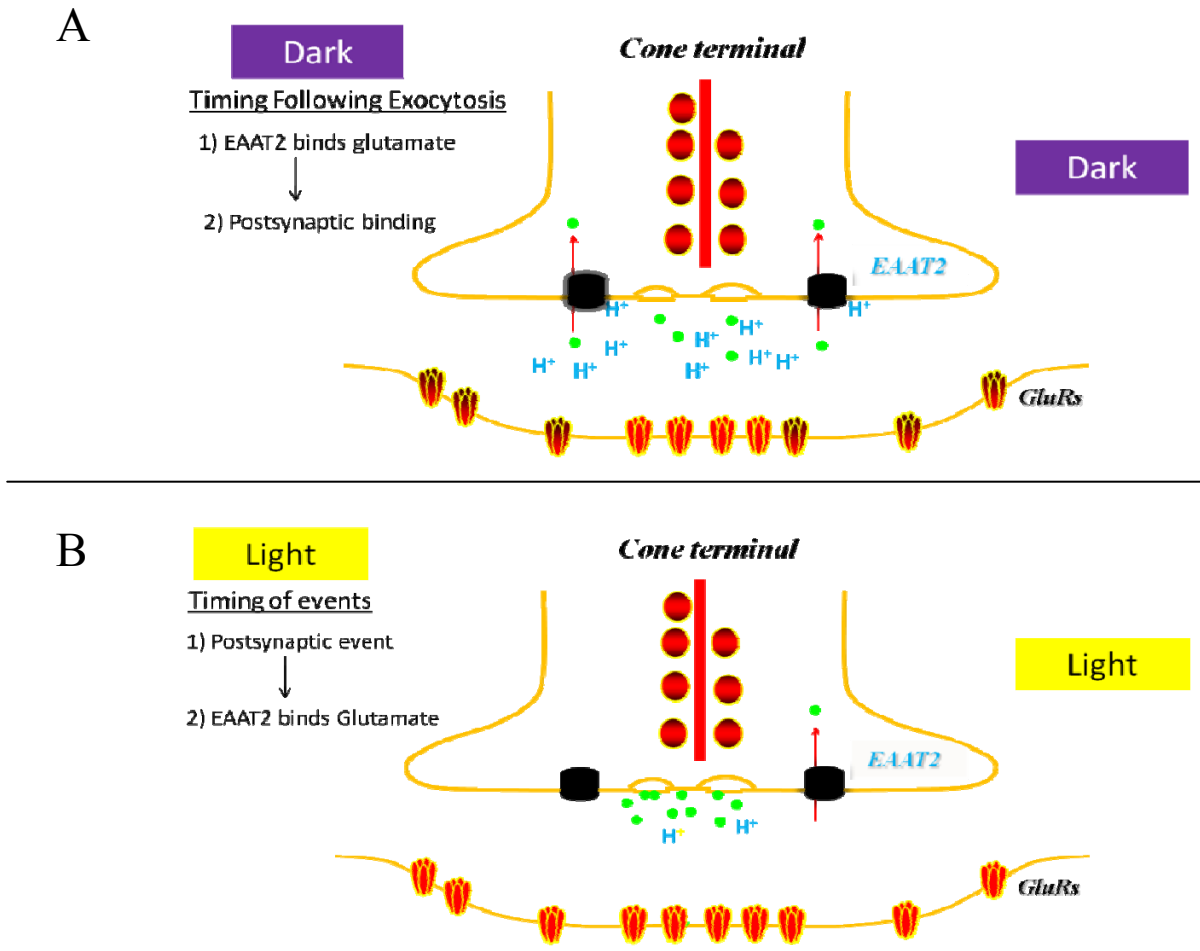


Figure 21. Proposed model for light-specific EAAT2 regulation

3.4 Metabotrophic Roles of Taurine and Glycine within OFF and ON Bipolar Cells of the Retina

3.4.1 K^+ Channels Subtypes of BPCs are regulated via Taurine and Glycine

Voltage-gated K^+ channels were specifically chosen to study the metabotropic regulation of glycine and taurine for multiple reasons. 1) It is already understood that glycine has a metabotropic activity which acts as a downstream pathway upon voltage-gated Ca^{2+} channels in retinal neurons (#). 2)Voltage-gated channels of neurons, including voltage-gated K^+ channels, are subject to various forms of regulation (see section 1.3.2 for review). A number of different voltage-gated K^+ channels are expressed within the outer retinal bipolar cells. The Kv1.3 antagonist margatoxin (50nM) has little effect on the transient portion of the K^+ current but blocks the majority of sustained K^+ currents in all bipolar cells and the third-order neurons, amacrine and ganglion cells (Bulley 2010). It is this sustained outward rectified voltage-gated K^+ channel that is regulated by taurine and glycine expresses in OFF-bipolar cells; activation of the channels with 10-20ms of a depolarizing step produced sustained currents. These currents, which were only observed in ill-defined subset of bipolar cells (data not shown), are likely A-type currents sensitive to 4-AP (Bulley 2010). With this background knowledge, the mechanisms behind the Taurine- and Glycinergic VGKC regulation may be better understood. Importantly for this dissertation overall, the regulation of postsynaptic VGKCs in postsynaptic cells of the OPL will lead to changes in the encoding of glutamatergic signal amplitudes and kinetics. As a matter of organization, bipolar cells were tested as two broadly distinct groups, OFF and ON, to test for differences in VGKC regulation.

3.4.3 Distinct regulation of voltage gated K^+ currents by Taurine and Glycine in Off Bipolar Cells

The first group of metabotrophically regulated bipolar cells that I will discuss is the OFF, or hyperpolarizing, bipolar cell group- deemed as such due to their hyperpolarizing signal with light. During the course of these experiments, a large amount of the cells patched were displaced OFF bipolar cells, their distinct morphology allows one to differentiate from ON bipolar cells before recording. All recordings were performed with intracellular Lucifer Yellow dye. After recording, and non-displaced OFF bipolar cells were confirmed as such due to their distinct axonal stratification within sublamina A of the IPL. Importantly, all recordings include 100μM Cd^{2+} in addition to the standard PTX/Strychnine cocktail in the bath; this was done to block any possible network effects of applying Glycine and Taurine. One of the most intriguing discoveries found concerning the metabotrophic properties of Taurine and Glycine within OFF BPCs is their opposite effect on sustained VGKC currents. Figure 22A depicts this data in two separate cells, one subjected to 80uM Taurine (red, left) and the other Glycine (blue, right). Cells were held at -60mV during recordings, and stepped from -100 through +60mV to observe VGKCs. Currents at very negative voltages did not change significantly with either taurine or glycine, indicating that under the conditions tested, these substances play little role in the regulation of inward rectifying currents.

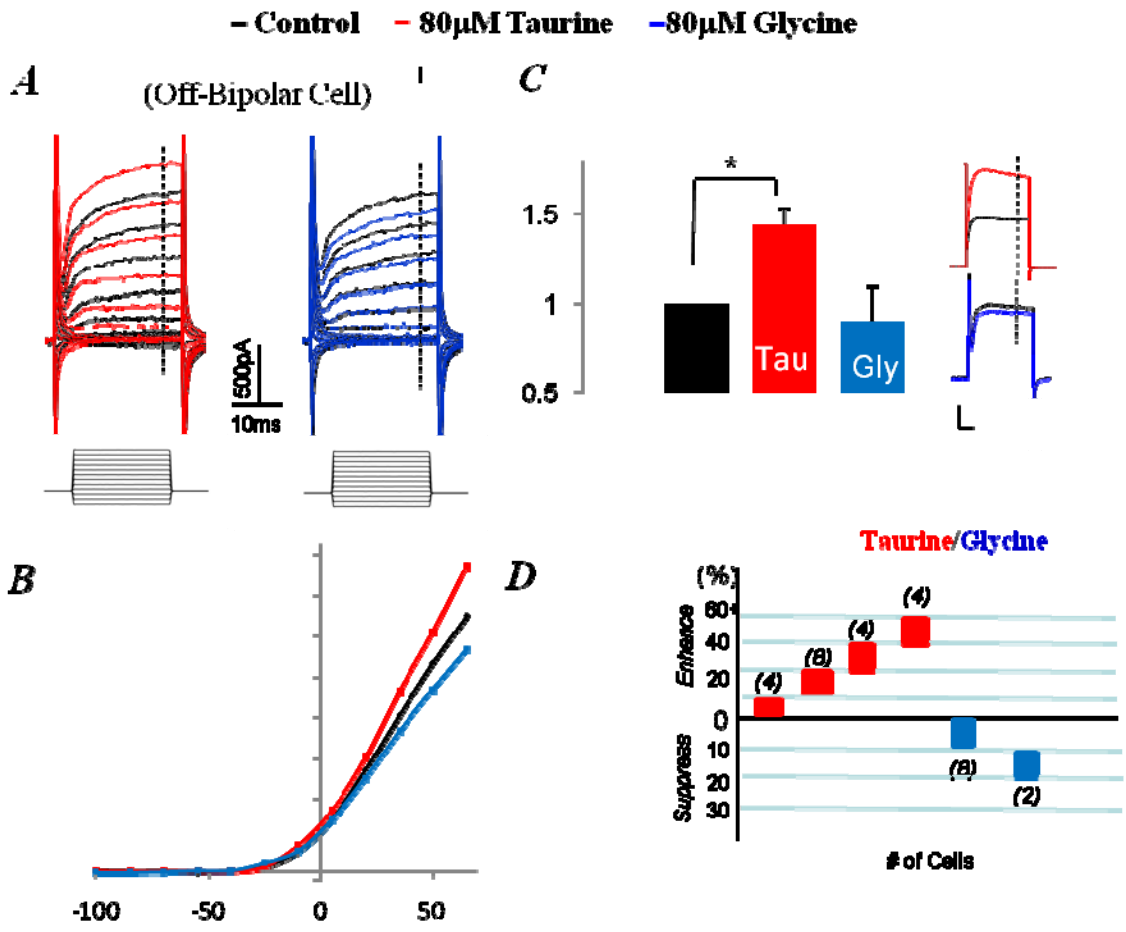


Figure 22. Distinct regulation of voltage gated K+ currents by Taurine and Glycine in Off Bipolar Cells

A, Current responses in Off Bipolar Cells stepped from -100 to +65mV, following bath perfusion of 80uM Taurine (red) and Glycine (blue).B, Current-Voltage relationship of Off Bipolar Cells following bath perfusion of 80uM Taurine (red) and Glycine (blue). Voltage Gated K+ currents are enhanced or inhibitied via Taurine and Glycine, respectively. C, Taurine enhancement of Voltage Gated K+ currents are statistically significant (p=.0004). D, Percentage enhancement and suppression of Voltage Gated K+ currents in Off Bipolar Cells via Taurine and Glycine. *Dotted marker conforms to current measured for the traces. Scale bar= 500pA, 5ms.*

These currents were greatly enhanced after Taurine application, while Glycine caused a slight inhibition. Figure 22B depicts the effects of within one recording. Taurine (*red*) was first applied after an established control IV curve (*black*), displaying an enhancement in outward VGKC current. The control was then reestablished by washing with control solution (*trace omitted for clarity*) a process that occurred within 1-2 minutes normally, and Glycine was then applied, causing an inhibition which could also be washed in the same cell. This experiment could also be repeated in reverse order (data not shown), confirming the distinct regulation of VGKCs via glycine and taurine. Figures C and D reference statistical analysis of this OFF BPC study. The histogram in Figure? C represents the absolute current at +60mV before and after taurine/glycine application. In OFF BPCs, Taurine caused an average 44.37%±8.5 increase in current ($n=20$; $p<.001$). This enhancement percentage was not always consistent, as shown in Figure 22 D (*red boxes*), varying from a 10-60% increase in outward VGKC current. Importantly, this enhancement was observed in OFF BPCs with severed axons, as discovered through post-hoc examination of intracellular staining. This fact should not be overlooked as it localizes the activity to the specific local in which taurine is naturally found in high concentrations, namely, the outer retina.

The Glycine inhibition was not as consistent, albeit averaging a 10% inhibition ($n=10$; $p<.05$). It is important to note that the overall average may disguise a subset of OFF bipolar cells which suppress more strongly with glycine, to 20%, as depicted in Figure 22D (*blue boxes*). Furthermore, no enhancement was ever noted with glycine in OFF BPCs.

3.4.4 Distinct regulation of voltage gated K^+ currents by Taurine and Glycine in On Bipolar Cells

The next group of metabotrophically regulated bipolar cells that I will discuss are the ON, or depolarizing, bipolar cell group- deemed as such due to their depolarizing signal with light. All recordings were performed with intracellular Lucifer Yellow dye. After recording, ON bipolar cells were confirmed as such due to their distinct axonal stratification within sublamina B of the IPL. As in the previous all relevant experiments, recordings include 100μM Cd^{2+} in addition to the standard PTX/Strychnine cocktail. As OFF and ON bipolar cells play distinct roles in signaling, it should come as no surprise that they should be regulated in differing ways. Indeed, it is found that Glycine and Taurine had different, although not completely opposite metabotrophic regulatory properties with respect to VGKCs. Figure 23A depicts this data in two separate cells, one subjected to 80uM Taurine (*red, left*) and the other Glycine (*blue, right*). As in OFF BPCs, cells were held at -60mV during recordings, and stepped from -100 through +60mV to observe VGKCs. Currents at very negative voltages- which were generally observed as larger in ON BPCs, did not change significantly with either taurine or glycine. This indicated that under the conditions tested, that glycine and taurine play no role in the regulation of inward rectifying currents. The large outward rectifying sustained currents seen at voltages more positive than 0mV were again the most regulated. These currents were well inhibited after Glycine application, while Taurine only caused a slight inhibition.

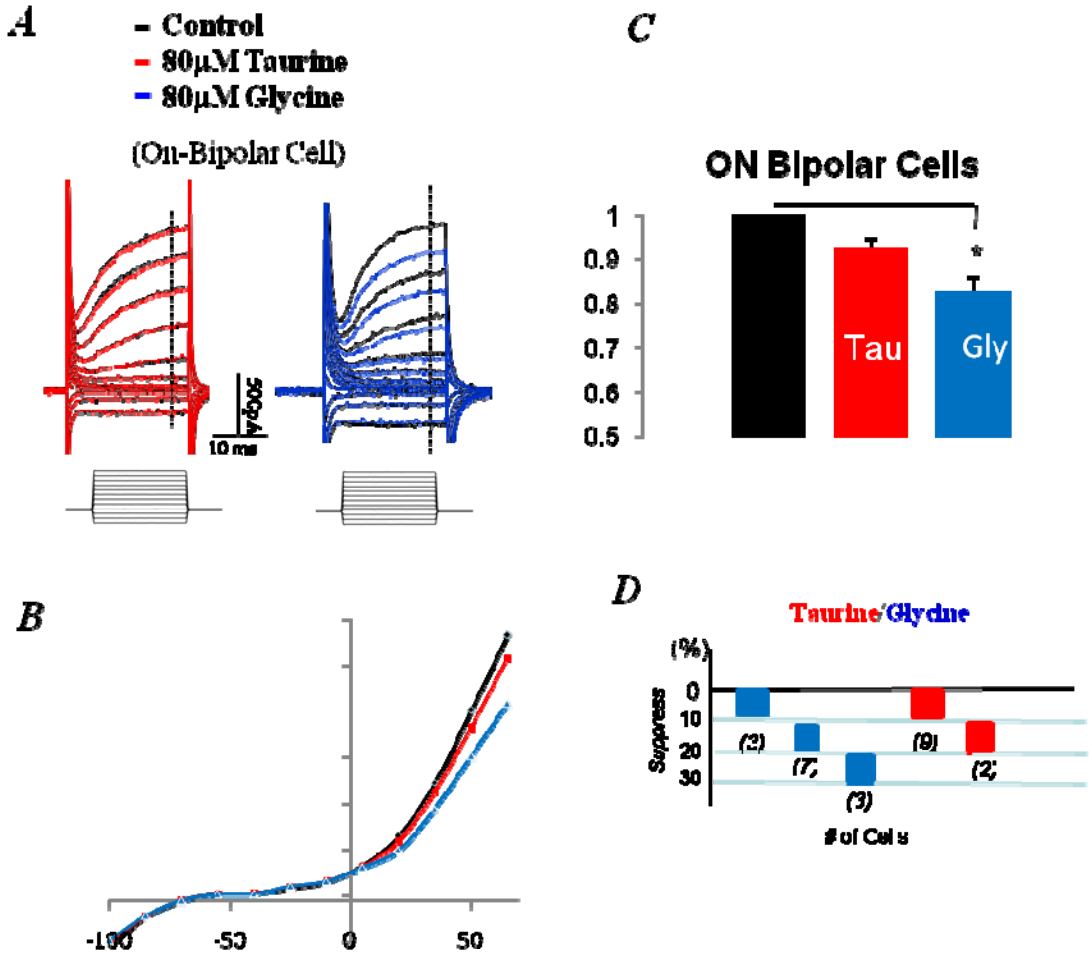


Figure 23. Regulation of voltage gated K+ currents by Taurine and Glycine in On Bipolar Cells

A, Current responses in Off Bipolar Cells stepped from -100 to +65mV, following bath perfusion of 80uM Taurine (red) and Glycine (blue).B, Current-Voltage relationship of Off Bipolar Cells following bath perfusion of 80uM Taurine (red) and Glycine (blue). Voltage Gated K+ currents are inhibited via Taurine and Glycine, although Glycine provides a much stronger suppression. C, Glycine inhibition of Voltage Gated K+ currents are statistically significant (p=.0004). D, Percentage inhibition of Voltage Gated K+ currents in Off Bipolar Cells via Taurine and Glycine.

This might be described as an expected result for a shared binding site, of which displays greater sensitivity for glycine. What is clear is that no enhancement ever occurs in ON BPCs under the tested condition. Figure 23B depicts the effects of within one recording. Taurine (*red*) was first applied after an established control IV curve (*black*), displaying a very slight inhibition of the outward VGKC current. The control was then reestablished over a short period of time (*trace omitted for clarity*), and Glycine was then applied, causing a more significant inhibition, which could later also be washed in the same cell. . Figures 23C & D reference statistical analysis of this ON BPC study. The histogram in Figure 23C represents the absolute current at +60mV before and after taurine/glycine application. In ON BPCs, Glycine caused an average 18.68%±3.1 decrease in control current (n=13; p<.002). This inhibition percentage was not always consistent, as for Taureringic enhamcents in OFF BPCs. Figure 23D (*blue boxes*), displays an inhibition varying from a 10-30% of the control outward VGKC current. The taurine inhibition was also consistent, but much smaller, averaging a 8.32%±2.3 inhibition (n=11; p<.01).

3.4.5 Enhancement of Voltage Gated K^+ currents by Taurine is insensitive to $GABA_B$ antagonists

To be certain that the VGKC current enhancement observed with taurine was not acting on $GABA_B$ receptors in OFF bipolar cells, as has been observed to occur in at higher concentrations in previous studies, the $GABA_B$ receptor antagonist CGP55845 was included in an experiment (Figure 24). The IV relationship depicts the enhancing effect of taurine (*red trace*). During taurine application, CGP55845 was co-applied. If $GABA_B$ receptors were involved in the process of enhancement, CGP should inhibit some or the entire tauinergic

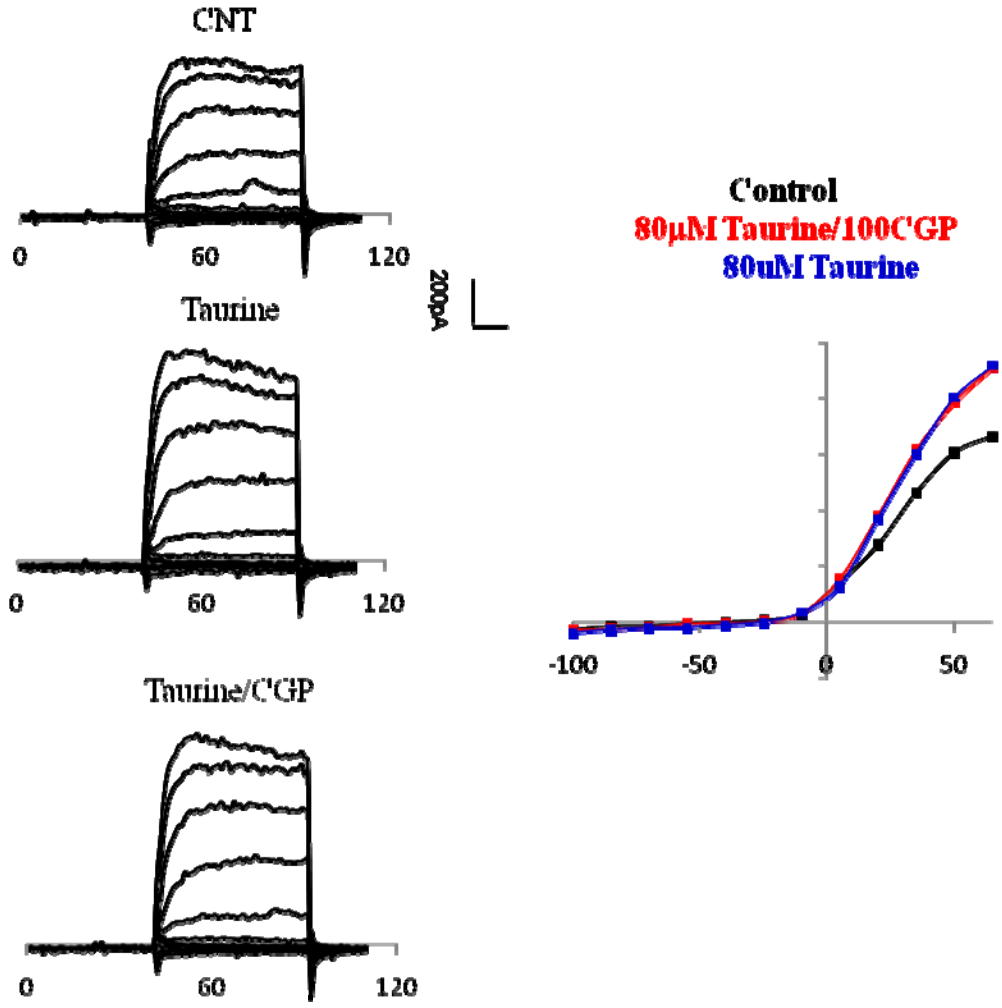


Figure 24. Enhancement of Voltage Gated K^+ currents by Taurine is insensitive to $GABA_B$ antagonists. *A,B*, Taurine enhancement persists with 100uM CGP. $GABA_B$ receptors likely do not play a role in the enhancement via Taurine.

current enhancement. It was found to have no effect, meaning that this VGKC enhancement process occurs at a site that is independent of $GABA_B$ receptors, lending credence to the idea that the binding site for taurine is distinct and more specific at the 80μM level, a physiologically realistic level.

3.4.6 Taurine and Glycine Voltage Gated K^+ current modulation is sensitive to distinct Protein Kinase inhibitors

To understand how these metabotrophic effects work, it was postulated that each distinct receptor site for taurine and for glycine might activate distinct protein kinase pathways. This hypothesis appeared highly likely, especially due to the opposite behavior of glycine and taurine within OFF bipolar cells, with respect to VGKC regulation. The cell type in which the glycine or taurine effect was strongest, ON and OFF bipolar cells, respectively, was chosen to study each internal pathway.

Results for these tests are depicted in Figure 25A and B, separated as groups of ON OFF BPCs, respectively. Although an entire spectrum of kinase inhibitors was not used, the data gathered should be considered sufficient to create a general understanding of the major internal pathways activated via glycine and taurine in ON and OFF bipolar cells, as the two specific kinase blockers, PKI and GF109203X, could eliminate their metabotrophic effect quite completely. These drugs are specific inhibitors of protein kinase A (PKA) and protein kinase C (PKC), respectively.

The inhibitory effect of glycine on VGKC conductances was greatly reduced (8%±3.0 vs. 18.68%±3.1 with PKI; Figure 25A) with the application of 50M PKI within the internal solution. Albeit the small inhibition, no significant difference in VGKC current was

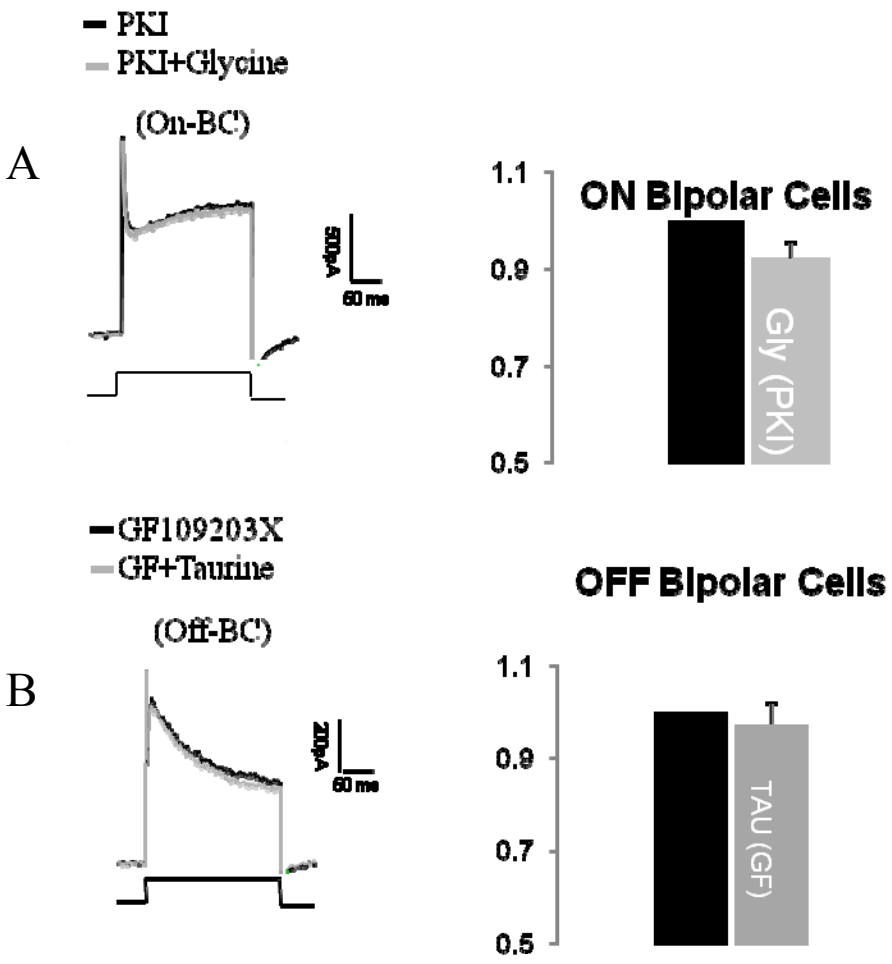


Figure 25. Taurine and Glycine Voltage Gated K^+ current modulation is sensitive to distinct Protein Kinase inhibitors.

A, Inhibition via Glycine in ON Bipolar cells is greatly reduced when internal PKA blocker PKI is included in the pipette ($p > .05$ when compared with control). B, Enhancement via Taurine in Off Bipolar cells is completely eliminated ($p > .05$ when compared with control) when internal PKC inhibitor GF 109203X is included in the pipette.

found when ON BPCs were PKI-infused (n=5; p>.05). If the slight inhibition of taurine in ON BPCs is compared with and without PKI internally, a significant difference is seen (8.32.± 2.3 control, n= vs. 7.01%±5.2, n=3with PKI).

This could be considered evidence for a shared receptor site, as well a shared internal pathway for glycine and taurine within ON BPCs. This is plausible as taurine has a known, albeit lesser, affinity for other glycinergic receptors; namely strychnine-sensitive ionotropic glycine receptors (Bulley 2010). The exact site in which Glycine and Taurine is working on in ON BPCs is completely unknown. Future work may shed light on the receptor itself, whether being a novel metabotropic receptor or a shared receptor.

The excitatory effect of taurine on VGKC conductance was greatly reduced by the external application of the membrane permeable, specific PKC inhibitor GF109203X. Within OFF bipolar cells the PKC inhibitor completely blocked any enhancement with taurine (Figure 25B) (n=3). No significant changes in current were observed 3.70%±4.5 in VGKC current was seen with taurine/ external GF109203X. This result strongly suggests that the internal pathway activated via taurine is PKC-specific within OFF bipolar cells. This is in contrast to the findings on glycine in ON bipolar cells, which was found to be through a PKA-directed path. The enhancing effect of taurine in OFF BPCs was still observed in conditions with internal PKI, as used previously in ON BPCs to block the glycine effect. Furthermore, the slight inhibition of VGKC current caused by glycine was also completely eliminated with internal PKI in OFF BPCs (n=3). It appears quite clear that two distinct glycinergic and taurinergic metabotropic pathways exist in OFF bipolar cells, in which glycine activates a VGKC inhibition through a PKA-directed signal, and in which taurine

activates a VGKC enhancement through a PKC-directed signal. The internal pathways distinct players within each specific bipolar cell subtype will need to be further elucidated.

3.4.7 Voltage waveforms in Off Bipolar Cells are shaped via changes in K^+ conductance

The physiological necessity for the specific regulation of VGKCs in bipolar cells at first seems paradoxical. Outward rectifying channels play an important role in the repolarization of action potentials, being activated at very positive voltages and allowing the flow of K^+ ions outward, therefore hyperpolarizing the cell to rest. BPCs cells to not receive or generate spikes, and are good at integrating light signals, the low voltages of which should not generate large outward rectifying K^+ currents. On the contrary- OFF bipolar cells, especially those postsynaptic to cones, receive huge multi-vesicular phasic signals as previously discussed. These signals likely will activate, although only transiently, outward rectifier VGKCs which are regulated via taurine. Therefore a dual hypothesis can be postulated- that outward rectifying VGKCs might regulate the repolarization of OFF bipolar cells following phasic release, and that this repolarization could be regulated following the metabotropic action of taurine.

To test these hypotheses, a current clamp paradigm was developed to simulate the phasic signal received within OFF bipolar cells. Extracellular Cd^{2+} was still necessary to block the network effects of bath applied taurine. Short, 5ms current injections were used to depolarize the cell to +20mV from resting. This short current injection should allow the cell to repolarize back to resting naturally.

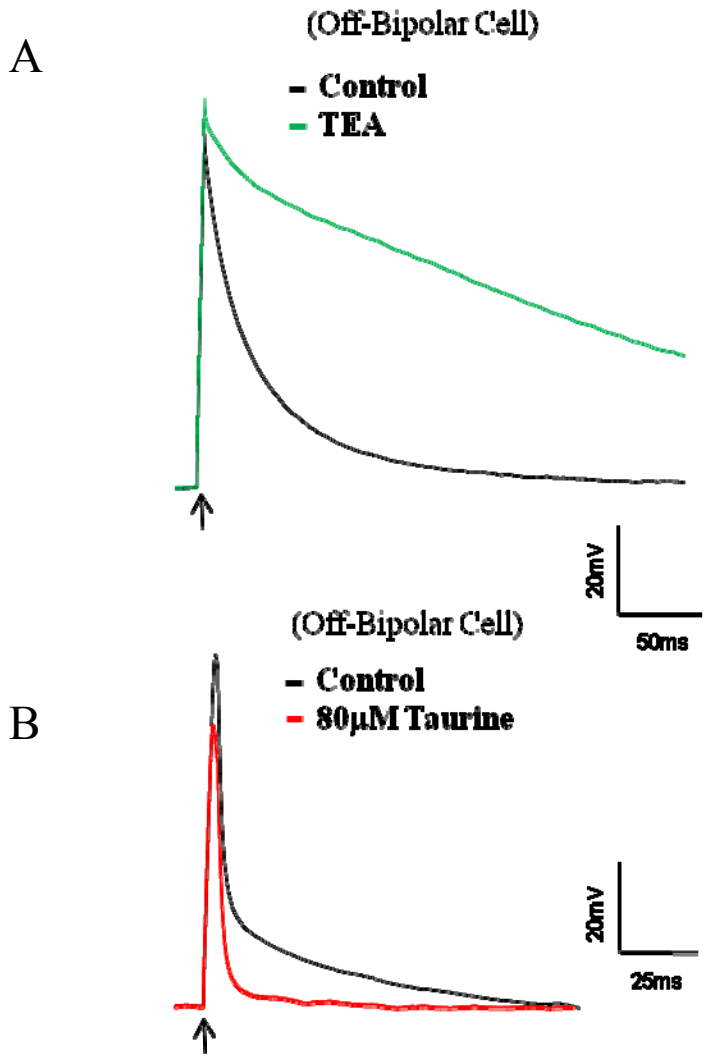


Figure 26. Voltage waveform in Off Bipolar Cells is shaped via changes in K^+ conductnce

A, Off Bipolar Cell under current clamp conditions. Cells were subjected to short 5ms current injections sufficient to depolarize the cell to +20 (black). After blocking K^+ conductance with external TEA (20mM), an identical current injection causes stronger voltage increases with an increase in decay. B, The voltage waveform is reduced in amplitude with 80uM Taurine, with an increase in decay, when compared with control.

In Figure 26, the experiment was performed in control conditions and following external application of the general K^+ blocker TEA (20mM). An expected result was obtained (*top traces; black control, green TEA*). Within most OFF BPCs, the control repolarization time following current injection decayed rapidly in a single exponential fashion, with a time constant of t=?±. This was in stark contrast to the repolarization phase with external TEA; the decay of which might be more suitably described as linear and at a far slower rate. In addition, the same amount of current could further depolarize the cell, a consequence of the higher resistance of the cell under conditions in which K^+ channels (or any pores) are blocked. The repolarization with TEA is on the order of 100s of milliseconds if not seconds, obviously not an ideal state to encode multiple large signals. Likely, the repolarization delay with TEA reflects the ability of VGKCs to ease the cell back to resting following short, strong stimuli. This study was not specifically limited to any particular subtype of VGKC, as TEA will block nearly any K^+-permissive pore. But it does give a basis for the underlying hypothesis that taurine might affect the repolarization signal.

The test was repeated again, (Figure26 *lower traces*) in control conditions and after application of 80μM taurine. Two specific hypotheses can be predicted for this signal with taurine. 1) Taurine either causes more K^+ channels to open at a given voltage, or prolongs their respective open times. This would limit the range to which the cell can depolarize, as more open K^+ channels will limit electrical propagation. 2) The signal should more rapidly decay, as the larger K^+ current will repolarize the cell more quickly following a depolarization. Each idea was experimentally determined to be correct. The control signal (*black trace*) is larger in amplitude, and decays more slowly when compared with that following taurine application (red trace). If taurine is acting upon these receptors in a light or

time-dependent manner in a physiological setting, than postsynaptic signal encoding will be regulated via VGKC modulation in the outer retina.

3.4.8 Evidence for a shared Taurinergic/Sertonergic site of action in Off Bipolar Cells

The results in section 3.4 describe a newly described receptor site for taurine within OFF BPCs; the activation of which leads to a PKC-driven enhancement of VGKCs. Whether or not the receptor itself is novel was undetermined, although the fact that taurine is known to act upon many different receptors, with varying affinities, leads to the initial presumption that taurine may be acting upon an already well described receptor site. Metabotropic receptor $GABA_B$ had been experimentally ruled out, although myriad receptors possibilities exist. Through a systematic deduction of the known receptors which activate PKC and cause a regulation of VGKCs, one clear choice was necessary to examine- $5HT_2$, a metabotropic serotonin receptor. The expression patterns of 5HT2 are completely unknown within the retina.

The effect of equimolar taurine and serotonin were tested in OFF BPCs. Both enhanced the outward VGKC current in a similar manner, increasing the probability of a shared metabotropic receptor. Furthermore, the specific $5HT_2$ antagonist MDL-11939 completely inhibited the taurinergic enhancement at a very specific concentration (500picoM). Taken together, these results strongly suggest that the taurine-induced enhancement of VGKCs in OFF bipolar cells originates at a non-specific metabotrophic receptor also sensitive to serotonin in the CNS. Serotonin may not work on this receptor in the OPL, at least in salamander (see discussion).

3.4.9 Possible network and intracellular model for taurine's regulation of VGKCs in OFF bipolar cells

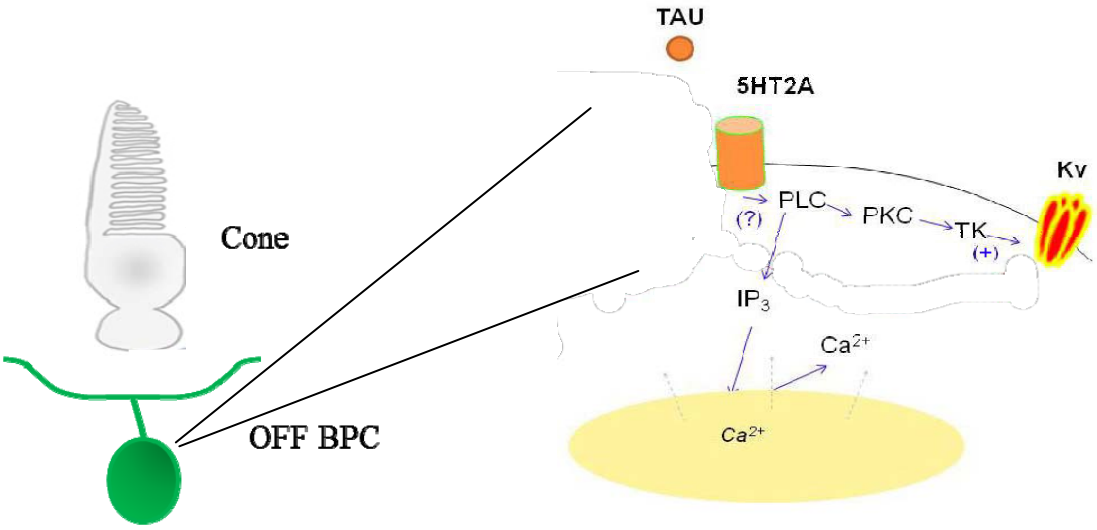


Figure 27. Model of Taurine action in the OPL

Chapter 4- Discussion

4.1 EAAT Roles in Cone Photoreceptors of the Retina

4.1.1 Possible function of the presynaptic glutamate transporter at cone synapses

Photoreceptors are unique in that they are able to transiently release glutamate in response to changing levels of light and dark. The rapid burst of transmitter release from cones at light offset has been reported as due to a buildup of vesicles on the synaptic ribbon in light. These stored vesicles undergo a rapid exocytosis at light offset (Jackson et al., 2009), causing the characteristic "off-overshoot" signal observed from cone dominant On- and Off-bipolar cells as well as from ganglion cells. Our study demonstrates that EAAT2 is involved in encoding these light offset signals in the distal retina. We find that EAAT2 inhibition enhances the amplitude of cone-mediated transient responses in bipolar cells. However, it is noteworthy that DHKA has no direct influence on Ca^{2+} channel currents or exocytosis-associated capacitance changes in cones, indicating that the signal increase in bipolar cells following sEAAT2A inhibition is due to an increase of glutamate input in bipolar cells, without a shift in vesicular output from cones. We postulate that inhibition of sEAAT2A causes excessive glutamate spillover, thereby activating more AMPA receptors in bipolar dendrites after rapid release from cones. Rapid reuptake of glutamate in cones at light offset appears necessary for cone vision to retain its high sensitivity to changing levels of illumination. Our results show that presynaptic sEAAT2A strongly reduces cone signal transmission to bipolar cells.

Fast reuptake of glutamate by presynaptic sEAAT2A may be critical in preventing glutamate depletion within cone terminals. Since glutamate is continuously released in darkness, transmitter reuptake into cone terminals would serve to prevent synaptic depression in photoreceptors and prevent over activation of post-synaptic receptors. Previous studies indicate that DHKA-sensitive transporters balance glutamate levels internally and externally in cones, and therefore help retain a steady tonic glutamate synapse in dark (Picaud et al., 1995; Gaal et al., 1998). Our results agree with these previous studies that DHKA-sensitive transporters are responsible for maintaining tonic glutamate levels in the OPL in dark; we suggest that EAAT2A activity is most important with respect to limiting excess glutamate buildup within the synaptic cleft.

4.1.2 Different types of EAATs control glutamate uptake in rod and cone terminals

It is generally accepted that EAAT uptake is tightly coupled with the associated glutamate transporter current, including the large, thermodynamically uncoupled anion current (Arriza et al., 1997; Otis & Jahr, 1998; Palmer et al., 2003). This Cl^- conductance is variable among EAAT subtypes; for example, sEAAT5 has a larger Cl^- conductance compared to sEAAT2. Previous studies have revealed that sEAAT5 is also present in photoreceptors (Eliasof et al., 1998), suggesting that glutamate uptake in salamander cones is performed by both sEAAT5 and sEAAT2A subtypes. As DHKA specifically blocks sEAAT2A, we could pharmacologically separate the currents of these two subtypes. We report that sEAAT2A mediates ~37% of the total glutamate transporter current. Clearly, sEAAT2A does not elicit the most significant anion current. Although sEAAT2A is less permeable to Cl^-, its contribution to glutamate clearance is highly significant, suggesting that despite its smaller Cl^- conductance, the kinetics and binding affinity of EAATs are

independent of the magnitude of their uncoupled Cl^- conductance, as previously observed (Amara 1998). Our finding also lends credence to the idea that different EAAT subtypes may play alternative roles in modulating synaptic activity.

Our study indicates that glutamate transporter currents are not blocked by DHKA in rods, suggesting that glutamate uptake in salamander rods is mediated by EAATs other than sEAAT2. Therefore, sEAAT2A appears to be cone-specific, with respect to photoreceptor signaling. To understand why different subtypes of EAATs engage in glutamate uptake in rod and cone synapses, the functional differences between these two distinct types of photoreceptors should be considered. Cones have a significantly faster releasable pool of vesicles (Cadetti et al., 2008). We suggest that EAAT2A allows the cone terminal to provide a more efficient mechanism with which to buffer glutamate output, thereby suppressing over activation of the post-synaptic cells and increasing contrast sensitivity in the cone system.

Although a previous immunocytochemical study indicates that sEAAT2A might also be present in Müller cells (Eliasof et al., 1998b), DHKA does not inhibit Müller cells (Barbour et al., 1991). Moreover, the processes of salamander Müller cells do not enter the invaginations of the cone terminal (Lasansky, 1973). Therefore, it appears that EAAT2A in Müller cells does not participate in DHKA-sensitive regulation of cone to bipolar cell synapses, especially after transient release. We agree with the perception from previous literature (Gaal et al., 1998; Furness et al., 2008) that glutamate uptake by Müller and glial cells is most likely a slow and inefficient process in regulating synaptic transmission. Vandenbranden et al. (2000) has previously reported EAAT2 localization in far from the synaptic ribbon in goldfish (*Carassius auratus*) cones. We must note that in their study, tissues were fixed after a 3 hour period of light adaptation. The localization of EAAT2 may

be in closer proximity to synaptic ribbons in cones of the dark-adapted retina, as is implied in our study.

4.1.4 Outcome of light-intensity driven regulation of EAAT2 in the OPL synapse

As I have shown, EAAT2 activity in dark adapted conditions can play a vital role in shaping synaptic gain control at the first visual synapse between cones and 2nd order OFF BPCs, particularly concerning phasic release from cones. As the mechanisms by which EAAT2 contributes to tonic signals in post-synaptic cells were not studied, it is currently not possible to hypothesize how this light modulation might affect these signals (single or low count quantal events particularly). The fact that the efficiency of this transporter might be modulated dependent upon the intensity of background light in highly significant in terms of contrast sensitivity, i.e., how animals might perceive dark signals imposed on different background intensities. More intense light signals are to be less filtered from cones via EAAT2, reducing the dynamic range of the signal.

The hypothesized mechanism for light intensity-dependent inhibition of EAAT2 raises more interesting questions. It appears that protons are necessary for the efficient binding of glutamate to EAATs after release from the synapse, as has already been observed in bipolar cell terminals (Palmer et al. 2003). Protons are already known modulators of Ca^{2+} channels within the cone synapse (Devries 2000), and known to be delivered from the pre-synaptic cone and postsynaptic horizontal cells, when depolarized (Cadetti and Thoreson 2006). The proposed mechanism for EAAT2 modulation via protons does not discriminate between pre- or postsynaptic areas, but it is likely not possible that the pH shift was specific to the cone recorded, as cones were held constantly at -70mV. Therefore, little vesicular release would occur, and less protons would be dumped into the synaptic cleft. Surrounding

photoreceptors, as I have shown, most certainly do chemically effect a center cone. It is therefore feasible that, over the 4 second light stimuli that a shutdown of proton buildup via surrounding photoreceptor hyperpolarization causes the mechanism to occur. Another possibility, or a concurrent event, may be that proton feedback from horizontal cells is ceased during full field stimulation. Future experiments with more specific light stimuli, i.e., true center/surround light experiments should be performed to learn more concerning this mechanism.

4.1.5 The postsynaptic transporter might be less important in control of cone synaptic gain

Molecular cloning studies have shown that EAAT2 exists in the salamander retina in two isoforms, sEAAT2A and sEAAT2B (Eliasof et al., 1998a), that are located at different pre- and post-synaptic sites in the OPL. Our results from axon-truncated bipolar cells suggest that post-synaptic sEAAT2B uptake in the OPL plays a less significant role compared with its pre-synaptic counterpart in cones. In addition, Figure $4A_3$ and $4B_3$ depict statistical differences of glutamatergic currents in cone-dominant and rod-dominant Off-bipolar cells in the presence of DHKA. Since salamander rods appear to lack sEAAT2A, inhibition of sEAAT2A via DHKA causes more glutamate accumulation in the synaptic clefts of cone-to-Off-bipolar cells than in rod-to-Off bipolar cells. The small glutamatergic currents in rod-dominated Off-bipolar cells could be due either to the blockage of sEAAT2B by DHKA in the Off-bipolar cells dendrites, or to a lower glutamate receptor density in the dendrites of rod -dominated Off-bipolar cells. Since sEAAT2B elicits a smaller uptake-mediated Cl^- conductance, we speculate that glutamate uptake via sEAA2B in Off-bipolar cell dendrites might cause a shift in membrane potential toward E_{Cl}. Although the dendritic EAAT2B current is small, sEAAT2B could theoretically act to counter the effect of

glutamate receptors, as the E_{Cl} is likely to be negative to the glutamate reversal potential in Off-bipolar dendrites.

In further agreement with previous studies, our results also denote sEAAT2B as an excellent marker for Off-bipolar cells in the salamander retina. Immunocytochemical results show that sEAAT2B labels the entire Off-bipolar cell, including dendrites and axon terminals (Fig. 2). Although a strong antibody labeling of sEAAT2B is observed by immunohistochemistry, our electrophysiological results indicate that the transporter has a smaller overall Cl^- conductance (Fig. 3) with respect to sEAAT2A in cones. Indeed, a previous study (Eliasof et al., 1998b) reported that no significant differences were observed between the overall conductances of sEAAT2A and sEAAT2B, which raises the possibility that most labeling in Off-bipolar cells represent an inactive form of sEAAT2B in the cytosol, rather than the active form expressed in the plasma membrane of cells.

Although sEAAT2B is the only neuronal-specific transporter found in salamander Off-bipolar cells, the function of sEAAT2B has yet to be determined. Nevertheless, there is a strong possibility that sEAAT2B acts as a presynaptic transporter in bipolar cell terminals, in much the same way that the sEAAT2A isoform acts presynaptically in cone terminals. Indeed, transient EAAT currents can be elicited from Off-bipolar cells following fast depolarizations (our unpublished observation). Therefore, sEAAT2B in Off-bipolar cell terminals may control glutamate uptake in the synapses of bipolar cells and third-order neurons. Since EAAT2 has been found as the sole glutamate transporter expressed in cone-linked Off-bipolar cells in mouse retinas (Hana & Calkins, 2007), these and future results obtained from salamander retina should have general importance for higher vertebrates. Furthermore, since EAAT2 is a major transporter responsible for glutamate uptake in the

CNS, using amphibian retina allows us to pursue the possible function of EAAT2 in pre- and postsynaptic locales. Thus, our findings on the roles of pre- and post-synaptic EAATs in the distal retina may provide a better understanding of the function of EAATs throughout the CNS where they may be of general importance in the control of fast synaptic signaling, as well as in synaptic facilitation and plasticity.

4.2 Consequences of the Distinct Roles and Localizations of EAAT2 and EAAT5 within cones

4.2.1 EAAT2 and EAAT5 are physically isolated and serve different roles in the same cell

Previously, it has been postulated (Eliasof 1998a,b) that EAAT2 and EAAT5 may be physically near to each other within the cone terminal, possibly even forming heterodimers. If true, each transporter subtype should be activated in a nearly simultaneous manner following natural glutamate release, and would be reflected in pharmacological studies using DHKA vs. TBOA. Interestingly, I propose that these two subtypes are isolated, and therefore play different roles as well.

EAAT2 has the highest binding affinity for glutamate, so functionally speaking it would serve well to localize this transporter to an area with a high concentration of release and/or with fast kinetics- namely, the areas surrounding cone ribbons. EAAT2 was largely activated following short depolarizations (2ms), and likely reached a peak during this time. In addition, the decay time for EAAT2 activity following this rapid exocytotic event was short, around 200ms. This time factor allows for the approximation of glutamate diffusion within the presynaptic active site. When I compare EAAT2 decay times of low glutamate release vs. high release, there is very little change in the EAAT decay time course, meaning

that EAAT2 is almost certainly found in high concentrations near the release site. Pharmacology has shown that nearly all the EAAT current activated following this rapid release from cones is EAAT2 specific, as DHKA inhibits the current. The role of the EAAT5 subtype does not appear to come into play under these circumstances.

EAAT5 is retina-specific, found in cone and rod terminals, as well as within rod bipolar cell terminals (Hasegawa et al. 2006;Wesinger et al. 2006). Its most distinct feature is a very large Cl^- conductance, with respect to EAAT2 (Eliasof et al. 1998a). Therefore its activation should theoretically be modulatory, dependent on the $E_{(Cl)}$ near the transporter. Curiously, very little EAAT5 current is activated in the cone terminal following rapid glutamate release. The only non-DHKA specific currents seen were spontaneous, apparently unitary-type EAAT currents. These EAAT currents were only inhibited with TBOA, making them pharmacologically distinct from EAAT2 currents activated with a depolarization. These occurred at a very low holding potential, -70mV, and therefore were unlikely involved with normal tonic release from the recorded cone. Through a number of subsequent experiments, it was confirmed that these EAAT5 currents were induced from surrounding photoreceptors, as recordings were performed in dark conditions. They represent unitary release from surrounding cones, and possibly involve rod signals as well, which must be more fully explored. The kinetics of these unitary EAAT5 events are interesting, with a sharp transient following by a sustained inward current before tapering off about 1 second after the initiation of the response. This indicates a significantly longer activation period with respect to EAAT2 activation following depolarization. One strong hypothesis is that EAAT5 is spread over a larger area within the basal or perisynaptic zone of cones. Another possibility may be a basal or ectopic release mechanism, different from that near the ribbon, may be responsible for the distinct kinetics. Regardless of the exact mechanism, EAAT5 is

available for chemical crosstalk between cones, and may be inhibitory or excitatory dependent on local Cl^-, which is not well understood, especially with respect to how it varies in different conditions of light and dark. This finding opens up a new paradigm of modulation within the 1st signal encoding neurons in the retina.

4.3 Implications of the specific Taurinergic and Glycinergic Regulation of BPCs

4.3.1 Taurine and Glycinergic Inputs to the distal BPCs processes

The finding that both Glycine and Taurine have modulatory effects through metabotrophic receptors in bipolar cells is interesting, but may not be relevant unless the requisite inputs for these neurotransmitters are available. Luckily, work on the possible inputs within the distal retina has already been undertaken. Glycine is delivered to the outer retina via interplexiform cells (Jiang and Shen 2010), which project an axon from a soma within the inner retina. Taurine is also found in high concentrations within cones, although it is unclear exactly how or when it is released within photoreceptors. Therefore inputs to sites within bipolar cell dendrites are highly likely.

4.3.2 Metabotrophic Taurine and Glycine and the modulation of signals of the outer retina

One of the most unexpected findings of the work on metabotrophic taurine and glycine effects was that each had a cell specific response, as well as a completely distinct modulatory effect of VGKCs. Earlier work has shown (Bulley and Shen 2010) that taurine and glycine both activate the described ionotropic glycine receptor within retinal third order neurons. Importantly, it appears that taurine is less specific for the site, as much greater concentrations are necessary to a achieve a similar ionotropic current It was expected that

taurine might also activate the proposed glycine metabotrophic site as well, perhaps in a non-specific manner similar to that of the ionotropic glycine receptor.

This study confirms the metabotrophic effect of glycine within the retina, as first proposed by Hou et al. 2008. Their study was concerned with the downstream effects at voltage gated Ca^{2+} channels; whist this study independently confirms a response at VGKCs. The response was most strongly observed in ON bipolar cells, in which glycine inhibited the sustained VGKC. The response was also largely eliminated by inhibiting PKA activity within these cells, in further agreement with the aforementioned study. Taurine also had a similar effect within ON Bipolar cells, but with significantly less VGKC inhibition at the same (80M) concentration. This finding suggests that taurine may non-specifically bind to the proposed glycinergic metabotrophic site, but with less affinity. Until a site specific antagonist is found for this receptor, and until it is elucidated as an already described or novel site, it is impossible to determine if these molecules act upon a similar receptor. Nonetheless, the glycinergic metabotrophic site now has at least two independent confirmations.

A novel metabotrophic site was found for taurine within OFF bipolar cells, and a complete distinction was made between it and earlier described metabotrophic GABA and glycine sites. Taurine led to the downstream activation of VGKCs, and appears to act through a PKC-dependent pathway within these cells. This activity is completely different then that found within ON bipolar cells, which apparently lack the receptor site for this PKC-dependent effect. (This hypothesis has now been further confirmed through the collaborative work with S. Bulley. Blocking PKA will normally increase the taurine enhancement in OFF bipolar cells; but in ON bipolar cells with PKA blocked, no taurinergic enhancement was

observed. Therefore it is unlikely that the PKC dependent, taurinergic receptor found within OFF bipolar cells is present in ON bipolar cells).

Further evidence for a novel metabotrophic site of taurine is seen is with new pharmacological evidence. The VGKC-enhancing effect of taurine in OFF bipolar cells was seen to be eliminated at very low concentrations of specific 5-HT_2 antagonist. In further agreement, serotonin and taurine have the same effect on VGKCs (at the same concentration) in OFF bipolar cells. Therefore it appears that taurine and serotonin share a metabotrophic receptor site, an interesting finding for more than just visual research. Serotonin is well known to have long term modulatory effect throughout the CNS, and taurine is found in relatively high concentrations throughout as well. It is reasonable to suggest that taurine has a more wide spread effect, similar to that of serotonin. As far as retina-specific consequences, I have described a mechanism by which taurine may in attenuate rapid signals in OFF BPCs. The enhancement of VGKCs, especially sustained outward currents, will affect the ability of the cell to repolarize following large EPSCs. This effect was observed in OFF BPCs, as taurine application attenuated a depolarizing response. This modulation would allow for an increased signal encoding rate, which is also limited by the degree of presynaptic release.

References

1. Arriza JL, Eliasof S, Kavanaugh MP & Amara SG (1997). Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci U SA* **94**, 4155-4160.

2. Barbour B, Brew H & Attwell D (1991). Electrogenic uptake of glutamate and aspartate into glial cells isolated from the salamander (Ambystoma) retina. *J Physiol **436***, 169-193.

3. Brew H & Attwell D (1987). Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. *Nature* **327**, 707-709.

4. Bulley, S., and Shen, W. (2010). Reciprocal regulation between taurine and glutamate response via Ca2+-dependent pathways in retinal third-order neurons. J. Biomed. Sci ***17*** *Suppl 1*, S5.

5. Cadetti L, Bartoletti TM & Thoreson WB (2008). Quantal mEPSCs and residual glutamate: how horizontal cell responses are shaped at the photoreceptor ribbon synapse. *Eur J Neurosci* **27**, 2575-2586.

6. Cadetti L, Tranchina D & Thoreson WB (2005). A comparison of release kinetics and glutamate receptor properties in shaping rod-cone differences in EPSC kinetics in the salamander retina. *J Physiol* **569**

7. Danbolt NC (2001). Glutamate uptake. *Prog Neurobiol* **65**, 1-105.

8. Werblin, F.S. & Dowling, J.E. Organization of the retina of the mudpuppy, Necturus maculosus. II. Intracellular recording. *J. Neurophysiol* **32**, 339-355 (1969).

9. Eliasof S & Werblin F (1993). Characterization of the glutamate transporter in retinal cones of the tiger salamander. *J Neurosci* **13**, 402-41.

10. Eliasof S, Arriza JL, Leighton BH, Kavanaugh MP & Amara SG (1998a) Excitatory amino acid transporters of the salamander retina: identification, localization, and function. *J Neurosci* **18**, 698-712.

11. Eliasof S, Arriza JL, Leighton BH, Amara SG & Kavanaugh MP (1998b). Localization and function of five glutamate transporters cloned from the salamander retina. *Vision Res* **38**, 1443-1454.

12. Fatechand R (1978). Suppression of cone signal in the dark-adapted frog retina as indicated by the electroretinogram. *Rev Can Biol* **37**, 101-113.

13. Frumkes TE, Lange G, Denny N & Beczkowska I (1992). Influence of rod adaptation upon cone responses to light offset in humans: I. Results in normal observers. *Vis Neurosci* **8**, 83-89.

14. Frumkes TE & Wu SM (1990). Independent influences of rod adaptation on cone-mediated responses to light onset and offset in distal retinal neurons. *J Neurophysiol* **64**, 1043-1054.

15. Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Gaal L, Roska B, Picaud SA, Wu SM, Marc R & Werblin FS (1998). Postsynaptic response kinetics are controlled by a glutamate transporter at cone photoreceptors. *J Neurophysiol* **79**,190-196.

16. Grant GB & Werblin FS (1996). A glutamate-elicited chloride current with transporter-like properties in rod photoreceptors of the tiger salamander. *Vis Neurosci* **13**, 135-144.

17. Hanna MC & Calkins DJ (2007). Expression of genes encoding glutamate receptors and transporters in rod and cone bipolar cells of the primate retina determined by single-cell polymerase chain reaction. *Mol Vis* **13**, 2194-2208.

18. Hasegawa J, Obara T, Tanaka K & Tachibana M (2006). High-density presynaptic transporters are required for glutamate removal from the first visual synapse. *Neuron* **50**, 63-74

19. Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D & Danbolt NC (2008). A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). *Neuroscience* **157**, 80-94.

20. Hou, M., Duan, L., and Slaughter, M. M. (2008). Synaptic inhibition by glycine acting at a metabotropic receptor in tiger salamander retina. The Journal of Physiology *586*, 2913 - 2926.

21. Hille B (2001) *Ion channels of excitable membranes*. Massachusetts: Sinauer

22. Innocenti B & Heidelberger R (2008). Mechanisms contributing to tonic release at the cone photoreceptor ribbon synapse. *J. Neurophysiol* **99**, 25-36 2008.

23. Jiang, Z., and Shen, W. (2010). Role of Neurotransmitter Receptors in Mediating Light-Evoked Responses in Retinal Interplexiform Cells. Journal of Neurophysiology ***103***, 924 - 933.

24. Jackman, S. L., Choi, S.-Y., Thoreson, W. B., Rabl, K., Bartoletti, T. M., and Kramer, R. H. (2009). Role of the synaptic ribbon in transmitting the cone light response. Nat. Neurosci *12*, 303-310.

25. Kandel ER, Schwartz, JH (1982) Molecular biology of learning: modulation of transmitter release. *Science.* 218: 433-443.

26. Kaczmarek LK, Levitan IB (1987) Neuromodulation: *The biochemical control of neuronal excitability.* New York: Oxford University Press.

27. Kuriyama K, Ida S, Nishimura C, Ohkuma S (1982) Distribution and function of taurine in nervous tissues: an introductory review. *Prog Clin Biol Res*. **125**: 127-140.

28. Jackman SL, Choi SY, Thoreson WB, Rabl K, Bartoletti TM, & Kramer RM (2009). Role of the synaptic ribbon in transmitting the cone light response. *Nat. Neurosci* **12**, 303-310.

29. Lasansky A (1973) Organization of the outer synaptic layer in the retina of the larval tiger salamander. Phil. Trans. Royal Soc. Biol. Science **265**. 472-789

30. Levy LM, Warr O & Attwell D (1998). Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na+-dependent glutamate uptake. *J Neurosci* **18**, 9620-9628.

31. Lombardini JB (1991) Taurine: retinal function. *Brain Res.* **16**, 151-169.

32. Maple BR, Zhang J, Pang JJ, Gao F & Wu SM (2004). Characterization of displaced bipolar cells in the tiger salamander retina. *Vision Res* **45**, 697-705.

33. Militante JD, Lombardini JB (2002) Taurine: evidence of physiological function in the retina. *Nutr Neurosci.* **5**, 75-90

34. Muramatsu M, Kakita K, Nakagawa K, Kuriyama K (1978) A modulating role oftaurine on release of acetylcholine and norepinephrine from neuronal tissues. *Jpn J Pharmacol*. **28**, 259-268.

35. Otis TS & Jahr CE (1998). Anion currents and predicted glutamate flux through a neuronal glutamate transporter. *J Neurosci* **18**, 7099-7110.

36. Palmer MJ, Taschenberger H, Hull C, Tremere L & von Gersdoff H (2003). Synaptic activation of presynaptic glutamate transporter currents in nerve terminals. *J Neurosci* **23**, 4831-4841.

37. Pang JJ, Gao F & Wu SM (2004). Stratum-by-stratum projection of light response attributes by retinal bipolar cells of *Ambystoma. J Physiol* **558**, 249-262.

38. Picaud SA, Larsson HP, Grant GB, Lecar H & Werblin FS (1995). Glutamate-gated chloride channel with glutamate-transporter-like properties in cone photoreceptors of the tiger salamander. *J Neurophysiol* **74**, 1760-1771.

39. Rabl, K., Cadetti, L. & Thoreson, WB (2005). Kinetics of Exocytosis Is Faster in Cones Than in Rods. *J. Neurosci* **25**, 4633-4640.

40. Rauen, T., and Kanner, B. I. (1994). Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. Neuroscience Letters *169*, 137-140.

41. Rauen T, Wiessner M, Sullivan R, Lee A & Pow DV (2004). A new GLT1 splice variant: cloning and immunolocalization of GLT1c in the mammalian retina and brain. *Neurochem Int* **45**, 1095-1106, 2004.

42. Schnapf JL & Copenhagen DR (1982). Differences in the kinetics of rod and cone synaptic transmission. *Nature* **296**, 862-864.

43. Sturman JA (1988) Taurine in development. *J Nutr.* **118**: 1169-1176.

44. Thoreson WB, Rabl K, Townes-Anderson E & Heidelberger R (2004). A highly Ca^{2+}-sensitive pool of vesicles contributes to linearity at the rod photoreceptor ribbon synapse. *Neuron* **42**, 595-60.

45. Veruki ML, Morkve SH & Hartveit E (2003). Functional properties of spontaneous EPSCs and non-NMDA receptors in rod amacrine (AII) cells in the rat retina. *J Physiol* **549**, 759-774.

46. Vandenbranden, CA, Yazulla, S., Studholme, KM., Kamphuis, W & Kamermans M (2000). Immunocytochemical localization of the glutamate transporter GLT-1 in goldfish (*Carassius auratus*) retina. *J. Comp. Neurol* **423**, 440-451, 2000.

47. Wadiche, J. I., Tzingounis, A. V., and Jahr, C. E. (2006). Intrinsic kinetics determine the time course of neuronal synaptic transporter currents. Proc. Natl. Acad. Sci. U.S.A ***103***, 1083-1087.

48. Wu SM (1988). The off-overshoot responses of photoreceptors and horizontal cells in the light adapted retinas of the tiger salamander. *Exp Eye Res* **47**, 261-268.

49. Wong KY, Cohen ED & Dowling JE (2005). Retinal bipolar cell input mechanisms in giant danio. II. Patch-clamp analysis of on bipolar cells. *J Neurophysiol* **93**, 94-107.

50. Wersinger E, Schwab Y, Sahel JA, Rendon A, Pow DV, Picaud S & Roux MJ (2006). The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. *J Physiol* **577**, 221-234.

51. Yang, J & Wu, SM (1997). Characterization of Glutamate Transporter Function in the Tiger Salamander Retina. *Vision Research* **37**, 827-838.