Effect of the Retinal Circadian Clock on Expression of GABA_\textsubscript{A} Receptors and Chloride Co-Transporters by Goldfish Cone Synaptic Terminals

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ABSTRACT

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) on cone synaptic terminals mediate GABA feedback from horizontal cells to cones and KCC2 expression determines the effect of GABA<sub>A</sub>R activation. Since GABA<sub>A</sub>R expression is dependent on intracellular cAMP and PKA, second messengers which are regulated by the retinal circadian clock, it seems reasonable to postulate that the circadian clock (24-hour) will increase GABA<sub>A</sub>R and KCC2 expression on cone synaptic terminals at night when intracellular cAMP and PKA are elevated. To elucidate the effect of the circadian clock on GABA-mediated feedback from cone horizontal cells to cones, immunohistochemistry was used to visualize the expression of GABA<sub>A</sub>R and KCC2 in goldfish retinas collected at night after dark adaptation and in the day following dark and light adaptation. The results support the hypothesis that the circadian clock increases the expression of GABA<sub>A</sub>Rs and KCC2 on cone terminals at night.

INTRODUCTION

The neurons in the retina that are primarily responsible for light detection are rod and cone photoreceptors. Rods mediate dim light detection in the dark at night (scotopic vision) while cones mediate bright light detection in the day (photopic vision) (Dowling, 2012). They are both active in intermediate light conditions (mesopic vision) that occur at dawn and at dusk (Heikkinen et al., 2011). The synaptic processes and neural pathways that underlie the transition from rod to cone vision and back again as ambient light levels increase at dawn and decrease at dusk are largely unknown.

Photoreceptors communicate with each other through electrical synapses and with other neurons through chemical synapses. Electrical synapses involve physical connections called gap junctions between the membranes of the two electrically coupled neurons. Chemical synapses involve the release of chemical agents called neurotransmitters from pre-synaptic cells into the synaptic cleft where the neurotransmitters interact with their corresponding receptors on post-synaptic cells. Cones form
chemical synapses with second order neurons such as horizontal cells (HCs) (Yazulla, 1986; Dowling, 2012). Since horizontal cells inhibit cones and release gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the retina, it logically follows that GABA receptors might be present on cone photoreceptors (Yazulla et al., 1997). However, most efforts to detect GABA receptors on cones have been unsuccessful. However, recent findings have suggested that GABA-mediated feedback to cones from horizontal cells may play a role in the transition between rod and cone vision that occurs at dawn and at dusk (Choi et al., 2012).

Figure 1. Chloride Co-transporters determine polarization of a neuron's response to \( \text{GABA}_A \) activation

\[ \text{NKCC} \] increases the intracellular concentration of chloride (\( \text{Cl}^- \)) ions, so there is an outflow of chloride ions when \( \text{GABA}_A \) Rs are activated by GABA; the outflow of negative \( \text{Cl}^- \) ions causes a positive change in the cell membrane potential (voltage) called a depolarization. In contrast to NKCC, KCC2 decreases the intracellular concentration of chloride ions, so there is an inflow of chloride ions when \( \text{GABA}_A \) Rs are activated by GABA; the inflow causes a negative change in the cell membrane potential called a hyperpolarization. In sum, the effect of \( \text{GABA}_A \) \( \text{R} \) activation on the membrane potential depends on the presence and activity of either KCC2 or NKCC. Modified from Thoreson and Mangel (2012).
One class of GABA receptor that is expressed in the outer plexiform layer, which is the synaptic layer containing photoreceptor terminals and processes from horizontal cells and bipolar cells, another type of second order neuron, is the Type A GABA receptor (GABA\textsubscript{A}R) (Yazulla et al. 1997). GABA\textsubscript{A}Rs are ligand-gated chloride channels; that is, activation of GABA\textsubscript{A}Rs by GABA opens chloride channels, increasing the flow of chloride ions either in or out of the cell (Fig. 1) (Wright et al., 2011). The direction of chloride ion flow (in or out) after GABA\textsubscript{A}R activation is determined by the intracellular concentration of chloride ions, which is primarily regulated by two chloride transporting proteins called the Na-K-2Cl (NKCC) and K-Cl (KCC2) co-transporters (Vardi et al., 2000). NKCC increases the intracellular concentration of chloride ions while KCC2 decreases it (Fig. 1). When the intracellular concentration of chloride ions is decreased by KCC2 activity so that the chloride reversal potential (the voltage at which the net driving force on Cl\textsuperscript{-} is zero) is more negative than the resting membrane potential, activation of GABA\textsubscript{A}Rs leads to the inflow of chloride ions and therefore to a negative change in voltage called a hyperpolarization (Vithlani et al., 2011; Choi et al. 2012). When the intracellular concentration of chloride ions is increased by NKCC activity so that the chloride reversal potential is more positive than the resting membrane potential, activation of GABA\textsubscript{A}Rs leads to the outflow of chloride ions and therefore to a positive change in voltage called a depolarization (Wright et al., 2011).

The expression and activity of GABA\textsubscript{A} receptors increase when intracellular levels of cAMP and protein kinase A (PKA) increase but the dependence of KCC2 and NKCC expression and activity on cAMP/PKA in the nervous system have not been studied (Jacob et al., 2008). The intracellular levels of cAMP and PKA are in turn dependent on whether dopamine D\textsubscript{1} or D\textsubscript{2} receptors are activated. Activation of D\textsubscript{1} receptors by dopamine increases intracellular cAMP and PKA, but D\textsubscript{2} receptor activation decreases cAMP and PKA (Ribelayga et al., 2002). In addition, D\textsubscript{2} receptors respond to low (nanomolar) levels of dopamine, but D\textsubscript{1} receptors require approximately 500 times higher levels of
dopamine for activation. Recent work has shown that the circadian (24-hr) clock in the retina increases dopamine levels enough during the day to activate the D$_2$ receptors on rods and cones, but not enough to activate D$_1$ receptors on the dendrites of bipolar cells and horizontal cells, and that bright light stimulation during the day increases dopamine levels enough to activate the D$_1$ receptors on bipolar cell and horizontal cell dendrites (Fig. 2) (Ribelayga et al., 2010).

The intracellular concentration of cAMP has also been linked to the coupling of the gap junctions between rods and cones (Ribelayga et al., 2002, 2008). It seems that an increase in cAMP leads to an increase in rod-cone coupling. Rod-cone coupling allows cones to transmit rod-mediated signals to second order neurons. Since cAMP levels in cones are higher in the dark at night, it means coupling is stronger at night in the dark, when rods are most active (Fig. 2). When the retina is exposed to mesopic light levels at dawn, the decrease in cAMP leads to the decoupling of rod-cone gap junctions (Fig. 2). The decoupling leads to an increase in cone-driven signals. This increase in cone-driven responses occurs slowly over a few minutes (Heikkinen et al., 2011). It is possible that the slowness results from the slow decrease in cAMP, which also leads to a decrease in the expression and activity of GABA$_A$Rs, so that cone responses to mesopic light stimuli increase in size. The reduction in rod-cone coupling as well as the reduction in the expression GABA$_A$R and KCC2 as mesopic light stimuli increase, which is typically at dawn, are ways that the circadian clock might enhance cone-driven signals as day approaches (Choi et al., 2012). Conversely, the circadian clock might enhance rod-mediated signals through cone circuitry as mesopic light stimuli decrease as night approaches by increasing rod-cone coupling and increasing the expression of GABA$_A$R and KCC2 on cone terminals.
Figure 2. Day-night difference in GABA\textsubscript{A}R expression in the OPL due to cAMP/PKA Activity

Dendrites of cone bipolar cells express dopamine D\textsubscript{1}Rs, unlike cone terminals which express dopamine D\textsubscript{2}Rs. D\textsubscript{1}Rs increase intracellular cAMP/PKA activity in the day following prolonged bright light exposure and D\textsubscript{2}Rs decrease intracellular cAMP/PKA activity in the day due to the activity of the retinal circadian clock. Since GABA\textsubscript{A}R expression is high when cAMP/PKA activity is high, GABA\textsubscript{A}Rs are expressed on cone bipolar cell dendrites in the day and on cone terminals at night when D\textsubscript{2}Rs are not activated. Rod-cone coupling is also dependent on cAMP/PKA activity in cones so it is strongly increased at night when cAMP/PKA activity is high in cones (i.e., D\textsubscript{2}Rs are not activated) and much reduced in the day when cAMP/PKA activity is low in cones (i.e., D\textsubscript{2}Rs are activated) (Mangel, unpublished observations).
I conducted an immunohistochemical study of KCC2 to test the hypothesis that the circadian clock increases the expression of GABA$_A$Rs and KCC2 on cone photoreceptor terminals at night and decreases the expression of GABA$_A$Rs and KCC2 on cone photoreceptor terminals in the day. The relative differences in the expressions of GABA$_A$Rs and KCC2 on cone terminals were investigated in dark-adapted goldfish retinas collected at night (which were exposed to prolonged darkness) as well as in dark and light-adapted (exposed to prolonged brightness) retinas collected in the daytime. I posited that the circadian clock increases the expression of GABA$_A$Rs and KCC2 as night approaches and decreases it as day approaches.

**MATERIALS AND METHODS**

**Preparation**

Goldfish (*Carassius auratus*), ~ 6 inches long, were maintained on a 12 hr light/ 12 hr dark cycle at constant temperature (20 °C). The fish were either dark-adapted (placed in complete darkness) or light-adapted (placed under bright light) for 1 hour in the subjective day but they were only dark-adapted in the subjective night. The fish were deeply anesthetized with tricaine methanesulfonate (Sigma) before decapitation and enucleation of the eyes. The excised eyes were hemisected and the posterior sections were immersed in hyaluronidase saline solution for 20 minutes to facilitate the removal of the vitreous humor. The tissues were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde solution for 1 hour. Then, the fixed tissues were immersed sequentially in 10%, 20% and 30% sucrose solutions until they sank. Afterward, the tissues were embedded in Optimal Cutting Temperature Compound (Sakura Finetek USA) and frozen. The frozen tissue blocks were sectioned (12 - 18 um) on a cryostat and the sections were collected on glass microscope slides. The slides were stored in a -20°C freezer until use. The care and handling of the fishes were in accordance with all federal guidelines and were approved by the Ohio State Institutional Animal Care and Use Committee.
Immunohistochemistry

Cone Labeling

The sections were rinsed once with 0.1 M phosphate-buffered saline (PBS) for 10 minutes. Next, the sections were incubated in blocking buffer, which contained 3% normal donkey serum (NDS), 10 mg/ml bovine serum albumin and 0.01% Triton X-100 in 0.1 M PBS, for 1 hr at room temperature. After blocking, the sections were rinsed in 0.1 M PBS three times for 10 minutes each at room temperature. The tissue sections were then incubated with mouse zpr-1 monoclonal antibody (Millipore) in diluent buffer (1:100), which includes 0.01% Triton X-100 and 1% NDS in 0.1M PBS, overnight at 4º C followed by 3 x 10 minute rinses at room temperature. The secondary antibody, Alexa Fluor 546 Donkey anti-mouse IgG (Invitrogen) in diluent buffer at 1: 200, was administered and after 1 hr, another set of 3 x 10 minute rinses was performed at room temperature.

Cone and GABA_A R Double Labeling

After the first secondary antibody incubation and rinses, two additional blocking steps were employed to prevent cross-reactions that could result from having two primary antibodies of the same species. First, the sections were incubated 5% normal mouse serum (Jackson ImmunoResearch) in diluent buffer for 1 hr at room temperature, followed by 3 x 10 min rinses. The second blocking step included Affinipure Fab fragment goat anti-mouse IgG (Jackson ImmunoResearch) at 20 μg/ml for 1 hr at room temperature followed by 3 x 10 min rinses. Then, the sections were incubated with Anti-GABA_A Receptor B2/3 clone 62-3G1 monoclonal antibody (Millipore) at 1:50 overnight at 4º C. A set of 3 x 10 min rinses were performed followed by incubation of Alexa Fluor 488 Donkey anti-mouse IgG (Invitrogen) in diluent buffer (1: 200) for 1 hr at room temperature. The final 3 x 10 min rinses were performed and the slides were mounted and cover-slipped.
Cone and KCC2 Double Labeling

After the first secondary antibody incubation and rinses, the sections were incubated with rabbit polyclonal B22 antibody (Upstate) in diluent buffer (1: 100) overnight at 4 °C. Another set of 3 x 10 min rinses was performed followed by a 1 hr incubation with Alexa Fluor 488 Donkey anti-rabbit IgG (Invitrogen) in diluent buffer at 1: 200. Then, the sections were rinsed three more times for 30 minutes (3 x 10 min). Finally, the sections were mounted and cover-slipped.

Controls

The primary antibodies, 62-3G1 and B22, were omitted.

Image Analysis

Images were acquired using Zen 2008 Software on the Zeiss LSM 510 META, a laser confocal microscope. The images were collected with the Plan-Apochromat 63x/1.40 Oil DIC M27 objective lens with a pixel dwell time of 1.60 µs and 4x averaging. Brightness and contrast settings were adjusted to reduce noise using Image J. The intensity of a cone terminal co-localized with either GABA_A R or KCC2 was obtained through Image J. In order to account for noise and background staining, the intensity at the co-localized terminal was divided by the intensity of a background spot. Nine dark-adapted cone terminals at night from one animal, five dark-adapted cone terminals in the day from another animal and six light-adapted cone terminals in the day from a third animal were used for GABA_A R image analysis. For KCC2 image analysis, five cone terminals were examined per retina and four retinas from four different animals were collected for each of the three conditions (Day Light-Adapted, Day Dark-Adapted, Night Dark-Adapted) for a total of twelve retinas and sixty data points. Student's unpaired t-test was used to determine the significance of the comparisons.
RESULTS

**GABA\_A Rs are present to a greater extent on cone terminals at night than in the day**

GABA\_A labeling was observed in the OPL in all three conditions (Fig. 3C, G, K). However, some of the staining observed in the day in both light-adapted (state of retina during prolonged exposure to bright light) and dark-adapted conditions (state of retina during prolonged exposure to darkness) can be attributed to neurons post-synaptic to the cone terminals. There also appears to be more GABA\_A localization on post-synaptic neurons in both conditions in the day than at night (Fig. 3D, H, L). GABA\_A labeling on cone terminals in the day is minimal and appears to be confined to the upper region of the cone terminal. In contrast, GABA\_A labeling on cone terminals at night is more prevalent and is localized throughout the cone terminals (Fig. 3 D). GABA\_A localization of nine dark-adapted cone terminals at night, five dark-adapted cone terminals in the day and six light-adapted cone terminals in the day revealed that there is significantly more GABA\_A labeling on cone terminals at night than in the day; there is significantly more GABA\_A labeling at night than in the day for both light-adapted and dark-adapted retinas (Fig. 4). There were no significant differences in the localization of GABA\_A on cone terminals between the dark-adapted cones and light-adapted cones in the day (Ishii and Mangel, unpublished observations).
Figure 3. GABA\(_A\)R labeling in the goldfish OPL

GABA\(_A\)R labeling in the goldfish OPL is apparent in dark-adapted (exposed to prolonged darkness) retinas at night (C) and in dark-adapted (K) and light-adapted (exposed to prolonged bright light) (G) retina in the day. Localization of GABA\(_A\)Rs is apparent throughout the cone terminals at night in dark-adapted (exposed to darkness at night) retinas (D) but is confined to the upper area of the cone terminals in the dark-adapted condition and light-adapted condition (exposed to bright light) in the day (H). GABA\(_A\)R labeling appears stronger in post-synaptic neurons in the OPL in both light-adapted (H) and dark-adapted (L) retinas in day than at night (D). Controls are not shown (Ishii and Mangel, unpublished observations).
Figure 4. There is significantly more GABA\(_A\)R labeling on cone terminals at night than in the day

The intensity of GABA\(_A\)R labeling on cone terminals (I) is divided by the intensity of background GABA\(_A\)R labeling (I\(_0\)). The difference in GABA\(_A\)R labeling between night dark-adapted cone terminals and day light-adapted cone terminals is significant (p < 0.05) as is the difference between night dark-adapted and day light-adapted cone terminals; however, the difference in GABA\(_A\)R labeling between light-adapted and dark-adapted retinas in the day was not significant. Nine night dark-adapted cone terminals from one animal, five day dark-adapted cone terminals from another animal and six day light-adapted cone terminals from a third animal were examined (Ishii and Mangel, unpublished observations). Statistical significance was determined using Student's unpaired t-test.
**KCC2 expression on cone terminals is higher at night than in the day**

KCC2 labeling is present in the OPL at night after dark adaptation and it is also present in the day regardless of adaptation level (Fig. 5 A, B, C). Although, KCC2 labeling is present in the OPL in all three conditions, KCC2 localization on cone terminals differs between the conditions. Like GABA<sub>A</sub>R, KCC2 is expressed to a greater extent on dark-adapted cone terminals at night than both dark-adapted and light-adapted cone terminals in the day. The differences in KCC2 localization between dark adapted cone terminals at night and both light-adapted and dark-adapted cone terminals in the day are significant (p < 0.01 and p < 0.01; Fig. 7). The comparison between KCC2 localization between light-adapted and dark-adapted cone terminals in the day yielded no significant difference. KCC2 localization on second-order neurons accounts for most of the labeling observed in day time conditions but only some of the labeling observed in the dark-adapted OPL at night could be attributed to second-order neurons (Fig. 6ii A,B,C).
**Figure 5. KCC2 is present in the goldfish OPL**

KCC2 labeling is present in the goldfish OPL in all three conditions: Night dark-adapted (A), day dark-adapted (B), and day light-adapted (C). The controls show no staining (D, E, F); however, there is some insignificant noise present in the light-adapted sections (C,F). Goldfish cone terminals were labeled with zpr-1.
Figure 6. KCC2 labeling in the goldfish OPL appears post-synaptic to cone terminals in the day

KCC2 labeling in the OPL in the day appears to be confined to neurons that are post-synaptic to cones. (iH, L; iiB, C).

However, at night, KCC2 labeling is present on cone terminals and the dendritic processes post-synaptic neurons (iD, iiA).

Note: Figure 6iH is the same as figure 6iiB. Figures 6iC,G, K are the same as figures 5 A-C.
Figure 7. KCC2 labeling is significantly higher in cone terminals at night than in the day

The intensity of KCC2 labeling on cone terminals (I) was divided by the intensity of background KCC2 labeling (I₀) and shown for each of three experimental conditions. KCC2 expression was significantly higher in dark-adapted cone terminals at night than in dark-adapted cone terminal in the day (p < 0.01); there was also significantly more KCC2 expression on dark-adapted cone terminals at night than on light-adapted cone terminals in the day (p < 0.01). The difference between KCC2 expression on dark-adapted and light-adapted cone terminals in the day was not significant (n.s.). Twenty cone terminals from four animals were examined for each of the three conditions. Student's unpaired t-test was used to calculate statistical significance.
DISCUSSION

The results show that GABA<sub>A</sub>R and KCC2 labeling are both expressed in the OPL at night after dark-adaptation (i.e., prolonged exposure to darkness) and in the day after dark and light-adaptation (i.e., prolonged exposure to bright light). However, there are significant day-night differences in GABA<sub>A</sub>R and KCC2 localization on cone terminals. Both GABA<sub>A</sub>Rs and KCC2 are expressed by cone synaptic terminals following dark adaptation at night to greater extent than in the day following light or dark adaptation. In contrast, the differences in GABA<sub>A</sub>R and KCC2 expression between light-adapted and dark-adapted cones in the day are not significant (Figs. 2 and 5). The results support the hypothesis that both GABA<sub>A</sub>R and KCC2 are expressed to greater extent at night than in the day due to the action of the retinal circadian clock.

The increase in GABA<sub>A</sub>R and KCC2 expression at night suggests that the clock reduces the effectiveness of the inhibitory GABA-mediated signal from cone horizontal cells in the day; it also suggests the clock enhances the GABA-mediated signal from cone horizontal cells to cones at night (Mangel et al., 2012). KCC2 decreases the intracellular concentration of chloride ions, so that when GABA<sub>A</sub>Rs on cone terminals are activated by the release of GABA from horizontal cells at night, there is a net influx of chloride ions into the cells and an inhibitory negative shift in voltage occurs due to the negative charge of chloride ions; this is a kind of negative feedback because the signal from cones to horizontal cells causes a positive change in the membrane potential of the horizontal cells, which leads to the release of GABA onto cone terminals and a negative change in the membrane potential of cones. So, increasing the strength of horizontal cell feedback to cones at night reduces cone signals because a negative change in membrane potential reduces the level of cone signaling. By reducing GABA<sub>A</sub>R and KCC2 expression in the day, the clock enhances cone signaling because the inhibitory signaling from horizontal cells to cones is reduced (Choi et al., 2012).

Cone-mediated signals in dark-adapted retinas gradually increase in response to increasing
mesopic light stimulation, which typically occurs at dawn. The mesopic light stimulation decreases rod-cone coupling, and consequently suppresses rod input to cones and increases cone responses as a result. The growth of cone-mediated responses as mesopic light stimulation increases takes much longer (~5 min) than would be expected if it were due to voltage-evoked changes in gap-junctional conductances (< 1 sec), which could occur when mesopic light hyperpolarizes rods, suggesting that a more slowly-acting mechanism, such as the circadian clock, underlies it (Heikkinen et al. 2011).

Since the circadian clock modulates rod-cone gap junctions through the dopaminergic cAMP/PKA pathway, it seems reasonable to postulate that the same pathway could account for the slow and gradual increase in cone signals in response to mesopic light stimulation. The retinal circadian clock, like mesopic background light, regulates the level of dopamine in the retina; the clock increases the level of dopamine in the retina during the day and decreases it at night (Iuvone et al., 2005; Ribelayga et al., 2003). Dopamine binds to D2Rs on cones and causes a decrease in the levels of intracellular cAMP and PKA. A decrease in the intracellular level of cAMP/PKA reduces gap-junctional coupling between rods and cones and the expression of GABA_A_R and KCC2 on cone terminals in the day. Conversely, an increase in the intracellular level of cAMP/PKA increases gap-junctional coupling between rods and cones and the expression of GABA_A_R and KCC2 on cone terminals at night (Ribelayga et al., 2008; Choi et al., 2012).

By regulating the expression of GABA_A_R and KCC2 on cone terminals and the level of rod-cone coupling, the circadian clock as well as light inputs in the mesopic range can facilitate the transition between rod vision to cone vision and back again at dawn and at dusk.
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driving force for GABA\textsubscript{A} receptors. Neural Plasticity
