Negative feedback from horizontal cells to cones. The mechanism and its modulation

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Chapter 2

Hemichannel mediated inhibition in the outer retina


An essential feature of the first synapse in the retina is a negative feedback pathway from horizontal cells to cones. We show here that at this synapse, connexin26 forms hemichannels on horizontal cell dendrites, near the glutamate release site of the cones. Blocking these hemichannels hyperpolarizes horizontal cells, modulates the Ca²⁺-channels of the cones and abolishes all feedback-mediated responses. We propose a feedback mechanism in which the activity of the Ca²⁺-channels and the subsequent glutamate release of the cones is modulated by a current through these hemichannels. Because the current through the hemichannels depends on the polarization of the horizontal cells, their activity modulates the output of the cones.

In all vertebrate retinas, photoreceptors project to horizontal cells (HCs) and bipolar cells (BCs). The synaptic complex of this interaction reveals a peculiar and conserved ultrastructure. The cone pedicles are characterized by a pre-synaptic ribbon, where neurotransmitter release takes place, centrally positioned BC dendrites and laterally positioned HC dendrites (Fig.2.1A). These lateral contacts are thought to be the origin of negative feedback from HCs to cones. In goldfish, this feedback modulates the Ca²⁺-current in the cones. Hyperpolarization of HCs shifts the Ca²⁺-current to more negative potentials, which increases the Ca²⁺-influx and subsequently leads to an increase in glutamate release. Various neurotransmitters have been proposed for this pathway, but this retrograde neurotransmitter has not yet been unequivocally identified (Kamermans and Spekreijse 1999).

In the carp retina, connexin26 (Cx26) immunolabel¹ (Janssen-Bienhold et al. 2001) was restricted to the membrane of the lateral processes of the HCs close to the voltage-dependent Ca²⁺-channels on the opposing cone membrane (Fig.2.1B and C) (Nachman-Clewner et al. 1999). Septilaminar structures indicative of gap-junctions between the cones and the HC are neither discernible at this site, nor have such structures been reported by physiological studies, suggesting that the immunolabel reflects the presence of hemichannels. That functional hemichannels are present on HCs and do not compromise cell viability has been shown in dissociated HCs (DeVries and Schwartz 1992; Lu and McMahon 1996).

The location of Cx26 immunolabel suggested that such hemichannels might

¹For details about the pre-embedding immunoelectron microscopy, and the Cx26 antibody see Janssen-Bienhold et al. (1998).
be involved in the synaptic interactions between HCs and cones. We thus studied the effect of carbenoxolone, a blocker of gap-junctional channels on this feedback pathway\(^2\) (Osborne and Williams 1996; Vaney et al. 1998). Figure 2.2A (left) shows the feedback-induced responses of a cone clamped at various membrane potentials\(^3\). The feedback-mediated responses in cones can be measured most effectively when the cone response is saturated with a white 20 \(\mu\)m diameter spot and the retina stimulated with a full-field light stimuli were used.

\(^2\)At present there is no specific blocker for Cx26 hemichannels. Therefore, carbenoxolone has blocked other gap-junctions in the retina. This has not influenced our results because full-field light stimuli were used.

\(^3\)To present there is no specific blocker for Cx26 hemichannels. Therefore, carbenoxolone has blocked other gap-junctions in the retina. This has not influenced our results because full-field light stimuli were used.
Figure 2.2 Carbenoxolone blocks feedback-mediated responses in both cones and HCs. a) Feedback induced response in a cone clamped at various potentials in control solution (left) and in carbenoxolone (right). Feedback-induced responses are maximal around -47 mV and diminished at both hyperpolarized and depolarized potentials. b) Feedback-induced response in a cone clamped at -47 mV in control (left), carbenoxolone (middle) and after 15 min wash (right). c) Carbenoxolone does not affect the response of a cone to light. d) Responses of a monophasic HC to 550 nm, full field, stimuli of 500 ms, which are separated by 500 ms, during which no stimuli were presented. Expanded light responses at different time points, indicated by the arrows are given below. e) Carbenoxolone blocks feedback-mediated responses in MHCs. Responses in control (left) and in carbenoxolone (right). f) Responses of a biphasic HC (BHC) in control (left) and carbenoxolone (right). Carbenoxolone blocks the depolarizing response to red light. The responses in b, c, d and f have been shifted such that the baseline is equal.

white light stimulus (Kamermans and Spekreijse 1999). Such a stimulus induces a shift of the Ca$^{2+}$-current in the cones to more negative potentials, which will be seen as an inward current in a voltage clamped cone. In the presence of 100 µM carbenoxolone, these feedback-induced responses disappeared (Fig.2.2A right; n = 13). The effect of

3 Retinas of light-adapted goldfish, Carassius Auratus, (12 - 16 cm standard body length) were used in the electrophysiological experiments. For details about the experimental procedures, see Fahrenfort et al. (1999). All experiments were done in the presence of 25 M SKF 89976A, a kind gift from Smith Kline Beecham, to block the influence of the GABAergic system in the outer retina without affecting the inner retina. Data are presented as mean ± sem.
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carbenoxolone could be washed out within 15 min (Fig.2.2B). During the application of carbenoxolone cones hyperpolarized by -4.6 mV ± 1.7 mV (n = 5) while their light response amplitude was unaffected (Fig.2.2C).

Because blocking the hemichannels led to the disappearance of the feedback-induced responses in cones, the feedback-mediated responses in HCs should also disappear. Carbenoxolone hyperpolarized HCs strongly and reduced their light responses (Fig.2.2D). Because of this large hyperpolarization, the effect of carbenoxolone on the feedback-mediated responses was studied before the HCs had hyperpolarized more than about 25% of their maximal hyperpolarization (Fig.2.2D; (3)). HC light-responses show a characteristic transient component (arrow Fig.2.2E, left), mainly attributable to negative feedback from HCs to cones (Witkovsky et al. 1995; Wu 1994). In monophasic HCs (MHCs), which hyperpolarize to light of all wavelengths, this pronounced transient component was blocked by carbenoxolone (Fig.2.2D, right; n = 8). Biphasic HCs (BHCs) hyperpolarize to full-field green light stimulation but depolarize upon red light stimulation (Fig.2.2F, left). The depolarizing responses are thought to originate from negative feedback from MHCs to middle wavelength sensitive cones (Kamermans and Spekreijse 1999; Kraaij et al. 2000a; Toyoda et al. 1982; Weiler and Wagner 1984; Witkovsky et al. 1995). Carbenoxolone blocked these depolarizing responses while the hyperpolarizing responses were almost unaffected (Fig.2.2F, right; n = 5).

Is the block of the hemichannels or the hyperpolarization of the HCs responsible for the block of feedback? Hyperpolarization of HCs with an intact feedback system shifts the Ca\(^{2+}\)-current to more negative potentials (Kamermans and Spekreijse 1999). The maximal light-induced shift of the Ca\(^{2+}\)-current to negative potentials is about -10 mV (Kraaij et al. 2000a). Blocking hemichannels with carbenoxolone also hyperpolarizes HCs, but now the Ca\(^{2+}\)-current was shifted to more positive potentials. The mean carbenoxolone-induced shift of the half-activation potential of the Ca\(^{2+}\)-current was 4.7 mV (ranging from 0.5 mV to 11.5 mV; n = 8). Although HCs hyperpolarize strongly under carbenoxolone, the Ca\(^{2+}\)-current is not shifted to negative potentials as would be expected if feedback were still intact. The observed positive shift of the Ca\(^{2+}\)-current shows that blocking the hemichannels, and not the HC hyperpolarization, is essential for the carbenoxolone-induced block of feedback responses in cones.

Hemichannels have a reversal potential around 0 mV (DeVries and Schwartz 1992; Lu and McMahon 1996). Blocking these channels should hyperpolarize HCs. The size of the carbenoxolone-induced hyperpolarization of the HCs was unexpectedly large (by -44.4 mV ± 3.0 mV; n = 8). This observation suggests that the hemichannel conductance is by far the largest conductance in the HCs. However, carbenoxolone not only blocks the hemichannels but also shifts the Ca\(^{2+}\)-current of the cone, leading to a reduction of glutamate release, which in turn closes the glutamate-gated channels\(^4\). We estimated that the glutamate conductance was eight times larger than that of the hemichannel\(^5\).

How do hemichannels mediate negative feedback from HCs to cones? An elec-

\(^4\) Blocking hemichannels resulted in a positive shift of 4.7 mV in the Ca\(^{2+}\)-current. This reduced the Ca\(^{2+}\)-influx into the cones and hyperpolarized them by -4.6 mV leading to a relative shift of membrane potential vs Ca\(^{2+}\)-current of about 9 mV. In conditions where feedback is not active, such a shift reduces synaptic transmission between cones and HCs by about 90% (Kraaij et al. 2000a). This accounts for the large hyperpolarization of the HCs and the reduction of the HC light-responses.
trical feedback mechanism has been suggested in which glutamate receptors on the HC dendrites in the cone synaptic terminal form a current sink by which the extrasynaptic potential could be modulated (Byzov and Shura-Bura 1986). Although this original hypothesis was not validated experimentally (Kamermans and Spekreijse 1999), a modified version can account for the observed effects (see appendix 1). Key elements of this mechanism are a relatively high resistance of the extracellular space in the synaptic terminal, and hemichannels at the tips of the HC dendrites. The extracellular potential near these hemichannels will become negative because of the current flowing through the high resistance of the extracellular space, via the hemichannels into the HCs. The voltage-dependent Ca\(^{2+}\)-channels of the cones will therefore sense a more positive membrane potential, causing an increase in the release of glutamate. Modulation of the HC membrane potential will change the current flowing through the hemichannels, which will subsequently modulate the glutamate release of the cones. In voltage clamp experiments, this modulation of the Ca\(^{2+}\)-current by the HCs will be seen as a negative shift of the Ca\(^{2+}\)-current in the cones.

Such an electrical feedback mechanism critically depends on a postsynaptic current sink, near the presynaptic Ca\(^{2+}\)-channels. This suggests that other channels present in the cone synaptic terminal could also form a current sink and should therefore also be able to mediate feedback. Therefore we studied the contribution of the glutamate-gated channels while hemichannels were blocked with carbenoxolone (Fig. 2.3). Recordings were made from a BHC during alternating green and red light stimulation. Application of carbenoxolone closed the hemichannels and greatly reduced the glutamate-gated conductance. To study the effect of current flowing through the glutamate-gated channels, we applied kainate. In a restricted time window, only some of the glutamate receptors will be activated by kainate while the others will be modulated by the glutamate released by the cones. In this time window the light responses and the feedback-mediated depolarizing response to red light stimuli reappeared (Fig. 2.3 (4); \(n = 3\)). This experiment showed that glutamate-gated conductances could, under certain conditions, also act as a current sink and contribute to electrical feedback. Because feedback does not depend on the type of channel on the HC dendrites, these experiments support the hypothesis that it is the current that generates the feedback signal. However, under physiological conditions with full field white light stimulation, the current through the glutamate-gated channels will reduce with HC hyperpolarization instead of increase. This happens because the cones

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5 In the dark, the resting potential of HCs is \(-34.7 \pm 3.6\) mV \((n = 12)\). Blocking the glutamate-gated conductances with DNQX (an antagonist of ionotropic, non-NMDA glutamate receptors) in the control condition induced a hyperpolarization of \(-36.7\) mV \(\pm 2.8\) mV \((n = 7)\) in HCs which is significantly less than the carbenoxolone-induced hyperpolarization \((-44.4 \pm 3.0\) mV\) (\(t\)-test, \(p < 0.05\)). Application of DNQX during carbenoxolone further hyperpolarized HCs by \(-3.6 \pm 1.3\) mV \((n = 5)\), to a final value of \(-82.7\) mV. Taking this as the reversal potential for the potassium conductance and 0 mV as the reversal potential for the cation- and hemichannel conductances, one can estimate, using Ohms law, that the hemichannel conductance is about one-eighth of the cation conductance.
reduce their glutamate release. This cascade of events turns the feedback signal via the glutamate-gated channels into a positive one. The contribution of the glutamate-gated channels to feedback is therefore negative in cones that are not stimulated by light, whereas it is positive when the cones are directly stimulated by light.

An electrical feedback mechanism strongly depends on the resistance of the extracellular space in the synapse, the conductance of the hemichannels, and their localization. Using morphological data, one can calculate that 13-400 hemichannels

The surface of the extracellular space at the base of the cone pedicle in goldfish has been estimated to be between 0.01 to 0.1 m² depending on the fixation procedure used (Vandenbranden, et al. 1996). The tips of the dendrites invaginate about 1 μm into the cone pedicle. Assuming that the mobility of the ions in the intersynaptic space is equal to that in the Ringer's solution, one can calculate that the resistance of the intersynaptic space is between 6 and 60 MΩ. The shift in the Ca²⁺-current is about -10 mV for a change in HC membrane potential of about -40 mV. Therefore, the total resistance of the hemichannels should be between 24 and 240 MΩ. The single channel conductance of a Cx26 hemichannel is about 270 pS (Suchyna et al. 1999), and is almost potential-independent over a voltage range of 60 mV (Oh et al. 1999). Various hemichannels are closed by high Ca²⁺-concentrations (9). It was estimated that with 1 mM extracellular Ca²⁺-concentration (the concentration used in the present experiments) the conductance of the channels is about 4% of the maximal conductance. Given these numbers, and assuming that the conductance of Cx26 hemichannels is also Ca²⁺-dependent, one needs between 400 and 4000 hemichannels in the entire terminal to generate enough current to shift the Ca²⁺-current by 10 mV. Since in goldfish-cones 5 to 16 synaptic ribbons are present with each at least 2 HC dendrites (Stell et al. 1982) per ribbon, one would need about 13 to 400 hemichannels per dendrite.
focally localized at the tips of the HC dendrites yield a large enough current sink\(^6\) for the proposed electrical feedback mechanism to function. In contrast to hemichannels that are focally localized, glutamate receptors are expressed more diffusely and extrasynaptically on the dendrites of HCs (Kloosterman et al. 2001; Schultz et al. 2001), reducing the efficiency with which they modulate the calcium channels of the cone.

We propose an electrical or ephaptic feedback mechanism in which hemichannels are key elements that form a current sink near the Ca\(^{2+}\)-channels of a pre-synaptic neuron, making the potential sensed by these Ca\(^{2+}\)-channels dependent on the activity of the post-synaptic neuron.

**Appendix 1**

Electrical feedback model. A schematic drawing of the cone synaptic terminal is given. In (A) the hemichannels (dark grey) are indicated in the HC dendrites and the Ca\(^{2+}\)-channels (white) in the cone membrane, close to the glutamate release site of the cones near the end of the synaptic ribbon (R). In (B), the equivalent electrical circuit is drawn in the condition in which the cones and HCs rest at -40 mV. Hemichannels (1) form a current sink in the HC dendrites near the voltage dependent Ca\(^{2+}\)-channels (2) in the cones. The small inward current through the hemichannels has to come from outside the synaptic complex and needs therefore to pass through the intersynaptic space. Because the resistance of this space (3) is appreciable, a voltage drop will occur, making the potential near the hemichannels slightly negative. The result is that, **locally**, the potential difference over the cone membrane will become **smaller**, leading to an increase in Ca\(^{2+}\)-current. (4) is the nonsynaptic membrane resistance of the HCs outside the cone synaptic complex. In this scheme the Ca\(^{2+}\)-channels are represented by a potentiometer because the activity of these channels (and therefore the amount of glutamate release) depends on the potential difference across the cone membrane. In (C) the equivalent circuit is drawn in a condition
in which the HCs have hyperpolarized to -80 mV while the cone remains at -40 mV. This represents the condition in which the receptive field surround of a cone is strongly stimulated while the cone itself is not. Now the current through the hemichannels (1) and therefore the potential drop along the resistance of the intercellular cleft (2) has increased. The result will be that the potential near the voltage-dependent Ca\(^{2+}\)-channels has become even more negative leading to a local reduction of the cone membrane potential. In this way hyperpolarization of the HCs leads to a local depolarization of the cone membrane potential, at the critical site where voltage-dependent Ca\(^{2+}\)-channels are located.

**Appendix 2**

Current flowing through glutamate-gated channels can also mediate feedback. Fig.2.4A shows an electronmicrograph of the cone/HC synapse stained with immunolabel against the Glu-R2 glutamate-receptor subunit. Label is found on the lateral processes of the HCs, indicating that glutamate-gated channels are present at a location in close proximity to the calcium channels of the cone. If negative feedback from HCs to cones is indeed ephaptic, current flowing through these channels should also be able to modify the output of the cone. That this is indeed the case is shown in chapter 2. In the absence of a hemichannel conductance, current flowing through glutamate-gated channels can also induce feedback-mediated responses (Fig.2.3).

At first glance, this might contradict the results of Jan Verweij, who showed that blocking the glutamate-gated channels with the ionotropic glutamate antagonist DNQX did not block negative feedback from HCs to cones. One has to realise that at that time the existence of hemichannels at the tips of the HC processes was not known. Can

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**Figure 2.4 Localisation of ionotropic glutamate-gated channels in the outer retina.** A) Electron micrograph of the cone-HC synapse. R=ribbon of the cone; HC= invaginating HC processes. Immunolabel against Glu-R2 is found in the tips of the invaginating HC dendrites. B) Electron micrograph of cone/HC synapse and the adjacent neuropil. Glu-R2 immunolabel is also present in dendrites far away from the cone synaptic release sites. Bars in (A) indicate 0.1 \(\mu\)m and (B) 0.5 \(\mu\)m.
we explain the DNQX experiments when we take the hemichannel conductance into account? Fig.2.5A, B shows the same electric equivalent circuit of the cone/HC synapse. In addition to the hemichannel resistance, a glutamate-gated resistance is given. Since glutamate-gated channels also have a reversal potential of about 0 mV, application of DNQX will hyperpolarize HCs. This will decrease the amount of current flowing through the glutamate-gated channels and increase the current flow through the hemichannels. Depending on the balance of these processes the total intersynaptic current will either increase or decrease. An increase in current results in a shift of the Ca\(^{2+}\)-current to negative potentials whereas a decrease of current leads to a shift of the Ca\(^{2+}\)-current to positive potentials. Since Fig.2.5C shows that application of DNQX shifts the Ca\(^{2+}\)-current to negative potentials, we can conclude that the glutamate-gated channels are not the major current source of negative feedback from HCs to cones. If the glutamate-gated channels had been the major current source, closing them would have resulted in a shift of the Ca\(^{2+}\)-current to more positive potentials.

How can hemichannels be the major current source of negative feedback from HCs to cones, if estimates about the relative conductances present in HCs indicate that the glutamate-gated conductance is 8 times larger than the hemichannel conductance (see footnote 5)? A possible answer to this question was given by ultrastructural studies done by Klooster and Yazulla (Klooster et al. 2001). Although glutamate-gated channels are present in the cone/HC synapse, much of the immunolabel was found far away from the release sites of the cone (Fig.2.4B). Current flowing through the glutamate-gated channels outside the terminal is not able to modify the intersynaptic potential near the calcium channels of the cone. So, although the hemichannel conductance is relatively small, their focal localisation makes them the major current source in the ephaptic negative feedback from HCs to cones.
Hemichannel-mediated feedback

Figure 2.5 Hemichannels are the major current source in negative feedback from HCs to cones. A) Electrical equivalent circuit of the cone/HC synapse. B) Closing either conductance will lead to a hyperpolarization of HCs. By using the position of the calcium current of the cone as a monitor for the extracellular synaptic potential one can determine which of these two conductances is the major current source in the negative feedback from HCs to cones. C) The calcium current of the cone in control (open circles) and after closing the glutamate-gated channels with DNQX (closed circles). DNQX shifts the calcium current of the cone to more negative potentials.