Chapter 7

General Discussion

This thesis describes the synaptic mechanism of negative feedback from horizontal cells (HCs) to cones in unprecedented detail. This mechanism has puzzled the retinal community for decades and many hypotheses have been proposed to account for it. In this thesis I show that feedback consists of two components, an ephaptic mechanism and a mechanism involving the release and extracellular hydrolysis of ATP. Furthermore, I present evidence for a new mechanism that modulates feedback; negative feedback can induce glutamate release in cones, which then spills over to neighbouring cones and inhibits them. In this discussion I will address issues that did not get enough attention in the preceding chapters. Since every new theory needs to also account for all the previous data collected, I will evaluate the data collected in earlier studies on feedback in light of my findings.

1. A GABA-mediated mechanism for negative feedback

Initially, it was proposed that negative feedback from HCs to cones is GABA-ergic (Wu and Dowling, 1980; Tachibana and Kaneko, 1984). The first evidence for such a mechanism came from studies showing that HCs can synthesise GABA and release it upon depolarisation (Schwartz, 1982; Ayoub and Lam, 1984). This release mechanism was unusual, as the HCs of cold-blooded animals have been shown to release GABA via GABA transporters working in the reversed direction (Schwartz, 1982, 1987; Yazulla, 1983; Yazulla and Kleinschmidt, 1983; Cammack and Schwartz, 1993; Kamermans and Werblin, 1992; Dong et al., 1994). Furthermore, electrophysiological and immunocytochemical studies showed that cones express ionotropic GABA receptors (GABARs) (Tachibana and Kaneko, 1984; Kaneko and Tachibana, 1986; Yazulla et al., 1989). Thus, the synthesis of, the release mechanism for and the targets for GABA are present in the outer retina.

Physiological evidence was gathered in support of the GABA hypothesis. Cones hyperpolarise when stimulated with a small light spot. When subsequently activating the surround with a larger spot, the hyperpolarised response reduces (Baylor et al., 1971). A prominent surround induced depolarising response can be seen when cones are saturated with a small bright spot preceding the surround stimulation (O’Bryan, 1973; Piccolino...
et al., 1981; Lasansky, 1981). Since HCs have large receptive fields, it was suggested that the surround induced responses were mediated by HCs. The surround-induced responses were shown to be mediated by a Cl-current in cones (Lasansky, 1981; Wu, 1991; Barnes and Deschênes, 1992; Thoreson and Burkhardt, 1991), suggesting the involvement of the GABA-gated Cl-current (I_{ClGABA}). Indeed the depolarising responses in cones were GABA-sensitive and could be reduced, or sometimes blocked by GABA receptor antagonists (Djamgoz and Ruddock, 1979; Murakami et al., 1982; Stone and Witkovsky, 1987; Wu, 1991). At that time, the hypothesis of a GABA-mediated mechanism underlying negative feedback seemed well supported.

However, others were unable to reproduce the effects of GABA agonists or antagonists on the surround-induced depolarisation in cones (Fish: Miller et al., 1981; Turtle: Thoreson and Burkhardt, 1990; Fish: Kamermans et al., unpublished results). Later it was demonstrated that negative feedback from HCs to cones was mediated by a GABA-independent modulation of I_{Cl} in cones (Fish: Verweij et al., 1996; Endeman et al., 2012; Monkey: Verweij et al., 2003). Upon hyperpolarisation of HCs, the activation potential of I_{Cl} shifted to more negative potentials (Verweij et al., 1996). This shift caused an increase in the influx of Ca^{2+} into the cone synaptic terminal and consequently an increase in glutamate release. The modulation of I_{Cl} itself, however, did not cause a significant depolarisation of the cone membrane potential (Verweij et al., 1996; Kraaij et al., 2000).

These findings left open two questions: what is the origin of the surround-induced depolarising response, and what is the role of GABA release from HCs?

**The origin of the surround-induced depolarisation**

Because the surround-induced depolarising response was shown to be mediated by an I_{Cl} and all the components for a GABAergic mechanism were present, it was assumed that I_{Cl} was I_{ClGABA}. However, the cone contains two other Cl-conductances, the Ca^{2+}-dependent Cl-current (I_{ClCa}) and the glutamate transporter associated Cl-current (I_{ClGluT}) (see chapter 5).

According to the GABA hypothesis, GABA release is high when the HCs are depolarised and decreases upon HC hyperpolarisation. Thus, feedback results in a reduction of I_{ClGABA}, leading to an increase in input resistance. Conversely, however, many studies have shown that surround stimulation leads to a decrease in input resistance of the cones (see also chapter 1; O’Bryan, 1973; Gerschenfeld and Piccolino, 1980; Lasansky, 1981; Thoreson and Burkhardt, 1991). This suggests that the depolarising response is caused by the opening, not closing, of a channel. Could one of the other Cl-currents mediate the surround induced depolarisation? Negative feedback from HCs to cones leads to an increase in I_{Cl} and thus an increase in the Ca^{2+}-concentration ([Ca^{2+}]) in the cones. This activates I_{ClCa} which leads to a decrease in input resistance and can depolarise cones if the equilibrium potential of Cl (E_{cl}) is more positive than the membrane potential. Whether the modulation of a Cl-current will lead to a surround induced depolarisation therefore depends on E_{cl}.

If E_{cl} is more negative than the cone membrane potential, feedback mediated via the GABA-ergic mechanism would result in a surround induced depolarisation, while it would result in a surround induced hyperpolarisation if I_{ClGABA} was involved. Thoreson and Bryson (2004) found that E_{cl} in physiological conditions was around the dark resting membrane potential of cones (-46 mV; Thoreson and Bryson, 2004). If cones are hyperpolarised by light, E_{cl} will be more positive than the cone membrane potential, suggesting that the surround-induced depolarising response is a modulation of I_{ClGluT}. Similar conditions might have occurred in other studies as well, as often a bright small spot focussed on the recorded cone was used to prevent direct light responses (O’Bryan, 1973; Piccolino et al., 1981; Lasansky, 1981; Kraaij et al., 2000) and as such, the cone being recorded from was therefore relatively hyperpolarised. Wu (1991) removed the outer segment from the cone being recorded, which is likely to also results in a hyperpolarisation of the cone. In the experimental conditions of all of these studies cones depolarise to surround stimulation, which is consistent with the modulation of I_{ClCa}. Furthermore, many of the early experiments were performed with sharp microelectrodes filled with a high concentration of KCl (1.5-3 M). Cl may have leaked from the pipette into the cone, shifting E_{cl} to more positive potentials (Djamgoz and Ruddock, 1979; Lasansky, 1981; Burkhardt et al., 1988; Thoreson and Burkhardt, 1991) enhancing a I_{ClGluT} mediated depolarising responses. Consistent with this, Kraaij et al. (2000) showed directly, in voltage clamp experiments, that the depolarising response is mediated by the activation of I_{ClGluT} and not by modulation of I_{ClGABA}.

In chapter 6 I showed that a depolarising response can also be induced by the activation of I_{ClGluT}, especially when cones are experimentally hyperpolarised to potentials outside the activation range of I_{Cl}. In such hyperpolarised conditions, the surround-induced shift of the activation potential of I_{Cl} does not result in an increased Ca^{2+}-influx into the cone terminal and, consequently,
**General discussion**

$I_{\text{Cl}}(\text{GABA})$ is not activated and can therefore not account for these depolarising responses. Given that in these strongly hyperpolarised conditions $I_{\text{Cl}}(\text{GluT})$ mediates the depolarisation, why then did some researchers find that GABA and GABA-antagonists affect feedback? One possible explanation is that the effect of GABA and GABA-antagonists could be indirect. The surround induced depolarising responses reported by Wu (1991) was partially blocked by bicuculline, a GABA receptor antagonist. Nonetheless, bicuculline did not result in a complete block. There are a number of complicating factors here. Firstly, cones are not the only neurons in the outer retina expressing GABARs as HCs also express ionotropic GABA-receptors (Kamermans and Werblin, 1992; Verweij et al., 1998; Yang, 2004). Secondly, HCs receive dopaminergic input from interplexiform cells (IPCs) (Dowling and Ehinger, 1975, 1978; Dowling, 1986; Yazzulla and Zucker, 1988), which modulates the gap junctions between HCs (Teranishi et al., 1983; Piccolino et al., 1984; Lasater and Dowling, 1985; Mangel and Dowling, 1985; DeVries and Schwartz, 1989; Dong and McReynolds, 1991; Xin and Bloomfield, 1999) and the sensitivity of the glutamate receptors on HCs (Knapp and Dowling, 1987). Finally, IPCs are under GABA-ergic inhibition. Modulation of each these different GABA-ergic and dopaminergic mechanisms will have different effects on HCs.

Direct effects of GABA antagonists on HCs will result in a hyperpolarisation of HCs (Kamermans and Werblin, 1992; Verweij et al., 1998) since $E_c$, in HCs is -20 mV (Miller and Dacheux, 1983; Djamgoz and Laming, 1987). Blocking the GABA-ergic inhibition on IPCs causes them to increase their dopamine release, which leads to an increase in sensitivity of the glutamate receptors on HCs and thus a depolarisation of HCs (Umomo and Dowling, 1991). Such depolarisation of HCs will result in a shift of $I_{\text{L}}$ of cones to more positive potentials. If the recorded cone is already relatively hyperpolarised, as $I_{\text{L}}$ previously proposed, a surround-induced shift of the activation potential of $I_{\text{L}}$ results in a much smaller increase of the Ca$^{2+}$-influx into the cone terminal than without bicuculline. The increase in $I_{\text{Cl}}(\text{GABA})$ is consequently smaller and thus the depolarising response is reduced. The bottom line is that the effect of GABA on the cone-HC system will strongly depend on the relative contribution of the various pathways and the adaptation state of the retina.

**The role of GABA in the outer retina**

GABA is still likely to play a role in negative feedback, but most likely functions as a neuromodulator instead of a neurotransmitter. GABA has recently been proposed to modulate negative feedback by shunting feedback via the activation of $I_{\text{Cl}}(\text{GABA})$ on the cone photoreceptor (Endeman et al., 2012). The GABARs on the cone are located within the synaptic cleft and can interfere with the circuitry responsible for ephaptic feedback. Hemichannels located on the top of the HC dendrites form large pores and thus, have a large conductance leading to a current flowing into the HCs. This current must also pass through the synaptic cleft, which is a very confined space and thus has a relatively large resistance. This causes the synaptic cleft to have a slightly negative potential relative to the extrasynaptic space. Activation of $I_{\text{Cl}}(\text{GABA})$ on the HC offers an alternative route for the current through the connexin (Cx) hemichannels and in this way reduces negative feedback. If the GABA concentration in the outer retina is high, the shunting by $I_{\text{Cl}}(\text{GABA})$ is larger and thus negative feedback will be smaller.

It has been known for a long time that feedback is reduced in the dark adapted retina (Witkovsky et al., 1988; Witkovsky et al., 1989). A possible mechanism underlying this modulation of the feedback strength may be the GABAergic system in the outer retina. The GABA release of HCs increases with dark-adaptation, which is a relatively slow process (Yazzulla and Kleinschmidt, 1982; O’Brien and Dowling, 1985; Yazzulla, 1985). GABA transporters are distributed over the whole HC (Klooster et al., 2004) and GABA is therefore released in the relatively large extrasynaptic volume, instead of only in the confined synaptic cleft. Changes in the GABA concentration are consequently slow and thus GABARs will only be modulated on a slow time scale. Furthermore, the amount of GABARs on cones has been shown to increase with dark-adaptation (Choi et al., 2011). Taken together, this suggests that GABA-mediated signalling is governed by slow mechanisms. This makes the GABAergic pathway a slow modulation pathway instead of a component of the fast feedback mechanism that underlies the surround responses of BCs. Activation of the GABAergic system in the dark adapted retina will reduce the efficiency of the feedback pathway from HCs to cones. In the light adapted retina the GABAergic system will not be active and feedback will be fully functional.

Unlike in fish, HCs in the mammalian retina release GABA via vesicles (Lee and Brecha, 2010; Hirano et al., 2011; Liu et al., 2013). Mammalian HC dendrites contain low quantities of vesicles (Dowling et al., 1966; Raviola and Gilula, 1975; Linberg and Fisher, 1988; Brandstätter et al., 1999) and the proteins necessary for vesicle docking and fusion are present in the tips of the HC dendrites (synaptotagmin, SNAP-25, Rab3A and SNAPRE; Brandstätter et al., 1996; Grabs et al., 1996; Lee and Brecha, 2010). Furthermore, mammalian HCs express the vesicular GABA transporter (VGAT) that fills vesicles with GABA and glycine (Cueva et al., 2002), suggesting the presence of a
mechanism involving vesicle release of GABA. Fish HCs contain considerably fewer vesicles (Wagner, 1980) and the expression of VGAT is much lower than in the mammalian retina (Klooster, unpublished data), so it is therefore unlikely that vesicle release is their primary mechanism for the release of GABA.

Liu et al. (2013) proposed that in the mammalian retina GABA mediates negative feedback from HCs to cones in a pH-dependent manner. They suggested that when HCs are depolarised, \( \text{Ca}^{2+} \) enters the HC via voltage-gated \( \text{Ca}^{2+} \) -channels and, as vesicle docking and fusion is dependent on \( \text{Ca}^{2+} \), GABA is released. GABA activates GABARs on the HC itself, which has been shown to happen in other systems (Kamermans and Werblin, 1992). They showed that GABARs are permeable for Cl\(^{-}\) and bicarbonate and went on to argue that a bicarbonate-flux through the GABARs influences the pH in the synaptic cleft and, as such, mediates feedback from HCs to cones. Of course, the functioning of this mechanism would be dependent on the equilibrium potential of bicarbonate (\( E_{\text{bicarb}} \)). Liu et al. (2013) proposed that the \( E_{\text{bicarb}} \) was more negative than the HC membrane potential. Under such conditions they speculated that bicarbonate flows into the HC, leading to an acidification of the synaptic cleft. Hyperpolarisation of HCs reduces the GABA release and thus the uptake of bicarbonate. This then leads to an alkalinisation of the synaptic cleft and thus induces a shift of \( I_{\text{ca}} \) to negative potentials. In this way HCs feed back to cones in a pH sensitive manner.

There are three arguments against this speculation. First of all, feedback is present in conditions where the GABA-receptors are blocked, both in lower vertebrates and in mammals. Secondly, the \( E_{\text{bicarb}} \) is most likely more positive than the dark resting membrane potential (Farrant and Kaila, 2007). Only in extremely depolarized conditions might \( E_{\text{bicarb}} \) become more negative than the membrane potential of HCs. This means that under physiological conditions bicarbonate will be released by the HCs via the GABARs and the resulting efflux of bicarbonate will make the synaptic cleft slightly alkaline. This efflux will reduce with hyperpolarisation of HCs, since they stop releasing GABA, and consequently the GABARs will close. This then leads to an acidification of the synaptic cleft and thus a shift of \( I_{\text{ca}} \) to more positive potentials, which is the opposite of what was found experimentally by Liu et al., (2013). Finally, the assumption that diffusion of bicarbonate through the GABARs into the HC would be more efficient than diffusion of bicarbonate out of the synaptic cleft seems far-fetched, given the much higher resistance of the GABARs compared to the cleft.

What would be the role of this GABA-ergic system? Let us assume that, just as in the lower vertebrates, GABA functions as a modulator in the mammalian outer retina. In the dark-adapted retina, GABA release is high and GABA receptors on HCs are activated. Bicarbonate is released, slightly alkalinising the synaptic cleft. Note that this will oppose the ATP mediated acidification. The net result will be that the pH gradient in the synaptic cleft will be reduced and thus negative feedback is reduced. In the light-adapted retina GABA release is low and the GABA-gated channels will be closed. Consequently, no bicarbonate will be release into the synaptic cleft, which will lead to a stronger acidification of the synaptic cleft due to the hydrolysation of ATP and, as a result, the pH gradient will be larger and thus feedback will become larger. In this mechanism, GABA is a neuromodulator inhibiting feedback from HCs to cones, instead of a neurotransmitter mediating feedback.

2. The ephaptic mechanism

The effect of carbenoxolone on the calcium current in cones

One of the proposed hypotheses to account for the feedback induced modulation of \( I_{\text{ca}} \) is the ephaptic hypothesis (see chapter 2). The key components in this mechanism are Cx hemichannels, which are located on the tips of HC dendrites (Kamermans et al., 2001; Shields et al., 2007; Klaassen et al., 2011). Part of the evidence supporting the ephaptic hypothesis is the blockage of the light-induced feedback response by the Cx and pannexin...
blocker carbenoxolone (CBX) (Kamermans et al., 2001). It was later shown that CBX may directly inhibit $I_{\text{Ca}}$ as well (Vessey et al., 2004). To account for the CBX-induced blockage of feedback it was suggested that CBX blocked $I_{\text{Ca}}$ and thus synaptic transmission of the cones. This would result in a blocking of the HC responses and thus a blockage of feedback.

Kamermans et al. (2001) performed an experiment that ruled out this possibility by showing that CBX does not block the cone synaptic transmission. The ephaptic feedback mechanism is dependent on the current flowing through the intersynaptic space into the HCs. In principle, the identity of the channel mediating this current does not matter. Therefore, the ephaptic hypothesis predicts that other channels should be able to fulfil the role of Cx hemichannels on the HC dendrites as well. The rationale for the experiment performed by Kamermans et al. (2001) was as follows: if Cx hemichannels are blocked with CBX and glutamate receptors are opened with kainate, the glutamate gated channel should form the current sink and feedback should be restored. The results of this experiment led to two conclusions: (1) CBX does not block glutamate release by cones and (2) other channels can take over the role of the Cx hemichannels.

Figure 1 shows the response of a biphasic HC to middle and long wavelength light stimulation. The depolarising response due to long wavelength stimulation is mediated by feedback from monophasic HCs to M-cones and can be used as a measure for negative feedback ①. First CBX is added, showing an elimination of the depolarising response ②, and finally a strong hyperpolarisation of the resting membrane potential of the HC due to Cx closure ③. Kainate is subsequently added and the glutamate receptors on the HC dendrites open, resulting in a depolarisation of the HC. After prolonged application, all glutamate receptors are fully open and the HC has depolarised completely and lost all its light responses ④. However, for a brief window in time a portion of the glutamate receptors are sustainably opened by kainate, while the remaining receptors are modulated by the glutamate release from cones ⑤. The result is that light responses reappear. During this time, the kainate-receptors take over the role of the Cx hemichannels (Kamermans et al., 2001; Klaassen et al., 2011) and feedback is restored. This shows that CBX does not block synaptic transmission and that other channels can take over the role of Cx hemichannels.

An additional argument against the suggestion that CBX blocks $I_{\text{Ca}}$ is that Vessey et al. (2004) reported that CBX blocked $I_{\text{Ca}}$ by only 37 ± 7% in isolated cones, while CBX blocks feedback completely (Kamermans et al., 2001). Therefore, it is unlikely that the elimination of feedback by CBX can be entirely attributed to a reduction in $I_{\text{Ca}}$.

**Figure 1** CBX does not block $I_{\text{Ca}}$ because HC responses that depend on $I_{\text{Ca}}$ can be temporarily restored by the additional application of kainate. See main text for explanation. Figure taken from Kamermans et al. (2001).

3. The proton-mediated feedback mechanism

**HEPES**

In 2003, Hirasawa and Kaneko showed that buffering of the pH in the synaptic cleft by 10 mM HEPES diminished the shift of the activation potential of $I_{\text{Ca}}$, providing evidence for the hypothesis that $I_{\text{Ca}}$ of cones is modulated by pH changes in the synaptic cleft. Alkalisation shifts the activation potential of $I_{\text{Ca}}$ towards more negative potentials and increases its amplitude (Barnes and Bui, 1991; Barnes et al., 1993), resulting in an increased influx of Ca$^{2+}$ into the cone and consequently an increased release of glutamate. Many researchers have confirmed that strong extracellular pH buffering by HEPES inhibits negative feedback from HCs to cones (Vessey et al., 2005; Cadetti and Thoreson, 2006; Jouhou et al., 2007; Davenport et al., 2008; Fahrenfort et al., 2009; Trenholm and Baldridge, 2010; Wang et al. 2014).
Using zebrafish retinas loaded with Ca\(^{2+}\)-indictors, Vessey et al. (2005) measured the modulation of [Ca\(^{2+}\)], in the cone synaptic terminal when cells were depolarised by increasing the external K\(^+\) concentrations. This depolarisation resulted in an increase in the Ca\(^{2+}\)-influx. They then compared the depolarisation-induced change in [Ca\(^{2+}\)], in conditions where HCs were hyperpolarised with DNQX or depolarised by kainate. Application of kainate or DNQX respectively decreased and increased the change in [Ca\(^{2+}\)], and these effects could be blocked by high concentrations of HEPES. They argued that the differences in the modulation of [Ca\(^{2+}\)], induced by kainate and DNQX were due to negative feedback from HCs to cones. Since HEPES could block these effects, it was concluded that negative feedback from HCs to cones was mediated by protons. Furthermore, Cadetti and Thoresen (2006) recorded feedback responses from a cone while directly polarising HCs by current injection. In this way they showed that hyperpolarisation of HCs shifted the activation potential of I\(_{\text{ca}}\) in cones towards more negative potentials. This shift disappeared after adding HEPES. Again, this was taken as evidence the feedback was mediated by protons.

Another generally used measure for negative feedback is the rollback in the HC response. Hyperpolarisation of the HC initiates negative feedback, which leads to an increase in the cone glutamate release. This can be seen in the HC response as a slight secondary depolarisation (but see chapter 5). Several studies have shown that a high dose of HEPES decreases the rollback response in HCs (Vessey et al., 2005; Davenport et al., 2008; Fahrenfort et al., 2009; Trenholm and Baldridge, 2010). Similar results were obtained with other pH buffers such as Tris (Vessey et al., 2005). Interestingly, intracellular acidification with 25 mM acetate, which is a very poor pH buffer, also leads to a reduction of the rollback response (Fahrenfort et al., 2009). Finally, the depolarising response of biphasic HCs, which is mediated by feedback from HCs to cones, is decreased by HEPES (Vessey et al., 2005; Fahrenfort et al., 2009).

A problem arose when HEPES was shown to cause intracellular acidification (Fahrenfort et al., 2009). Since Cx hemichannels are blocked by intracellular acidification (Malchow et al., 1993; Trexler et al., 1999; Ripps et al., 2002), it became clear that the HEPES experiments were unsuitable for discriminating between the ephaptic and the proton mediated mechanisms. Furthermore, many arguments in favour of a purely proton mediated feedback hypothesis are based on measurements of the rollback response in HCs. In chapter 5 I showed that the rollback response in HCs is an unreliable measure for feedback and proposed that it is generated by the inactivation of the HC inward rectifying K\(^+\) current (I\(_{\text{hk}}\)). Since this current is sensitive to both intracellular and extracellular H\(^+\), HEPES may affect the rollback response independently of negative feedback.

In this thesis I have shown that negative feedback is mediated by both an ephaptic and a pH dependent mechanism. With this in mind, can the effect of HEPES on feedback be accounted for? First of all, it has to be noted that HEPES does not block feedback completely. It reduces the maximal inducible shift of the activation potential of I\(_{\text{ca}}\) to about 40%. Experimentally it has been shown that HEPES removes both the fast component of feedback (primarily Cx-mediated) as well as the slow component (pannexin 1-mediated) (Chapter 6, figure 2). The fast component is most likely reduced because of the intracellular acidification and subsequent closure of Cx hemichannels. The slow component may either be reduced because pannexin 1 (Panx1) is also affected by the intracellular increase in pH, or because the increased pH buffer capacity in the synaptic cleft by HEPES makes modulation of the pH in the synaptic cleft impossible. In chapter 4 I showed that blocking Panx1 channels with probenecid caused a hyperpolarisation of the HC dark resting membrane potential (Chapter 4, figure 5). Adding probenecid in the presence of HEPES resulted in an even larger hyperpolarisation, suggesting that intracellular acidification does not block Panx1.

**Measurements of extracellular pH**
The proton-mediated feedback hypothesis predicts that HC depolarisation makes the synaptic cleft more acidic, but is there direct evidence for this? Jouhou et al. (2007) measured the external pH of isolated HCs using the pH-sensitive lipophylic dye 5-hexa-decanoylaminofluorescein (HAF). They showed that depolarisation of HCs induced an external acidification. However, Molina et al. (2004) used self-referencing H\(^+\)-sensitive electrodes and came to the opposite conclusion (Molina et al., 2004; Kreitzer et al., 2007; Kreitzer et al., 2012). To resolve this discrepancy, Jacoby et al. (2012) used both methods simultaneously, replicating the contradictory results, but also identifying the source of the discrepancy. Contrary to what Jouhou et al. (2007) suggested, the HAF dye was localised on the intracellular, instead of extracellular, side of the HC plasma membrane. Therefore, the fluorescence imaging experiments showed an acidification of the intracellular compartment, instead of the extracellular compartment. Overall, these experiments show that HC depolarisation leads to an alkalinisation of the extracellular medium.
In the pH-mediated feedback hypothesis, it is assumed that the pH-changes can be induced by a proton source or sink specifically located on the HC dendritic tips that invaginate the cone terminal. This may be independent of pH-controlling mechanisms on the rest of the HC. Measuring extracellular pH changes of the whole HC may, therefore, not correspond with the extracellular pH changes on the very specialized dendritic tips. The best way to approach this problem is to measure the pH changes in intact synaptic clefts.

Recently, Wang et al. (2014) measured pH-changes of the cone synaptic spaces in the intact retina. They designed a pH-sensitive dye, pHfluorin, fused to the extracellular domain of the voltage dependent Ca\(^{2+}\)-channel, which is expressed close to the cone synaptic ribbon. A transgenic zebrafish line was developed to express this protein in the cone synapse, allowing for measurements of the pH changes in the synaptic cleft. Using repeated two-photon scanning, these pH changes in the cleft could be monitored. They then applied a full field light stimulation during which measurements were obscured, but immediately after the illumination the fluorescence was increased and returned to baseline. In this way, they found that HC hyperpolarisation leads to an alkalinisation of the synaptic cleft and that the time constant of the recovery from this pH change was about 200 ms.

These results are fully in line with the results described in chapter 4. There I described a mechanism involved in negative feedback that is mediated by modulating the pH buffer capacity of the synaptic cleft. When HCs are depolarised and Panx1 channels are consequently opened, ATP is released into the synaptic cleft. ATP is hydrolysed by ecto-ATPases, forming a buffer with a pKa of 7.2 and thus acidifying the synaptic cleft. Hyperpolarisation of HCs due to a light stimulation results in the closing of Panx1 and thus an alkalinisation of the synaptic cleft. I found that the time constant for this mechanism is \(\sim 190\) ms, which is comparable to the time constant of \(\sim 200\) ms found by Wang et al. (2014).

**The involvement of V-ATPase in negative feedback**

The original proton-mediated feedback hypothesis requires the presence of a proton source or sink to change the proton concentration in the synaptic cleft. One of the mechanisms that has been proposed to serve this function is the vacuolar type H\(^+\) pump (V-ATPase) (Jouhou et al., 2007; Hirasawa et al., 2012). Hyperpolarisation of the HCs would reduce the amount of protons pumped into the synaptic cleft by the V-ATPases, which would then result in an alkalinisation of the cleft and a disinhibition of the cone I\(_{Ca}\).

Wang et al. (2014) showed an acidification of the synaptic cleft at light-off when HCs depolarise. They hypothesised that this was due to V-ATPases pumping protons into the synaptic cleft. To test this, they added the V-ATPase blocker bafilomycin A1 (BFA1) and showed a decrease in the acidification. V-ATPase is, however, also located in cone glutamate vesicles. A general function of V-ATPases is to generate a proton gradient in vesicles such that they can be loaded with neurotransmitters. Therefore, blocking V-ATPase with BFA1 might have affected the glutamate release of the cones and, as a consequence, negative feedback may have been reduced.

To address this possibility, Wang et al. (2014) developed a zebrafish line that not only expressed the pH sensitive Ca\(^{2+}\)-channels in cones, but also an FMRFamide (FMRFa) gated Na\(^+\)-channel in the HCs. Adding FMRFa caused HCs to depolarise, resulting in an acidification of the synaptic cleft that was solely due to the polarisation of the HCs. This condition, BFA1 prevented the FMRFa-induced acidification of the synaptic cleft, suggesting the involvement of V-ATPase. However, the reversal potential of Na\(^+\) is very positive, so the FMRFa-induced HC depolarisation was likely to be far outside the physiological range, calling into question how applicable this result is to typical conditions. Therefore, I tested directly whether BFA1 could block the light induced slow component of feedback and found that it affected neither the slow nor the fast component, suggesting that V-ATPases are not involved in generating the light induced feedback responses in cones.

Wang et al. (2014) also studied the effect of the Cx and Panx1 blocker meclofenamic acid (MFA) on the pH changes in the synaptic cleft. The acidification of the synaptic cleft at light-off was largely reduced, but the FMRFa-induced acidification of the synaptic cleft remained unchanged. Wang et al. (2014) suggested that the role of Cx hemichannels is to clear the synaptic cleft of protons and as such, light-induced alkalinisation would be dependent on this function of the Cx hemichannels; so blocking these channels would prevent the alkalinisation. This would mean, however, that clearance of protons by the hemichannels is much more efficient than the diffusion of protons from the synaptic cleft to the extrasynaptic space. Estimates of the time constants for diffusion of neurotransmitters out of the synaptic cleft make this very unlikely (Vandenbranden et al., 1996) and consequently the role that Cx channels are speculated to have in the clearance of protons from
the synaptic cleft seems doubtful. A better explanation for the MFA results obtained by Wang et al. (2014) is that Panx1 channels were inhibited by MFA, blocking the release of ATP and thus preventing the modulation of the pH buffer capacity in the synaptic cleft.

4. The ATP-mediated mechanism

Panx1 channel activity far outside the physiological range

Many studies in which Panx1 channels are characterized focus mostly on positive potentials (0–100 mV) (Bruzzone et al. 2003; Pelegrin and Surprenant, 2006; Ma et al., 2009; Wang et al., 2013). This range of membrane potentials is, however, not physiological. What is the relevance of these results for Panx1 function under typical conditions? In general it seems that Panx1-channels are potentiated by prolonged or repetitive strong depolarisation (Bruzzone et al. 2003; Gründken et al., 2011). Locovei et al. (2006) showed that Panx1 opening is dependent on [Ca²⁺] by measuring the opening of the channel in an ‘inside out’ patch clamp configuration while changing the Ca²⁺ concentration in the perfusion medium. Strong depolarisation of the cells opens Panx1 channels, leading to the influx of Ca²⁺ and thus to a further opening of Panx1, resulting in an increase in the Panx1 currents. Gründken et al. (2011) reported a similar effect using a series of ramp protocols that depolarised the cell up to 80 mV. This procedure led to a significant increase in the Panx1 currents. It might have been that the cell’s [Ca²⁺], had increased as a result of the depolarising ramp protocols, thereby causing potentiation of Panx1 channels. Along similar lines, Thompson et al. (2006) showed that after ischemia-induced depolarisation of cells, Panx1 channels open and the influx of Ca²⁺ promotes further Panx1 opening, eventually leading to cell death (see also chapter 1).

How does this relate to the Panx1 function in more physiological conditions? Panx1 in the HC dendritic tips is located close to the glutamate receptors. Since Panx1 opening is dependent on [Ca²⁺], and the activation of glutamate receptors leads to the influx of Ca²⁺, Panx1 activity may depend on the activity of the glutamate receptors. To mimic such conditions in neuroblastoma 2a (N2a) cells overexpressing Panx1, I kept the free [Ca²⁺] relatively high. In this way, I showed that Panx1 was not only open at substantially depolarised potentials, as previous work has shown, but also within the range of physiological membrane potentials (Chapter 3; Prochnow et al., 2009).

The involvement of Panx1 in ephaptic feedback

Panx1 forms large pores in the membrane of the dendritic tips of HCs, just like Cx hemichannels. The Cx hemichannels function as a current sink in the ephaptic mechanism. It seems inevitable that Panx1 channels, apart from their role in ATP-release, also contribute to the ephaptic mechanism. In chapter 4 I confirmed this. The reduction in amplitude of the cone feedback response after adding the Panx1 blocker probenecid was larger than would have been expected from a reduction in the slow component alone. This suggests that probenecid also affects the fast ephaptic component of feedback. Since the drugs that affected ATP hydrolysis, but not Panx1 channels, only influenced the slow component of feedback, it is highly likely that Panx1 also functions as a current sink in the ephaptic mechanism.

This has consequences for the effect that Panx1 channels have on the shift of the activation potential of I_Ca. If Panx1 is only involved in ATP-release, one would expect that blocking Panx1 would lead to a reduction in ATP release and thus an alkalinisation of the synaptic cleft, causing a resulting shift in the activation potential of I_Ca to more negative values (Barnes and Bui, 1991). If, on the other hand, Panx1 is only involved in the ephaptic mechanism, blocking Panx1 would lead to a shift of the activation potential of I_Ca towards more positive values. I found that blocking Panx1 did not significantly shift the activation potential of I_Ca, suggesting that the contribution of Panx1 in the ATP mediated feedback component and the ephaptic feedback component was about equal. Furthermore, in a genetically modified zebrafish line lacking Cx55.5 hemichannels in the HC dendritic tips, Panx1 expression was up-regulated (Klaassen et al., 2011), suggesting that Panx1 is able to partially compensate for the role of Cx55.5 in the ephaptic mechanism.

On the other hand, the possibility that Cx hemichannels play a role in ATP release cannot be excluded, as in other systems Cx hemichannels have been proposed to function as ATP release channels (Kang et al., 2008; Sonntag et al., 2009; Orellana et al., 2011). It may be, therefore, that the two components of the negative feedback pathway cannot be separated completely at a molecular level. Both Cx55.5 and Panx1 may contribute to both components, just to different extents. The generation of a zebrafish line lacking Panx1 would be required in order to resolve this issue.
5. Why are there two feedback components?

Natural images contain a lot of redundant information. In the spatial domain, large homogenous surfaces like the sky do not contain much important information. In the temporal domain, stationary objects contain less information than moving ones. As such, reducing the amount of redundancy in both domains improves the efficiency of the visual system by preserving only the most salient information.

How does this selection occur? The first step is taken in the outer retina where HCs integrate signals over a large area and feed the resulting signal back negatively to cones. The result is that the HC response is subtracted from the cone response, resulting in a reduction of the spatial redundancy. To do this effectively even for moving stimuli, the underlying synaptic mechanism has to be very fast. If this were not the case, the surround response would lag the centre response, inducing a kind of ‘motion smear’. In chapter 4, I showed that negative feedback from HCs to cones consists of two components. The ephaptic component is very fast, having no delay, and does not filter temporal information. This mechanism effectively reduces the redundancies in the spatial domain, but is incapable of doing so in the temporal domain.

The slow feedback component however, is very suitable for removing redundancies in the temporal domain. This mechanism is mediated by the release and extracellular hydrolysation of ATP and has a time constant of ~190 ms. Incoming signals are averaged in time and subtracted from the cone response, leading to temporal redundancy reduction. To allow for temporal redundancy of small objects, as well as large, the mechanism needs to work locally. The ephaptic mechanism is wholly dependent on the membrane potential of the HC and changes in this potential spread quickly over a large area, making this mechanism particularly suited for reducing spatial redundancy. However, the ATP/Panx1 mechanism most likely depends on the \([\text{Ca}^{2+}]\) in the HC dendritic tips, which in turn depend on the activation HC glutamate receptors. This chemical, rather than electrical, dependency is what gives this mechanism its more local processing properties.

A small stationary object will only activate a small number of cones, which will not induce a significant membrane polarisation in HCs. However, it will change the amount of glutamate release from the stimulated cones. The result may be that the slow feedback mechanism is activated, and thus that temporal redundancy is reduced. This would mean that the two feedback systems are likely to differ in both their temporal as well as their spatial properties. This prediction still needs experimental proof however.

6. summary

I have shown that negative feedback from HCs to cones is a synthesis of an ephaptic mechanism, a Panx1/ATP-mediated mechanism and an additional component involving glutamate spillover and the activation of \(I_{\text{Cl(GluT)}}\). The three hypotheses that were previously proposed over the years (the GABA-mediated, ephaptic, and pH-mediated mechanism) all covered certain properties of the system. The GABA-mediated mechanism is a modulatory pathway of feedback, instead of a mediator. Surround-induced depolarising responses that were thought to be mediated by \(I_{\text{Cl(GABA)}}\) are actually mediated by the other \(I_{\text{Cl(Ca)}}\) and \(I_{\text{Cl(GluT)}}\), where the latter accounts for responses in strongly hyperpolarised cones. The ephaptic mechanism has the largest contribution to the feedback response and is responsible for spatial redundancy reduction. The involvement of pH changes in the synaptic cleft has also been confirmed, although the mechanism seems to operate in a very different manner than was previously thought; ATP released via Panx1 channels on the HCs is hydrolysed by ectoATPases, which results in the increase of the pH buffer capacity and acidification of the synaptic cleft. Together these newly elucidated mechanisms account for most, if not all, of the findings of previous literature dealing with negative feedback from HCs to cones.
References


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Section 7: General discussion


