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Feedback-induced glutamate spillover enhances negative feedback from horizontal cells to cones

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Key points

- In the retina, horizontal cells feed back negatively to cone photoreceptors.
- Glutamate released from cones can spill over to neighbouring cones.
- Here we show that cone glutamate release induced by negative feedback can also spill over to neighbouring cones.
- This glutamate activates the glutamate transporter-associated chloride current in these neighbouring cones, which leads to a change in their membrane potential and thus modulates their output.
- In this way, feedback-induced glutamate spillover enhances negative feedback from horizontal cells to cones, thus forming an additional feedback pathway.
- This effect will be particularly prominent in cones that are strongly hyperpolarized by light.

Abstract Inhibition in the outer retina functions via an unusual mechanism. When horizontal cells hyperpolarize the activation potential of the Ca^{2+} current of cones shifts to more negative potentials. The underlying mechanism consists of an ephaptic component and a Panx1/ATP-mediated component. Here we identified a third feedback component, which remains active outside the operating range of the Ca^{2+} current. We show that the glutamate transporters of cones can be activated by glutamate released from their neighbours. This pathway can be triggered by negative feedback from horizontal cells to cones, thus providing an additional feedback pathway. This additional pathway is mediated by a Cl^- current, can be blocked by either removing the gradient of K^+ or by adding the glutamate transporter blocker TBOA, or low concentrations of Zn^{2+}. These features point to a glutamate transporter-associated Cl^- current. The pathway has a delay of 4.7 ± 1.7 ms. The effectiveness of this pathway in modulating the cone output depends on the equilibrium potential of Cl^- (E_{Cl}) and the membrane potential of the cone. Because estimates of E_{Cl} show that it is around the dark resting membrane potential of cones, the activation of the glutamate transporter-associated Cl^- current will be most effective in changing the membrane potential during strong hyperpolarization of cones. This means that negative feedback would particularly be enhanced by this pathway when cones are hyperpolarized. Spatially, this pathway does not reach further than the direct neighbouring cones. The consequence is that this feedback pathway transmits information between cones of different spectral type.

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Abbreviations ATP, adenosine triphosphate; DNQX, 6,7-dinitroquinoxaline-2,3-dione; E_{Cl}, Cl^--equilibrium potential; GABA, γ-aminobutyric acid; GluT, glutamate transporter; HC, horizontal cell; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonicacid; I_{Ca}, Ca^{2+} current; $I_{Cl(Ca)}$, Ca^{2+}-dependent Cl^- current; $I_{Cl(GABA)}$, GABA-gated Cl^- current; $I_{Cl(GluT)}$, glutamate transporter-associated Cl^- current; L cone, long wavelength sensitive cone; M cone, middle wavelength sensitive cone; Panx1, pannexin 1; PC, primary surround-induced current; SC, secondary surround-induced inward current; S cone, short wavelength sensitive cone; TBOA, DL-*threo*-β-Benzyloxyaspartic acid; THC, triphasic horizontal cell.

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Introduction

Cones hyperpolarize when stimulated with a spot of light. This hyperpolarization reduces when the spot size is increased (Baylor *et al.* 1971), suggesting that cones are inhibited by surround stimulation. This is most prominent when cones are strongly hyperpolarized by a small spot of light and subsequently stimulated by an additional annulus or full field stimulus (O'Bryan, 1973; Lasansky, 1981; Piccolino *et al.* 1981). In such conditions, cones will depolarize. These depolarizing responses are mediated by feedback from horizontal cells (HC) to cones and depend on a Cl^- current (Lasansky, 1981; Wu, 1991; Barnes & Deschênes, 1992; Thoreson & Burkhardt, 1991) (Fig. 1, ②). It has been suggested that the underlying current was a GABA-gated Cl^- current ($I_{Cl(GABA)}$) (Wu, 1991) or a Ca^{2+}-dependent Cl^- current ($I_{Cl(Ca)}$) (Barnes & Deschênes, 1992). Later experiments, however, indicated that HCs feed back to cones by modulating the Ca^{2+} current (I_{Ca}) in cones (Verweij *et al.* 1996) leading to the activation of $I_{Cl(Ca)}$ (Kraaij *et al.* 2000). The underlying mechanism of this feedback pathway involves an ephaptic and a Panx1/ATP-mediated component (Kamermans *et al.* 2001; Klaassen *et al.* 2011; Vroman *et al.* 2014).

The ephaptic/Panx1/ATP feedback mechanism can account for the surround-induced depolarizing responses and their dependency on Cl^-. Negative feedback from HCs to cones shifts the activation potential of I_{Ca} in cones to more negative potentials (Verweij *et al.* 1996). This induces an increase in Ca^{2+} influx into the cone synaptic terminal, leading to activation of $I_{Cl(Ca)}$. Depending on the Cl^--equilibrium potential (E_{Cl}) in cones, this may lead to a depolarization of the cone. However, this mechanism does not account for the finding that surround-induced depolarizing responses are most prominent when cones are strongly hyperpolarized (O'Bryan, 1973; Lasansky, 1981; Piccolino *et al.* 1981). When cones are strongly hyperpolarized, their membrane potential is almost outside the activation range of the Ca^{2+} channels (Verweij *et al.* 1996), meaning that the feedback-induced shift in the activation potential of I_{Ca} will hardly lead to modulation of the Ca^{2+} influx. $I_{Cl(Ca)}$ will therefore not be activated.

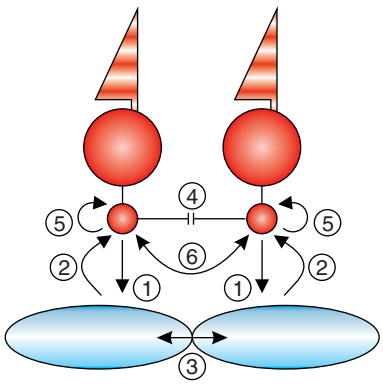

Figure 1. Schematic drawing of the pathways by which cones interact with each other
① Glutamatergic pathway from cones (red) to horizontal cells (blue). ② Ephaptic/Panx1/ATP mediated negative feedback pathway. ③ Gap junction coupling of horizontal cells. ④ Gap junction coupling of cones at the tips of the telondendria. ⑤ Glutamatergic auto-feedback. Glutamate released by the cone activates the cone's own glutamate transporters. ⑥ Glutamate spillover. Glutamate released by one cone activates glutamate transporters in neighbouring cones.

Cones can interact with each other in various ways (Fig. 1). Cones project to HCs via a sign preserving pathway ①. These signals are integrated in HCs because they are strongly electrically coupled by gap junctions ③ before they are fed back negatively to the cones ②. In addition, cones are electrically coupled by gap junctions at the tips of their telondendria ④. In the dark-adapted retina this coupling is pronounced but (almost) absent in the light-adapted retina (Ribelayga *et al.* 2008; Li & DeVries, 2011; Jin *et al.* 2015). Furthermore, Szmajda and DeVries (2011) showed that glutamate release by one cone not only activates its own glutamate transporters (Picaud *et al.* 1995) ⑤, but can spill over to the neighbouring cone and activate the glutamate transporters in that cone ⑥. How these pathways interact is not fully resolved.

In this study, we show that this glutamate spillover pathway interacts with the negative feedback pathway from HCs to cones. We studied negative feedback from HCs to cones in strongly hyperpolarized cones and found a novel feedback pathway particularly efficient at hyperpolarized cone membrane potentials. The glutamate released by cones, either dark-induced or feedback-induced, spills over to neighbouring cones and activates the glutamate transporter-associated Cl^- current ($I_{Cl(GluT)}$). This leads to a depolarization of the cone, enhancing negative feedback from HCs to cones. If cones are hyperpolarized to such an extent that modulating I_{Ca} does not lead to an increase in Ca^{2+} influx, this pathway can bring the cone membrane potential back into the operating range of I_{Ca}. This process operates over short distances, as only directly neighbouring cones are affected.

Methods

Experimental animals and isolated retina preparation

Animal experiments were performed under the responsibility of the ethical committee of the Royal Netherlands Academy of Arts and Sciences acting in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Goldfish, *Carassius auratus*, were killed by decapitation and the eyes enucleated. The retina was isolated and placed, receptor side up, in the recording chamber.

Solutions

Control Ringer's solution contained (in mM): 102.0 NaCl, 2.6 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 28.0 $NaHCO_3$, 5.0 glucose. One hundred μM picrotoxin was added to exclude that GABA-mediated mechanisms modulated the strength of feedback and in that way interfered with our measurements (Endeman *et al.* 2012). The Ringer's solution was continuously gassed with 2.5% CO_2 and 97.5% O_2 to yield a pH of 7.6. Concentrations of the drugs used are indicated in the text and legends. Pipette solution (in mM): 87 potassium gluconate, 10 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 Hepes, 10 ATP-K_2, 1 GTP-Na_3, 20 phosphocreatine-Na_2, 50 units ml^{-1} creatine phosphokinase, adjusted with NaOH to pH 7.3 and resulting in a E_{Cl} of -55 mV. For other values of E_{Cl} the concentration of potassium gluconate and KCl of the pipette solution were adjusted accordingly. All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands), except for Hepes and acetate, which was supplied by Merck KGaA (Darmstadt, Germany) and DL-*threo*-β-Benzyloxyaspartic acid (TBOA), which was obtained from Tocris (Bristol, UK).

Electrodes and recording set-up

Electrophysiological recordings of cones were performed following published methods (Kraaij *et al.* 2000). Patch pipettes (resistance 3–5 MΩ) were pulled from borosilicate glass capillaries (GC-150T-10; Harvard Apparatus Ltd, Cambridge, UK) using a Brown Flaming Puller (Model P-87; Sutter Instruments Company, Novato, California, USA). Pipettes were connected to an Axopatch 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA, USA; four-pole low pass Bessel filter setting: 2 kHz). All data shown are corrected for the junction potential. Data were digitized and stored with a personal computer using a CED 1401 AD/DA converter at 4KHz using Signal software (v. 3.07; Cambridge Electronic Design (CED), Cambridge, UK) to acquire data, generate voltage command outputs and drive light stimuli.

Light stimuli

A 17 μm white light spot (1 log) was focused through a ×60 water immersion objective on the cone outer segment. A 4500 μm 'full field' white spot (0 log) was projected through the microscope condenser. The light stimulator consisted of two homemade LED stimulators based on a three-wavelength high-intensity LED (Atlas, Lamina Ceramics Inc., Westhampton, NJ, USA). The peak wavelengths of the LEDs were 624, 525 and 465 nm, respectively, with bandwidths smaller than 25 nm. An optical feedback loop ensured linearity. The output of the LEDs was coupled to the microscope via light guides. White light consisted of equal quantal output of the three LEDs. Log intensity at zero was 3.8×10^{15} quanta $m^{-2}s^{-1}$.

Data analysis

Data were analysed using Office Excel 2010. Fitting of the data was performed using Origin Pro (v8, Origin Lab Corporation). Significance was tested using a two-tailed paired *t*-test. The ANOVA was performed using IBM SPSS Statistics v21. All error bars represent the standard error of the mean.

Results

Feedback from HCs to cones was recorded in cones using whole cell voltage clamp techniques (Verweij *et al.* 1996). The direct light response of the cone that we recorded from was saturated with a 17 μm spot of white light for the full duration of the protocol. A 500 ms full field stimulus was used to hyperpolarize the cones in the rest of the retina leading to hyperpolarization of HCs. HCs feed back to cones by shifting the activation range of I_{Ca} of cones towards more negative potentials (Verweij *et al.* 1996). The resulting Ca^{2+} influx leads to an increase in glutamate release, making this pathway a negative feedback pathway. The feedback-induced modulation of I_{Ca} can be seen as an inward current when the cone is clamped at a potential within the activation range of I_{Ca} (Fig. 2*Ba*). Studies have shown that outside the activation range of I_{Ca}, the full field stimulus does not induce a current (Verweij *et al.* 1996; Hirasawa & Kaneko, 2003), suggesting that only I_{Ca} is modulated by feedback. Although this pathway is not mediated by GABA (Verweij *et al.* 1996), GABA modulates the strength of this pathway (Endeman *et al.* 2012). Furthermore, HCs also express $GABA_A$-receptors. As E_{Cl} in HCs is more positive than the dark resting membrane potential of HCs, application of GABA will depolarize HCs (Kamermans & Werblin, 1992; Verweij *et al.* 1998). To prevent interference of these GABAergic pathways, all experiments were performed in the presence of the GABA antagonist picrotoxin.

Recently, we slightly modified the procedure to isolate the retinas. Instead of removing a large number of rods from the retina to visualize the cone more easily (Fig. 2*Aa*) (Verweij *et al.* 1996), we left the tissue more intact (Fig. 2*Ab*). In such a preparation, the cones could be detected as slightly lighter areas beneath the rods (Fig. 2*Ab*, right). Negative feedback measured in this preparation differed to some extent from that measured in a preparation where rods were removed (Fig. 2*Bb*). In both preparations, the modulation of I_{Ca} was visible and the maximum amplitude of feedback occurred at about –46 mV (Fig. 2*B*, red arrowheads). We will call this current the primary surround-induced current (PC). However, in the more intact preparation a secondary surround-induced inward current (SC) was also present (Fig. 2*Bb*, blue arrowhead). This current was most prominent at potentials more negative than the activation range of I_{Ca} and its amplitude increased towards more

negative potentials. Both PC and SC were observed in all cones studied in this preparation, whereas PC was present and SC was absent in the preparation used by Verweij *et al.* (1996). The nature of PC has been the subject of many studies and is due to a combination of an ephaptic and Panx1/ATP-mediated modulation of I_{Ca} (Kamermans *et al.* 2001; Hirasawa & Kaneko, 2003; Klaassen *et al.* 2011; Wang *et al.* 2014; Vroman *et al.* 2014). However, what is the nature of SC?

The secondary surround-induced inward current is mediated by horizontal cells

First, we determined whether SC resulted from activation of second order neurons or was an intrinsic property of the cones themselves. Application of DNQX (50 μM), a glutamate receptor antagonist, led to a near complete block of this current (Fig. 3*A*, black trace: control; red trace: DNQX). On average DNQX reduced PC and SC about equally (PC: 78.8 ± 8.3%; $n = 6$; $P < 0.001$; SC: 72.1 ± 11.5%; $n = 5$; $P < 0.001$). This shows that both PC and SC were generated via a glutamatergic pathway, probably via modulation of HCs. If this were the case then blocking the output of HCs should also block both PC and SC. Hepes (20 mM) and acetate (25 mM) block the feedback from HCs to cones (Hirasawa & Kaneko, 2003; Fahrenfort *et al.* 2009) probably via intracellular acidification and by buffering the pH in the synaptic cleft. Indeed both PC and SC were reduced when feedback was blocked by these substances (Fig. 3*A*, black traces: control; red traces: Hepes or acetate). On average Hepes reduced PC and SC by 86.7 ± 6.6% ($n = 6$; $P < 0.001$) and 73.9 ± 7.6% ($n = 6$; $P < 0.001$) respectively and acetate by 88.6 ± 5.1% ($n = 5$; $P < 0.001$) and 75.3 ± 4.3% ($n = 5$; $P < 0.001$) respectively (Fig. 3*B*). Finally, we tested the intensity range over which SC could be modulated (Fig. 3*C*). Responses to light stimuli of increasing intensities were measured (Fig. 3*Ca*) and the normalized peak response amplitude was plotted as a function of intensity (Fig. 3*Cb*, open symbols). A Hill function was fitted through the data points (Fig. 3*Cb*, red line). The dynamic range of SC spanned about 2.5 log units, which is similar to the dynamic range of HCs (Fig. 3*Cb*, blue line) (Kraaij *et al.* 2000). These results show that both PC and SC are generated by HC activity.

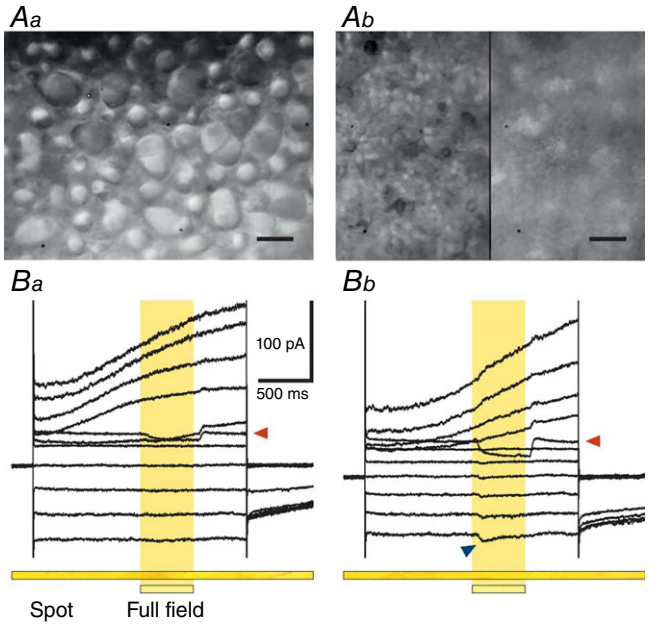

Figure 2. Negative feedback induced currents in cones
Aa, microscopy image of the preparation as described by Verweij *et al.* (1996). *Ab*, preparation as used in this study. The focus is on the rod outer segments in (*Aa*) and on the cone inner segments in (*Ab*). As the rods are not removed, the cones are barely visible, making it harder to target cones for patch clamp experiments. The scale bars represent 10 μm. *Ba*, responses of a voltage clamped cone using a step protocol from –110 to –10 mV. A spot with a diameter of 17 μm was focused on the recorded cell during the whole protocol. One second after the start of a membrane potential step, a full field stimulus was applied for 500 ms. *Bb*, responses using the same stepping protocol as for (*Ba*), but now with the retina prepared according to the new procedure. Apart from negative feedback (red arrowhead), another surround-induced current is visible that has a transient character and increases towards more hyperpolarized potentials (blue arrowhead).

Primary surround-induced current and secondary surround-induced inward current are mediated by different mechanisms

Next, we determined whether SC is mediated by the same mechanism as PC. PC is the result of a feedback-induced shift in the activation potential of I_{Ca} to more negative potentials. SC, on the other hand, was active well outside the activation range I_{Ca} (Fig. 2*Bb*) making it unlikely that SC is mediated by modulation of I_{Ca}. In addition, the kinetics of the PC and SC differed significantly (Fig. 4*A*). SC responses were transient, peaking at 120.2 ± 27.4 ms after stimulus onset whereas PC responses were sustained. This suggests that these two inward currents (PC and SC) have different origins. Apart from the difference in kinetics, PC and SC differ in their rundown behaviour during a whole cell recording. After an initial increase, I_{Ca} tends to run-down over time (Barnes & Hille, 1989), leading to a reduction of the amplitude of PC (Fig. 4*B*, closed symbols and 4*C*). SC, on the other hand, did not show a decrease in amplitude over time (Fig. 4*B*, open symbols and 4*C*). In fact, it often slowly increased until it finally stabilized. If the underlying mechanism of PC and SC were the same, similar rates of run-down or run-up would be expected. Next, we determined whether PC and SC had a similar delay relative to the light flash by comparing the times at which PC and SC reaches 5% of their peak amplitudes and found that SC was delayed

relative to PC by 4.7 ± 1.7 ms (n = 22; P = 0.011) (Fig. 4*D*). These results show that PC and SC are mediated by separate mechanisms.

Secondary surround-induced inward current shares characteristics with the glutamate transporter-associated chloride current

We next set out to identify what type of current SC is. Figure 5*Aa* shows the whole cell IV relation of the peak SC current when E_{Cl} was set at –55 mV (open symbols). An exponential function was fitted through the data points. To prevent interference from PC, the potential range where I_{Ca} is most active was excluded (–70 mV to –10 mV). The fit shows that SC is an inwardly rectifying current. The estimated reversal potential was –63 ± 6 mV (n = 6), which is close to E_{Cl}. When E_{Cl} was set at –28 mV (Fig. 5*Aa*, closed symbols), the reversal potential shifted to –34 ± 3 mV (n = 8), suggesting that SC is an inward rectifying Cl^- current.

Is the transient character of SC an intrinsic feature of SC or does it indicate that SC is the sum of two processes?

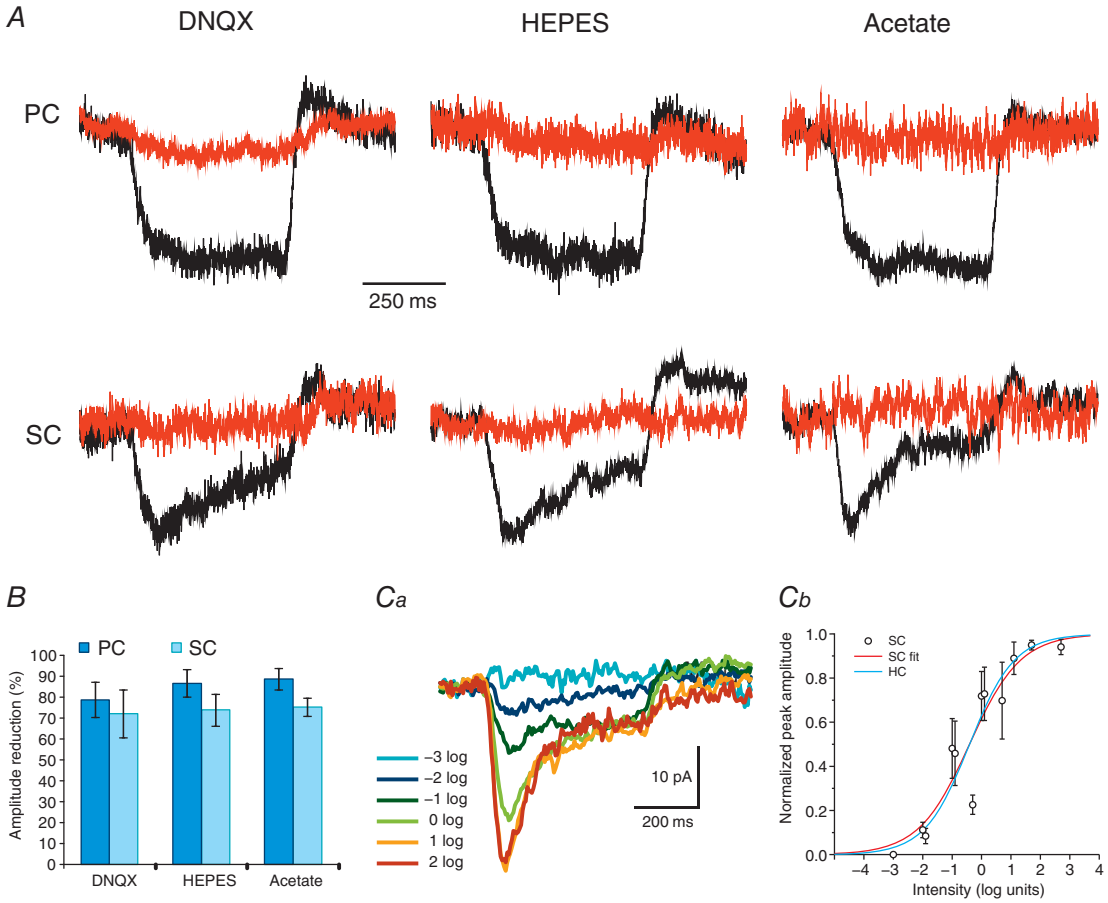

Figure 3. The SC can be reduced by known blockers of negative feedback and is light intensity dependent
A, DNQX (50 μM), a glutamate receptor antagonist, reduced PC (holding potential: –46 mV) by 78.8 ± 8.3% (n = 5; P < 0.001) and SC (holding potential: –96 mV) by 72.1 ± 11.5% (n = 6; P < 0.001). The negative feedback blockers Hepes (20 mM) and acetate (25 mM) reduced PC by respectively 86.7 ± 6.6% (n = 6; P < 0.001) and 88.6 ± 5.1% (n = 5; P < 0.001) and SC by respectively 73.9 ± 7.6% (n = 6; P < 0.001) and 75.3 ± 4.3% (n = 5; P < 0.001) Traces represent averages of five to six experiments in different cones before and after application of the drug. Black traces: control; Red traces: drug application. Each measurement is normalized to the minimum value in control conditions before averaging, no vertical axis is therefore shown. *B*, histogram of the results from (*A*). Reduction of the amplitude of the surround responses is shown for the different pharmacological agents used. *Ca*, SC responses (holding potential: –96 mV) to different intensities of the full field stimulus. Intensities are expressed in relative log units. Similar results were observed in six cells. *Cb*, intensity response relation of SC as function of intensity (open symbols). The red line is the Hill curve fitted through the data point (slope factor = 0.50). For comparison the intensity response relation of HCs is given (blue line; slope factor = 0.57) (taken from Kraaij *et al.* 2000). HC, horizontal cells; PC, primary surround-induced current; SC, secondary surround-induced inward current.

To obtain insight into this, we plotted the IV relations of the sustained part of SC (Fig 5*Ab*, E_{Cl} = –55mV: open symbols; E_{Cl} = –28 mV: closed symbols) and fitted an exponential function in the same way as before. Both the peak and the sustained IV relation showed similar inwardly rectifying characters and had similar reversal potentials that shifted with E_{Cl}. For the sustained IV relation these values were –73.9 ± 10.2 mV (n = 6) for E_{Cl} = –55 mV and –36.1 ± 7.4 mV (n = 8) for E_{Cl} = –28 mV, which did not differ significantly from the values obtained for the peak IV relation, –63 ± 6 mV (n = 6) and –34 ± 3 mV (n = 8) for E_{Cl} = –28 mV (P = 0.192 and P = 0.771 respectively). These results suggest that SC consists of a single inwardly rectifying Cl^- current.

The inwardly rectifying character of SC resembled that of the $I_{Cl(GluT)}$ (Picaud *et al.* 1995). To test whether SC is the modulation of $I_{Cl(GluT)}$, we applied the specific glutamate transporter blocker TBOA. Blocking glutamate transporters will have two effects. First, it will block the reuptake of glutamate, resulting in an increase in the extracellular glutamate concentration that will ultimately lead to saturation of the glutamate receptors on the post-synaptic cells. Such saturation will block the signal flow between pre- and postsynaptic neurons (Vandenbranden *et al.* 1996; Jabaudon *et al.* 1999; Izumi *et al.* 2002). Secondly, blocking the glutamate transporter will block $I_{Cl(GluT)}$, which will be visible as a closure of a Cl^- current in the cells expressing the glutamate transporter.

In the next experiments E_{Cl} was set at –28 mV to increase the amplitude of the SC. Figure 5*B* shows that prolonged (>5 min) application of 280 μM TBOA blocks both PC and SC whereas a shorter application of TBOA (2–3 min) hardly affected PC while SC was reduced by 46.6 ± 8.5% (P = 0.014, n = 7) (Black traces: control; red traces: TBOA). The complete block of both PC and SC after prolonged application of TBOA is probably due to saturation of synaptic transmission due to the inhibition of glutamate reuptake. Short-term application of TBOA will not saturate synaptic transmission but will block GluTs and their associated $I_{Cl(GluT)}$. Indeed we found that after

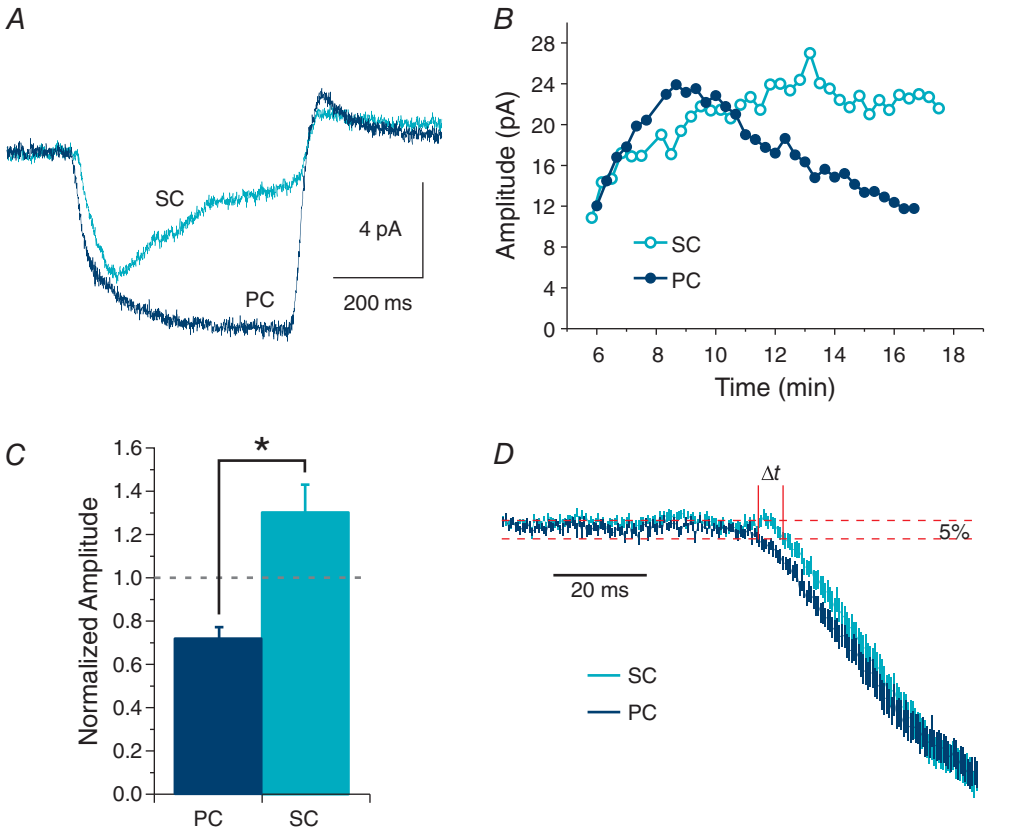

Figure 4. PC and SC are mediated by different mechanisms
A, average PC (dark blue trace, n = 7) and SC (light blue trace, n = 13). E_{Cl} = –55 mV. *B*, PC (dark blue, filled symbols) shows run-down, while SC (light blue, open symbols) does not. *C*, normalized average amplitude of PC and SC 15 min after gaining access to the cell (n = 5). PC and SC were normalized to the amplitude of PC and SC at the time PC reached its maximum. PC run-down (P = 0.004) while SC run-up (P = 0.067). *D*, SC is delayed in relation to PC. (Δt = 4.7 ± 1.7 ms; n = 22; P = 0.011). The difference in response time was measured by determining the time at which 5% of the peak response was reached. PC, primary surround-induced current; SC, secondary surround-induced inward current.

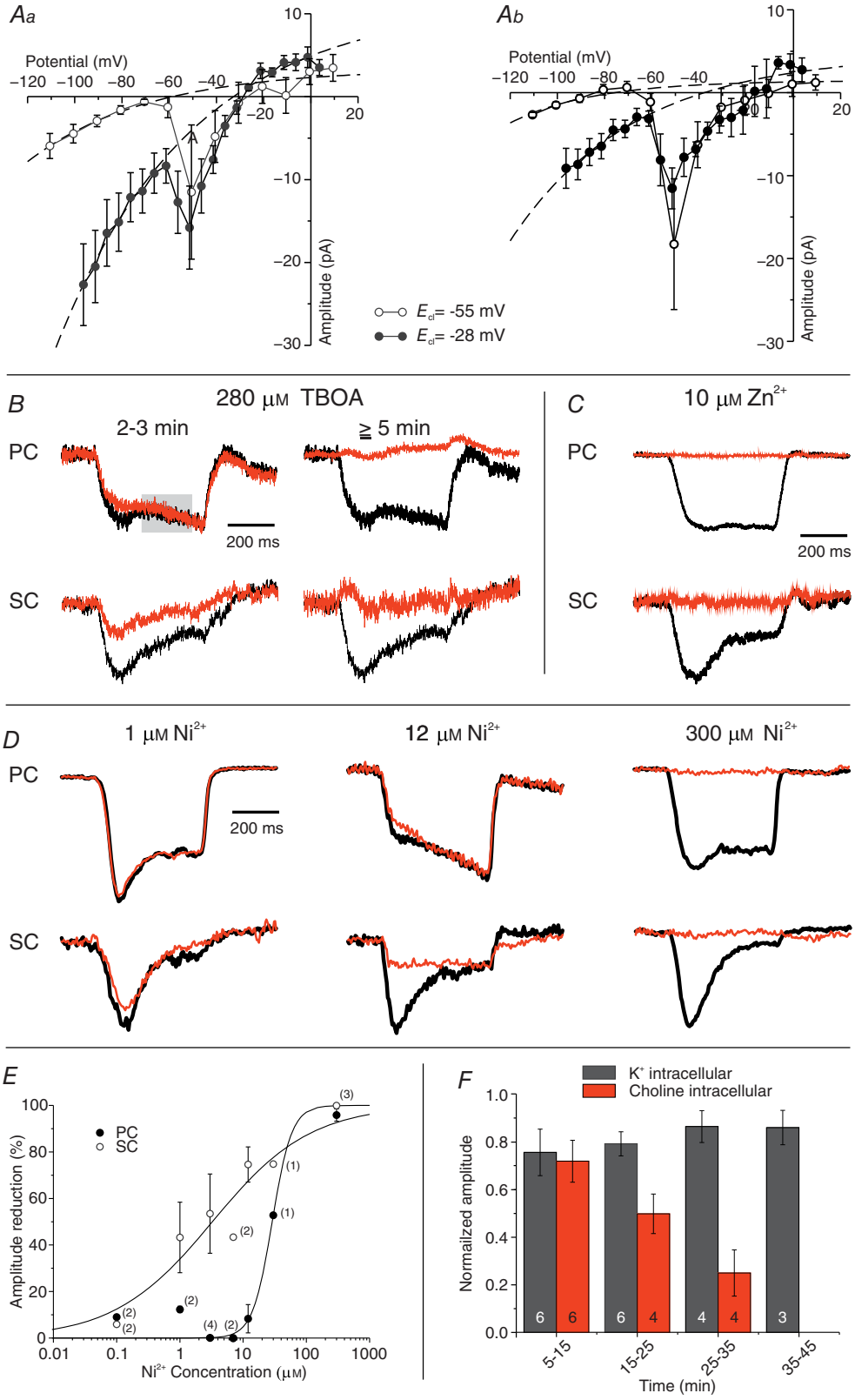

Figure 5. SC has the characteristics of the $I_{Cl(GluT)}$
Aa, IV relations of the amplitude of the surround-induced currents; the amplitude was determined by subtracting the current just before the full field light stimulus from the peak of surround-induced current (open symbols:

short-term application of TBOA, PC was hardly affected while SC was reduced strongly, suggesting that SC is mediated by $I_{Cl(GluT)}$.

GluT activity depends on the intracellular K^+ concentration (Picaud *et al.* 1995). Does this hold for SC as well? Figure 5*F* shows that the amplitude of SC decreased with time after breaking in with a pipette filled with a solution in which K^+ was replaced by choline (red bars). Such a decrease in amplitude was absent when the pipette solution contained a K^+ intracellular solution (black bars). The time it took to abolish the response completely was about 30 min, which is about the time it takes for a complete exchange of the cytosol with the pipette solution (Kraaij *et al.* 2000). These results show that SC depends on the K^+ gradient and are consistent with the notion that SC is the activation of $I_{Cl(GluT)}$.

To study $I_{Cl(GluT)}$ in more detail we set out to find a pharmacological tool that did inhibit $I_{Cl(GluT)}$ but did not lead to saturation of synaptic transmission. Divalent ions are known to affect neuronal systems in a number of ways. One of them is Zn^{2+}, which has been shown to block $I_{Cl(GluT)}$ of the cones (Spiridon *et al.* 1998). We found that 10 μM Zn^{2+} blocked both PC and SC (91.2 ± 4.9%; $n = 3$ and 93.0 ± 1.0%; $n = 3$ respectively) (Fig. 5*C*, black trace: control; red trace: 10 μM Zn^{2+}). Zn^{2+}-induced block of $I_{Cl(GluT)}$ was about equal to that induced by TBOA making it difficult to use this drug to study PC and SC independently of each other. Next, we tested whether another divalent ion, Ni^{2+}, could separate PC and SC (Fig. 5*D*). Ni^{2+} 12 μM reduced the peak of SC by 74.6 ± 7.5% ($P = 0.009$; $n = 7$; partial washout in four cells) while having no significant effect on PC ($P = 0.956$; $n = 6$) (Fig. 5*D*, black traces: control; red traces: Ni^{2+}). The effect of Ni^{2+} did not change over time, suggesting that 12 μM Ni^{2+} does not saturate synaptic transmission while it still affects $I_{Cl(GluT)}$. A dose–response curve of the effect of Ni^{2+} on PC and SC was constructed (Fig. 5*E*). The IC_{50} for Ni^{2+} on PC and SC was 28.8 ± 2.8 μM ($n = 22$) and 3.3 ± 1.2 μM ($n = 23$) respectively. In addition, we found that the reversal potential of the Ni^{2+}-blocked current was –54.9 ± 4.4 mV ($n = 4$), when E_{Cl} was set at –50 mV suggesting that Ni^{2+} inhibits a Cl^- current. As 12 μM of Ni^{2+} did not block PC, even after prolonged application, we conclude that glutamate uptake was minimally affected. Although non-specific actions of Ni^{2+} cannot be fully excluded, the results presented so far are consistent with the hypothesis that 12 μM Ni^{2+} only blocked $I_{Cl(GluT)}$ while leaving the transport of glutamate intact. In line with this is the finding of Wadiche *et al.* (1995) who showed that the transport of glutamate is independent of the associated Cl^- current.

To test whether Ni^{2+} had direct effects on I_{Ca}, we determined the shift of the half activation potential of I_{Ca} because of 12 μM Ni^{2+}. To avoid activation of GluTs, the whole retina was stimulated with a bright full field light stimulus to block glutamate release of all photoreceptors in the retina. In this way we prevented modulation of $I_{Cl(GluT)}$ to interfere with this measurement. We found no effect of 12 μM Ni^{2+} on I_{Ca}. The half activation potential shifted on average –0.1 ± 0.4 mV ($n = 5$), which is not significantly different from zero ($P = 0.752$). Ni^{2+} 12 μM did not significantly affect the peak amplitude of I_{Ca} (control: –136.4 ± 25.0 pA; 12 μM Ni^{2+}: –133.3 ± 22.3 pA; $P = 0.455$; $n = 5$), suggesting that other non-specific actions of Ni^{2+} are not prominently present.

Low concentrations of Ni^{2+} have been shown selectively to block T-type Ca^{2+} channels (Lee *et al.* 1999), but these Ca^{2+}-channels are not expressed in cones (Barnes & Hille, 1989). However, to be sure that the effect we observed could not be attributed to T type Ca^{2+} channels we tested the effect of the selective T type Ca^{2+} channel blocker mibefradil (Martin *et al.* 2000) on SC and found no significant reduction in amplitude ($P = 0.26$; $n = 9$; data not shown). These results show that 12 μM Ni^{2+} did not affect I_{Ca} directly and that Ni^{2+} at this concentration can be used as a blocker for $I_{Cl(GluT)}$.

$E_{Cl} = -55$ mV; filled symbols: $E_{Cl} = -28$ mV. Exponential functions were fitted through the data points outside the range of the cone I_{Ca} and the reversal potentials were estimated. The average reversal potential for $E_{Cl} = -55$ mV was 63 ± 6 mV ($n = 6$) and for $E_{Cl} = -28$ mV, 34 ± 3 mV ($n = 8$). *Ab*, IV relations from the same cells, only now measured in the sustained part of the surround induced current. The reversal potentials for $E_{Cl} = -55$ mV is –73.9 ± 10.2 mV and for $E_{Cl} = -28$ mV is –36.1 ± 7.4 mV. *B*, glutamate transporter blocker 280 μM TBOA caused a reduction in the SC amplitude (black traces: control; red traces: TBOA). The two columns show measurements at different times after onset of wash-in of the pharmacological agent (left column: 2–3 min; right column: ≥5 min). PC is normalized to the highlighted area in control (grey box). SC is normalized to the peak response in control. 2–3 min after wash-in of TBOA, there was no significant reduction in PC ($P = 0.128$; $n = 7$). For SC, however, a significant reduction in amplitude was observed of 46.6 ± 8.5% ($P = 0.014$; $n = 7$). At ≥5 min both PC and SC were blocked. E_{Cl} was set at –28 mV. *C*, application of 10 μM Zn^{2+} (red traces) caused a complete block of both PC and SC ($n = 3$). The traces represent the average. Measurements are normalized as described in (*B*). *D*, PC and SC responses before (black traces) and after (red traces) application of different concentrations of Ni^{2+}. Traces are normalized as described in (*B*). *E*, dose–response curve for the effect of Ni^{2+} on PC and SC. The IC_{50} for PC was 28.8 ± 2.8 μM ($n = 22$) and for SC was 3.3 ± 1.2 ($n = 23$). *F*, intracellular substitution of K^+ for choline blocked the SC over a course of ∼30 min (black bars: control; red bars: K^+ substituted for choline). The numbers in the bars indicate the number of cells analysed at that time interval. PC, primary surround-induced current; SC, secondary surround-induced inward current.

What is the source of glutamate that activates $I_{Cl(GluT)}$

So far we have shown that SC depends on HC hyperpolarization, and is mediated by $I_{Cl(GluT)}$. SC could be measured at membrane potentials well outside the activation range of I_{Ca}, potentials where cones do not release glutamate. Therefore, the voltage clamped cone cannot provide the glutamate that activates GluT. The glutamate has to originate from a different source. Photoreceptors are the only source of glutamate in the outer retina. As we used light intensities well above the operating range of rods, the neighbouring cones are the only possible source for glutamate. As glutamate transporters are mainly found in the bottom outer surface of the cone synaptic terminal (Vandenbranden *et al.* 2000) interactions via the telondendria seem improbable. In a possible scenario, glutamate released by these cones spills over to the recorded cone and activates $I_{Cl(GluT)}$, which can be seen as SC. To test this, we determined the spatial reach of this glutamate spillover. The rationale for the current experiment was as follows. In the dark condition, glutamate release from neighbouring cones spills over to the centre cone and activates the $I_{Cl(GluT)}$ (Szmajda & DeVries, 2011). In whole cell configuration, the direct light response of a cone runs down in about 5–10 min (Fig. 6*A*, closed symbols) while SC runs up (open symbols). When the light response had run-down, the recorded cone and its direct neighbours were stimulated with a small spot of light. In this condition, the spot will not induce a direct light response in the recorded cone, but will hyperpolarize neighbouring cones and reduce their glutamate release. The consequence is that the glutamate spilled over from the neighbouring cones to the cone recorded from will diminish, leading to a reduction of $I_{Cl(GluT)}$. By increasing the diameter of the spot and measuring the amplitude of $I_{Cl(GluT)}$, the spatial extent of the glutamate spillover can be estimated.

In the next experiment we investigated the activation of $I_{Cl(GluT)}$ due to sustained glutamate spillover in the dark instead of surround-induced spillover evoked by light stimulation. We determined the IV relation in the dark and when a spot was present, in a condition when the direct light response had fully run down. We used two spot sizes. The smallest spot (17 μm) covers the recorded cone and its direct neighbours while the larger spot (140 μm) covers a radius of about 11 surrounding cones. Figure 6*B* shows the resulting IV relations. We subtracted the IV relations with and without the 17 μm spots (Fig. 6*B*, closed symbols) and fitted an exponential function through the result (Fig. 6*B*, dashed line). The subtracted current had a reversal potential of –31.5 ± 3.7 mV ($n = 10$) ($E_{Cl} = -28$ mV), consistent with the modulation of $I_{Cl(GluT)}$. No additional reduction of $I_{Cl(GluT)}$ occurred when the 140 μm spot was used (Fig. 6*B*, open symbols; ANOVA; $F_{1,10} = 0.064$; $P = 0.805$; $\mu^2 = 0.006$; $n = 6$). This suggests that most of the glutamate reaching the recorded cone spills over from its direct neighbours and very little originates from cones that are further away.

Feedback-induced activation of the $I_{Cl(GluT)}$ modulates the cone output

Does the activation of $I_{Cl(GluT)}$ affect the cone membrane potential and thus the cone's output? To study this, we

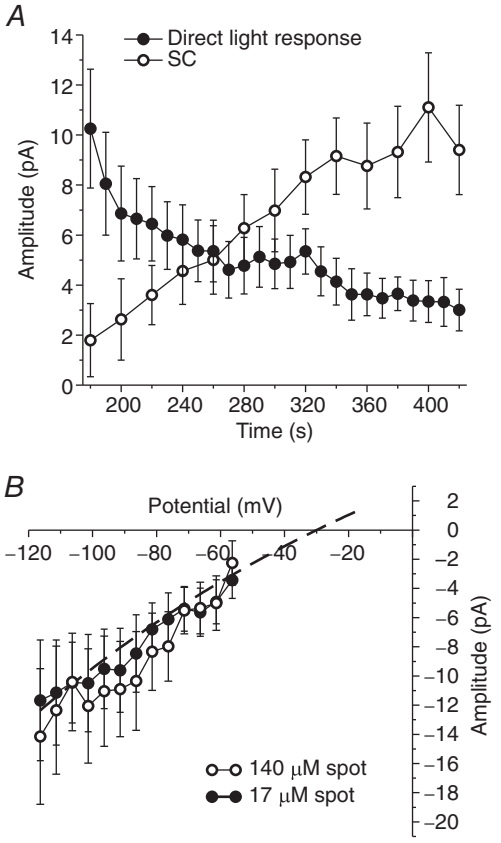

Figure 6. Glutamate spillover reaches only the synapses of neighbouring cones
A, as the direct light response to the spot reduces due to bleaching (holding potential: –60 mV), the amplitude of SC increases (holding potential: –96 mV). *B*, difference of the IV relations determined with a 17 μm spot and in the dark (filled symbols). In the dark, the centre cone receives glutamate spilled over from neighbouring cones. When using the spot, however, the neighbouring cones do not release glutamate. The difference IV plot therefore shows the $I_{Cl(GluT)}$ activated by glutamate spilled over from neighbouring cones. Exponential functions were fitted through the individual IV relations and the mean fit was plotted as a dotted line. The estimated reversal potential was –31.5 ± 3.7 mV with $E_{Cl} = -28$ mV ($n = 10$). The difference IV plot using the 140 μm spot (open symbols; $n = 6$). To test whether glutamate spilled over from cells further away than the direct neighbours is also activating the GluT-associated I_{Cl}, we plotted an IV relation of measurements in dark conditions, using the 17 μm spot and using the 140 μm spot. The difference IV plot for the 140 μm spot for the 17 μm spot do not differ, showing that glutamate spillover does not reach further than one cone. SC, secondary surround-induced inward current.

saturated the cone being recorded from with a small spot of light, flashed a full field light stimulus and determined the cone voltage response. As SC is maximal at very negative potentials, current was injected to hyperpolarize the cone's dark membrane potential outside the activation range of I_{Ca}. This procedure also excluded interference of PC with the cone voltage response. The amount of injected current was adjusted stepwise such that the cone voltage response to the full field stimulus was obtained for various cone membrane potentials. The light-induced response was always depolarizing, had a transient character and increased towards more negative membrane potentials (Fig. 7*A*). To test if the depolarization was caused by $I_{Cl(GluT)}$, we shifted E_{Cl} from –50 mV to –2 mV. The extrapolated reversal potential of the light response with E_{Cl} at –50 mV was –55.1 ± 5.6 mV ($n = 6$) and with E_{Cl} at –2 mV the reversal potential was –15.4 ± 8.7 mV ($n = 6$) indicating that the light response was mediated by a Cl^- current, presumably $I_{Cl(GluT)}$. Next we blocked $I_{Cl(GluT)}$ with 12 μM Ni^{2+} ($E_{Cl} = -50$ mV), which inhibits $I_{Cl(GluT)}$ without saturating the outer retina with glutamate (see above) and found that the surround-induced depolarizing response at a membrane potential of –67.4 ± 1.2 mV was reduced by 55.5 ± 8.2% ($n = 5$; $P = 0.003$) (Fig. 7*B*). In three of five cells tested, a partial washout was observed. Together these results show that activation of $I_{Cl(GluT)}$ by glutamate released from neighbouring cones causes modulation of the central cone's membrane potential and thus its output.

Discussion

In this study, we show that the output of cones is modulated through activation of a GluT by the glutamate released from their direct neighbouring cones. This pathway can be activated by negative feedback from HCs to cones, thus providing an additional feedback pathway. We have shown that this pathway can be blocked by TBOA and low concentrations of the divalent ion Zn^{2+} and by removing the gradient of K^+ from the recorded cone. Furthermore, the pathway was mediated by a Cl^- current. These features suggest that it is mediated by activation of $I_{Cl(GluT)}$ in cones. This pathway is much slower than the ephaptic feedback pathway (Vroman *et al.* 2014) and has a slight delay (4.7 ± 1.7 ms). The effectiveness of the pathway will therefore depend on E_{Cl} and, as E_{Cl} is close to the dark resting membrane potential of cones, it will enhance negative feedback particularly when cones are strongly hyperpolarized.

Activation of the $I_{Cl(GluT)}$ by negative feedback from horizontal cells to cones

We have shown that glutamate released from cones can diffuse to neighbouring cones and activate $I_{Cl(GluT)}$. This is in agreement with the results of Szmajda and DeVries (2011) who demonstrated glutamate spillover between cones. In their experiments, glutamate release was triggered by artificially polarizing cones or by a dark flash. Here we show that a light flash can also induce glutamate spillover. In this case, glutamate release induced by negative feedback from HCs to cones spills over to neighbouring cones. This feedback-induced spillover will be present under any stimulus condition, but in most conditions, the current it induces will be masked by the reduction in glutamate release due to direct light stimulation. One needs to realize that spillover of glutamate may have two effects on the surrounding cones: (1) it activates GluT and thus induces SC, and (2) potentially it activates glutamate receptors on dendrites innervating the surrounding cones. This latter effect will not be visible in the cones, but will be physiologically highly relevant. To detect SC, the presence of the 17 μm spot covering the centre cone and its direct neighbours is essential (Fig. 8*A*). None of the cones covered by the spot will respond directly to the full field stimulus, but

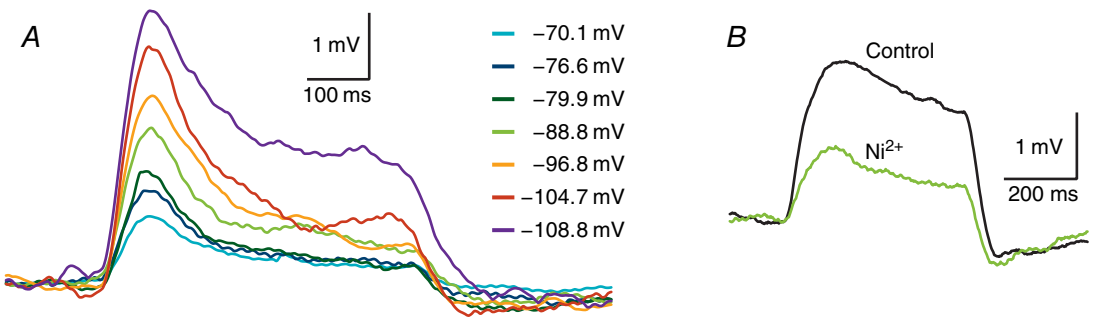

Figure 7. Glutamate spillover modulates the cone membrane potential
A, surround-induced cone responses in current clamp (constant saturating spot, 500 ms full field stimulus). By injecting current, the membrane potential was hyperpolarized to the indicated values. $E_{Cl} = -50$ mV. *B*, Ni^{2+}, a divalent ion we used to block the $I_{Cl(GluT)}$, inhibited the spillover induced depolarization by 55.5 ± 8.2% ($n = 5$; baseline at –70 mV). Partial washout was observed in three cells. The traces represent the average, measurements are normalized to the peak values of the control conditions.

will be affected by negative feedback leading to an increase in their glutamate release. This glutamate will spill over to the centre cone and, with a slight delay, activate $I_{Cl(GluT)}$ (Fig. 8*B*).

The absence of the SC in measurements from previous studies seems to be due to the difference in tissue preparation. In our procedure, the retina was isolated without removing the rods, leaving the retina in a more physiological condition. This might have prevented glutamate from diffusing too quickly into the perfusion medium. This makes it highly probable that SC is absent in retinal slices.

Feedback-induced activation of the GluT-associated I_{Cl} depolarizes the cone

It has been shown that GluT activation due to glutamate spillover can modulate synaptic transmission in different systems in the central nervous system (Tzingounis & Wadiche, 2007). Whether activation of the $I_{Cl(GluT)}$

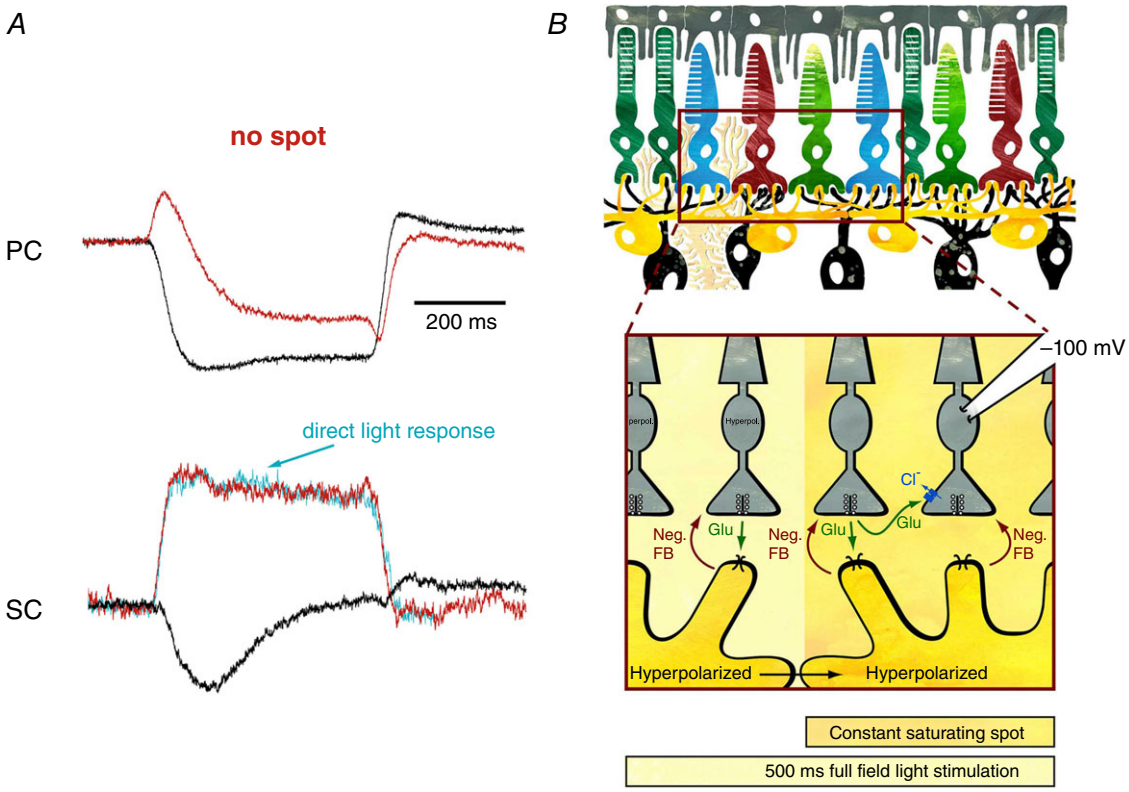

Figure 8. The proposed mechanism
A, spot is crucial for the presence of SC, but not for PC. When only the full field light stimulus is used and no spot, one can still measure PC (top, red trace; holding potential: –46 mV), although it is mixed with a direct light response. The SC, however, is dependent on the saturating spot. When only a full field stimulus is used, the response (bottom, red trace; holding potential: –96 mV) is identical to a direct light response (light blue trace). The direct light response is measured using a spot of the same intensity as the full field stimulus. Black traces are measurements in the same cells using the saturating spot. SC and PC are normalized to the peak response in control conditions (black traces). Traces represent the averaged measurements from six cells. *B*, schematic representation of the light stimulation used and the proposed underlying mechanism of SC. The top illustration of the retina shows the innervation of the HC dendritic tips (dark yellow) into the cone terminals, forming a highly specialized synapse. The bottom schematic is simplified and spaced out to show the mechanisms involved in SC. A constant saturating light stimulation is focused on the recorded cone and its direct neighbours, preventing them from releasing glutamate. A 500 ms full field light stimulation is then applied to hyperpolarize surrounding cones. This leads to the hyperpolarization of the HCs (dark yellow). The HCs then send a negative feedback signal to all cones, including the cones within the saturating spot (red arrows). This leads to an increased release of glutamate (green arrows) from all cones except for the one being recorded from because the clamping potential is set outside the range of I_{Ca}. The glutamate release from the cone adjacent to the recorded cone spills over and activates the glutamate transporters and consequently $I_{Cl(GluT)}$ (blue). Schematic drawing of the first layers of the retina. Photoreceptors release glutamate (red) on to HCs and bipolar cells. Glutamate release is not restricted to the synaptic terminals of cones by spill-overs to neighbouring cones and activated glutamate transporters on the neighbouring cones, which can be seen as an inward current (traces). Glu, glutamate; HCs, horizontal cells; Neg. FB, negative feedback; PC, primary surround-induced current; SC, secondary surround-induced inward current.

modulates the membrane potential of cones and thus its output depends on the exact value of E_{Cl}. Estimates of E_{Cl} vary from –42 to –55 mV. Estimates of Thoreson and Bryson (2004; salamander) show that E_{Cl} is very close to the cone dark resting membrane potential (–46 mV). Kraaij *et al.* (2000; goldfish) found a more hyperpolarized value for E_{Cl} (–55 mV). Other estimations put the E_{Cl} at –42 mV (Thoreson & Burkhardt, 1991; turtle), between –34 and –43 mV (Sarantis *et al.* 1988; salamander), at –31 (Szmajda & DeVries, 2011; ground squirrel) and at –47 mV (Kaneko & Tachibana, 1986; turtle). Taken together, it seems probable that E_{Cl} is very close to the dark membrane potential of cones. Therefore, modulation of $I_{Cl(GluT)}$ in the dark will hardly affect the membrane potential. However, when cones are hyperpolarized, activation of $I_{Cl(GluT)}$ will depolarize cones. In addition, activation of $I_{Cl(GluT)}$ may lead to shunting inhibition of the cones, thus reducing the gain of the synaptic transmission from cones to second order neurons. Consistent with this notion are the results of Veruki *et al.* (2006) who showed that activation of $I_{Cl(GluT)}$ in bipolar cells by glutamate spillover from neighbouring BCs reduced glutamate release as a result of shunting inhibition (Veruki *et al.* 2006). We tested directly whether activation of $I_{Cl(GluT)}$ could modulate the membrane potential of cones. With E_{Cl} at –50 mV, cones depolarized due to activation of $I_{Cl(GluT)}$ by glutamate spillover from neighbouring cones. This shows that, at least when cones are hyperpolarized, glutamate spillover modulates the cone membrane potential and thus its output.

Cl^- dependence of surround-induced responses

Many researchers have shown that negative feedback from HCs to cones modulates a Cl^- current in cones (Lasansky, 1981; Thoreson & Burkhardt, 1991; Wu, 1991; Barnes & Deschênes, 1992). Potentially there are three Cl^--mediated currents that could be involved: (1) $I_{Cl(GABA)}$; (2) $I_{Cl(Ca)}$; and (3) $I_{Cl(GluT)}$. Surround-induced responses have been proposed to be mediated by $I_{Cl(GABA)}$ (Murakami *et al.* 1982*a*,*b*; Wu, 1991). Wu (1991) showed that the depolarizing response could be blocked by bicuculline, a $GABA_A$ receptor antagonist. However, the effect of bicuculline was only partial and may have been due to its depolarizing effect on the HCs (Umino & Dowling, 1991). Furthermore, others found no effect of GABA agonists or antagonists on the surround-induced depolarization in cones (Miller *et al.* 1981; Thoreson & Burkhardt, 1990). We and others have found that $I_{Cl(GABA)}$ is not mediating negative feedback but rather plays a modulatory role (Gilbertson *et al.* 1991; Xiong-Li & Wu, 1993; Verweij *et al.* 1996; Kamermans *et al.* 2001; Endeman *et al.* 2012). It seems that the surround-induced depolarizing responses in cones are caused by the activation of $I_{Cl(Ca)}$ (O'Bryan, 1973; Lasansky, 1981; Piccolino *et al.* 1981; Kraaij *et al.* 2000). Such activation can lead to prolonged depolarizations (Burkhardt *et al.* 1988; Thoreson & Burkhardt, 1991).

As we have shown here, negative feedback can also act via $I_{Cl(GluT)}$. The pathway is particularly active when cones are hyperpolarized outside the operational range of the Ca^{2+} channels. At those potentials, activation of $I_{Cl(GluT)}$ underlies the surround-induced depolarizing responses in cones. We propose the following interaction between I_{Ca}, $I_{Cl(Ca)}$ and $I_{Cl(GluT)}$. The activation of $I_{Cl(GluT)}$ is prominent only when cones are strongly hyperpolarized and will depolarize the cones back in the operating range of I_{Ca}. By doing so it will increase the efficiency of the ephaptic and Panx1/ATP feedback systems, which will lead to an increased influx of Ca^{2+} into the cone terminal and an activation of $I_{Cl(Ca)}$, leading to a further depolarization.

Functional implications of the surround-induced activation $I_{Cl(GluT)}$

What is the functional consequence of feedback-induced activation of $I_{Cl(GluT)}$? The first notion would be that glutamate spillover might induce spatial blur (Szmajda & DeVries, 2011). In principle this will happen, but given the distance glutamate spills over, this effect is very small and may even be insignificant. However, as observed before by Szmajda and DeVries (2011), glutamate spills over to neighbouring cones of a different spectral type. The functional consequences of this effect will be substantial. In fish, cones are arranged in a very regular array (Fernald, 1981; Bowmaker & Kunz, 1987; Beaudet *et al.* 1997; Li *et al.* 2009), where L cones flank M and S cones and M cones flank L and ultraviolet light-sensitive cones. Glutamate released from cones will therefore spill over to cones of different spectral sensitivity, substantially affecting the spectral sensitivity of second order neurons. For instance, L cones are direct neighbours of S cones. Glutamate released by L cones will thus spill over to S cones and vice versa. As L cones have larger synaptic terminals than M and S cones (Li *et al.* 2009), it is probably that they are releasing more glutamate than S cones. Therefore, the influence of L cones on S cones will be more prominent than the other way around.

Fish have three types of cone driven HCs, all with a specific spectral sensitivity. Monophasic HCs hyperpolarize to light of all wavelengths, biphasic HCs hyperpolarize to short and middle wavelength light and depolarize to long wavelength light, and triphasic HCs (THCs) hyperpolarize to short and long wavelength light and depolarize to middle wavelength light. These responses are thought to be generated via a cascade of feed forward and feedback connections (Stell *et al.* 1975; Kamermans *et al.* 1990). Although the spectral sensitivity of the HCs is well described by this model, some discrepancies remain. The cascade model makes a

very specific prediction. The hyperpolarizing response to long wavelength light of the THC should be generated by negative feedback from the biphasic HCs to the S cones. As the biphasic HCs depolarize to long wavelength light, negative feedback, due to long wavelength light stimulation in the S cone, should be inverted. This prediction has been tested directly and could not be confirmed (Kraaij *et al.* 1998) leaving the hyperpolarizing response to long wavelength light in THCs unexplained. Can spillover of released glutamate resolve this discrepancy between the morphological and physiological connectivity? In the dark, L, M and S cones release glutamate continuously, causing spillover. When stimulated with long wavelength light L cones will hyperpolarize and reduce their glutamate release. Consequently, the glutamate concentration around the synaptic terminal of adjacent S cones will reduce, leading to a reduction of the glutamate concentration sensed by the THCs. This will induce the hyperpolarizing response observed in the THC cells without requiring a sign reversed negative feedback response in the S cones. Such a scenario would solve a long-standing controversy in retinal research and shows that morphological and functional connectivity may not always fully overlap.

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Additional information

Competing interests

The authors declare that no competing interests exist.

Author contributions

R.V. and M.K. conceived and designed the experiments, interpreted the data and wrote the paper. R.V. performed the experiments and analysed the data.

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