

# Silent-substitution stimuli silence the light responses of cones but not their output

Sizar Kamar

Netherlands Institute for Neuroscience,  
Amsterdam, the Netherlands

Marcus H. C. Howlett

Netherlands Institute for Neuroscience,  
Amsterdam, the Netherlands

Maarten Kamermans

Netherlands Institute for Neuroscience,  
Amsterdam, the Netherlands  
Academic Medical Center, University of Amsterdam,  
Amsterdam, the Netherlands



Chromatic vision starts at the retinal photoreceptors but photoreceptors are themselves color-blind, responding only to their effective quantal catch and not to the wavelength of the caught photon per se. Mitchell and Rushton (1971) termed this phenomenon the *univariance concept*, and it is widely used in designing silent-substitution stimuli to test the unique contributions of specific photoreceptor types to vision. In principle, this procedure controls the effective quantal catch of photoreceptors well and hence works at the phototransduction-cascade level of vision. However, both phototransduction-cascade modulation and the horizontal-cell-mediated feedback signal determine photoreceptor output. Horizontal cells receive input from, and send feedback to, more than one photoreceptor type. This should mean that silent-substitution stimuli do not silence horizontal-cell activity, and that this activity is fed back to the silenced cones. This in turn will modulate the output of silenced cones, making them not so silent after all. Here we tested this idea and found that silent-substitution stimuli can adequately silence cone-membrane potential responses. However, these cones still received a feedback signal from horizontal cells, which modulates their  $\text{Ca}^{2+}$  current and thus their output. These feedback-induced  $\text{Ca}^{2+}$ -current changes are substantial, as they are of the same order of magnitude as  $\text{Ca}^{2+}$ -current changes that occur when cones are directly stimulated with light. This illustrates that great care needs to be taken in interpreting results obtained with silent-substitution stimuli. In the discussion, we outline two basic types of interpretation pitfalls that can occur.

## Introduction

Retinal photoreceptors are the environmental-neural interface of the visual system, converting a change in photon flux into alterations of membrane potential and ultimately into modulation of the glutamate release sensed by the retina's second-order neurons. This process starts when a photopigment in the photoreceptors absorbs a photon. Photopigments absorb photons over a wide range of wavelengths, but the efficiency with which they do so depends on both the photon's wavelength and the photopigment's absorption spectrum. For multichromatic species, their various photopigments have absorption spectra that differ in peak absorption wavelength and strongly overlap (see, e.g., Lennie & D'Zmura, 1988). This arrangement of broad and overlapping absorption spectra has the advantage that photons are efficiently absorbed over a large part of the visible spectrum while still allowing for good wavelength discrimination by comparing the relative activation of the various pigments. However, for scientists wishing to study contributions of one photoreceptor class to visual processing, overlapping absorption spectra are problematic, as it is virtually impossible to stimulate a photoreceptor type in isolation. To circumvent this problem, silent-substitution stimuli are often used (Crook et al., 2009; Dacey, Lee, Stafford, Pokorny, & Smith, 1996; Estevez & Spekreijse, 1974, 1982; Kamermans, van Dijk, & Spekreijse, 1991; Kremers, 2003; Kuchenbecker, Greenwald, Neitz, & Neitz, 2014).

Silent substitution is based on the univariance concept of Mitchell and Rushton (1971, p. 1041): "For

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each class of receptor the result of light depends upon the effective quantum catch, not upon what quanta are caught.” In other words, two photons of differing wavelength captured by the same photoreceptor will elicit the same response. Hence, two light sources with different wavelengths but matched absorption likelihoods will be indistinguishable to the photoreceptor. The silent-substitution stimulus usually consists of two stimuli modulated in counterphase, whose wavelengths and radiances are chosen such that they present steady excitation in one photopigment type while others are modulated. However, while univariance holds at the photopigment levels, it does not necessarily hold true at the photoreceptor-output level. Although this critical point is acknowledged by some authors (Estevez & Spekrijse, 1974, 1982; Kamermans et al., 1991; Kremers, 2003), in many other studies it is often ignored (see, e.g., Dacey et al., 2005; Kuchenbecker et al., 2014).

The absorption of a photon by the photopigment activates the phototransduction cascade, which eventually hyperpolarizes the photoreceptor membrane potential. This reduces the  $\text{Ca}^{2+}$  current in the synaptic terminal, leading to decreased neurotransmitter release, to which horizontal cells (HCs) and bipolar cells (BCs) subsequently respond (see, e.g., Chalupa & Werner, 2003). HCs receive input from many cones and form a laterally interconnected network with other HCs via strong electrical coupling. The signal they feed back to photoreceptors modulates the photoreceptor  $\text{Ca}^{2+}$  current-activation function (Verweij, Kamermans, & Spekrijse, 1996). As the steepest part of the  $\text{Ca}^{2+}$  current-activation function corresponds with the physiological range of photoreceptor membrane potentials, even small shifts in its function will substantially change photoreceptor output (Fahrenfort, Habets, Spekrijse, & Kamermans, 1999). Hence, photoreceptor output strongly depends on both the photons it absorbs and the HC feedback signal it receives.

In all animals with more than one cone type, HCs receive input from more than one spectral cone type. For instance, the H2-type HC of trichromatic primate retina (humans and old-world monkeys) contacts all three cone types (Ahnelt & Kolb, 1994a, 1994b). Consequently, cones receive a spectrally mixed feedback signal. The same holds for the tetrachromatic goldfish retina, where each cone type receives spectrally mixed feedback signals from the HC network (Kraaij, Kamermans, & Spekrijse, 1998). This connectivity of multiple cone types to HC means that a silent-substitution stimulus for a specific cone type will not silence HCs and that HC activity generated by nonsilenced cone types is fed back to silenced cones. Since the output of photoreceptors is determined both by the modulation of the phototransduction cascade and by the feedback signal from the HCs, we wondered

how much silent-substitution stimuli affect photoreceptor output.

In this article, we show that cone light responses at the level of the phototransduction cascade are silenced with silent-substitution stimuli. However, these same stimuli still modulate the cone  $\text{Ca}^{2+}$  current—a direct marker of cone output. These results show that great care is required when studying post-phototransduction-cascade processes with silent-substitution stimuli.

## 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 July 22 (2003/65/CE) and the ARVO Animal Statement. Goldfish, *Carassius auratus*, were dark-adapted for about 10 min, euthanized, and their eyes enucleated. The retina were isolated and placed, photoreceptor side up, in the recording chamber. All procedures were conducted under condition of dim red light.

### Solutions

The control Ringer’s solution contained (in mM) 102.0 NaCl, 2.6 KCl, 1.0  $\text{MgCl}_2$ , 1.0  $\text{CaCl}_2$ , 28.0  $\text{NaHCO}_3$ , and 5.0 glucose. It was continuously gassed with 2.5%  $\text{CO}_2$  and 97.5%  $\text{O}_2$  to yield a pH of 7.6. The pipette solution contained (in mM) 87 K-gluconate, 10 KCl, 1  $\text{MgCl}_2$ , 0.1  $\text{CaCl}_2$ , 1 EGTA, 10 HEPES, 10 ATP- $\text{K}_2$ , 1 GTP- $\text{Na}_3$ , and 20 phosphocreatine- $\text{Na}_2$ , as well as 50 units/ml creatine phosphokinase, adjusted with NaOH to a pH of 7.3 and resulting in a  $\text{Cl}^-$  equilibrium potential ( $E_{\text{Cl}}$ ) of  $-50$  mV. All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). We set  $E_{\text{Cl}}$  at  $-50$  mV to minimize the interference of secondary processes like the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current in our  $\text{Ca}^{2+}$ -current measurements.

### Electrodes and recording setup

Electrophysiological recordings of cones were performed following published methods (Kraaij, Spekrijse, & Kamermans, 2000). Patch pipettes (resistance: 3–6 M $\Omega$ ) were pulled from borosilicate glass capillaries (GC-150T-10; Harvard Apparatus, Cambridge, UK) using a Brown Flaming Puller (Model P-97; Sutter Instruments

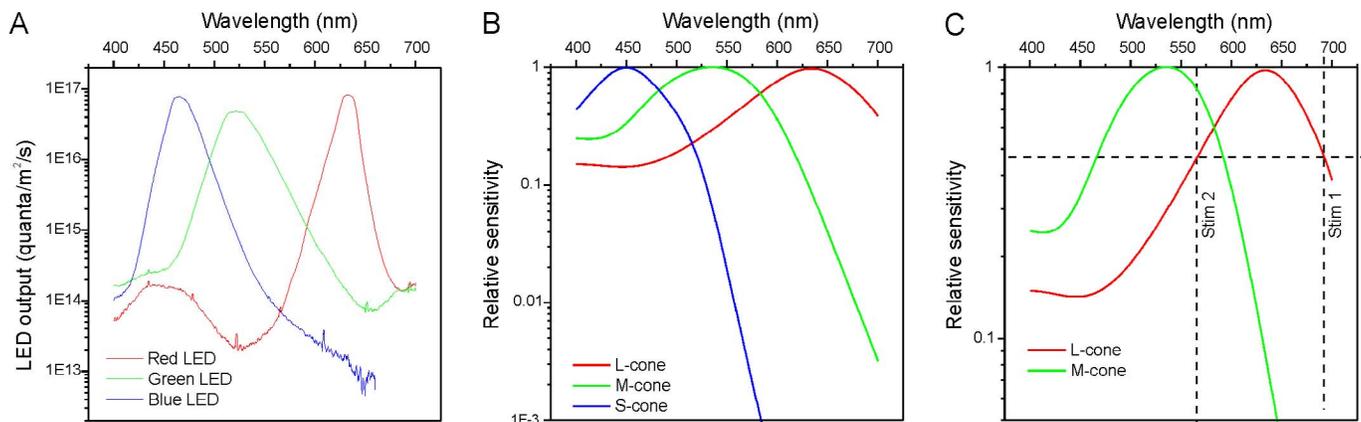


Figure 1. Design of the silent-substitution stimuli. (A) Spectral output of the LEDs used in this study, in quanta/m<sup>2</sup>/s. (B) The spectral-sensitivity plots of goldfish cones are based on the data of Palacios, Varela, Srivastava, and Golds (1998) and were fitted with the template of the absorbance of photopigments from Govardovskii et al. (2000). (C) Explanation of a silent-substitution stimulus. For clarity we considered only a system with two cone types. To silence the L cones, a red and a green light stimulus are exchanged. The intensities of the two light stimuli are adjusted such that they both stimulate the L cone pigment equally. The red stimulus (Stim 1) will stimulate only the L cones, while the green stimulus (Stim 2) will stimulate both the L and the M cones. Since both stimuli will activate the L-cone pigment equally, an exchange of those stimuli will modulate only the M-cone pigment. The L cones are silenced.

Company, Novato, CA). Pipettes were connected to a Dagan 3900A patch clamp amplifier (Dagan Corp., Minneapolis, MN). Data were digitized and stored with a PC using a CED 1401 AD/DA converter at 1 kHz using Signal software (Version 3.07; Cambridge Electronic Design, Cambridge, UK) to acquire data, generate voltage command outputs, and drive light stimuli.

## Current-voltage relation of the Ca<sup>2+</sup> current

Cones were voltage clamped at a holding potential of  $-40$  mV and stepped to potentials between  $-100$  and  $+20$  mV in 5-mV steps for 100 ms, and the mean current was determined between 10 and 90 ms after step onset. Using such short voltage steps prevented activation of the cone's calcium-activated chloride conductance. The Ca<sup>2+</sup> current was further isolated from the whole-cell current by subtracting the leak current, which was estimated using the potential range from  $-100$  to  $-70$  mV.

## Light stimuli

### Light source

Full-field chromatic stimuli ( $4,500 \mu\text{m}$ ) were projected through the condenser of the microscope. The light stimulator consisted of a homemade LED stimulator based on a three-wavelength high-intensity LED (Atlas, Lamina Ceramics, Westhampton, NJ). The peak wavelengths of the LEDs were 624, 525, and 465 nm. A 12-bit DA converter was used to drive the LEDs. An optical feedback loop ensured linearity over

the full 12-bit range. The output of the LEDs was coupled to the microscope via light guides. The maximal output of each LED channel was  $3.8 \times 10^{15}$  quanta/m<sup>2</sup>/s.

### Relative stimulus efficiency

To determine the efficiency of an LED at stimulating a specific cone type, we measured the spectral output of the LEDs  $O_n(\lambda)$  with an Avantes PC2000-ISA spectrometer (Avantes BV, Apeldoorn, the Netherlands). The output in watts was converted to quanta/m<sup>2</sup>/s (Figure 1A) and multiplied by the spectral sensitivities of the short- (S), mid- (M) and long- (L) wavelength sensitive cone types  $S_m(\lambda)$  (Palacios, Varela, Srivastava, & Goldsmith, 1998). The cone spectral-sensitivity curves were fitted with the template of the absorbance of photopigments from Govardovskii, Fyhrquist, Reuter, Kuzmin, and Donner (2000) (Figure 1B). The final curve was integrated over the wavelength, yielding the relative stimulus efficiency of the LED ( $R_{n,m}$ ) for each cone type (Table 1):

$$R_{n,m} = \int_{400}^{700} O_n(\lambda) \cdot S_m(\lambda) \cdot d\lambda. \quad (1)$$

### The stimuli

Figure 1C explains the concept of a silent-substitution stimulus using a simplified scheme consisting of L and M cones and two chromatic light sources. As we use goldfish in the present article, which have four cone

	Relative stimulus efficiency		
	Blue LED	Green LED	Red LED
S cone	0.9413	0.1736	0.0047
M cone	0.6019	1.0000	0.0943
L cone	0.1824	0.3111	0.9027

Table 1. Relative stimulus efficiency  $R_{n,m}$ .

types, Figure 1C is meant only as an explanatory aid and does not correspond directly with any stimulus used here. To silence or isolate cone responses, we used three LED sources (624, 525, and 465 nm). Since this light source has no significant output in the ultraviolet range, we included only the L, M, and S cones in our study. All stimuli presentations were 2,000 ms long and consisted of the exchange of a chromatic full-field stimulus (Stim 1) with another chromatic full-field stimulus (Stim 2) for 500 ms. Here we controlled the relative activation levels of the various photopigments by adjusting the intensities of the three LED sources. Contrast experienced by photoreceptors under the differing stimulus conditions was calculated as

$$\text{Contrast} = \frac{I_{\max} - I_{\min}}{I_{\max} + I_{\min}}. \quad (2)$$

## Data analysis

Data were analyzed using Excel 2010 and MATLAB (MathWorks, Natick, MA). Fitting of the absorption spectra was performed using Origin Pro (Version 8; Origin Lab Corporation, Northampton, MA).

## Results

Goldfish have tetrachromatic vision but here we focused on L, M, and S cones, as our light stimulator lacked sufficient ultraviolet output to engage the

ultraviolet-sensitive cones. We excluded rod light responses by using stimuli with photopic intensities. We used two silent-substitution paradigms: cone-isolating stimuli, where the quantal catch of only a single targeted cone type changes, and cone-silencing stimuli, where the quantal catch of only a single targeted cone type remains unchanged. Tables 2 and 3 give the relative LED intensities, photopigment stimulations, and contrasts of these stimuli.

First, we tested whether we could isolate the light response of the L, M, and S cones with the stimuli we designed. We measured the membrane potential of cones under the various stimulus conditions (see Table 4 for mean resting membrane potentials in the dark). Figure 2A shows the responses of representative L, M, and S cones to the cone-isolating stimuli. The S cone (Figure 2A, top traces) hyperpolarized to the S-cone-isolating stimulus (left trace) but did not respond at all to the M- or L-cone-isolating stimuli (middle and right traces, respectively). The same holds for the L and M cones: The cones respond exclusively to their isolating stimuli. We found this for all cones tested this way (nine L cones, six M cones, six S cones). These results indicate that the silent-substitution method can adequately isolate a specific cone type at the membrane-potential-response level.

Next we tested whether we could effectively silence a specific cone type. Figure 2B shows that the L, M, and S cones were silenced by their corresponding stimuli (Table 3), whereas the other two cone types responded strongly to that same stimulus. For example, the S cone shown in Figure 2B (top traces) hyperpolarized in response to L- and M-cone-silencing stimuli (middle and right-traces, respectively) but did not respond to S-cone-silencing stimuli (top left trace). Conversely, the S-cone-silencing stimulus elicited hyperpolarizing responses in M cones (middle left trace) and L cones (bottom left trace). The same holds for the L and M cones. These experiments show that we can adequately silence the membrane potential response of a specific cone type with the appropriate silencing stimuli. We

	Isolating stimuli								
	S-cone photopigment			M-cone photopigment			L-cone photopigment		
	Stim 1	Stim 2	Contrast (%)	Stim 1	Stim 2	Contrast (%)	Stim 1	Stim 2	Contrast (%)
Blue LED	0.0000	0.9931		0.2001	0.0198		0.0000	0.0140	
Green LED	0.9735	0.3752		0.0000	0.9855		0.1027	0.0000	
Red LED	0.0000	0.0055		0.3304	0.0271		0.0000	1.0000	
S cone	0.1689	1.0000	71	0.1899	0.1899	0	0.0178	0.0178	0
M cone	0.9735	0.9735	0	0.1516	1.0000	74	0.1027	0.1027	0
L cone	0.3029	0.3029	0	0.3347	0.3347	0	0.0319	0.9052	93

Table 2. Relative LED intensities, photopigment stimulations, and contrasts for isolating stimulus conditions. Notes: Only one cone type experienced a contrast change.

	Silencing stimuli								
	S-cone photopigment			M-cone photopigment			L-cone photopigment		
	Stim 1	Stim 2	Contrast (%)	Stim 1	Stim 2	Contrast (%)	Stim 1	Stim 2	Contrast (%)
Blue LED	1.0000	0.8200		0.0000	1.0000		0.0000	0.0000	
Green LED	0.0000	1.0000		0.7000	0.0000		0.0000	1.0000	
Red LED	0.0000	1.0000		0.0000	1.0000		1.0000	0.6600	
S cone	0.9413	0.9501	0	0.1215	0.9460	77	0.0047	0.1766	95
M cone	0.6019	1.5878	45	0.7000	0.6962	0	0.0922	1.0618	84
L cone	0.1824	1.3634	76	0.2178	1.0850	67	0.9069	0.9027	0

Table 3. Relative LED intensities, photopigment stimulations, and contrasts for silencing stimulus conditions. *Notes:* One cone type experienced no contrast change.

found the same result for all cones tested this way (six L cones, four M cones, six S cones).

Having established that we can successfully isolate cone types at the level of their membrane potential response, we next asked whether the cone’s output was isolated as well. To test this, we switched to whole-cell voltage-clamp experiments and measured cone current responses under the various stimulus conditions at holding potentials of either  $-40$  or  $-70$  mV (Figure 3). At  $-70$  mV, the membrane potential is outside the cone  $Ca^{2+}$  current-activation range and current responses are dominated by cyclic nucleotide-gated channel modulation in the cone outer segment. Hence, a change in current at  $-70$  mV indicates a direct light response by the cone to the stimulus. In contrast, current responses absent at  $-70$  mV but present at  $-40$  mV signify indirect light responses resulting from HC negative feedback-induced shifts of the cone  $Ca^{2+}$  current (Verweij et al., 1996).

Figure 3A shows the mean ( $\pm$  SEM) responses of L, M, and S cones to the cone-isolating stimuli. As Figure 2A indicates, these stimuli should modulate only the targeted cone type. Indeed, when clamped at  $-70$  mV there was no indication of direct light responses in nontargeted cones (black traces). However, at  $-40$  mV the same stimuli induced an inward current response (red traces) in these same cones, indicating that their  $Ca^{2+}$  current was being modulated by HC feedback.

A comparable outcome occurred using cone-silencing stimuli (Figure 3B). In this case the targeted cone type showed no direct light responses when clamped at  $-70$  mV (black traces), confirming the outcomes shown in Figure 2B. However, at  $-40$  mV (red traces) a prominent inward current response occurred in these same cones. Hence, even though the stimuli silenced the

	$V_{rest}$
L cone	$-45.25 \pm 6.27$ ( $n = 9$ )
M cone	$-44.95 \pm 7.25$ ( $n = 6$ )
S cone	$-45.33 \pm 3.38$ ( $n = 4$ )

Table 4. Mean ( $\pm$  SEM) resting membrane potential  $V_{rest}$  (mV) of cones in the dark.

targeted cone’s direct light response, these cones still received a strong feedback signal from the HC network, which modulated their output.

Next we addressed the question how these feedback-induced changes in the  $Ca^{2+}$  current of the nontargeted cones (about 4 pA) compare to the changes in membrane potential of the targeted cone (about 5 mV). Are these feedback-induced responses merely a biological curiosity with little physiological relevance, or is this a significant confound? The simplest way to assess this would be to compare the  $Ca^{2+}$ -current change induced by feedback when a cone’s membrane potential response is silenced with that produced by the cone’s direct light response. However, it is not possible to isolate the cone’s  $Ca^{2+}$  current from its cyclic nucleotide-gated conductances. Instead, we addressed this issue in the following way. We first determined the current-voltage relation of the  $Ca^{2+}$  current of the cones used in this study by stepping the holding potential from  $-40$  mV to various potentials for 100 ms (Figure 4A), then determined the mean current between 10 and 90 ms after the voltage step onset. The leak current was estimated in the potential range from  $-100$  to  $-70$  mV and subtracted from the whole-cell current. We then fitted the resulting mean  $Ca^{2+}$  current current-voltage relation (Figure 4B, red line  $\pm$  SEM) with Equation 3 (Figure 4B, dashed black line; the parameters of this fit are given in Table 5):

$$I_{Ca} = \frac{1}{1 + e^{-\frac{(V-K)}{n}}} (V - E_{Ca})g_{Ca}, \quad (3)$$

where  $I_{Ca}$  = cone  $Ca^{2+}$  current (pA),  $V$  = cone membrane potential (mV),  $E_{Ca}$  = reversal potential of

	Parameter
$E_{Ca}$	41.01 mV
$g_{Ca}$	3.66 nS
$K$	$-13.44$ mV
$n$	9.34 mV

Table 5. Estimated Ca-current parameters.

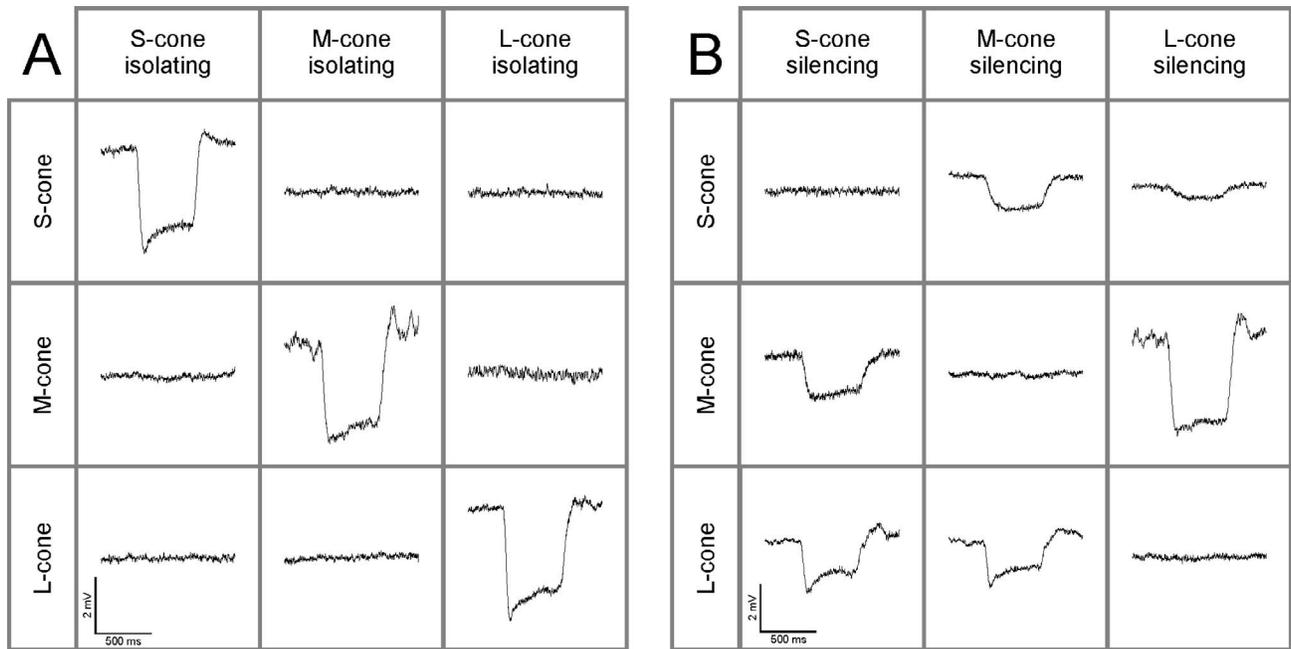


Figure 2. Membrane potential responses to cone-isolating and cone-silencing stimuli. (A) Membrane potential light responses of an L, an M, and an S cone to L-, M-, and S-cone-isolating stimuli. L, M, and S cones respond with a fast hyperpolarizing response followed by a sag-back to the cone-isolating stimuli specific for their spectral type. None of the cones responded with a membrane potential response to the cone-isolating stimuli specific for the other cone types. (B) Membrane potential light responses of an L, an M, and an S cone to L-, M-, and S-cone-silencing stimuli. All cone types could be fully silenced by their specific silencing stimulus but responded to all other stimuli.

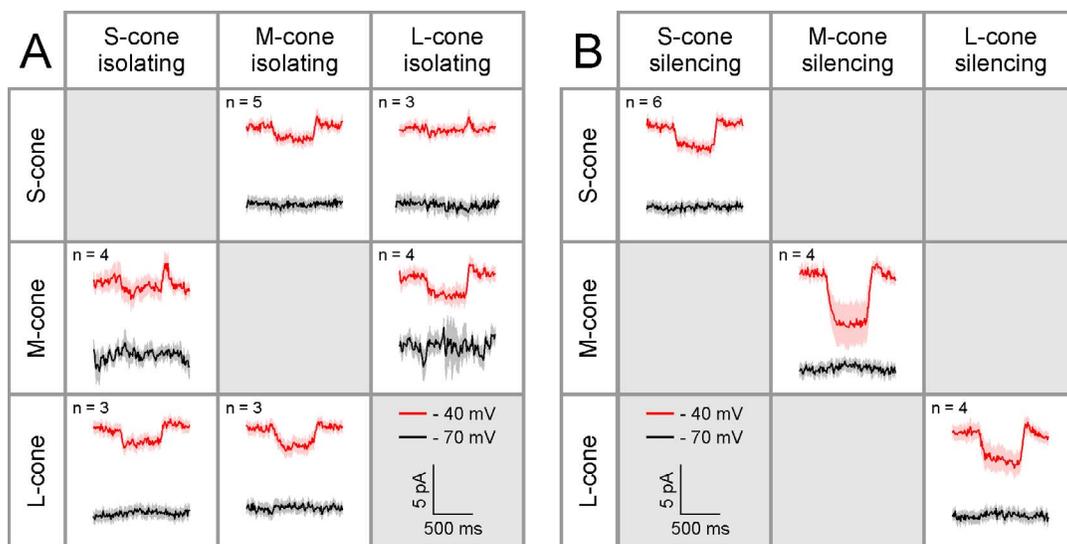


Figure 3. Current responses to cone-isolating and cone-silencing stimuli. (A) Mean current responses of L, M, and S cones to the cone-isolating stimuli. The responses recorded at  $-40$  mV (red traces) are mediated by feedback from horizontal cells to cones, while the responses at  $-70$  mV are the direct light responses (black traces). As expected, the black traces show no direct light response and so follow the same pattern as the membrane potential responses in Figure 2A. On the other hand, the red traces show significant responses for all stimuli that did not induce a membrane potential light response, indicating that cone-isolating stimuli generate a feedback response in the nonisolated cones. (B) Mean current light responses of L, M, and S cones to cone-silencing stimuli. The responses recorded at  $-40$  mV (red traces) are mediated by feedback, while the traces at  $-70$  mV are direct light responses of the cones. As expected, direct light responses are absent for the cone-silencing stimuli. However, feedback induces a significant response in these silenced cones. Data shown are means  $\pm$  standard error of the mean.

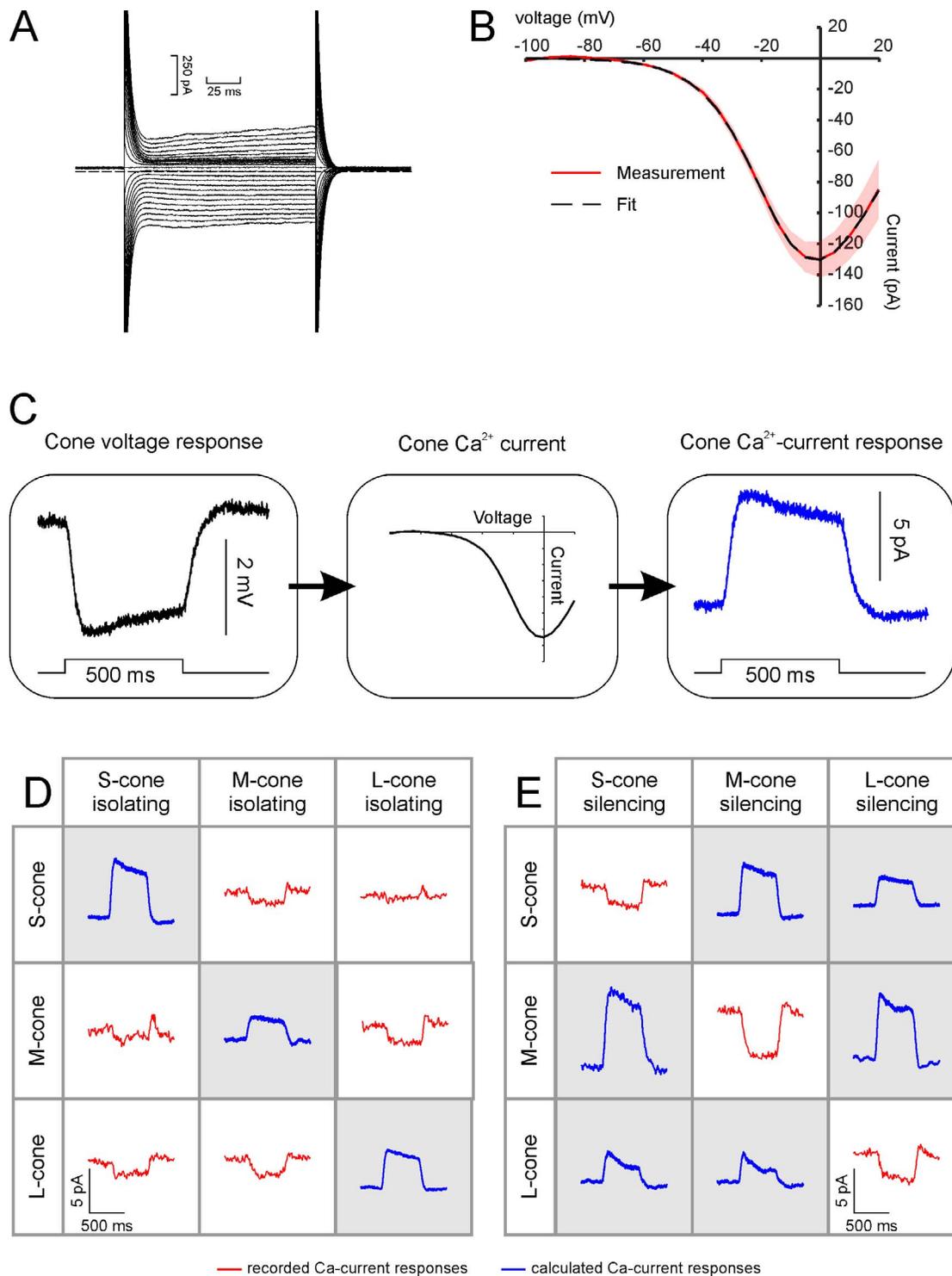


Figure 4. Comparison of cone output due to direct light stimulation and to feedback. (A) Whole-cell currents in a membrane-potential-clamped cone held at  $-40$  mV and stepped for 100 ms to potentials ranging from  $-100$  to  $+20$  mV in steps of 5 mV. (B) Mean ( $\pm$  SEM;  $n = 34$ ) leak-subtracted  $\text{Ca}^{2+}$ -current current-voltage relation (red line) and fitted  $\text{Ca}^{2+}$  current using Equation 3 and the parameters given in Table 5 (dashed black line). (C) Schematic depicting the conversion of the direct light response of a cone into a change in  $\text{Ca}^{2+}$  current—that is, the output of the cone. The cone’s membrane potential light responses (left) were fed into the fitted  $\text{Ca}^{2+}$ -current current-voltage relation (center) to determine the corresponding  $\text{Ca}^{2+}$ -current response (right). (D) The measured mean changes in  $\text{Ca}^{2+}$  current of targeted L, M, and S cones to the cone-isolating stimuli (red lines) and calculated changes in  $\text{Ca}^{2+}$  current due to the same stimuli (blue lines). (E) The measured mean changes in  $\text{Ca}^{2+}$  current of targeted L, M, and S cones to the cone-silencing stimuli (red lines) and calculated changes in  $\text{Ca}^{2+}$  current due to the same stimuli (blue lines).

the  $\text{Ca}^{2+}$  channel (mV),  $g_{\text{Ca}}$  = conductance of the  $\text{Ca}^{2+}$  channels (nS),  $K$  = half activation potential of the  $\text{Ca}^{2+}$  current (mV), and  $n$  = slope factor (mV).

With Equation 3 and the values of Table 5, we can now convert the membrane-potential changes during a direct light response into changes in  $\text{Ca}^{2+}$  current (Figure 4C). Figure 4D compares these mean calculated changes in  $\text{Ca}^{2+}$  current of the targeted cones (blue lines) with the measured feedback responses of non-targeted cones (red lines) for the cone-isolating stimuli. As the figure shows, both hyperpolarization- and feedback-induced changes in  $\text{Ca}^{2+}$  current are of the same order of magnitude. The same analysis performed for the cone-silencing stimuli shows this outcome even clearer (Figure 4E). The feedback-mediated  $\text{Ca}^{2+}$ -current change in cones with silenced membrane potential responses (red) is about equal to—but opposite in amplitude from—that of the nontargeted cones with direct light responses (blue). These figures illustrate two important issues. First, a hyperpolarizing light response of about 5 mV induces a change in  $\text{Ca}^{2+}$  current of only a few picoamperes. Secondly, in both cone-isolating and cone-silencing stimulus paradigms, the output of cone types with silenced membrane potential responses changes by the same order of magnitude as that of the cone types with nonsilenced membrane potential responses.

## Discussion

In this article we have shown that it is possible to silence and isolate the phototransduction cascade of specific cone types using silent-substitution stimuli. However, even though silencing stimuli did not induce a direct light response in targeted cone types, they nonetheless modulated their  $\text{Ca}^{2+}$  current. Similarly, isolating stimuli modulated the  $\text{Ca}^{2+}$  current of non-targeted cone types even though they did not elicit a direct light response in these cones. As cone glutamate release fully depends on the activity of the  $\text{Ca}^{2+}$  current (Barnes & Kelly, 2002; Copenhagen & Jahr, 1989; Schmitz & Witkovsky, 1997), this means that even though a cone itself has “seen nothing,” the signal it transmits to second-order neurons is changing in a stimulus-dependent manner. This situation arises as the spectral sensitivities of HCs differ from those of cones (Kamermans et al., 1991; Kraaij et al., 1998; Spekrijse & Norton, 1970; Stell, Lightfoot, Wheeler, & Leeper, 1975), which is the case for all animals with more than one cone type, and limits the usefulness of the silent-substitution stimulus method.

How is the cone output modified without modulating its membrane potential? The mechanism of negative feedback from HCs to cones is rather unconventional

(Cenedese et al., 2017; Hirasawa & Kaneko, 2003; Kamermans et al., 2001; Thoreson & Mangel, 2012; Verweij et al., 1996; Vroman et al., 2014; Wang, Holzhausen, & Kramer, 2014). HCs modulate the cone’s  $\text{Ca}^{2+}$  current by changing the extracellular synaptic environment via at least two separate mechanisms (Vroman et al., 2014). These changes shift the voltage sensitivity of cone voltage-sensitive  $\text{Ca}^{2+}$  channels. When HCs hyperpolarize, it leads to a shift of the cone  $\text{Ca}^{2+}$  current-activation function to more negative potentials (Verweij et al., 1996). For a cone with a membrane potential of  $-40$  mV, this negative shift of the  $\text{Ca}^{2+}$  current-activation function increases the number of  $\text{Ca}^{2+}$  channels likely to be open at this potential, thereby increasing the  $\text{Ca}^{2+}$  influx. However, since the  $\text{Ca}^{2+}$  current is small relative to the photocurrent, these changes in  $\text{Ca}^{2+}$  current do not in themselves significantly affect the cone membrane potential.

Changing levels of  $\text{Ca}^{2+}$  influx resulting from HC feedback could potentially affect the cone membrane potential via the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current present in the cone synaptic terminal. Indeed, this process is thought to cause depolarizing responses sometimes found in cones when a surround stimulus is used (Barnes & Deschenes, 1992; Baylor, Fuortes, & O’Bryan, 1971; Kraaij et al., 2000; Lasansky, 1981; Maricq & Korenbrot, 1988; O’Bryan, 1973; Thoreson & Burkhardt, 1991). However, changes in the cone membrane potential brought about by the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current depend on the  $\text{Cl}^-$  equilibrium potential ( $E_{\text{Cl}}$ ). Estimates of the physiological  $E_{\text{Cl}}$  position are close to, or slightly negative compared to, the dark resting membrane potential (Kaneko & Tachibana, 1986; Miller & Dacheux, 1983). In addition, the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$ -current ion pore is not perfectly selective for  $\text{Cl}^-$  (Barnes & Hille, 1989). Consequentially, the reversal potential of the current flowing through the channel is slightly more positive than  $E_{\text{Cl}}$ . In our experiments, we set  $E_{\text{Cl}}$  very close to the dark resting membrane potential, such that the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current would not induce membrane potential changes. This makes the cone’s direct light response, and the feedback response received by the cone, rather independent.

In principle, silent substitution is an elegant method to modulate the phototransduction cascade in some cone types while leaving it unchanged in others. However, as we show, this does not hold at the level of photoreceptor output. The changes in output of cones with direct membrane potential light responses, and of cones with silenced membrane potential responses, are of the same order of magnitude. Since the changes in output of the silenced cones are mediated by HC feedback, these responses are sign inverted relative to the change in Ca current due to direct light stimulation.

The silenced cones will therefore signal a decrease in light intensity, whereas the targeted cones will sense an increase in light intensity. Hence, caution is required, as the results obtained via silent substitution are highly sensitive to misinterpretation. We next illustrate two basic types of interpretation pitfalls: the connectivity pitfall and the response-kinetics pitfall.

### Connectivity pitfall

Using silent-substitution stimuli, Dacey et al. (2005) showed that macaque intrinsic photosensitive M1 ganglion cells (GCs) receive ON input from rods and L and M cones and, surprisingly, OFF input from S cones. ON inputs to the M1 GCs occur via ectopic synapses between BCs and M1 GCs in the inner plexiform layer (Dumitrescu, Pucci, Wong, & Berson, 2009), whereas the source of OFF inputs from the S cones remains unclear. However, our results suggest a possible source for the OFF S-cone input. In trichromatic primates, H2 HCs receive L-, M-, and S-cone input. The S-cone-isolating stimulus will therefore modulate the H2 HCs, which then feed this signal back to the silenced L and M cones. This results in a sign-inverted output of the L and M cones and thus an OFF response in the M1 GCs, even in the absence of direct S-cone input. If this is true, then HC feedback could underlie the paradoxical phenomena whereby pupils dilate with increased intensity of S-cone-targeting stimuli (Spitschan, Jain, Brainard, & Aguirre, 2014).

More generally, it is a risky endeavor to use cone-isolating stimuli to determine retinal circuitry such as cone–BC–GC connectivity. For instance, in primates an S-cone-isolating stimulus will induce an H2-HC-mediated feedback signal that will modulate the output of L and M cones. As the feedback signal is sign inverted, the L and M cones will signal a decrease in light intensity in response to increased intensity of S-cone-isolating stimuli. Hence, any GC type receiving only inputs from L- or M-cone pathways will respond as if it also has an OFF S-cone pathway. Indeed, there are a number of occasions where opponent S-cone input is found compared to the L- and M-cone input. Thus, independent confirmation of such direct inverted S-cone input is needed, since the silent-substitution paradigm allows for different connectivity interpretations (see, e.g., Crook et al., 2009).

### Response-kinetics pitfall

Silent substitution is often used in electroretinogram studies to isolate the S-cone response (Kuchenbecker et al., 2014). Cones generate the electroretinogram a-wave, while the b-wave represents the activity of ON

BCs. Following the previous discussion, S-cone-isolating stimuli will generate inverted outputs in L and M cones. Thus, S-cone-isolating stimuli will generate b-waves driven by the S-cone output together with the inverted L- and M-cone output. The combined signal will be both complex and hard to interpret. Indeed, the S-cone-isolated signal reported by Kuchenbecker et al. (2014) is very complex and seems to consist of a combination of different signals. Hence, caution is needed when interpreting such a signal, and making strong claims about delays and time constants of the S-cone responses measured in this way seems unrealistic.

## Conclusion

Silent-substitution stimuli can silence specific cone types at the phototransduction level, but they fail to silence the output of the cones. This illustrates that great care needs to be taken in interpreting results obtained with silent-substitution stimuli.

*Keywords:* silent substitution, retina, photoreceptors, horizontal cells

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Corresponding author: Maarten Kamermans.

Email: m.kamermans@nin.knaw.nl.

Address: Netherlands Institute for Neuroscience, Amsterdam, the Netherlands.

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