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1 **Evidence for functional GABA_A but not GABA_C receptors on mouse cone photoreceptors**

2

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19

1 Title: Evidence for functional GABA_A but not GABA_C receptors on mouse cone photoreceptors

2 Abstract:

3 At the first retinal synapse, horizontal cells contact both photoreceptor terminals and bipolar cell
4 dendrites, modulating information transfer between these two cell types to enhance spatial contrast
5 and mediate color opponency. The synaptic mechanisms through which these modulations occur are
6 still debated. The initial hypothesis of a GABAergic feedback from horizontal cells to cones has been
7 challenged by pharmacological inconsistencies. Surround antagonism has been demonstrated to
8 occur via a modulation of cone calcium channels through ephaptic signaling and pH changes in the
9 synaptic cleft. GABAergic transmission between horizontal cells and cones has been reported in some
10 lower vertebrates like the turtle and tiger salamander. In these reports, GABA is released from
11 horizontal cells through reverse transport and target GABA receptors are located on cone terminals.
12 In mammalian retinas, there is growing evidence that horizontal cells can release GABA through
13 conventional vesicular transmission, acting both on autaptic GABA receptors and on receptors
14 expressed at the dendritic tips of bipolar cells. The presence of GABA receptors on mammalian cone
15 terminals remains equivocal. Here, we looked specifically for functional GABA receptors on mouse
16 photoreceptors by recording in the whole-cell or amphotericin/gramicidin perforated patch-clamp
17 configurations. Cones could be differentiated from rods through morphological criteria. Local GABA
18 applications evoked a Cl⁻ current in cones but not in rods. It was blocked by the GABA_A receptor
19 antagonist bicuculline methiodide and unaffected by the GABA_C receptor antagonist TPMPA. The
20 voltage dependency of the current amplitude was as expected from a direct action of GABA on cone
21 pedicles, but not from an indirect modulation of cone currents following the activation of the GABA
22 receptors of horizontal cells. This supports a direct role of GABA release from horizontal cells in the
23 control of cone activity in the mouse retina.

24

25 Keywords: GABA, feedback, cone photoreceptor, horizontal cell

26

1 INTRODUCTION

2 Our ability to detect edges in the visual world is enhanced through a process called lateral
3 inhibition, mediated by horizontal cells (HCs) which exert both a negative (Baylor *et al.*, 1971) and a
4 positive (Jackman *et al.*, 2011) feedback onto cone photoreceptors. The underlying synaptic
5 mechanisms are still not fully understood. Inconsistencies in the pharmacology and ions underlying
6 the negative feedback current have challenged the initial hypothesis of a GABAergic transmission
7 between HCs and cones (reviewed in Piccolino, 1995; Thoreson & Mangel, 2012). A modulation of
8 cone calcium channels (Verweij *et al.*, 1996, 2003), by either hemichannel-mediated ephaptic
9 communication (Kamermans *et al.*, 2001; Pottek *et al.*, 2003; Fahrenfort *et al.*, 2009; Klaassen *et al.*,
10 2011; Kemmler *et al.*, 2014) or through pH changes in the synaptic cleft (Hirasawa & Kaneko, 2003;
11 Davenport *et al.*, 2008; Wang *et al.*, 2014) has arisen as the main mechanism for the negative
12 feedback. Far from being mutually exclusive, both ephaptic and pH modulations are likely to shape
13 cone calcium signals (Kemmler *et al.*, 2014).

14 Many of the molecular and physiological underpinnings required for a GABAergic
15 transmission from HCs to cones are however present in various species. On the presynaptic side, in
16 lower vertebrates, GABA can be released in a Ca²⁺-independent manner via membrane transporters
17 (Schwartz, 1987). In the mammalian outer retina, the vesicular transporter VGAT/VIAAT was localized
18 in HC tips in mouse, guinea pig, human and macaque retinas (Haverkamp *et al.*, 2000; Cueva *et al.*,
19 2002; Jellali *et al.*, 2002; Guo *et al.*, 2010); HCs were reported to express the synaptic proteins
20 required for vesicular release in rabbit and guinea pig (Hirano *et al.*, 2005; Lee & Brecha, 2010).
21 Though HCs were previously reported not to contain GABA in rodents (Agardh *et al.*, 1986; Versaux-
22 Botteri *et al.*, 1989; Fletcher & Kalloniatis, 1997), we have demonstrated that this was due to GABA
23 loss during tissue preparation, that could be compensated by supplementing the medium with a
24 GABA precursor, such as glutamine or glutamate, and with pyridoxal phosphate, a cofactor of the
25 glutamate acid decarboxylases (Deniz *et al.*, 2011). Preventing GABA release from HCs by targeted

1 deletion of VGAT removed the feedback to cone photoreceptors (Hirano *et al.*, 2016), possibly
2 through an autaptic action of GABA on HCs, changing the membrane permeability to HCO_3^- and
3 hence affecting the pH in the synaptic cleft (Liu *et al.*, 2013). If GABA receptors are expressed in cone
4 terminals, they could modulate cone output both by influencing directly the membrane potential and
5 by affecting the pH in the synaptic cleft, as those expressed on HCs.

6 On the postsynaptic side, while the presence of GABA receptors on cone terminals has been
7 reported in many lower vertebrates (reviewed in Wu, 1992), it remains controversial regarding
8 mammalian cones. *In situ* hybridization in rat showed signal in the ONL for β_1 but not for several
9 other subunits of GABA_A receptors (α_{1-4} , β_{2-3} , γ_2 et δ) (Greferath *et al.*, 1993, 1995). β_1 and β_2 mRNA
10 were detected in the rat ONL by PCR (Grigorenko & Yeh, 1994). *In situ* hybridization for the GABA_A α_1
11 subunit was also negative in the rabbit retina (Brecha *et al.*, 1991). α and β subunits were detected in
12 cone pedicles but not in rod spherules by immunohistochemistry in the cat (Vardi *et al.*, 1992).
13 Further electron microscopy studies in macaque and rabbit retinas suggested that the GABA receptor
14 subunit staining in the OPL might be attributed solely to the strongly labelled bipolar cell dendritic
15 tips (Vardi & Sterling, 1994; Greferath *et al.*, 1994). α_1 and ρ subunits were not detected in mouse
16 cones by immuno electron microscopy (Kemmler *et al.*, 2014). A GABA-evoked current was detected
17 in only a fraction of cones in macaque flatmount retina, without pharmacological characterization
18 (Verweij *et al.*, 2003); both GABA_A and GABA_C receptors were reported by combining
19 electrophysiology and immunohistochemistry in porcine cones in culture (Picaud *et al.*, 1998) and in
20 putative mouse cones from flatmount *rd1* retinas or dissociated from WT C57BL/6J retinas (Pattnaik
21 *et al.*, 2000). These apparent discrepancies may be due to variations between species, a difficult path
22 for GABA to reach the cone terminals in WT flatmounts, low levels of expression of GABA receptor
23 subunits in mammalian cones, difficulty in identifying dissociated cones, and/or changes in
24 expression during retinal degeneration or in culture. To circumvent those issues, we have assessed
25 directly the presence of GABA receptors in photoreceptors on acute slices of adult mouse retinas,
26 using either whole-cell or perforated patch clamp recordings.

1 MATERIAL AND METHODS

2 *Retinal slice preparation*

3 Procedures involving animals and their care were conducted in agreement with the ARVO Statement
4 for the Use of Animals in Ophthalmic and Vision Research, the French Ministry of Agriculture and the
5 European Community Council Directive no. 86/609/EEC, OJL 358. Mice strains used in this study were
6 either C57BL/6J or Balb/c ByJ bred at the Mouse Clinical Institute animal house (Illkirch, France). As
7 results from both strains were similar, results were pooled together. Mice were maintained on a
8 12h/12h light-dark cycle, with light ON from 7 AM to 7 PM. Light-adapted, adult (9-19 weeks) mice
9 were killed by cervical dislocation in the morning (9-10 AM). The eyes were enucleated and
10 immediately put in ice-cold bicarbonate-buffered saline (BBS), composed of (in mM): NaCl 126, KCl
11 2.5, CaCl₂ 2.4, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 18, glucose 11 previously bubbled with 95% O₂ / 5%
12 CO₂. The cornea, lens and vitreous were removed. The retina was detached from the pigmented
13 epithelium and embedded in agarose 1.5% prepared in PBS (0.1 M; pH 7.4) kept at 42°C. After
14 agarose solidification on ice, the retina was cut in 150 or 200 µm thick slices using a Leica VT1000S
15 vibratome (Leica, Wetzlar, Germany). The slices were kept at room temperature in bubbled BBS for
16 at least half an hour before recording, in normal lighting conditions.

17 *Patch-clamp recordings*

18 Slices were observed under infrared differential interference contrast (DIC) using a 63x objective and
19 a C8484 camera (Hamamatsu, Massy, France) on a Leica DMLFS microscope (Leica, Wetzlar,
20 Germany). The preparation was continuously perfused at ~2 ml / minute with bubbled BBS. Pipettes
21 (6-8 MΩ) were pulled from GC150TF borosilicate glass capillaries (Harvard Apparatus, Les Ulis,
22 France) on a horizontal puller (DMZ Universal Puller, Zeitz Instrumente, Munich, Germany). Two
23 different intracellular solutions were used: one containing (in mM) KCl 42, K gluconate 98, EGTA-Na₄
24 10, MgCl₂ 1, HEPES 5, ATP-Na₂ 5 ($E_{Cl} = -28.9$ mV, junction potential of 12.4 mV, referred to in the text
25 as $E_{Cl} = -29$ mV) or KCl 138, EGTA-Na₄ 10, MgCl₂ 3, CaCl₂ 1, HEPES 10, ATP-Na₂ 3, GTP-Na₃ 0.5 ($E_{Cl} =$

1 1.9 mV, junction potential of 3.9 mV, referred to in the text as $E_{Cl} = 2$ mV). For perforated patch
2 recordings, amphotericin B (80-180 $\mu\text{g/ml}$) or gramicidin (80-100 $\mu\text{g/ml}$) was added to the pipette
3 solution from a 120 mg/ml stock solution in DMSO. All solutions contained 10 μM of Alexa Fluor®
4 (488 or 594) hydrazide (Molecular Probes, Eugene, OR), and pH was adjusted to 7.4 with NaOH. The
5 extracellular solution was the BBS described above, continuously bubbled with 95% O_2 / 5% CO_2 .
6 Potentials were corrected post-recording for the calculated junction potential. For simplicity,
7 potentials indicated in the text are rounded to integer values. All experiments were performed at
8 room temperature (20-25°C), between 11 AM and 8 PM for the successful cone recordings, with no
9 evident circadian variation (Supplementary Figure 4). Data were acquired using a Multiclamp 700A
10 amplifier, a Digidata 1322A interface and the pClamp9 software (Molecular Devices, Sunnyvale, CA).
11 Data were filtered prior to digitization at a frequency of 1/2 or 1/5th of the acquisition frequency,
12 which was 200 Hz for puffed GABA (γ -aminobutyric acid) and 10 kHz for depolarizing pulse
13 experiments. Agonists were applied locally with a puffer pipette connected to a Picospritzer III
14 (Parker Hannifin, Fairfield, NJ) and antagonists were applied via bath application. TPMPA ((1,2,5,6-
15 Tetrahydropyridin-4-yl)methylphosphinic acid) was obtained from Tocris (Ellisville, MI), all others
16 chemicals were obtained from Sigma-Aldrich (Lyon, France). All values are indicated as mean \pm S.E.M.

1 RESULTS

2 *Targeting cone photoreceptors on mouse retinal slices*

3 To determine if mouse photoreceptors express functional GABA receptors, we recorded from
4 cones and rods in the whole-cell patch-clamp configuration, on acute slices from mouse retinas.
5 Cones, which represent only 3% of the photoreceptor population in mice (Jeon *et al.*, 1998), have
6 slightly larger and more oval cell bodies than rods. This morphological difference allowed for a
7 partially targeted rather than “blind” cone recording. Although most of the cone cell bodies were
8 located close to the outer limiting membrane (Fig. 1A), some of them were also found close to the
9 OPL (Fig. 1B). As an Alexa dye was included in the pipette solution, cone identity could be confirmed
10 at the end of the recording by visualizing the synaptic terminal, as previously reported by Cangiano *et al.*
11 *al.*(2012). Cone pedicles (Fig. 1A-C, supplementary figure 1) are much larger than the small rounded
12 terminals of rods (Fig. 1D-E, supplementary figure 2). The long outer segment of rods (Fig. 1D-E,
13 supplementary figure 3) was also more readily observed than the shorter one from cones. While the
14 formation of a gigaseal could be readily obtained with both types of photoreceptors, most of the
15 cells were lost when attempting to go into the whole-cell configuration, possibly due to the fact that
16 the nucleus occupies most of the cell soma volume. Some rods were recorded after contacting them
17 at the level of the inner segment (Fig. 1D), which allowed for an easier transition to whole-cell mode.
18 Some recordings were obtained using amphotericin B perforated-patch, which allowed for a higher
19 success rate than whole-cell patch. Cone identity could be confirmed by dye diffusion after
20 membrane rupture at the end of the recording.

21 To compare the responses to GABA applications in cones with and without terminals, we
22 needed a criterion to distinguish the latter from terminal-lacking rods. To this end, we compared the
23 current-voltage (I-V) relationships obtained from photoreceptors. We have more specifically focused
24 on I-V curves recorded with the $E_{Cl} = -29$ mV pipette solution during 100 ms voltage steps, conditions
25 for which we had the most cells in the different categories. For well-identified, terminal-bearing

1 photoreceptors, currents evoked by voltage steps above -50 mV were larger in cones than in rods
2 (Fig. 2A-D), as were the following tail currents (Fig. 2A-C, E) (13 rods and 11 cones in D and E),
3 requiring in some cones over a second to fully de-activate after a 100 ms depolarizing step (Fig. 2C).
4 These currents were similar to the chloride currents described in cones from the larval tiger
5 salamander (Barnes & Bui, 1991) and the ground squirrel (Szmajda & DeVries, 2011). The
6 depolarization-evoked current was maximal at +26 mV (Fig. 2D), while the tail current peaked when
7 returning from a step to +6 mV (Fig. 2E). The individual values of these currents are represented in
8 Figure 2 F-G, distinguishing five groups of cells. Cones (n = 11) and rods (n = 13) that could be
9 identified through the morphology of their synaptic terminal are represented with green and grey
10 symbols, respectively. Rods identified as such through the presence of a typical outer segment but
11 with no visible synaptic terminal are represented with orange symbols (n = 11). The remaining cells
12 were tagged either as putative cones (red symbols, n = 8) or putative rods (cyan symbols, n = 5)
13 depending on the shape of their soma as observed prior to recording – oval for cones (see stars in
14 Fig.1B), rounded for rods. The presence of a short outer segment was not used to tag cones, as such
15 a segment could correspond to a damaged/collapsed rod outer segment. For both types of currents,
16 the range of amplitudes were comparable between identified and putative cones, and between
17 identified and putative rods (Fig. 2F). However, there was some overlap between the cone and rod
18 populations, thus neither the plateau nor tail current amplitude alone could be used to
19 unambiguously identify as cones some of the terminal-less cells. When considering both currents
20 together, there was very little overlap between the cone and rod populations, all identified and
21 putative rods being included in the range [$I_{\text{tail}} < 100$ pA, $I_{\text{plateau}} < 350$ pA], while all but one of the
22 identified and putative cones were outside this range (Fig. 2G).

23 *Cones response to GABA applications*

24 GABA (1 mM) was puffed nearby the synaptic terminal of the recorded photoreceptors (5 to
25 250 ms duration). A GABA-evoked current was detected in 34 out of 35 cones with pedicles. The

1 amplitude of the current evoked by a 100 ms puff of 1 mM GABA on 14 pedicle-bearing cones
2 recorded with the $E_{Cl} = 2$ mV intracellular solution ranged between -12.5 and -166 pA at -64 mV
3 (-51.4 ± 46.5 pA, n = 14) (Fig. 3A; left). As GABA was applied onto the surface of the slice, the current
4 amplitude was at least in part dependent upon the depth of the pedicle in the slice. Notably, a cone
5 with an intact morphology but with a pedicle deeper than 30 μm (estimated from the
6 epifluorescence focus) did not respond to GABA when puffed on the slice surface, but responded
7 when the puff pipette was dipped into the slice. As the response kinetics were similar to those of
8 other cones after GABA puffs on the slice surface, it was most probably a true response to GABA
9 rather than a mechanical artifact. No GABA-evoked current was detected from cells tentatively
10 identified as pedicle-less cones through the current amplitude criteria presented in Figure 2G (n = 4),
11 in line with a GABA receptor localization restricted to the synaptic terminal, as in turtle retina
12 (Tachibana & Kaneko, 1984). No current was evoked in rods either (Fig. 3A, right), whether they had
13 their spherule (n = 7) or not (n = 8). This was an additional control against a possible mechanical puff-
14 induced response. Bicuculline methiodide (100 μM) blocked 87.5 ± 3.2 % (n = 11) of the GABA evoked
15 current (Fig. 3B, left). For cells in which bicuculline methiodide did not fully block the response to
16 GABA, the presence of the antagonist did not change the kinetics of the GABA-evoked current, as can
17 be seen in Figure 3C, which presents both the raw traces (left) and currents normalized to their peak
18 (right). This suggests that the remaining current corresponds to an incomplete blockade, rather than
19 to a kinetically slower GABA_C component. Consistent with this hypothesis, the GABA_C antagonist
20 TPMPA (50 μM) had little effect (Fig. 3B, right) on the GABA-induced current with a mean blockade of
21 4.7 ± 4.7 % (n = 3). We could not systematically apply both inhibitors, as most of the cone recordings
22 were short-lived. As expected for currents mediated by ionotropic GABA receptors, the GABA-evoked
23 current reversed at a potential close to the Cl⁻ equilibrium potential (E_{Cl}): when E_{Cl} was set at -29 mV,
24 the current reversed at -30.3 ± 4.1 mV (n = 5, Fig. 3D). When E_{Cl} was set at 2 mV, the current
25 reversed at 0.7 ± 1.7 mV (n = 7, Fig. 3E).

26 *E_{Cl} in mouse cones*

1 The physiological role of the GABA-evoked current depends on its reversal potential. To
2 assess this parameter, we used gramicidin perforated-patch recordings. The success rate of such
3 recordings on cones was very low. In 4 cells with adequate intracellular access, GABA evoked very
4 small currents (-3.4 ± 1.7 pA at -94 mV, -1.5 ± 0.8 pA at -64 mV). It is probable than these cells were
5 cones rather than rods, as in whole-cell, we did not record any GABA response from identified rods
6 (0/7) in opposition to cones (34/35), as mentioned above. The GABA-evoked current changed linearly
7 with the membrane potential, reversing at -36.9 ± 6.3 mV ($n = 4$, Fig. 3F). .

8

9 **DISCUSSION**

10 There is contradictory evidence for the presence of functional GABA_A receptors on cone
11 synaptic terminals, depending on species. GABA_A receptor-mediated currents were reported in many
12 lower vertebrates, including in the turtle (Kaneko & Tachibana, 1986) and the salamander (Wu,
13 1986). Atypical ionotropic GABA receptors as well as GABA_B receptors were detected in the bullfrog
14 retina (Liu *et al.*, 2005, 2006). There are however very few published electrophysiological recordings
15 from mammalian cones. Small GABA responses were detected in only a fraction of macaque cones
16 on flatmount retinas (5/12), with a polarity matching the one expected for a Cl⁻ current in 4 out of
17 the 5 responding cones (Verweij *et al.*, 2003). No pharmacology was performed to determine if those
18 responses could be due to the activation of GABA receptors. Large GABA_A and GABA_C currents were
19 recorded both in primary culture of pig cones (Picaud *et al.*, 1998), and on putative mouse cones,
20 either on flatmount *rd1* mouse retinas or dissociated from C57BL/6J retinas (Pattnaik *et al.*, 2000).

21 We report here for the first time, in morphologically identified cones on acute mouse retinal
22 slices, the presence of GABA-evoked currents with a GABA_A pharmacology. Cone identification was
23 obtained through the inclusion of a morphological dye into the patch pipette, allowing the
24 visualization of the large cone synaptic terminal. Moreover, cones with terminals displayed currents
25 comparable to the calcium-activated chloride currents / glutamate transporter currents detected in

1 cones from other species (Barnes & Hille, 1989; Verweij *et al.*, 2003; Szmajda & DeVries, 2011). As no
2 synaptic transmission blocker was present in our recording conditions, the GABA-evoked current
3 could correspond to a secondary current following a GABA action on horizontal cell, as observed in
4 the rat retina (Liu *et al.*, 2013) rather than a direct effect on GABA_A receptors present on cone
5 pedicles. It is however unlikely: the recorded current was reversing close to E_{Cl} with a voltage-
6 dependence not compatible with a modulation of the activation curve of cone calcium channels. If
7 that was the case, the maximum effect would have occurred in the -35 to -45 mV range, and minimal
8 effect at potentials below -60 mV or over 0 mV. However, the maximal (absolute) amplitude of the
9 GABA-evoked currents was systematically recorded at the most negative potential tested, which was
10 between -102 and -64 mV depending on the recording conditions (Fig. 3 D-F). In addition, outward
11 currents could be recorded above 0 mV for many cones. The most likely explanation is thus that
12 functional GABA_A (and not GABA_C) receptors are expressed on mouse cone pedicles. While such a
13 conclusion would have been expected some decades ago when lateral inhibition was considered to
14 result from a GABAergic inhibition of cones, it is now dissonant with a number of published studies,
15 either on the presence of functional GABA receptors on mammalian cone pedicles or on the indirect
16 response of cones to GABA, mediated by horizontal cells.

17 The apparent discrepancy between our results and the reported presence of both GABA_A and
18 GABA_C currents in cultured pig cones (Picaud *et al.*, 1998) and in mouse cones from flatmount *rd1*
19 retinas or freshly dissociated from C57BL/6J retinas (Pattnaik *et al.*, 2000) can be tentatively
20 explained. In the former case, it could come either from a difference between mouse and pig cones,
21 or a partial dedifferentiation of pig cones after 2 to 10 days in culture. In flatmount *rd1* retinas, cone
22 identity was deduced from the localization in the thin remaining outer nuclear layer at an age at
23 which most rods have degenerated. The recording of both GABA_A and GABA_C currents, larger than
24 the GABA_A-only current reported here, could thus be due to a partial dedifferentiation of cones, or to
25 morphological rearrangements occurring during degeneration, with the presence of bipolar cells at
26 the distal surface of the degenerated *rd1* retinas. The former hypothesis is supported by

1 transcriptome studies from *rd1* cones at more advanced ages (110-220 days), suggesting that the
2 GABA_C ρ_1 and ρ_2 subunit mRNAs are expressed by surviving cones (Busskamp *et al.*, 2010) (see also
3 GEO Series GSE22338). The latter is consistent with the current-voltage relationships of the recorded
4 cells, reminiscent of those of rod bipolar cells. The four freshly dissociated cells from C57BL/6J
5 retinas considered as cones in the same report, may similarly have been bipolar cells with partially
6 damaged/collapsed processes, morphologically close to cones once their pedicle and inner/ outer
7 segments have collapsed, which occurs quickly after dissociation – their current-voltage relationships
8 are also matching those from rod bipolar cells, both in kinetics and in amplitude.

9 Besides these initial reports, more recent studies suggest that mammalian cones do not
10 express GABA receptors. The lack of a systematic GABA response of macaque cones on flatmount
11 retinas (Verweij *et al.*, 2003), seconded by similar results from David Schneeweis obtained on
12 macaque retinal slices (personal communication reported in Verweij *et al.*, 2003), indicate that
13 macaque cones do not consistently respond, directly or indirectly, to GABA applications. Similarly,
14 GABA-evoked current were not detected in ground squirrel cones, over a large range of potentials
15 (-110 to +40 mV, Sercan Deniz and Steven DeVries, personal communication). In rat (Liu *et al.*, 2013)
16 and mouse (Kemmler *et al.*, 2014) retinal slices, the effect of GABA on cone calcium signals is not
17 direct but occurs through horizontal cells. In the macaque retina, as surround antagonism was
18 observed in only 20% of the cones (Verweij *et al.*, 2003), the lack of an indirect action of GABA on
19 cones in macaque (and ground squirrel) could be due to the lability of the secondary mechanism
20 relaying horizontal cell membrane potential changes to cones in these species. This mechanism could
21 be more robust in rodent retinas, allowing for a more reliable detection of responses in rat and
22 mouse cones – however not in our case. Both Liu *et al.* (2013) and Kemmler *et al.* (2014) prepared
23 slices using a tissue chopper, while we used agarose embedding and vibratome sectioning. Even if it
24 is slower and possibly more mechanically demanding on the tissue due to the blade vibrations, it
25 shouldn't weaken the horizontal cell to cone contact sufficiently to prevent feedback. Studies that
26 did not detect an indirect GABA effect on mammalian cones (Verweij *et al.*, 2003, David Schneeweiss,

1 Steven DeVries, ours) used patch-clamp recordings of cone currents, while those which did (Liu *et al.*,
2 2013; Kemmler *et al.*, 2014) relied on calcium imaging. The indirect effect is expected to be mediated
3 by pH changes in the synaptic cleft (Liu *et al.*, 2013), as well as by hemichannels (Kemmler *et al.*,
4 2014), both resulting in a modulation of the calcium channel activation curve. Whole-cell recordings
5 of cones may perturb this indirect effect, due to the run-down of calcium channel currents. However,
6 we recorded some cones using amphotericin (crossed symbols in Fig. 3E) or gramicidin-perforated
7 patch (Fig. 3F), which should better preserve calcium channel activity over the time course of the
8 experiments. Those cones responded to GABA similarly to those recorded in whole-cell
9 configuration, with no sign of a non-linearity in the potential range affecting calcium channel
10 activation. Another difference between our conditions and those of Liu *et al.* (2013) and Kemmler *et*
11 *al.* (2014) is that we used fully light-adapted retinas. This could tentatively explain the discrepancy
12 between their results and ours, as lateral inhibition is affected by the ambient light level and the
13 light-adaptation state of the retina (reviewed in Thoreson & Mangel, 2012). In Kemmler *et al.* (2014),
14 the conclusion that “GABA is unlikely acting directly at the [mouse] cone terminal, but instead may
15 modulate cone output by controlling other feedback mechanisms”, was based 1) on the fact that the
16 observed changes in calcium signal evoked by GABA puffs in cone pedicles were in contrast to what is
17 expected from a GABAergic inhibition, and 2) on the lack of immunostaining for α_1 and ρ_1 subunits.
18 Neither of these arguments can rule out the presence of GABA_A receptors on cone pedicles: 1) the
19 direct action of GABA on cones depends on E_{Cl} , which most reports point as close to the dark
20 potential: -34 mV in the salamander (Thoreson & Bryson, 2004), -31 mV in the ground squirrel
21 (Szmajda & DeVries, 2011), -37 mV in the putative mouse cones we recorded in the gramicidin
22 perforated-patch configuration. Only a small change in membrane potential is thus expected from
23 the activation of GABA receptors. Moreover, this effect could be masked by the indirect effect
24 mediated by horizontal cells. 2) the presence of the α_1 subunit is not required to have functional
25 GABA_A receptors (reviewed in Olsen & Sieghart, 2008).

1 The group of Stuart Mangel reported in the recent years an increase in the expression of
2 GABA receptor subunits in rabbit cones during the night (Mangel S, et al. IOVS 2013;54:ARVO E-
3 Abstract 404, Mangel S, et al. IOVS 2015;56:ARVO E-Abstract 1340, Mangel S, IOVS 2016;57:ARVO E-
4 Abstract 588). There was however no recording from rabbit cones to demonstrate that this increase
5 is associated with the presence of functional receptors on the cone terminals. While rabbits are
6 endogenously nocturnal animals, they become predominantly diurnal in animal-house conditions
7 (Jilge, 1991). Thus, similar circadian changes in receptor expression may explain the absence of
8 GABA-evoked current in macaque and ground squirrel cones during the day. Nocturnal animals as
9 rats and mice could be expected to have opposed variations, with GABA_A receptor expression during
10 the day. Our GABA responses recordings from mouse cones were obtained during the day (12 PM – 8
11 PM), with no evidence of a circadian influence on the current amplitude (Supplementary Figure 4).
12 This time period may have been too short to detect it, but it should also be kept in mind that both
13 mouse lines used in this study are melatonin-deficient (Ebihara *et al.*, 1986), which may prevent
14 circadian changes in receptor expression.

15 Since the initial demonstration of horizontal cell to cone negative feedback in the vertebrate
16 retina (Baylor *et al.*, 1971), identifying the underlying mechanism(s) has been an active field of
17 research. There is now growing evidence that lateral inhibition results from the compound action of
18 various pathways modulating the pH in the synaptic cleft (Vroman *et al.*, 2014; Warren *et al.*, 2016a),
19 including horizontal cell GABA receptors (Liu *et al.*, 2013), together with an ephaptic mechanism
20 (Kamermans *et al.*, 2001; Kamermans & Fahrenfort, 2004; Warren *et al.*, 2016b; reviewed in
21 Thoreson & Mangel, 2012; Chapot *et al.*, 2017). The causes of many apparent discrepancies have
22 been progressively identified, but many others still have to be understood. Regarding the influence
23 of GABA on cone responses to center-surround illumination, a confounding factor may have been the
24 low capacity of horizontal cells to counteract the GABA loss happening during retinal harvesting for
25 electrophysiology, as described in the mouse retina (Deniz *et al.*, 2011). This may apply to other
26 species, including those with both a well-established GABAergic nature of horizontal cells and

1 expression of GABA receptors on cone terminals, as the turtle. If a GABA-dependent feedback was
2 detected only in presence of pentobarbital in slices (Tatsukawa *et al.*, 2005), it may be occurring
3 more robustly *in vivo*. Besides discrepancies, it is unclear why a diversity of modulatory mechanisms
4 coexist in the horizontal cell to cone synapse, or in other words what is the exact contribution of
5 each mechanism depending on the type of stimuli. It is notably hard to predict the role of cone GABA
6 receptors. As mentioned above, many reports indicate that the cone E_{Cl} is close to the dark potential:
7 opening or closing of GABA receptors in response to changes in the GABA release from horizontal
8 cells should then have little direct effect on the cone membrane potential. This effect will be more
9 substantial when the cone E_{Cl} is more negative than the dark potential, as in the turtle or the goldfish
10 (Kaneko & Tachibana, 1986; Kraaij *et al.*, 2000). As cones are graded-potential neurons, even small
11 changes in membrane potential can change the glutamate release. GABA receptors could also
12 contribute through their influence on the synaptic cleft pH, as proposed for those of horizontal cells
13 (Liu *et al.*, 2013), through their permeability to HCO_3^- . Changes in intracellular Cl^- may also affect
14 release, either through the modulation of calcium channels (Thoreson *et al.*, 2000; Babai *et al.*, 2010)
15 or through changes in osmotic tension (Chavas *et al.*, 2004).

16

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8

9 **Abbreviations:**

10 BBC: bicarbonate-buffered saline

11 DIC: Differential Interference Contrast

12 GABA: γ -amino butyric acid

13 GCL: ganglion cell layer

14 HC: horizontal cell

15 INL: inner nuclear layer

16 ONL: outer nuclear layer

17 OPL: outer plexiform layer

18 TPMPA: (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid

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- 5

1 **FIGURE LEGENDS**

2 **Figure 1: Distinguishing cones from rods through their morphology.** (A-B) Superposition of DIC
3 images from retinal slices and fluorescence images, and fluorescence images only, of recorded cones,
4 filled with Alexa Fluor 488. Cones had an oval soma (s) and a large terminal (t). Stars (*) indicate
5 putative cone somas. (C) Confocal image from a cone filled with Alexa Fluor 488 acquired after
6 recording. (D-E) Superposition of DIC images from retinal slices and fluorescence images, and
7 fluorescence images only, of recorded rods, filled with either Alexa Fluor 488 or 594. The smaller
8 rounded somas and terminals contrast with those of cones. Scale bars: 10 μm in C, 20 μm in other
9 panels.

10

11 **Figure 2: Electrophysiological signature of cones and rods.** (A-B) Representative currents evoked by
12 voltage-jumps in a cone (A) and in a rod (B), from a holding potential of -74 mV, with 100 ms steps to
13 potentials ranging from +46 mV to -134 mV by 20 mV decrements ($E_{\text{Cl}} = -29$ mV intra-pipette
14 solution). The magenta horizontal bar indicates the period considered to measure the “plateau”
15 current, as plotted in D, F and G; the green arrow indicates the time at which the tail current was
16 measured, 13 ms after repolarization, as plotted in panels E, F and G. (C) Currents evoked by voltage-
17 jumps in a cone as in A, with a longer time scale to show the full deactivation of the Ca^{2+} -dependent
18 Cl^- current. The interval between steps was 10 s to allow for a full deactivation of this current. (D)
19 Current-voltage relationship of the current evoked by 100 ms voltage jumps, from a holding potential
20 of -74 mV, to potentials ranging from +46 mV to -134 mV by 10 mV decrements ($E_{\text{Cl}} = -29$ mV intra-
21 pipette solution), measured from 97 to 99 ms following depolarization, for morphologically identified
22 rods (black circles, $n = 13$) and cones (open circles, $n = 11$). (E) Current-voltage relationship of the tail
23 current measured 13 ms after returning to the holding potential of -74 mV, following 100 ms voltage
24 jumps to potentials ranging from +46 mV to -134 mV by 10 mV decrements ($E_{\text{Cl}} = -29$ mV intra-
25 pipette solution), for rods (black circles, $n = 13$) and cones (open circles, $n = 11$). (F-G) Individual

1 values of the “plateau” current measured at +26 mV (F) or the tail-current measured after a
2 depolarization at +6 mV (G) for rods (grey symbols when spherule is present, n = 13; orange symbols
3 for rods for which no spherule could be seen at the end of the recording but identified as rods
4 through their external segment, n = 11), putative rods (cyan symbols, for cells considered as putative
5 rods due to their soma appearance prior to patching, n = 5), cones (green symbols, presence of a
6 pedicle, n = 11) or putative cones (red symbols, cells for which no pedicle could be seen at the end
7 of the recording, but with a soma appearance suggestive of a cone prior to recording, n = 8).
8 Triangles are used for rods in which an intact outer segment could be identified at the end of the
9 recording, circles are used for other cells. The dotted line delimits the [$I_{\text{tail}} < 100$ pA, $I_{\text{plateau}} < 350$ pA]
10 range, which includes all identified and putative rods, and only one of the identified or putative
11 cones.

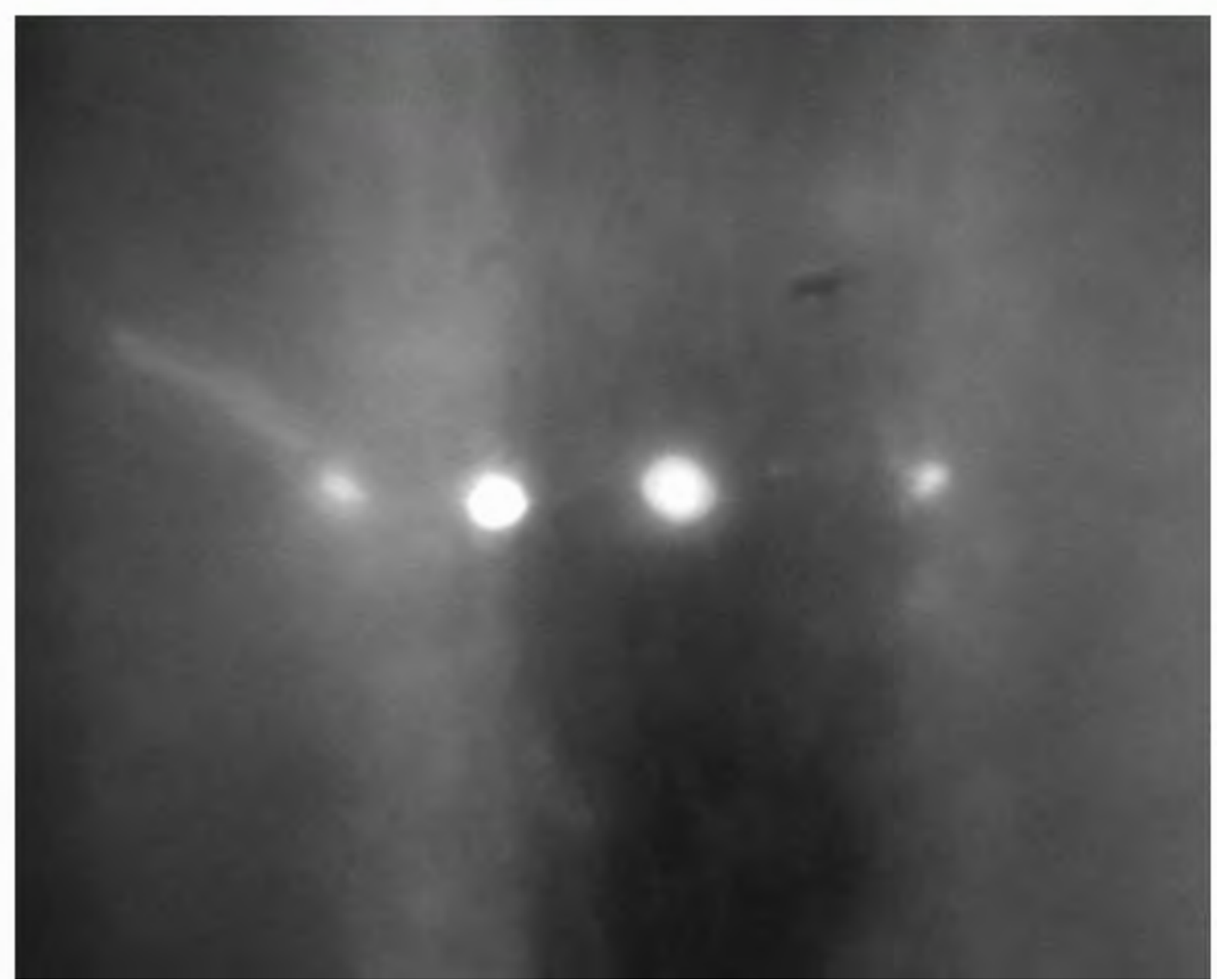
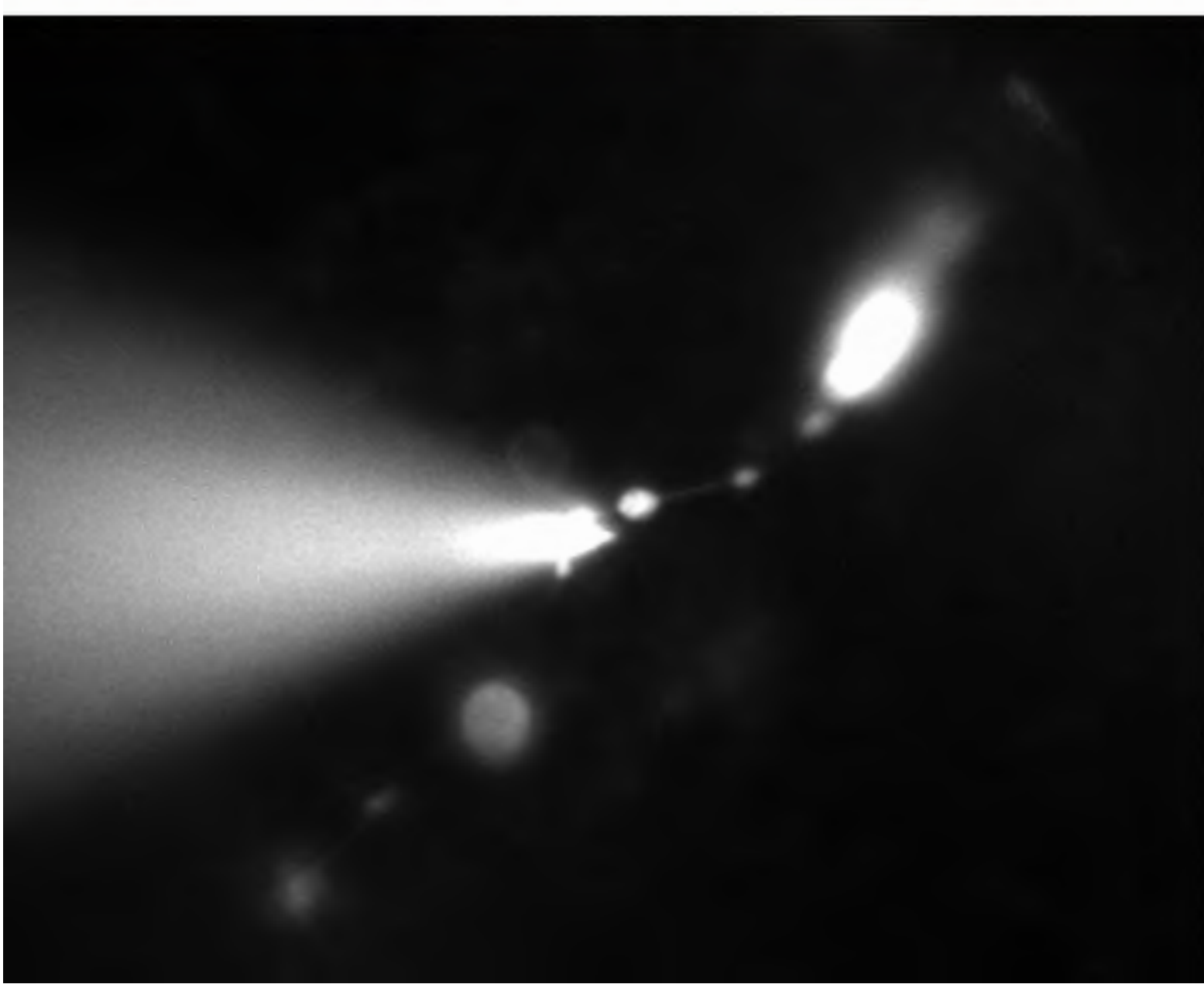
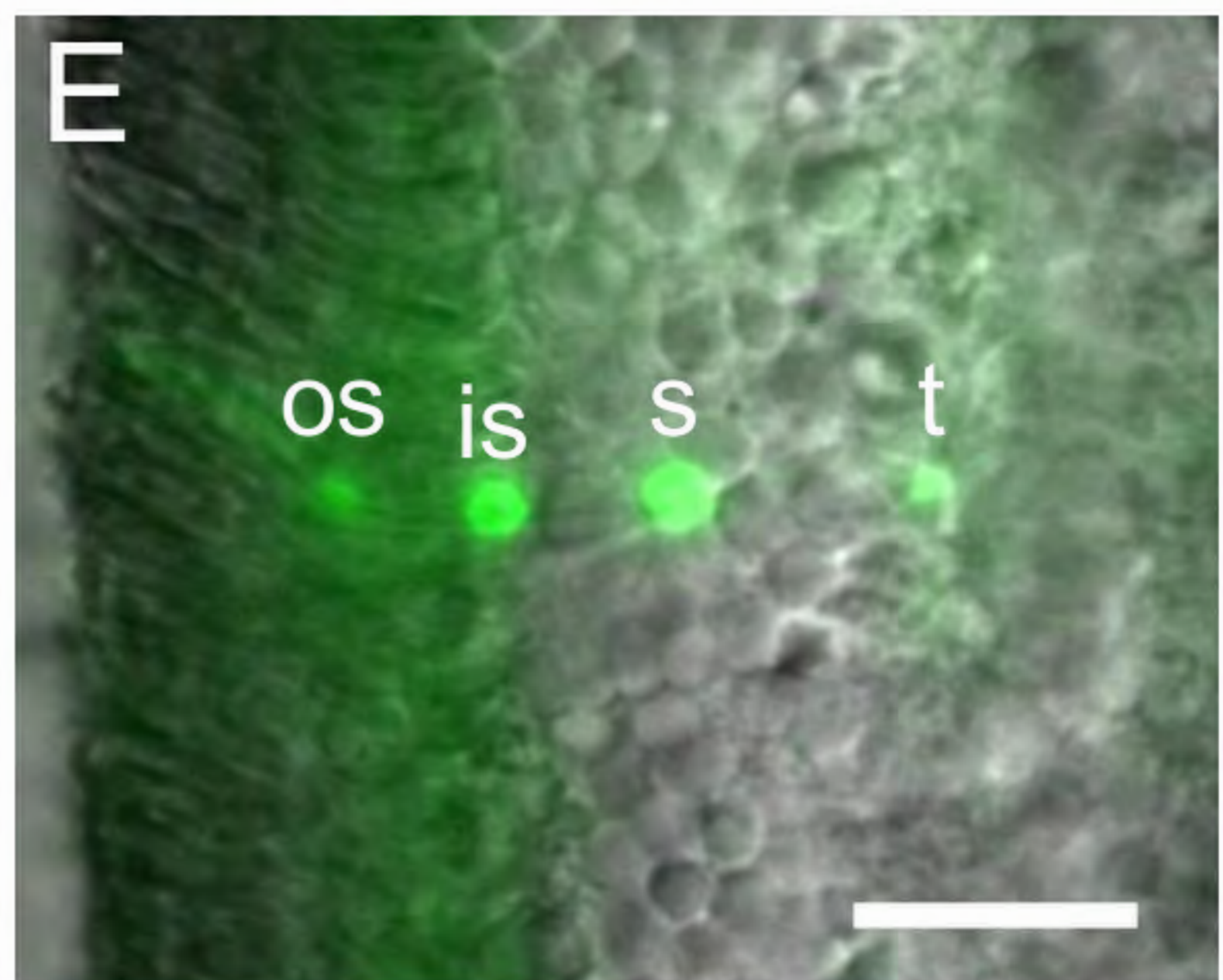
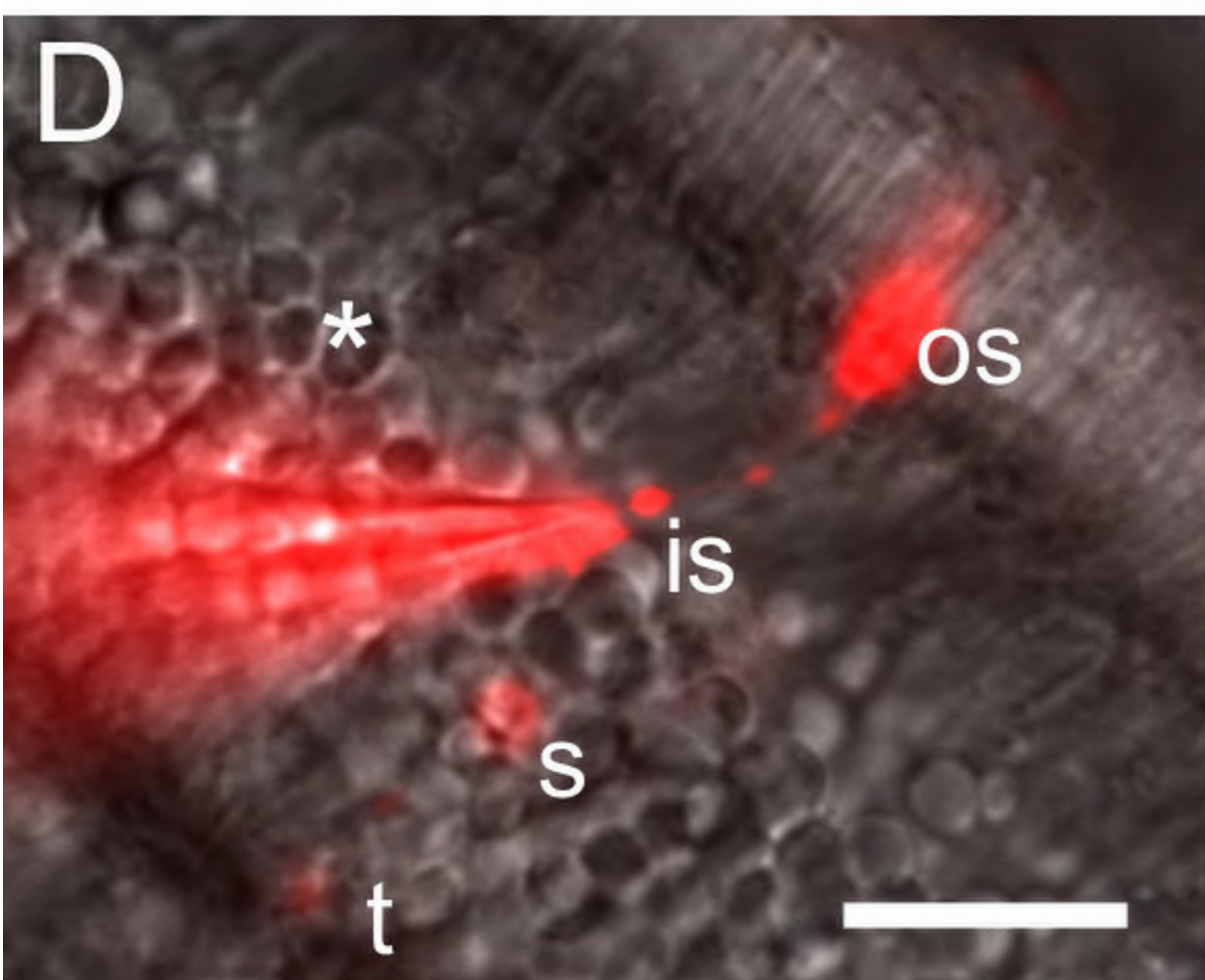
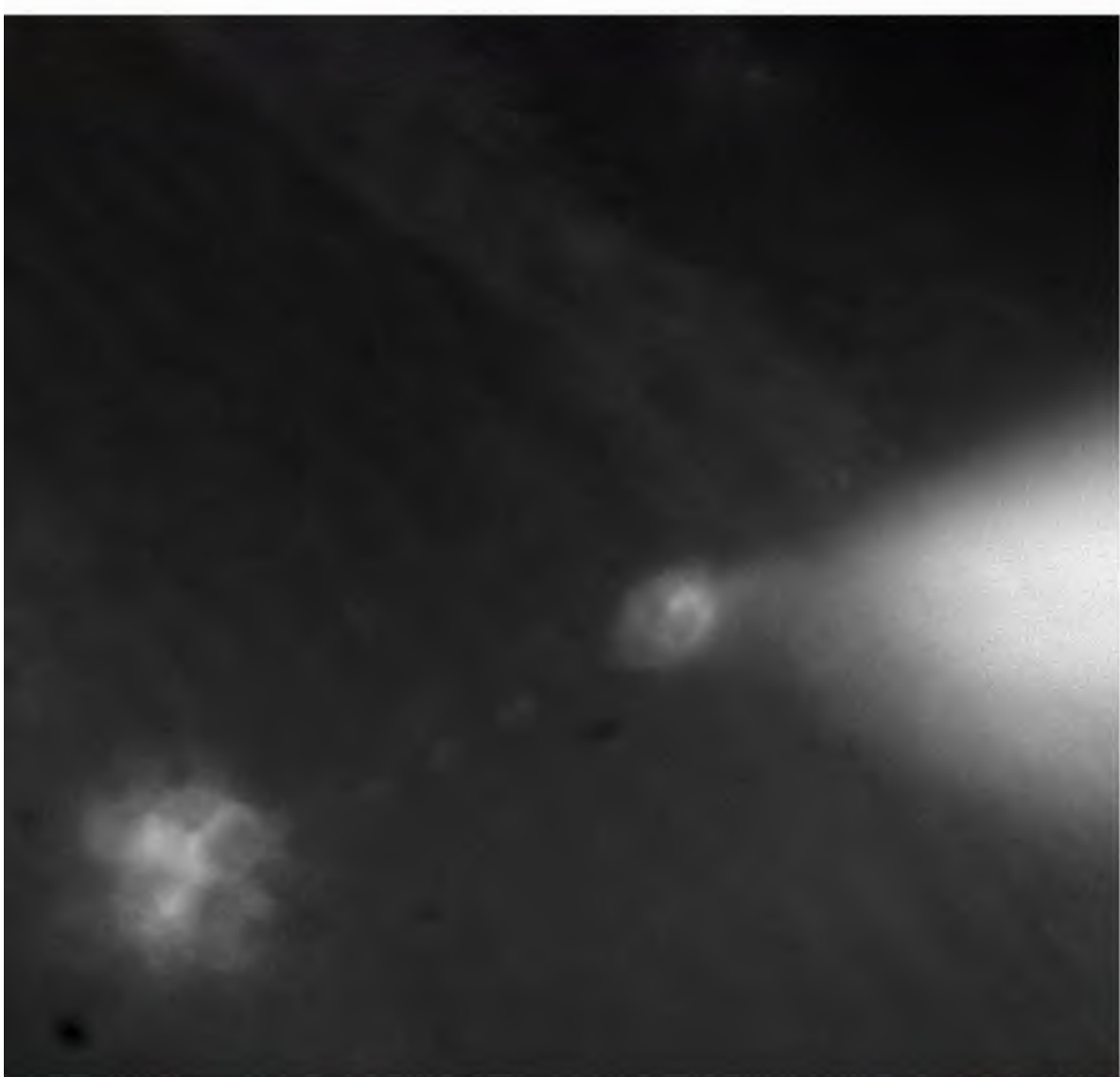
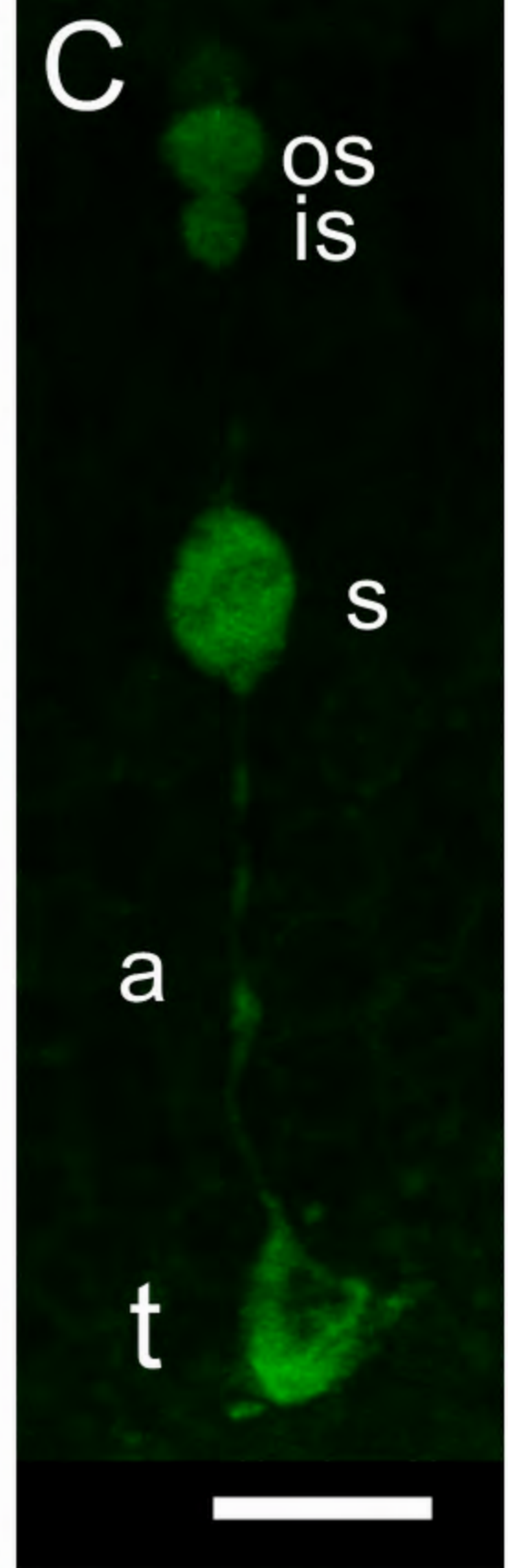
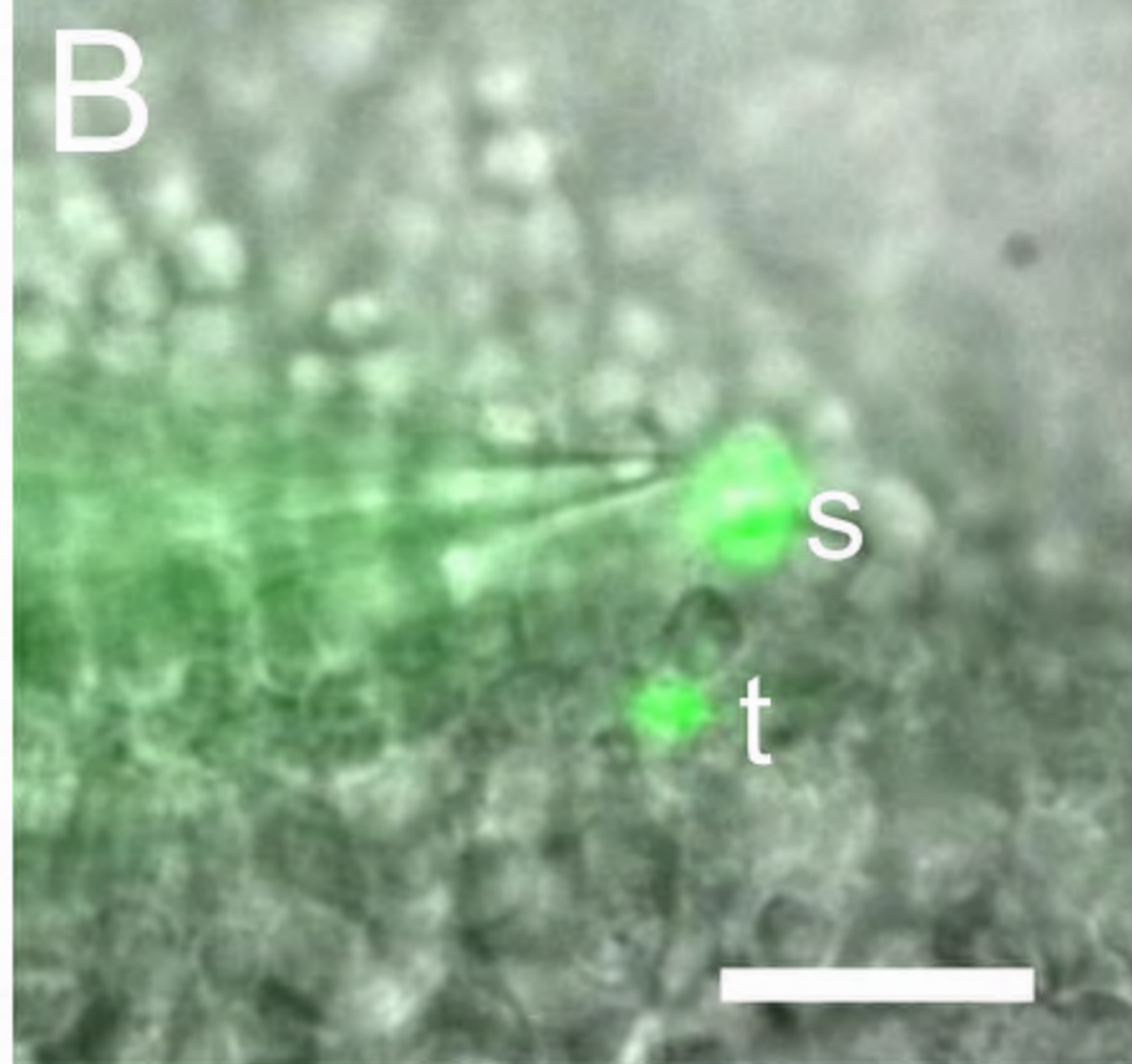
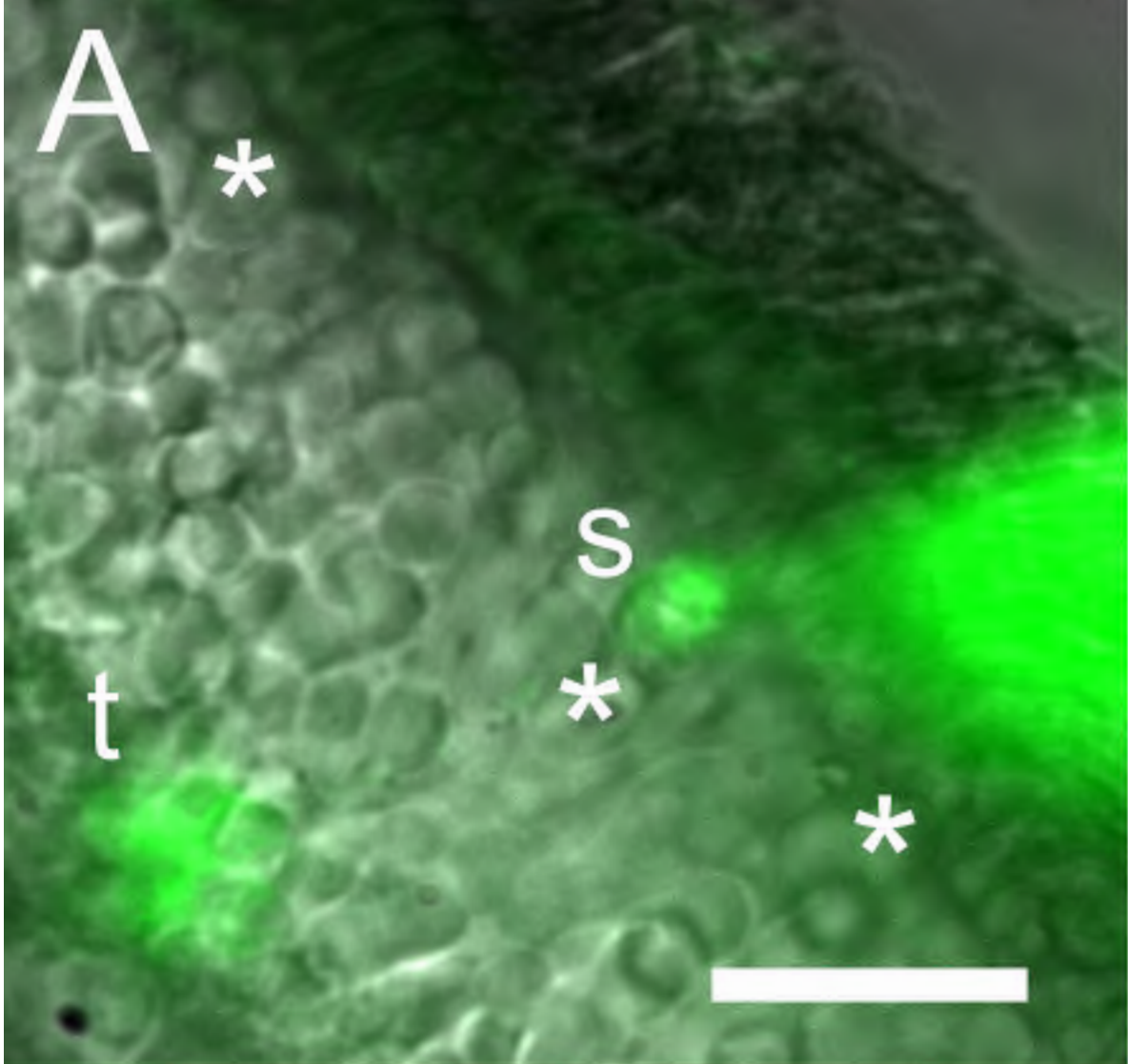
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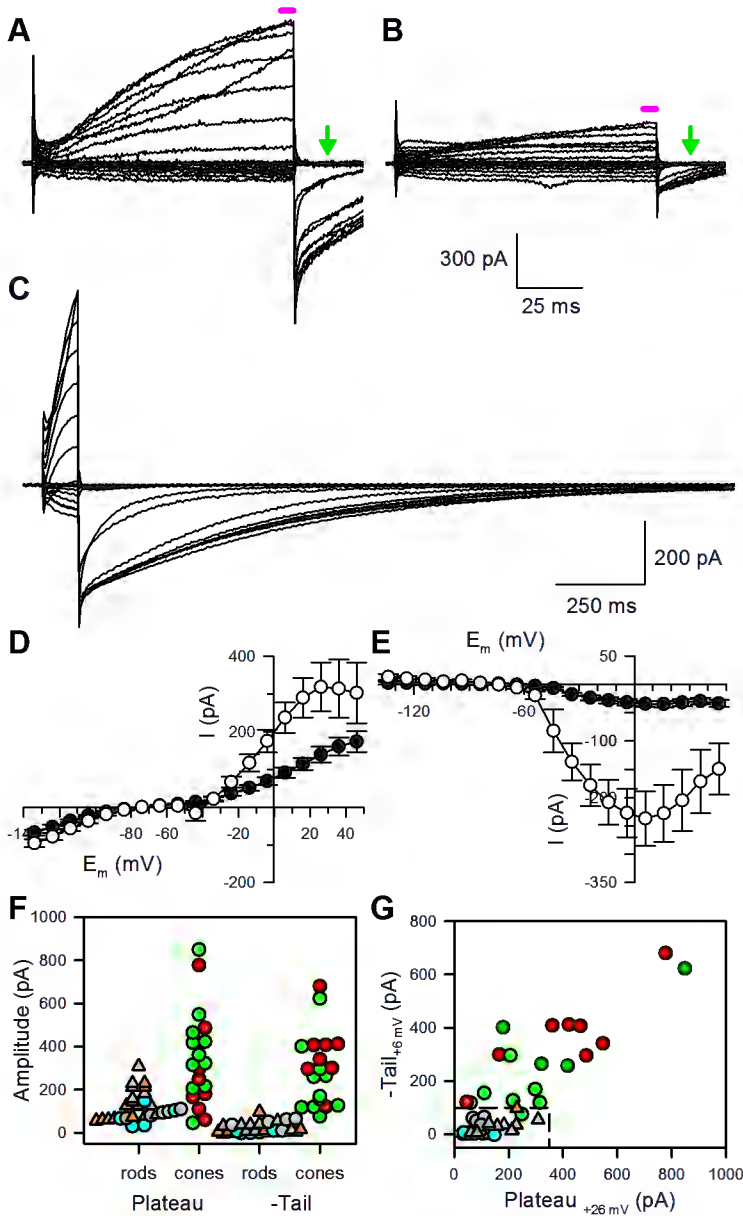
13 **Figure 3: Cone responses to GABA have a GABA_A pharmacology.** (A) Puff application of GABA (100
14 ms, 1 mM) in the vicinity of a photoreceptor terminal evoked a current in cones (left) but not in rods
15 (right). The holding potential was -74 mV, as in B and C. (B) Cone responses to puff application of
16 GABA (100 ms, 1 mM, control trace in black) were blocked by 100 μ M bicuculline methiodide (left,
17 red trace) but not by 50 μ M TPMPA (right, blue trace). Traces obtained following wash out of the
18 applied drug are shown in grey. (C) As in B, but for a cone which GABA response was not fully blocked
19 by 100 μ M bicuculline methiodide (left, red trace). The right panel represents the traces from the left
20 panel normalized to their peak, showing that the remaining component has the same kinetics as the
21 control and wash currents. (D-E) Currents evoked by GABA reversed close to E_{Cl} , whether it was set
22 close to -29 mV (D, 5 cells) or 2 mV (E, 6 cells), depending on the intrapipette solution. The calculated
23 E_{Cl} is represented by a red line for reference. Each cell is plotted with a given color and distinct
24 symbols linked by a dashed line. (F) GABA-evoked currents as a function of membrane potentials for

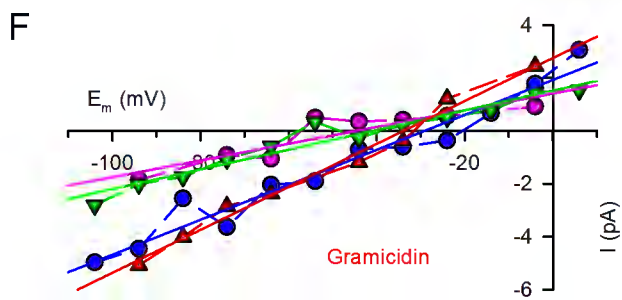
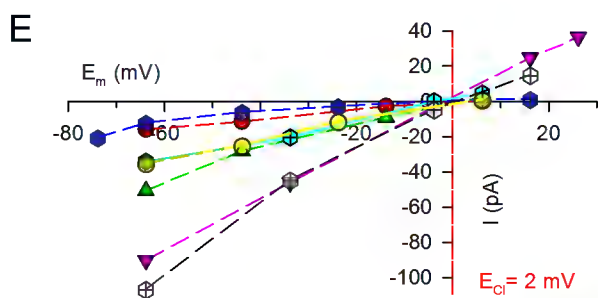
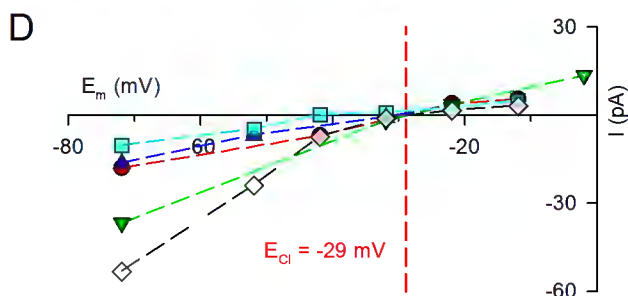
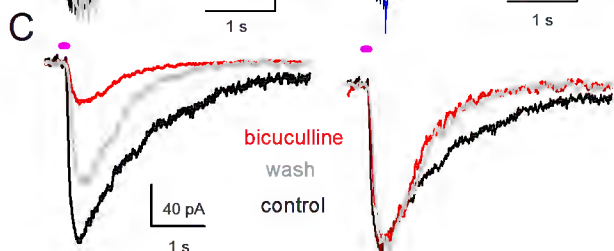
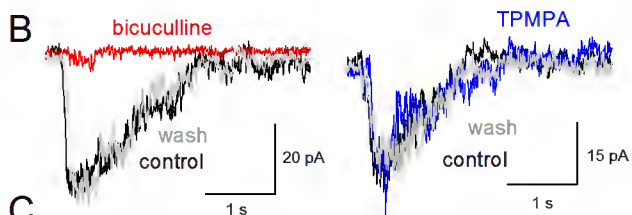
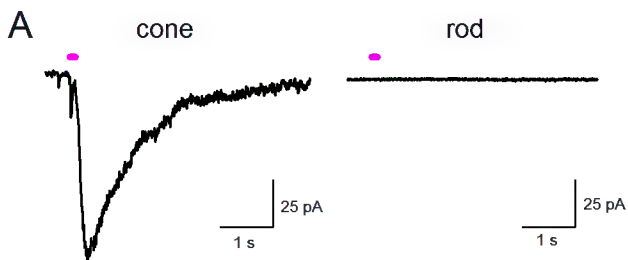
1 4 cones recorded using gramicidin perforated patch. Data points of a given cell are linked by a dashed
2 line; solid lines are linear regressions to the data points.

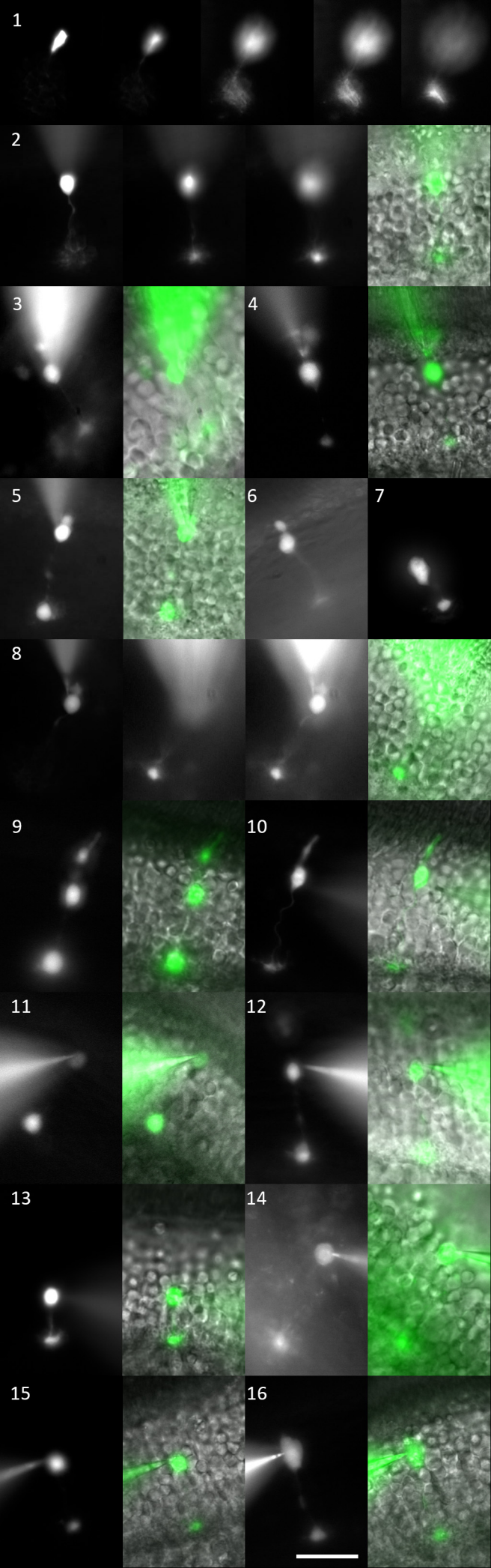
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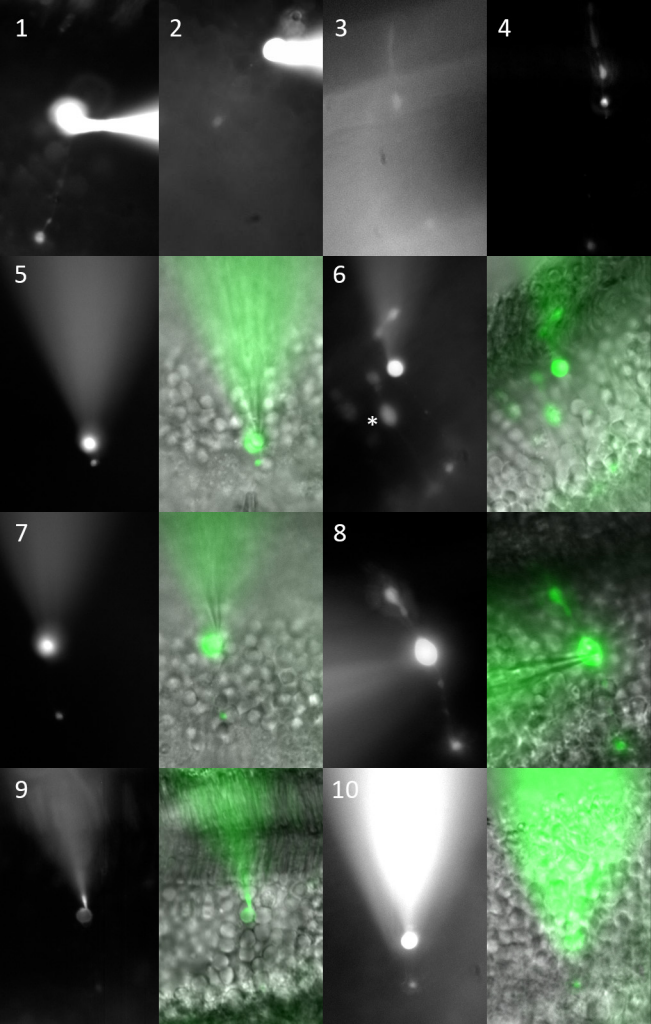
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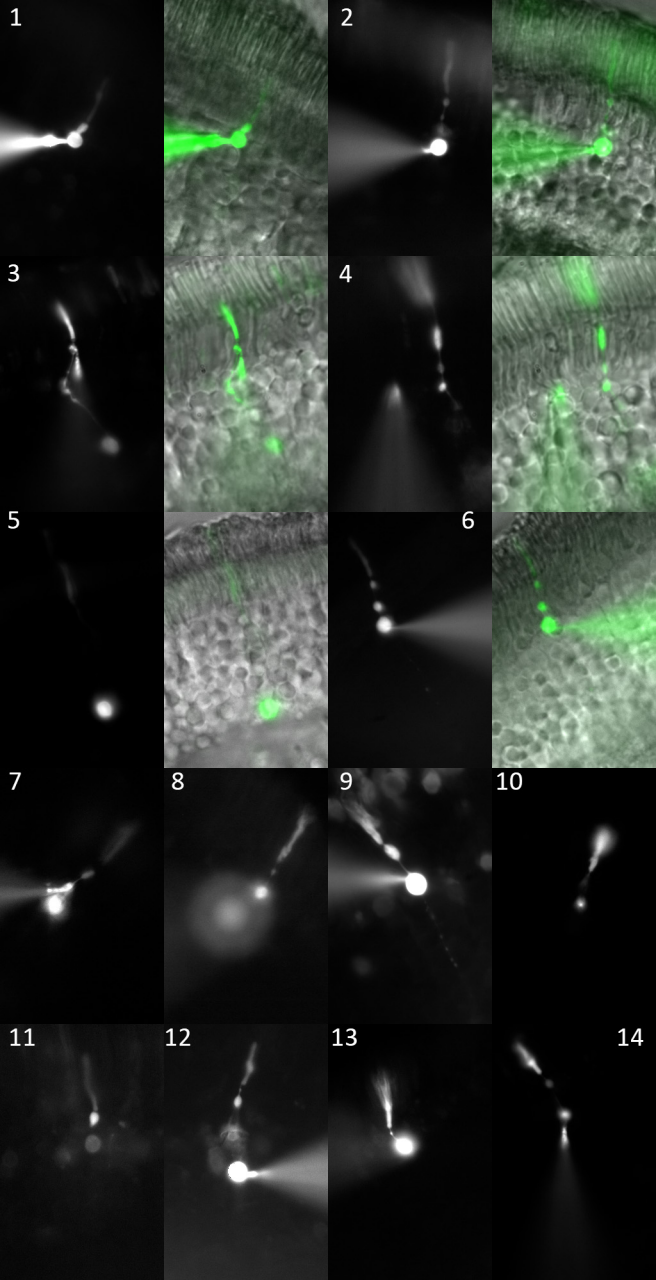


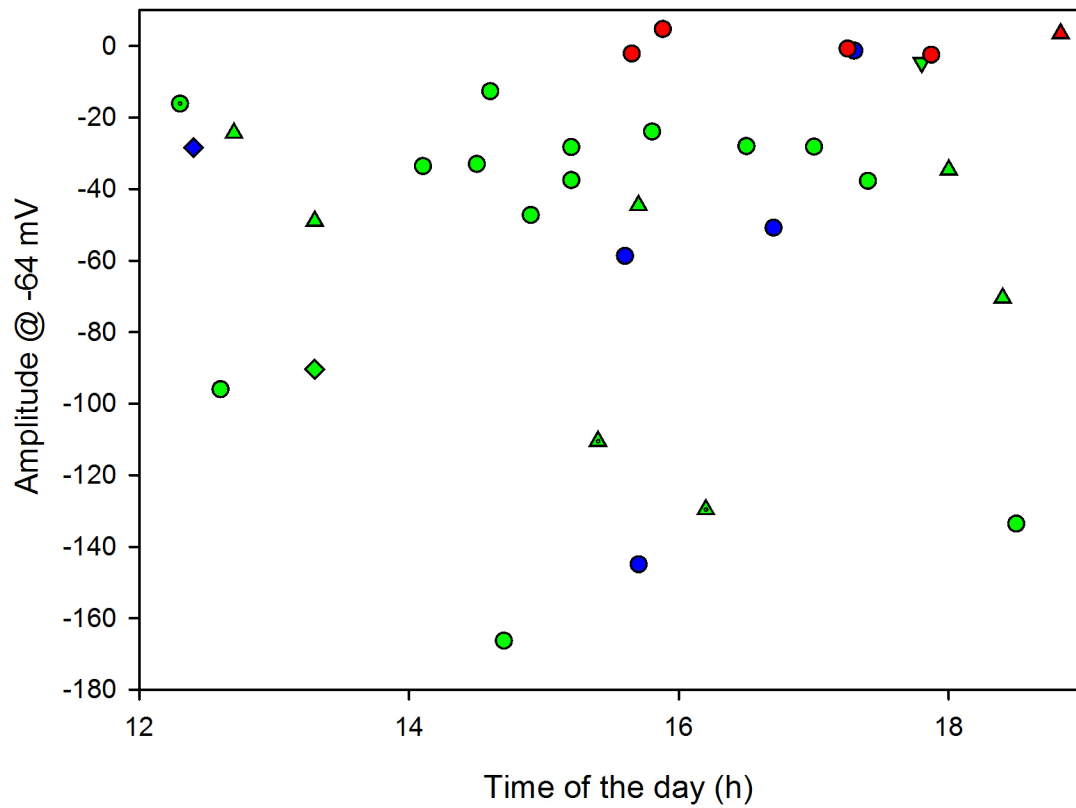












- $E_{Cl} = 0$ mV
- $E_{Cl} = -30$ mV
- Gramicidin

Legends of Supplementary Figures

Supplementary Figure 1: Cones with pedicle

Morphology of 16 recorded cones with their pedicle, obtained through the inclusion of an Alexa dye in the intrapipette solution. For cones 1,2 and 8,, images taken at different depth of focus are represented. For 13 of them, superimposition with a DIC image of the ONL/OPL is also presented. Scale bar in 16: 30 μm .

Supplementary Figure 2: Rods with spherule

Morphology of ten recorded rods with their spherule. For rods 2 and 3, the image was taken after retrieval of the patch pipette, to which the cell soma remained attached. In 6, a cone previously recorded is labeled with a *, next to the brighter rod.

Supplementary Figure 3: Rods with outer segment but without spherule

Morphology of 14 recorded rods identified through the presence of a long, thin outer segment, but which had lost their spherule during the slicing process.

Supplementary Figure 1: Amplitude of the GABA-evoked current as a function of the time of recording.

Amplitude of the current evoked by a 1 mM GABA puff of duration 5-10 (\diamond), 50 (\triangle), 100 (\circ) or 250 ms (∇). Recordings were obtained with intrapipette solutions with $E_{\text{Cl}} = -30$ mV (blue symbols) or $E_{\text{Cl}} = 0$ mV (green symbols, open for whole-cell, dotted symbols for amphotericin perforated patch). For cones not recorded with the $E_{\text{Cl}} = 0$ mV intracellular solution, the current amplitude was corrected for the difference in driving force. Recordings obtained using gramicidin perforated patch are indicated by red symbols, and were left uncorrected