Mechanisms and circuitry underlying directional selectivity in the retina

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In the retina, directionally selective ganglion cells respond with robust spiking to movement in their preferred direction, but show minimal response to movement in the opposite, or null, direction[1,2]. The mechanisms and circuitry underlying this computation have remained controversial[3]. Here we show, by isolating the excitatory and inhibitory inputs to directionally selective cells and measuring direct connections between these cells and presynaptic neurons, that a presynaptic interneuron, the starburst amacrine cell, delivers direct inhibition to directionally selective cells. The processes of starburst cells are connected asymmetrically to directionally selective cells: those pointing in the null direction deliver inhibition; those pointing in the preferred direction do not. Starburst cells project inhibition laterally ahead of a stimulus moving in the null direction. In addition, starburst inhibition is itself directionally selective: it is stronger for movement in the null direction. Excitation in response to null direction movement is reduced by an inhibitory signal acting at a site that is presynaptic to the directionally selective cell. The interplay of these components generates reduced excitation and enhanced inhibition in the null direction, thereby ensuring robust directional selectivity.

Directionally selective (DS) movement detection was first characterized in rabbit retina in a series of classical experiments[2], which suggested that directional selectivity was mediated by an interaction of excitatory and inhibitory signals, whereby inhibition suppressed excitation for movement in the null direction. This role of inhibition was supported by later studies showing that directional selectivity is lost in the presence of antagonists of GABA (γ-aminobutyric acid), an inhibitory transmitter[4,5]. Other studies have suggested that starburst amacrine cells supply this crucial inhibitory input to DS cells because they co-fasciculate with the processes of DS cells[6,7] and release GABA[8–10].

But the results of some other studies have been contradictory about the role of starburst cells: laser ablation of starburst cells did not eliminate directional selectivity in rabbit[11], but eliminating starburst cells with neurotoxin did eliminate it in mouse[12]. The site of interaction between excitation and inhibition is also controversial: one report suggests a postsynaptic interaction at the DS cell dendrites[13], another suggests a presynaptic location[14]. Here we have addressed each of these issues to yield a comprehensive description of the synaptic inputs and circuitry that endow the DS cell with directional selectivity.

The excitatory and inhibitory input currents (Fig. 1b) underlying the DS spiking output (Fig. 1a) were measured in on–off DS cells in response to preferred and null direction movement. We isolated these currents by patch clamping at the appropriate reversal potentials (Methods). Preferred direction movement always ($n = 22/22$) elicited larger excitatory currents than did null direction movement (mean 2.5-fold, range 1.1–5.3), whereas the inhibitory currents were always larger for movement in the null direction (mean 1.6-fold, range 1.2–3.7). The same asymmetries were measured when the movement was confined within the receptive field of the DS cell ($n = 10/10$; Fig. 1c). Thus, similar to the spiking output[2], directional selectivity of the inputs is achieved by a local computation that does not require crossing of the receptive field boundaries. The asymmetries in magnitude of the input currents suggest that directional selectivity may be formed at sites that are presynaptic to the dendrites of DS cells. Our results show that both inputs are themselves already directional (but see refs 13, 14), but they are delivered to the dendrites of the DS cell where there is presumably further interaction.

A clue to how these asymmetries in magnitude are formed can be found in the spatial profile of the inhibitory input. This profile was offset with respect to the dendritic field of the DS cell. We observed this by correlating the positions at which excitation and inhibition were generated with the dendritic field of the DS cell in response to an edge moving in the preferred direction (Fig. 1d and Methods). The excitatory response profile fell symmetrically around the dendritic field of the DS cell, but the inhibitory response profile was shifted towards the null side of the DS dendritic field. This was not a consequence of the direction of movement: a similar offset in the inhibitory profile was seen for a stimulus moving in the null direction (Fig. 1e). Nor were these profiles a consequence of movement at all: a similar offset of the inhibitory profile could be generated by mapping the receptive field with stationary test flashes moved to different positions along the preferred–null axis of the DS cell receptive field (Fig. 1f).

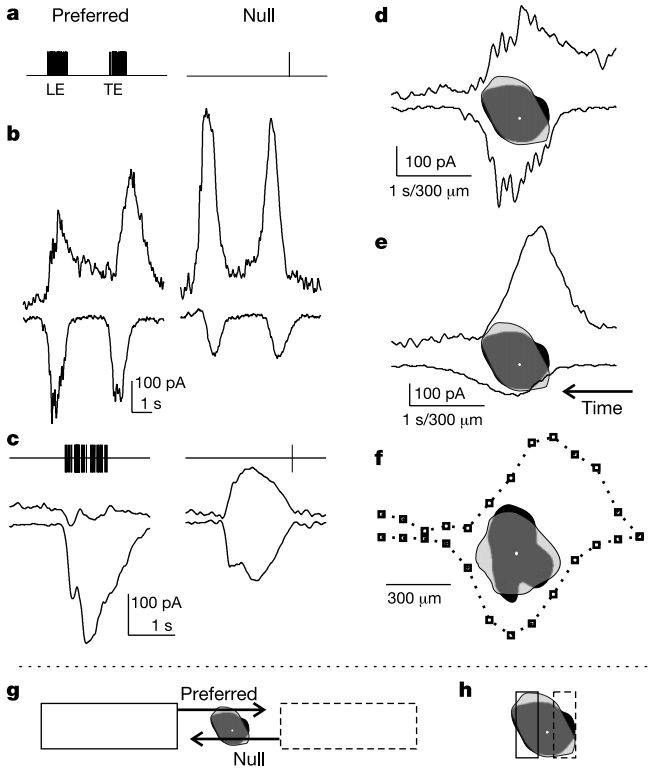

Figure 1 Excitatory and inhibitory inputs to DS ganglion cells: asymmetries in magnitude and space. **a**, Spiking response to movement in the preferred and null directions (the stimulus is shown in **g**). Leading (LE) and trailing (TE) edges of the stimulus generate spiking. **b**, Excitatory (downward deflections) and inhibitory (upward deflections) inputs in response to the same stimulus. **c**, Spiking, inhibition and excitation in response to a stimulus constrained within the dendritic field (depicted in **h**). **d**, **e**, Excitation and inhibition (from **b**) plotted as a function of the stimulus edge position (see Methods) for preferred (LE, **d**) and null direction movements (TE, **e**). Shaded regions indicate off (dark) and on (light) dendrites. Bright spot indicates soma. Current traces in **e** are reversed to reflect right to left movement (indicated by time arrow). **f**, Profiles of excitation and inhibition in response to 100-μm square stationary flashes along the preferred–null axis. **g**, The stimulus bar (300 × 900 μm) used in **a**, **b**, **d** and **e**. Outlines indicate start and end positions. **h**, The stimulus bar (300 × 100 μm) used in **c**. In all figures, axes are aligned so that the preferred direction is from left to right.

These three measurements of excitatory and inhibitory response profiles suggest that excitation is symmetrically arranged around the dendritic field of the DS cell but that there is a region outside the dendritic field of the DS cell on the null side, an 'inhibitory lobe', that is populated by a separate set of neuronal elements that detect movement and project inhibition laterally to the dendrites of the DS cell. A consequence of this inhibitory lobe is that, for null direction movement, the inhibitory signal arrives at the DS cell before excitation (Fig. 1b, e). For the cell shown in Fig. 1f, the inhibitory lobe was offset by about 150 μm (range 50–200 μm, $n = 6$).

We thought that starburst amacrine cells were likely to populate the inhibitory lobe. Their dendritic arbour is broad enough (about 300 μm)[15] to carry signals laterally by more than 150 μm, which was the dimension of the inhibitory offset shown in Fig. 1f. To test whether starburst cells do provide direct inhibition to the DS cells, we simultaneously patched a DS cell and a starburst cell whose soma was located in the inhibitory lobe of the DS cell. Depolarization of such a null-side starburst cell elicited inhibition in the DS cell, confirming that the inhibitory lobe is comprised of starburst cells ($n = 3/3$; Fig. 2b). There were variations in the time course of the inhibitory currents for each cell pair (data not shown), but starburst depolarizations above −20 mV always elicited inhibition. We did not see any excitation from starburst cells in this lobe. We made similar attempts to measure connectivity from starburst cells on the preferred side of the DS cell (Fig. 2a, $n = 3$), but found neither excitatory nor inhibitory activity. Figure 2c summarizes the results of the null-side cell pairs. The steady-state inhibitory currents measured in the DS cell are plotted as a function of starburst cell depolarization for each cell pair. For comparison, a typical *I–V* curve for a preferred-side cell pair is also shown.

The measurements showing a lack of excitatory input from starburst cells to DS cells were unexpected. It is known that starburst cells release acetylcholine[16–18], and that DS cells show cholinergic sensitivity[4,19]. If the excitatory connectivity exists then it is probably sparse, being expressed by cells other than the ones we measured (starburst cells overlap greatly[20]), or it is very subtle as compared with the inhibitory inputs. The results of our double patch experiments are, however, consistent with the existence of only an inhibitory lobe on the null side and the lack of any offset lobe on the preferred side of the DS dendrites (Fig. 1f).

Notably, there was an anatomical correlation with our physiological measurements: the cell pairs on the null side showed 17.5 ± 1.4% (mean ± s.d.) co-fasciculation (Fig. 2e), whereas those on the preferred side (Fig. 2d) showed only 6.4 ± 2.9% co-fasciculation ($P < 0.01$, unpaired *t*-test).

Could directional selectivity originate in the starburst cells themselves? Previous recordings from starburst cells detected no obvious directionality of their light response[21], but several groups have theorized that starburst cells might release transmitter asymmetrically[22,23]. The inhibitory input from starburst cells can be measured in isolation in the DS cell by confining the movement of a stimulus within the inhibitory lobe, which lies completely outside the dendritic field of the DS cell in a region where there is no excitatory input (Fig. 2g). Within this lobe, movement in the null direction elicited a larger inhibitory current than did movement in the preferred direction (Fig. 2f). Starburst cells in this lobe contact DS cells with only their left processes. This suggests that starburst cells release more inhibitory transmitter if a stimulus moves outward along these processes.

We draw three conclusions from the results shown in Fig. 2. First, the connectivity between a starburst cell and a DS cell depends on

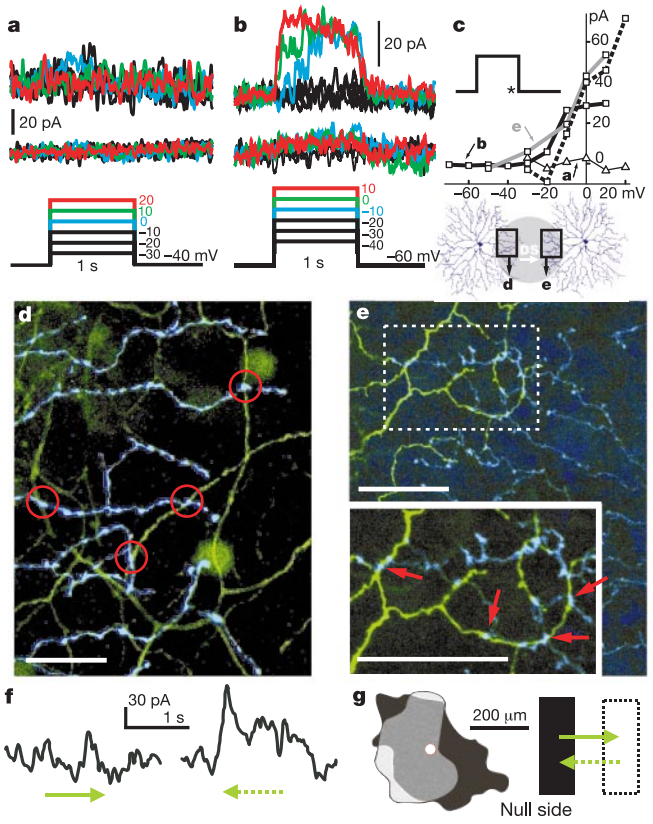

Figure 2 Starburst cells on the null side supply directionally selective inhibition to DS cells. **a**, **b**, Inhibitory (top traces) and excitatory (middle traces) input currents measured in DS cells when neighbouring starburst cells on the preferred side (**a**) or null side (**b**) were stimulated with depolarizing voltage steps (bottom traces). **c**, Inhibitory input currents measured in DS cells at the indicated time (asterisk) as a function of the stepping voltage of a starburst cell for all null-side (squares) and one preferred-side (triangles) cell pairs. *I–V* curves from the cells shown in **a**, **b** and **e** are indicated. **d**, **e**, Confocal image slices (~1.5-μm thick) of the contact region between DS (green) and starburst (cyan) pairs. There is co-fasciculation on the null side (**e**, red arrows; inset shows magnified view of outlined region), but mainly crossings on the preferred side (**d**, red circles). **f**, Inhibitory input currents to a DS cell when a stimulus contained within the inhibitory lobe (depicted in **g**) is moved in the preferred (left) or null (right) directions. **g**, The stimulus black bar (300 × 100 μm) used in **f**. Shaded regions indicate off (dark) and on (light) dendrites. Bright spot indicates soma. Scale bars, 20 μm (**d**, **e**).

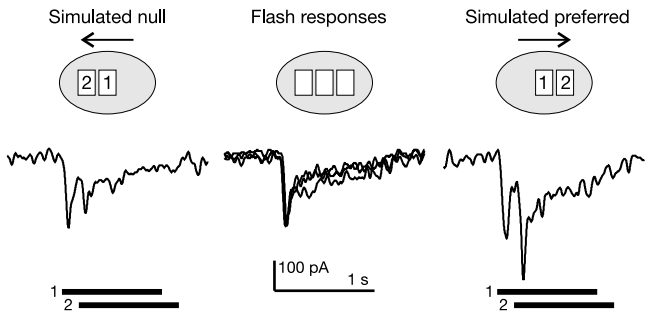

Figure 3 Null movement vetoes excitatory input to the DS cell. Middle, three superimposed excitatory responses to flashed bars (100 × 60 μm) at three locations (separated by 60 μm) within the dendritic field of the cell. Flashes at each of the three locations generate similar responses. Right, excitatory response to two flashes (1 then 2), simulating preferred direction movement. Left, excitatory response to two flashes simulating null direction movement. Grey ovals indicate the dendritic field of the DS cell. White rectangles indicate the spatial positions of the flashes. Horizontal bars at bottom of the left and right panels indicate the timing sequence of the two flashes (167-ms delay between onsets of 1 and 2).

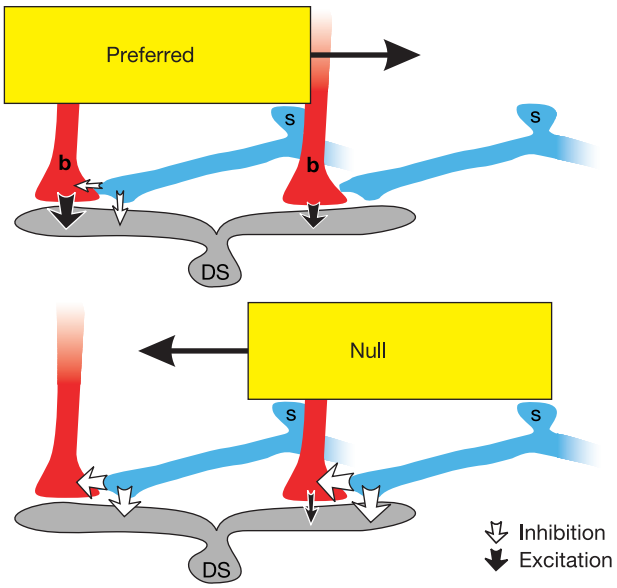

Figure 4 Proposed circuitry underlying the DS response. The processes of starburst cells ('s', blue) that point in the null direction provide inhibition to DS cell dendrites ('DS', grey). Starburst processes respond best to movement away from the cell body, which makes the inhibitory input delivered to DS cells larger for movement in the null direction (bottom panel) than for movement in the preferred direction (top panel). An additional inhibition acts presynaptically to reduce excitation for null direction movement. Although this presynaptic inhibition is depicted as coming from starburst cells, our results do not rule out the existence of another type of cell. The excitatory input to DS cells probably comes from bipolar cells ('b', red) and may also have a cholinergic component from other starburst cells. For movement in the null direction, the inhibitory input reaches each subregion of the DS cell ahead of the stimulus edge and therefore before excitation. For movement in the preferred direction, inhibition lags behind excitation.

the relative location of the starburst cell: starburst cells on the null side, but not the preferred side, supply inhibition, an idea that was proposed previously in the co-transmission model[24]. Second, starburst cells detect movement at a site that is displaced from the site where they deliver inhibition to the DS cell, consistent with the proximo-distal segregation of input and output sites[25]. Third, this inhibition delivered by starburst cells is itself directionally selective, a finding that is complemented by studies showing that a stronger depolarization is measured at the starburst soma in response to centrifugal stimuli (ref. 26 and T.A.M., S.I.F and F.S.W., unpublished data), and that a larger Ca^{2+} signal is measured in starburst cell processes in response to outward motion[26].

Could the directional selectivity inherent in the inhibitory input contribute to directional selectivity in excitation? To examine this relationship, we tested the effect of inhibition on excitation by simulating movement with a paired sequence of flashes (Fig. 3). The first flash was presented at the centre of the dendritic field of a DS cell. We measured its effect on a second flash, presented to either side of the first flash. When the second flash was presented to the right, simulating movement in the preferred direction, the excitation elicited by the second flash simply added to that of the first flash. But when the second flash was presented to the left, simulating movement in the null direction, the excitatory response to the second flash was suppressed completely. We quantified this difference by extracting the maximum amplitude of excitation for each recording and then comparing simulated motion to the sum of the two flashes presented individually. The simulated null motion was 47 ± 19% (mean ± s.d.) of the sum of the individual flashes, whereas the simulated preferred motion was 97 ± 33% of the sum of the individual flashes ($n = 6$, data not shown). These results suggest that inhibition is broadcast only to the left of the stimulus to interfere with excitation for movement in the null direction. This inhibition seems to act presynaptically, modulating the excitatory input before it arrives at the DS cell. It therefore confers directional selectivity to the excitatory input. In the preferred direction, a few cells showed a supralinear summation (data not shown), suggesting a facilitatory mechanism may exist.

Directional selectivity seems to be mediated by complementary mechanisms, as shown in the proposed circuitry in Fig. 4. The starburst cells themselves provide a directionally selective inhibitory input to the DS cell at a site displaced in the null direction from the site of movement. A similarly displaced inhibition, possibly also from starburst cells, suppresses the excitatory signal before excitation arrives at the DS cell, so excitation also seems to be directionally selective. Robust directional selectivity is generated by a combination of increased inhibition and suppressed excitation in the null direction.

Our study moves the unknown issues related to directional selectivity to sites upstream of the DS cells and raises new questions. How do starburst cells come to favour centrifugal motion? Is it a simple biophysical property of the cell, or does it involve synaptic interactions? What is the role of acetylcholine in directional selectivity? Perhaps even more intriguing is the issue of how the spatially asymmetric connectivity between starburst cells and DS cells is established during development. □

Methods

Electrophysiology

We recorded from 40 'on–off' DS ganglion cells and 6 pairs of 'on' starburst amacrine and DS cells in light-adapted whole-mount retinas of 2.5-kg New Zealand white rabbits using EPC-7 amplifiers (Heka). The retina was continuously superfused at 7–10 ml min^{-1} with Ames solution (Sigma; pH 7.4, 36 °C), equilibrated with 95% O_2 and 5% CO_2. Spiking was recorded with a loose cell-attached electrode (3–4 MΩ), filled with Ames solution. For DS cell recordings, whole-cell electrodes (5–8 MΩ) were filled with (in mM): 112.5 $CsMeSO_4$, 1 Mg SO_4, 7.8×10^{-3} $CaCl_2$, 0.5 BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrapotassium salt), 10 HEPES, 4 ATP-Na_2, 0.5 GTP-Na_3, 5 lidocaine *N*-ethyl bromide (QX314-Br), 7.5 neurobiotin chloride (pH 7.2). For starburst cells, we used 1.5 mM EGTA instead of BAPTA, and the concentrations for $CaCl_2$ and neurobiotin chloride were 0.5 and 6.7 mM, respectively. Before double patch experiments, the tissue was incubated for 10–15 min in 2–10 μM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) to stain starburst cell bodies[27]. We measured input currents as described[28] by voltage clamping the DS cell at the reversal potential for chloride channels (−60 mV) or ligand-gated nonspecific cation channels (0 mV) to measure excitation or inhibition, respectively. We assessed the effectiveness of space clamp in control experiments (data not shown) by applying SR-95531, a $GABA_A$ antagonist, which blocked the inhibitory signal. Clamping at 0 mV showed no input currents, as expected in the presence of a good space clamp. To plot the *I*–*V* curves (Fig. 2c), we averaged the inhibitory currents measured in the DS cells over a 200 ms window at the end of the depolarizing pulse to the starburst cell. The data acquisition software (RED) was written by M. Wang, T. Lan and D. Handwerker. Data were analysed in Mathematica (Wolfram Research) or Matlab (MathWorks).

Light stimulus

Light stimuli were projected onto the retina as described[28]. The stimulus for the experiments in Fig. 1a, b, d and e was a bar of 300 × 900 μm (velocity 300 μm s^{-1}). The long axis of the bar was oriented parallel to the preferred-null axis of the cell. The centre of the bar started 900 μm from the soma, moved 1,800 μm in the preferred direction, and then back in the null direction to the original starting point. The stimulus for the experiments in Fig. 1c and 2f was a bar of 100 × 300 μm (velocity 300 μm s^{-1}). The long axis of the bar was oriented perpendicular to the preferred-null axis of the cell. The travel distance of the bar was 200 μm. For the experiment in Fig. 1f, 100-μm squares were flashed for 1 s. Thirteen successive flashes were presented 50-μm apart along the preferred-null axis with flash number 7 centred over the soma. At each position the maximum amplitude of excitation and inhibition ('on' response) were determined and used to represent the strength of the input receptive field at that location. The lines in Fig. 1f connect the maxima from each of the 13 flash locations. For the experiment in Fig. 3, bars of 100 × 60 μm were flashed for 1 s. The long axis was perpendicular to the preferred-null axis of the cell. Flashes were spaced by 60 μm. The second flash followed 167 ms after the onset of the first flash. The stimulus contrast was 200% for each experiment.

Fluorescence imaging

Alexa Fluor 488(Molecular Probes) was added to the intracellular electrode for fluorescent imaging. Cell morphology was obtained by fluorescently imaging the dendrites after electrophysiology had been completed. The image was captured and stored for later analysis using an intensified camera (SIT 66, Dage-MTI) and a video capture board

(PVR2500, Perception). By tracing the dendrites in each sublamina, we obtained the dendritic field limits. The on and off sublamina were imaged by adjusting the depth of focus. In Figs 1 and 2, the extent of the on and off sublaminas are represented by light grey or black outlines, respectively.

Space–time alignment

We obtained Fig. 1d and e by correlating the spatial position of the dendritic field of the DS cell with the time response of the input currents. The position of the stimulus was known at all points in time and could therefore be aligned with the position of the dendrites. The horizontal scale of the figure was adjusted using the stimulus velocity such that the scale bar represents both 300 μm and 1 s. Therefore, any vertical line through the figure would give the position of the leading (Fig. 1d) or trailing (Fig. 1e) edge of the stimulus and the corresponding response magnitudes. The current trace in Fig. 1e is reversed to take into account the right-to-left stimulus movement.

Confocal reconstruction

After the whole-cell patch-clamp recordings, the retinas were fixed, permeabilized and incubated with streptavidin-conjugated Alexa Fluor 488 conjugate to stain the neurobiotin-filled cells. During the double patch experiments, the starburst cell pipette additionally contained Lucifer Yellow (lithium salt, Molecular Probes), which was recognized with a rabbit antibody against Lucifer Yellow (Chemicon International; used at a dilution of 1:100) and visualized with a Rhodamine-Red-conjugated donkey antibody against rabbit IgG (Jackson ImmunoResearch; used at a dilution of 1:200). Images of the whole-mount retina were taken at two different wavelengths (488 nm for ganglion cell morphology, 568 nm to image the starburst cell) on a Zeiss LSM 510 confocal microscope. We determined the relative amount of co-fasciculation between recorded starburst and DS cells by dividing the length of co-fasciculating processes by the total length of starburst dendrites in the region of overlap.

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Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons

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The rules by which neuronal activity causes long-term modification of synapses in the central nervous system are not fully understood. Whereas competitive or correlation-based rules result in local modification of synapses, homeostatic modifications allow neuron-wide changes in synaptic strength, promoting stability[1,2]. Experimental investigations of these rules at central nervous system synapses have relied generally on manipulating activity in populations of neurons[1,3–6]. Here, we investigated the effect of suppressing excitability in single neurons within a network of active hippocampal neurons by overexpressing an inward-rectifier potassium channel. Reducing activity in a neuron before synapse formation leads to a reduction in functional synaptic inputs to that neuron; no such reduction was observed when activity of all neurons was uniformly suppressed. In contrast, suppressing activity in a single neuron after synapses are established results in a homeostatic increase in synaptic input, which restores the activity of the neuron to control levels. Our results highlight the differences between global and selective suppression of activity, as well as those between early and late manipulation of activity.

Activity is thought to play a role in the formation and modification of neural circuits[3,7]. The specific manner in which activity modifies synaptic strength remains a matter of speculation, and is controversial even in relatively well-studied systems[8–12]. Two general classes of synaptic modification have emerged as targets of concerted investigation. Hebbian modification has been studied at the level of single neurons or single synapses over a timescale of hours[13,14]. In contrast, homeostatic modifications have been studied over longer timescales, but have involved uniform changes in activity in entire networks of central neurons[4,5,15]. The relation between these two rules of synaptic modification has not been studied experimentally.

We sought to determine the consequences of chronic suppression of activity in single neurons within an active network (Fig. 1a). To this end, we used the inward-rectifier potassium channel Kir2.1, which has been used to suppress excitability in mammalian superior cervical ganglion cells and chick hair cells[16,17]. Overexpression of this channel hyperpolarizes the neuron and decreases its resting

