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Bistratified starburst amacrine cells in the *Sox2* conditional knockout mouse retina display ON and OFF responses

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ABSTRACT

Cell-intrinsic factors, in conjunction with environmental signals, guide migration, differentiation and connectivity during early development of neuronal circuits. Within the retina, inhibitory starburst amacrine cells (SBACs) comprise ON-types with somas in the ganglion cell layer (GCL) and dendrites stratifying narrowly in the inner half of the inner plexiform layer (IPL), and OFF-types with somas in the inner nuclear layer (INL) and dendrites stratifying narrowly in the outer half of the IPL. The transcription factor Sox2 is crucial to this subtype specification. Without Sox2, many ON-type SBACs destined for the GCL settle in the INL while many that reach the GCL develop bi-stratified dendritic arbors. This study asked whether ON-type SBACs in Sox2-conditional knockout retinas exhibit selective connectivity only with ON-type bipolar cells, or whether their bi-stratified morphology allows them to connect to both ON and OFF bipolar cells. Physiological data demonstrate that these cells receive ON and OFF excitatory inputs, indicating that the ectopically stratified dendrites make functional synapses with bipolar cells. The excitatory inputs were smaller and more transient in Sox2-conditional knockout compared to wild-type, however inhibitory inputs appeared largely unchanged. Thus, dendritic stratification, rather than cellular identification, may be the major factor that determines ON versus OFF connectivity.

New and Noteworthy

Conditional knock-out of the transcription factor Sox2 during early embryogenesis converts a mono-stratifying starburst amacrine cell into a bi-stratifying starburst cell. Here we show that these bistratifying starburst amacrine cells form functional synaptic connections with both ON and OFF bipolar cells. This suggests that normal ON versus OFF starburst connectivity may not require distinct molecular specification. Proximity alone may be sufficient to allow formation of functional synapses.
Introduction

The retina provides a convenient model to analyze the role of transcription factors in orchestrating neural determination, which ultimately guides neural migration, differentiation, and connectivity (for review see Reese, 2011). Here we focus on the starburst amacrine cells (SBACs), a well-defined neural population that is easily recognized as the only cholinergic neurons in the retina (Masland and Mills 1979). Mirror symmetric ON and OFF populations of SBACs are distinguished by the localization of their somata in the ganglion cell layer (GCL) or the inner nuclear layer (INL) respectively (Hayden et al. 1980; Vaney et al. 1981), and by their sharp dendritic stratification in the ON and OFF strata in the inner plexiform layer (IPL) (Famiglietti 1983b; Vaney 1984). Within those narrow strata, SBAC dendrites exhibit strong self-avoidance. The dendrites of an individual cell rarely cross over one another, giving rise to their characteristic “starburst” morphology (Tauchi and Masland 1984; Vaney 1984; Lefebvre et al. 2012), however, adjacent cells display a high degree of dendritic overlap (Tauchi and Masland 1984; Vaney 1984; Keeley et al. 2007) and form reciprocal inhibitory synapses with one another (Lee et al. 2010). The spatially asymmetric inhibitory connections that SBACs make with cofasciculating direction-selective ganglion cells (DSGCs) form the basis of the directional responses of these cells (Fried et al. 2002; Taylor and Vaney 2002; Lee et al. 2010; Briggman et al. 2011; Wei et al. 2011).

The ON and OFF SBAC dendritic strata are established before the arrival of the axon terminals of retinal bipolar cells (Stacy and Wong 2003; Morgan et al. 2006). Development is guided at least in part by semaphorin6A signaling via plexinA2 receptors and Megf10-signalling between homotypic SBACs (Sun et al. 2013; Ray et al. 2018). Recent evidence demonstrates that the transcription factor Sox2 is also critical for the control of SBAC development, including soma positioning and dendritic stratification. When Sox2 is conditionally eliminated, migration of the ON and OFF SBACs into the GCL and INL is altered; somata are added to the INL and a corresponding number are lost from the GCL (Whitney et al. 2014). This disruption likely reflects a deficit in subtype specification, rather than defective migration because although many ChAT-positive cells exhibit bistratified dendritic arbors the dendrites stratify within the ON and OFF sublaminae of the IPL at levels appropriate for SBACs. Moreover, the SBACs with somas in the INL, which are normally OFF-type cells, lack expression of the purine receptor P2X2, which is normally expressed heavily in OFF SBACs (Whitney et al. 2014). Together these observations indicate that differentiation of both ON and OFF SBACs is abnormal when Sox2 expression is eliminated.
ON and OFF bipolar cells normally make synaptic connections with ON and OFF SBACs respectively (Famiglietti 1983a). Here we asked whether neuronal connectivity between both ON and OFF bipolar cells is established with bistratified SBACs. Bipolar cells express different cadherins, which are believed to confer laminar specificity and post-synaptic target connectivity (Duan et al. 2014). Might bistratified SBACs in the GCL lacking Sox2 still retain their sub-type (ON) specificity through selective wiring mediated by molecular recognition, or do these cells now receive input from both ON and OFF bipolar cells? To address this question, we compared the excitatory synaptic inputs to SBACs in wild-type and Sox2 conditional knockout mice (Sox2-CKO). The data show that unlike wild-type SBACs, the nominally ON-type SBACs in Sox2-CKO retinas exhibit ON-OFF excitatory responses, suggesting that the laminar positioning of an SBAC dendritic arbor is sufficient to confer bipolar cell connectivity. Curiously, the magnitude and time-course of the ectopic OFF-inputs and the “normal” ON-inputs are smaller and more transient that seen in wild-type retinas, suggesting that Sox2 also modulates other properties of synaptic physiology in SBACs.

Materials and Methods

All procedures involving animals complied with the National Institutes of Health guidelines for animal use and a protocol approved by the Institutional Animal Care and Use Committees at Oregon Health & Science University. Three Sox2-WT and 10 Sox2-CKO animals were transferred from the Animal Resource Center at the University of California, Santa Barbara to OHSU. Sox2-CKO mice were generated by crossing breeders homozygous for both Chat-Cre and Rosa-YFP reporter, and heterozygous for the floxed Sox2 allele, thereby yielding Sox2-WT and Sox2-CKO mice from the same litters (mice heterozygous for the floxed Sox2 allele were not used) (Whitney et al., 2014). As described previously, every SBAC in the Sox2-CKO retina retains its cholinergic status, even if their somata are slightly smaller than those in Sox2-WT retinas. In wholemount preparations, these cells in the GCL (Fig. 1A, B), like those in the INL (not shown), have lost their Sox2-immunopositive status in the Sox2-CKO retina, amidst a collection of Sox2-immunopositive Müller end-feet, arising from the population of Sox2-immunopositive Müller cells situated in the inner nuclear layer (Fig. 1C, D).

All mice were between two and four months of age at the time of each experiment. Animals had ad libitum access to food and water and were kept on a 12/12 light/dark cycle. Experiments were performed during the circadian day. For the physiological studies, mice were dark-adapted for at least 1 h prior to isolating the retinas. The animals were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (0.25 ml, 50 mg ml⁻¹) and killed by cervical

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dislocation immediately following enucleation. Retinas were isolated and maintained under dim red or infra-red illumination for all subsequent procedures.

**Immunofluorescence and single cell injections**

For immunolabeling studies, mice were given a lethal injection of sodium pentobarbital, and then intracardially perfused using 2-3 ml of physiological saline followed by 50 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2 at 20°C). Eyes were removed and fixed by immersion for an additional 15 mins. Whole retinas were dissected from the eyes and then immunostained using antibodies to choline acetyltransferase (ChAT; 1:50; Millipore, #AB144P) and to Sox2 (1:200; Abcam, #AB97959), all as previously described (Whitney et al., 2014). For single cell injections, whole retinas were dissected from deeply anesthetized mice and then fixed by immersion in the same fixative for 20 mins. Single starburst amacrine cells were impaled with a micropipette filled with Alexa 546 dye in order to label the dendritic arbor, then retinas were subsequently immunostained for the presence of ChAT to reveal the ON and OFF starburst plexuses, all as previously described (Whitney et al., 2014).

**Electrophysiology**

Whole-cell patch recordings from SBAC somata, targeted due to their small spherical somata and lack of action potentials, were performed with borosilicate glass electrodes with a resistance of 4–8 MΩ. Electrodes were filled with an intracellular solution containing (in mM): 128 methane sulfonate, 6 CsCl, 10 Na-HEPES, 1 EGTA, 2 Mg-ATP, 1 Na-GTP, 5 phosphocreatine, 3 QX-314, and 0.1 spermine. The pH was adjusted to 7.4 with CsOH. Reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated. To improve voltage-clamp at positive potentials, potassium was replaced by caesium in order to suppress current through voltage-gated potassium channels. Voltages were adjusted by –10 mV to correct for the liquid junction potential.

Radial cuts were made on retinal halves to facilitate flat mounting on Whatman Anodiscs, which were then placed in a recording chamber and held in place with a harp. The preparation was constantly perfused with Ames medium (United States Biological, Salem, MA, USA) at a flow rate of 3–4 ml min⁻¹ maintained at 32–34°C. Alexa 488, 594, or a mix was added to the pipette to visually confirm the identity of SBACs at the end of recordings.

**Light stimulation and recording**

Stimuli generated on a CRT computer monitor at a refresh rate of 60 Hz were projected through the ×20 water-immersion microscope objective (NA = 0.95) onto the photoreceptor outer
segments. Percentage contrast was defined as $C = 100 \frac{(L_{\text{Stim}} - L_{\text{Back}})}{(L_{\text{Back}})}$, where $L_{\text{Stim}}$ and $L_{\text{Back}}$ were the stimulus and background intensities, respectively. The contrast of all stimuli was 80%. The retina was adapted to photopic levels by setting the screen intensity to $\sim 10^5$ photons $\mu$m$^2$ s$^{-1}$ throughout the experiment. Center-surround sizes in Figure 3 were measured by recording responses to stimulus spots with a range of diameters. The amplitude of the current responses as a function of spot diameter were fitted with a difference-of-Gaussians, to estimate the center and surround extents (Rodieck 1965). Light-evoked excitatory and inhibitory synaptic conductances were estimated from the current-voltage relations of the net light-evoked postsynaptic currents, as described previously (Borg-Graham 2001; Taylor and Vaney 2002; van Wyk et al. 2006; Venkataramani and Taylor 2010).

**EPSC analysis**

Data analysis and figure preparation were performed with custom procedures in Igor Pro (Wavemetrics, Tigard, OR, USA). Excitatory inputs were recorded by holding at the chloride reversal potential (-70 mV). The power spectral density (PSD, Figure 5B) functions were calculated from 96 seconds of data in 6 Sox2-WT and 126 seconds of data in 6 Sox2-CKO cells. The PSD was calculated from the Fourier transform of the current records using routines implemented in the Igor Pro software. Fourier transforms were performed on multiple 1.5 s segments of continuous baseline current. The PSDs were calculated from the Fourier transforms for each cell, and then averaged across cells to produce the data in Figure 5.

The same data used for the power spectral density analysis was analyzed to detect spontaneous excitatory postsynaptic currents (sEPSCs). sEPSCs were isolated in a two-step process. First, the large, slow fluctuations in the membrane current, particularly prominent in the wild-type cells, were removed by high-pass filtering (e.g. see Fig. 5A black versus grey). Second, the fast sEPSCs were identified as events that crossed a negative threshold of -15 pA in the filtered trace (see Fig. 5A right panel). The same threshold was used for both WT and CKO cells. Average EPSCs were generated from 200 ms segments excised from the original, unfiltered records. Average sEPSCs in each cell were calculated from between 228 and 706 events excised from the current recordings in each of the 6 Sox2-WT cells. Similarly, the average sEPSC in each of the Sox2-CKO cells was calculated from between 165 and 854 events in each cell. A total of 2695 and 2530 events contributed to the overall averages for the Sox2-WT and Sox2-CKO data in Figure 5.
Results

**ON-type SBACs in Sox2-CKO mice receive input from ON and OFF bipolar cells**

The ON-type SBACs, with somata in the ganglion cell layer, were targeted based on the relatively small soma size and an absence of spike responses during extracellular recordings. SBACs were more difficult to find in Sox2-CKO retinas as their somata were smaller than in Sox2-WT retinas (Whitney et al., 2014), making them harder to distinguish from other non-spiking, amacrine cells. Cells were confirmed to be SBACs based on the dendritic morphology revealed by filling the cells with fluorescent dye (Alexa) during the recordings. As previously described (Whitney et al. 2014), such filled cells frequently show a less radially symmetric dendritic field (Fig. 2A, B), with a variable proportion of the dendritic arbor mis-positioned to the outer portion of the inner plexiform layer. Figure 2C and D (top) show the boxed region indicated in Fig. 2A and B, with the ON and OFF strata shown separately, and include the immunopositive cholinergic plexus, along with 3 other cells, for both Sox2-WT and Sox2-CKO retinas. Together, they show these two prominent alterations, namely, a reduction in the extent of the dendritic arbor in the ON stratum, and the addition of ectopic dendrites ramifying in the OFF stratum.

Our first goal was to determine whether these cells received excitatory inputs from bipolar cells appropriate for the cell-type (ON, defined by somal positioning), or whether they received excitatory inputs from both ON and OFF bipolar cells. To this end, we isolated excitatory postsynaptic currents (EPSCs) by holding the cells close to the chloride reversal potential. We stimulated the cells with light or dark spots (relative to a constant grey background, contrast was 80%) and measured the amplitudes of the EPSCs. We used a range of stimulus diameters to obtain estimates of the spatial extent of the centre excitation, and any inhibitory surround effects. Wild-type SBACs displayed sustained EPSCs, either during a bright spot presentation or upon termination of a dark spot (Fig. 3A,C, black). The magnitude of the EPSCs in Sox2-CKO cells were smaller and more transient than those seen in Sox2-WT cells (Fig. 3A,C, red). For bright spot stimuli, EPSCs in the Sox2-WT and Sox2-CKO cells were evoked only at the onset (ON response) of the flashes (Fig 3A,B). An inward current was seen in the Sox2-WT cells at the fixed measurement time-point 100 ms after termination of the bright flash (Fig. 3B solid black). This current is probably not a separate OFF-response but appears to result from slow turn off of the ON-EPSC activated during the light-step. It was not seen in the Sox2-CKO cells (Fig. 3B solid red), presumably due to faster shut-off of the light-evoked response. For dark spots, EPSCs were evoked at both the onset (OFF response) and
termination (ON response) of the flashes (Fig 3C,D, red). Note the suppression of the tonic inward current during the dark stimulus for Sox2-WT cells (Fig. 3C,D solid black), as has been reported previously (Taylor and Wässle 1995; Stincic et al. 2016).

Previous anatomical analysis has shown that the stratification of ON SBACs in Sox2-CKO mice is disrupted, with asymmetric ON and OFF arbors, however, the total area enclosing both dendritic arbors is similar to that seen for the ON arbors in normal mice (Whitney et al., 2014). The area-response curves illustrated in Figure 3B,D allowed us to compare the physiological receptive field sizes calculated from the EPSCs. In wild-type cells, the ON-response elicited by the onset of a bright spot (Fig 3B, left), or the termination of a dark spot (Fig 3D, left), could be fit by a difference-of-Gaussians (DOG) function, with centre sizes (2*sigma) of 190 and 240 µm, respectively. Centre sizes of 230 and 280 µm were measured in Sox2-CKO cells (Fig. 3B,C red open symbols). The data-set is not sufficient to establish whether the larger estimates for centre-size are significant, however, the previous morphological analysis indicates that the anatomical spread of the dendrites is not larger in Sox2-CKO cells (Whitney et al. 2014). The corresponding surround sizes measured in Sox2-WT cells of 610 and 500 µm were smaller than the surrounds of 890 and 1060 µm measured in Sox2-CKO cells.

In addition to excitatory inputs from bipolar cells, SBACs receive inhibitory inputs from other SBACs, and also potentially from other amacrine cells (Taylor and Wässle 1995; Lee and Zhou 2006; Chen et al. 2016; Ding et al. 2016). In order to examine the magnitude and kinetics of both excitatory and inhibitory synaptic inputs to the SBACs we recorded light-responses over a range of holding potentials (Figure 4). Average whole cell leakage currents in SBACs were about 2-fold smaller in the Sox2-WT cells (Fig. 4A). Since the total dendritic length of SBACs in Sox2-WT and Sox2-CKO cells is approximately the same (Whitney et al. 2014), the smaller leak currents suggest an increase in the specific membrane resistivity in the latter. Along with the increased input resistance, the excitatory conductance was smaller in Sox2-CKO cells relative to Sox2-WT cells (Fig. 4B), in line with the data shown in Figure 3. On the other hand, the amplitude of the inhibitory conductance in Sox2-WT and Sox2-CKO cells was similar (Fig. 4C).

Spontaneous release is altered in Sox2-CKO SBACs

SBACs display spontaneous EPSCs under steady background illumination (Taylor and Wässle 1995; Peters and Masland 1996; Petit-Jacques et al. 2005; Vlasits et al. 2014). In the recordings presented here the spontaneous input appeared to comprise slow fluctuations with rapid events superimposed. Visual inspection suggested that the spontaneous EPSCs were qualitatively...
different in the Sox2-WT and Sox2-CKO cells. The Sox2-WT cells displayed slow fluctuations in the membrane current that were not evident in the Sox2-CKO cells (Fig 5A). We quantified this difference by measuring the power spectral density in 6 Sox2-WT and 6 Sox2-CKO cells (Fig. 5B). The power at low frequencies was lower in the Sox2-CKO recordings consistent with the loss of the slow fluctuations observed in the raw data. In addition to the slow fluctuations, rapid spontaneous EPSCs were evident in both Sox2-WT and Sox2-CKO cells. In order to examine these events in more detail, we isolated them using a threshold-crossing criterion (see Methods). The Sox2-WT data was high-pass filtered to remove the slow fluctuations and allow isolation of the rapid events. For each threshold crossing time, we excised a 200 ms segment from the original current record and added it to the average. The resulting average spontaneous EPSCs (sEPSCs) are shown in Figure 5C for Sox2-WT and Sox2-CKO cells. The time-courses of the fast events detected by the threshold crossing were measured as the time-constants for exponential fits to the decay phases of the sEPSCs in Figure 5C (blue overlays, right panel).

The time-constants were 0.45 and 0.34 ms for Sox2-WT and Sox2-CKO cells, respectively, similar to a time-constant of 0.33 ms measured for similar events in rabbit SBACs (Taylor and Wässle 1995). Similarly, the amplitudes of the fast sEPSCs measure from the exponential fits were essentially the same at -29 and -24 pA for Sox2-WT and Sox2-CKO cells. The slow component in Sox2-WT cells had a 10-90% rise-time of ~19 ms and an initial decay time-constant of ~26 ms. The analysis revealed the presence of a slower component to the sEPSCs in the Sox2-WT cells that was much reduced in the Sox2-CKO cells. The amplitude of this slow component, measured from the sEPSCs 5ms after the peak of the fast events (see arrows Fig. 5C, left), was -16.8 pA for the Sox2-WT cells but only -2.8 pA in the Sox2-CKO cells. In summary, analysis of the spontaneous excitatory inputs to the SBACs revealed the presence of fast and slow EPSC components. The fast component appeared to be similar in amplitude and time-course in the Sox2-WT and Sox2-CKO cells while the slow component appeared to be substantially reduced in the Sox2-CKO retinas.

Discussion

Sox2 is an essential transcription factor during mammalian embryogenesis. It serves to maintain early cells in a pluripotent state, and in concert with a few other transcription factors, is sufficient for re-establishing pluripotency from otherwise differentiated cells (Feng and Wen 2015). It is also critical for organogenesis, including normal eye development, as mutations in Sox2 yield anophthalmia and other ocular dystrophies (Bardakjian and Schneider 2011). Curiously, Sox2 is down-regulated as retinal development proceeds, yet is retained into maturity.
in retinal Muller glia (Surzenko et al. 2013), astrocytes (Kautzman et al. 2018) and the two populations of SBACs. We have previously shown that Sox2 is critical for the sub-type specification of the two populations of SBACs that differ in their somal positioning, dendritic stratification, and distinct P2X2 receptor expression patterns (Whitney et al. 2014). SBACs in the Sox2-CKO retina retain many wild-type properties (including their cholinergic status, their total number, their positioning within the INL and GCL, and the targeting of their dendrites to their normal depths within the IPL), yet lack features which fully discriminate them. In the absence of Sox2, although these cells stratify only within the ON and OFF cholinergic strata in the IPL, they lose the ON versus OFF specificity for dendritic targeting and many cells become bistratified. Nevertheless, they might retain other intrinsic instructions critical for producing two distinct populations in the two cellular layers. For example, they may retain an intrinsic instruction to connect selectively with ON versus OFF bipolar cells.

The primary goal of this study was to determine whether ectopically bistratifying SBACs receive functional synaptic inputs from both ON and OFF-type bipolar cells. The physiological analysis supports four key conclusions. First, SBACs located in the ganglion cell layer, which are normally ON-type cells, receive both ON and OFF light-evoked excitatory inputs in Sox2-CKO retinas, consistent with input from ON and OFF bipolar cells. Second, compared with Sox2-WT cells, the excitatory inputs are more transient in Sox2-CKO cells, suggesting that the synaptic connections that form in either ectopically- and entopically-localized dendrites are not normal. Analysis of spontaneous EPSCs confirmed the loss of a slow component that was tightly correlated with fast sEPSCs. Third, the magnitude of the excitatory synaptic inputs to Sox2-CKO cells is reduced compared with Sox2-WT cells. And finally, unlike the excitatory synapses, the inhibitory synaptic inputs to SBACs are largely similar in the Sox2-CKO and Sox2-WT cells.

The smaller excitatory inputs activated by light (ON-responses) in the Sox2-CKO cells compared with WT cells (Fig. 3A) may be due in part to the reduction in the dendritic arbor positioned within the ON stratum of these bistratifying SBACs (Whitney et al. 2014). The reduced ON-arbor would present fewer opportunities to make connections with ON-type bipolar cells yielding a reduction in the average peak ON-response. Similarly, the size of the ectopic OFF-arbor in these cells may limit the size of the OFF-response. Homeostatic mechanisms could also contribute. The input resistance of the Sox2-CKO cells was higher than Sox2-WT cells, as evident from the smaller leak currents observed in Figure 4A. A higher input resistance will mean a larger voltage-change per unit synaptic current. Perhaps the smaller
synaptic currents in the Sox2-CKO cells result in part from homeostatic mechanisms that down-
regulate synaptic gain in order to regulate overall excitability (Wefelmeyer et al. 2016). Moreover, if the total dendritic length and membrane area of SBACs in Sox2-CKO and Sox2-
WT mice were similar (Whitney et al. 2014), then the change in input resistance might indicate
changes in the specific membrane resistivity.

Analysis of the light-evoked synaptic inputs demonstrated that the sustained ON-
excitation seen in Sox2-WT cells was replaced with more transient ON and OFF excitation in the Sox2-CKO cells (Fig. 3). The shift from sustained to more transient excitation inputs was echoed in the analysis of the spontaneous excitatory currents observed in the absence of light-
stimulation. Background spontaneous activity has been documented in SBACs from rabbit
(Taylor and Wässle 1995; Peters and Masland 1996) and mouse (Petit-Jacques et al. 2005; Vlasits et al. 2014). Both species display fast sEPSCs along with slower fluctuations in the inward current, in line with the current results (Fig. 5). Our analysis of fast sEPSCs reveals for
the first time that these fast sEPSCs are temporally correlated with slower spontaneous fluctuations in the membrane current (Fig. 5C). After filtering out the slow current fluctuations (compare black and grey traces Fig. 5A), and thresholding to detect fast sEPSCs (Fig. 5A, right), we generated the average fast sEPSC by averaging epochs from the original unfiltered records. If the fast and slow currents were uncorrelated, the average fast sEPSC should have appeared on a flat baseline with an inward current offset equal to the mean membrane current over the analyzed data. Instead the average fast sEPSC revealed the presence of temporally correlated slower EPSC-like current fluctuations (Fig. 5C). It remains unclear whether there are tightly correlated fast and slow glutamate release processes, or a single process with complex kinetics. In any case, using identical analysis procedures, the slow component was not seen in the Sox2-CKO cells (Fig. 5 red), suggesting a fundamental difference in the properties in excitatory synapses in these retinas. A comparison of other studies in mouse suggests that the balance between sustained and transient excitation to SBACs can vary considerably, from mostly transient EPSCs (Petit-Jacques and Bloomfield 2008; Hoggarth et al. 2015), to a more balanced mix of transient and sustained (Vlasits et al. 2014; Stincic et al. 2016), similar to the current data. The reasons for these differences in time-course remain unclear. It is possible that the same underlying mechanisms are activated but to different extents due to differences in adaptation state or stimulus conditions. It is also interesting to note that the light-evoked OFF-
EPSCs that appear in the Sox2-CKO cells are activated at the onset of a dark-spot, but not at
the termination of a bright-spot (Fig. 3, solid red), whereas the ON-EPSCs are activated both at
the onset of a bright-spot and the termination of a dark-spot. This asymmetry between the ON
and OFF inputs seems consistent with the difference in rectification for synaptic transmission in the ON and OFF pathways (Chichilnisky and Kalmar 2002; Liang and Freed 2010). Under this scenario, ON bipolar cells tend to rest above release threshold during background illumination (and modulate output above and below a tonic baseline level, see Fig. 3A,C, 4B), while OFF bipolar cells tend to rest below release threshold. Thus, upon return to background at the termination of a non-preferred contrast stimulus, ON bipolar cells exceed release threshold and increase glutamate release, while OFF bipolar cells do not. In this context it is interesting to note that OFF-responses have been observed in ON-SBACs under some conditions (Vlasits et al. 2014), and the authors proposed that such OFF-responses resulted from differential adaptation effects produced by localized rod saturation combined with horizontal-cell mediated surround signals arising from cones. We failed to observe similar adaptation effects in our experiments possibly due to our lower stimulus contrast.

Recent work has established that ON SBACs in the mouse receive excitatory inputs from sustained Type-7 and more transient Type-5 ON bipolar cells that form synaptic contacts at different distances from the soma (Kim et al. 2014; Ding et al. 2016). It is tempting to speculate that the present results reflect differences in the ability of the sustained bipolar cells to make synaptic connections with SBACs in the Sox2-CKO retinas. Under this scenario, both Type-5 and Type-7 cells would contribute fast sEPSCs, while Type-5 cells would contribute additional slow events, which would be highly correlated with some or all of the fast events. The loss of the slow events in Sox2-CKO cells might be explained by an impaired ability of SBACs to form synapses with Type-5 ON-bipolar cells. Furthermore, the results indicate that the off-inputs on ectopically located dendrites avoid connections with sustained OFF bipolar cell subtypes (e.g. Type-2) (Greene et al. 2016). Since we could not directly detect the slow spontaneous EPSCs, it remains possible that they can appear in the absence of the fast sEPSC. Further work is needed to test this hypothesis.

In contrast to the excitatory input, the amplitude of the inhibition appeared to be similar in the Sox2-WT and Sox2-CKO cells (Fig. 4). Moreover, the area-response analysis revealed the presence of surround-suppression of excitatory inputs to Sox2-CKO cells (Taylor and Wässle 1995). The spatial extent and strength of the surround also appeared to be relatively normal in the Sox2-CKO cells (Fig. 3B, scaled red). These results suggest that both the feed-forward inhibition onto the SBACs, and the inhibitory connections onto the bipolar cells presynaptic to the SBACs are intact and functioning fairly normally. Altogether, the results indicate pronounced
changes in the direct excitatory inputs to the SBACs, but milder effects on inhibitory circuits in
the absence of Sox2.

The processes of SBACs, like other retinal neurons, are thought to form selective
synaptic contacts by virtue of their targeted distribution within the IPL, where they engage in
homophilic or heterophilic molecular interactions with their synaptic partners (Yamagata and
Sanes 2008; Duan et al. 2014; Krishnaswamy et al. 2015). SBACs in the GCL have recently
been shown to express Cntn5, and its expression in these ON SBACs is critical for the
formation of the inner stratum of dendrites to form in bistratifying DSGCs (Peng et al. 2017).
Cntn5 is also expressed by those same ON-OFF DSGCs and is regulated by Satb1. Loss of
this transcriptional regulator yields a loss of Cntn5 from those DSGCs, yielding a comparable
reduction of the inner processes of their bistratifying dendritic arbors, rendering them mono-
stratified, producing only OFF responses (Peng et al. 2017). The present study, by contrast,
has shown that the loss of Sox2 has turned a mono-stratifying ON amacrine cell into a bi-
stratifying ON-OFF one. It remains to be seen whether these SBACs in the GCL also have lost
Cntn5 expression, and whether there is any downstream consequence for the DSGC.

A recent study suggests that ON and OFF bipolar cells are attracted to the strata
occupied by their respective ON and OFF SBACs, even when those SBACs generate ectopic
micro-stratifications in association with their primary ON or OFF strata (Ray et al., 2018). The
present results suggest that specification of laminar position, orchestrated by Sox2 well before
bipolar cell differentiation, is enough to generate ON versus OFF bipolar cell connectivity. Other
factors direct bipolar cells to the ON versus OFF divisions in the IPL (Duan et al. 2014), and
once in position, they make connections with any SBAC dendrites, since the SBACs (in the
Sox2-CKO retina) lack specificity for a bipolar cell’s ON versus OFF status. In this scenario,
normal Sox2-dependent ON versus OFF specification may be due to gene expression that
prevents dendrites from entering the OFF versus ON divisions of the IPL, respectively, perhaps
mediated by repulsive factors (e.g. (Matsuoka et al. 2011)). Once a bipolar terminal has reached
one of the cholinergic strata, other Sox2-dependent factors intrinsic to the SBACs may be
important for forming functionally normal synapses.
Figure legends

**Figure 1:** Sox2 is selectively eliminated from SBACs. Panels A and B show that SBACs retain their cholinergic status (red) in the Sox2-CKO retina, while only the SBACs in the Sox2-WT retina retain their Sox2-immunopositive status (cyan). Panels C and D confirm that Müller glial somata remain Sox2-immunopositive, giving rise to the population of immunopositive Müller glial endfeet in panels A and B. Calibration bar 20 µm.

**Figure 2:** Dendritic arbors of SBACs in the Sox2-CKO retina are bistratified. Panels A and B show examples of single injected SBACs from Sox2-WT and Sox2-CKO retinas, showing the altered dendritic morphology of SBACs deprived of Sox2. Panels C and D show portions of the dendritic arbor that lie within the two cholinergic immunolabeled plexes for these same two cells (being the highlighted box in panels A and B), and for three additional cells in each condition. In Sox2-WT cells, all of the dendritic arbor is contained within the ON stratum (shown in red, panel C), while Sox2-CKO cells show a reduced density of its dendritic arbor in the ON stratum (shown in red, panel C) and ectopic dendrites present in the OFF stratum (shown in green, panel D). The entire cholinergic plexus was immunolabeled as well (shown in blue), confirming that the bistratified dendritic arbors localize to the ON and OFF cholinergic strata. Calibration bar 20 µm.

**Figure 3:** Average responses to spot stimuli. Membrane current recorded in SBACs at a holding potential of -70 mV in Sox2-WT (black) and Sox2-CKO cells (red). A, Average current traces for 6 cells. The bars above the traces show light stimulus timing and contrast. The size of the stimulus spot, centered on the receptive field, is indicated above the timing bars. Shaded regions show standard errors. B, EPSC amplitudes from A measured as a function of stimulus spot diameter at the time-points indicated by the symbols. Smooth lines show fits to a difference-of-Gaussians. Re-scaling the Sox2-CKO data (red dashed line) to match the peak magnitude of Sox2-WT data demonstrates the largely unchanged spatial properties. C,D, Average EPSCs for 5 cells during negative contrast (dark spot) stimulation. Format and layout identical to A,B.

**Figure 4:** Calculated excitatory and inhibitory conductances activated by 300 µm diameter centered spots of positive and negative contrast. A. Average membrane currents recorded at a range of membrane potentials between -90 and +30 mV. Stimulus contrast and timing indicated beneath the traces. B,C. Excitatory and inhibitory conductances calculated for the data shown in A (see Methods).
**Figure 5:** Spontaneous EPSCs recorded at a holding potential of -70 mV in Sox2-WT (black) and Sox2-CKO SBACs (red). **A.** Representative segments of baseline currents. The grey trace shows the control (black) after high-pass filtering to remove the slow fluctuations. This allowed for efficient threshold-detection of the fast sEPSCs as illustrated in the right panel. The asterisks mark 4 detected sEPSCs that crossed the threshold. **B.** Average power spectra were calculated from 96 seconds of data in 6 Sox2-WT and 126 seconds of data in 6 Sox2-CKO cells. Shading shows the standard deviation. **C.** Average spontaneous EPSCs calculated from average sEPSCs derived from the same 6 cells (see Methods). The arrows in the left panel show the estimation of the amplitude of the slow sEPSC components. The right panel shows the fast component on an expanded time-scale. The solid blue lines show exponential fits to the decay phase, with time-constants of 0.45 ms (Sox2-WT) and 0.34 ms (Sox2-CKO). The corresponding amplitudes obtained from these exponential fits were -29 and -24 pA. The shading shows the standard deviations calculated for the average sEPSCs across the 6 cells, after subtraction of the mean offset current.
References


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A

Sox2-WT  Sox2-CKO

B

ON response

OFF response

C

D

Amplitude (pA)

Spot diameter (µm)

Spot diameter (µm)

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