Inhibitory inputs tune the light response properties of dopaminergic amacrine cells in mouse retina

G.S. Newkirk¹, M. Hoon², R.O. Wong², and P.B. Detwiler¹*

University of Washington, ¹Department of Physiology & Biophysics and Program in Neurobiology & Behavior, ²Department of Biological Structure, Seattle, Washington 98195

*Corresponding author: P.B. Detwiler, University of Washington, Department of Physiology & Biophysics, Seattle, Washington 98125; detwiler@uw.edu

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Abstract

Dopamine (DA) is a neuromodulator that in the retina adjusts the circuitry for visual processing in dim and bright light conditions. It is synthesized and released from retinal interneurons called dopaminergic amacrine cells (DACs), whose basic physiology is not yet been fully understood. To investigate their cellular and input properties as well as light responses, DACs were targeted for whole cell recording in isolated retina using two-photon fluorescence microscopy in a mouse line where the dopamine receptor 2 promoter drives GFP expression. Differences in membrane properties gave rise to cell-to-cell variation in the pattern of resting spontaneous spike activity ranging from silent to rhythmic to periodic burst discharge. All recorded DACs were light sensitive and generated responses that varied with intensity. The threshold response to light onset was a hyperpolarizing potential change initiated by rod photoreceptors that was blocked by strychnine, indicating a glycinergic amacrine input onto DACs at light onset. With increasing light intensity the ON response acquired an excitatory component that grew to dominate the response to the strongest stimuli. Responses to bright light (photopic) stimuli also included an inhibitory OFF response mediated by GABAergic amacrine cells.
driven by the cone OFF pathway. DACs expressed GABA (GABA_{A_{1α}} and GABA_{A_{3α}})
and glycine receptor (GlyR{α_2}) clusters on soma, axon and dendrites consistent with the
light response being shaped by dual inhibitory inputs that may serve to tune spike
discharge for optimal DA release.
**Introduction**

Dopamine (DA) is a modulatory neurotransmitter that is present in the peripheral and central nervous system of all vertebrates. In the retina, it is synthesized and released from a subset of amacrine interneurons called dopaminergic amacrine cells (DACs) and plays a key role in numerous events that accompany the switch from rod (dim light) to cone (bright light) mediated vision as night turns to day (Witkovsky, 2004). In spite of their importance in orchestrating the diurnal changes in the performance of the visual system, *in situ* functional studies of DACs have been hindered by the fact that they are sparsely distributed, 20 - 30 per mm^2, ~450/ mouse retina (Gustincich et al., 1997) and are indistinguishable amongst the myriad of other similar-looking neurons in the intact retina. They can, however, be labeled and specifically targeted for electrical recording using transgenic methods in which the promoter for tyrosine hydroxylase, the rate-limiting enzyme for dopamine biosynthesis, directs the selective expression of a reporter, either alkaline phosphatase (Gustincich et al., 1997) or fluorescent protein (Zhang et al., 2004).

Such methods have been used most commonly to identify DACs isolated from enzymatically dissociated retina for studies of their biophysical properties and repertoire of neurotransmitter receptors using pharmacology and immunohistochemistry (Gustincich et al., 1997; Feigenspan et al., 1998; Gustincich et al., 1999; Feigenspan et al., 2000; Steffen et al., 2003; Xiao et al., 2004). This approach has also been used to fluorescently tag DACs in whole mount retina for recording purposes (Zhang et al., 2007; Zhang et al., 2008). These studies were based, however, on extracellular recorded spike activity and used only intensely saturating light stimuli. For these
reasons they provide an incomplete description of the cellular physiology of DACs and the stimulus dependence of their light responses. In this study, we use whole cell current- and voltage-clamp recording to present an in-depth study of the functional properties of DACs in the intact retina and the synaptic inputs that give rise to their responses to a full range of light intensities.

Amacrine cells were targeted for whole cell recording using two-photon laser scanning fluorescence microscopy in a BAC transgenic mouse line in which the promoter for dopamine receptor 2 (Drd2) drove expression of GFP. In this line, two distinct populations of amacrine cells express GFP. One population comprised exclusively DACs, which could be identified on the basis of characteristic morphological features and co-localization with tyrosine hydroxylase (TH) labeling. The DAC population was morphologically homogeneous but physiologically diverse. DACs exhibited spontaneous activity with a stable spike pattern that ranged from nearly silent, to rhythmic firing at a steady rate, to firing in periodic bursts. These differences were accompanied by cell-to-cell differences in membrane properties. All of the DACs in the recorded sample generated light responses that depended on stimulus strength. At light onset threshold stimuli evoked glycinergic inhibition whereas stronger stimuli evoked a net excitatory response that was followed by a GABAergic inhibitory response at light offset. Furthermore, the postsynaptic receptor composition of these inhibitory inputs and their distribution across the DAC were identified by immunolabeling.
Methods

All experiments were conducted in accordance with institutional and national guidelines for animal care. We used P21-P50 day old Gensat BAC transgenic mice (RP23-161H15) crossed into C57/B6 background. In this line, green fluorescent protein (GFP) transgene was inserted immediately after the ATG start codon of the dopamine receptor 2 promoter. Animals were housed in University of Washington approved facilities on a 12-hour light/dark cycle with ad libitum access to food and water.

Tissue Preparation

Experiments began during the animal subjective day, approximately 5 hours into their daily light cycle. After 2 to 3 hours of dark adaptation, mice were killed by cervical dislocation and eyes were removed in the dark using infrared illumination with image converters, placed in Carbogenated (95% O₂ and 5% CO₂) Ames’ medium (Sigma, St. Louis, MO) at room temperature and hemisected. The posterior half of the eyecup was cut into 3 to 5 smaller pieces. Retina was isolated from each of the pieces as needed and adhered photoreceptor side down to a translucent Anodisc filter (Whatman, Florham Park, NJ) by wicking away excess solution and transferred to a recording chamber fixed to the stage of a custom-built two-photon laser scanning fluorescence microscope. The mounted retina was perfused with warmed (30 –34°C) Carbogenated Ames’ medium at a rate of 5-7 ml/min and viewed with a CCD camera using infrared illumination.
Cell Identification

In the Drd2-GFP BAC transgenic mouse line, GFP expression was visualized in whole mount retina using two-photon microscopy (Denk et al., 1990; Denk and Detwiler, 1999; Euler et al., 2009). The light source for two fluorescent excitation was a pumped laser (Mira, Coherent) that delivered ~ 100 femtosecond laser pulses of 930nm light at 100 MHz with estimated average power at the retina of 4 - 8 mW. Fluorescence emission was collected by a 40X 1.0NA water-immersion objective (Nikon). Custom band pass (BP) filters (Chroma Technology) directed green (535 BP50nm) and red (622 BP36nm) fluorescence to two independent photomultiplier tubes (Hamamatsu). The green channel was used to visualize GFP-positive cells in the inner nuclear layer (INL), and the red channel was used to visualize the recording pipette filled with an intracellular recording solution containing 100 μM Alexa-594 (Invitrogen).

Retinal photoreceptors are not blind to the pulses of long wavelength (930 nm) light used to excite fluorescence by two-photon absorption in laser scanning microscopy (Euler et al., 2009). The light sensitivity of alpha RGCs was used to assess the effect of laser exposure on retinal function. All recordings were done in the presence of 2 Rh+/rod/s background illumination. After a 2- 3 minute period of laser scanning that mimicked the conditions used to target DACs using GFP fluorescence, the threshold intensity (500 ms step of 440 nm light) for a just detectible change in extracellularly recorded spike activity (loose cell attached patch) was increased by about 1 -1 .5 log unit for 20 to 40 s before returning to a control step sensitivity of ~ 1 Rh+/rod/step. At the onset of laser scanning, with the focal plane in the inner plexiform layer (IPL), alpha RGCs were initially sensitive to the excitation light and fired spikes in synchrony with the rate of image scanning as the laser spot swept across the receptive field of the cell.
With continued scanning, the retina adapted to the stimulus and the laser-evoked spike activity disappeared. Spike response to test flashes as well as laser exposure returned with the recovery of light sensitivity following the termination of laser scanning. These observations indicate that the two-photon imaging methods we have used to target DACs for whole cell recording did not have a persistent effect on retinal function as appraised by light sensitivity.

**Electrophysiology**

GFP labeled cells in the INL were accessed on a diagonal trajectory from a micro-dissected hole in the internal limiting membrane, 50-75 μm from the targeted cell (Margolis and Detwiler, 2007). Patch clamp recordings were obtained using 3-5 MΩ electrodes and signals were amplified using an Axopatch 200B amplifier (Axon Instruments). For current clamp recordings, the standard internal solution contained (in mM): K-gluconate 122, Na-HEPES 10, KCl 6, K-EGTA 6, Mg-ATP 3, Tris-GTP 0.2 and brought to pH 7.4 with NaOH. The internal solution used for voltage-clamp recordings contained (in mM): Cesium Methyl Sulfonate 105, TEA-Cl 10, K-EGTA 10, QX-314 2, Mg-ATP 5, Tris-GTP 0.5, brought to pH 7.3 with ~ 35mM CsOH. Voltages were corrected for a -10 mV liquid junction potential that was determined experimentally (Neher, 1992). Series resistances ($R_s$), as measured from the average response to trains of -5 mV steps, were 10 to 20 mΩ and holding potentials were corrected offline by subtracting the product of leakage current and $R_s$ from the applied voltage. Voltage and current signals were filtered at 2 kHz and digitized at a sampling interval of 0.1 ms via an ITC-16 interface (Instratech) using custom software written in Igor Pro (WaveMetrics) by Fred Rieke (University of Washington, Seattle, WA).
Receptor antagonists

To block synaptic transmission, neurotransmitter receptor antagonists were added to the extracellular solution. These included (in μM): 40 Gabazine (SR-95531), 50-75 L-APB (L- (+) -2-Amino-4-phosphonobutyric acid), 20-40 NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), 50 TPMPA ((1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid hydrate), 1 Strychnine. Chemicals were purchased from Sigma or Tocris (Ellisville, MO).

Light Stimuli

An optical bench (Baylor and Hodgkin, 1973) with a quartz-halogen light source was used with a substage condenser to deliver focused visual stimuli to the photoreceptor layer of the retina. Unless otherwise noted, stimuli were 500 ms steps of 720 μm diameter (full-field) circular spots of 440 nm light centered on the receptive field of the cell with an unattenuated intensity of 2.1 x 10^6 photons μm^-2 s^-1, which corresponds to 9 x 10^5 Rh*/rod/0.5 s step using an effective collecting area of 0.85 μm^2 for mouse rods (Lyubarsky et al., 2004). All experiments were done in the presence of a background light intensity equivalent to 2 Rh*/rod/s to provide a defined and reproducible baseline level of illumination and consistent light-adaptational state.

Immunohistochemistry

Retinas were isolated in cold oxygenated mouse artificial cerebrospinal fluid (mACSF, pH 7.4) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1 NaH₂PO₄, 11 glucose, and 20 HEPES, and mounted retinal ganglion cell side up on black membrane
filters (Millipore, HABP013). The retina and filter paper were then immersed for 20 minutes in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), rinsed with PBS, pre-incubated in PBS with 5% donkey serum and 0.5% triton overnight, and then incubated for 3 nights with primary antibodies. For vibratome sections, 60 μm slices were made post-fixation. Antibodies used were directed against TH (mouse monoclonal, 1:500, Chemicon), GFP (rabbit polyclonal, 1:1000, Chemicon), GABA<sub>A</sub> receptor α3 subunit (guinea pig polyclonal, 1:3000, kindly provided by J.M. Fritschy), GABA<sub>A</sub> receptor α1 subunit (guinea pig polyclonal, 1:5000, kindly provided by J.M. Fritschy), GABA<sub>C</sub> receptor ρ subunit (rabbit polyclonal, 1:500, kindly provided by R. Enz), Glycine receptor α2 subunit (goat polyclonal, 1:300, Santa Cruz), and VIAAT (rabbit polyclonal, 1:1000, Synaptic Systems). Secondary antibodies utilized were either anti-isotypic Alexa Fluor conjugates (1:1000, Invitrogen) or DyLight conjugates (Jackson ImmunoResearch). Secondary antibody incubation was carried out in PBS overnight, and the retinas were subsequently mounted in Vectashield (Vector labs).

For DAC fills, 5 mM neurobiotin was used and retinas were post-fixed for 20 mins and processed thereafter for receptor labeling. To amplify the neurobiotin signal, streptavidin conjugated to Alexa Fluor 568 (1:200, Invitrogen) was included with the secondary antibody.

**Image Analysis**

Images were acquired on an Olympus FV1000 laser scanning confocal microscope, using a 1.35NA 60X oil objective. Raw image stacks were processed using MetaMorph (Universal Imaging) and Amira (Mercury Computer Systems) software. For volume estimation, the neurobiotin signal was used to mask in 3D, the DAC dendrites or
axon using the 'label field' function in Amira. Alternatively, the TH signal was used to mask the DAC soma in 3D. Thereafter, the DAC mask was multiplied to the receptor channel to isolate the receptor signal exclusively within the mask. A constant threshold, chosen subjectively to exclude background (average fluorescence intensity of non-clustered pixels), was then applied to the receptor-labeled channel to isolate receptors within the mask. The volume of the pixels above this threshold was expressed as a percentage of the total volume occupied by the pixels within the mask. To determine whether or not immunolabeled receptor clusters represent inhibitory synaptic sites, we quantified the extent to which GABA$_3$$\alpha$3 receptor clusters (most abundant on DAC processes) were apposed to inhibitory presynaptic terminals. To do so, we performed triple immunostaining for the receptors ($\alpha$3), vesicular inhibitory amino acid transporter (VIAAT) and TH positive process in retinal slices. Receptor puncta within the TH mask were isolated, the VIAAT signal thresholded, and apposition of both signals was judged by eye upon rotation of the image volumes in 3D. Each GABA receptor cluster was considered to be apposed to a VIAAT positive terminal if the two signals showed overlap of pixels (>1 pixel overlap) at all angles of the 3D rotation.
Results

Cell identification and targeted recording

In the Drd2-GFP transgenic mouse line, the promoter sequence for dopamine receptor 2 drives selective GFP expression in two separate populations of retinal amacrine cells that can be distinguished on the basis of soma size and density (Fig. 1A). The smaller cells, with soma diameters of 7 - 9 μm, were distributed numerously in the inner quarter of the inner nuclear layer (INL). They had dendrites that branched out first as a narrow band extending laterally along the outer boundary of the inner plexiform layer (IPL) and then diffusely in the middle third of the IPL. These cells are the subject of an ongoing investigation and will not be discussed further. The second population of Drd2-GFP amacrine cells had large (13 - 18 μm in diameter) spherical somas that were distributed sparsely along the inner boundary of the INL. In all regions (dorsal, ventral, temporal and nasal) of the Drd2-GFP Gensat retina, cells that were immunolabeled with TH antibodies also expressed GFP (n=76 cells, n=4 retinas; Fig. 1A, B). Images of neurobiotin filled cells (Fig. 1C) showed somas with 2 to 3 mono-stratified primary dendrites that spread out tangentially in the outer most (S1) strata of the IPL over an area roughly 600 μm diameter. The dendrites gave rise to two different kinds of secondary extensions. One was a small number (1 to 3) of axon-like processes that were beaded with varicosities and coursed laterally for long distances (1 -2 mm) across the retina (Fig. 1D). The primary dendrites also gave rise to a number of faint interplexiform processes that projected vertically into the outer plexiform layer (OPL)
(Fig.1E). There were no apparent differences in the gross morphology of DACs targeted for intracellular dye-filling at randomly selected retinal eccentricities.

On the basis of these anatomical features (Versaux-Botteri et al., 1984; Dacey, 1990; Kolb et al., 1991; Gustincich et al., 1997), along with the fact that these cells (unlike the GFP positive cells with small somas) colocalized with tyrosine hydroxylase (TH) labeling (Fig. 1), the large soma Drd2-GFP cells were classified as interplexiform dopaminergic amacrine cells (DACs); also referred to as type 1 catecholamine cells. Our observations suggest that in the transgenic mouse used in our study, fluorescent protein expression in cells with large somata represent a single population of dopaminergic neurons based on their morphology.

[Figure 1]

Resting properties of DACs

In whole cell current clamp recordings, DACs had resting potentials in the vicinity of -30 to -65 mV (mean 54 ± 1.04 mV, n = 81) and generated action potentials spontaneously (in such cells resting potential was defined as the baseline voltage at spike threshold). The pattern of spontaneous spike activity was not the same in all cells (Fig. 2). In a set of experiments on 59 DACs, in which the analysis focused on spontaneous activity in darkness, only five (8%) were quiet cells that generated spikes infrequently (< 0.1 Hz) at irregular intervals (Fig. 2A1). In all other cells in this sample spontaneous spike discharge occurred more frequently, at either a steady rate of ~ 2 to 8 Hz (in 25% of the cells) (Fig. 2B1) or in bursts (in 62% of the cells) that were intermingled with a more regular rate of ongoing spike activity (Fig. 2C1). In 5% (3/59)
of the recorded cells discrete high-frequency bursts of action potentials were generated at regular (~ 1 to 5 s) intervals on an otherwise quiet background of spike activity (Fig. 2D). The cell-to-cell variation in the pattern of spontaneous spike activity was not associated with observable differences in DAC gross morphology or cellular health, as judged by the stability of resting membrane potential and the presence of action potentials. There were no significant differences in the resting potentials of cells that exhibited the different categories of activity, with mean values that ranged from -52 to -48 mV in quiet and periodic bursting DACs, respectively. The characteristics of the spontaneous activity in a particular cell did not change over the course of a typical whole cell recording lasting 30 to 45 mins. In cells having a mixture of single spikes and bursts of activity, hyperpolarizing current injection accentuated the bursting component of the activity and revealed an underlying "pacemaker-like" periodicity (Fig. 2 C1, C2).

Bursting spike activity and the unmasking effect of hyperpolarization persisted in the presence of a combination of excitatory and inhibitory synaptic blockers (Fig. 2E), consistent with generation by an intrinsic mechanism. In quiet cells that displayed little to no spontaneous activity the voltage response to a hyperpolarizing step of current was flat and showed no evidence of relaxing from an initial peak hyperpolarization (Fig. 2A2). In contrast, the hyperpolarizing step response in cells with regular and/or bursts of spike activity exhibited a prominent sag as voltage relaxed from an initial negative peak back towards a less negative steady state potential with a time constant of 1 - 2 s (Fig. 2B2). This suggests a hyperpolarization activated inward current (I_{hi}) may play a role in the biophysical mechanism that gives rise to the periodic bursts of action potentials, similar to what has been reported for several other types of neurons.
exhibiting bursting behavior (Alonso and Llinas, 1989; McCormick and Pape, 1990a; Yung et al., 1991; Mercuri et al., 1995).

[Figure 2]

**Light response properties of DACs**

Over the course of this study, we have recorded from more than 300 DACs and have yet to find one that did not respond to light. This result differs sharply from earlier reports where a large fraction (40%) of DACs did not respond to light (Zhang et al., 2007; Zhang et al., 2008).

The minimally detectable full field (720 μm diameter) scotopic stimulus evoked a hyperpolarizing potential change and a transient suppression of spontaneous spike activity at the onset of steps of 440 nm light with intensities that ranged from 9 to 70 Rh+/rod across cells (Fig. 3A, B). In many but not all cases, the recovery from the hyperpolarizing response evoked by dim light included a brief increase in spike activity caused by a delayed excitatory after potential (Fig. 5A). This is a feature of responses evoked at the end of hyperpolarizing voltage steps in neurons that express I_H (Robinson & Siegelbaum, 2003).

With increasing step intensity, the DAC light response grew to become dominated by excitatory input at light onset and a strong hyperpolarizing response at light offset (Fig. 3A,C). The ON and OFF components of the DAC response to a bright stimuli are apparent in a raster plot as an initial burst of spikes followed by an inhibition of spike activity at light offset (Fig. 3C). Small diameter (35 μm) spots of bright light evoked similar light responses when centered on the soma as when displaced from it by
up to 300 μm (data not shown), suggesting that DACs do not receive antagonistic synaptic input from a surround that extends over that area.

In a minority of cells, the strongest stimuli recruited an additional excitatory input that overcame the inhibitory OFF response and extended the step response well beyond the duration of the stimulus (Fig 3D). The prolongation of the response grew with further increase in light intensity, resulting in responses to the brightest light that typically lasted 15 to 20 s; 7 to 10-fold longer than the responses evoked by the same stimulus in the majority of DACs. Long lasting responses such as these have been reported previously in extracellular recordings of DACs and attributed to there being a subset of DACs that receive excitatory synaptic input from intrinsically photosensitive retinal ganglion cells (ipRGCs) (Zhang et al., 2008). Recordings from these types of cells were infrequent (~ 12% of recorded DACs) and since they obscured the inhibitory response evoked at light offset our experiments concentrated on GFP labeled DACs that did not exhibit any evidence of ipRGC input.

As the amplitude of the depolarizing ON response grew with increasing light intensity, spike threshold was exceeded and action potentials were triggered that declined in amplitude over the course of the excitatory response (Fig. 3A). With further increase in stimulus strength the frequency of the evoked spike discharge increased whereas the accompanying decline in spike amplitude accelerated resulting in the failure of action potential generation (Fig. 3A). The rapid inactivation of spike production by depolarization block is a characteristic feature of DAC electrophysiology as well as an established attribute of midbrain dopaminergic neurons-(Bunney and Grace, 1978; Grace and Bunney, 1986). It limits the response evoked by intense stimuli to an initial
transient burst of spikes at the onset of a strongly depolarizing synaptic potential (Fig 3A,D).

Depolarization block shapes the response to steady illumination in a similar manner (Fig 4A). Action potentials evoked at the onset of a step of bright light decline rapidly in amplitude to the point of spike failure after a short-lived burst of activity. The absence of spike activity persists until the underlying depolarizing potential decays for sufficient relief of inactivation for the generation of spikes that first appear as miniature (~ 5 mV peak amplitude) spikes and grow to full amplitude (~ 90 mV) at an irregular firing rate. Depolarization block also plays a role in shaping the responses evoked by a train of repetitive flashes of light (Fig 4B). Stimuli that flicker ON and OFF at a low rate (1 - 3 Hz) evoke a brief burst of spikes at the onset of the periods of light exposure that are quickly silenced by depolarization block. Under these conditions the hyperpolarizing (inhibitory) OFF response accelerates the recovery from depolarization block and serves to remove spike inactivation in time for the next flash in the stimulus train.

*Light-evoked synaptic currents*

The underlying excitatory and inhibitory synaptic currents that gave rise to the DAC light response were revealed by recording stimulus-activated currents in voltage-clamped cells held at either -60 or 0 mV, i.e. respectively at potentials where net current
flow through inhibitory or excitatory transmitter gated ion channels would be expected to be eliminated (Fig. 5). Dim light evoked a net outward current when the cell was held at 0 mV, consistent with the DAC threshold light response being a hyperpolarizing voltage change in current clamp recordings. In cells held at -60 mV, stimuli in the same intensity range had no effect on the recorded current whereas stronger stimuli evoked an inward current response that grew in amplitude and duration with further increase in intensity. In cells held at 0 mV, the amplitude of the outward current evoked by dim stimuli remained constant until the stimuli reached the intensity level that had first evoked an inward current response when the cell was held at -60 mV. From that point on, the outward response declined with increasing light intensity to become a net inward current response when stimulated with the brightest light (Fig. 5). These changes in the amplitude and polarity of the response suggest that the responses to all but the weakest stimuli are the sum of inhibitory and excitatory inputs with relative strengths that change with light intensity. This may arise as a consequence of voltage-clamp error due to the well-established inability to control dendritic voltage by clamping somatic voltage (Armstrong and Gilly, 1992; Williams and Mitchell, 2008); a particularly acute problem in cells, such as DACs, with extensive dendritic arbors. Under these conditions setting the somatic voltage at the expected reversal potential for excitatory synaptic current would not uniformly nullify the current throughout the dendritic tree. This would allow the contribution of inward currents from excitatory dendritic inputs to the net stimulus-activated current recorded in the soma to increase with stimulus intensity. This can also account for the fact that the hyperpolarizing potential change that is associated with the offset of steps of bright light in current clamp recording is not apparent in voltage clamp as an outward current at $V_{\text{hold}} = 0$ mV. The presence of the inhibitory OFF response is
however apparent as an outward current when the contamination by light-activated inward current has been eliminated by blocking excitatory input with L-APB (Fig. 7B, & 8C).

Taken together, there are three distinguishing components to the DAC light response: a dim step ON inhibition, a moderate to bright step ON excitation and an OFF inhibition. These intensity dependent features of the light response were observed in DACs that had a low rate of resting spike activity (quiet cells) as well as in DACs that spontaneously discharged spikes at either a steady rate or intermingled with bursts of activity. The intensity dependence of the light responses in the relatively small number (~ 5%) of DACs that fired spikes in discrete periodic bursts (Fig. 2D) could not be clearly demonstrated on the background of the pacemaker-like fluctuations in cell voltage that generated the periodic bursts of activity.

[Figure 5]

**Synaptic origin of light response components**

To investigate the synaptic inputs that underlie the DAC light responses we next examined the three light response components with the help of pharmacological agents.

**Dim step ON inhibition:** Bath application of 1 μM strychnine eliminated the hyperpolarizing voltage response as well as the outward current response evoked by dim light in current (V_{rest} @ I_{zero}) - and voltage -clamp (V_{hold} = 0 mV) recordings (Fig. 6; n=7 in current clamp, n=3 in voltage clamp recordings). The inhibitory input stimulated by low intensity steps persists in the presence of GABA_{zine} (40 μM) and TPMPA (50 μM) and is blocked by either L-APB (50 μM) or NBQX (40 μM), indicating that DACs receive light driven glycinergic input from an amacrine cell type that is excited by ON
rod bipolar cells. The glycinergic input to the DACs at light onset does not appear to be mediated by AII amacrine cells, which are an established source of glycinergic inhibition under scotopic conditions (Bloomfield and Dacheux, 2001). This is because the inhibitory input evoked by bright light is blocked by NBQX (Fig. 6C,) showing that glycinergic input is not able to be driven by electrical coupling with ON cone bipolar cells, as would be the case if the amacrine in question were an AII (Xin and Bloomfield, 1999; Veruki and Hartveit, 2002; Pang et al., 2007).

Bright step ON excitation: Bath application of L-APB blocked the transient spike discharge triggered by the onset of a bright step of light in current clamp recordings (Fig. 7A1, 2; n=6) as well as an inward current response in voltage clamped cells at light onset (Fig. 7B1, 2; n= 4). That the DAC excitatory ON response is also blocked by NBQX alone (data not shown) indicates that it is mediated by glutamate release from ON bipolar cells acting on postsynaptic ionotropic glutamate receptors. This is in agreement with experiments showing that solitary DACs express AMPA receptors activated by either glutamate or kainate (Gustincich et al., 1997). It appears that the excitatory input to the DAC maybe driven by light activation of both cone and rod pathways. The switch of the DAC light response from net inhibition at light onset to net excitation occurs when the step intensity is sufficiently bright to excite cones, suggesting that DACs receive excitatory input from ON cone bipolar cells (CBCs). As the threshold response in cells held at 0 mV is an outward current that decreases with increasing stimulus intensity before reaching photopic (bright) light levels, suggests that scotopic
(dim light) stimuli are too weak to directly excite cones, and instead give rise to stimulus-evoked currents that are the sum of rod mediated excitatory and inhibitory synaptic currents (Fig. 5).

[Figure 7]

**Bright step OFF inhibition:** In current clamp recordings, the inhibitory OFF response causes a transient suppression of spike activity at light offset and was not affected by bath application of strychnine or TPMPA (Fig. 8A; n=4 for strychnine, n=3 for TPMPA), ruling out mediation by glycineergic input or by GABAergic drive mediated by GABAC receptors. The duration of the depolarizing light response evoked by moderate to bright light was increased by more than two-fold (Figure 8B, B1; n=9) in the presence of GABAzine, a selective GABA_A antagonist. This is consistent with the elimination of inhibitory synaptic input at light offset that would normally curtail the light response by opposing the excitatory ON response. GABAzine treatment was associated with the generation of a spontaneous voltage oscillation that trigger bursts of spikes that were followed by a silent period of suppressed spike activity. This made it difficult to use light-evoked spike activity to evaluate the effect of GABAzine on the inhibitory OFF response as in the experiments with strychnine and TPMPA (Fig. 8A). To circumvent this problem we tested the effect of GABAzine on the inhibitory OFF response in voltage clamp (Fig. 8C; n=3). In the presence of a high concentration (75 μM) of L-APB, a step of bright light evoked an inward current (∆Vhold = 0 mV) that developed slowly during the step and was followed by a larger and faster outward current at light offset (Fig. 8C).
Both components of the response were eliminated by GABAzine, suggesting that the
DAC receives tonic GABAergic input in darkness from an unidentified amacrine cell
(AC) that is excited by OFF CBCs, as postulated previously (Critz and Marc, 1992;
Gustincich et al., 1997, see however Zhang et al., 2007). Light, by inhibiting OFF
bipolar cells, extinguishes the GABAergic input to the DAC causing a reduction of the
outward current that appears as a slow activation of a net inward current during the step
of light. At light offset the OFF pathway is disinhibited, which excites the GABAergic
amacrine cell in the circuit causing, in turn, an outward current response at the
termination of the stimulus (Fig 8C).

These results can be summarized by a synaptic wiring diagram in which the DAC
receives excitatory and inhibitory input via the ON pathway and inhibition via the OFF
pathway (Fig. 9). In the proposed circuit the threshold response to scotopic stimuli is
generated by inhibitory input at light onset from an unidentified glycinergic amacrine cell
that is activated by excitatory input from rod driven ON bipolar cells. Photopic stimuli
evoke a net depolarizing ON response that is produced by direct excitatory input to the
DAC from cone ON bipolar cells. The inhibitory OFF response that is a characteristic
feature of the response to photopic stimuli includes GABAergic input to the DAC from
an unidentified amacrine cell that is excited by OFF cone bipolar cells. This limb of the
circuit provides tonic GABAergic inhibition to the DAC in the dark that is suppressed by
light and re-excited at light offset. While the glycinergic and GABAergic amacrine cells
in the proposed diagram are unidentified they are likely to have narrow and wide-field
dendrites, respectively based on the type of neurotransmitter they release (Wassle et al., 1998).

**Inhibitory receptor subtypes expressed on mouse DACs**

Our pharmacological analysis suggests that distinct inhibitory inputs shape the DAC response to light onset and light offset (ON-glycinergic, OFF-GABAergic), and that different GABA receptor subtypes (GABA$_A$ and not GABA$_C$) are involved. To correlate the physiological observations with expression of GABA and glycine receptors on DACs, we performed immunolabeling using antibodies directed against specific GABA and glycine receptor subunits. In the IPL of the rodent retina individual GABA$_A$ and glycine receptor clusters contain specific $\alpha$-subunits (\(\alpha_{1-3}\)) (Koulen et al., 1996; Wassle et al., 1998; Wassle et al., 2009). TH-immunopositive DAC processes exhibited numerous clusters of $\alpha_1$-GABA$_A$ and $\alpha_3$-GABA$_A$ receptors, some $\alpha_2$-glycine receptor clusters but few, if any, GABA$_C$ receptors clusters. To quantify the relative expression levels of the various GABA and glycine receptor types, DACs in the Drd2-GFP whole mount retina were intracellularly filled with neurobiotin and afterward immunolabeled for GABA$_A\alpha_3$, GABA$_A\alpha_1$, GlyR$\alpha_2$, and GABAC$\rho$ receptor subunits (Fig. 10A). Three-dimensional masks of the dendritic and axonal processes were generated to exclude receptor staining outside the DAC processes of interest (Fig. 10B, C) and estimate the percent volume occupied by a particular receptor type (Fig. 10E). This was then used to compare the relative abundance of each receptor type on DAC axonal and dendritic processes. GABA$_A\alpha_3$ receptors were the most abundant inhibitory receptor type expressed on both DAC dendrites and axonal processes (Fig. 10E), followed by
GABA$_A$$\alpha_1$, and to a much lesser extent GlyR$_\alpha 2$ receptor clusters. GABA$_C$ receptors were minimally expressed on both the axonal and dendritic compartments of the DAC.

[Figure 10]

Receptor expression on DAC soma was estimated in a similar way, but after generating a 3-D mask of TH immuno-positive somata (Fig 10D) rather than using neurobiotin filled cells in which the soma was stained strongly and generated a saturating fluorescence signal. GABA$_A$$\alpha 1$ and GABA$_A$$\alpha 3$ were equally abundant on the soma (Fig. 10E) in contrast to DAC processes that express higher levels of receptors with $\alpha 3$ than $\alpha 1$ subunits. This suggests that the DAC uses different GABA$_A$ receptor subsets to process dendritic/axonal versus somatic inhibition. The soma, like the DAC dendrites and axons, exhibited very few receptor clusters that were GABA$_C$ positive (Fig. 10E). These results are consistent with the persistence of the inhibitory OFF response in the presence of bath applied TPMPA, a selective GABA$_C$ receptor antagonist (Fig. 8).

Because of the dense labeling of receptors in the IPL, two strategies were used to evaluate the specificity of receptor labeling within DAC processes. First, DACs were filled with neurobiotin and the retina double-labeled with an antibody against the C-terminal-binding protein 2 (CtBP2). We chose CtBP2 because it is densely expressed throughout the IPL. Also CtBP2 is localized at synaptic ribbons of excitatory glutamatergic presynaptic terminals in the retina (Schmitz et al., 2000; Soto et al., 2011) and thus should not be within DAC processes. Upon quantification, the % occupancy of CtBP2 puncta on DAC dendritic and axonal processes was found to be only 0.98 ± 0.17 % and 0.64 ± 0.15 % (N= 2 cells) of the available dendritic and axonal volume,
respectively. This suggests that the probability of random association of receptor clusters with DAC processes is less than 1 in 100. Second, we determined the percentage of GABA<sub>A</sub>α<sub>3</sub> (most abundant receptor type on DACs) clusters on TH immuno-positive processes that were apposed to terminals labeled with antibodies directed against vesicle inhibitory amino acid transporter (VIAAT) (Sagne et al., 1997; Soto et al., 2011) (Fig. 11A). TH positive processes were masked to isolate GABA<sub>A</sub>α<sub>3</sub> receptor clusters specifically within the DAC neurite; we found that 87.9 ± 2.4% (N=152 GABA<sub>A</sub>α<sub>3</sub> puncta, 2 animals) of the GABA<sub>A</sub>α<sub>3</sub> clusters were apposed to VIAAT positive presynaptic terminals (Fig. 11B). Thus, we conclude that the vast majority of immunolabeled receptor puncta on DAC processes represent sites of inhibitory synaptic input.

Discussion

Functional diversity of DACs

DACs appear to be a morphologically homogeneous population of cells but exhibit cell-to-cell differences in their functional properties. These are apparent in the cell-to-cell variation of their spontaneous activity in darkness. The characteristics of their spike activity fell into four categories: quiet cells that generated spikes infrequently at random intervals, rhythmic cells that fired spikes at a maintained rate, and cells that generated bursts that were either mixed in with single spikes or occurred in discrete bursts at regular intervals (Fig. 2). The existence of two categories of DAC bursting
activity agrees with Zhang et al. (2007), as does the presence of a minority of DACs that are not spontaneously active. The presence of cells in the DAC population that exhibit the observed categories of spiking activity is also consistent with the range of cell-to-cell differences in spontaneous spike discharge exhibited by midbrain dopaminergic neurons (Grace and Bunney, 1984a, b). The differences in spike patterns ranging from infrequent discharge of single spikes to periodic bursts may participate in setting the basal tone of DA release; the only function that DACs and dopaminergic midbrain neurons have in common. A diversity in resting DA release across the DAC population might have the advantage of offering a richer selection of options for regulation than if all the cells had the same level and mode of DA release. In any case, the variability in spike activity appears to be a feature of DACs in the intact retina since solitary DACs isolated from enzymatically dissociated mouse retina generate spikes rhythmically at a steady rate (3 - 9 Hz) that is remarkably uniform from one cell to the next and devoid of periodic burst firing (Gustincich et al., 1997; Feigenspan et al., 1998; Steffen et al., 2003; Xiao et al., 2004). The difference between quiet and active DACs is not due to differences in cell health, recording time or experimental conditions but it is associated with differences in their responses to hyperpolarizing current injection (Fig. 2). Active, but not quiet, DACs express a voltage-dependent cation conductance that is turned on by hyperpolarization. Since $I_{\text{H}}$ is a known contributor to pacemaking and oscillatory spike discharge in a variety of cell types, including midbrain dopaminergic neurons (Alonso and Llinas, 1989; McCormick and Pape, 1990a; Yung et al., 1991; Mercuri et al., 1995), it reasonable to suggest that it plays a role in the mechanism that generates burst firing in active DACs. In this regard it is relevant to note that spontaneous spike activity in solitary DACs is
silenced by a 5 to 10 mV hyperpolarizing step that does not exhibit a voltage sag ($I_{H}$), or unmask pacemaker activity as it does with DACs in the intact retina (Gustincich et al., 1997; Feigenspan et al., 1998; Steffen et al., 2003; Xiao et al., 2004). The reason for the differences between the intrinsic properties of solitary DACs and DACs in the intact retina are not clear. It could be that the ion conductances that are the source of biophysical differences between solitary and intact DAC reside in the neurites that are shorn off in the dissociation process and constitute the bulk of surface area of the DAC. It is also possible that the retina milieu includes neuromodulators that are absent from the saline solution in which solitary cells are maintained.

The explanation for the cell-to-cell differences in membrane properties of in-vivo DACs is not known. It is possible that the DAC population is divided into physiologically discrete subtypes with fixed differences in their intrinsic properties. It is also possible that DACs exhibit physiological differences depending on the strength of neuromodulatory control and/or circadian regulation. Neuromodulators, such as dopamine, are known to influence the repertoire of ion channel expression in a variety of neuronal cell types and affect their pattern of spontaneous spike activity (Lacey et al., 1987; Pape and McCormick, 1989; McCormick and Pape, 1990b; Stefani et al., 1995). Furthermore, DACs contain clock genes (Dorenbos et al., 2007) and dopamine release oscillates in tune with the circadian cycle (Mangel and Dowling, 1987; Weiler et al., 1997; Ribelayga et al., 2004; Storch et al., 2007). Although our present observations do not discriminate between these possibilities, our findings do raise the need for future experiments to correlate the spontaneous spike patterns of DACs with their release of dopamine.
DAC light responses and distinct modulatory roles for GABA and glycine

The DAC light response depends on stimulus intensity. Onset of dim light evoked a hyperpolarizing response that was initiated by rods and mediated by inhibitory synaptic input from an unidentified glycinergic amacrine cell (AC) that appears not to be an AII amacrine (Fig. 6C). At brighter light intensities the DAC ON response switched from being inhibitory to net excitatory. This was attributed to the recruitment of cones and direct excitatory input to DACs from ON cone bipolar cells (CBCs). While we have assumed that the glycinergic AC responsible for the inhibitory response evoked by dim light is excited by direct synaptic input from ON rod bipolar cells (Fig. 9), we cannot exclude the possibility that rod signals evoked by weak stimuli can electrically spread to cones via gap junctions. Such coupling would allow glycinergic ACs to be excited by ON CBCs. Since inhibition evoked by dim light is eliminated by strychnine without unmasking a residual depolarizing response, the ON CBCs that are excited by cones coupled to rods would have different properties than the ON CBCs providing direct excitatory input to DACs in response to bright light. The segregation of ON CBC signals evoked by rod to cone coupling from those evoked by light activated cones could be based on differences in the ON CBCs, such that weak signals in the cone stimulate ON CBCs that excite glycinergic AC whereas strong signals stimulate ON CBC that excite DACs directly.

The DAC OFF response is evoked by stimuli in the same intensity range as the stimuli associated with the switch in the ON response from being inhibitory to net excitatory at light levels that begin to activate cones as well as rods. The OFF response evoked by moderate to bright light is generated by strong inhibitory synaptic inputs from unidentified GABAergic ACs that are excited by cone driven OFF CBCs. The synaptic
circuit inferred from the intensity dependence and pharmacology of the DAC light response (Fig 9) is consistent with immunohistochemistry showing the expression of GABA\(_{A}\alpha 1\), GABA\(_{A}\alpha 3\) and GlyR\(\alpha 2\) receptor clusters on DAC processes. The distinct receptor subtypes were not confined to specific cellular compartments; each receptor subtype was present on the soma, axon and dendrites. This indicates that glycinergic inhibition evoked by dim light onset and GABAergic inhibition at bright light offset are not correlated with a differential distribution of GABA and glycine receptors. While DAC somata possess similar amounts of GABA\(_{A}\alpha 1\) and \(\alpha 3\) receptor clusters, GABA\(_{A}\alpha 3\) receptors were relatively more abundant in the dendritic and axonal processes. That gating kinetics are slower and the agonist affinity higher in GABA\(_{A}\) receptors with \(\alpha 3\) than with \(\alpha 1\) subunits (Gingrich et al., 1995) suggests that GABAergic regulation of DAC activity is complex and tuned by a variety of factors. The presence of inhibitory input on dendrites may modulate excitatory synaptic input whereas the inhibitory input on the axonal processes, which have not been described previously in retina, may serve to modulate the fidelity of spike mediated communication between the DAC soma and its wide field synaptic targets. The exclusive expression of glycine receptors with \(\alpha 2\) receptors contrasts with previous observations in the primate retina showing that DACs preferentially express GlyR\(\alpha 3\) receptors (Jusuf et al., 2005). The functional significance of the species difference in GlyR\(\alpha\)-subunit expression in the same type of amacrine cell is not known.

The DAC is thus a novel example of a retinal neuron that receives inhibitory input at light onset and offset using different transmitters. The physiological consequences of this arrangement are not clear but it is reasonable to consider it in the context of DA release, which is the central DAC function. Our results show that DACs exhibit diverse
intrinsic properties and receive a repertoire of synaptic contacts that regulate strength of excitatory input and axonal output. These cellular attributes of DAC physiology may act together to fine tune spike triggered DA release in a manner that would not be possible if the cell behaved as a simple on/off switch. Numerous studies based on quantitative chemical analysis of DA levels in retina superfusate have established that DA release is increased by light (Nichols et al., 1967; Kramer, 1971; Iuvone et al., 1978; Morgan and Kamp, 1980; Kamp and Morgan, 1981; O'Connor et al., 1987; Ishita et al., 1988; Kirsch and Wagner, 1989), in which case rod mediated glycinergic inhibition may act to suppress spike activity and DA release in darkness. Previous studies have also demonstrated that DA release is increased more strongly by light that flickers ON and OFF at a low frequency (1 - 3 Hz) than by steady illumination (Dowling and Watling, 1981; Kirsch and Wagner, 1989; Kolbinger and Weiler, 1993; Weiler et al., 1997). Our results, along with evidence that DA release from isolated DACs is spike dependent (Puopolo et al, 2001), provide an explanation for the difference in the effectiveness of these two stimulus procedures. Light that flickers ON and OFF triggered strong bursts of spikes in phase with periods of light exposure (Fig. 4B), whereas steady light evoked an initial transient burst of spikes at light onset that was rapidly silenced by the depolarizing block and remained silent until the depolarizing excitatory response declined to a level that relieved spike block at which point spike generation resumed at an irregular rate not that different from the cell's rate in darkness (Fig. 4A); consistent with reports that DA release is not increased over its level in darkness by continuous light exposure (Godley and Wurtman, 1988; Kolbinger and Weiler, 1993; Weiler et al., 1997). The inhibitory OFF response participates in the response to flicker by hyperpolarizing the cell during periods of darkness thereby relieving depolarizing spike
block in time for the generation of action potentials at the onset of the next period of light exposure.

The reason for the difference in the effectiveness of flicker and steady light in triggering DA release is not known, but seems likely to be the result of facilitated vesicular release that in other neuronal cell types is commonly associated with repetitive firing and increased intracellular Ca2+ due to summated influx (Fioravante and Regehr, 2011). This is in agreement with evidence that neuromodulator release is evoked more strongly by phasic than tonic spike generation (Dutton and Dyball, 1979; Gonon, 1988; Floresco et al., 2003). The inhibitory OFF response evoked by moderate to bright light rapidly terminates spike discharge after a period of light exposure and thus benefits the generation of responses characterized by transient increases in spike discharge. In the midbrain DA system tonic and phasic spike activity control different higher level behaviors (Floresco et al., 2003; Zweifel et al., 2008; Tsai et al., 2009) and have been modeled in terms of changes in the balance between DR1 and DR2 signaling pathways (Dreyer et al., 2010). It is possible that DA signaling in the retina is influenced in a similar manner and that a mixture of inhibitory inputs are important controlling factors in this process, a suggestion that remains to be explored.

Comparison with previous studies

The results presented here differ in several significant ways from the results presented in the only two previous papers on in situ DACs that were identified and targeted for single cell recording in intact retina using fluorescence protein expression in a transgenic mouse line (Zhang et al. 2007, 2008). The earlier studies reported that 40% of DACs do not respond to light and that the remaining light sensitive cells
belonged to two distinct classes in which light onset evoked either a transient burst or a sustained train of spikes with no evidence of an OFF response in either. The ON-sustained response, which was recorded from 44% of the subpopulation of light sensitive DACs, was attributed to excitatory input from ipRGCs.

In contrast, over the course of our experiments we found that 100% of the recorded DACs responded to light and whether they generated a sustained or transient spike response depended on stimulus intensity (Fig. 3A). Sustained spike trains were evoked by weak stimuli whereas depolarization block limited the ON response evoked by bright light to an initial transient burst of spikes at response onset. This was also the case in a minority of DACs in our sample that generated exceptionally prolonged large depolarizing responses to strong stimuli, which we considered to be the result of direct excitatory input from ipRGCs (Fig. 3D). In addition, our results show clear evidence of inhibitory ON and OFF responses, which contradicts previous reports that neither GABAergic nor glycinergic OFF-channel signals were involved in DAC light responses (Zhang et al. 2007, 2008).

Whereas both the earlier studies and our study were based on recording from DACs in different transgenic mouse lines, this seems unlikely to be an explanation for the difference in the results since in the Drd2-GFP Gensat line used here all amacrine cells that were label with antibodies to TH also express GFP. This would presumably encompass population of DACs that were targeted by using the TH promoter to drive red fluorescent protein expression in the studies by Zhang and colleagues. It seems more likely that discrepancies between our results and those of Zhang et al (2007, 2008) are related to important differences in methodology. In the earlier studies DACs were targeted using fluorescence excited by one-photon absorption of short wavelength
light. Although no specific information is given about the intensity or illumination area of
the excitation light it was presumed to have reduced the light sensitivity of the retina
(Zhang et al. 2008). Changes in the adaptational state of the retina were avoided in the
present study by exciting fluorescence using 2-photon absorption of infrared light (see
methods). Furthermore the results of the earlier studies were based on extracellularly
recorded spike activity evoked using only intense photopic stimuli that would saturate
the photoresponses of both rods and cones. The use of saturating light exposure to
evoke responses that can only be detected if they are super-threshold for action
potential production provides a severely limited description of the light response
properties and fails to fully characterize the dynamic range of DAC light sensitivity and
the subthreshold synaptic inputs that give rise to the intensity dependent properties of
the DAC light response.

In the Zhang studies, a reduction in the light sensitivity from the use of short
wavelength light to excite fluorescence for cell targeting and from repeated exposure of
the retina to steps of saturating light could account for the presence of cells that do not
generate a strong enough response to evoke spikes and thus are classified as non-
responsive (null) DACs. In any case, the absence of light-evoked spike activity in a
retinal neuron is not sufficient evidence to conclude that it does not respond to light.
Light-adaptational changes in the retina might also serve to reduce the amplitude of the
depolarizing response evoked by excitatory input to a level that is not sufficient to cause
a depolarizing spike block accounting for the presence of a sub-population of DACs in
which the response to saturating light is a sustained spike train rather than a transient
burst as seen in our recording.
In view of the fundamental differences in the experimental methods used in this and the earlier studies it is not surprising that there is limited agreement in the results. The main points of accordance include cell-to-cell variation in the pattern of spontaneous spike activity (with disagreement about the extent of the differences) and the elimination of the transient excitatory ON response by L-APB.

In summary our experiments on *in situ* DACs characterize the intensity dependence of the synaptic inputs that give rise to their response to light. The onset of dim stimuli evoked an inhibitory response that with increasing light intensity become a net depolarizing response that is truncated at light offset by GABAergic inhibition via the OFF pathway. The interplay between light intensity and the strength and timing of multiple excitatory and inhibitory inputs determine the DAC response to light and the role it plays in modulating retina function through the release of dopamine.
References


Lacey MG, Mercuri NB, North RA (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. The Journal of physiology 392:397-416.


Xin D, Bloomfield SA (1999) Comparison of the responses of AII amacrine cells in the

Yung WH, Hausser MA, Jack JJ (1991) Electrophysiology of dopaminergic and non-
dopaminergic neurones of the guinea-pig substantia nigra pars compacta in vitro. The

dopaminergic neurons underlying their multiple roles in vision. The Journal of

genetically labeled catecholamine neurons in the mouse retina. Neuroreport 15:1761-
1765.

Intraretinal signaling by ganglion cell photoreceptors to dopaminergic amacrine

Zweifel LS, Argilli E, Bonci A, Palmiter RD (2008) Role of NMDA receptors in dopamine
neurons for plasticity and addictive behaviors. Neuron 59:486-496.
Figure Legends

Figure 1. Morphology of dopaminergic amacrine cells in Drd2-GFP transgenic. (A) Distribution of GFP expressing cells across a Drd2-GFP retina. Higher magnification of four representative regions immunolabeled for tyrosine hydroxylase (TH) in different parts of the Drd2-GFP retina (D, dorsal; V, ventral; T, temporal and N, nasal. ONH, optic nerve head). All GFP expressing cells with large somata are TH positive. (B) Higher magnification view showing a large TH positive, GFP expressing cell. (C) Morphology of a large GFP positive cell as revealed by intracellular filling with neurobiotin. Its cell body lies in the inner nuclear layer (INL) and processes stratify in the outermost part of the inner plexiform layer (IPL; inset 1). An axonal process is highlighted by arrows and shown at higher magnification in inset panel 2. (D) A neurobiotin filled DAC with varicosities along its axonal process (arrows). (E) TH immunolabeling in retinal whole-mounts shows a fraction of dopaminergic amacrine processes (arrow in inset panel) reaching the outer plexiform layer (OPL).

Figure 2. Cellular properties of DACs. Resting spontaneous spike activities in four different cells provide examples of DACs that are nearly silent (A1), continuously active (B1), steady discharge with intermixed bursts of spikes (C1) or periodic bursts (D). Resting membrane potentials (mV) are indicated by labels left of each trace. The voltage responses evoked by constant current steps in the same cells are shown by traces in A2, B2, and C2. Negative current steps in A2 and B2 were 20, 30 and 40 pA. Periodic burst firing persists in the presence of a mixture of excitatory and inhibitory
synaptic blockers (in μM): APB (50), AP5 (50), CNQX (50), picrotoxin (50), and strychnine (1). Labels to the left of each recording identify the resting potential (mV) relative to the voltage trace using the provided voltage scale.

Figure 3. DAC light response properties. (A) DAC light response evoked by 500 ms steps of full field (700 μm diameter spot) 440 nm light of indicated intensities (log$_{10}$ Rh*/rod/step). (B & C) Spike rasters and example trace showing responses to light steps in different cells. (D) An example of DAC that exhibited a prolonged response to a bright full field step of 440 nm light (9x10$^5$ Rh*/rod/0.5s). Labels to the left of each recording indicate the resting potentials in mV relative to the voltage trace using the voltage scales provided.

Figure 4. DAC responses and steady and flickering light. (A) 20 s step of light evoked a transient burst of spikes at light onset that gave way to irregular lower frequency spike discharge as the cell adapted to the steady light (n=4). The termination of action potential discharge at the leading edge of the response by depolarization spike block is shown on an expanded time base in the insert (arrow). The traces (B) show the entrainment of DAC spike responses to periodic 500 ms stimulus (3.94 log$_{10}$ Rh*/rod/0.5s) that flickers ON and OFF at 0.7 Hz on two different time scales. Labels to the left of each recording indicate the resting potentials in mV relative to the voltage trace using the voltage scales provided.

Figure 5. Light response intensity series in voltage clamp. Responses evoked by 500 ms steps of full field 440 nm light at the intensities (central column) expressed as log$_{10}$
Rh*/rod/0.5s in a representative DAC held at a voltage close to the reversal potential for inhibitory synaptic input (-60 mV; left column) and excitatory synaptic input (0 mV; right column) in Ames’s solution.

Figure 6. Inhibitory ON response evoked by a dim step of light is blocked by strychnine. (A) Current clamp recordings of responses in weak steps of light in absence and presence of 1 μM strychnine (n=7). Average response is shown in black and individual responses are displayed in gray. Labels to the left of each recording indicate the resting potentials in mV relative to the voltage trace using the voltage scale provided. (B) Voltage clamp recordings of outward current response at 0 mV holding potential evoked by dim steps in absence and presence of 1 μM strychnine. Stimulus intensities are reported as log10 Rh*/rod/0.5s for full field 440 nm light and associated with the traces showing the timing of the light step.

Figure 7. Synaptic origin of the excitatory ON response evoked by bright light. Spike raster plots and example single traces (below) show the response evoked by bright step of light in absence of glutamatergic reagents (A1) and in the presence of L-APB, a metabotropic glutamate receptor agonist that quenches the excitatory light response in ON bipolar cells alone (A2) and with the addition of NBQX (A3). The results show that the control response had an excitatory ON response that was eliminated by L-APB and an inhibitory OFF response that was blocked by the addition of NBQX (n=6). B1 and B2 show the currents evoked by bright light in voltage clamp recordings at the indicated holding potentials (midline labels) in the absence (control) and presence of L-APB (B2). The ON pathway antagonist eliminated the inward current evoked at light onset but not
the outward current at light offset (n=4). Light stimuli: 500 ms full field steps of 440 nm light equivalent to 5.0 log_{10} Rh*/rod/0.5s.

**Figure 8.** GABAergic inhibitory OFF response evoked by bright light. (A) Spike rasters showing the persistence of the inhibitory OFF response in the presence of strychnine (n=4), a glycine receptor antagonist, and TPMPA (n=3), a GABA_C receptor antagonist. (B) Bath application of GABAzine (red trace) prolonged the depolarizing response to light, shown on an expanded time base (B1) that compares superimposed responses in absence (black) and presence (red) of GABAzine (n=9). Labels to the left of each recording indicate the resting potentials in mV relative to the voltage trace using the voltage scales provided. (C) In voltage clamp recordings (V_{hold} = 0 mV) in the presence of L-APB there was a slowly increasing negative (inward) current during the light step that was supplanted by a large positive (outward) current at light offset. Both components of the step response were eliminated by GABAzine and partially reversed upon washout (n=3). Light stimuli: 500 ms full field steps of 440 nm light equivalent to 3.94 (A, B) and 5.95 (C) log_{10} Rh*/rod/0.5s.

**Figure 9.** DAC synaptic wiring diagram. In the proposed schematic DACs receive excitatory glutamatergic input from ON cone bipolar cells as well as from ipRGCs in a subset (~ 15%) of DACs. In addition, DACs received inhibitory input from two sources. One is an unidentified glycinergic amacrine cell that is driven by excitatory input from rod bipolar cells and the other is an unidentified GABAergic amacrine cell that is driven by excitatory input from OFF cone bipolar cells. Red and green terminals represent
ionotropic (AMPA / kainate) and metabotropic (mGluR) glutamatergic synapses, respectively.

**Figure 10.** Inhibitory receptor density on DACs. (A) Neurobiotin fills (NB-568) of dopaminergic amacrine cells in the Drd2-GFP line co-labeled for specific inhibitory receptor subsets: GABA_A receptors (α3 or α1 subunit), glycine receptor (α2 subunit) and GABA_C receptors (ρ subunit). (B) Higher magnification of a stretch of dendrite and receptor labeling. Receptors localized within the volume of the dendrite are visualized by first creating a 3-D ‘dendrite mask’ of the NB-568 signal, and then digitally excluding signal from the receptor immunolabeling outside this mask. A threshold is imposed above which receptor signal intensities are quantified. (C) Receptor labeling within the axon mask. (D) Receptor labeling within the soma mask. Somata labeled here by anti-TH. (E) Quantification of the percentage of volume of the dendrite, axon or soma occupied by receptors containing different subunits.

**Figure 11.** GABA receptor puncta on TH immuno-positive processes are apposed to inhibitory presynaptic terminals. (A) Dopaminergic amacrine processes labeled with TH in retinal slices, together with labeling for GABA_Aα3 receptors and the inhibitory presynaptic marker, VIAAT (IPL, inner plexiform layer). Maximum intensity projection of confocal image stack representing 0.9 µm thicknesses. (B) To quantify GABA_Aα3-VIAAT appositions, TH labeled processes were masked in thicker image stacks (4.8 µm). GABA_Aα3 and VIAAT signal within the masks were isolated to reveal appositions in 3D. The majority of GABA_Aα3 receptor puncta were apposed to VIAAT (exception,
arrowhead, merged panel). Plots show quantification of the appositions identified by 3D rotation of the merged images.
A
Control
1 µM Strychnine
50 µM TPMPA

B
20 mV
2 s
-50-
Control
40 µM Gabazine

B1
20 mV
0.5 s
-50-

C
+75 µM L-APB
+75 µM L-APB +80 µM Gabazine
wash to 75 µM L-APB

500 ms
20 pA