Materials and Methods

Immunotoxin application

Injection protocol was identical to that previously described (*1*): P0 ferrets were anesthetized with inhalant isofluorane anesthesia and 2µl solution of immunotoxin: an antibody directed against the Vesicle Acetylcholine Transporter (VAChT) conjugated to saporin (VAChT-SAP), was injected into the vitreal chamber of the eye using a 33 gauge needle attached to a Hamilton microsyringe (final concentration of immunotoxin=70ng/µl in 0.9% saline). Control ferrets received equal volume injections of saline. Details of the immunotoxin have been described previously (*2*). All procedures involving animals were in accordance with NIH guidelines and institutional protocols.

Quantification of starburst amacrine cells following toxin treatment

Ferrets were perfused either 48 hours or 10 days following the toxin injection. Retinae were hemisected, cryosectioned at 12µm, and processed for Choline Acetyl Transferase (ChAT) immunohistochemistry (to label Starburst amacrine cells), DAPI (to label cytoarchitecture), and in some cases, parvalbumin or tyrosine hydroxylase (to label horizontal and a subset of non-starburst amacrine cells) using previously described protocols (*2*). Tissue from age-matched control and toxin-treated retinae were immmunostained in parallel and the number of ChAT-immunopositive cells/mm retina in the ganglion cell and inner nuclear layers was quantified on P2 and P10 (48 hours and 10 days after injection, respectively) from serial sections of the central-half and peripheral-half of all 4 retinal quadrants, by an observer blind to experimental treatment. All areas of the recorded retinae were embedded, then sectioned and immunostained for ChAT as described. Whole-mount staining of ChAT immunoreactivity was not employed because it is unreliable in young (<P15) ferret retinae (especially for cells located in the inner nuclear retina) and, even with confocal microscopy, it only allows reliable quantification of starburst cells in the ganglion cell layer (half of all starburst cell bodies reside in the inner nuclear layer). When applied to the P0 ferret retina, a 2µl of the 70ng/µl concentration produces the maximal reduction of starburst amacrine cells without compromising other cell types. Therefore, this concentration was applied *in vivo* on P0 prior to patch-clamp recordings and retinogeniculate anatomy. In 3 animals, the toxin

treatment did not appear to work in one retina (numbers of starburst amacrine cells appeared normal). These cases were excluded from the study.

Calcium Imaging

Calcium imaging of spontaneous retinal activity using Fura2-AM (10μm; Molecular Probes) loaded retinae was performed according to previously described protocols (*1,3-5*) on normal P3-7 ferret retinae, and P3-7 retinae that were treated with VAChT-SAP immunotoxin. A 1.331 X 1.206mm area of retina was imaged using a low magnification (5X) objective which, at the ages described here, encompassed approximately 1 retinal quadrant; multiple quadrants from each retina were examined. Each recording lasted 10-20 minutes. Fluorescence intensity/time traces were averaged from a 20.8 X 20.8μm area in each retinal quadrant (IGOR software) and normalized for background bleaching (ORIGIN software). Calcium transient amplitudes were calculated by dividing the change in fluorescence (ΔF) by the baseline fluorescence level preceding the wave event (F). Waves normally range from 2-20% ΔF/F (*1, 3-5*). Calcium transient frequency was determined by dividing the total number of 2% or greater ΔF/F events/time.

Patch-clamp recordings

Recording, current injection and cell filling methods were identical to those previously described (*6, 7*) except that retinae were age P2-9 and were immunotoxin or saline injected on P0 as described above. Cells were recorded from 2-4 quadrants of each retina. 2-4 retinae were recorded per age/treatment. Recordings were carried out at equal distances (soma-soma recording distance was less than 25μm), over the same duration following tissue dissection, at equal temperatures and blind to experimental treatment.

Characterization of single-cell activity patterns

All normal and toxin treated cells were categorized into one of four groups based on their pattern of activity. "Silent" cells were those that showed no slow depolarizations or spiking activity for the entire recording session (10 minutes or more). "Depolarizing-only" cells showed periodic slow depolarizations but no action potentials. Cells that did show action potentials were divided into two groups: "bursting", and "spikes not

confined to bursts". To make this characterization, spikes were identified from raw patch clamp recording traces and inter-spike intervals (ISIs) were calculated using Minianalysis software (Synaptosoft; Decatur GA.). "Bursting" cells were defined as cells where less than 25% of ISIs fell in the range of 1-30 sec. ISI's in the 1-30 second range likely represent spikes occurring between bursts (ISIs of less than one second could represent two spikes within the same burst, ISIs of greater than 30 seconds could represent two spikes in different bursts). Cells with greater than 25% of ISIs falling between 1 sec and 30 sec were characterized as "spikes not confined to bursts". This quantitative characterization was in excellent agreement with characterizations of the traces by eye.

Correlation Analysis of spiking activity

Cross-correlations of spikes (discriminated using Spike 2 software) from the entire recording session were calculated in MATLAB using standard cross-correlation functions/code. Bin size was 10 ms. This analysis controls for differences in mean firing rate. The statistical significance (P value) of the resulting Pearson correlation coefficients values was determined by consulting a statistical table (in: J.H. Zar's Biostatistical Analysis, Prentice-Hall, INC.) Random spike processes were generated by cross-correlating the random-time shuffle of one cells spike raster with the activity of the other cell in the pair.

Correlation Analysis of membrane potential

Cross-correlation of membrane potentials were calculated in MATLAB using standard functions/code. Membrane potentials were sampled at a rate of 4000 Hz, and the resulting signals were corrected for slow (10 seconds or greater) changes in DC offset (to control for any slow drifts in resting potential). The statistical significance (P value) of the resulting Pearson correlation coefficient r-values was determined by consulting a standard statistical lookup table (in: J.H. Zar's Biostatistical Analysis, Prentice-Hall, INC.). Random processes were generated by cross-correlating the random-time shuffle of one cells membrane activity with that of the other cell in the pair.

Labeling of Retinogeniculate Afferents

Cholera Toxin-β (CTβ) conjugated to Alexa dye 488 (green label) was injected into the vitreal chamber of the right eye, and CTβ conjugated to 594 (red label) was injected into the vitreal chamber of the left eye according to the eye-injection protocol described previously (*1*) (3-5µl depending on the age of the animal; 0.5% in sterile physiological saline; Molecular Probes: Eugene OR; CTβ has no biological activity). 24 hours later, animals were transcardially perfused with 4% paraformaldyhyde. Thalamic tissue was postfixed overnight, cryoprotected and sectioned horizontally at 50µm. Ages in text correspond to age at sacrifice.

Blockade of Spontaneous Retinal Activity

Animals received intravitreal injections of epibatidine HCL (1mM in sterile saline; dose=1µl on P3 and increased by 0.25µl for each subsequent injection; Sigma) every 48 hours from P3-P9 according to the eye injection procedure described (*1*). At concentrations of 1-10nM, epibatidine blocks all spontaneous increases in intracellular calcium and all ganglion cell spiking in P1-10 ferret retinae *in vitro* (*1,4*); 1mM epibatidine has previously been shown to prevent eye-specific segregation in the ferret *in vivo* (*1,4*).

Imaging and Quantification of Retinogeniculate Projections

Image acquisition and quantification was identical to that previously described (*1*). Briefly, images of the labeling pattern in the dlgn were digitally acquired with a CCD camera (SPOT Diagnostic); Universal gains were established for each label at a given magnification. Raw images of the dlgn were imported to Photoshop (Adobe), cropped to exclude the optic tract and medial intralaminar nucleus and then set to a threshold 30% above background (background was designated as a non-retinorecipient portion of the tissue slice 1mm lateral to the midline of the thalamus). The 30% value is based on previous studies (*1,4,5*) and evaluation of signal:noise in tissue from different age animals. Threshold images were then set to black (<30% above threshold) or white (>30% above threshold). Measurements of the area of the dlgn occupied by the contralateral or ipsilateral eye projections were calculated by automatically selecting all

white pixels within the image frame (Scion Image). Measurements of overlap were calculated by multiplying the thresholded image of the contralateral eye inputs to the dlgn, with the thresholded image of the ipsilateral eye inputs to the same dlgn. Thus, in the resulting image, white pixels correspond only to locations where the contralateral and ipsilateral afferents were both present; overlap area was calculated by automatically selecting all the white pixels within the image frame (using Scion Image software). 4-6 sections through the middle 200-300μm portion of the dlgn were analyzed for each animal (depending on the animal's age). Retinal labeling of ChAT immunopositive neurons was used to confirm that the immunotoxin depleted starburst amacrine cells (see above). Note: Serial sections from both eyes were processed and quantified for ChAT immunohistochemistry. Tissue from animals in which retinal ganglion cell or retinogeniculate labeling appeared incomplete, or in which fewer than 70% of ChAT+ cells were eliminated from both eyes, were excluded from the study. For preparation of photomicrographs, images were imported to Photoshop (Adobe) for cropping, resizing and alignment.

Supporting text

Calcium Imaging

Fluorescence imaging of intracellular calcium provides an efficient way to assess the activity patterns across large populations of cells. However, this technique provides only an indirect measure of neural activity and cannot distinguish ganglion cells (the neurons that send axonal projections to the dlgn) from displaced amacrine cells, because both of these cell types participate in retinal waves (*3,8*). Ganglion cell spiking comprises a large percentage of the calcium signal (*8*), but periodic waves of reduced magnitude and frequency persist throughout the retina even in the complete absence of ganglion cell action potentials (*8*). Thus, the reduced/absent calcium transients we observed following toxin treatment likely reflect altered ganglion cell spiking. We observed waves (albeit of reduced amplitude and frequency) through P5. A large percentage of eye-specific segregation is completed by P6/7 *(9)* (A.D.H and B.C unpublished observations). Thus, it is possible that propagating calcium waves play a role in eye-specific segregation via a mechanism(s) other than by synchronizing ganglion cell action potentials.

Patch-clamp recordings

That 11/46 ganglion cells from control retinae showed periodic slow-depolarizations but did not spike is consistent with previous work from our laboratory on the maturation of intrinsic membrane properties of retinal ganglion cells in the ferret and cat (*10, 11*). In paired recordings from control retinae, these non-spiking slow depolarization events were highly correlated with the bursting/depolarizing activity of the neighboring ganglion cell (Table S2)

Reliability of Toxin Treatment within retinae

We recorded from multiple sites across each retina including quadrant-matched locations of control versus toxin-treated retinae. The wide variation of single cell responses exhibited by neighboring ganglion cells following toxin treatment indicates that large regions of the retina were not either silent or overactive, but rather that cells with different activity patterns were randomly distributed across the retina. The immunohistochemical quantification of starburst amacrine cell depletion was conducted on serial sections of the central-half and peripheral-half from all 4 retinal quadrants (Fig. 1 main text; and see above). This confirmed that starburst amacrine cell depletion was uniform (i.e., there were no significant differences in starburst amacrine cell density as a function of eccentricity; see Fig. 1 main text).

Reliability of Toxin Treatment between retinae

For all animals used for retinogeniculate anatomy, the efficacy of the immunotoxin ablation (70%+ reduction in ChAT+ cells from the entire retina; see methods above) was confirmed for both retinae. Retinal physiology was carried out on 2-4 retinae per age. At all ages (P2-9), spiking as well as non-spiking ganglion cells were found in each retina.

Supporting Tables

Table 1: Cross-correlation of spiking activity in neighboring ganglion cells

Cell pair# (and age)	Correlation coefficient	Significance level
Control		
2001 (P3)	r=0.17	P<0.001
2002 (P2)	r=0.16	P<0.001
2003 (P2)	r=0.16	P<0.001
2005 (P2)	r=0.11	P<0.001
4001 (P4)	r=0.16	P<0.001
4002 (P5)	r=0.27	P<0.001
4004 (P4)	r=0.21	P<0.001
4005 (P5)	r=0.09	P<0.005
6000 (P9)	r=0.12	P<0.001
6001 (P7)	r=0.18	P<0.001
6002 (P6)	r=0.20	P<0.001
Toxin		
1002 (P2)	r=0.02	P>0.500
1005 (P2)	r=0.04	P>0.200
1006 (P2)	r=0.03	P>0.200
2002 (P4)	r=0.03	P>0.200
3008 (P7)	r=0.02	P>0.500

Table 2: Cross-correlation of membrane potential (for ganglion cell pairs in which one cell did not spike)

Cell pair# (and age)	Correlation coefficient	Significance level
Control		
2006 (P3)	r=0.465	P<0.001
2008 (P2)	r=0.411	P<0.001
4000 (P4)	r=0.297	P<0.001
4006 (P5)	r=0.135	P<0.001
Toxin		
1000 (P3)	r=0.060	P>0.050
1007 (P2)	r=0.021	P>0.500
2000 (P3)	r=0.040	P>0.100
2004 (P5)*	NA	NA
2006 (P5)	r=0.049	P>0.100
2008 (P4)	r=0.087	P>0.050
3000 (P7)	r=0.040	P>0.200
3002 (P6)	r=0.037	P>0.200
3004 (P6)	r=0.026	P>0.200
3006 (P9)	r=0.047	P>0.200

*This cell pair did not show any depolarization, hyperpolarization or spiking activity on either electrode for the entire recording duration.

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