GABAergic control of retinal ganglion cell dendritic development

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Abstract

Developing GABAergic neurons mature long before excitatory neurons, and early GABA activity exerts important paracrine effects while neurons extend dendrites and axons and they establish neural connections. One of the unique features of early GABA activity is that it induces membrane depolarization and Ca$^{2+}$ influx and it shifts to inhibition when networks mature. Although it has been demonstrated in several systems that early GABA signalling plays a fundamental role in guiding neurite outgrowth, it has never been investigated in the retina. Here we show that chronic GABAergic activity is required for the stabilization and maintenance of newly formed dendritic branches in developing turtle retinal ganglion cells (RGCs) in ovo. Blocking GABA$_{	ext{A}}$ receptors with bicuculline or inhibiting GABA synthesis with L-allylglycine have contrasting effects on dendritic growth and branching in biocytin-labeled RGCs. Dendritic arbor reconstruction shows that bicuculline induces dendritic branch loss without global change in the extent of dendritic fields whilst L-allylglycine causes the entire tree to shrink. At the same time, multielectrode array recordings and Ca$^{2+}$ imaging show that L-allylglycine has similar effects to bicuculline (Leitch et al., 2005) on overall network excitability, preventing the disappearance of immature retinal waves of activity and the GABAergic polarity shift. This study demonstrates for the first time that GABA plays an important role in vivo in stabilizing developing dendrites into mature arbors in the retina. However, the way GABA influences dendritic growth appears to be driven by complex mechanisms that cannot be explained solely on the basis of overall network activity levels.

Keywords: retina, retinal wave, GABA, dendrite, multielectrode array, glutamic acid decarboxylase.
Highlights

- Retinal GABA synthesis can be blocked *in ovo* with the GAD inhibitor L-Allylglycine.
- GABA synthesis inhibition prevents maturation of the retinal network.
- Chronic GABAAR blockade induces dendritic loss in developing ganglion cells.
- GABA synthesis inhibition induces dendritic loss and global dendritic retraction.
- Both those effects occur during persistent enhanced retinal network activity.
1. Introduction

Although GABA is the main inhibitory neurotransmitter in the mature CNS, early GABAergic signaling is depolarizing and even excitatory (LoTurco et al., 1995), exerting important epigenetic functions during the wiring of neural networks, including dendritic growth (for review see Represa and Ben-Ari, 2005; Ben-Ari et al., 2007; Wang and Kriegstein, 2009; Sernagor et al., 2010; Ben-Ari et al., 2012). GABA promotes dendritic development during late embryonic stages in hippocampus, cortex and cerebellum (Barbin et al., 1993; Maric et al., 2001; Borodinsky et al., 2003; Cancedda et al., 2007), and it also triggers dendritic proliferation (Gascon et al., 2006) and maturation (Duveau et al., 2011) as well as synaptic integration (Ge et al., 2006, 2007) during adult neurogenesis. These questions have not been investigated in the retina.

GABA-mediated Ca\(^{2+}\) signals modulate dendritic proliferation by stabilizing dendritic growth cone lamellipodia (Gascon et al., 2006). However, Ca\(^{2+}\)-induced signaling is a common feature of synaptic activity-driven dendritic development (Chen and Ghosh, 2004; Konur and Ghosh, 2005). It is therefore difficult to draw firm conclusions about the GABAergic specificity of these effects and to decipher whether these mechanisms involve specific pathways and/or rely upon global neural activity levels.

Using an \textit{in ovo} turtle model (Sernagor and Grzywacz, 1996; Leitch et al., 2005), we have investigated the involvement of GABA in the process of dendritic development during late embryonic and early postnatal stages, when arbors are refined through dendritic pruning and stabilization (Mehta and Sernagor, 2006a,b). Immature RGCs undergo spontaneous bursts of activity that spread between neighbors in a wave-like fashion across the RGC layer (Meister et al., 1991; Wong et al., 1993; Feller et al., 1996; Sernagor et al., 2000, 2003; Zhou and Zhao, 2000). Gestation in turtles lasts two months, and retinal waves start at embryonic Stage 22 (S22), ~5 weeks post-fertilization (3 weeks before hatching) (Sernagor et al., 2003). Waves
stop propagating and become stationary patches of synchronized activity towards hatching (S26), and these patches completely disappear during the first month post hatching (PH).

During the period of retinal waves, there is intense RGC dendritic growth and remodeling (Mehta and Sernagor, 2006a,b). Chronic cholinergic blockade of the waves from S22 results in small, undeveloped trees, whilst increasing spontaneous activity (by rearing newly hatched turtles in the dark) enhances dendritic growth and branching (Mehta and Sernagor, 2006b). Dendritic growth peaks when GABAergic activity starts shifting to inhibition, one week before hatching (at S25) (Sernagor et al., 2003), and is followed by a maturational pruning process (Mehta and Sernagor, 2006a, b).

In this study, GABAergic activity was chronically altered from S24-25 (at the onset of the GABA polarity shift and dendritic pruning, 10-7 days before hatching) until several weeks PH. This was done by blocking either GABA_A receptors or GABA synthesis. We have already demonstrated that chronic GABA_A receptors blockade in the embryonic turtle retina causes waves to persist and GABA responses to remain excitatory at one month PH (Leitch et al., 2005). Here we show that GABA synthesis inhibition results in efficient GABA depletion, and, similarly to receptor blockade, prevents the disappearance of correlated spontaneous activity and depolarizing GABAergic responses within the same age range. However, despite these physiological similarities, the effects of these treatments upon dendritic growth vary, suggesting that while the retina develops, GABA plays multiple roles that are not necessarily related to global network activity patterns.
2. Experimental Procedures

All animal procedures were conducted under the UK Home Office, Animals (Scientific procedures) Act 1986.

This study was done using the turtle species Pseudemys Scripta Elegans. Embryonic ages were determined according to specific staging criteria (Yntema, 1968). S22 and S25 respectively correspond to 3 and 1 week before hatching, and hatching process is at S26 (see also Sernagor and Grzywacz, 1995). Animals were anesthetized by hypothermia, decapitated and enucleated prior to retinal isolation.

2.1 Preparation and implantation of Elvax 40W

Elvax beads (duPont Chemicals) were washed in 100% ethanol for 3-4 days at -20ºC and subsequently dissolved in methylene chloride. 10 µL of either the GABA<sub>A</sub> receptor antagonist bicuculline (10 mM, see Leitch et al., 2005) or the glutamic acid decarboxylase inhibitor L-allylglycine (Horton et al., 1978) solution (1M, see Results) were added to the liquid Elvax along with 10 µL of 0.1% Fast green (allowing visualization of the drug inside the plastic). The Elvax-drug solutions were frozen in dry ice for 10 min and then kept in the freezer for 1-2 weeks, until complete evaporation of the solvent. Implantations were performed around S24-S25 (10-7 days before hatching). Embryos were anesthetized by hypothermia and a small piece of Elvax was introduced in the left eye. The implanted eyes were treated for five days with ophthalmic antibiotic drops (chloramphenicol).

2.2 Calcium imaging

RGCs were back-labeled with 20% Ca<sup>2+</sup> green dextran (CGD) as in previous studies (Sernagor et al., 2000, 2003; Leitch et al., 2005). Injected eyecups were kept overnight in oxygenated Ringer’s solution at room temperature to allow retrograde loading of RGCs. The composition of Ringer’s solution was (in mM): NaCl, 96.5; KCl, 2.6; MgCl<sub>2</sub>, 2; NaHCO<sub>3</sub>, 31.5; glucose, 10; Hepes, 10 and CaCl<sub>2</sub>, 4 (recordings were performed in 4.9 mM KCl to increase network
excitability as in previous studies). Retinas were isolated and flattened on nitrocellulose paper with the RGC layer facing up and transferred to the experimental chamber onto the stage of an upright microscope (Olympus AX70). The chamber was continuously perfused (2–5 ml/min) with oxygenated Ringer's solution kept at 26–28 °C. GABA was applied with a micropipette in single puffs of 50µl of a 5mM solution, resulting in an approximate concentration of 100µM in the bath (Sernagor et al., 2003). The imaging technique and analysis of Ca\(^{2+}\) transients are fully described in previous studies (Sernagor et al., 2000, 2003; Leitch et al., 2005). Briefly, fluorescence changes were detected using a Video Rate Intensified CCD camera (Princeton Scientific Instruments, Monmouth Junction, NJ, USA) and continuously recorded onto video tape (25 frames/s). Episodes of activity were digitized using MetaMorph imaging software (Universal Imaging, Downingtown, PA, USA). To calculate cellular recruitment during waves, we included all RGCs participating in the production of one wave of activity. When the activity was patchy, later in development, we calculated cellular recruitment by sampling spontaneous activity occurring during consecutive 5 s bins (Sernagor et al., 2003). To calculate relative-onset plots, a cell that was activated early in a wave was chosen as the reference cell. Then, for every other active cell, we plotted the difference in onset time for that cell and the reference cell as a function of the distance between them. When there is propagation, relative-onset plots exhibit clear oblique lines, indicating increasing delays with distance from the reference cell. When the activity is patchy, relative-onset plots exhibit clusters of horizontal lines, with each line representing one patch of neighboring coactive RGCs. When local correlations disappear with development, relative onset plots exhibit no specific pattern.

2.3 Multielectrode arrays recordings
To assess the levels of spontaneous activity, in addition to Ca\(^{2+}\) imaging we have also performed MEA recordings using a 60-channels planar MEA system (Multi Channel Systems,
Retinas were placed, RGC layer facing down, onto the surface of MEAs consisting of 60 titanium nitride electrodes (30 μm diameter, 200 μm spacing) arranged in an 8x8 grid without corners on indium tin oxide substrate (Multi Channel Systems, Reutlingen, Germany). Reliable coupling between the tissue and the electrodes was achieved by placing a small piece of polyester membrane filter (5 μm pores) (Sterlitech, Kent, WA, USA) on the retina followed by a slice anchor holder (Warner Instruments, Hamden, CT, USA). The retina was perfused and heated as for Ca$^{2+}$ imaging experiments.

Signals were amplified (gain 1,200) and acquired using a 128-channel analogue to digital converter (Multi Channel Systems MC_Card, Reutlingen, Germany). Signals were digitized at 25 kHz and acquired without filtering using the software MC_Rack (Multi Channel Systems, Reutlingen, Germany). The time of occurrence of spontaneous spikes was threshold-detected with MC_Rack (spikes are mostly downwards deflections, and the threshold was typically set at a signal amplitude that was 3 times below the average baseline noise). We did not perform spike sorting between RGCs recorded by the same electrode. Hence, the firing rate on each electrode reflects the overall activity level generated by all RGCs on that same electrode (typically 4-5 cells). Using the software MC_Data Tools (Multi Channel Systems, Germany), the times of spike occurrence were converted into text files for further analysis using the program SJEMEA written in R (Demas et al., 2003). When individual electrodes on the array were too noisy to allow reliable spike detection, they were removed from the analysis. The mean firing rate was estimated in 1 s bins for each electrode. Correlation indices were calculated between all pairs of electrodes on the array (Wong et al., 1993). The correlation index indicates how likely two electrodes are to detect a spike within a given window, normally 50 ms. Hence, for a propagating wave, correlation indices are higher for electrodes in
close vicinity to each other than for distal pairs. For a pair of spike trains that are uncorrelated, the correlation index will be close to 1.

2.4 Immunostaining

Eye cups were fixed for 1 hour at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) and cryoprotected overnight with 30% sucrose in 0.1 M PBS at 4 °C. Eyecups were frozen in OCT embedding medium and cryosectioned at 20µm. Sections were pre-blocked and incubated overnight at 4 °C in rabbit anti-GABA (1:50) (Chemicon, Temecula, CA) diluted in 0.1 M PBS containing 10 % normal goat serum, 5 % sucrose, and 0.3 % Triton X-100. Sections were then washed 3 times in 0.1M PBS for 30 min and incubated in CY3 donkey anti-rabbit (1:5:100) (Jackson ImmunoResearch), followed by 3 washes in 0.1M PBS for 30 min and finally they were mounted in Vectashield hard set with DAPI (Vector laboratories, Burlingame, CA). Samples were viewed at 40X magnification using a Leica (Nussloch, Germany) DMRA fluorescence microscope. All images were captured at the same exposure and gain with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) using MetaMorph.

2.5 Biocytin labeling

Biocytin dextran (20%) was injected in the optic nerve of freshly isolated eye cups (incubated overnight in oxygenated Ringer’s solution). Alternatively, Biocytin was injected in several locations in the retina with a broken micropipette and introduced into cells by electroporation (Axoporator, Axon Instruments) (20 V/200 ms bipolar pulses at 1Hz for 10 sec). Retinas were then fixed overnight at 4 °C in 1% paraformaldehyde in 0.1 M PBS at pH 7.4, washed 3 times in 0.1 M PBS for 20 min and permeabilized with 0.5 % Triton X-100 in 0.1 M PBS for 1 hour at room temperature. Retinas were then incubated in the Vecta-stain Avidin Biotinylated enzyme Complex (ABC, Vector laboratories, Burlingame, CA) diluted at 1:100 in 0.1 M PBS and 0.5 % Triton X-100 for 2 hours at room temperature. Retinas were then washed 3 times in
0.1 M PBS (pH 7.4) for 1 hour and 2 times in 50 mM TRIS-HCl (pH 7.4) for 10 minutes. Retinas were then preincubated in a 50 mM TRIS-HCl solution containing 0.1 % diaminobenzidine and 1 % CoCl$_2$ at pH 7.4 for 30 min at room temperature. 1% H$_2$O$_2$ solution was then added to the solution and the reaction was visually monitored under a dissecting microscope until the dark reaction product was sufficiently visible. The reaction was stopped by transferring the retinas into 50 mM TRIS-HCl. After 2 washes in 50mM TRIS-HCl for 20 min, the retinas were flattened on glass slides and left to dry overnight, washed in Histoclear 1&2 and mounted in Histomount solution (Invitrogen).

2.6 RGC dendritic arbor reconstruction
Labeled RGCs were traced using the neuron reconstruction software Neurolucida (MicroBrightField, Inc, Colchester, VT). Only RGCs that were well stained and sufficiently isolated from other labeled cells were used for drawings. By focusing at different planes through the tissue, all visible dendrites were traced to reconstruct the dendritic tree in two dimensions. The following dendritic parameters were calculated using Neuroexplorer (part of Neurolucida) and used for further analysis: 1) dendritic field area (a line was traced around the dendritic arbor, encompassing all most distal dendrites); 2) total number of dendritic branches; 3) total dendritic length (the length of all branches joined end-to-end); 4) the mean dendritic segment length (the total dendritic length divided by the total number of dendritic branches). To look at the profile of dendritic branching along the tree, we also used the Sholl analysis, calculating the total number of dendritic intersections crossing 10µm-spaced concentric circles centered on the soma.

2.7 Statistical analysis
Comparison between experimental groups was done using an Analysis of Variance (ANOVA) test, followed by Tukey’s multiple comparison post-hoc test with 95% confidence interval.
When comparing only two groups, we used unpaired 2 tailed t-tests with 95% confidence interval.

To look at changes in dendritic parameters, standard descriptive statistics (mean and standard error of the mean) were used to quantify the number of dendritic branches and the total dendritic length.

A linear regression fit was used to segregate between dendritic features (dendritic field area versus total number of dendritic branches or versus total dendritic length) in various experimental groups. Both the slope (m) and the goodness of fit ($r^2$) are given for each experimental group.
3. Results

3.1 Inhibition of GABA synthesis by L-allylglycine reduces retinal GABA expression and induces the re-emergence of spontaneous correlated activity

Before undertaking chronic experiments, we have tested the potency of L-allylglycine (2-4 mM) in acutely blocking GABA synthesis (Horton et al., 1978). To this aim, we have both looked at GABA expression in retinal sections and recorded spontaneous activity using Ca²⁺ imaging in neonatal retinas, once waves have been replaced by small patches of correlated activity due to GABA gradually shifting to becoming inhibitory (Sernagor et al., 2003). Figure 1 illustrates how effectively L-allylglycine depletes the retina from endogenous GABA.

GABA immunofluorescence was investigated 24h following incubation in L-allylglycine to ensure that enough time was allowed for the drug to exert its action (see next paragraph). Figure 1A illustrates GABA expression at 4 days PH following 24 hours incubation in normal Ringer’s solution. Staining is strong in amacrine cells somata in the inner part of the inner nuclear layer (INL) as well as in their dendritic processes in sublamina of the inner plexiform layer (IPL). Conversely, GABA immunofluorescence is almost absent following 24h incubation in L-allylglycine (2mM) (figure 1A, middle panel). Residual labeling can be seen in scattered somata in the inner part of the INL and in a single band in the upper portion of the IPL. These findings confirm that L-allylglycine is a potent pharmacological tool to induce retinal depletion of endogenous GABAergic stores.

RGC activity was monitored in 5 days PH retinas using Ca²⁺ imaging. Figure 1B shows calcium transients recorded from nine cells that were co-activated during one patch of spontaneous activity. The hotspot activity patterns that are characteristic of that period of development (Sernagor et al., 2003) are clearly illustrated in the relative onset plot for the same dataset (Figure 1C, left panel), showing sets of horizontal lines (patches) (see Methods for further explanation). We did not expect L-allylglycine to exert an immediate effect when added
to the bath because depletion of endogenous retinal GABA stores is a gradual process. Indeed, retinas kept exhibiting normal stationary patches of activity. However, when the same retinas were incubated in L-allylglycine (2 mM) overnight, on the next day they expressed much stronger spontaneous activity, including propagating waves (figure 1D, left panel). These effects were remarkably similar to those previously reported in the presence of bicuculline (Sernagor et al., 2003; see Figure 5). Cellular recruitment (CR) increased from 17.2±2.3% (n=12) (measured 2-3 hours after the initial exposure to L-allylglycine) to 53.9±12.1% (n=28) (p<0.0001; N=4 retinas) (figure 1C, right panel). These findings confirm that like previously shown for bicuculline, L-allylglycine efficiently reduces GABAergic inhibition in neonatal retinas, reverting stationary patches of activity to propagating waves.

We have also tested whether L-allylglycine is capable of acutely re-establishing correlated spontaneous activity in PH one month retinas (around the age chosen to test the effect of chronic exposure to the drug, see below) when normally RGCs no longer fire in synchrony (Sernagor et al., 2003). Following 24 hours incubation in 4mM L-allylglycine, 5 weeks old retinas exhibited spontaneous patches of correlated activity as well as propagating waves (figure 1D, right panel), with CR increasing to 39.2±11.5 % (compared with values obtained from control conditions in 4-weeks old retinas; p<0.0002, n=32, N=4 retinas) (figure 1C, right panel).

The above experiments were all performed in vitro, within 24 hours after initiating retinal exposure to L-allylglycine. To determine the optimal concentration of L-allylglycine in Elvax implants for chronic in ovo treatments, implants containing 400 mM, 600 mM and 1 M L-allylglycine were inserted in 1 week old hatchling eyes and left to exert their effect on retinal activity for one week. RGCs were subsequently labeled for Ca$^{2+}$ imaging to measure spontaneous activity levels. The effect of L-allylglycine was concentration-dependent, with 1M being the most efficient (figure 1E). Retinas treated with 1M L-allylglycine exhibited frequent
patches of activity with CR of 42±15.6% (n=10, N=3 retinas; figure 1E). The effect was weaker for 600 and 400 mM L-allylglycine (CR 36.3±5 %, n=10, N=2 retinas; p>0.05 for 600 mM; CR 27±3.7%, n=7, N=3 retinas; p<0.001 (compared with 1M L-allylglycine values) for 400 mM) (figure 1E). These effects were entirely due to L-allylglycine released from Elvax implants, as there was no L-allylglycine in the perfusate. We have used implants containing 1M L-allylglycine for subsequent chronic treatments.

Chronic L-allylglycine treatments where carried out from S24-25 and the effects were tested at 4-6 weeks PH. Immunolabeling performed at 6 weeks PH revealed that GABAergic stores were greatly reduced in L-allylglycine-treated retinas (figure 1F). GABA expression was virtually non-existent, except for few cell bodies in the INL (presumably amacrine cells) projecting to the upper edge of the OFF sublamina of the IPL (figure 1F), suggesting that a subset of GABAergic amacrine cells did not respond to the L-allylglycine treatment.

### 3.2 Chronic blockade of GABA<sub>A</sub> receptors causes dendritic branch loss

Chronic exposure to bicuculline causes waves to persist at 1 month PH (Leitch et al., 2005) although they normally disappear within the first postnatal month (Sernagor et al., 2003). We have investigated the effects of this treatment on dendritic arborization in 53 bicuculline-treated RGCs (N=11 retinas) and compared them to 50 control RGCs (N=7 retinas) aged 5-7 weeks PH (figures 2, 3). Although the main dendritic arbor structure of bicuculline-treated cells is similar in size and shape to normal, they exhibit a significant loss of high order branches (figures 2, 3).

To quantify differences in dendritic arbor structure between both conditions, the total number of dendritic branches, dendritic field area, total and average dendritic length were calculated for all pooled RGCs (Figure 3). An average decrease of 46.9% (p<0.001) in the total number of dendritic branches was observed (Figure 3A). As RGC dendritic peak growth and branching occurs around the start of treatment (S24-25) (Mehta and Sernagor, 2006a, b), this bicuculline-
induced effect must be due to overall dendritic loss. On the other hand, dendritic field areas were unaffected (figure 3B). As a result of overall dendritic branch loss, the total dendritic length decreased by 27.1% \((p<0.001)\) (Figure 3C). Finally, the mean dendritic segment length (total dendritic length divided by the total number of branches) significantly increased by 37% \((p<0.001)\) (figure 3D). This increase may originate from an increase in the ratio between long and short dendritic branches.

In order to compare equivalent samples between different retinas and to detect more subtle changes in dendritic parameters, all cells were classified according to their retinal eccentricity and dendritic field size. RGCs within the distal half between the optic disc and the periphery were considered as peripheral, and those situated in the proximal half were classified as proximal. For each experimental group, RGCs exhibiting dendritic field areas above median values (within that same group, e.g. Ctl, BIC, ALYL) were categorized as large field RGCs, and those with values below the median were categorized as small field RGCs. RGC subtypes from all quadrants were pooled for the analysis. The results are presented in Table 1. The results were in general similar to those reported for the pooled data, demonstrating that the effect of blocking GABA\(_A\) receptors impact dendritic growth across the retina and equally affect all RGC subtypes. Closer inspection revealed one small difference, namely that dendritic field areas were insignificantly larger in small field proximal and large field peripheral cells \((p>0.05)\) (Table 1). Hence, substantial dendritic elongation, especially in large-field peripheral cells, may contribute to the increase in mean dendritic length.

### 3.3 Effect of chronic depletion of endogenous GABA on RGC dendritic refinement

Forty two allylglycine-treated RGCs \((N=5\) retinas) from 6 weeks PH retinas were analyzed. Figure 2 shows the effect of GABA synthesis inhibition on RGC dendritic arbor structure. Overall, dendritic arbors shrink drastically, and at the same time, these shorter dendritic trunks exhibit numerous short branches. RGC dendritic trees are well developed and branched at the
start of the treatment (S24-25) (Mehta and Sernagor, 2006a,b), hence we can reasonably assume that it is the lack of retinal GABA that triggered dendritic retraction. Despite the presence of numerous small order branches, the total number of dendritic branches decreased by 29.8% compared to control cells ($p<0.001$) (figure 3A). This decrease was significantly less pronounced than in bicuculline-treated cells ($p<0.001$) (figure 3A). Dendritic field areas shrank dramatically, with an average decrease of 56.3% ($p<0.001$) (figure 3B). This appears to result from the collapse of the main structure of the tree as well as from dendritic retraction. The total dendritic length was reduced by 51%, significantly more than in bicuculline-treated cells ($p<0.001$) (figure 3C). Dendritic retraction resulted in a significant decrease of 30.9% ($p<0.001$) in the mean dendritic length (figure 3D). Inspection of RGCs according to their subtype classification (Table 1) revealed no further hidden trend, once again demonstrating that the effect of depleting the retina of GABA has global effects on RGC dendritic growth and remodeling.

3.4 Comparison of the effect of bicuculline and L-allylglycine treatments on RGC dendritic arbor structure

Both bicuculline and L-allylglycine cause dendritic loss, although it is much less pronounced in the latter, especially in view of the overall shrinkage of the entire tree. The differences in dendritic elongation and branching patterns between the three experimental groups are emphasized in the dendrograms of three example cells in Figure 4. The bicuculline-treated cell has fewer and longer dendrites, covering a wider field around the soma. The allylglycine-treated cell, on the other hand, has a small tree with many very small dendritic appendages. It has the highest density of branches on the tree. Figure 5A shows the number of dendritic branches as a function of their order in the tree for all RGCs, revealing the extent of dendritic loss. The profile of dendritic organization is bell-shaped for control RGCs, with a peak around order 10. Both bicuculline- and L-allylglycine-treated cells exhibit a smaller and narrower
distribution. The decrease in peak values is attributed to an overall branch loss, whilst the loss of the right tail of the distribution (slightly more pronounced for bicuculline-treated RGCs) suggests that the loss is specific to high-order branches. Indeed, for both groups, there is no change in the distribution of proximal dendrites for the treated cells, whilst there are fewer branches from dendritic order 5 and above, towards distal parts of the tree.

Figure 5B shows the spatial distribution of dendritic branches across the tree (Sholl analysis), illustrating the number of branches as a function of eccentricity from the soma. The dendritic distribution in control RGCs is bell-shaped with a peak around 180 µm from the soma. Bicuculline-treated cells exhibit a similar distribution with a peak around the same eccentricity, but with fewer dendrites. Surprisingly, bicuculline-treated cells branch further away from the soma than control ($p<0.001$, Two way ANOVA). This argues for an elongation of the main structure of the tree as suggested by the small increase in dendritic field areas (Table 1). For L-allylglycine-treated cells, the extent of the distribution is smaller, with fewer branches than control from about 60µm away from the soma, their number peaking at about 100µm eccentricity, and a maximal spatial extent of 350µm attributed to dendritic retraction.

Plotting dendritic field areas as a function of the total number of dendritic branches for all RGCs clearly illustrates the overall effect of the treatments (figure 5C). The three groups (control, bicuculline- and allylglycine-treated cells) are segregated from each other, following a positive correlation between both parameters. The bicuculline population has a steeper slope ($m=1,387 \ \mu m^2$/branch, $r^2=0.7485$; linear regression fit) than control ($m=448.8 \ \mu m^2$/branch, $r^2=0.4322$) attributed to fewer dendritic branches, while dendritic areas remain either similar or occasionally larger than normal. Indeed, RGCs with the largest dendritic areas belong to this group. The L-allylglycine population slope is slightly smaller than control ($m=381 \ \mu m^2$/branch, $r^2=0.6310$), but with lower values because of the decrease in dendritic field areas. Moreover, values are clustered to the left because of dendritic branch loss. RGCs with the smallest
dendritic areas do indeed belong to this population. Plotting dendritic field areas as a function
of the total dendritic length reveals a steeper slope for the bicuculline population (m=24.62 μm,
$r^2=0.7574$ compared to $m=14.86 \mu m$, $r^2=0.5642$ for control) with a compression towards
shorter dendritic lengths for equivalent or larger dendritic areas than normal (figure 5D). The
L-allylglycine population is clustered in the bottom left part of the plot due to both dendritic
areas and length decrease ($m=17.95 \mu m$, $r^2=0.8861$). The largest bicuculline-treated cells have
an equivalent dendritic length to the largest control cells although they have fewer dendritic
branches, indicating dendritic elongation in these cells. On the other hand, L-allylglycine-
treated cells have the shortest dendritic lengths.

3.5 Chronic inhibition of GABA synthesis by L-allylglycine prevents the disappearance of
embryonic correlated spontaneous activity

When GABA$_A$ receptors are chronically blocked with bicuculline from S24-25 (just before
GABA starts shifting from excitation to inhibition (Sernagor et al., 2003)) until one month PH,
correlated spontaneous activity persists and GABA does not switch to inhibition (Leitch et al.,
2005). We found that chronic exposure to L-allylglycine during the same developmental period
has a similar effect on global retinal activity (Figure 6). MEA recordings from the RGC layer
revealed that retinas that had been chronically exposed to L-allylglycine from S24 until one
month PH still exhibited spontaneous waves, just like following chronic exposure to
bicuculline (Leitch et al., 2005). Figure 6A (top panel) illustrates the raster plot of a
spontaneous wave recorded in a chronically treated retina freshly excised from a 4 week-old
hatchling (there is no L-allylglycine in the perfusate). The bottom panel of Figure 6A shows
the raster plot of spontaneous activity recorded from the contralateral control eye (there was no
Elvax implant in that eye) of the same animal. Waves were never detected in control conditions
at that age (see also Sernagor et al., 2003). To estimate CR (for comparison with our previous
findings obtained with Ca$^{2+}$ imaging on retinas chronically treated with bicuculline, where we
calculated the CR based on Ca\textsuperscript{2+} transients from individual labeled RGCs in 5s bins (see Methods), we measured how many electrodes recording from treated retinas fired above the average control level (from untreated retinas). This was done in 2 animals, where one eye was chronically exposed to L-allylglycine from S24 until one month PH and the other eye remained intact. The average firing level (calculated in 0.5s bins) was first calculated for the control eye. We then counted how many electrodes fired on average above that level, both for the control and the treated eye. Figure 6B shows that for both animals, most electrodes recording from treated retinas fired above the average control firing rate (96.5±3.4%, N=59 electrodes in total; p<0.0096, 2-tailed t test). These observations are proportionally very similar to those we have reported for bicuculline-treated eyes using Ca\textsuperscript{2+} imaging (Leitch et al., 2005). Figures 6E and F illustrate the overall spontaneous firing rate averaged across all electrodes during several minutes for an L-allylglycine treated retina (E) and its control counterpart (F). The L-allylglycine-treated retina reveals 5 global episodes of strong activity whereas the control retina does not exhibit global periodic bursting. Moreover, bursting was synchronized between neighboring electrodes in that same L-allylglycine-treated retina, as shown by correlation index plots (figure 6G). The degree of correlation was high between adjacent electrodes and decreased progressively with distance, suggesting wave propagation. In contrast, in the control eye there was virtually no correlation (figure 6H).

Ca\textsuperscript{2+} imaging was performed on another set of chronic animals at 6 weeks PH. Figure 6C shows that these retinas expressed large patches of synchronized activity, with an average CR of 13.6±7.9 % (n=17, N=6 retinas), which was significantly higher than in PH 4 weeks control retinas (p<0.0001). Finally, GABA puffs (100 µM) in the vicinity of the tissue elicited large, slowly propagating and long lasting Ca\textsuperscript{2+} transients in 5 retinas (N=6) (Figure 6D). Similar events have been reported in retinas harvested during perinatal weeks (Sernagor et al., 2003) and also in one month-old bicuculline-treated retinas (Leitch et al., 2005), and they most
probably reflect immature, excitatory GABAergic responses (see Discussion). Overall, these results demonstrate that when endogenous GABA is reduced, spontaneous correlated activity persists beyond normal and GABA responses remain immature.
4. Discussion

We have shown for the first time that disrupting GABAergic signaling has profound effects on dendritic growth in the developing retina. Chronic blockade of GABA$_A$ receptors with bicuculline induces dendritic branch loss without global changes in the extent of dendritic fields whilst chronic blockade of GABA synthesis with L-allylglycine causes the entire tree to shrink. Moreover, depleting retinal GABAergic stores with L-allylglycine prevents the disappearance of embryonic correlated activity and the maturation of GABAergic inhibition. Just as in bicuculline-treated (Leitch et al., 2005) and dark-reared (Sernagor et al., 2003) retinas, GABA puffs induced large Ca$^{2+}$ transients in retinas that have been depleted of endogenous GABA. Similar responses have been observed in young spinal neurons (Cordero-Erausquin et al., 2005) and in adult hippocampus (Staley et al., 1995) where they have been attributed to large Cl$^-$ influxes that induce a collapse of the driving force for Cl$^-$, unmasking a depolarizing bicarbonate efflux through GABA$_A$ receptors that ultimately leads to an increase in extracellular K$^+$(Kaila et al., 1997; Smirnov et al., 1999).

4.1 Chronic GABA$_A$ receptors blockade

RGC dendrites develop while the retina generates spontaneous correlated activity (Sernagor et al., 2001). Chronic GABA$_A$ receptors blockade with bicuculline was started at S24-25, around the time of peak dendritic growth and branching in RGCs (Mehta and Sernagor, 2006a,b), coinciding with the time GABA begins to modulate retinal waves, a few days before GABAergic inhibition starts to down-regulate spontaneous correlated activity (Sernagor et al., 2003). We have previously shown that chronic blockade of GABA$_A$ receptors (Leitch et al., 2005) as well as dark-rearing (Sernagor et al., 2003) prevent the disappearance of correlated spontaneous activity. Dark-rearing promotes dendritic proliferation by inducing an increase in dendritic elongation and branching in large RGCs (Mehta and Sernagor, 2006a). This effect is
blocked by chronic exposure to the cholinergic antagonist curare (Mehta and Sernagor, 2006a) that blocks the generation of spontaneous correlated activity (Sernagor and Grzywacz, 1996, 1999). A similar need for neural activity in promoting dendritic growth has been reported in the developing hippocampus (Groc et al., 2002). However, here we have demonstrated that RGCs undergo dendritic loss rather than addition of new branches (as they do as a result of dark-rearing) when GABA_A receptors are blocked, and this occurs despite the persistence of correlated spontaneous activity.

GABA_A activity has long been known to promote neurite outgrowth in neonatal neurons in vitro (Barbin et al., 1993; Represa and Ben-Ari, 2005; Ben-Ari et al., 2007; Wang and Kriegstein, 2009; Sernagor et al., 2010). Indeed, early GABAergic signaling generates membrane depolarization and induces Ca^{2+} transient influx that is necessary for dendritic growth and branching. Studies on neonatal cortical neurons (Cancedda et al., 2007; Wang and Kriegstein, 2008) and hippocampal adult neurogenesis (Ge et al., 2006) have confirmed the neuritogenic effect of excitatory GABA_A transmission in vivo. Indeed, turning GABAergic excitation into inhibition by manipulating Cl\(^-\) transport activity during the period of dendritic growth strongly suppresses further growth. In agreement with our findings, a study on frog optic tectum has shown that when GABAergic synaptic activity is reduced by modifying the γ2 subunit of GABA_A receptors, tectal neurons have more sparsely branched dendritic arbors that expand over larger areas (Shen et al., 2009).

It has been reported in cultures that spontaneous cholinergic nicotinic activity triggers the GABAergic shift from excitation to inhibition through KCC2 upregulation, resulting in dendritic pruning (Liu et al., 2006). Once again, this confirms that depolarizing GABA has a neuritogenic effect and the link to cholinergic nicotinic signalling may be of particular relevance to the retina because retinal waves are generated through cholinergic nicotinic signaling (Feller et al., 1996; Sernagor et al., 1996, 2003). In turtle, RGC dendritic pruning
starts precisely when cholinergic retinal waves break down (at S25, see Mehta and Sernagor, 2006a) and GABA shifts to inhibition (Sernagor et al., 2003).

Importantly, in this study we have shown that GABA<sub>A</sub> activity continues to be involved in dendritic development and remodeling during late embryonic and early postnatal stages. The effect is complex and cannot be explained only by global network activity levels or patterns, as other conditions (dark-rearing, GABA synthesis blockade) yielding similar activity patterns have a different impact on dendritic growth. As shown by the dendritic order plots, only high order dendrites are affected, leaving the main structure of the tree and the dendritic area intact.

On the other hand, increased dendritic elongation as reported in subgroups of bicuculline-treated RGCs has also been observed in large RGCs from dark-reared retinas (Mehta and Sernagor, 2006a), and may therefore be due to enhanced global neural activity.

The role of GABA<sub>A</sub> signaling in dendritic development may involve specific mechanisms rather than mere membrane depolarization. Specific effects of neurotransmission on RGC dendritic refinement have been reported in a previous study (Lohmann et al., 2002), showing that while blockade of global activity does not affect the structure of the dendritic tree, inhibition of local Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in well-developed trees induces retraction of distal, but not proximal dendrites. Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release mostly originates from local amacrine cholinergic nicotinic transmission (responsible for wave generation). As immature amacrine cells co-release acetylcholine and GABA (Zheng et al., 2004), depolarizing GABA may participate in local Ca<sup>2+</sup> signaling, hence influencing dendritic stabilization. Indeed, it has been proposed that the neuritogenic effect of GABA<sub>A</sub> activity is due to its ability to stabilize newly formed dendritic segments (Gascon et al., 2006). This would explain why, in our model, cholinergic nicotinic driven activity in the presence of GABAergic activity (during dark-rearing), but not in its absence, promotes dendritic branching. Hence, in our model, when GABA<sub>A</sub> receptors are blocked chronically during the period of spontaneous cholinergic
activity, dendritic growth and branching is arrested prematurely. On the other hand, when
spontaneous cholinergic and depolarizing GABAergic activities are both prolonged in dark-
reared turtles, dendrites continue to grow and branch, resulting in larger trees with significantly
more dendrites (Mehta and Sernagor, 2006a).

4.2 Chronic inhibition of GABA synthesis

We have demonstrated that removing endogenous GABA has similar effects (both acutely and
chronically) on global network activity as previously shown for GABA\textsubscript{A} receptor blockade
(Leitch et al., 2005). However, the effects on dendritic growth are profoundly different.
The main structure of the dendritic tree has retracted and high order branches have
disappeared. Dendritic loss may be due to the overall absence of GABAergic signaling
following the depletion of retinal GABA content as shown with immunolabeling. Interestingly,
even if they experience substantial dendritic loss compared to control, L-allylglycine-treated
RGCs have significantly more dendritic branches than bicuculline-treated RGCs. In fact, L-
allylglycine-treated RGCs exhibit a loss of “middle-order” dendritic branches similar to
bicuculline-treated RGCs, but they have numerous dendrites branching high up in the tree.
Moreover, we noticed the presence of very small branches covering the entire tree.
Unfortunately, due to their very small size we were not able to visualize them with sufficient
accuracy for quantification. Nonetheless, these numerous high-order branches suggest that L-
allylglycine-treated RGCs may compensate for the retraction of their dendritic trees by
generating additional branches. Indeed, it is known that developing dendritic trees keep their
global length stable by equilibrating addition and retraction of dendritic filopodia (Wong et al.,
2000). As for the addition of high-order branches, the collapse and retraction of the dendritic
tree is L-allylglycine-specific. High-order branches have strongly retracted and primary and
secondary branches have come closer together, so that the dendritic tree is compressed and does not cover the periphery of the cell to a smaller field than normal.

How can we explain that bicuculline and L-allylglycine have such different effects on dendritic growth while their impact on global retinal network excitability looks similar (and both look similar to activity patterns reported in dark-reared turtles (Sernagor et al., 2003))? One possibility is that the activity patterns are actually not identical in these experimental groups, but that the differences are too subtle to be detected with our recording approaches. Indeed, the field of view is small with Ca\(^{2+}\) imaging (approximately 0.25 mm\(^2\)), hence it is not possible to record simultaneously from large retinal areas. Moreover, Ca\(^{2+}\) transients are slow and do not allow us to characterize firing patterns accurately. Perhaps there are differences in propagation patterns at the pan-retinal scale, and/or in RGC bursting properties in these experimental groups. In this study we have also used MEAs to record global network activity from retinas chronically treated with allylglycine, allowing us to record from larger retinal areas (2.56 mm\(^2\)), albeit at much lower spatial resolution (200 μm separation between the electrodes). Our MEA recordings indicate that waves are still present in these retinas chronically treated with allylglycine, although Ca\(^{2+}\) imaging from retinas that have undergone a similar treatment do reveal large stationary patches of activity rather than propagating waves (see Figure 6). These differences allude to the limitations imposed by different recording approaches and therefore conclusions regarding network activity patterns in the different experimental groups (including dark-rearing in our previous studies) must be drawn with caution. Future studies should endeavor to use a large scale, high resolution recording approach such as the Active Pixel Sensor MEA that allows simultaneous near-cellular resolution recording from 4096 channels encompassing an area of 7.12 mm\(^2\) (Berdondini et al., 2009). Nevertheless, in our hands,
activity patterns recorded from these different experimental groups with Ca\textsuperscript{2+} imaging look similar; hence at this point we cannot draw additional conclusions about this important issue.

The fact that L-allylglycine-treatment affects the entire dendritic tree argues for a more global, possibly paracrine, bicuculline-insensitive GABAergic action. During development, before becoming transmitted phasically by developing synapses, ambient GABA is important for tonic activation of developing neurons (Ben-Ari, 2002). Such paracrine activity may not be reflected in synaptic network function (e.g. in networks involved in the generation and modulation of retinal waves). Some studies suggest that ambient concentration of GABA produced by local inhibitory networks can serve as an integrative signal in adult neurogenesis (Ge et al., 2006; Bortone and Polleux, 2009). Hence, GABA may play a similar role in the retina by directing dendritic growth towards synaptic partners, allowing activity-dependent dendritic growth. The exact nature of the GABAergic pathway involved in this mechanism remains to be determined.

It is possible that it requires extra-synaptic GABA\textsubscript{A} receptors (Wang et al., 2007) with low affinity for bicuculline (Farrant and Nusser, 2005) or different types of GABAergic receptors such as bicuculline-insensitive GABA\textsubscript{C} receptors (Zhang and Slaughter, 1995). Functional GABA\textsubscript{B} receptors are also found in the developing retina, although their activation is known to downregulate embryonic Ca\textsuperscript{2+} transients (Catsicas and Mobbs, 2001). Interestingly, GABA has been shown to act as a chemo-attractant, promoting neuronal migration through the activation of GABA\textsubscript{B} receptors \textit{in vitro} (Behar et al., 2001).

Embryonic neural activity plays a crucial role in the formation of synaptic circuits. Afferent neurotransmission drives dendritic growth and instructively refines synaptic connections. Our model allows local pharmacological blockade of GABAergic activity \textit{in ovo} without directly affecting the overall activity level in the brain. Here we have demonstrated that in the turtle retina, GABA is a major contributor to dendritic development, and that it may act independently of global retinal network activity generated in concert with other excitatory
neurotransmitters. Depolarizing GABA$_A$ activity allows the formation of new dendritic branches and GABA is required at a more general level to maintain the structure of the developing tree.
Acknowledgements

This work was supported by the Anatomical Society of Great Britain and Ireland and by a grant from the Engineering and Physical Sciences Research Council (EP/E002331/1). We thank members of the CARMEN (Code Analysis, Repository & Modelling for e-Neuroscience) consortium for providing infrastructure for data sharing. SJEMEA code for MEA data analysis is freely available at http://www.damtp.cam.ac.uk/user/sje30/. We thank Christopher Adams for his technical assistance with MEA recordings, David Cox and Claudia Racca for their help with immunolabeling and biocytin protocols and Matthias Hennig for his comments on the manuscript.
Author contributions

F.C. designed and performed all experiments and analyzed the data except for the MEA experiments and contributed to writing the manuscript. S.J.E. wrote the codes to analyze the Ca$^{2+}$ imaging and MEA data and contributed to data analysis. E.S. designed the experiments, performed Ca$^{2+}$ and MEA recordings and analyzed MEA data and finalized the manuscript preparation.

All authors have seen and approved the final manuscript.
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Figures captions

Figure 1: L-allylglycine depletes GABA stores from the retina and induces the reemergence of spontaneous correlated activity in RGCs. A: GABA immunolabeling reveals that 24 hours of L-allylglycine treatment in vitro greatly reduces retinal GABA content. In control retinas, GABA immunofluorescence is found in cell bodies in the lower part of the INL and in IPL sublaminas. In L-allylglycine-treated retinas, only few GABA-expressing cells are detected. Scale bar: 50µm. NPA: no primary antibody. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Allyl, L-allylglycine; ctl, control. B: Spontaneous Ca\(^{2+}\) transients recorded from 9 RGCs in a 5 days old retina. The traces show the changes in fluorescence compared to baseline. C: Left panel: relative onset plot for an episode of spontaneous activity recorded from the same 5 days post-hatching (PH5d) retina as in Panel B. Right panel: cellular recruitment (CR) in various experimental groups. Columns are mean values with S.E.M. bars. There is a significant increase in CR in retinas treated with L-allylglycine. D: Relative onset plots of spontaneous activity recorded with Ca\(^{2+}\) imaging from PH5d and 5 weeks (PH5w) retinas incubated during 24 hours in 2 and 4 mM L-allylglycine respectively. Waves reappear, as indicated by a linear increase of onset times with distance from the reference cell. E: Relative onset plots of spontaneous activity recorded with Ca\(^{2+}\) imaging following chronic treatments with increasing concentrations of L-allylglycine in Elvax implants. Rightmost panel: CR during activity recorded with increasing L-allylglycine concentrations in the implant. Columns are mean values with S.E.M. bars. Horizontal bar indicates Tukey post-test between 400 mM and 1 M. F: GABA immunolabeling in 6 weeks old retinas (PH6w) is present in cell bodies in the lower part of the INL and in IPL sublaminas. There is only weak residual staining in age-matched retinas treated with L-allylglycine from S24. Scale bar: 50µm.
Figure 2: The effect of chronic bicuculline and L-allylglycine treatments on RGC dendritic arbor structure. Examples of reconstructed RGCs labeled with biocytin dextran from control retinas, from 6-9 weeks PH bicuculline-treated and 6-7 weeks PH L-allylglycine-treated retinas. Scale bar: 100µm.

Figure 3: Quantitative analysis of dendritic parameters from control (Ctl), bicuculline- (Bic) and L-allylglycine (Allyl)-treated RGCs summarizing results over different classes of RGCs. A, B, C and D respectively illustrate the total number (mean and S.E.M) of dendritic branches, dendritic field areas, total dendritic length and mean dendritic segment length for all three experimental groups. Statistical significance (p and asterisks) above columns relate to Tukey post-hoc test compared to control values. Horizontal lines relate to Tukey post-tests comparing Bic and Allyl values.

Figure 4: Examples of three reconstructed RGCs (control, chronic bicuculline and L-allylglycine) with their corresponding dendrograms (primary dendrites to left). For the dendrograms, increasing distances from the soma to the periphery is from left to right. Each first order dendrite and its corresponding tree are represented in a different color. Brackets represent branching points and the length of each individual dendrite is reflected by the length of the corresponding line between brackets. For each cell, the numbers above the dendrogram indicate the total number of branches, the total dendritic length and the average dendritic length (in parentheses) and the total dendritic field area. Scale bar: 100µm.

Figure 5: Bicuculline and L-allylglycine-treatments have contrasting effects on the structure of RGC dendritic trees. A: Dendritic order shown as line plots of the number of dendritic branches as a function of their order in the tree. Blue, control cells; red, bicuculline treated cells; green, L-allylglycine treated cells. B: Sholl analysis shown as line plots of the number of dendritic intersections crossing equally spaced (every 10µm) concentric circles centred at the soma. Plot conventions as for A. C: Scatter plots of dendritic field areas as a function of the
total number of dendritic branches for pooled RGCs for each experimental condition. Straight lines represent the linear regression fit for each experimental group. Colors are the same as for A and B. D: Scatter plots of dendritic field areas as a function of the total dendritic length. Graph conventions as for C.

**Figure 6:** Chronic depletion of retinal GABA content prevents the disappearance of spontaneous correlated activity. A: Raster plots of spontaneous activity recorded from both eyes of a one month old hatchling. The top plot shows a wave of activity spreading across the retina excised from the eye that had been exposed to L-allylglycine from S24. The bottom plot shows the activity recorded from the control retina. No correlated activity is detected. B: Plot of the percentage of electrodes on the MEA firing above average control rate calculated in 2 animals. Symbols are mean values with S.E.M. bars. ** indicates \( p < 0.0096 \) (2-tailed t test). C: Relative onset plots of spontaneous activity recorded with Ca\(^{2+}\) imaging in PH6w retinas chronically treated with 1M L-allylglycine from S24. D: example of a GABA-evoked wave in a PH6w retina chronically treated with L-allylglycine from S24. The trace represents the fluorescence intensity over time averaged from all RGCs taken into consideration. Arrow indicates the time of GABA addition to the chamber. E: Average firing rate from all recording electrodes in the MEA (global firing rate) shows periodic bursts of spikes in an L-allylglycine-treated retina (4 weeks PH). F: Same as E, but for the control eye from the same animal. The inset box illustrates the average firing rate of episodes of activity recorded from the L-allylglycine-treated and control retinas from 2 animals. G: Correlation index plot illustrated as scatter plots for all values (black symbols) as well as mean values and S.E.M. (red symbols) for the L-allylglycine treated retina, showing high correlation index values for adjacent electrodes. H: Correlation index plot for the normal retina showing low values for adjacent electrodes.

**Table 1:** Summary of dendritic parameters for all analyzed RGCs from bicuculline-, L-allylglycine-treated and control retinas. The results are presented separately for each group of
RGCs, classified according to their dendritic field size and retinal eccentricity. \( n \) is the number of RGCs. Values are expressed as mean±S.E.M. \( p \) indicates the significance level (Tukey post-test). NS: non-significant. Ctl: Control. BIC: bicuculline. ALLYL: L-allylglycine.
Figure
Click here to download Figure: Chabrol Fig2.eps

Control

Bicuculline

L-allylglycine
control

bicuculline

allylglycine

Figure
Click here to download Figure: Chabrol Fig 4.eps
Figure

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Note: The table represents statistical data comparing the effects of different treatments (Ctl, BIC, ALLYL) on the total number of dendrites, dendritic field area, and total dendritic length. The p-values indicate statistical significance with *p < 0.05*, **p < 0.01**, and ***p < 0.001*** indicating levels of significance.