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# GABAergic CONTROL OF RETINAL GANGLION CELL DENDRITIC DEVELOPMENT

F. P. CHABROL,[a] S. J. EGLEN[b] AND E. SERNAGOR[a]*

[a] *Institute of Neuroscience, Faculty of Medical Sciences, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK*

[b] *Cambridge Computational Biology Institute, Centre for Mathematical Sciences, University of Cambridge, Wilberforce Road, Cambridge CB3 0WA, UK*

**Abstract—Developing GABAergic neurons mature long before excitatory neurons, and early $GABA_A$ activity exerts important paracrine effects while neurons extend dendrites and axons and they establish neural connections. One of the unique features of early $GABA_A$ 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 $GABA_A$ signaling 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 $GABA_A$ 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 while 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.** 

**Key words: retina, retinal wave, GABA, dendrite, multielectrode array, glutamic acid decarboxylase.**

## 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, 2012; Wang and Kriegstein, 2009; Sernagor et al., 2010). 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 lamelipodia (Gascon et al., 2006). However, $Ca^{2+}$-induced signaling is a common feature of synaptic activity-driven dendritic development (Konur and Ghosh, 2004; Chen 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 *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 retinal ganglion cells (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 toward 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, while

*Corresponding author. Tel: +44-191-2228541; fax: +44-191-2225227.
E-mail address: Evelyne.sernagor@ncl.ac.uk (E. Sernagor).

*Abbreviations:* Allyl, L-allylglycine; ANOVA, analysis of variance; Bic, bicuculline; CR, cellular recruitment; Ctl, control; INL, inner nuclear layer; IPL, inner plexiform layer; PBS, phosphate-buffered solution; PH, post-hatching; RGC, retinal ganglion cell.

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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 $GABA_A$ receptor 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.

## 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.

### Preparation and implantation of Elvax 40W

Elvax beads (duPont Chemicals) were washed in 100% ethanol for 3–4 days at −20 °C and subsequently dissolved in methylene chloride. 10 μL of either the $GABA_A$ receptor antagonist bicuculline (10 mM, see Leitch et al., 2005) or the glutamic acid decarboxylase inhibitor L-allylglycine (Horton et al., 1978) solution (1 M, see Results) was 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).

### Calcium imaging

RGCs were back-labeled with 20% $Ca^{2+}$ 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; $MgCl_2$, 2; $NaHCO_3$, 31.5; glucose, 10; Hepes, 10 and $CaCl_2$, 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 50 μL of a 5 mM solution, resulting in an approximate concentration of 100 μM 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.

### 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, Reutlingen, Germany) (Hennig et al., 2009, 2011). 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 8 × 8 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 1200) and acquired using a 128-channel analog 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 downward 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