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The role of activity in the development of inhibition in the mouse retina

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The role of activity in the development of inhibition in the mouse retina

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

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2009
The Dissertation of William Benjamin Barkis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009
This work is dedicated to my parents,

whose love and support have made this possible.
We work in the dark – we do what we can – we give what we have. Our doubt is our passion and our passion is our task.

Henry James
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ABSTRACT OF THE DISSERTATION

The role of activity in the development of inhibition in the mouse retina

by

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Neural circuits rely on the interaction between excitation and inhibition to process information and require an appropriate balance between the two to avoid pathological consequences. Gamma-aminobutyric acid (GABA) is the principal neurotransmitter mediating inhibition in the adult central nervous system (CNS). However, during development GABAergic transmission is excitatory. Understanding the transition from excitatory to inhibitory GABA provides insight into the development of mature brain function as well as devising treatments for diseases involving imbalanced excitation and inhibition.
A leading hypothesis is that neural activity plays a critical role in the transition from excitatory to inhibitory GABAergic transmission. Here I studied the role of neural activity in regulating the timing of the GABA switch in neurons in the ganglion cell layer of the mouse retina. I used three different knockout mice to demonstrate that the timing of the GABA switch is independent of nicotinic cholinergic activity, retinal waves, and glutamatergic activity.

To further explore the role of activity in regulating the GABA switch, I developed a retinal explant model system in which neural activity could be pharmacologically manipulated. Blockade of GABA receptor-mediated activity did not prevent the transition from excitatory to inhibitory GABA, in contrast to previous observations in hippocampus and retina. Further, sustained blockade of glutamate receptors, GABA receptors, nicotinic acetylcholine receptors, glycine receptors, voltage-gated sodium channels, gap junctions, and L-type voltage-gated calcium channels (VGCCs) did not alter the transition. These results suggest that neuronal activity is not required for the transition from excitatory to inhibitory GABA in the mouse retina.

To determine if the GABA switch is independent of signaling from other cell types, I cultured purified retinal ganglion cells (RGCs) in isolation. I found that purified RGCs did not develop an inhibitory response to GABA. These results indicate that unlike other parts of the nervous system, the transition from excitatory to inhibitory GABA signaling in mouse RGCs does not depend on activity but requires activity-independent signaling from another cell type.
I. Introduction

The action of GABA switches from excitatory to inhibitory during development

Though the amino acid gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mature central nervous system (CNS), it is a robust source of excitation during development (Owens and Kriegstein, 2002). Activating GABA$_A$ receptors (GABA$_A$Rs) on immature neurons leads to excitation through depolarization while activating GABA$_A$Rs in mature neurons leads to inhibition through hyperpolarization. This shift from an excitatory to an inhibitory action of GABA has been observed in many vertebrate species and in a number of different brain areas (reviewed in Ben-Ari, 2002).

Although GABA$_A$ receptors are ion channels permeable to both chloride and bicarbonate, intracellular chloride concentrations ([Cl$^{-}$]) predominantly determines whether the activation of GABA$_A$Rs is depolarizing or hyperpolarizing (Reichling et al., 1994; Owens et al., 1996; Rohrbough and Spitzer, 1996; Farrant and Kaila, 2007). Indeed, it is estimated that a change in the concentration of internal chloride of approximately 12 mM is sufficient to shift the polarity of the actions of GABA between excitation and inhibition (Staley and Smith, 2001).

Intracellular chloride concentrations are established primarily by the cation chloride cotransporter proteins encoded by the Slc12a1-9 gene family (reviewed in Delpire, 2000; Blaesse et al., 2009). These proteins form three functional groups: two are Na-K-2Cl cotransporters (NKCC1 and NKCC2), one is a Na-Cl cotransporter (NCC), and four are K-Cl cotransporters (KCC1-KCC4). The functions of the
proteins encoded by the two remaining genes, *Slc12a8* and *Slc12a9*, are currently unknown. The cotransporters that are expressed in the nervous system consist of two functional types: NKCC1 accumulates intracellular chloride by using the sodium and potassium gradients to drive in two Cl\(^{-}\) ions with one sodium and one potassium ion, establishing an \(E_{\text{Cl}}\) more positive than the resting membrane potential (\(V_{\text{rest}}\)) and KCC1-KCC4 which extrude chloride using the potassium gradient to drive chloride out of the cells, lowering \([\text{Cl}]_i\) and establishing \(E_{\text{Cl}}\) more positive than the \(V_{\text{rest}}\) (reviewed in Delpire, 2000; Blaesse et al., 2009). NKCC2 and NCC are expressed predominantly in the kidney and not in the nervous system.

Two cation chloride cotransporters, NKCC1 and KCC2, have been implicated in mediating the excitatory to inhibitory switch in action of GABA during development. NKCC1 is the predominant isoform expressed in developing neurons and is down-regulated during development (Plotkin et al., 1997). In early postnatal rat neocortical neurons, NKCC1 mRNA expression correlates positively with high \([\text{Cl}]_i\) and a GABA reversal potential (\(E_{\text{GABA}}\)) that was depolarizing. Further, blockade with the NKCC1-selective antagonist bumetanide induces a shift in the \(E_{\text{GABA}}\) of approximately -15 mV in early postnatal neurons that expressed mRNA for NKCC1 while having no effect on \(E_{\text{GABA}}\) in late postnatal neurons that expressed mRNA for KCC2 (Yamada et al., 2004).

Several observations indicate that KCC2 is also a key player in the developmental switch from GABA mediated excitation to inhibition. KCC2 is the principal transporter for chloride extrusion in neurons and while KCC1, KCC3,
KCC4 have all been found in the CNS, they have limited expression in neurons (reviewed in Payne et al., 2003). KCC2 is up-regulated late in development causing a shift in $E_{\text{GABA}}$ to more negative potentials (reviewed in Ben-Ari, 2002). KCC2 knockout mice (Hubner et al., 2001) and KCC2 knockdown through RNA interference (Rivera et al., 1999) show an extended excitatory response to GABA. In addition, KCC2 knockout mice exhibit hyperexcitability in juvenile hippocampal slices (Woo et al., 2002) indicating that delaying the hyperpolarizing action of GABA affects network function. Over-expression of KCC2 expression in hippocampal (Fiumelli et al., 2005) or cortical neurons (Lee et al., 2005) induces an early shift to an inhibitory response to GABA. Hence, expression of KCC2 is necessary and sufficient for the maturation of GABAergic inhibition.

**Regulation of KCC2 expression by neuronal activity**

There is evidence in both mature and immature neurons that activity regulates KCC2 function. In mature neurons, coincident pre- and postsynaptic stimulation, tetanic stimulation, repeated postsynaptic spiking, rebound bursting following hyperpolarizing current injection, and epileptic activity lead to depolarizing GABAergic transmission that persists for tens of minutes (reviewed in Fiumelli and Woodin, 2007). There are several lines of evidence that indicate this acute shift in $E_{\text{GABA}}$ is mediated by changing KCC2 function. First, this activity-dependent shift in the action of GABA is prevented by pharmacological blockade of KCC2. Second, the positive shift in $E_{\text{GABA}}$ was larger in mature neurons that express KCC2 at high levels. Third, young neurons that expressed KCC2 at low levels did not have this effect.
Exogenous expression of KCC2 in young neurons showed a shift comparable to that of mature neurons, indicating that this acute effect was mediated by KCC2.

How does activity modulate KCC2 function in mature neurons? The activity-dependent shift in $E_{GABA}$ in adult was prevented by buffering intracellular Ca$^{2+}$ with BAPTA and by blocking either voltage-gated calcium channels or blocking Ca$^{2+}$ release from internal stores. Inducing Ca$^{2+}$ release from internal stores mimicked the effect of activity, indicating that increased intracellular calcium ([Ca$^{2+}]_i$) was sufficient to cause the shift. Pharmacological blockade of KCC2 prevented the shift in $E_{GABA}$ induced by Ca$^{2+}$ released from internal stores. Together, these observations indicate that in mature neurons, calcium signaling triggers a decrease in KCC2 function.

Does activity regulate the maturation of KCC2 in immature neurons? The current model is that in contrast to acute changes observed in mature neurons, activity during development increases KCC2 function. Several lines of evidence indicate that the timing of the switch of the action of GABA during development requires the activation of excitatory ionotropic neurotransmitter receptors, though different types of activity have been implicated in different systems (reviewed in Ben-Ari et al., 2007).

First, excitatory GABA$\text{A}_\text{R}$-mediated activity itself has been implicated in regulating the maturation of GABAergic inhibition. In dissociated rat hippocampal neurons, maintaining cultures in GABA$\text{A}_\text{R}$ antagonists prevented the increase in KCC2 mRNA expression and extended the time period of depolarizing GABA, while
increased GABA activation induced the switch earlier (Ganguly et al., 2001). Blocking voltage-gated sodium channels with tetrodotoxin (TTX) does not affect the developmental GABA switch (Ganguly et al., 2001) or the up-regulation of KCC2 (Ludwig et al., 2003), suggesting that action potentials are not necessary for the regulation of the $E_{Cl}$ shift mediated by activation of GABA$_{A}$Rs.

In developing turtle retina, chronic in vivo application of the GABA$_{A}$R antagonist bicuculline prolonged the developmental period of spontaneous wave activity indicating a delayed GABA switch (Leitch et al., 2005). These effects also correlated with an inhibition of the up-regulation of KCC2 expression in dendrites in the inner plexiform layer. This is the first study to demonstrate that GABAergic activity regulates its own excitatory to inhibitory developmental switch in vivo.

However, the role that GABA$_{A}$R-mediated activity plays in the regulation of the GABA switch remains controversial. The developmental up-regulation of KCC2 in mouse dissociated hippocampal cultures and in organotypic hippocampal slices occurred in the absence of spiking activity, glutamatergic activity, and GABAergic activity – individually and in concert (Ludwig et al., 2003). Further, rat midbrain neurons cultures maintained in the presence of GABA$_{A}$R antagonists switched their response to GABA from excitatory to inhibitory at the same age as untreated cultured neurons (Titz et al., 2003).

Second, nAChR-mediated activity has been implicated in the developmental GABA switch. Knockout mice lacking $\alpha$7-subunit containing nicotinic acetylcholine
receptors (α7 nAChRs) have an extended period of depolarizing GABA in dissociated hippocampal neurons and show higher expression of NKCC1 and lower expression of KCC2 than wild-type mice (Liu et al., 2006). Further, blockade of α7- and/or α3-containing nAChRs in the developing chick embryo with antagonists prevented the loss of depolarizing GABA responses in the ciliary ganglion and spinal cord (Liu et al., 2006). Interestingly, over-expression of KCC2 to eliminate GABAergic excitation earlier resulted in striking changes in morphology and synaptic development chick neurons both in culture and in vivo. Importantly, these changes were blocked when neurons were treated with nicotinic antagonists, indicating that GABAergic inhibition and nicotinic excitation must cooperate to produce the changes and that GABAergic inhibition is important for later stages of development.

Here we explore whether neural activity influences the timing of the switch in the developing retina. Evidence indicates that the action of GABA_ARs switches from being depolarizing to hyperpolarizing in neurons in the ganglion cell layer (GCL) of the mouse retina during development. First, calcium imaging showed that application of GABA induced an increase in intracellular calcium until postnatal day 6 (P6) and that by P8 GABA_AR activation either had no effect or reduced [Ca^{2+}]_i. Second, gramicidin perforated patch recordings demonstrated a developmental shift in E_{GABA} from approximately -45 mV at P1-3 to -60 mV at P7-10. Third, chloride imaging showed developmental declines in [Cl^-] from 29 mM to 14 mM, corresponding to a change in E_{Cl} from -39 mV to -58 mV (Zhang et al., 2006). These developmental changes persisted in a CO_2-/bicarbonate-free HEPES-based medium, indicating that
the action of GABA was primarily mediated by chloride flux. RGCs do not express NKCC1 early in development and NKCC1 KO mice have a normal GABA depolarization and GABA switch in the retina (Zhang et al., 2007; Li et al., 2008), suggesting that RGCs accumulate chloride by a different mechanism than NKCC1 action, perhaps through the anion exchanger AE3 (Gonzalez-Islas et al., 2009). However, KCC2 expression in the retina follows the same developmental pattern seen elsewhere: immunostaining and Western blots for KCC2 show increased expression gradually after P1 reaching adult levels by approximately P12-P14 (Vu et al., 2000; Zhang et al., 2007).

The retina affords several advantages over other systems used to study the role of neuronal activity in the GABA switch. First, the sources of neuronal activity during the developmental period of switching action of GABA are well understood. Prior to vision, the developing retina exhibits spontaneous correlated activity, termed retinal waves. Hence, in addition to studying the role of specific signaling mechanisms in the GABA switch, we can also test for the role of correlated network activity in driving the developmental switch. Second, the retina is a thin, isolated sheet of tissue and therefore is possible to study the physiological properties of neurons in vitro with the entire circuit intact in contrast to hippocampus where cells need to be either dissociated or cut in slices. Third, purified retinal cell cultures are excellent model systems for studying biological processes in vitro. Finally, it is important to determine whether the observations in other parts of the nervous system can be generalized to all circuits in the brain.
II. The maturation of GABAergic inhibition in neurons in the GCL is independent of retinal waves, activation of nicotinic acetylcholine receptors, and ionotropic glutamatergic receptors

Introduction

Despite the importance of the transition from excitatory to inhibitory action of GABA, little is known about the mechanisms controlling the timing of the transition. The current model is that during development, ionotropic neurotransmitter receptor-mediated activity depolarizes neurons leading to calcium influx via voltage-gated calcium channels. This calcium influx leads to changes in the expression of chloride cotransporters resulting in chloride transport switching from net accumulation to net extrusion and a consequent change of polarity in the driving force for chloride. The current hypotheses about the source of the activity and its means of action are controversial and evidence suggests that the transition may be mediated by different neurotransmitter systems in different parts of the CNS (Ganguly et al., 2001; Ben-Ari, 2002; Leitch et al., 2005; Liu et al., 2006; Ben-Ari et al., 2007).

The first hypothesis that we are going to test is that activity mediated by nAChRs regulates the timing of the transition from excitatory to inhibitory GABA. Nicotinic cholinergic activity could mediate its effect on the excitatory to inhibitory GABA transition in three ways. First, cation flux through nAChRs could depolarize neurons sufficiently to activate calcium influx and subsequent regulation of protein expression. Second, nAChRs can directly flux calcium, particularly α7 subunit containing nAChRs which are highly calcium permeable. Third, spontaneous,
correlated network activity requiring nAChRs may drive large, coordinated bursts of depolarizing activity from convergent neurotransmitter receptor systems. Knockout mice lacking α7/β2 nAChR subunits and knockout mice lacking only β2 nAChRs allowed us to differentiate these two possibilities.

The second hypothesis that we are going to test is that spontaneous network activity regulates the timing of the transition from excitatory to inhibitory GABA. Spontaneous activity in developing circuits plays a multitude of important roles (reviewed in Goodman and Shatz, 1993; Feller, 1999). In the developing retina, RGCs fire periodic, spontaneous bursts of action potentials that propagate from one cell to the next in a wavelike manner (reviewed in Wong, 1999). This highly correlated, propagating activity is called “retinal waves” and is largely mediated by synaptic transmission.

Retinal waves are present during late embryonic/early postnatal retinal development when the retina is rapidly maturing (Sernagor et al., 2001; Morgan, 2006). The mechanisms that mediate waves change over this time period and are divided into three stages (Torborg and Feller, 2005). Stage I waves (embryonic day 16 to P0 in mice) are blocked by gap-junction blockers but are only weakly affected by antagonists to fast neurotransmitter receptors. Stage II waves, which take place during the period in which the transition from excitatory to inhibitory action of GABA takes place (P0–P10) are blocked by nAChR antagonists and are unaffected by glutamate receptor antagonists. Stage III waves occur after the maturation of inhibitory GABA (P10–P14) and are blocked by ionotropic glutamate receptor
antagonists but are unaffected by nACh antagonist. Knockout mice lacking β2
nAChRs have altered stage II retinal waves, but maintain non-correlated spontaneous
activity, allowing us to test the hypothesis that retinal waves regulate the transition.

The third hypothesis that we are going to test is that glutamatergic activity
regulates the timing of the transition from excitatory to inhibitory GABA. Glutamate
is the major excitatory transmitter in the mature vertebrate brain, but it plays an
important role in many aspects of development, including synapse formation,
maturity, and plasticity (Huang, 2009). In the hippocampus, GABAergic and
 glutamatergic circuitry appear to interact in a highly coordinated manner in the
development of neuronal circuits with GABAergic depolarization driving the
activation of NMDARs in “silent synapses” that predominate early glutamatergic
synapse development (Ben-Ari et al., 1997).

The glutamatergic circuitry in the retina matures during the same
developmental period as the transition from the excitatory to inhibitory action of
GABA. In the mouse retina, the first conventional glutamatergic synapses in the inner
retina are detected at P3 though the first ribbon synapses are not observed in the inner
retina until P11 (Fisher, 1979). The vesicular glutamate transporter VGLUT1 is
expressed in the outer retina by P3 and in the inner retina by P5 (Johnson et al., 2003;
Sherry et al., 2003). In addition, functional glutamatergic currents were recorded in
mouse retina as early as P7, an age when bipolar terminals are clearly present in the
inner plexiform layer (Johnson et al., 2003).
Synaptic release from glutamatergic neurons requires vesicular glutamate transporters (VGLUTs) to load synaptic vesicles with glutamate from the cytoplasm. There are three isoforms of the vesicular glutamate transporter, VGLUT1-3. VGLUT1 is the predominant form of vesicular glutamate transporter in retinal glutamatergic synapses: VGLUT1 is expressed exclusively in bipolar cells and photoreceptors (Fremeau et al., 2004; Wassle et al., 2006). VGLUT1 is also required for glutamatergic retinal waves and for the cessation of the preceding stage of cholinergic retinal waves (Blankenship et al., 2009). VGLUT2 is expressed only in RGCs and therefore does not contribute to signaling within the retina. VGLUT3 is only expressed in one type of amacrine cell (Johnson et al., 2004) and is not required for glutamatergic retinal waves nor for the termination of cholinergic retinal waves (Blankenship et al., 2009). Thus, VGLUT1 knockout mice lack the predominant glutamatergic inputs onto RGCs. We used VGLUT1⁻/⁻ mice to test the role of glutamatergic activity in regulating the transition from excitatory to inhibitory GABA.

Materials and Methods

Animals

C57Bl/6 mice obtained from Harlan were used for all wild-type experiments. We used transgenic mice that were generous gifts from several labs. β2 knockout mice were generated as previously reported (Xu et al., 1999) and were originally provided by Dr. Art Beaudet from Baylor College of Medicine. These mice were bred after backcrossing onto a C57Bl/6 strain for many generations. α7/β2 double
knockout mice were generated as previously reported (Marubio and Paylor, 2004) and were generously provided by Kerri Massey and Darwin Berg at the University of California, San Diego. VGLUT1 knockout mice were generated as reported previously (Fremeau et al., 2004) and were generously provided by Juliette Johnson and David Copenhagen at the University of California, San Francisco. Genotypes were determined according to the published protocols for the β2−/− (Xu et al., 1999), α7β2−/− (Marubio and Paylor, 2004) and VGLUT1−/− (Fremeau et al., 2004). All animal procedures were approved by the University of California, San Diego and the University of California, Berkeley Institutional Animal Care and Use Committees and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Public Health Service Policy, and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

*Whole-mount Retinal Preparation*

Postnatal day 2-12 (P2-P12) mice were anesthetized with isoflurane and decapitated. Retinas were isolated in artificial cerebrospinal fluid (ACSF) (in mM: 119.0 NaCl, 26.2 NaHCO₃, 11 glucose, 2.5 KCl, 1.0 K₂HPO₄, 2.5 CaCl₂, 1.3 MgCl₂) and mounted RGC side up on nitrocellulose filter paper (0.45 μm, Millipore, Billerica, MA) with a hole cut to allow more transmitted light to pass through. Retinas were incubated at room temperature in bubbled (95% O₂/5% CO₂) ACSF until transfer to the recording chamber where they were constantly perfused with bubbled ACSF at 32°C–34°C.
**Imaging**

Whole-mount and explant retinas were loaded using the multicell bolus loading technique (Stosiek et al., 2003; Blankenship et al., 2009). Briefly, Oregon Green BAPTA-1 AM (Invitrogen, Carlsbad, CA) was made at 0.8 mM in 2% pluronic acid plus 8% dimethyl sulfoxide (DMSO) in puffing solution containing (in mM) 150 NaCl, 2.5 KCl, 10 HEPES. The OGB-1 AM solution was pressure ejected from a borosilicate glass micropipette (Garner Glass, Claremont, CA) using a PV-820 Pneumatic PicoPump (World Precision Instruments, Sarasota, FL). Dye injections were made just under the inner limiting membrane in multiple, non-overlapping locations in the RGC layer for 1-1.5 sec. at a pressure of 10 – 20 psi. Epifluorescent calcium imaging was performed on an Olympus BX61WI microscope using a 60x water immersion objective (Olympus LUMPlanFl/IR) or a Zeiss Axioscop 2 FS Plus microscope with a 40x water immersion objective (Zeiss Achroplan) with illumination provided by a Sutter Lambda LS controlled by a Uniblitz shutter (Sutter Instruments, Novato, CA). Images were acquired at 1 Hz using Metamorph software (Molecular Devices, Sunnyvale, CA). Short applications of muscimol [100 μM] in puffing solution or high-K\(^+\) external solution (in mM: 105 KCl, 3 CaCl\(_2\), 2 MgCl\(_2\), 10 glucose, and 10 HEPES) were delivered to neurons in the GCL through a glass electrophysiology electrode (~2 μm tip size, located near the edge of the field of view and <50 μm above the GCL in whole-mounts and explants or <50 μm from cultured RGCs) using a PV820 Pneumatic PicoPump at 6-12 psi, 100-500 ms pulse duration with two pulses separated by a 60 sec. interval. Regions of interest (ROIs) were
manually drawn around all cells. The fractional change in fluorescence intensity $\Delta F/F = (F(t) - F(\text{avg}))/F(\text{avg})$ was computed for each ROI for each frame, where $F(t)$ is the fluorescence intensity at time $t$ and $F(\text{avg})$ is the fluorescence intensity at each ROI averaged over 10 frames immediately before the stimuli and after correction for bleaching. Cells were classified as “responsive” if the application of muscimol induced a $\Delta F/F$ above a threshold set at seven standard deviations above baseline noise. Analysis was carried out using Metamorph and IgorPro (Wavemetrics, Inc.).

**Pharmacology**

6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), SR-95531 hydrobromide (gabazine), strychnine, Dihydro-beta-erythroidine hydrobromide (DH$\beta$E), and carbenoxolone stock solutions were prepared at $\geq1000x$ concentration in water. CGP54626 (10,000x) and nifedipine (1,000x) stocks were prepared in DMSO. The stocks were then added to ACSF. DNQX, D-AP5, SR-95531, DH$\beta$E, and CGP54626 were purchased from Tocris Bioscience (Ellisville, MO); TPMPA, strychnine, carbenoxolone, and nifedipine were from Sigma-Aldrich (St. Louis, MO).

**Statistics**

Unpaired t tests were used for comparisons between two groups. For multiple comparisons, a one-way ANOVA with Tukey-Kramer post-hoc test was used. Results with $p < 0.05$ were considered significant. Values reported are mean ± standard
deviation unless otherwise noted. Statistical analysis was carried out using InStat (GraphPad Software, Inc., La Jolla, CA).

Results

Retinal ganglion cells are depolarized by GABA\textsubscript{A}R activation until P7

To determine the age at which the transition from excitatory to inhibitory GABAergic activity occurs, we measured GABA\textsubscript{A}R-mediated depolarization in neurons in the GCL across development. In the mouse retina, the GCL is comprised of retinal ganglion cells (RGCs) and displaced amacrine cells. Calcium imaging was used to characterize the depolarizing action of GABA since increases in intracellular calcium concentrations via activation of voltage-gated calcium channels are a sensitive indicator of depolarization. This indirect measure of depolarization has two advantages over whole cell recording: it does not perturb the concentration of chloride inside cells and it allows us to record the activity of many neurons simultaneously.

Whole-mount retinas were prepared acutely from postnatal day 2 (P2) to P11 wild-type mice. Cells in the RGC layer were loaded with the calcium indicator Oregon Green BAPTA-1-AM using the multicell bolus loading method (Stosiek et al., 2003) (Figure II-1A). Fractional fluorescence changes were recorded in response to brief applications of the GABA\textsubscript{A} receptor (GABA\textsubscript{A}R) agonist muscimol (500 ms, 2 times at 60 sec. interval, 6-10 psi, 100 \(\mu\)M) (Figure II-1B). Experiments were
performed in the presence of antagonists to the non-GABA_A ionotropic neurotransmitter receptors to ensure direct activation of neurons in the GCL: AMPA and kainate receptors were blocked with DNQX [20 μM], NMDA-Rs were blocked with D-AP5 [50 μM], glycine receptors were blocked with strychnine [4 μM], nAChRs were blocked with DHβE [8 μM], and GABA_BRs were blocked with CGP54626 [2 μM]. In addition, the muscimol-induced changes in ΔF/F were blocked by the GABA_AR antagonist gabazine [25 μM] and the GABA_CR antagonist TPMPA [25 μM], which were included in both the applied muscimol solution and the bath ([2.5 μM] and [25 μM], respectively) (Figure II-1B). The fraction of cells that were depolarized in response to muscimol was determined by computing the ratio of the number of cells in the imaging area that exhibited a significant increase in ΔF/F in response to muscimol to the number of cells that exhibited a significant increase in ΔF/F in response to brief applications of a depolarizing, high potassium solution (KCl, 500 ms, 2 times at 60 sec. interval, 6-10 psi, 100 mM) (Figure II-1B).

Nearly all neurons in the GCL exhibited an increase in [Ca^{2+}] in response to muscimol between P2 and P6 (P2-P6: 99.1 ± 0.9%, n = 13 mice; Figure II-1C). Between P7 and P9 there is a gradual reduction in the percentage of cells that depolarized in response to muscimol and at P10 almost no neurons in the GCL responded to muscimol (2.7 ± 6.6%, n = 3 mice, p < 0.0001, unpaired t test versus P2-P6; Figure II-1C). In addition, the average amplitude of the ΔF/F in response to muscimol was reduced at older ages, suggesting that there was a gradual reduction in the strength of the GABA_A R-mediated depolarization (2.10 ± 0.35%, n = 6 mice, P9-
P10, versus 12.53 ± 3.13%, n = 13 mice, P2-P6, p < 0.0001, unpaired t test; Figure II-1C). Hence, we conclude that responses in neurons in the GCL to GABAergic activation have switched from an excitatory to an inhibitory action by P9.

**Neurons in the GCL develop inhibitory GABA at the normal age in mice lacking nicotinic acetylcholine receptors**

The transition from excitatory to inhibitory action of GABA in various parts of the CNS is regulated by nicotinic acetylcholine receptor (nAChR) mediated activity: mice lacking α7 nAChRs show a delayed shift in response to GABA in hippocampus and spinal cord and blocking α7-nAChRs or α3-heteromeric nAChRs with antagonists in the chick *in ovo* extended the period of GABAergic excitation as well (Liu et al., 2006).

To determine if the GABA switch in neurons in the GCL is dependent on activation of nAChRs, we looked at double knockout mice lacking both α7 and β2 nAChRs. Acute, whole-mount retinas were prepared from the α7/β2−/− mice at P9-P11, ages immediately after neurons in the GCL cease to be depolarized by GABA in wild-type mice. Cells in the GCL were loaded with the multicell bolus method and GABAₐR-mediated depolarization was assayed as in wild-type retinas. Knocking out nicotinic cholinergic activity (α7/β2−/−) did not prevent the GABA switch, with 2.4 ± 3.9% of cells responding to muscimol (20 of 1030 cells, n = 4 mice, P9-P11; Figure II-2), similar to the wild-type controls (12.9 ± 25%, n = 5 mice, P9-P10, p = 0.594, one-way ANOVA). The average amplitude of the ΔF/F in response to the depolarizing, potassium solution was not different from wild-type mice (22.0 ± 4.8%, n = 4 mice,
$\alpha 7/\beta 2^{-/-}$, versus $29.2 \pm 8.0\%$, $n = 6$ mice, wild-type, $p = 0.34$, one-way ANOVA; Figure II-2), indicating that the decrease in the number of neurons exhibiting a depolarizing response to muscimol was not due to a loss of depolarization-induced calcium influx.

These data suggest that the total lack of nAChR signaling does not prevent the timing of the switch of the action of GABA from excitatory to inhibitory. However, mice lacking the $\beta 2$ subunit of the nAChR have altered cholinergic retinal waves (Bansal et al., 2000; McLaughlin et al., 2003). Therefore the possibility remains that retinal waves regulate the timing of the GABA switch, but only in the presence of a signaling via $\alpha 7$ nAChRs. Hence, we repeated these experiments in $\beta 2$-nAChR single knockouts and found that the action of GABA switched at the normal age, with no cells responding to muscimol (0 of 889, $n = 3$ mice, P9-P11, $p = 0.594$, one-way ANOVA; Figure II-2). The average amplitude of the $\Delta F/F$ in response to the depolarizing, potassium solution was not different from wild-type mice ($18.6 \pm 2.5\%$, $n = 3$ mice, $\beta 2^{-/-}$, versus wild-type, $p = 0.34$, one-way ANOVA; Figure II-2), indicating that the decrease in the number of neurons exhibiting a depolarizing response to muscimol was not due to a loss of depolarization-induced calcium influx. Hence, neither normal cholinergic waves nor signaling via nAChRs is necessary for the timing of the excitatory action of GABA.
Neurons in the GCL develop inhibitory GABA at the normal age in mice lacking glutamatergic signaling

The glutamatergic circuitry in the retina matures at a developmental period coincident with the transition from the excitatory to inhibitory action of GABA (Morgan, 2006). To determine if the excitatory to inhibitory GABA switch in neurons in the GCL is dependent on glutamatergic activity, we examined mice lacking the predominant vesicular glutamate transporter in the retina, VGLUT1. Acute, whole-mount retinas were prepared from the VGLUT1−/− mice at P9-P11 and GABA_AR-mediated depolarization was assayed with calcium imaging. Almost no cells responded (0.2 ± 0.4%, 1 of 707 cells, n = 3 mice, P9-P11, p = 0.594, one-way ANOVA; Figure II-2), indicating that that GABA switch occurs independent of glutamatergic activity. The average amplitude of the ΔF/F in response to the depolarizing, potassium solution was similar to wild-type mice (24.7 ± 9.1%, n = 3 mice, p = 0.34, one-way ANOVA; Figure II-2), indicating that the decrease in the number of neurons responding was not due to a loss of depolarization-induced calcium influx.

Discussion

Here, we have explored the role of three types of activity in signaling the transition from excitatory to inhibitory GABAergic transmission. We first showed that wild-type mice transition from excitatory to inhibitory GABAergic transmission between P7 and P10 (Figure II-1). We then used three different transgenic mice to
explore the role of specific types of activity in signaling the transition. First, knockout mice lacking nAChRs (α7/β2−/−) did not have an extended period of excitatory GABA (Figure II-2), demonstrating that activity mediated by nicotinic acetylcholine receptors is not necessary for signaling the switch in the action of GABA. Second, knockout mice with altered correlated activity but intact uncorrelated activity in the retina (β2−/−) did not have an extended period of excitatory GABA, demonstrating that correlated network activity is not essential for signaling the switch. Finally, knockout mice lacking the primary source of glutamatergic activity in the retina (VGLUT1−/−) did not have an extended period of excitatory GABA. Thus they too showed a normal developmental switch in the action of GABA, demonstrating that activity mediated by glutamate is not necessary for signaling the switch.

Our results in the mouse retina contrast Liu and colleagues' results in mouse hippocampus and chick spinal cord and ciliary ganglion. In α7 knockout mice, Liu and colleagues showed that the transition of transition to inhibitory GABA was delayed in many cells in the hippocampus (Liu et al., 2006). One possibility is that nicotinic cholinergic activity alters the timing of the excitatory to inhibitory GABA transition by down-regulating the expression of NKCC1, the chloride cotransporter that accumulates intracellular chloride in immature neurons. RGCs do not express NKCC1 and thus must accumulate high intracellular chloride early in development through other means, potentially the anion exchanger AE3 (Zhang et al., 2007; Li et al., 2008; Gonzalez-Islas et al., 2009). This is consistent with their results showing that NKCC1 protein expression remains approximately four-fold higher in the
presence of antagonists to nACHRs in ovo. However, this cannot fully explain the difference, because α7 nACHR knockout also led to decreased KCC2 expression at later ages. A more parsimonious explanation is that KCC2 expression in the retina in contrast to other parts of the nervous system is not modulated by neural activity.
Figure II-1: Neurons in the Ganglion Cell Layer transition from excitatory to inhibitory response to GABA_A receptors activation during early postnatal development

A) Fluorescent image of an acutely isolated, whole-mount P5 mouse retina loaded with the Ca^{2+} indicator Oregon Green BAPTA-1 AM via the bolus loading method. 4 example ROIs are shown. Scale 20 µm.  B) Timecourse of fractional change in fluorescence (ΔF/F) averaged over each ROI. Example traces were smoothed to remove noise. Arrows: 500 ms applications of the GABA_A agonist muscimol [100 µM] or KCl [100 mM]. C) Summary of muscimol-induced Ca^{2+} transients in acutely isolated wild-type retinas. Left y-axis (open squares): % responsive to muscimol application. Right y-axis (black circles): average amplitude of ΔF/F to muscimol in responsive cells. Error bars are +/- SEM. In all experiments antagonists to glutamatergic, cholinergic, glycinergic, and GABA_B receptors (DNQX [20 µM], D-AP5 [50 µM], DHβE [8 µM], Strychnine [4 µM], CGP54626 [2 µM]) were included in the bath to block indirect depolarization of neurons.
Figure II-2: Knockout mice with disrupted cholinergic or glutamatergic activity show a normal maturation of inhibitory GABA in neurons in the GCL.

Summary of calcium imaging experiments in acutely isolated P9-P11 wild-type, \(\alpha 7/\beta 2^{-/-}\), \(\beta 2^{-/-}\), and VGLUT1\(^{-/-}\) mice. Left y-axis (open squares): % responsive cells to muscimol-induced Ca\(^{2+}\) transients; threshold ΔF/F of seven standard deviations above baseline noise. Right y-axis (black circles): average amplitude of ΔF/F to KCl application in all cells. Error bars are +/- SEM. In all experiments antagonists to glutamatergic, cholinergic, glycinegic, and GABA\(_{\mu}\)Rs (DNQX [20 µM], D-AP5 [50 µM], DHβE [8 µM], Strychnine [4 µM], CGP54626 [2 µM]) were included in the bath to block indirect depolarization of neurons.
III. The maturation of GABAergic inhibition in neurons in the GCL is independent of GABAergic activity, ionotropic neurotransmitter receptor mediated activity, action potentials, and spontaneous calcium transients

Introduction

Several types of activity have been implicated in regulating the GABA switch. One of the predominant hypotheses is that GABAergic activity itself regulates its own maturation. In dissociated rat hippocampal neurons, GABA_A R blockade prevented the increase in KCC2 expression and extended the time period of depolarizing GABA, while increased GABA activation induced the switch to occur earlier ((Ganguly et al., 2001), but see (Ludwig et al., 2003) and (Titz et al., 2003)). GABA_A R blockade in turtle retinas retina in vivo extended the period of propagating waves (Leitch et al., 2005), indicating that GABA_A R mediated activity itself plays an important role in the switch of GABA_A responses from excitatory to inhibitory.

Explants of brain tissues that are cultured in vitro are a powerful system for studies of how altering the environment can alter brain wiring. They have several advantages over knockout mice. First, knocking out certain types of activity leads to premature death. Second, knockouts may exhibit compensatory mechanisms for generating activity. Further, knocking out multiple types of activity in concert is often unfeasible for technical reasons relating to the number of different proteins that would need to be knocked out.
Essential development processes often have built-in redundancy in which absent or impaired functions are compensated for by the changed action of remaining processes. Blocking the activity of one neurotransmitter system throughout development, whether through genetic knockout or pharmacological treatment, could thus lead to compensation by other neurotransmitter systems. The use of a model system in which multiple types of activity can all be controlled simultaneously allows us to remove all processes which are likely to compensate for the lost function of others.

Materials and Methods

Retinal explant preparation

Whole-mount retinas were isolated from P2 wild-type mice as described in Chapter 2 with two slight modifications. First, the dissection was done in a sterile hood with tools that had been cleaned with 70% ethanol and irradiated with UV light for 15 minutes. Second, to provide better contact for mounting, the nitrocellulose filter paper onto which the pieces of retina were mounted did not have a one large hole to allow transmitted light to pass through but instead had many small holes punched with the tip of a razor blade. After isolation, whole-mount retinas were then cultured as “explants” on a feeding layer of P1 dissociated superior colliculus (SC) cells plated the day before. The feeding layer of neurons and glia from the superior colliculus (SC) was isolated by dissecting the SC of P1 wild-type mice. The tissue was digested
for 30 minutes at 34°C in a solution 0.25% trypsin-EDTA (Sigma-Aldrich) in DMEM/F12 (Invitrogen). After digestion, the SC tissue was rinsed three times in SC plating medium, consisting of 5% horse serum (Invitrogen) and 5% fetal bovine serum (Invitrogen) in DMEM/F12. Cells were tritivated in SC plating medium using a series of three increasingly small tipped, fire-polished Pasteur pipettes until dissociated (tip size decreased from full size to ½ to 1/3 of the original tip diameter). Cells were then plated in poly-D-lysine (Sigma-Aldrich) coated, tissue culture plastic 24-well plates (BD Falcon) at high density (approximately 2.5-5 x 10^5 cells per well). The SC cultures were incubated overnight in a humidified atmosphere of 5% CO2 at 34°C.

Whole-mount retinas were isolated from P2 wild-type mice to make retinal explants. Serum containing media on the SC feeding layer was replaced the following day with warmed, equilibrated serum-free culture medium (SFCM-A) consisting of Neurobasal-A medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 0.6% glucose, 2 mM glutamax (Invitrogen), 10 mM HEPES, 1 mM Na-Pyruvate, 50 mg/mL penicillin G, 50 units/mL, streptomycin, 2.5 mg/mL Insulin, and 10 ng/mL CNTF (Peprotech, Rocky Hill, NJ). Retinal explants were then added to the SC feeding layer and cultured for 2-11 days in vitro (DIV). All reagents are from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell purification and culture

Retinal ganglion cells (RGCs) were purified and cultured from P2 wild-type mouse retinas essentially as previously described (Barres et al., 1988; Watkins et al.,
Briefly, retinas were dissociated with papain and sequential immunopanning was used to deplete macrophages (BSL I, Vector Labs, Burlingame, CA) and isolate RGCs (α-Thy1.2, Serotec, Oxford, UK). RGCs were plated onto glass coverslips at 1-10 x 10³ cell/cm² and grown in a serum-free, Neurobasal-based medium containing B27, selenium, putrescine, triiodo-thyronine, transferrin, progesterone, pyruvate, glutamine, CNTF (10 ng/ml, Peprotech), BDNF (50 ng/ml, Peprotech), insulin (5 mg/ml), and forskolin (10 μM) as defined previously (Meyer-Franke et al., 1995). RGC cultures were incubated in a humidified atmosphere of 5% CO₂ at 34°C for up to 25 DIV.

Calcium imaging in cell cultures

Purified RGCs were loaded by bath application of Oregon Green BAPTA-1 AM made at 0.8 mM in 2% pluronic acid plus 8% dimethyl sulfoxide (DMSO) in puffing solution containing (in mM) 150 NaCl, 2.5 KCl, 10 HEPES then added 1:100 to RGC growth media for 10 minutes in an incubator with a humidified atmosphere of 5% CO₂ at 34°C. Coverslips were removed from the incubator and placed in a recording chamber constantly perfused with bubbled ACSF (95% O₂/5% CO₂) at 32°C–34°C. Calcium imaging was then performed as with whole-mount retinas.

Electrophysiology

Whole-cell voltage-clamp recordings were made from neurons in the GCL in retinal explants. Retinas were visualized with differential interference contrast (DIC) optics on an Olympus BX61WI microscope using a 60x water immersion objective
(Olympus LUMPlanFl/IR). Neurons in the GCL were targeted under control of a micromanipulator (MP-225; Sutter Instruments). Recording pipettes (Garner Glass, Claremont, CA or Sutter Instruments) were pulled (PP-830; Narishige, Tokyo, Japan) with a tip resistance of 3-4 MΩ and filled with a cesium gluconate internal solution (in mM: 100 gluconic acid, 100 CsOH, 1.7 CsCl, 40 HEPES, 10 EGTA, 5 MgCl₂, 1 QX-314, 2 Na₂ATP, and 0.3 Na-GTP; pH adjusted to 7.25 with CsOH; $E_{Cl} = -60$ mV). Data were acquired using pCLAMP 10 recording software and a Multiclamp 200B amplifier (Molecular Devices, Sunnyvale, CA), sampled at 10 kHz and low-pass filtered at 1 kHz.

**Results**

**Extended blockade of activity in retinal explants did not significantly disrupt functional retinal circuits**

To pharmacologically control specific as well as combined forms of neuronal activity, we developed an *in vitro* retinal explant system. First, we established that retinal explants were a viable model system to study the maturation of inhibition in the retina. Retinal explants were made by preparing whole-mount retinas from P2 wild-type mice and culturing them on a feeding layer of dissociated P1 superior colliculus cells plated the previous day (see Materials and Methods). Retinal explants were then cultured for up to 11 days *in vitro* (DIV) with or without a cocktail of ionotropic neurotransmitter receptor antagonists plus TTX [2.5 µM] to block voltage-gated
sodium channels: AMPA and kainate receptors were blocked with DNQX [20 μM], NMDA-Rs were blocked with D-AP5 [50 μM], glycine receptors were blocked with strychnine [4 μM], nicotinic acetylcholine receptors were blocked with DHβE [8 μM], and GABARs were blocked with gabazine [25 μM], CGP54626 [2 μM], and TPMPA [25 μM].

To image spontaneous calcium transients in the explants, we placed explants in a bath of regular ACSF and loaded cells in the GCL of explants with the multicell bolus method (Figure III-1A). For at least 11 DIV, neurons in the GCL of retinal explants exhibited spontaneous, correlated calcium transients with a frequency of approximately once a minute, qualitatively similar to retinal waves (Figure III-1B, left panel), suggesting that the in vitro network remained capable of synchronous activity qualitatively similar to what has been observed in vivo. Spontaneous calcium transients were blocked by a cocktail of neurotransmitter receptor antagonists, indicating that they were mediated by a similar network to that observed in acutely isolated tissue.

To establish that neurons in the GCL in explants maintained in the presence of neurotransmitter receptor antagonists continue to express functional GABA<sub>A</sub>Rs after 11 DIV, we performed whole-cell voltage clamp recordings from neurons in the GCL. GABA<sub>A</sub>R-mediated currents were recorded at different holding potentials in response to brief applications of muscimol (200 ms, 100 µM) (Figure II-1C). Muscimol-induced currents reversed near the E<sub>Cl</sub>, which we estimate to be -60 mV (n = 6). Muscimol-induced currents were blocked by the presence of the GABA<sub>A</sub>CR
antagonists gabazine [2.5 μM, 25 μM] and TPMPA [25 μM, 25 μM] in the bath and in the puffing solution, respectively (Figure III-1C), confirming they were mediated by ionotropic GABARs.

Retinal explants recapitulate the timing of the transition from excitatory to inhibitory action of GABA in vitro

To determine the timing of the GABA switch in retinal explants, we loaded cells in the RGC layer of explants with the multicell bolus method and GABA$_A$R-mediated depolarization was assayed as in wild-type retinas with calcium imaging in response to muscimol-induced depolarization. The timing of the GABA switch in retinal explants closely recapitulated the in vivo timeline: neurons in the GCL in retinal explants are depolarized by muscimol through 4 DIV (93.0 ± 9.8% responded, 495 of 532 cells, 2-4 DIV, n = 8 mice) and cease to be depolarized by 8-11 DIV (4.5 ± 5.2% responded, 12 of 264 cells, 8-11 DIV, n = 5 mice, P < 0.0001, unpaired t test versus 2-4 DIV) (Figure III-2). We observed that the average amplitude of the ΔF/F in response to muscimol was reduced at these timepoints (3.92 ± 1.2%, n = 3 mice, 8-11 DIV, versus 7.7 ± 4.3%, n = 8, 2-4 DIV, p = 0.17, unpaired t test; Figure III-2), suggesting that there was a gradual reduction in the strength of the GABA$_A$R-mediated depolarization, similar to the reduction seen in vivo (Figure II-1).

Neurons in the GCL developed inhibitory GABA at the normal age in the absence of GABAergic activity
GABA$_A$R-mediated activity itself has been directly implicated in regulating the transition from excitatory to inhibitory GABA in two different systems (Ganguly et al., 2001; Leitch et al., 2005). Does GABA$_A$R-mediated activity modulating the timing of the action of GABA in neurons in the GCL? To determine if the excitatory to inhibitory GABA switch is regulated by GABA$_A$R-mediated activity itself, we cultured retinal explants in the presence of the GABA$_A$R antagonist gabazine [12.5 μM], GABA$_B$R antagonist CGP54626 [2 μM], and GABA$_C$R antagonist TPMPA [50 μM]. GABA$_A$R-mediated depolarization was assayed as in control explants. At 7-11 DIV (approximately equivalent to P9-P13), 4.5 ± 4.5% of cells exhibited an increase in intracellular calcium in response to short applications of muscimol which was not significantly different to control explants (8 of 176 cells, n = 2 explants, p > 0.05, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-3), indicating that blockade of GABAergic activity did not prevent the GABA switch from occurring.

**Neurons in the GCL develop inhibitory GABA in the absence of combined ionotrophic neurotransmitter receptor mediated activity and spiking activity**

We have demonstrated that nicotinic cholinergic, ionotropic glutamatergic, or GABAergic signaling alone does not influence the timing of the switch of GABA. However, there is the possibility that the mode of activity is not important and that any signaling pathway is sufficient. To test this possibility, we took advantage of the pharmacological control provided by our explant model system and blocked the
activity of multiple neurotransmitter systems and voltage-gated ion channels simultaneously.

First, to determine if the excitatory to inhibitory GABA switch is independent of all forms of ionotropic neurotransmitter receptor mediated activity that neurons in the GCL participate in, we cultured explants for up to 11 DIV in a cocktail of ionotropic neurotransmitter receptor antagonists plus that included: DNQX [20 μM] and D-AP5 [50 μM] to block AMPA/Kainate and NMDA-type glutamate receptors respectively, DHβE [8 μM] to block nicotinic acetylcholine receptors, strychnine [4 μM] to block glycine receptors, and a combination of gabazine [12.5 μM], CGP54626 [2 μM], TPMPA [50 μM] to block GABA<sub>A</sub>Rs, GABA<sub>B</sub>Rs, and GABA<sub>C</sub>Rs respectively. We also added TTX [2.5 μM] to block action potentials in neurons in the GCL. This combination of pharmacological agents is referred to as “synaptic block.”

GABA<sub>A</sub>R-mediated depolarization was assayed as in control explants. Surprisingly, at 7-11 DIV only 9.0 ± 3.3% of cells responded to muscimol (36 of 402 cells, n = 6 mice, “Synaptic Block”, p > 0.05, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-3), indicating that the GABA switch is independent of activity mediated by ionotropic neurotransmitter receptors and spiking activity. The decrease in the number of neurons responding was not due to a loss of depolarization-induced calcium influx, because the average amplitude of the ΔF/F in response to the depolarizing, potassium solution was not statistically different from control explants (16.27 ± 9.9%, n = 6 mice, “Synaptic Block”, 13.23 ± 7.4%, n = 5 mice, “Control”, p
> 0.05, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-3). Hence, neither synaptic signaling nor action potentials are necessary for the maturation of the inhibitory action of GABA.

**Neurons in the GCL develop inhibitory GABA in the absence of spontaneous calcium transients**

Studies of development processes often face the fundamental issue of compensation in which the changed functions of surviving processes compensate for those functions normally provided by absent processes. Blocking the activity of one neurotransmitter system throughout development, whether through genetic knockout or pharmacological treatment, could lead to compensation by other neurotransmitter systems or by changes in other properties controlling activity in the developing circuit. This makes it difficult to interpret studies in which processes have been removed throughout development in systems where other processes may be able to compensate for lost function.

Culturing explants with the synaptic block antagonist cocktail acutely prevents all spontaneous calcium transients, but culturing cells in antagonists for an extended period of time might lead to compensatory activity through other means such as increased gap junction coupling or changes in intrinsic properties. If the retina is able to compensate for the lost activity of the blocked systems, this compensatory activity might be sufficient to regulate the maturation of inhibitory GABA.
To determine if any compensatory activity developed in the presence of the synaptic block cocktail, we measured spontaneous calcium transients in explants that had been cultured in the synaptic block antagonist cocktail for 7-11 DIV. Explants were placed in the recording chamber in a bath of ACSF containing the same synaptic block antagonist cocktail. Cells in the GCL of explants were loaded with calcium indicator using the multicell bolus method as before and spontaneous activity was monitored. Surprisingly, explants cultured in the synaptic block antagonist cocktail showed infrequent and uncorrelated calcium transients (Figure III-3, left inset), suggesting that the cultures adapted to the synaptic block with an increase in calcium transients mediated by non-spiking, non-synaptic mechanisms.

Are these infrequent, uncorrelated spontaneous calcium transients sufficient to regulate the transition from excitatory to inhibitory GABA in neurons in the GCL? To answer this question, we cultured explants in conditions that blocked these calcium transients completely. We blocked the calcium transients that develop during synaptic block by culturing the explants in a “total block” drug cocktail containing the synaptic block antagonists with the addition of the L-type voltage gated calcium channel (VGCC) antagonist nifedipine [100 μM] and the gap junction blocker carbenoxolone [100 μM]. Culturing explants in the presence of these antagonists prevented all spontaneous calcium transients in cultures for at least 7-11 DIV (Figure III-3, right inset).

Total block of spontaneous calcium transients in explants did not prevent the GABA switch from occurring: no cells responded to muscimol at 7-11 DIV (0 of 168,
n = 9 mice, p > 0.05, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-3). Again, the decrease in the number of neurons responding was not due to a loss of depolarization-induced calcium influx, because the average amplitude of the $\Delta F/F$ in response to the depolarizing, potassium solution was similar to wild-type mice (8.42 ± 2.5%, n = 9 mice, p > 0.05, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-3). Finally, the decrease in the number of cells responding was not due to a down-regulation of functional GABA$_\text{A}$Rs in explants cultured for at least 11 DIV in the either the synaptic block cocktail or total activity block cocktails because whole-cell voltage clamp recordings in which we briefly applied muscimol at different holding potentials showed large GABA$_\text{A}$R-mediated currents (Figure III-1C). Hence, we conclude that neural activity – either synaptically generated or intrinsic to the neurons themselves – did not play a role in the timing of the maturation of the inhibitory action of GABA in neurons in the GCL.

**Purified RGCs continue to be depolarized by GABA for at least 3 weeks in cell culture**

The independence of the transition from excitatory to inhibitory GABA from neural activity suggests that the switch might occur in a cell-autonomous manner. To determine if the GABA switch is regulated cell-autonomously, we purified RGCs from P2 mouse retinas using immunopanning and cultured them for up to 25 DIV in serum-free, Neurobasal-based, defined growth media (see Materials and Methods). Cells were bath loaded with calcium indicator and GABA-mediated depolarization
was assayed by briefly applying muscimol onto RGCs. In contrast to neurons in the GCL in acute retinas and in explants under all culture conditions, isolated RGCs responded to muscimol for at least 25 DIV: 82% at 1-2 DIV (18 of 22 cells), 86% at 9-10 DIV (6 of 7 cells), 81% at 15-18 DIV (13 of 16 cells), and 79% at 21-25 DIV (11 of 14 cells) (Figure III-4C). The average amplitude of the ΔF/F in response to muscimol measured was 35.4 ± 7.6% at 1-2 DIV, 57.8 ± 20.4% at 9-10 DIV, 38.8 ± 9.4% at 15-18 DIV, and 29.2 ± 7.8% at 21-25 DIV, suggesting that there was not a significant reduction in the strength of the GABA_A-mediated depolarization across the 25 days in culture (p > 0.05 for all comparisons except 9-10 DIV, which is statistically from all others, p < 0.001, one-way ANOVA with Tukey-Kramer post-hoc test; Figure III-4C). Hence, RGCs do not undergo the switch from excitatory or inhibitory GABA_A-mediated action in the absence of other cell types. In future experiments, we will determine if the relevant signals that mediate the switch are from other retinal neurons or glial cells.

Discussion

Here, we have explored the roles of multiple types of neuronal activity alone and in concert in signaling the transition from excitatory to inhibitory GABA_A-mediated activity. First, we developed an *in vitro* model system for studying this transition using retinal explants. We showed that these explants, cultured in the presence of antagonists to various forms of activity, maintain spontaneous network activity and continue to express functional GABA_ARs throughout culture (Figure III-
1). We then used our model system to demonstrate that GABAergic activity is not necessary for the maturation of inhibitory GABA in mouse retinal ganglion cells.

The manipulation of GABAergic activity has been shown to alter the transition from excitatory to inhibitory GABAergic transmission in two different systems. In dissociated hippocampal neurons, maintaining cultures in GABA\textsubscript{A}R antagonists delayed the transition from excitatory to inhibitory response to GABA and increased exposure to GABA in culture led to an early transition (Ganguly et al., 2001; Leitch et al., 2005). In turtle retina, chronic *in vivo* application of the GABA\textsubscript{A}R antagonist bicuculline prolonged the developmental period of spontaneous wave activity indicating a delayed GABA switch (Leitch et al., 2005). However, other evidence suggests that GABAergic activity does not play a role in regulating the timing of the GABA switch. The up-regulation of KCC2 in mouse dissociated hippocampal cultures and in organotypic hippocampal slices occurred in the absence of GABAergic activity, glutamatergic activity, and spiking activity – individually and in concert (Ludwig et al., 2003). Further, rat midbrain neurons cultures maintained in the presence of GABA\textsubscript{A}R antagonists switched their response to GABA from excitatory to inhibitory at the same age as untreated cultured neurons (Titz et al., 2003).

Our results indicate that GABAergic activity is not involved in regulating the timing of the transition in mouse retina. Dissociated hippocampal neurons *in vitro* have very different inputs compared to those they receive *in vivo*, and one possibility is that neurons in retinal explants have other sources of activity that can compensate for the lost GABAergic excitement in the presence of GABA\textsubscript{R} antagonists. There is
also the possibility that the retina develops differently than hippocampus. The results from Leitch and colleagues are from an in vivo study of the retina, but the comparison of their results with ours is complicated by the fact that GABAergic depolarization was indirectly assayed by its affect on spontaneous, correlated, propagating network activity three weeks after the end of GABAergic excitation. The excitatory to inhibitory transition of GABA action occurs six days after hatching in turtle retina (Sernagor et al., 2003). GABAergic activity was blocked for 28 days after hatching and then correlated propagating network activity was assayed. One possibility is that the transition from excitatory to inhibitory GABA was unaffected by blockade of GABAergic excitation, but that the extended period of correlated propagating activity observed was due to other effects of the blockade of excitatory GABA during the first 6 days post-hatching or to the blockade of inhibitory GABA for the 21 days after the switch occurs.

We next showed that the maturation of inhibitory GABA occurred in retinal explants in the combined blockade of ionotropic neurotransmitter receptor activity, including GABARs, glutamate receptors, nAChRs, and glycine receptors, as well as the spiking activity of neurons in the GCL. Generally depolarizing dissociated hippocampal cultures by maintaining them in a high potassium extracellular solution drove the transition earlier, indicating that the source of depolarization may not be important (Ganguly et al., 2001). Our results indicate that the GABA switch is independent of depolarizing activity in the retina. This is in contrast to the model
proposed by Ganguly and colleagues that general depolarization may be sufficient to drive the switch in the absence of GABAergic signaling.

The chronic blockade of L-type voltage-gated calcium channels with nimodipine delayed the transition from excitatory to inhibitory GABA in dissociated hippocampal cultures (Ganguly et al., 2001; Leitch et al., 2005), suggesting that depolarization leads to the influx of calcium through voltage-gated calcium channels. Thus the current model is that independent of the source of the ionotropic receptor-mediated activity, the activity serves as a source of depolarization causing calcium influx via voltage-gated calcium channels. This calcium influx is essential for regulating the timing of the transition from excitatory to inhibitory GABA action, but a delayed transition still occurred in the dissociated hippocampal cultures even without L-type VGCC activity.

Is calcium influx through voltage-gated calcium channels necessary for the transition of excitatory to inhibitory GABA in the mouse retina? We showed that the maturation of inhibitory GABA occurred in retinal explants maintained in the presence of antagonists that blocked all spontaneous calcium transients. These results indicate that in the retina the transition from excitatory to inhibitory GABA is an activity-independent process and suggest that the transition is regulated by either a cell-autonomous process or by cell-cell signaling.

Activity-independent signaling is important for the excitatory to inhibitory GABA switch
Purified RGCs in culture have been used to study a number of processes in the function of neurons: axons and dendrites outgrowth, cell survival and development, cell-cell interactions and synapses formation (Meyer-Franke et al., 1995; Finlay et al., 1996; Goldberg et al., 2002; Ullian et al., 2004; Romano and Hicks, 2007). Here, we have used purified RGC cultured in serum-free defined medium to ask if the developmental transition from excitatory to inhibitory GABA is a cell-autonomous process or if it is dependent on signaling from other cells. We found that RGCs isolated from P2 mice and cultured for up to three weeks continued to be depolarized by GABAAR activation. We take this as evidence that a signal from another cell type is needed for the transition. The results, taken together with the in vivo and in vitro activity blockade results, lead us to conclude that the developmental transition from excitatory to inhibitory GABA is independent of activity but requires signaling from other retinal cells or from target cells in the superior colliculus.

One likely candidate for the source of the cell-cell signal regulating the timing of the transition from excitatory to inhibitory GABAergic transmission is astrocytes. Astrocytes differentiate at the same times as neurons during late embryonic/early postnatal development in the mammalian CNS and neuron-astrocyte interactions are critical to several aspects of neuronal differentiation, neuronal migration, neurite outgrowth, and axon guidance. Astrocytes have also been found to regulate both electrical and chemical forms of membrane excitability differentiation on embryonic central neurons, and provide an environment conducive to synapse formation in vitro (reviewed in Li et al., 1999)). Glial cells in culture and in vivo are known to express
GABAARs with properties similar to those in neurons (Kaila, 1994) and it has been shown that astrocytes accelerate the developmental change in the chloride gradient in neurons without influencing changes in the intrinsic properties of the channels (Li et al., 1998).

Broader implications of understanding the mechanisms underlying the transition from excitatory to inhibitory GABAergic transmission

In the developing brain, GABAergic excitation and subsequent inhibition are essential for a number of important processes and GABAergic signaling develops very early in the cortex. GABAAR-mediated depolarization regulates a number of key developmental steps early in CNS development: cell proliferation, migration, division, and neuronal differentiation; cell survival; neurite outgrowth; and synapse formation (reviewed in Owens et al., 1999). GABAergic inhibition is important during development as well as it has been shown to interact synergistically with cholinergic activity to guide the development of the morphology of neurons and the synaptic contacts they receive (Liu et al., 2006).

In the adult brain, the exquisite balance between excitation and inhibition is not only a fundamental mediator of neuronal information processing, it is an important physiological condition that must be maintained to avoid pathological consequences. Antagonists of GABAergic transmission generate seizures while enhancers of
inhibition have sedative, anticonvulsant, and anxiolytic actions. At the same time, excessive excitatory synaptic transmission leads to excitotoxicity and devastating neurological disorders (Kaila, 1994; Payne et al., 2003; Represa and Ben-Ari, 2005; Farrant and Kaila, 2007).

Brain traumas from concussion, epileptic seizures, or hypoxia-ischemia have been shown to cause changes in GABA$_A$R-mediated responses: after trauma, GABA$_A$R activation has been shown to be depolarizing, resulting from an increase in intracellular chloride coinciding with significant decreases of 60-80% of KCC2 expression (reviewed in van den Pol et al., 1996; reviewed in Payne et al., 2003; Represa and Ben-Ari, 2005). Elevated intracellular chloride seems to be primarily caused by changes in KCC2 expression, since no change in NKCC1 transcript or protein was detected in two trauma models. An increased concentration of intracellular chloride was also seen in spinal cord motor neurons after axonal injury and coincided with a significant reduction in KCC2 mRNA (Nabekura et al., 2002). In human hippocampal slices made from individuals with temporal lobe epilepsy, depolarizing GABA-mediated responses in pyramidal neurons in the subiculum were involved in the generation of spontaneous, interictal-like discharges (Cohen et al., 2002). There is also evidence that neuronal stressors, such as oxidative stress, hyperexcitability, and the induction of seizure activity, directly modulate KCC2 activity by rapid changes in the phosphorylation of the protein that precedes decreases in protein or mRNA expression (Wake et al., 2007).
The mechanisms that regulate the excitatory and inhibitory actions of GABA during development may provide insight into the effects of many antiepileptic, psychotropic, and sedative drugs that could potentially perturb brain maturation through their actions on GABA function. Understanding the developmental transition from excitatory to inhibitory action of GABA might also provide insight into the physiological function of the adult brain and into the pathological consequences of modulating GABAergic transmission.
Figure III-1: Neurons in the GCL exhibit spontaneous correlated activity and express functional GABA<sub>A</sub>Rs after prolonged activity blockade in retinal explants

A) Fluorescence image of a retinal explant isolated at P2 and maintained for 10 DIV before bolus loading of Oregon Green BAPTA-1-AM. This retinal explant was cultured in the presence of antagonists for glutamate receptors (DNQX, 20 µM; D-AP5, 50 µM), nAChRs (DHβE, 8 µM), glycine receptors (strychnine, 4 µM), GABA-A-Rs (Gabazine, 12.5 µM), GABA-B-Rs (CGP54626, 2 µM), and GABA-C-Rs (TPMPA, 50 µM) as well as voltage gated sodium channels (TTX, 2.5 µM). Scale bar = 20 µm.

B) Timecourse of spontaneous changes in ΔF/F for 6 example ROIs following the rinse (LEFT: “ACSF”) and re-application (RIGHT: “NT antag + TTX”) of the antagonists in which the explant in panel A was cultured.

C) Whole-cell voltage clamp recording from an RGC in a retinal explant isolated at P2 and maintained for 10 DIV in antagonists listed in A. Currents were recorded in response to short applications (200 ms) of musimol (TOP traces, 100 µM) or muscimol (100 µM) plus gabazine [25 µM] and TPMPA [50 µM] (BOTTOM traces). The cell was held at -60 mV and stepped to a holding potential between -100 mV and +20 mV (in 20 mV increments) for 7 sec.
**A**

**B**

**ACSF**

1  
2  
3  
4  
5  
6  

**C**

- **Muscimol**
  - Control
  - +20 mV
  - -100 mV
  - 500 pA
  - 400 ms

- **+Gbz + TPMPA**
  - +20 mV
  - -100 mV

**NT antag + TTX**

\[ \Delta F/F = 5\% \]

\[ 60\% \]
Figure III-2: Retinal explants recapitulate the GABA switch in vitro

Summary of calcium imaging experiments in retinal explants isolated at P2 and imaged after culture for X DIV. Left y-axis (open squares): % responsive cells to muscimol-induced Ca$^{2+}$ transients; threshold ΔF/F of seven standard deviations above baseline noise. Right y-axis (black circles): average amplitude of ΔF/F to muscimol application in all cells. Error bars are +/- SEM. In all experiments antagonists to glutamatergic, cholinergic, glycnergic, and GABA$_B$Rs (DNQX [20 µM], D-AP5 [50 µM], DHβE [8 µM], Strychnine [4 µM], CGP54626 [2 µM]) were included in the bath to block indirect depolarization of neurons.
Figure III-3: Activity blockade in retinal explants does not delay the maturation of inhibitory GABA

Summary of calcium imaging experiments in retinal explants isolated at P2 and imaged after culture for 7-11 DIV in antagonists to various forms of activity. GABA-mediated activity was blocked (“GABA Block”) with antagonists of GABA_α, GABA_β, and GABA_γRs (Gabazine, 12.5 µM; CGP54626, 2 µM; TPMPA, 50 µM). Synaptic activity was blocked (“Synaptic Block”) with antagonists for glutamate receptors (DNQX, 20 µM; D-AP5, 50 µM), nAChRs (DHβE, 8 µM), glycine receptors (strychnine, 4 µM), GABA_α/β/γRs (listed in GABA Block), as well as voltage gated sodium channels (tetrodotoxin, 2.5 µM). All Ca^{2+} transients were blocked (“Total Block”) using “synaptic block” with the addition of the gap junction antagonist carbenoxolone [100 µM], and the L-type voltage-gated calcium channel antagonist nifedipine [100 µM].

Left y-axis (open squares): % responsive cells to muscimol-induced Ca^{2+} transients. Right y-axis (black circles): average amplitude of ΔF/F to KCl application in all cells. Error bars are +/- SEM.

INSET: Fluorescence transients recorded in an 11 DIV retinal explant. LEFT: cultured in “synaptic block” antagonist cocktail. RIGHT: cultured in “total block” antagonist cocktail. Vertical scale bar = 5% ΔF/F, horizontal scale bar = 60 sec.
7-11DIV

Spontaneous Ca$^{2+}$ transients in explants

- Synaptic Block
- Total Activity Block

% responsive neurons (%)

%ΔF/F to KCl Puff (°)

CTRL  GABA Block  Synaptic Block  Total Block
Figure III-4: Purified RGCs remain depolarized by GABA

A) Differential interference contrast image of an RGC purified at P2 and cultured in serum-free medium for 24 DIV. Scale bar = 10 μm. B) Timecourse of ΔF/F at the soma of an RGC bath loaded with Oregon Green BAPTA-1 AM. Arrows: 500 ms applications of KCl [100 mM] or the GABA\(_{A/R}\) agonist muscimol [100 μM]. C) Summary of muscimol-induced Ca\(^{2+}\) transients in purified RGCs. Left y-axis (open squares): % responsive to muscimol application. Right y-axis (black circles): average amplitude of ΔF/F to muscimol in responsive cells. Error bars are +/- SEM.
IV. References


