THE UNIVERSITY OF CALGARY

GABA and Retino-Tectal Development

by

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ABSTRACT

Gama-aminobutyric acid (GABA), a neurotransmitter, regulates neurite outgrowth of a number of neuronal cell types *in vitro*. This study examined its role in neurogenesis in the developing *Xenopus laevis* visual system. Wholemount immunohistochemistry of the embryonic *Xenopus* brain revealed a population of GABAergic cells within the developing optic tract and optic tectum. I showed that GABA expression in the optic tract coincides spatially and temporally with developing retinal ganglion cell (RGC) axons. I demonstrated that developing RGC growth cones express GABA-A and GABA-B receptors. Stimulation of neurite outgrowth was observed when RGC cultures were incubated in either GABA or the GABA-B receptor agonist baclofen. Muscimol, a GABA-A receptor agonist reduced neurite outgrowth in RGC cultures. *In vivo*, exogenous application of GABA resulted in shortened optic projections, an effect mimicked by baclofen. Taken together, these results support the hypothesis that GABA acts as an extrinsic factor for developing RGC axons.

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This thesis is dedicated to my wife Tricia and my two sons Ewan and Kade.

My success is a reflection of the selfless love and dedication you give to me.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
Bon	basal optic nucleus
BSA	bovine serum albumin
CACA	cis-4-aminocrotonic acid
CAM	Cell adhesion molecule
cAMP	cyclic AMP
cGMP	cyclic GMP
ch	optic chiasm
CMZ	ciliary marginal zone
CNS	central nervous system
DAB	diaminobenzidine
Di	diencephalon
DTT	1,4-Dithithreitol
ECM	Extracellular matrix
FITC	Fluoroscein isothiocyanate
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GBR1	GABA-B receptor1
GBR1a	GABA-B receptor 1a
GBR1b	GABA-B receptor 1b
GBR2	GABA-B receptor 2

Hb	hindbrain
HRP	horseradish peroxidase
Hy	hypothalamus
INL	inner nuclear layer
IPL	inner plexiform layer
kDa	kilodalton
Mb	midbrain
MBS	Modified Barth's Saline
mot	mid-optic tract
NT3	neurotrophin-3
OCT	Optimal Cutting Temperature
ON	optic nerve
OPL	outer plexiform layer
ONH	optic nerve head
ONL	outer nuclear layer
ot	optic tract
PBS	phosphate-buffered saline
PBT	phosphate-buffered saline/ triton/bovine serum albumin
PE	pigment epithelium
Pin	pineal body
pPD	*p*-phenylene diamine
RGC	retinal ganglion cell
RGCL	retinal ganglion cell layer

RITC	Rhodamine isothiocyanate
SC	spinal cord
SDS	sodium dodecyl sulfate
Tec	optic tectum
Tel	telencephalon
TEMED	tetramethylethylenediamine
TPMA	[(1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid]

OBJECTIVES AND HYPOTHESIS:

The neurotransmitter GABA appears early in development where it functions as a trophic signal for developing neurons (for reviews see Meier et al., 1991, Belhage et al., 1998, Sandell, 1998). The purpose of my study was to examine its role in the development of the *Xenopus laevis* visual system. Wholemount immuno-histochemistry of the embryonic *Xenopus* brain revealed a population of GABAergic cells within the developing optic tract and optic tectum (Roberts et al., 1987). Developing RGC axons extend through the optic tract towards the optic tectum, possibly using a GABA signal. To test this idea, I: 1) determined if GABA was expressed in the optic tract during the period of RGC axon growth (Chapter 3); 2) determined if RGC growth cones express GABA receptors (Chapter 3); 3) tested the effects of GABA and its receptor agonists both *in vitro* and *in vivo* by using RGC cultures and an exposed brain preparation (Chapter 4). The majority of previous studies that have investigated developmental roles for GABA have been performed in culture (Meier et al., 1991, Belhage et al., 1998, Sandell, 1998). This is one of the first studies testing the functions of GABA *in vivo*.

General Hypothesis

RGC axons require GABA for directed growth towards their central target the optic tectum.

Specific Hypothesis I

GABA expression in the optic tract coincides spatially and temporally with developing RGC axons Further, that developing RGC axons and growth cones express GABA receptors.

Specific Hypothesis II

GABA functions as a cue for developing Xenopus RGCs in vitro and in vivo.

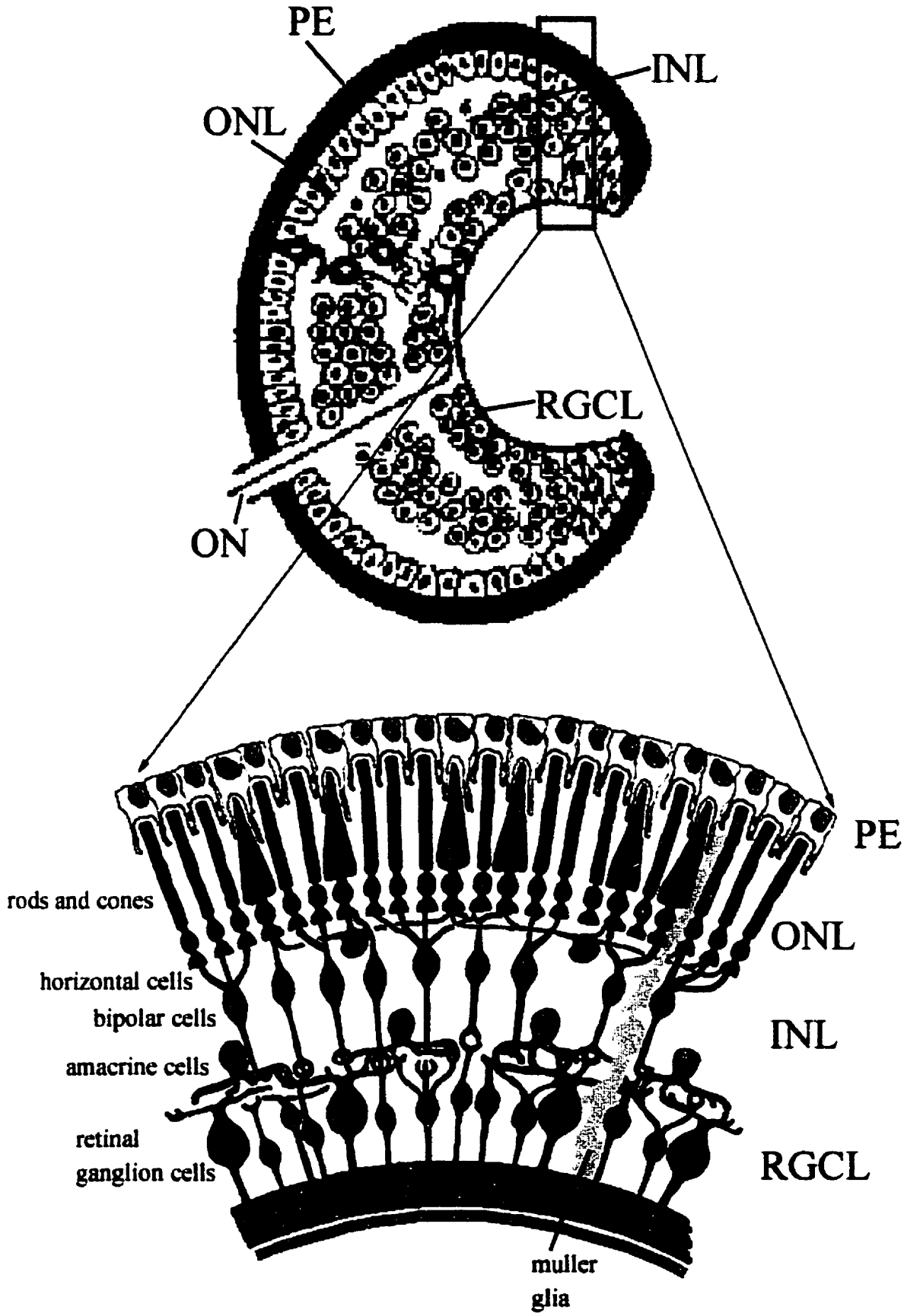
CHAPTER 1: INTRODUCTION

Vertebrate retinotectal system development:

During development of the vertebrate retina, newly generated neurons and glia migrate to their final relative positions to form a distinct layered structure (Cepko et al., 1996). The cells that make up the layers of the retina as well as their connectivity is depicted in Figure 1. The outer nuclear layer (ONL) of the retina is composed of rod and cone photoreceptor cells, which are responsible for the conversion of light energy into chemical energy. The rods and cones transduce signals through synapses formed with two types of interneurons, bipolar and horizontal cells of the inner-nuclear layer (INL). Further connections between interneurons within the INL are formed between bipolar cells and amacrine cells. In addition to these interneurons, the major glial cell of the retina, the Müller glia is found in the INL. The amacrine cells complete the retinal circuit by synapsing onto the dendrites of retinal ganglion cells (RGCs), which along with some displaced amacrine cells make up the final layer of the retina, the retinal ganglion cell layer (RGCL) (Cepko et al., 1996).

During its development, not only is the retina faced with generating the appropriate cells that make up these distinct layers, but it must also develop precise connections with the brain to create a functional visual system. To accomplish this task, newly generated RGCs initiate and extend long axons that gather at the optic nerve head and exit the eye forming the optic nerve. These RGC axons enter the brain and grow along the optic tract toward the visual centers of the brain; the superior colliculus in higher vertebrates and optic tectum in lower vertebrates (Tuttle et al., 1998; Shatz and

Figure 1: Transverse section drawing of a Stage 40 *Xenopus laevis* retina. Abbreviations are as follows: ONL (outer nuclear layer) is composed of rod and cone photoreceptor cells; INL (inner nuclear layer) is composed of horizontal, bipolar, and amacrine cells, as well as Müller glial cell bodies; RGCL (retinal ganglion cell layer); ON (optic nerve); PE (pigment epithelium). (Adapted from Holt et al., 1988).


PE
INL
ONL
RGCL
ON
PE
rods and cones
ONL
horizontal cells
bipolar cells
INL
amacrine cells
retinal
ganglion cells
RGCL
muller
glia

Kliot, 1982; Holt and Harris, 1983). RGC axons then innervate these regions and form synaptic connections with their appropriate cellular targets.

Xenopus retinotectal development

Xenopus laevis, the South African claw-toed frog, is a good model for studying RGC and retinotectal system development for several reasons (Chien and Harris, 1994). First, fertilization of eggs occurs *ex utero* and thus allows for easy, observable development and manipulation of embryos. Second, the development of the embryo is very rapid. Within three days post-fertilization (stage 40) (Nieuwkoop and Faber, 1994), the embryonic retino-tectal system has fully developed and the embryo can respond to simple visual stimuli. The developmental time course of the optic projection to the tectum has been resolved in *Xenopus* through the use of anatomical tracing methods. RGC axons grow very close to the surface of the brain, allowing their development to be easily visualized using anterogradely-transported markers (see Chien and Harris, 1994 for references). RGCs are generated at approximately one day of development (stage 24) (Chien and Harris, 1994; Holt et al., 1988), and they begin to initiate axons that extend across the surface of the retina towards the optic nerve head during the second day of development (stages 28-31). The axons exit the eye and enter the brain at the ventral diencephalon where they will then project dorsally through the optic tract (stages 32-34). By the third day of development, the RGC axons reach the mid-optic tract where they make a characteristic caudal turn towards the optic tectum (stages 35-38). Near the end of the third day the axons reach the optic tectum where they will make precise synaptic

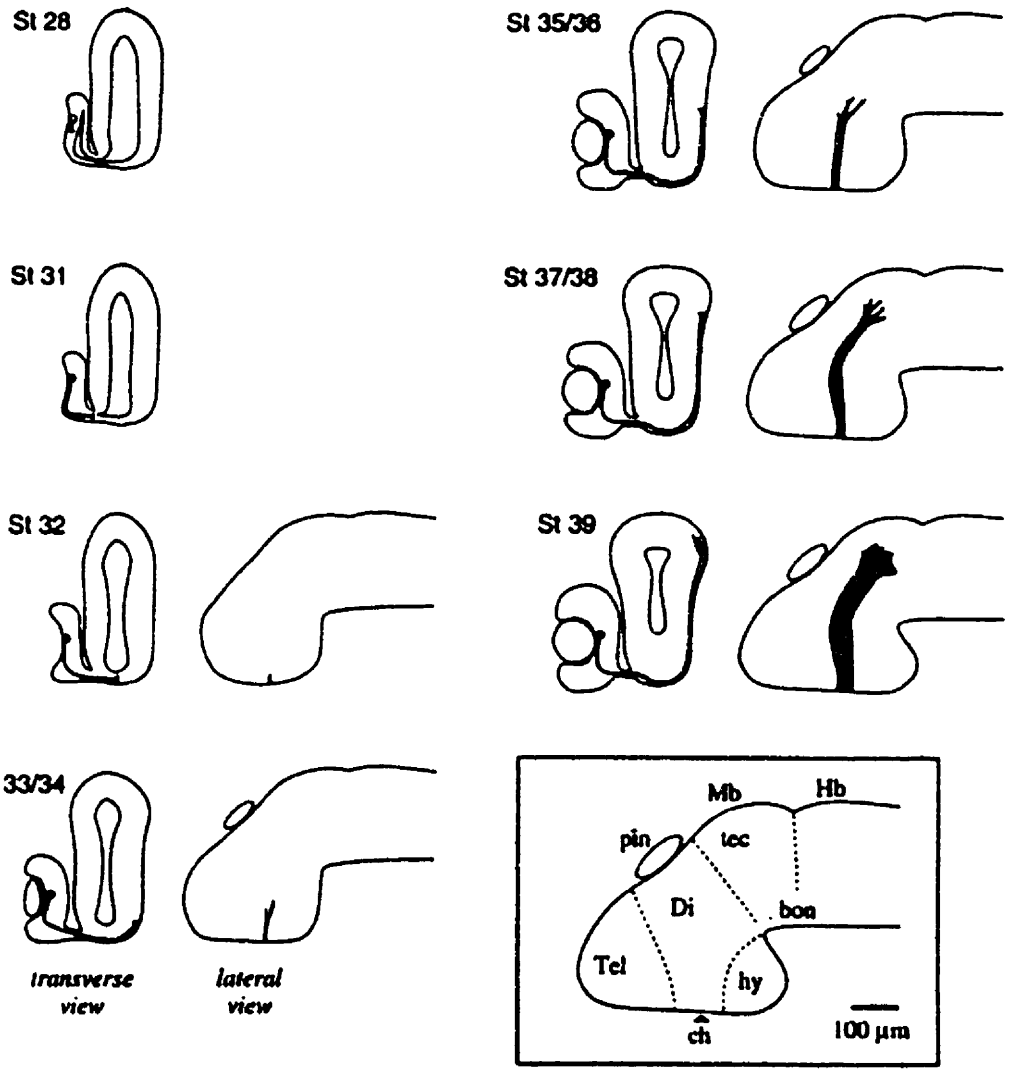
connections (Chien and Harris, 1994, Harris et al., 1985) (Figure 2). Time-lapse video recordings of live RGC axons labeled with the fluorescent membrane dye DiI have demonstrated that these axons grow at a constant rate, slowing down at critical choice points such as before they enter the optic tectum (Harris et al., 1987). From these studies with *Xenopus* it is apparent that the vertebrate retino-tectal system develops in a stereotypical and precise fashion with very few errors.

Axon guidance and neural connectivity

A critical stage in the precise neural connectivity of RGCs, or neurons in general, involves the growth of axons through complex environments toward their correct synaptic targets (Goodman, 1996, Tessier-Lavigne and Goodman, 1996, Mueller, 1999). The ability of axons to steer through their environment until they reach their proper target is called pathfinding. Axons can precisely pathfind because they are equipped with a specialized growth cone structure at their extending tip. The environment through which developing axons extend is laden with extrinsic cues that may promote axon extension (growth), and/or provide directional information (guidance) for growing axons. It is the growth cone that has a central role in detecting the extrinsic cues necessary for axon growth and guidance.

Growth cones are considered sensory, locomotory organelles and are active sites of membrane assembly. The body of the growth cone is rich in mitochondria, endoplasmic reticulum, vesicular structures, and microtubules that give structural support for axoplasmic transport (Levitan and Kaczmarek, 1997). Surrounding the body of the

Figure 2: Optic projection development in the *Xenopus* embryo: Stages 28-39. Transverse view through the brain and eye shows retinal ganglion cell (RGC) axonal development. The transverse view shows the position of RGC axons as they enter into the contralateral brain ventrally and extend toward their midbrain target, the optic tectum (tec). Lateral view (stages 32-39)(anatomical areas indicated in box) is shown once RGC axons cross into the contralateral brain (stage 32). The lateral view at stage 35/6 shows that once RGC have reached the mid-diencephalon, they turn toward their midbrain target the optic tectum. Additional abbreviations are as follows: Tel (telencephalon); Di (diencephalon); hy (hypothalamus); bon (basal optic nucleus); Hb (hindbrain); Mb (midbrain); pin (pineal body); ch (optic chiasm). (Adapted from Chien and Harris, 1994).


St 28
St 35/36
St 31
St 37/38
St 32
St 39
St 33/34
transverse view
lateral view
Mb
Hb
pin
tec
Di
bon
Tel
hy
ch
100 µm

growth cone is a region rich in actin protein that forms a web-like structure called lamellipodia, with finger-like extensions called filopodia (Figure 3). These actin-rich structures respond quite sensitively to extrinsic signals due to their relatively small intracellular volume (Kater et. al., 1994). Growth cones detect extrinsic cues by expressing specific receptors for a particular cue that when activated can lead to structural changes in the growth cone s cytoskeleton and ultimately changes in motility. A growth cone advances when filopodia extend, then remain in place while the lamellipodia advance toward the distal end of the filopodia. The rate at which axonal growth cones extend toward their targets depends upon the substrate and extrinsic factors expressed along the pathway and how the growth cone responds to them.

In general, extrinsic cues encountered by growth cones are considered either positive or negative, and promote or inhibit growth respectively (Figure 4). Positive cues can also span the range of being merely a permissive substrate for axon growth, to acting as an attractive guidance signal that directs the growth cone towards its source. Negative cues can vary from being simply repulsive (i.e. growth cones are repelled but continue to grow away from the cue), to strongly inhibitory (i.e. stop growth cone motility, or in some cases growth cones collapse and retract) (Goodman, 1996, Tessier-Lavigne and Goodman, 1996). Some molecular cues are attached to the substrate or cell membrane, and require a short-range direct membrane contact by the growth cone to exert their actions. Alternatively, extrinsic cues may be diffusible, acting in long-range chemoattactant or chemorepulsive gradients (Goodman, 1996, Tessier-Lavigne and Goodman, 1996, Mueller, 1999). This apparently distinct classification of cues is often blurred as extrinsic cues that act in a long-range diffusible manner could be converted

Figure 3: Schematic of a neuronal growth cone. The body of the growth cone is rich in microtubules that provide structural support. Surrounding the body of the growth cone is an actin filament region that gives rise to lamellipodia and thin finger-like filopodia. (Adapted from Mueller, 1999).

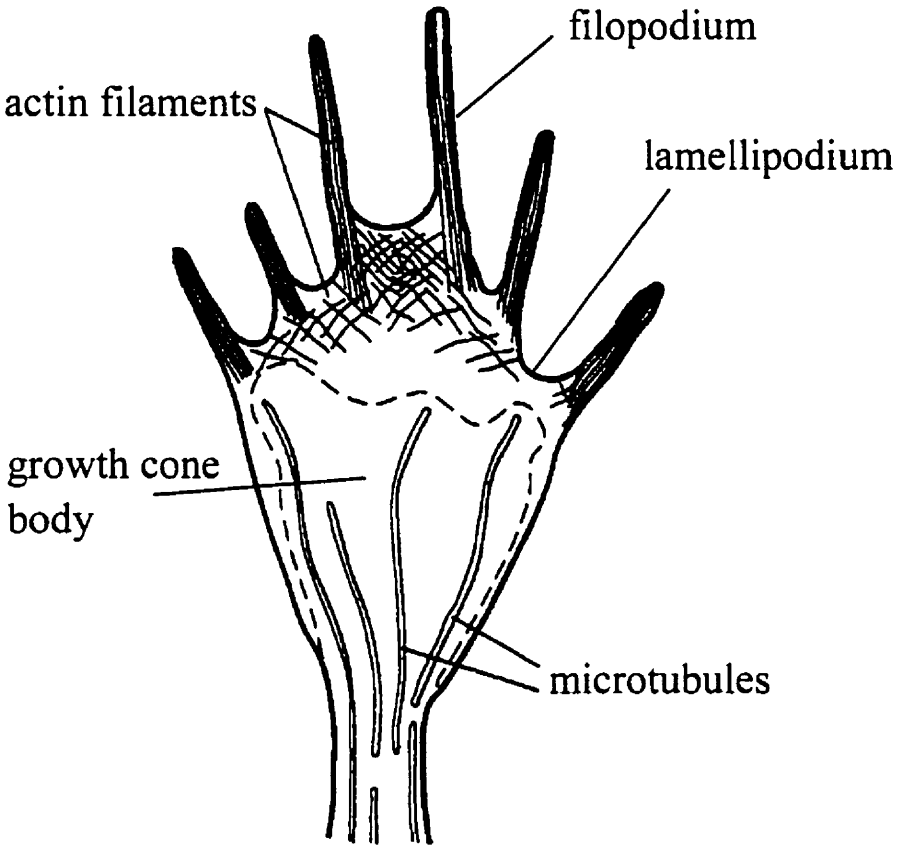
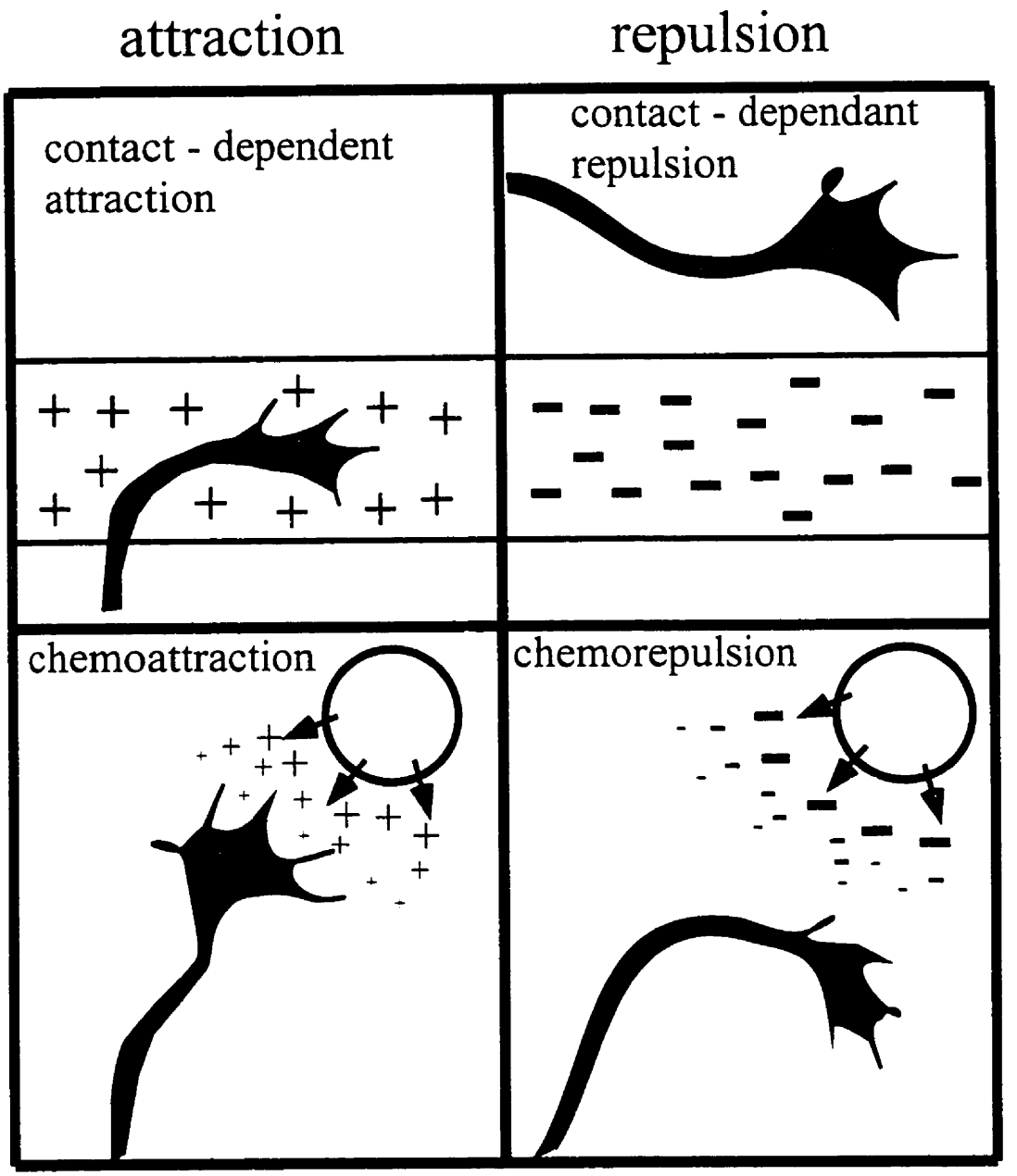

filopodium
actin filaments
lamellipodium
growth cone
body
microtubules

Figure 4: General mechanisms of growth cone guidance. Mechanisms are classified as attractive or repulsive, and acting over long range- (chemoattraction or chemorepulsion) or short range (contact-dependent attraction or repulsion). Positive and negative signs indicate attraction and repulsion respectively. Signs decreasing in size indicate a decreasing concentration gradient from a point source (circle with arrows).(Adapted fromTessier-Lavigne and Goodman, 1996, Goodman, 1996).


attraction
repulsion
contact - dependent attraction
contact - dependant repulsion
chemoattraction
chemorepulsion

into short-range and contact-dependent if the cue becomes bound to the substrate or cell surface machinery. In addition, substrate or cell membrane-attached cues could be spatially arranged in a gradient thus acting similar to long-range diffusible cues (Goodman, 1996, Tessier-Lavigne and Goodman, 1996, Mueller, 1999).

For any extrinsic molecule to be considered a cue important for neuronal connectivity, a number of specific criteria must be met (McFarlane and Holt, 1996). First, the molecule should be expressed both during the period of neuronal development (temporal expression) and within the regions where neurons are developing (spatial expression). Second, the specific receptors that bind a particular cue(s) should be expressed on the developing neuronal processes (axons and/or dendrites) and growth cones. Third, the molecule should have an effect on the extension of neurites in culture, and/or act attractively or repulsively to direct outgrowth (chemotropism) of these neurons. Finally the last criterion to be met is that changing the expression of the molecule or altering the signaling through its receptors should affect the development of the neurons *in vivo*. Table 1 represents a list of some molecules that appear to function as extrinsic cues for axonal outgrowth.

Neurotransmitters

Classically, neurotransmitters are used for communication in the mature nervous system, functioning as chemical messengers between specific electrically excitable cells that make up distinct neuronal pathways (Redburn and Rowe-Rendleman, 1996). Generally neurons terminate their axons onto the dendrites or cell bodies of neighbouring (postsynaptic) cells forming synapses. Mature neurons secrete neurotransmitters at their

Table 1: A list of some classes of molecules that have been identified as possible extrinsic cues for developing axons. Their effect on growing axons is also reported. Neurotransmitters such as acetylcholine have been shown to inhibit neurite outgrowth in some developing neurons in culture, whereas GABA can induce neurite outgrowth in certain neuronal cultures. Acetylcholine has also been shown to act as a chemoattractant molecule for embryonic *Xenopus* spinal neurons *in vitro*. (Adapted from McFarlane and Holt, 1997).

Guidance molecule	Effects on growing axons	Reference
Cell adhesion molecules (CAMs)		
Cell-cell		
Cadherins	Stimulate neurite outgrowth *in vitro*. Expressed *in vivo*.	Bixby et al., 1991 Keynes and Cook, 1995
Cell-matrix		Bixby et al., 1991
Extracellular matrix (ECM)- i.e. tenacin, fibronectin, laminin,etc.	Support or inhibit neurite outgrowth. Expressed *in vivo*.	Tessier-Lavigne and Goodman, 1996 Keynes and Cook, 1995
Netrins		Tessier-Lavigne
Netrin-1	Bifunctional- acts as chemoattractants and chemorepellents.	and Goodman, 1996 Goodman, 1996 Mueller, 1999
Semaphorins		
Sema-III Sema-D	Chemorepellant	Tessier-Lavigne and Goodman, 1996 Goodman, 1996 Mueller, 1999
Ephrins		
Ephrin-A-2 Ephrin-A-5	Chemorepellant	Tessier-Lavigne and Goodman, 1996 Mueller, 1999
Neurotransmitters		
Acetylcholine	Chemoattractant can also inhibit neurite outgrowth.	Zheng et al., 1994 Owen and bird, 1995
GABA	Stimulate neurite outgrowth.	Spoerri, 1988

axon terminals, where it is packaged in membrane vesicles and made ready for release through calcium-mediated exocytosis (Kandell, Schwartz, and Jessell, 1991). Neurotransmitter release occurs when the cell is stimulated sufficiently to open calcium ion channels. As calcium enters the pre-synaptic terminal, the neurotransmitter-filled vesicles fuse with the membrane and release their contents into the synapse. The neurotransmitter diffuses towards its synaptic partner and binds to receptors expressed on the membrane surface of the postsynaptic cell. Transmitter receptors are either ligand-gated ion channels that directly allow the passage of ions into or out of the cell, or alternatively, metabotropic receptors (composed of seven membrane spanning subunits) that couple to ion channels indirectly. This second type of receptor binds the neurotransmitter and activates G proteins anchored in the membrane, which then interact with an ion channel causing them to open. The actions of metabotropic G-protein linked receptors are typically slower than the actions of the direct receptor ligand-gated channels. The result of neurotransmitters binding to their receptors is the passage of ions into and out of the cell, which produce the electrical activity of the cell, as well as generating and modulating action potentials that make up the language of the nervous system (Thompson, 1993).

In general, neurotransmitters are classified as either excitatory or inhibitory depending upon their ability to depolarize or hyperpolarize neuronal membranes (Levitan and Kaczmarek, 1997). Since resting membrane potential is generally around -60 mV, excitatory neurotransmitter actions result in a less negative potential (depolarization) that crosses the threshold for firing action potentials. Inhibitory neurotransmitter actions

produce a more negative potential (hyperpolarization) resulting in a cell that is less likely to fire action potentials.

Neurotransmitters are further classified by their chemical structures, as well as through their unique pathways for synthesis and degradation. For example, acetylcholine, one of the first known excitatory neurotransmitters is synthesized from acetyl-CoA and choline. The enzyme choline acetyltransferase facilitates the synthesis. Once released into the synapse, excess acetylcholine can be degraded by another enzyme acetylcholinesterase (Levitan and Kaczmarek, 1997). Antibodies generated against the synthesis and degradative enyzmes of neurotransmitters in general have assisted in identifying cells and regions of the CNS that express neurotransmitters.

Neurotransmitter regulation of neuronal outgrowth

It has been postulated that neurotransmitters also play a role in neural connectivity, specifically in the survival and regulation of axonal outgrowth of developing neurons (Lipton and Kater, 1989, Redburn and Rowe-Rendleman, 1996, Sandell, 1998, Erskine and McCaig, 1995). Neurotransmitters as well as their receptors are present very early in the developing nervous system (Lauder, 1988, 1993, Chen et al., 1995, Redburn and Rowe-Rendleman, 1996). These findings led to experiments testing the effects of several different neurotransmitters upon neurons developing *in vitro*. In neuronal cell cultures, several different neurotransmitters cause inhibition of growth or retraction of extending neurites (Haydon et al., 1984, McCobb et al., 1988, Lankford et al., 1988, Mattson et al., 1988, Owen and Bird, 1995). For example, when identified neurons from the snail *Helisoma* are exposed to the amine neurotransmitters serotonin

and dopamine in culture, filopodia retract, lamellipodial surface area decreases, and neurite outgrowth is inhibited (Haydon et al., 1984,1985, McCobb et al., 1988). In vertebrates, hippocampal pyramidal neurons isolated from embryonic rats display inhibition of dendritic outgrowth when cultured in the presence of sub-toxic levels of the excitatory amino acid neurotransmitter glutamate (Mattson et al., 1988). In addition, embryonic mouse spinal cord neurons cultured in the presence of 10 μM glutamate show inhibition of neurite outgrowth and reduced axon motility (Owen and Bird, 1997). Further, adding a specific glutamatergic kainate/AMPA receptor antagonist blocks these effects (Owen and Bird, 1997). Experiments with the excitatory neurotransmitter acetylcholine produce similar effects on neurite outgrowth and motility as those seen with glutamate (Owen and Bird, 1995). One interesting finding common to these studies is that the actions of these excitatory neurotransmitters are inhibitory to neurite outgrowth. Perhaps these effects are attributable to the types of neurons used in these studies. Other neurons could possibly have different responses to these neurotransmitters.

Neurotransmitter involvement in growth cone guidance

In addition to regulating neurite outgrowth, neurotransmitters also function as chemotropic molecules (Zheng et al., 1994, Zheng, Wan, and Poo, 1996, Kuffler, 1996). For example, Zeng and colleagues (1994) cultured isolated embryonic spinal cord neurons from *Xenopus* and demonstrated through a growth cone turning assay that these growth cones respond positively by orienting themselves toward an extracellular gradient of acetylcholine. This finding is significant in the context of this thesis since it suggests that if neurotransmitters are expressed along specific axonal pathways, they may serve as

chemoattractants for growth cone guidance. To further support this claim, experiments using patches of acetylcholine receptor-rich membranes to detect acetylcholine release demonstrated that isolated embryonic neurons from chick ciliary ganglion release acetylcholine from their growth cones (Hume et al., 1983). Similarly, growth cones of *Xenopus* embryonic neurons (Young and Poo, 1983) and developing central neurons from *Drosophila* (Yao et al., 2000) can also release acetylcholine that is detectable with receptor-rich membrane patches. These studies suggest that a wide variety of neurons may use neurotransmitter release for growth cone development and guidance (Erskine and McCaig, 1995).

Neurotransmitters and the developing vertebrate retina

Neurotransmitters are potentially important in the development of the retina since they, as well as their receptors, appear at the earliest stages of vertebrate retinal development (see reviews: Lipton and Kater, 1989, Redburn and Rowe-Rendleman, 1996, and Sandell, 1998 for references). It has also been shown that neurotransmitters can regulate outgrowth of embryonic retinal neurons in culture through the activation of specific receptors (Lankford et al., 1988). For example, neurons from embryonic chick retinae were cultured 1-7 days in medium containing the neurotransmitter dopamine (Lankford et al., 1988). Upon examination, 25% of the neurons had a reduction in filopodia activity that was followed by a flattening of the growth cone and a retraction of neurites. Neurite retraction was greatest in younger cultures. In addition, the effects of dopamine on these growth cones could be reversed by the addition of the specific D_1-type receptor antagonist haloperidol.

Acetylcholine release, which is apparent in various neuronal cultures (Hume et al., 1983, Young and Poo, 1983, Yao et al., 2000), also occurs in cultures of developing vertebrate retinal neurons (Lipton et al., 1988, Lipton, 1988). This endogenous release of acetylcholine in culture resembles the acetylcholine leak in the intact retina (Masland and Livingstone, 1976, Masland and Cassidy, 1987). The source of this acetylcholine has been shown to originate from amacrine cells that produce the neurotransmitter and synapse onto RGC dendrites (Masland and Mills, 1979, Masland et al., 1984). In this acetylcholine-rich medium, dissociated RGCs from neonatal rat pups show a significant reduction in the ability to produce neurites (Lipton et al., 1988). When antagonists that block specific receptors to acetylcholine are added to the culture medium, the number of RGCs producing neurites as well as the individual neurite lengths increase (Lipton et al., 1988). This apparent inhibition of process outgrowth, coupled to the evidence that acetylcholine leaks in the intact retina, suggests that it may be a possible mechanism used *in vivo* to delay or stabilize RGC dendrite outgrowth in order to allow appropriate synapse formation between amacrine and RGCs to occur (Lipton and Kater, 1989).

GABA the neurotransmitter and its receptors

Of the many neurotransmitters identified, gamma-aminobutyric acid (GABA) has been extensively studied in both the developing and the mature nervous system. GABA is the major inhibitory neurotransmitter in the mature vertebrate CNS and is part of the amino acid neurotransmitter family that includes excitatory glutamate and inhibitory glycine. GABA was first established as an inhibitory neurotransmitter through studies involving a crustacean muscle synapse (Kravitz et al., 1963, Levitan and Kaczmarek,

1997). It gained favourable attention as a possible neurotransmitter when since it is expressed in mammalian brains at greater levels than in any other tissue (Levitan and Kaczmarek, 1997). Twenty to fifty percent of CNS neurons use GABA at their synapses (Bloom and Iverson, 1971, Young and Chu, 1990). GABA is synthesized from glutamate in a catalytic reaction where the enzyme glutamic acid decarboxylase (GAD) cleaves off a carbon dioxide group from glutamate (Kandel, Schwartz, and Jessell, 1991). Early identification of GABAergic neurons resulted from the positive identification of GAD within these neurons (Levitan and Kaczmarek, 1997). Studies show that GABA is present at high concentrations within many regions of the CNS, where it is expressed by a number of different inhibitory interneurons (see Sivilotti and Nistri, 1991). Some of the inhibitory interneurons that release GABA include: the granule cells in the olfactory bulb, Purkinje cells in the cerebellum, basket cells from both cerebellum and hippocampus, and amacrine cells in the retina (Sivilotti and Nistri, 1991,Kandell, Schwartz and Jessell, 1991). As mentioned earlier, inhibitory neurotransmitters such as GABA function to hyperpolarize neuronal membranes.

The physiological inhibitory actions of GABA are produced via a family of GABA receptors, which are named GABA-A, GABA-B, and GABA-C:

GABA-A receptors are transmembrane ligand-gated chloride channels composed from various subunits. These include α_1-α_6, β_1-β_3, γ_1-γ_3, δ, ε, π, and ρ_{1-3}. The channel is a pentameric assembly from a combination of these various subunits (Mehta and Ticku, 1999). The channels are opened by GABA, as well as by the specific agonists muscimol and isoguvacine, and are inhibited by bicuculline, gabazine (SR 95531), and (+)-β-hydrastine (Mehta and Ticku, 1999). GABA-A receptor channel activity is also

modulated by a variety of drugs (Sivilotti and Nistri, 1991, Macdonald and Olsen, 1994, Sieghart, 1995, Mehta and Ticku, 1999). These receptors are widely distributed throughout the brain and spinal cord where they act to convey fast synaptic transmission important in mediating neuronal inhibition. GABA-A receptor inhibition results from a flow of negatively charged chloride ions into the cell causing a hyperpolarized potential.

GABA-B receptors are metabotropic G protein-linked receptors that are coupled to Ca^{2+} or K^+ channels (Bormann, 1988; Bowery, 1993). GABA-B receptors are activated by GABA, (−)-baclofen, (±)-4-amino-3- (5-chloro-2-thienyl) butanoic acid, and 3-aminopropyl- (methyl) phosphinc acid (SKF 97541), and are inhibited by phaclofen, saclofen, and 2-hydroxysaclofen (Mehta and Ticku, 1999). The early identification of these receptors showed that they existed in two isoforms, GBR1a and GBR1b (GABA-B receptor 1a and b), with molecular weights of 130 and 100 kilodaltons (kDa) respectively (Kaupmann et al., 1997). Recently, three independent groups have shown that the GABA-B receptor exists as a heterodimer with a second identified GABA-B receptor (GBR2), with a molecular weight of 110 kDa, that links up to the C-terminal region of GBR1 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). GABA-B receptors show a wide distribution throughout the brain and spinal cord (Clark et al., 1998). *In situ* hybridization studies show that GBR1 and GBR2 mRNA have overlapping expression patterns within the vertebrate CNS (Jones et al., 1998), indicating that corresponding proteins might be expressed in identical regions. GABA-B receptor activation increases K^+ channel conductance associated with postsynaptic sites (Luscher et al., 1997) and decreases Ca^{2+} channel conductance at presynaptic receptor sites (Chen and van den Pol, 1998, Takahashi et al., 1998). The postsynaptic flow of K^+ out of the cell results in a

long-lasting neuronal hyperpolarization, whereas the presynaptic decrease of Ca^{2+} into the terminal results in a decrease of neurotransmitter release (Misgeld et al., 1995, Bowery and Enna, 2000).

GABA-C receptors are similar to the GABA-A receptors in that they are ligand-gated Cl^- channels. However, they differ from both GABA-A and GABA-B receptors, as they are insensitive to both bicuculline and baclofen (Johnston, 1994, 1996, Bormann and Feigenspan, 1995, Bormann, 2000). The GABA-C receptor can be activated selectively by CACA (cis-4-aminocrotonic acid) and antagonized selectively by TPMA [(1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid] (Johnston, 1996,Bormann, 2000). In addition, GABA-C receptors are activated at much lower concentrations of GABA, the channels stay open for a longer period of time, and are less easily desensitized when compared to the other GABA receptors (Johnston, 1996). GABA-C receptors are composed of ρ-subunits that form homooligomeric chloride channels. The majority of localization studies of GABA-C receptors in the CNS have focused on the retina, where the receptors are localized on most retinal neurons (see Bormann, 2000 for references).

GABA as an excitatory neurotransmitter in development

Interestingly, evidence suggests that GABA may function as an excitatory neurotransmitter in early development (Cherubini et al., 1991, Owens et al., 1999), opposite to its inhibitory role in the mature CNS. During early stages of cortical development, glutamatergic-mediated excitation is present, with little inhibition (Agmon and O'Dowd, 1992, Agmon et al., 1996, Burgard and Hablitz, 1993, Kim et al., 1995, Luhmann and Prince, 1991). This lack of inhibition was thought to be a result of the

delayed maturation of a GABAergic signaling system (Owens et al., 1999), however there is accumulating evidence suggesting that GABA-mediated signaling develops quite early in the cortex (Owens et al., 1999). GABA, GAD, and various GABA-A receptor sub-units have been identified in embryonic cortical tissue (Cobas et al., 1991; Lauder et al., 1986; Laurie et al., 1992; Ma and Barker, 1995; Van eden et al., 1989). Studies in developing neo-cortex showed that membrane depolarization occurs when GABA-A receptors are activated (Agmon et al., 1996; LoTurco et al., 1995; Owens et al., 1996; Yuste and Katz, 1991).

One explanation for this depolarization is a relatively high concentration of chloride ions inside the developing neurons (Clayton et al., 1998; Owens et al., 1996; Rivera et al., 1999). Thus when GABA-A receptors are activated by GABA this results in an efflux of Cl^- ions and depolarization. This depolarization through GABA receptor signaling is a transient effect since intracellular Cl- concentrations decrease perinatally (Owens et al., 1999). Alternatively, the depolarizing effects of GABA-A receptors may be a result of developmental changes in channel structure or assembly (Cherubini et al., 1991). Previously it was shown that the minimal assembly for a functional GABA-A receptor is the composition of α, β, and γ_2 subunits (Sigel et al., 1990). It has also been shown that the $\alpha 1$ subunit of the GABA-A receptor is expressed later in development (Mishina et al., 1986). Since the functional properties of ion channels depend upon precise sub-unit composition (Levitan and Kaczmarek, 1997), a different combination and assembly of GABA receptor sub-units could possibly contribute to the depolarizing effects of GABA-A receptors in development.

Neurotrophic effects of GABA

Both GABA and its receptors are present during early vertebrate CNS development (Baclar and Johnston, 1987, Rozenberg et al., 1989, Van Eden et al., 1989 Laurie et al., 1992; Poulter et al., 1992), well before GABAergic synapses are formed (Coyle and Enna, 1976). A number of studies have established GABA as an important neurotrophic signal in the developing nervous system (for reviews see Meier et al., 1991, Belhage et al., 1998, Sandell, 1998). In a variety of developing neurons, GABA can modulate the mitosis of neuronal precursors, enhance synapse thickening, promote the expression of GABA receptors, enhance protein synthesis, increase the density of organelles, and increase neurite outgrowth (Spoerri and Wolff, 1981, Eins et al., 1983, Meier et al., 1984, 1991, Spoerri, 1988, Michler, 1990, Hansen et al., 1991, Wolff et al., 1993, Abraham et al., 1994, Ben-Ari et al., 1994, Loturco et al., 1995, Behar et al., 1994, Liu et al., 1997, Belhage et al., 1998). For example, when embryonic rat brainstem monoamine neurons are cultured in the presence of GABA, both survival and neurite growth were enhanced. This effect was blocked by the addition of bicuculline, a specific GABA-A receptor antagonist (Liu et al., 1997). In a separate study, cultured postnatal rat hippocampal neurons in the presence of bicuculline, showed a reduction in the number of primary neurites, branching points, and total neuritic length (Barbin et al., 1993). These studies support the idea that GABA functions as a molecular cue for developing neurons. Further, it has been shown that GABA is released from isolated rat neuronal growth cones (Lockerbie et al., 1985; Taylor et al., 1991), and from developing mouse hypothalamic neuronal growth cones (Gao and van den Pol, 2000). These findings

suggest that GABA may act similar to acetylcholine in assisting in axonal pathfinding. However, growth cone turning assays with GABA have not yet been performed.

GABA and receptor expression in the vertebrate visual system

GABA has been extensively studied in the adult vertebrate retina, where it was one of the first neuroactive substances isolated (Kuriyama et al., 1968). In the adult mammalian retina, it is expressed in multiple subclasses of amacrine cells (Ehinger, 1970; Brandon et al., 1979; Koontz and Hendrickson, 1990), and in one or more classes of horizontal cells (Marc et al., 1995). GABA has also been localized in the retinal ganglion cell layer (see Wilson et al., 1996 for references), where it has been suggested to occur in displaced amacrine cells (Wassle et al., 1990). However, several studies using retrograde cell labeling and RGC antibodies show that these GABAergic cells are RGCs (Yu et al., 1988, Gabriel et al., 1992). In the adult vertebrate retina, GABA functions as an inhibitory neurotransmitter to block acetylcholine release from amacrine cells (Massey and Redburn, 1982), as well as inhibit dopamine turnover in dopaminergic amacrine cells (Marshburn and Iuvone, 1981). In addition, GABA acts postsynaptically to inhibits RGCs via hyperpolarizing currents (Zhang et al., 1997).

All three types of GABA receptors have been found in the retina (for review see Lukasiewicz and Shields, 1998). GABA-A receptors are ubiquitously expressed in the retina, whereas both GABA-B and GABA-C receptors have distinct expression patterns (Lukasiewicz and Shields, 1998). GABA-A receptors cause a Cl^- conductance that provides a strong inhibition of excitatory currents (Tian and Slaughter, 1994). GABA-B receptors are localized on Müller glia and on bipolar, horizontal, amacrine, and ganglion

cells (Maguire et al., 1989, Koulen et al., 1998, Lukasiewicz and Shields, 1998, Zhang and Yang, 1999). In addition, GABA-B receptor subtypes show a differential expression on pre- and postsynaptic sites, possibly causing inhibition of neurotransmitter release as well as postsynaptic inhibition (Koulen et al., 1998). GABA-B receptors close Ca^{2+} channels on both bipolar cells and RGCs (Maguire et al., 1989, Zhang et al., 1997, Bindokas and Ishida, 1991), and open K^+ channels on amacrine and RGCs (Slaughter and Bai, 1989). GABA-C receptors are distinctly localized on terminals of rod and cone photoreceptor cells and bipolar cell bodies (Koulen et al., 1997, Shields et al., 2000), where they open Cl^- channels.

Developmental expression of GABA and its receptors in the vertebrate visual system

GABA is also expressed during retinal development in several vertebrate species (Sandell et al., 1994; Schnitzer and Rusoff, 1984; Messersmith and Redburn, 1992; Hendrickson et al., 1994). Its expression has been localized to amacrine, horizontal, and RGCs (Messersmith and Redburn, 1992). One of the common findings from these studies is that GABA expression is higher during early retinal development than in the mature retina. In fact, in the rabbit there is an approximate 50% decrease in the density of GABAergic cells in the retinal layers between birth and post-natal day 20 (Redburn, 1992; Messersmith and Redburn, 1992, 1993). Although all three GABA receptor types have been identified within the mature vertebrate retina (Friedman and Redburn, 1990; Grunert and Hughes, 1993; Greferath et al., 1993,1994; Qian and Dowling, 1994), much less is known about the developmental expression of GABA receptors in the retina

(Sandell, 1998). Recently it was shown in the developing rabbit retina, that GABA-A receptor subunits are transiently expressed in cone photoreceptors (Mitchell et al., 1999). Using an antibody that labels GABA-A receptor subunits, immunoreactivity decreased within cone photoreceptor somata after postnatal day 5, and in cone processes after day 7 (Mitchell et al., 1999). The transient expression of GABA as well as GABA-A receptors suggests that GABA may function as an important developmental regulator of cone photoreceptor cells.

GABA is also expressed transiently in the developing optic nerve and optic tract in several species (Sakatani et al., 1991,1992, Sandell et al., 1994, Rogers and Pow, 1995, Messersmith and Redburn, 1992). For example, GABA immunoreactivity is observed in axons and glial cells of the rat optic tract before the axons are myelinated but not after myelination (Sakatani et al., 1992). In addition, GABA-A and GABA-B receptors are present within the developing optic nerve (Sakatani et al., 1991, 1992; Sun and Chiu, 1999). These studies also showed that GABA as well as specific GABA-A receptor agonists could cause depolarization of neonatal optic nerves, an effect blocked by the GABA-A receptor antagonist bicuculline. Further, adult optic nerves showed no significant effects when GABA, or its GABA-A receptor agonist, were applied (Sakatani et al., 1991, 1992). In addition, developing rat optic nerve axons show fast calcium transients when depolarized (Lev-Ram and Grinvald, 1987). This influx of calcium can be inhibited when baclofen, a specific GABA-B receptor agonist is applied to the developing optic nerve axons (Sun and Chiu, 1999).

Summary

The evidence that GABA is present early in development of the vertebrate visual system supports the hypothesis that GABA may have a role in the development of neurons within the retina and the optic tract. Trophic actions of GABA upon the retina have been seen both *in vitro* and *in vivo* (Spoerri, 1988; Madtes and Redburn, 1983). When embryonic chick retinae were cultured in the presence of GABA, retinal neurons proliferated, and both the length and branching of neurites increased when compared to control cultures (Spoerri, 1988). In addition, when GABA was exogenously applied to neonatal rabbit retina *in vivo*, GABA receptor expression was upregulated (Madtes and Redburn, 1983).

In the embryonic retinotectal system of *Xenopus laevis*, Roberts and colleagues (1987) demonstrated through immunohistochemistry that a population of mid-optic tract cells and tectal cells express GABA. The onset of GABA expression within the optic tract and tectum appears at about stage 29/30 (2 days post-fertilization) (Roberts et al., 1987). By stage 37/38, cells in the optic tract, as well as the tectum are intensely stained with GABA (Roberts et al., 1987). During these stages, RGC axons are extending through the optic tract towards the optic tectum. Thus, GABA expressed in the optic tract at these developmental stages may influence the development of RGC axons.

The majority of the studies that have investigated developmental roles for GABA have been performed in culture. *In vivo* testing of early developmental functions of GABA needs to be addressed. *I therefore hypothesize that RGC axons require GABA for directed growth towards their central target the optic tectum.* Addressing the following specific hypotheses will test this hypothesis:

Specific Hypothesis #1:

GABA is expressed in the optic tract when RGC axons are entering and pathfinding through the optic tract toward the tectum. Further, that developing RGC axons and growth cones express GABA receptors.

Specific Hypothesis #2

GABA functions as a molecular cue for developing Xenopus RGCs in vitro and in vivo.

CHAPTER 2: MATERIALS AND METHODS

Embryos:

Embryos were obtained from our breeding colony of *Xenopus laevis* at the University of Calgary. Pairs of male and female frogs were induced to mate by injecting both male and female frogs with the hormone Human Chorionic Gonadotropin (Sigma). The embryos were collected and reared in 10% Holtfreters solution (Holtfreter, 1943) at room temperature and were staged according to Nieuwkoop and Faber tables (Nieuwkoop and Faber, 1994). Stage 24-40 embryos were used for the experiments.

HRP Labeling of the Optic Projection:

To visualize the optic projection, horseradish peroxidase (HRP) (Sigma) fills were performed as described previously (Cornel and Holt, 1992). Briefly, stage 32-40 embryos were anesthetized in Modified Barth's Saline (MBS) containing 0.4 mg/ml ethyl 3-aminobenzoate methanesulfonic acid (MS-222, Aldrich, Milwaukee, WI), 50 mg/ml gentamicin (Sigma), and 10 mg/ml Phenol red (Sigma). The embryos were pinned down in a Sylgard dish (Dow Corning Corporation, Midland, Mich.), and the lens of right eye was removed with a 0.1 mm minutien pin instrument (Fine Science Tools Inc.). A bolus of HRP was dissolved in 1% lysolecithin and placed in the eyecup. At least two boli of HRP were administered to the eye and then given sufficient time (20 minutes) to anterogradely label the RGC axons. The embryos were fixed overnight in 4% paraformaldehyde. After fixation, the embryos were rinsed in phosphate-buffered saline (PBS) (3 x 5 minutes) and pinned in a Sylgard dish containing PBS. The brains were

dissected, rinsed with PBS containing 0.5% Triton X-100 (PBT) (BDH), and reacted with a diaminobenzidine kit (DAB) (urea tablet and DAB tablet dissolved in 0.5% PBT) (Sigma), which turns the HRP-filled optic projections brown. Following the DAB reaction, the brains were rinsed in PBS (3 x 5 minutes) and then post-fixed in 1% gluteraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were then dehydrated through a graded series of alcohol (1x 2 minutes each in 50%, 75%, and 95% ethanol, and 2 x 2 minutes in 100% ethanol) and cleared in a 2:1 benzyl benzoate:benzyl alcohol solution. The brains were mounted and coverslipped in Permount (Fisher Scientific) and observed using Nomarski optics attached to a Zeiss microscope. Images were acquired with a Spot digital camera and processed with Adobe Photoshop software. *Camera lucida* drawings that outline the brains and optic projections were also made using a Zeiss microscope.

Retinal Explant and Dissociated cell cultures:

Eye primordia from stage 24 embryos were used in these cultures since at this stage RGCs are post-mitotic, but have not yet initiated axons. The embryos were anesthetized in MBS containing MS-222 and the eye primordia were dissected from embryos under sterile surgical conditions.

Explant Cultures: Following dissection, eye primordia explants were rinsed with L15 supplemented with glutamine (Gibco, Life Technologies, Burlington, ON) and then plated onto glass coverslips, pre-treated with 1 mg/ml poly-l-ornithine (Sigma) and 10 µg/ml mouse laminin (Sigma). The coverslips were in 35 mm petri dishes (Falcon, Becton Dickinson, Franklin Lakes NJ) containing 2-3 mls of culture medium. Culture

medium was 60% L15 containing 5% fetal bovine serum (Gibco), 0.5% gentamycin sulfate (Sigma, Oakville, ON), and 1% embryo extract (Harris et al., 1985). Explant cultures were grown for 24 hours at room temperature. Such cultures normally have numerous processes extending radially from the explant. Since RGCs are the only retinal cells to initiate and extend axons out of the eye, these processes likely originated from RGCs. Previous work in our lab has shown that these processes can be immunolabeled with an antibody against neurofilament protein. The cultures were fixed overnight at 4°C in 2% paraformaldehyde and then processed for immunocytochemistry. Immunolabeled RGC axons and growth cones extending from the explant were identified using a Zeiss microscope and an oil immersion objective at 100X magnification. Images were taken using a Spot Digital camera and processed using Adobe Photoshop.

Dissociated Retinal cultures: Eye primordia were dissociated and cultured as previously described (Harris et al., 1985, Harris and Messersmith, 1992). Briefly, primordia were dissociated in dissociation medium (NaCl, KCl, Tris Base, EDTA, and 1 % Phenol Red (Sigma)) and cultured as described above. Culture medium was 60% L15 with 0.2% bovine serum albumin BSA (BDH) alone or supplemented with either 100 uM GABA (Sigma), the GABA-B receptor agonist Baclofen (100 uM) (Research Biochemicals, RBI), or the GABA-A receptor agonist Muscimol (150 μM) (RBI). Dose response experiments were also performed with GABA concentrations ranging from 1 μM to 1 mM. Cultures were maintained for 24 hrs at room temperature and fixed overnight at 4°C in 0.5% gluteraldehyde. Following fixation, the cultures were rinsed and mounted onto glass slides with Aquamount (Fisher, Lerner Laboratories, Pittsburg, PA). Cultures were examined using a 40X phase objective and RGCs were identified

based on their round phase bright soma with 1-3 neurites extending from the soma (Worley and Holt, 1996). For neurite measurements, images of RGCs were taken using the Spot digital camera and measured using the Spot software. Neurites were measured from the edge of the soma to the distal tip of the growth cone. If RGCs had 2 or more neurites, the longest continuous neurite was measured. The lengths of neurites from each condition were recorded in a spreadsheet (Excel, Microsoft Office). The average neurite length for each condition was generated and analysis of variance (ANOVA) was performed to determine statistical significance between experimental and control conditions.

Antibodies:

Antibodies and their concentrations used were as follows: 1) An affinity-purified rabbit polyclonal antibody against the neurotransmitter gamma-aminobutyric acid (GABA) (1:3000) (Sigma). 2) Affinity-purified goat polyclonal antibodies against both the GABA-A receptor (GABA-A Rα2 (N-19) 1:500) as well as the GABA-B receptor (GABA-B R1 (R-20) 1:500) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The GABA-A antibody specifically recognizes the alpha-2 receptor sub-unit and is not reactive to other alpha sub-units. The GABA-B antibody reacts with both GABA-B1 receptor isoforms. Blocking peptides that correspond to both the GABA-A Rα2 (N-19) and GABA-B R1 (R-20) were obtained from Santa Cruz Biotechnology. Primary antibodies were pre-absorbed in a 5-fold volume of blocking peptides for either 2 hrs at room temperature or overnight at 4°C. 3) A guinea pig polyclonal antibody against the GABA-B receptor (1:500) was obtained from BD Pharmingen (Oakville, Ontario). This

antibody recognizes both GABA-B1 receptor isoforms. 4) A mouse monoclonal anti-HRP antibody (1:500) (Sigma). 5) Fluoroscein or Rhodamine isothiocyanate-conjugated (FITC or RITC) secondary antibodies (1:500) (Jackson Laboratories) were used to fluorescently label and visualize primary antibody staining.

Immunocytochemistry:

Wholemount Brains: Double-labeling of stage 32-39 embryonic brains for GABA and the optic projection was performed. In order to visualize the optic projection the optic fibres were HRP-filled as described earlier. The embryos were then fixed in 4% paraformaldehyde either for three hours at room temperature or at 4°C overnight. After tissue fixation, the embryos were rinsed in PBS (pH 7.4) (3 x 5 minutes). Brains were dissected from the embryos and processed for immunohistochemistry. The brains were rinsed in PBT (PBS containing 1% Triton X-100, 0.2% BSA) (4 x 1 hour), and then blocked (4 x 1hour) in a 1:1 volume ratio of PBT and 10% normal goat serum. The brains were incubated for two days at 4°C with the polyclonal anti-GABA antibody and the monoclonal anti-HRP antibody. Antibodies were diluted in 1:1 PBT and 10% goat serum. FITC and RITC conjugated secondary antibodies were used to visualize the GABA expression and the HRP-labeled optic projection respectively. The brains were mounted in *p*-phenylene diamine (pPD glycerol, Sigma), an anti-bleaching agent. Fluorescent images of the immunolabeled brains were taken with a Spot digital camera mounted on a Zeiss microscope. Images were processed using Adobe Photoshop software.

Tissue Sections: Following fixation and rinsing, embryos were immersed in 30% sucrose in PBS for at least 30 minutes to cryoprotect the tissue. The embryos were

embedded in Optimal Cutting Temperature (OCT, Baxter) and quick-frozen at -20°C. Transverse tissue sections were cut at 12μm using a cryostat. The sections were mounted on gelatin-coated slides and allowed to dry. The samples were then rinsed in PBT (0.4% Triton X-100 was used) for 1 hour (3 x 10 minutes and 1 x 30 minutes), and incubated overnight at 4°C with primary antibodies diluted in 1:1 PBT and 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc.). Following rinses with PBT (3 x 10 minutes and 1 x 30 minutes), samples were incubated with FITC-conjugated secondary antibodies for 1hr at room temperature. After rinsing the samples with PBT, samples were then mounted and coverslipped with pPD glycerol and slides were sealed with clear nailpolish.

Explant cultures: Following overnight fixation at 4°C in 2% paraformaldehyde, cultures were rinsed in PBS (3 x 5 minutes) and in PBT with a reduced Triton X-100 (0.2%) (4 x 5 minutes and 1 x 20 minutes). Cultures were then blocked in 1:1 PBT and 10% normal donkey serum for 20 minutes and incubated overnight at 4°C in primary antibodies diluted in blocking solution. The cultures were rinsed (4 x 5 minutes and 1 x 20 minutes) and FITC-conjugated antibodies diluted in blocking solution were applied at room temperature for 45 minutes. After rinsing the samples with PBT, the cultures were mounted on glass slides with pPD glycerol and sealed with clear nailpolish.

Western Blot Analysis

Two hundred microlitres of RIPA buffer (0.25M Tris (Omnipur, EM Science, Gibbstown, NJ), 0.12M sodium deoxycholate (Sigma), 0.75M sodium chloride (Sigma), 1% Triton X-100, 0.5% sodium dodecyl sulfate (SDS) (BDH), protease inhibitors

(Sigma) (ph 7.4)) was added to 50 μg of experimental embryo heads (stages 37-40) frozen on dry ice. Samples were homogenized using a Tissue Tearor (Biospec Products Inc.) and centrifuged at 4°C, and 15,000 rpm for 5 minutes. Ten microlitre samples of the supernatant were diluted in SDS-sample buffer (SDS, bromophenol blue (LKB, Broma, Sweden), Tris-HCL, and glycerol (BDH)) containing DTT (1,4-Dithithreitol) (Sigma) and boiled for 5 minutes at 100°C before being loaded on a SDS-polyacrylamide gel consisting of a 5% stacking gel and a 7.5% resolving gel (acrylamide, bis acrylamide, SDS, ammonium persulfate, Tris-HCL, and tetramethylethylenediamine TEMED (Omnipur products)). Ten microlitres of pre-stained molecular weight standards (Biorad) was loaded simultaneously in order to resolve sample protein band weights. Gel electrophoresis of the samples was run at 30 mAmps through the stacking gel and 50-80 mAmps through the resolving gel using a Biorad power supply. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Biorad) overnight at 30 V in transfer buffer (Tris-HCL, glycine (BDH), and methanol (BDH)). The membranes were then blocked 1-2 hours in Tris-Buffered Saline (TBS) containing 0.2% Tween-20 (BDH), 0.5% NP-40 (Amersham, Arlington Heights, IL), and 3% BSA. Primary antibodies were diluted in blocking solution and membranes were incubated overnight at 4°C with rocking. Both the GABA-B receptor antibodies as well as the GABA-A receptor antibody were used at a 1:500 dilution. Following incubation in primary antibodies, the membranes were rinsed in TBS (1-2 hours) and incubated in peroxidase conjugated secondary antibodies (Jackson Laboratories) diluted in TBS (1: 10,000) for 1 hour. Protein bands were detected with ECL solutions (Amersham), as outlined in the manufacturer's instructions, and the membranes were exposed to

Hyperfilm (Amersham). Molecular weights of the observed protein bands were determined by comparison to the pre-stained molecular weight standards run alongside sample protein lanes. The exposed films were scanned into the Macintosh G4 computer using a Umax 1200s flatbed scanner and figures were produced using Adobe Photoshop software.

Bathing Media Brain Exposures:

The brain exposure preparation is performed as described previously (Chien et al., 1993). Briefly, stage 33/34 embryos were used since RGC axons are just entering the contralateral brain at this stage (Chien and Harris, 1994). The embryos were anesthetized in MBS containing MS-222, and then pinned down in a Sylgard dish (Dow Corning Corporation, Midland Mich.). The skin and dura covering the brain, as well as the eye were removed on the left side of the embryo. The procedure results in one side of the brain exposed while the other side is still covered. After the surgery, the embryos were randomly divided and allowed to develop in either control or experimental solutions for 20 hours until they reached stage 40. The control solution was the MBS with MS-222. The experimental bath solutions were prepared from stock solutions of GABA (Sigma), or the GABA-B receptor agonist baclofen (RBI). Each stock solution was prepared by adding the chemical to autoclaved double-distilled water. Volumes of these stock solutions were then added to the control bath solution for final concentrations of 100 μM GABA, and 200 μM baclofen. These concentrations are comparable to those previously used with isolated *Xenopus* embryonic spinal neurons (Rohrbough and Spitzer, 1996, Wall and Dale, 1993,1994).

Quantification of the Optic Projection:

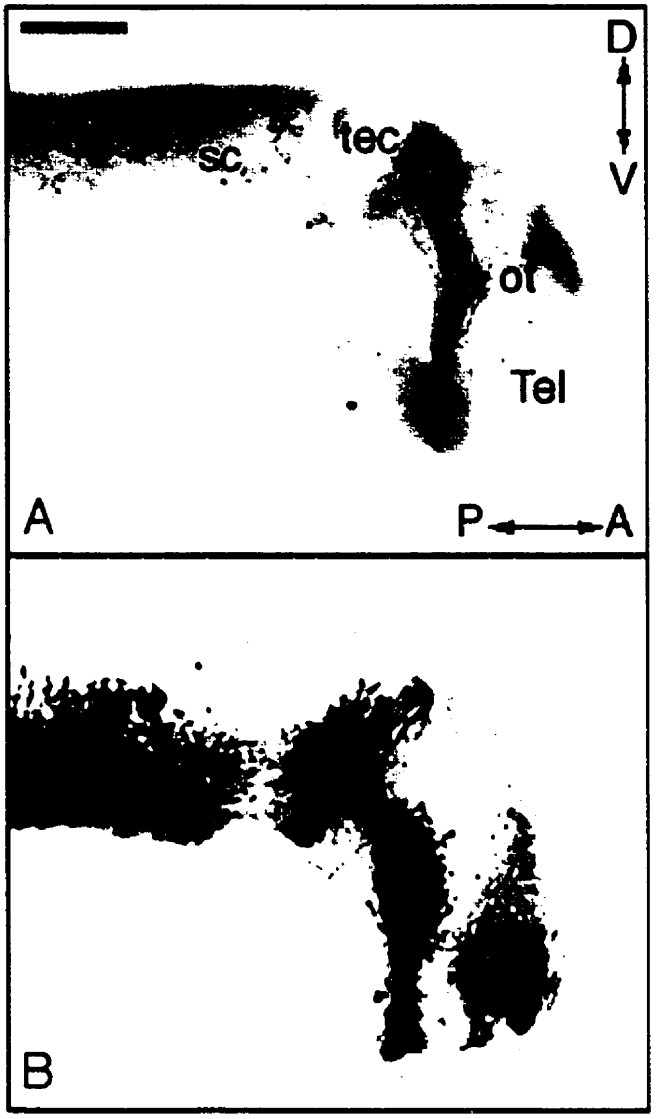
The *in vivo* effects of GABA, and the GABA-B receptor agonist baclofen, were quantified by first counting the number of brains in each condition that had normal projections and those with defects (i.e. shortened optic projections). The number of brains showing projection defects in each condition was statistically analyzed using a Pearson's chi-square contingency table to determine significance (Systat, SPSS). To further quantify optic projection lengths of experimental and control brains, *camera lucida* drawings of the whole-mount brains were scanned into digital images using UMAX Astra 1200s flatbed scanner. The brains were analyzed using an NIH image analysis package (Chien et al., 1993). Briefly, the brains were first oriented in the same direction and then normalized to a similar size. To normalize the brains, the images are scaled to a line that extends from the anterior optic chiasm to the midbrain-hindbrain isthmus. This line matches to a standard reference line that corresponds to a length of 620 μm in an unfixed brain (Chien et al., 1993). The optic projection length is measured from the optic chiasm to the end of the projection that has at least 10 axons. The optic projection lengths of brains from each condition were compiled in a spreadsheet (Excel, Microsoft Office), and the average optic projection length was determined. Statistical analysis using ANOVA was performed to determine significant differences between experimental and control brains.

CHAPTER 3: IMMUNOLOCALIZATION OF GABA AND ITS RECEPTORS IN THE DEVELOPING VISUAL SYSTEM

Introduction

GABA, an inhibitory neurotransmitter in the mature vertebrate CNS, is present early in the developing CNS before the formation of synapses (Coyle and Enna, 1976), where it may function as a trophic factor for developing neurons (for reviews see Meier et al., 1991, Belhage et al., 1998, Sandell, 1998). Roberts et al., (1987) previously demonstrated through immunohistochemistry, that a population of mid-optic tract cells and tectal cells express GABA in the *Xenopus laevis* embryo. The onset of this expression appeared around stage 29/30 (2 days post-fertilization) and intensified by stage 37/38. Although not discussed in the Roberts et al. (1987) paper, GABA appears to be expressed in the optic tract during early developmental periods when RGC axons are pathfinding towards the optic tectum. A striking comparison is observed when the GABA-expressing brains from the Roberts et al. (1987) study are compared to wholemount brains where the optic projections have been HRP-labeled (Figure 5). GABA expression in the optic tract spatially matches the labeled optic projection quite remarkably. I hypothesize that GABA in the optic tract at these early stages is required for the proper development of RGC axons. For GABA to be considered an important cue for developing RGC axons, it must therefore meet specific criteria as outlined by McFarlane and Holt (1996). The first two criteria are addressed here. First, GABA should be expressed both during the period of RGC development and within the regions that RGC cell axons are developing. Second, GABA receptors should be expressed on developing RGC processes and growth cones.

Figure 5: Cells expressing GABA delineate the pathway that RGC axons take in the *Xenopus* optic tract (ot). A) Lateral view of a stage 37/8 whole-mount brain with a labeled optic projection. RGC axons were anterogradely labeled by removing the lens of the contralateral eye and filling the eyecup with several boli of horseradish peroxidase (HRP, Sigma) (for detailed methods see Chapter 2). B) Lateral view of a stage 37/8 whole-mount brain immunolabeled for the neurotransmitter GABA (Adapted from Roberts et al., 1987). GABA expression is found throughout the optic tract and optic tectum (tec) at this stage, as well as in the telencephalon (Tel), and spinal cord (sc). Additional abbreviations are as follows: D (dorsal), V (ventral), A (anterior), and P (posterior). Scale is 100 μM.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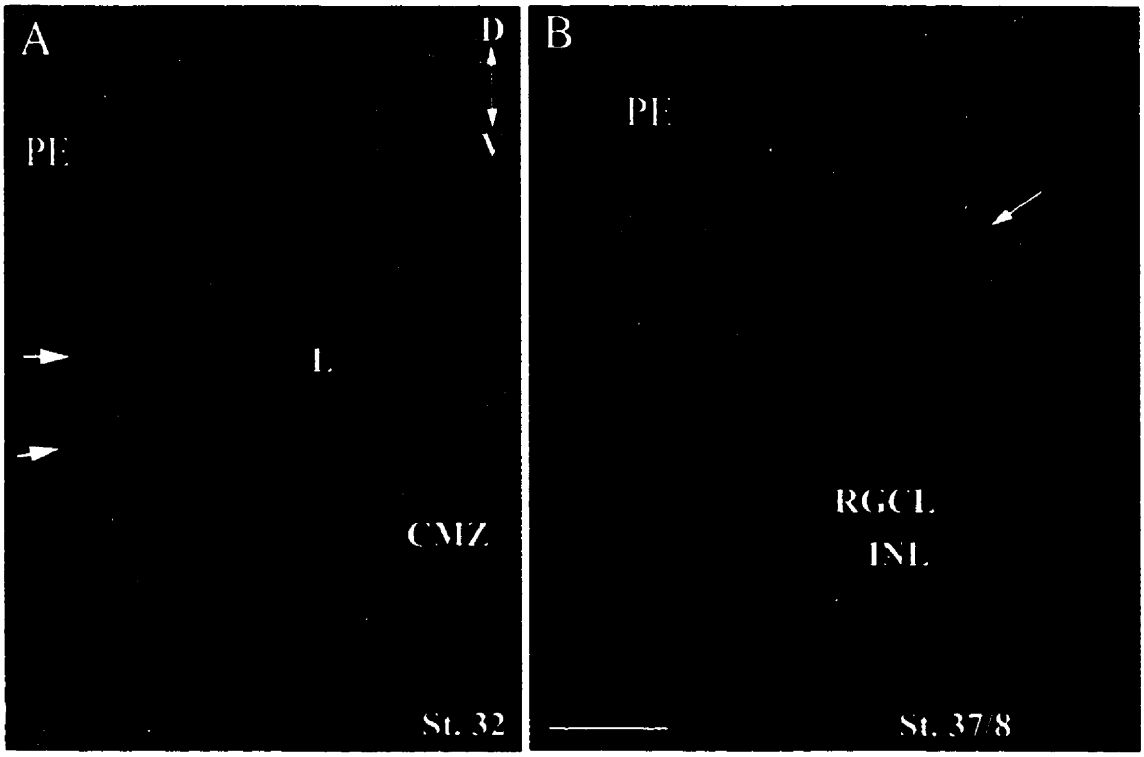
GABA expression in the developing Xenopus retina

GABA is expressed in the developing retina of several vertebrate species (Sandell et al., 1994; Schnitzer and Rusoff, 1984; Messersmith and Redburn, 1992; Hendrickson et al., 1994). I performed immunohistochemistry with the anti-GABA antibody on 12 μM frozen transverse sections through the eye of *Xenopus* embryos (stages 32- 39) to determine the onset and pattern of GABA expression in the developing *Xenopus* retina. GABA expression in the embryonic *Xenopus* retina is first detected at stage 32 (Figure 6) where it is expressed in the ciliary marginal zone (CMZ), a region of the amphibian retina where active cell proliferation occurs throughout life (Perron and Harris, 2000). The expression of GABA in the CMZ persists throughout the stages examined here. In addition, a few cells within the central retina also express GABA at stage 32 (see arrows in Figure 6A). GABA expression in the central retina increases in stage 35/36 embryos, and by stage 37/38, expression is found at high levels in the INL as well as in the RGCL (Figure 6B). Expression of GABA in the late stages (37-39) of developing *Xenopus* retina matches other reports of GABA expression in the vertebrate retina (see Sandell, 1998 for references). For example, amacrine and horizontal cells of the INL as well as RGCs in the developing rabbit retina are reported to express GABA (Messersmith and Redburn, 1992). GABA expression in the *Xenopus* retina is also seen in the processes of the inner plexiform layer (IPL), where amacrine cells synapse onto RGC dendrites (Cepko, 1996).

Xenopus RGC axonal development and GABA expression

To satisfy the first criterion and to examine the expression of GABA in the optic tract with respect to developing RGC axons, double wholemount immunohistochemistry

Figure 6: GABA expression in stage (St.) 32 and stage 37/38 *Xenopus* eyes as shown by immunocytochemistry. A) A transverse section through the eye of a stage 32 embryo. GABA expression is found in the ciliary marginal zone (CMZ) and in a few cells in the central retina (see arrows). B) A stage 37/38 transverse eye section showing staining in the retinal ganglion cell layer (RGCL) and inner nuclear layer (INL). Expression also persists in the CMZ at this stage (see arrow). Additional abbreviations are: PE (pigment epithelium). L (lens), D (dorsal), V (ventral). Scale bar is 50 μM.


A
D
V
PE
L
CMZ
St. 32
B
PE
RGCL
INL
St. 37/8

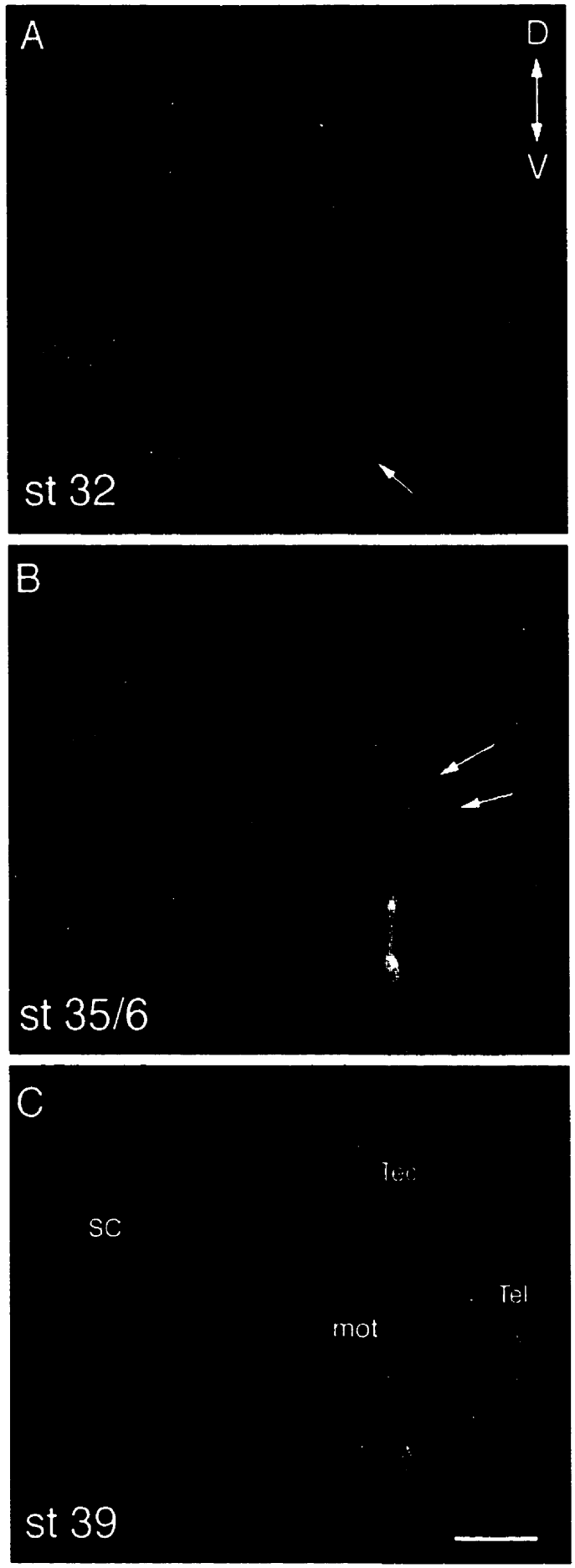
of the HRP-labeled optic projection and GABA was performed on brains from *Xenopus* embryos (stages 32-39) as outlined in chapter 2.

RGC axons enter the contralateral brain at the ventral diencephalon during the second day of embryonic development (stage 32) (Chien and Harris, 1994). At stage 32, a small population of GABA-expressing cells is found in the mid-optic tract and tectal regions of the embryonic *Xenopus* brain (Figure 7A). GABA expression in these regions appears to be cellular since distinct cell bodies are intensely stained. This expression increases in both the optic tract and tectal regions of stage 35/36 embryonic brains (Figure 7B). At this stage, labeled RGC axons have extended dorsally toward the GABAergic cells lining the mid-optic tract. This is also the point where RGC axons make a characteristic caudal turn towards the optic tectum (see arrows in Figure 7B). By stage 39, the labeled RGC axons have completed their navigation and entered the tectal region where GABA is intensely expressed (Figure 7C). Increasing immunoreactivity for GABA is also found in the telencephalon and spinal cord throughout these developmental stages. Similar expression of GABA along the optic tract is found during development in several vertebrate species such as rat (Sakatani et al., 1991,1992, Sandell et al., 1994, Rogers and Pow, 1995), rabbit (Rogers and Pow, 1995), cat (Rogers and Pow, 1995), and chick (Messersmith and Redburn, 1992).

Western blotting of GABA receptor antibodies

The GABA-A receptor is a pentameric assembly composed of various combinations of subunits (Mehta and Ticku, 1999). Reports have indicated that many native GABA-A receptor channels are assembled from α, β, and γ subunits. The molecular weights of these subunits have previously been determined at 51-58

Figure 7: Developmental expression of GABA in the optic tract coincides spatially and temporally with the developing optic projection as shown by wholemount double immunohistochemistry. GABA is in green and the labeled optic projection is in red. A) Lateral view of a stage (St.) 32 wholemount *Xenopus* brain. GABA expression is found in the mid-optic tract (mot) and tectum (tec) as well as in the spinal cord (SC). The optic projection at this stage is just crossing into the ventral brain on this side (arrow). B) GABA expression increases within the mot and tec at stage 35/36. RGC axons have reached the mot region of GABA expression (arrows). C) Stage 39 wholemount brain. At this stage RGC axons are entering the tectal region of GABA expression. Additional abbreviations are as follows: Tec (tectum), Tel (telencephalon), D (dorsal), V (ventral). Scale bar is 100 μM.


A
D
V
st 32
B
st 35/6
C
Tec
SC
Tel
mot
st 39

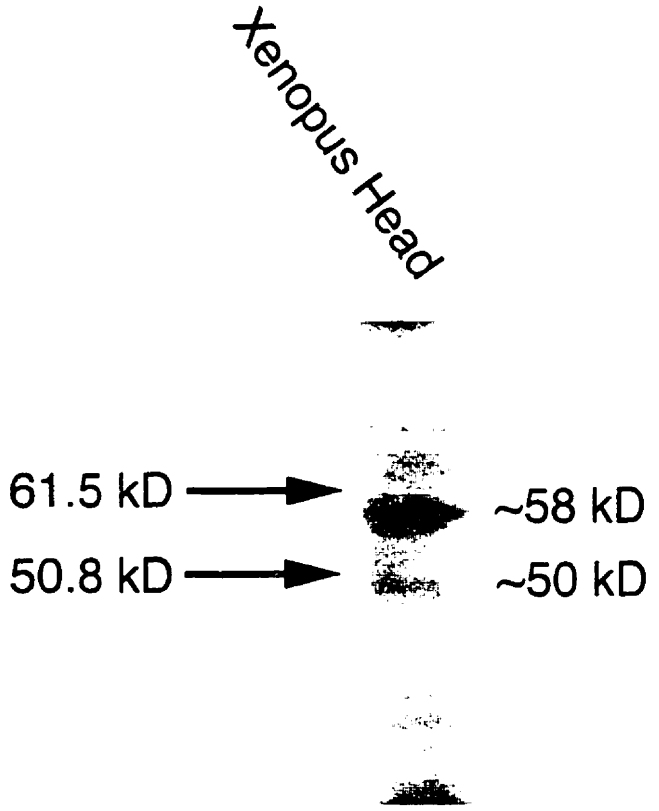
kilodaltons (kDa), 56-58 kDa, and 45 kDa respectively (see MacDonald and Olsen, 1994 for references). GABA-B receptors are metabotropic G protein-linked receptors originally shown to exist in two isoforms, GBR1a and GBR1b (GABA-B receptor 1a and b), with molecular weights of 130 and 100 kDa respectively (Kaupmann et al., 1997). In addition, a second GABA-B receptor (GBR2) has an identified molecular weight of 110 kDa (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Antibodies that react to either GABA-A or GABA-B receptors are commercially available, whereas no GABA-C receptor antibodies are presently commercially available. In order to test the specificity of the obtained GABA-A and GABA-B receptor antibodies on developing *Xenopus* CNS tissue, western-blotting techniques were employed (see chapter 2).

The affinity-purified goat polyclonal GABA-A Rα2 (C-20) receptor antibody was raised against the carboxyl terminus of the GABA-A receptor α2 subunit of human origin. The antibody is also reactive to α subunits 1-5 from mouse, rat and human. When this antibody was used to probe the membrane containing protein extract from stage 35-38 embryonic *Xenopus* head tissue, multiple protein bands were observed. Noteably, two prominent bands were found at approximately 58 and 50 kDa (Figure 8). These molecular weights were determined from comparison to molecular weight standards run along the same gel. The molecular weights of these bands are within the accepted range for GABA-A receptor α- subunits (see MacDonald and Olsen, 1994 for references).

A GABA-B receptor antibody obtained from Santa Cruz Biotechnology was used throughout these studies, and also tested for its specificity when applied to embryonic *Xenopus* CNS tissue. This affinity-purified goat polyclonal GABA-B R1 (R-20) antibody was raised against the carboxyl terminus of the GABA-B R1a receptor from rat. The

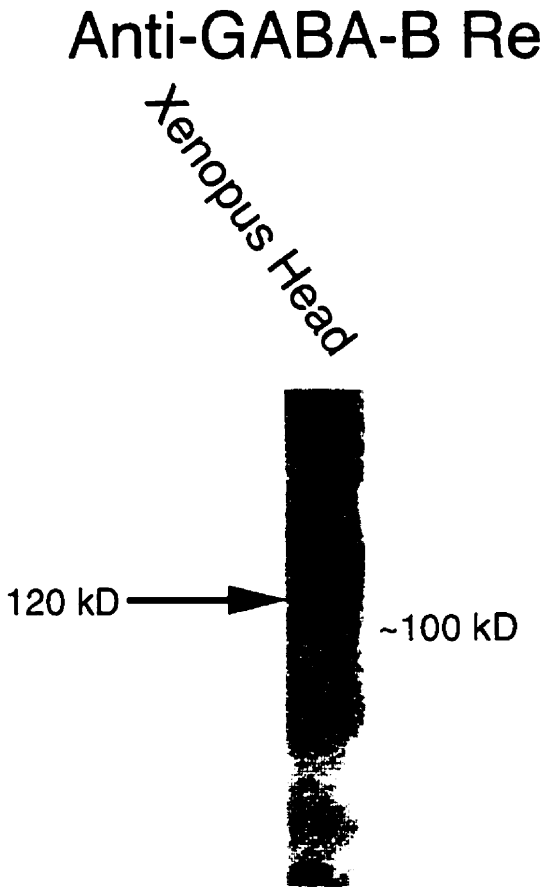
Figure 8: Western blot of stage 35-38 embryonic *Xenopus* head tissue showing GABA-A receptor protein expression. Arrows indicate molecular weight based on standard run at the same time as the sample tissue. Two protein bands with approximate molecular weights of 50 and 58 kilodaltons (kDa) were observed, which correspond to known GABA-A receptor α-subunits.

Anti-GABA A Receptor



antibody reacts to GBR1a and GBR1b from mouse and rat. Western blot experiments using this antibody upon membranes containing protein extract from stage 35-38 embryonic *Xenopus* head tissue were performed. Repeated experiments using this antibody detected no visible protein bands. A possible explanation for this result is that the appropriate conditions for western blotting with this antibody were not satisfied, or simply that the antibody is not suited for this procedure. A second anti-GABA-B receptor antibody from Pharmingen International was obtained since it was recently used in a study that identified GABA-B receptors in Müller glia of the bullfrog (*Rana calesbeiana*) (Zhang and Yang, 1999). This polyclonal guinea pig antibody also reacts to GBR1a and GBR1b from rat. When applied to membranes containing protein extract from embryonic *Xenopus* head tissue, a prominent band with an apparent molecular weight of 100 kDa was observed (Figure 9). This protein band corresponds to previous reports identifying the GABA-BR1b protein (Kaupmann et al., 1997). No discernable band (130 kDa) for the GBR1a isoform was detected. One explanation for this result is perhaps that this receptor isoform is expressed at undetectable levels in *Xenopus* at these stages. A previous report using this antibody in a western blot analysis of adult rat CNS tissue yielded a similar result (Sloviter et al., 1999). A distinct GBR1b band (100 kDa) was present along with a diffuse band with a comparable molecular weight to the GBR1a isoform (130 kDa). Another explanation could also be that the antibody has a greater affinity to the GBR1b isoform, thus binding selectively to this receptor. Nonetheless, the results from these experiments demonstrate that GABA-A and GABA-B receptors can be detected in embryonic *Xenopus* CNS tissue. GABA-C receptors were not addressed in this study since at this time, no GABA-C receptor antibodies were obtained.

Figure 9: Western blot of stage 35-38 embryonic *Xenopus* head tissue showing GABA-B receptor protein expression. Arrow indicates the molecular weight standard run at the same time as the sample tissue. The Pharmingen GABA-B receptor antibody was used for this western. A single band with a molecular weight of 100 kilodalton (kDa) was observed, which corresponds to the reported weight of the GABA-B receptor 1b isoform.


Anti-GABA-B Receptor
Xenopus Head
120 kD
~100 kD

Expression of GABA receptors in the developing *Xenopus* retina

Previously, all three GABA receptors have been identified within the adult vertebrate retina (see Redburn and Rowe-Rendleman, 1996, Lukasiewicz and Shields, 1998 for references). GABA-A receptors are ubiquitously expressed throughout the retina, whereas GABA-B and GABA-C receptors have distinct expression patterns (Lukasiewicz and Shields, 1998). For example, GABA-B receptors are localized on bipolar, horizontal, and amacrine cells of the INL, as well as retinal ganglion cells, and Mueller glia (Maguire et al., 1989, Koulen et al., 1998, Lukasiewicz and Shields, 1998, Congxiao et al., 1998, Zhang and Yang, 1999). In addition, GABA-B receptor subtypes show a differential expression on pre- and postsynaptic sites (Koulen et al., 1998). Less information is known about the expression of GABA receptors during the development of the retina. Recently, experiments revealed that GABA-A receptors are transiently expressed on cone photoreceptor cells during early postnatal developmental stages (Mitchell et al., 1999). To investigate GABA-A and GABA-B receptor expression in the developing embryonic *Xenopus* retina, immunocytochemistry was performed on 12 μM frozen transverse sections through the eye of stage 32-39 *Xenopus* embryos.

Immuno-like reactivity for the GABA-A receptor was found in stage 32 retina, although no discernable expression pattern was observed (Figure 10). A significant amount of background staining was also observed in all stages of retinal sections examined with this antibody. Since this GABA-A receptor antibody only recognizes a few receptor sub-units, this could contribute to the poor immuno-like reactivity observed. Alternatively, since the antibody was generated against a small peptide from the rat GABA-A receptor, a possibility exists that the antibody recognizes an unrelated protein

Figure 10: Immuno-like reactivity of GABA-A receptors in the embryonic *Xenopus* retina as revealed by immunocytochemistry. GABA-A receptor antibody was obtained from Santa Cruz Biotechnology. A) Ubiquitous expression was observed in transverse sections of stage (St.) 32 retinas. B) A stage 32 control retina; primary antibody incubation step was omitted. C) Expression in the stage 37/38 retina was found at greater levels in the outer plexiform layer (OPL) and within the inner nuclear layer (INL) (see arrows). D) Expression was not detected when the primary antibody incubation step was omitted. Additional abbreviations are as follows: L (lens), PE (pigment epithelium), D (dorsal), V (ventral). Scale bar is 50 μM.

in *Xenopus*. At later stages such as 35/36 and 37/38 GABA-A receptors were expressed throughout the retina with increased levels. At these stages, greater expression was found in the outer plexiform layer (OPL), INL, and RGCL. In some sections, the optic nerve was also labeled for GABA-A receptors. This expression pattern in the developing *Xenopus* retina is in agreement with previous studies showing developmental GABA-A receptor expression in the vertebrate retina (Mitchell et al., 1999).

Immuno-like reactivity for GABA-B receptors was also found early in the retina (i.e. at stage 32) (Figure 11). Both the Santa Cruz and the Pharmingen GABA-B receptor antibodies revealed expression in the retina at this stage. A greater level of expression was also found in the proliferative CMZ. At stage 37/38, GABA-B receptors were expressed within retinal cells of the INL and RGC. A lower level of expression was observed in the CMZ, as well as in the OPL at this stage (Figure 12). In addition, the optic nerve head was also immunolabeled. This expression pattern within the developing *Xenopus* retina was specific since it was not observed following pre-incubation of the antibody with a specific blocking peptide (Figure 12B). Moreover, similar regions of GABA-B receptor expression in the stage 37/38 retinas were found when using either the Santa Cruz or the Pharmingen GABA-B receptor antibody (Figure 13). In addition, differential staining between these antibodies was observed. For example, the Santa Cruz GABA-B receptor antibody immunolabeled the optic nerve head (Figure 12) and the Pharmingen antibody intensely labeled the lens (Figure 13). One explanation for the differences in expression observed may be due to the antibodies preferentially detecting different isoforms of the GABA-B receptors. Regardless, the expression observed here matches previous reports of GABA-B receptor expression in the vertebrate retina

Figure 11: Immuno-like reactivity of the GABA-B receptor in the stage (St.) 32 embryonic *Xenopus* retina as revealed by immunocytochemistry. (Pharmingen GABA-B receptor antibody). A) Transverse section through a stage 32 *Xenopus* eye. Expression was found in the retina, with greater levels in the peripheral ciliary marginal zone (CMZ). B) Stage 32 control retina; Primary antibody incubation step was omitted. Additional abbreviations are as follows: C (central retina), L (lens), D (dorsal), V (ventral). Scale bar is 50 μM.

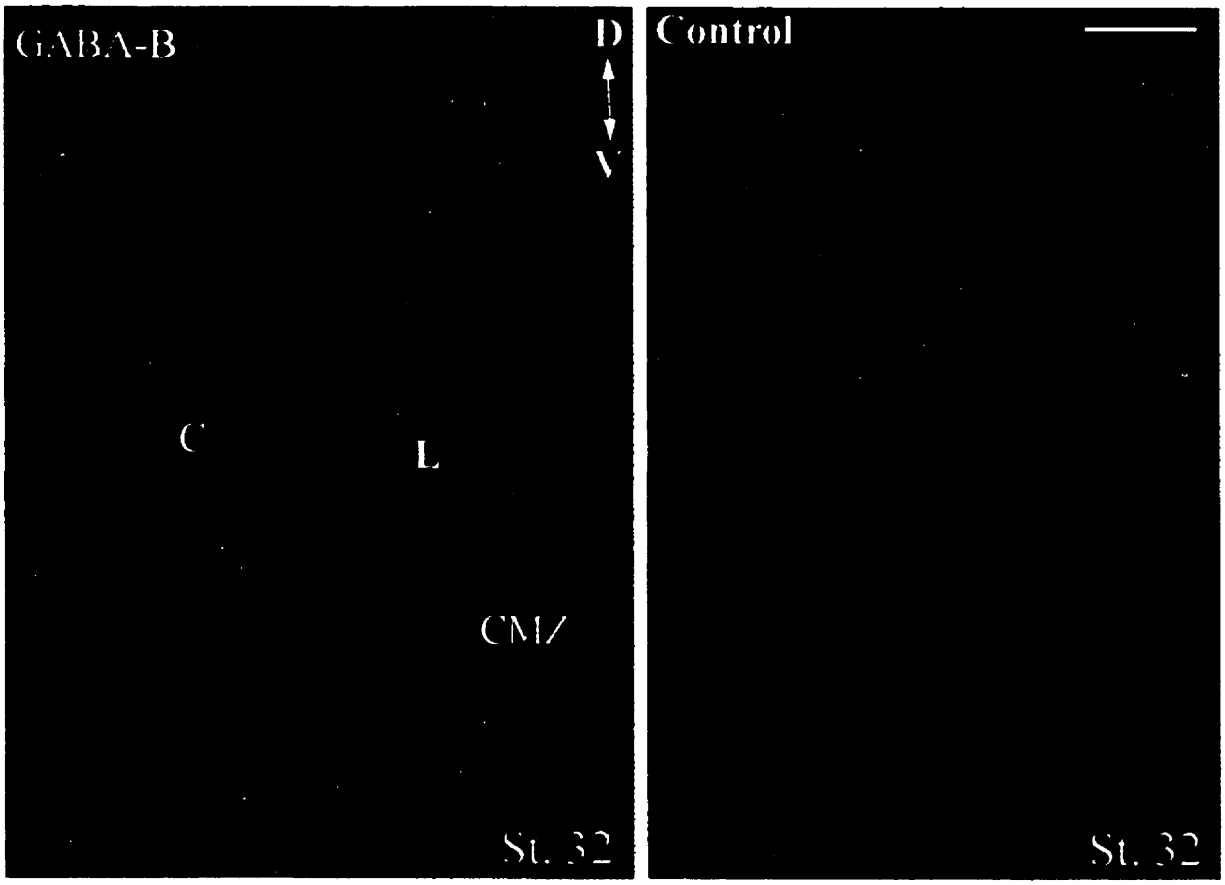

GABA-B
D
V
C
L
CMZ
St. 32
Control
St. 32

Figure 12: Immuno-like reactivity of GABA-B receptors in the stage (St.) 37/38 embryonic *Xenopus* retina as revealed by immunohistochemistry. (Santa Cruz GABA-B antibody). A) Transverse section through the stage 37/38 *Xenopus* eye. Expression in retinal layers was observed in the inner nuclear layer (INL), retinal ganglion cell layer (RGCL), and the outer plexiform layer (OPL). The optic nerve head (ONH) also expressed GABA-B receptors. B) A control stage 37/38 retina. Pre-incubating the primary antibody in a blocking peptide (b.p.) obtained from Santa Cruz Biotechnology blocked expression in the retina. Additional abbreviations are as follows: L (lens), PE (pigment epithelium), D (dorsal), V (ventral). Scale bar is 50 μM.

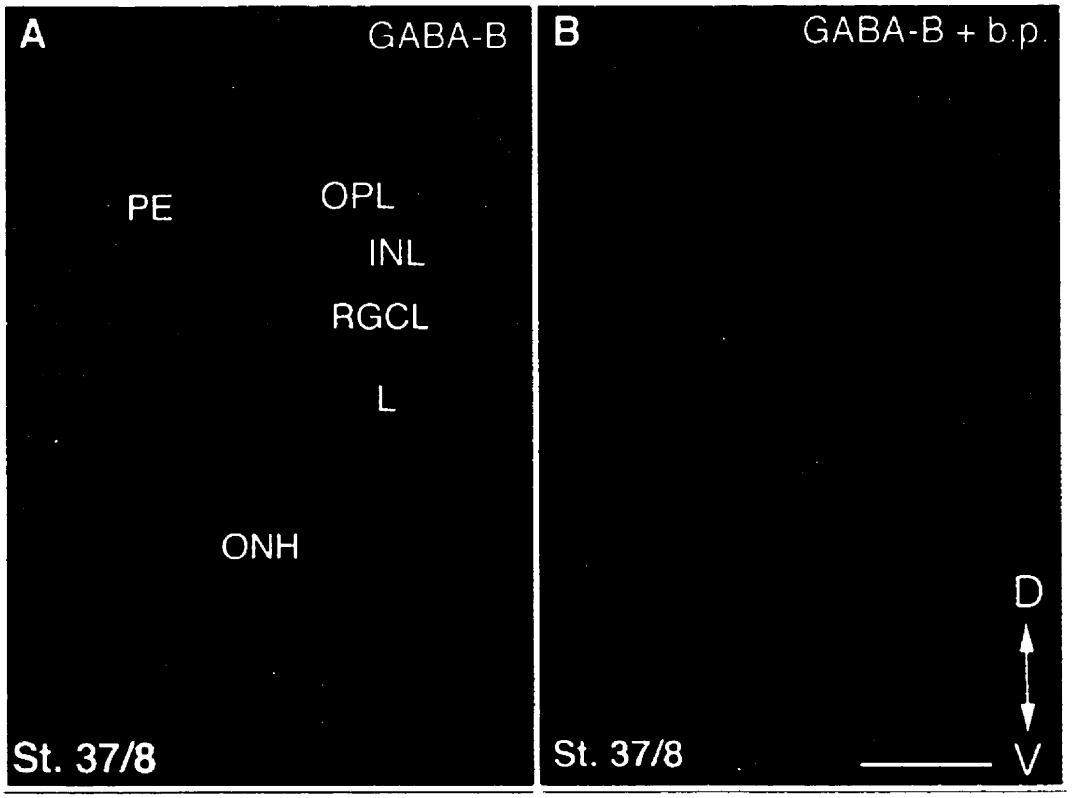
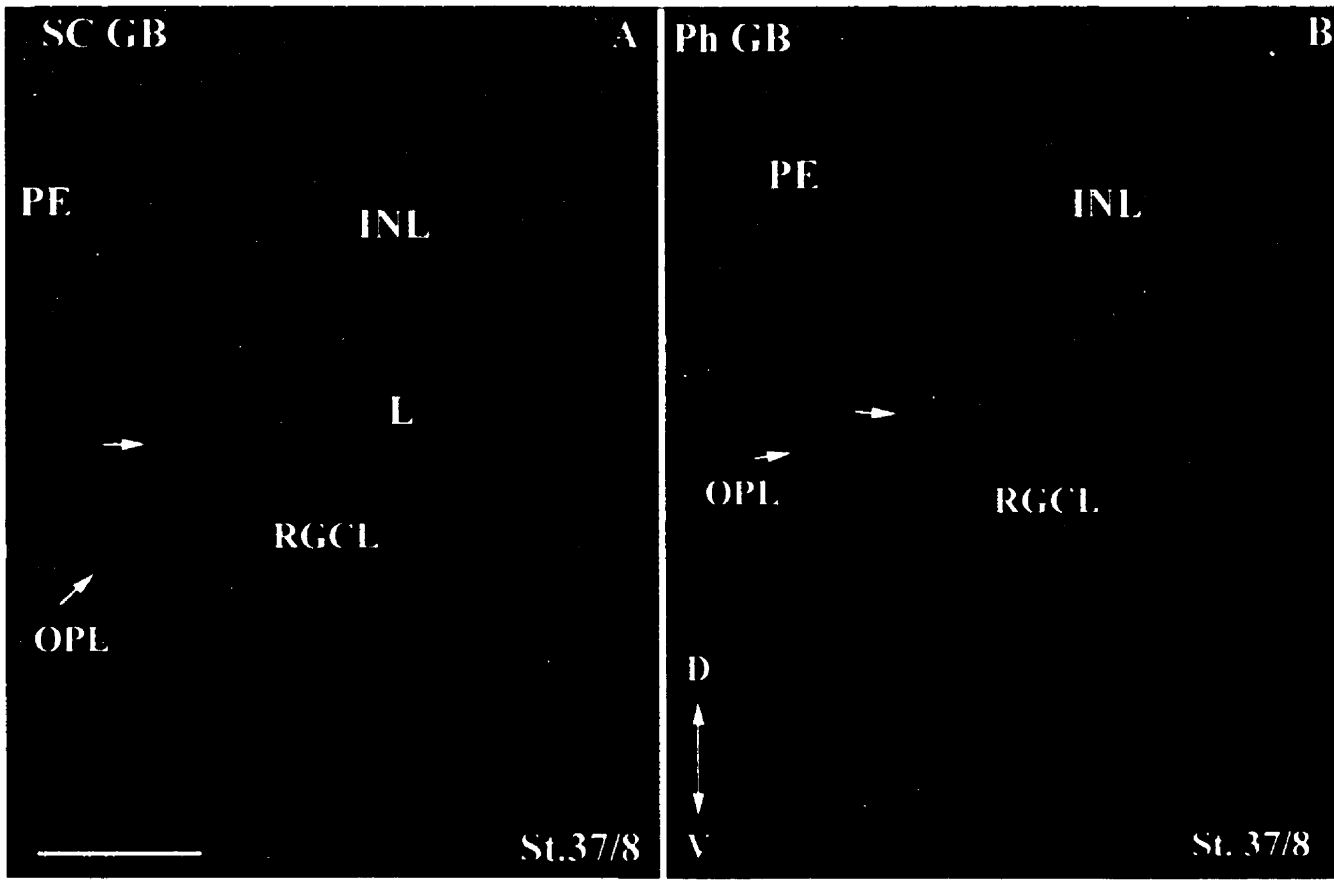

A
GABA-B
PE
OPL
INL
RGCL
L
ONH
St. 37/8
B
GABA-B + b.p.
D
V
St. 37/8

Figure 13: A comparison of immuno-like reactivity of GABA-B receptors in the stage (St.) 37/38 retinas as a result of using either the Santa Cruz or the Pharmingen GABA-B receptor antibody. In panels A and B, similar regions of GABA-B receptor expression include: the inner nuclear layer (INL), retinal ganglion cell layer (RGCL), and outer plexiform layer (OPL) (see arrows). High expression in the lens was observed with the Pharmingen GABA-B receptor antibody. Additional abbreviations are as follows: SC GB (Santa Cruz GABA-B antibody), Ph GB (Pharmingen GABA-B antibody), L (lens), PE (pigment epithelium), D (dorsal), V (ventral). Scale bar is 50 μM.

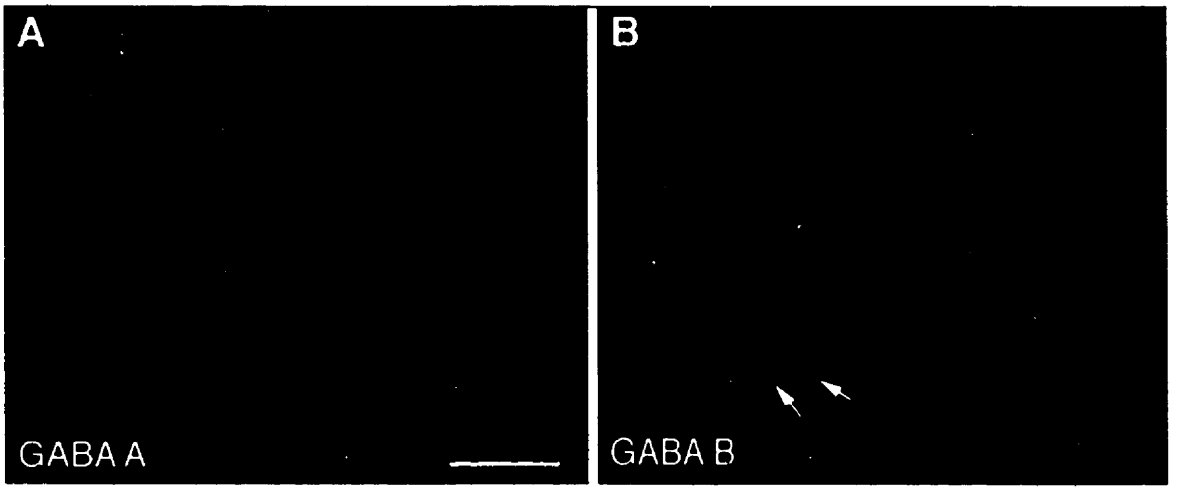

SC GB
A
PE
INL
L
RGCL
OPL
St.37/8
Ph GB
B
PE
INL
OPL
RGCL
D
V
St. 37/8

(Lukasiewicz and Shields, 1998), and suggest that GABA-B receptors are expressed in the developing *Xenopus* retina.

RGC growth cone labeling with GABA-A and GABA-B receptor antibodies

For GABA to play a role in the pathway of developing RGC axons, the axons and growth cones must express GABA receptors. Although embryonic *Xenopus* retinal sections were labeled with the GABA-A and GABA-B receptor antibodies, growth cone labeling cannot be detected in this preparation. To investigate the presence of GABA receptors in RGC axons and growth cones, stage 24 *Xenopus* eye buds were dissected and cultured as explants. At this stage, RGCs are newly generated, but have not yet begun to extend axons. In addition, since RGCs are the only retinal cells to exit the eye, neurites extending from the retinal explant originate from RGCs. After developing for 24 hours, these explant cultures were processed for immunocytochemistry with the GABA-A and GABA-B receptor antibodies. Immuno-like reactivity for both GABA-A and GABA-B receptors was found on RGC axons and growth cones (Figure 14). GABA-B receptors were expressed in the body, axon, and filopodia of RGC growth cones. GABA-A receptor expression was also observed in growth cones, although at a lesser intensity compared to the GABA-B receptor expression. Previous reports have demonstrated the presence of GABA-A receptors in growth cones of developing neurons (Fukura et al., 1996, 1999). The expression of GABA-A and GABA-B receptors in developing RGC axons and growth cones indicates that these axons are capable of sensing GABA in their environment.

Figure 14: Cultured RGC growth cones immuno-like reactivity for GABA-A and GABA-B receptors as demonstrated by immunocytochemistry of retinal explant cultures. A) Expression of GABA-A receptors was observed in greater levels in the body of growth cones. B) Intense labeling of GABA-B receptors was found in the body and filopodia (see arrows) of RGC growth cones as well as their axons. Scale bar is 10 μM.


A
GABA A
B
GABA B

Summary

These experiments demonstrate that GABA is present during the time that RGC axons are pathfinding, and that GABA is expressed in a spatially significant manner for these developing processes. The data also show that developing RGC axons and growth cones express appropriate GABA receptors. The specificity of the GABA receptor antibodies was verified using western blot techniques. Together, the results from these experiments are significant since they suggest that developing RGCs possess the appropriate receptor machinery to sense and respond to an early GABA signal within the optic tract.

CHAPTER 4: NEURITE LENGTH MEASURES AND EXPOSED BRAINS

Introduction

My data showing that GABA is present early in *Xenopus* visual system development, and that RGCs express both GABA-A and GABA-B receptors, support the hypothesis that GABA has a role in the development of these retinal neurons. The remaining criteria (McFarlane and Holt, 1996) for GABA to be considered an important molecular cue for developing RGCs are: 1) that GABA affects the growth of neurites in culture, and 2) that changing the expression of the GABA or altering the signaling through GABA receptors should affect the development of the RGCs *in vivo*.

Previous studies have identified trophic actions of GABA on developing neurons (Spoerri and Wolff, 1981, Eins et al., 1983, Meier et al., 1984, 1991, Spoerri, 1988, Michler, 1990, Hansen et al., 1991, Wolff et al., 1993, Abraham et al., 1994, Ben-Ari et al., 1994, Loturco et al., 1995, Behar et al., 1994, Liu et al., 1997, Belhage et al., 1998). More specifically, trophic actions of GABA upon retinal cells have been reported both *in vitro* and *in vivo* (Spoerri, 1988; Madtes and Redburn, 1983). For example, when extracellular GABA levels were increased in early postnatal rabbit retinae, GABA receptor expression was upregulated (Madtes and Redburn, 1983). Further, addition of exogenous GABA to embryonic chick retinal cultures resulted in the proliferation of retinal neurons (Spoerri, 1988). When neurite outgrowth was measured in these GABA supplemented chick retinal cultures, both the length and branching of neurites increased when compared to control cultures. Although these studies demonstrate a neurotrophic effect of GABA upon retinal neurons in general, specific effects of GABA on RGCs were not addressed.

Trophic actions of GABA upon RGCs in culture

As a first measure to determine if GABA could influence the development of *Xenopus* RGCs, I performed dissociated retinal cultures using stage 24 embryos (see chapter 2). At this stage RGCs are newly generated but have not yet begun to extend processes. Therefore we can study the development and not the re-generation of neuritic processes. RGCs were identified based on their morphology (i.e. a round phase bright cell body with 1-3 neurites extending from the soma) (see Figure 15) (Worley and Holt, 1996). Individual neurites were measured from the edge of the soma to the distal tip of the growth cone in order to obtain the average neurite length of RGCs in each condition. A dose-response experiment was first performed to determine an appropriate concentration of GABA to be used in subsequent experiments (see Figure 16). An increase in neurite outgrowth from cultured RGCs was apparent with specific concentrations of GABA. No effects on neurite outgrowth were observed with 1 μM GABA. However, 100 μM GABA increased neurite outgrowth of RGCs when compared to control cultures. Although a greater increase of neurite elongation was observed with 1 mM GABA, I decided to use a concentration of 100 μM in the subsequent experiments since it increased neurite outgrowth when compared to controls. In addition, whole-cell patch clamp recordings demonstrated that this concentration of GABA was effective at depolarizing isolated *Xenopus* embryonic spinal neurons (Rohrbough and Spitzer, 1996).

Subsequently I compared the neurite length of cultured RGCs in control media to that of cultured RGCs in media supplemented with 100 μM GABA. The data from three separate experiments was pooled and the average neurite length in each condition was obtained (Figure 17). An additional two experiments were not included in this analysis

Figure 15: RGC from a control dissociated eye explant. RGCs in culture have typically large phase bright cell bodies with one to three long extending neurites. Neurite measurements are made from the cell body to the tip of the growth cone. Scale bar is 50 μM.

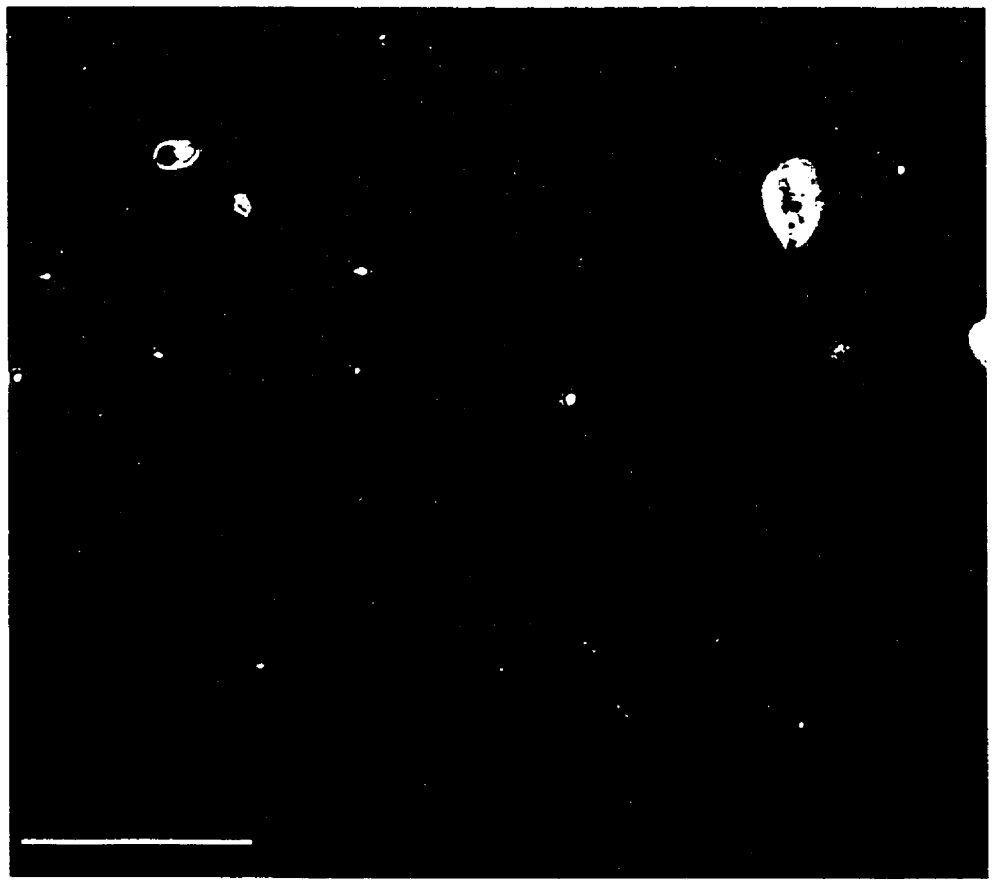


Figure 16: Dose response of RGC neurite length (μm) to increasing concentrations of GABA *in vitro*. Neurite length of RGCs was measured following a 24 hour incubation in various concentrations of GABA. Control media was L15. Average neurite length was plotted for each concentration. Error bars are s.e.m.; n= number of neurites measured.

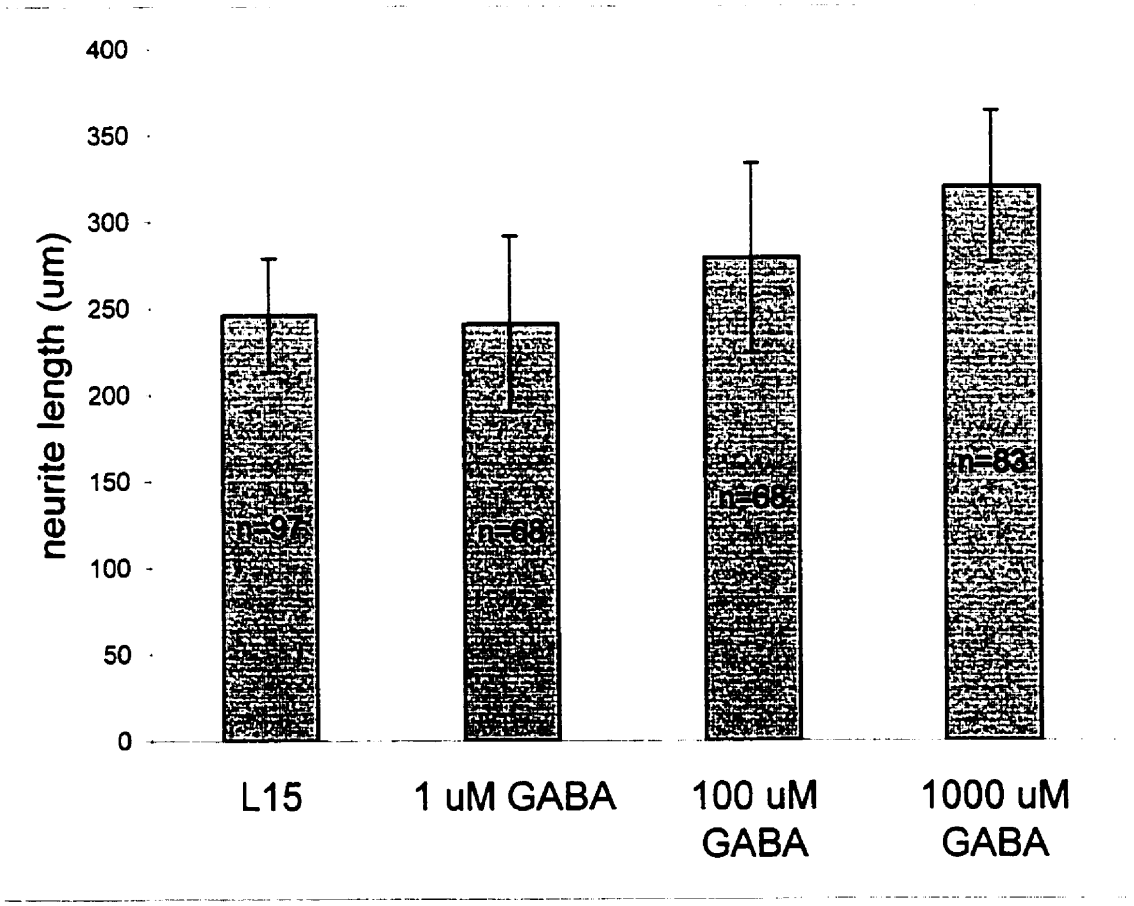
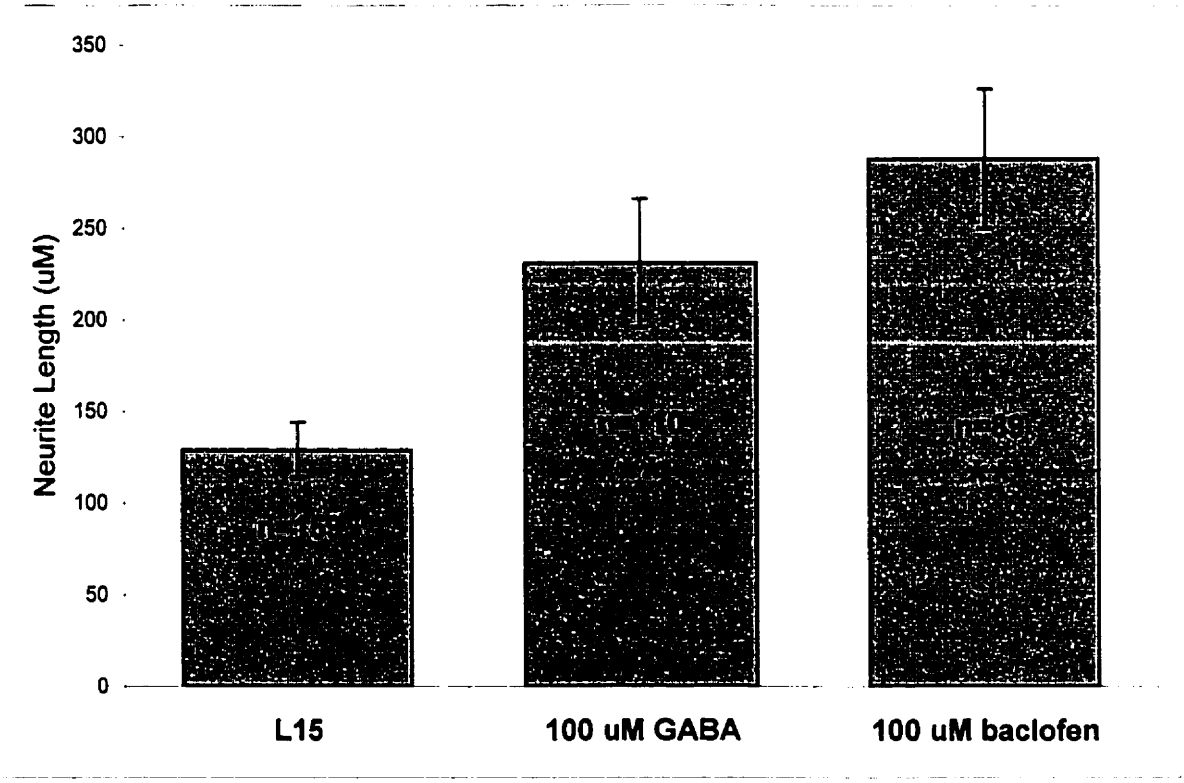

400
350
300
250
200
150
100
50
0
neurite length (um)
n=97
n=68
n=66
n=63
L15
1 uM GABA
100 uM GABA
1000 uM GABA

Figure 17: GABA and baclofen stimulate neurite outgrowth in cultured RGCs. Cultures were incubated in either L15 medium (control), 100 μM GABA, or 100 μM baclofen for a period of 24 hrs. Average neurite length of RGCs was measured in each condition and data from three separate experiments was combined. A significant stimulation of neurite outgrowth was observed as a result of incubation with either GABA or baclofen. Error bars are s.e.m.; n=number of neurites measured; (**, $P<0.02$; non-parametric analysis of variance (ANOVA), Scheffè's post-hoc test).


Neurite Length (uM)
350
300
250
200
150
100
50
0
L15
100 uM GABA
100 uM baclofen

due to excess variability of neurite lengths in control cultures. In these two experiments, sister cultures had average neurite lengths that differed greater than two-fold, such that the average neurite length could not be measured accurately.

The results from the pooled experiments demonstrated a trend consistent with the data obtained for the dose response experiment, that is, 100 μM GABA increased the average neurite length of RGCs when compared to control cultures (Figure 17). The average neurite length of RGCs cultured in the presence of 100 μM GABA (n=110) was 231 μm ± 35 μm (s.e.m) compared to 129 μm ± 15 μm from control cultures (n=159). To statistically compare this result, an analysis of variance (ANOVA) Scheffé's test was performed (Table 2A). The significance of Scheffé's test is that it compares the group means from each condition. In addition, Scheffé's test is more conservative than other ANOVA tests, since a larger difference between means is required for significance. Statistical analysis revealed a greatly significant effect when cultures were supplemented with 100 μM GABA, compared to control conditions ($p<0.02$).

GABA receptor agonists have different effects on neurite outgrowth

GABA functions by activating its specific receptors. Therefore, to determine if activation of specific receptor types could mimic the effects of GABA upon RGCs in culture, specific receptor agonists were applied. Concentrations of receptor agonists used in these experiments are comparable to those previously used with isolated *Xenopus* embryonic neurons (Rohrbough and Spitzer, 1996, Wall and Dale, 1993,1994). Sister cultures from the above experiments were supplemented with 100 μM of the GABA-B receptor agonist baclofen. Baclofen also significantly increased RGC neurite outgrowth

Table 2: GABA and baclofen significantly stimulate neurite outgrowth as determined through ANOVA non-parametric analysis using Scheffè's post-hoc test. A) Summary of ANOVA testing for significant differences between the following culture conditions: Control, and 100 μM GABA.. A significant difference ($P<0.02$) was found in comparison of neurite length of 100 μM GABA with controls. B) Summary of ANOVA testing for significant differences between the following culture conditions: Control, 100 μM baclofen. A significant difference was ($P<0.02$) found when comparing neurite length of 100 μM baclofen treated cultures with controls Abbreviations are as follows *SS* (sum of squares), *df* (degrees of freedom), *MS*. (mean of squares), *F* (F- distribution), *F crit* (critical error).

A

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	159	20539	129.1761	12126.159
100 uM GABA	110	25427	231.15455	35454.994

ANOVA

Variation source	*SS*	*df*	*MS*	*F*	***P-value***
Between Groups	676167.51	1	676167.51	31.231878	**0.00000**
Within Groups	5780527.4	267	21649.916		
					F crit
Total	6456695	268			3.8765222

B

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	159	20539	129.1761	12126.159
100 uM baclofen	89	25664	288.35955	30343.847

ANOVA

Variation source	*SS*	*df*	*MS*	*F*	***P-value***
Between Groups	1445876.8	1	1445876.8	77.555784	**0.00000**
Within Groups	4586191.6	246	18643.055		
					F crit
Total	6032068.3	247			3.8795349

($p<0.001$) (Figure 17 and Table 2B). In addition, the neurite growth-promoting effect of baclofen was significantly stronger than the effect of GABA on RGC cultures ($p<0.02$). The average neurite length of sister cultures from the same experiment supplemented with 100 μM baclofen (n=89) was 288 μm ± 38 μm (s.e.m). In a separate preliminary experiment, 150 μM of the GABA-A receptor agonist muscimol was added to dissociated RGC cultures (see Figure 18). When muscimol supplemented cultures were compared to sister control cultures, a significant decrease in the average neurite length was observed ($p<0.001$) (Table 3). The average neurite length of cultures supplemented with 150 μM muscimol (n=101) was 202 μm ± 32 μm (s.e.m) compared to control cultures (n=63) that measured 307 μm ± 67μm (s.e.m.). The effect of muscimol in cultured RGCs is opposite to the effect observed with baclofen. Interestingly, the activation of GABA-A and GABA-B receptors has previously been shown to have opposite effects on neurite outgrowth of embryonic chick tectal neurons (Michler, 1990).

Brain Exposures to GABA and Baclofen

The results obtained from the experiments *in vitro* suggest that GABA can influence the growth of RGC neurites. Since the *in vitro* model is a simplified system, it is necessary to test the effects of altering GABA levels *in vivo*. To accomplish this, I performed brain exposures on stage 33/34 embryos and exposed developing RGCs to exogenous GABA. This stage was used since RGC axons have just crossed into the contralateral brain (Chien and Harris, 1994). The skin and dura covering the brain are removed from one side, and subsequently the brain is exposed to control or experimental media (Figure 19).

Figure 18: Muscimol significantly reduces neurite outgrowth in cultured RGCs. (preliminary result). Cultures were incubated in control L15 medium, with or without 150 μM muscimol for a period of 24 hrs. Neurite length of RGCs was determined for each condition. Error bars are s.e.m.; n=number of neurites measured; (**, $P<0.02$; non-parametric analysis of variance (ANOVA), Scheffè's post-hoc test).

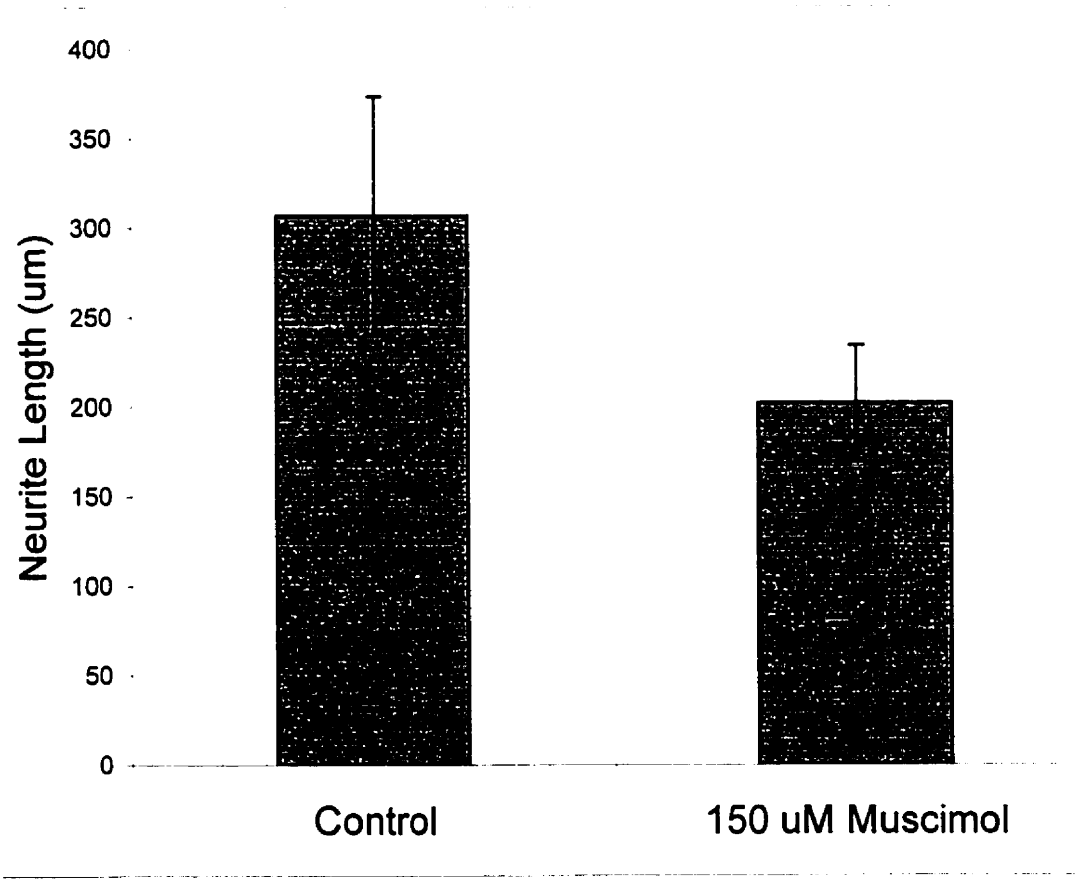

Neurite Length (um)
400
350
300
250
200
150
100
50
0
Control
150 uM Muscimol

Table 3: Muscimol significantly reduced neurite outgrowth in RGC cultures as determined through ANOVA non-parametric analysis using Scheffès post-hoc test. A significant difference ($P<0.02$) was found when comparing neurite length from 150 μM muscimol cultures with neurite length from controls. Abbreviations are as follows *SS* (sum of squares), *df* (degrees of freedom), *MS*. (mean of squares), *F* (F- distribution), *F crit* (critical error).

A

Anova: Single Factor

SUMMARY

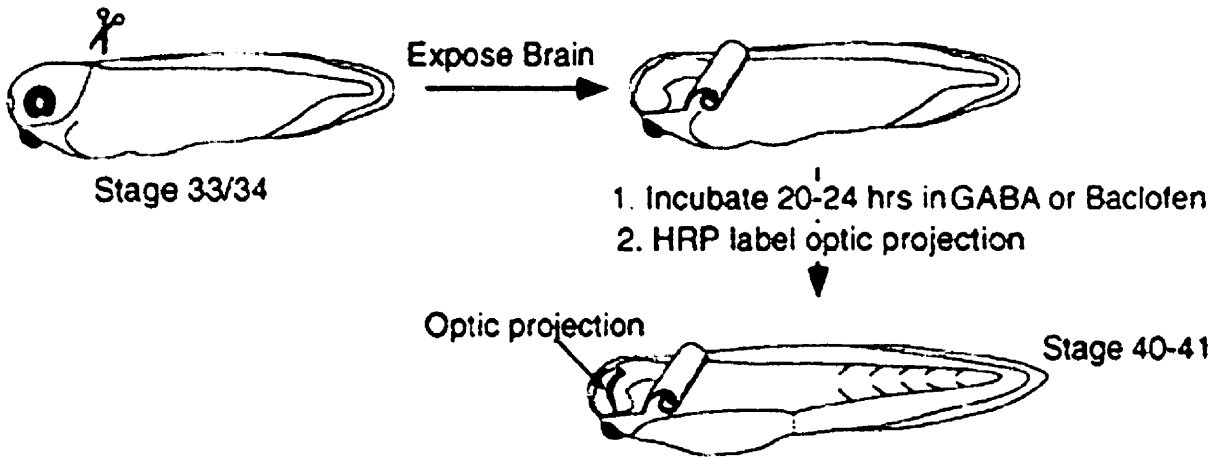
Groups	*Count*	*Sum*	*Average*	*Variance*
Control	64	19672	307.375	69829.79
150 uM Musc.	102	20557	201.5392	26096.9

ANOVA

Variation source	*SS*	*df*	*MS*	*F*	***P-value***
Between Groups	440491.085	1	440491.1	10.26864	**0.0016262**
Within Groups	7035064.34	164	42896.73		
					F crit
Total	7475555.43	165			3.8987764

Figure 19: Schematic of an exposed brain preparation. The skin and dura are removed from one side of stage 33/4 embryos. The embryos are incubated for 24 hours in control or experimental media. To visualize effects of exposure, horseradish peroxidase (HRP) fills are performed. For a complete description see chapter two. Optic projections of GABA-treated and sister control embryos were labeled with HRP at stage 40.

EXPOSED BRAIN PREPARATION



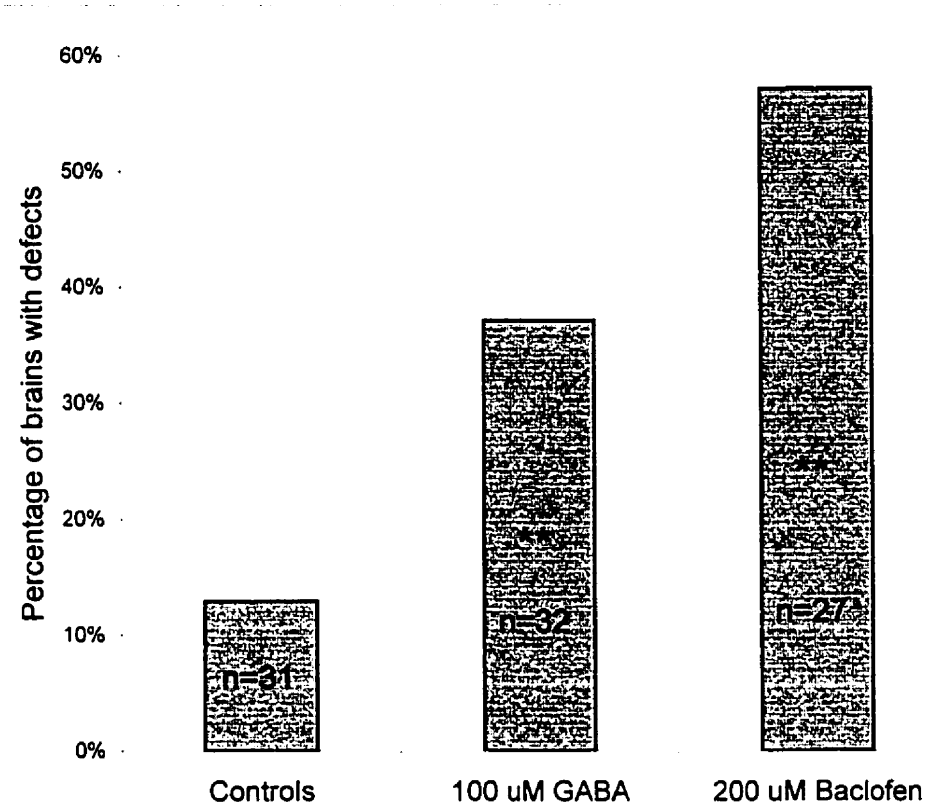
Brain exposures to GABA

To test the *in vivo* effects of exogenous GABA upon developing RGCs, embryos with exposed brains were incubated in medium containing 100 μM GABA. The embryos were allowed to develop until stage 40. To observe any effects of GABA, optic projections of GABA-treated as well as sister control embryos were labeled with HRP at stage 40

The analysis of the exposed brains revealed that 100 μM GABA did have an effect on optic projection development. Defects in the development of the optic projection were found. Data collected from 7 individual experiments showed that 37% of brains exposed to 100 μM GABA displayed optic projection defects (Figure 20A). This result was statistically significant ($p< 0.02$) when compared to controls showing defects (13%) (Figure 20B). The most common defect observed was a shortened optic projection when compared to controls. Thirteen of thirty-five brains exposed to exogenous GABA (Figure 20B) had very few RGC (< 10) axons that extended beyond the mid-optic tract (see arrows in Figure 21). Data from the 7 individual experiments were collected and the average length of the optic projections from each condition was quantified using an NIH image analysis package (see Chapter 2) (Chien et al., 1993). The average optic projection length of brains exposed to 100 μM GABA was 401 μm ± 48 μm (s.e.m.) compared to 484 μm ± 38 μm (s.e.m.) from controls (Figure 22). When ANOVA was performed, a significant difference was observed between GABA exposed brains and controls ($p<0.02$) (Table 4A).

Figure 20: Percentage of all brains showing optic projection defects as a result of brain exposure to control media, 100 μM GABA, and 200 μM baclofen. The projection defect examined here was a shortened optic projection (<10 axons crossing the mid-optic tract). Data represents seven individual experiments. A) Projection defects were observed in 37% of brains exposed to GABA and in 57% of brains exposed to baclofen. Thirteen percent of control brains had optic projection defects. B) A contingency table using a Pearson chi-square test demonstrated a significant effect ($P< 0.02$) of GABA and baclofen. Abbreviations are as follows: df (degrees of freedom), n= number of optic projections examined, (**, $P<0.02$).

A

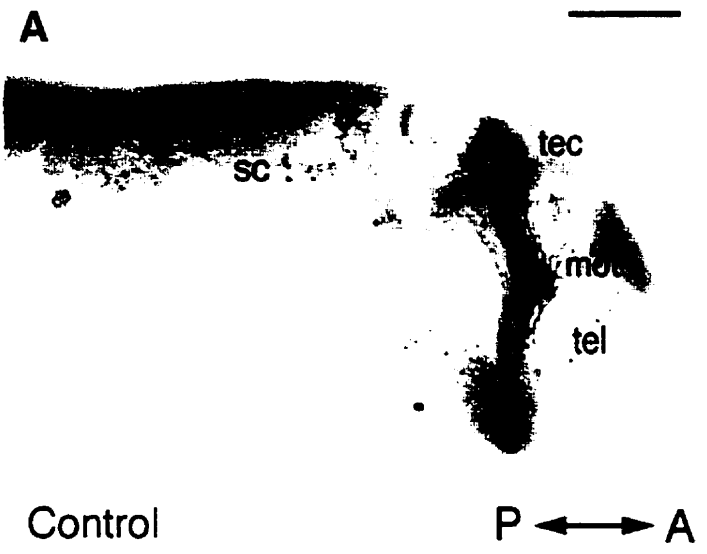


B

	DEFECT	NO DEFECT	Total
Control	4	27	31
Baclofen	16	12	28
GABA	13	22	35
Total	33	61	94

Test statistic	Value	df	p-value
Pearson Chi-s	12.74	2	**0.002**

Figure 21: Brain exposure to GABA or baclofen results in a shortened optic projection. Brains were exposed for 24hrs and HRP fills were performed to visualize the optic projection. Wholemount brains are positioned laterally. A) Control brain exposure performed simultaneously with exposures to GABA and baclofen. B) Representative brain exposed to 100 μM GABA which had very few (<10 axons) extending beyond the mid-optic tract (mot) (see arrow). C) Representative brain exposed to 200 μM baclofen had similar shortened optic projection (see arrow). In addition de-fasiculation of RGC axons was commonly seen in brains exposed to baclofen. Additional abbreviations are as follows: tec (optic tectum), tel (telencephalon), sc (spinal cord), A (anterior), P (posterior). Scale bar is 100 μm.


A
sc
tec
tel
Control
P
A


B
100 uM GABA
C
200 uM Baclofen

Figure 22: Average optic projection length of brains exposed to 100 μM GABA and 200 μM baclofen were shorter when compared to the average length of control optic projections. Data represents seven separate experiments. The average optic projection lengths of brains exposed to GABA or baclofen were significantly shorter when compared to controls. Error bars are s.e.m.; n=number of neurites measured; (**, $P<0.02$; non-parametric analysis of variance (ANOVA), Scheffè's post-hoc test).

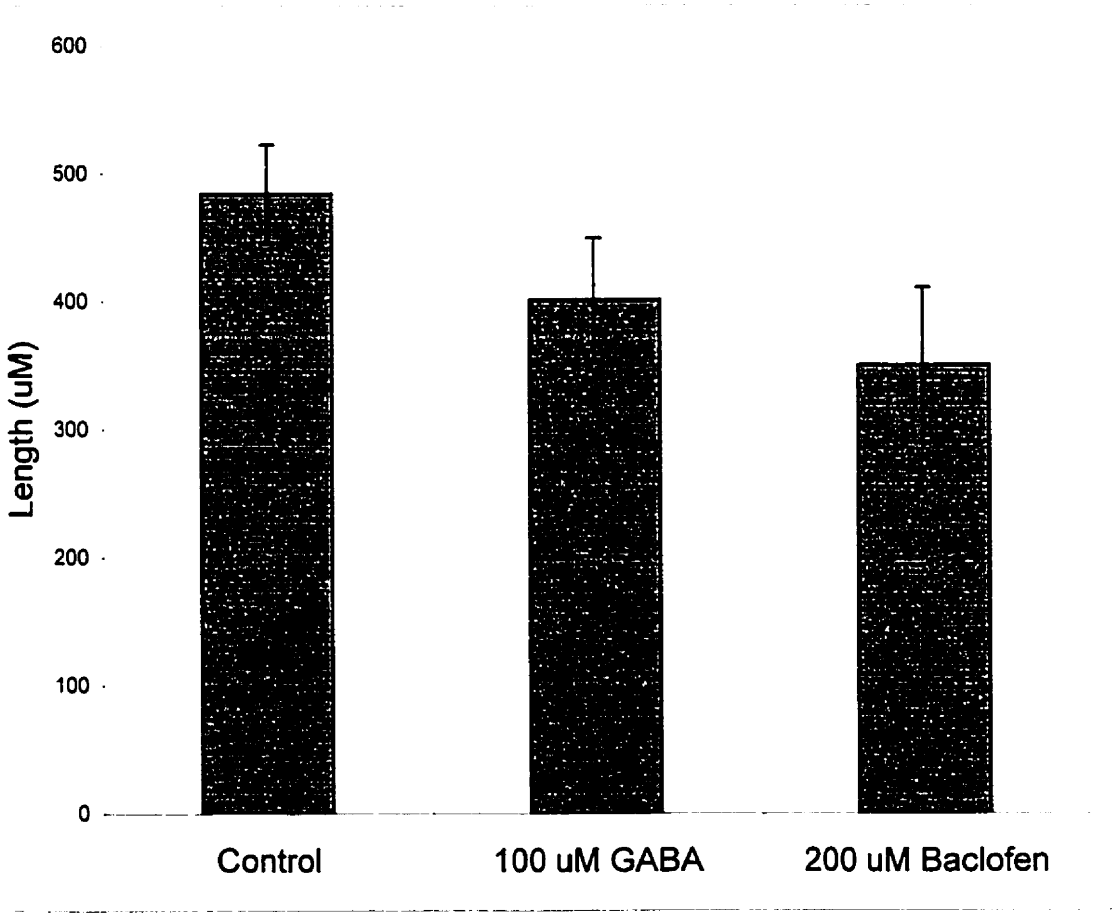

600
500
400
300
200
100
0
Length (uM)
Control
100 uM GABA
200 uM Baclofen

Table 4: Brain exposures to GABA and baclofen significantly shortened optic projection length as determined through ANOVA non-parametric analysis using Scheffè's post-hoc test. A) Summary of ANOVA, testing for significant differences between the following exposed brain conditions: Control, and 100 μM GABA. A significant difference ($P<0.02$) was found when comparing optic projection lengths of brains exposed to 100 μM GABA with control exposures. B) Summary of ANOVA, testing for significant differences between the following exposed brain conditions: Control and 200 μM baclofen. A significant difference ($P<0.02$) was found when comparing optic projection lengths of brains exposed to 200 μM baclofen with control exposures. Abbreviations are as follows *SS* (sum of squares), *df* (degrees of freedom), *MS*. (mean of squares), *F* (F- distribution), *F crit* (critical error).

A

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	31	7261.68	234.24774	2682.8385
100 uM GABA	35	6789.35	193.98143	4773.6852

ANOVA

Variation source	*SS*	*df*	*MS*	*F*	***P-value***
Between Groups	26654.439	1	26654.439	7.026158	**0.0101115**
Within Groups	242790.45	64	3793.6008		
					F crit
Total	269444.89	65			3.9909196

B

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	31	7261.68	234.24774	2682.8385
200 uM Baclofen	28	4747.14	169.54071	6029.4008

ANOVA

Variation source	*SS*	*df*	*MS*	*F*	***P-value***
Between Groups	61598.568	1	61598.568	14.432478	**0.0003551**
Within Groups	243278.98	57	4268.0522		
					F crit
Total	304877.54	58			4.0098769

Brain exposures to baclofen

Embryos were also exposed to 200 μM baclofen. A higher concentration of baclofen was used *in vivo* than *in vitro* to correct for possible problems with penetrance of baclofen into the brain. As a result of exposure to baclofen, defects in the development of the optic projection were also found (Figure 20A). A significant percentage ($p<0.02$) of brains exposed to baclofen displayed optic projection defects (57%) (Figure 20B). Similar to effects observed with GABA, optic projections exposed to 200 μM baclofen were shorter when compared to controls, with few RGC axons extending past the mid-optic tract region (see arrows in Figure 21). The average optic projection length of brains exposed to baclofen measured 350 μm ± 61 μm (s.e.m) (Figure 22). A significant difference in average optic projection length was observed following ANOVA analysis ($p< 0.02$) between controls and brains exposed to 200 μM baclofen (Table 4B).

Summary

A number of previous studies have demonstrated trophic actions of GABA on developing neurons (Spoerri and Wolff, 1981, Eins et al., 1983, Meier et al., 1984, 1991, Spoerri, 1988, Michler, 1990, Hansen et al., 1991, Wolff et al., 1993, Abraham et al., 1994, Ben-Ari et al., 1994, Loturco et al., 1995, Behar et al., 1994, Liu et al., 1997, Belhage et al., 1998). The purpose of my *in vitro* experiments was to first demonstrate that GABA could affect neurite outgrowth in cultured *Xenopus* RGCs. The results from these experiments demonstrate a specific neurite outgrowth-promoting effect of GABA upon RGCs. Moreover, applying the GABA-B receptor agonist baclofen mimicked this

effect. Preliminary experiments with the GABA-A receptor muscimol showed an opposite effect by decreasing neurite outgrowth from RGCs.

Previous to my study, experiments demonstrating an effect of GABA on developing neurons have been performed mostly in culture (see Meier et al., 1991, Belhage et al., 1998, Sandell, 1998 for references). The *Xenopus* brain exposure preparation allows for analysis of the effects of GABA on RGC and optic projection development in the intact embryo. My experiments demonstrated that GABA applied exogenously to exposed brains, resulted in a shortened optic projection when compared to controls. Further, this effect could be replicated by bath exposure to the GABA-B receptor agonist baclofen. A possibility exists that the results from the exposed brain preparation are due to toxic effects from bath application of these agents. A few pieces of evidence suggest that these results however are not due simply to toxicity. First, if the lens is removed from the *Xenopus* eye and the RGC bodies are exposed to GABA or baclofen, RGC axons project normally to the optic tectum. Second, GABA and baclofen stimulated neurite outgrowth *in vitro*, which also points to a non-toxic response. In conclusion, the results from these experiments suggest that GABA can act as an extrinsic cue for developing RGCs and the optic projection *in vivo*.

CHAPTER 5: GENERAL DISCUSSION

There is a large literature demonstrating that neurotransmitters like GABA are present quite early during nervous system development, where they function as trophic factors for developing neurons (eg. Redburn and Rowe-Rendleman, 1996; Erskine and McCaig, 1995, Lauder et al., 1998). In this study, I have provided several pieces of evidence suggesting that GABA acts as an early developmental signal for RGCs. First, GABA expression in the *Xenopus* optic tract coincides temporally and spatially with RGC axon development. Second, growth cones of developing RGC axons express GABA-A and GABA-B receptors. Third, GABA stimulates neurite outgrowth in cultured RGCs, an effect that is also observed with the GABA-B receptor agonist baclofen. In a separate preliminary experiment, when I applied the GABA-A receptor agonist muscimol to RGCs *in vitro*, neurite outgrowth was reduced when compared to sister control cultures. Finally, I demonstrated *in vivo* that exogenous GABA as well as baclofen caused a shortening of the optic projection. Taken together, the results from these experiments support the idea that GABA acts as an extrinsic factor for developing *Xenopus* RGC axons.

A possibility exists that the expression of GABA within the optic tract of *Xenopus* may serve as a marker of cells that express other extrinsic cues necessary for directing the growth of RGC axons toward the tectum. However, evidence that I have provided here, argue that GABA does have a role in the development of RGC axons. RGC axons have the necessary receptors to respond to GABA, and are affected by exogenous GABA both *in vitro* and *in vivo*.

In the exposed brain experiments, both RGC axons as well as the surrounding tissue are exposed to the bathing media. This raises the question as to whether the effects of exposure to GABA or baclofen may be a result of a direct action on RGC axons, or an indirect action upon the surrounding cellular substrate. I have evidence suggesting that the effects of GABA and baclofen are a result of direct action on RGC axons. First, isolated RGC axons and growth cones express GABA-A and GABA-B receptors. Second, GABA as well as its receptor agonists can alter neurite outgrowth of cultured RGCs. GABA and baclofen stimulated neurite outgrowth *in vitro*, whereas muscimol reduced neurite outgrowth. This observed effect of muscimol is a preliminary result and needs to be repeated. Nonetheless the *in vitro* data suggest that the observed effects were a result of direct action of GABA on RGC axons. To further support this claim, in the future we will need to demonstrate that no alterations to the cellular substrate surrounding the optic projection resulted from exposure to GABA or baclofen.

An interesting observation from this study is that the *in vivo* effects of GABA and baclofen were opposite to those found in *in vitro* experiments. These differences are likely explained by the fact that *in vitro* conditions are much simpler than the actual conditions within the intact embryo. During development, growth cones encounter many different cues along their specific pathways that contribute to their overall growth and guidance. Thus the presentation of one extrinsic cue *in vitro* may not result in the same effects observed *in vivo*. For instance, evidence suggests that GABA as well as its receptor agonists can have different effects on developing neurons in culture, depending upon constituents of the culture medium (Michler, 1990). Embryonic chick tectal neurons grown in serum containing medium in the presence of GABA showed increases in neurite

length and process branching. If these neurons were grown in serum free medium supplemented with GABA, neurite growth was substantially inhibited (Michler, 1990). The growth enhancing effects of GABA in serum containing medium were reproduced when the GABA-A receptor agonist muscimol, was added in place of GABA. Baclofen, the GABA-B receptor agonist did not stimulate neurite extension in serum containing medium, rather it was able to mimic the inhibitory effects of GABA on neurite growth in serum free medium (Michler, 1990). These findings are interesting, since GABA can either stimulate or inhibit neurite growth depending upon the conditions in which neurons develop.

Another possible explanation for the different responses of these neurons to GABA and baclofen, comes from evidence that the internal state of the growth cone is critical for determining the effects of any particular cue (Ming et al., 1997, Song et al., 1997). It has been demonstrated that growth cones can respond to the same diffusible factor with an attractive or a repulsive response, depending upon cyclic AMP (cAMP) or cyclic GMP (cGMP) levels in the growth cone (Ming et al., 1997, Song et al., 1997). Diffusible factors used in these studies included the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), as well as the neurotransmitter acetylcholine, and netrin-1 (Ming et al., 1997, Song et al., 1997, 1998). More recently, it was shown that *Xenopus* RGC axons sense the attractive guidance cue netrin as repulsive when netrin was presented to growth cones with laminin (Höpker et al., 1999). Thus *in vivo*, where many extrinsic cues can act on RGC growth cones, GABA may have different effects than those observed in the simpler culture system.

The mechanism(s) behind the actions of GABA upon developing RGCs in culture or within the intact embryo have not yet been identified. One possibility I will discuss draws from evidence showing that GABA increases intracellular calcium ($[Ca^{2+}]_i$) in developing neuronal growth cones (Obrietan and Van Den Pol, 1996), as well as in all retinal neurons of early postnatal rabbits (Huang and Redburn, 1996). As described in the introduction, GABA functions as an excitatory neurotransmitter in early development to depolarize neuronal membranes (see Cherubini et al., 1991 for review). A number of studies have shown that activation of the GABA-A receptor results in an efflux of Cl^- ions due to a relatively depolarized Cl^- reversal potential (Ben-Ari et al., 1989, Chen et al., 1996). The depolarizing effects of GABA are reported in developing neurons from a variety of different CNS areas including the hippocampus (Ben-Ari et al., 1989, Cherubini et al., 1990), spinal cord (Wu et al., 1992, Wang et al., 1994, Reichling et al., 1994, Rohrbough and Spitzer, 1996), cerebellum (Conner et al., 1987, Brickley et al., 1996), olfactory bulb (Serafini et al., 1995), hypothalamus (Chen et al., 1996), and retina (Yamashita and Fukuda, 1993). The efflux of Cl^- ions triggers a membrane depolarization and activation of voltage-sensitive Ca^{2+} channels, resulting in an increase in $[Ca^{2+}]_i$ (Yuste and Katz, 1991, Yamashita and Fukuda, 1993, Obrietan and van den Pol, 1995). In addition to increases in $[Ca^{2+}]_i$ levels in the growth cone as a result of GABA-A receptor activation, GABA-B receptor modulation of N-type calcium channels was observed in axons of neonatal rat optic nerve (Sun and Chiu, 1999). In this system, activation of GABA-B receptors by baclofen inhibited calcium influx into axons.

It is widely accepted that neuronal growth cones are sensitive to changes in $[Ca^{2+}]_i$ (see Goldberg and Grabham, 1999 for review). Substantial increases in $[Ca^{2+}]_i$

caused by neurotransmitter binding or depolarization may result in an inhibition of neurite outgrowth and growth cone collapse (Kater and Mills, 1991). In other cases however, an increase in $[Ca^{2+}]_i$ can stimulate growth cone motility and neurite outgrowth (Davenport and Kater, 1992). The role of Ca^{2+} in the regulation of neurite growth is supported by the evidence that growth cones of embryonic frog spinal neurons display brief increases in $[Ca^{2+}]_i$ (termed waves) that affect the speed of neurite extension in culture (Gu and Spitzer, 1995). As the frequency of calcium waves increased, the speed at which neurites grew decreased. In contrast, suppression of waves by removal of extracellular Ca^{2+} led to faster extension rates. Regulation of neurite outgrowth by Ca^{2+} was also demonstrated *in vivo* by this same group (Gomez and Spitzer, 1999). In this study, changes in $[Ca^{2+}]$ were imaged during suppression or induction of Ca^{2+} waves in the intact embryonic frog spinal cord using photorelease of caged Ca^{2+} chelators and donors respectively. Their data indicate that during growth and extension phases, growth cones have very few if any Ca^{2+} waves, whereas waves are associated with periods of slow or stalled growth. Slow or stalled growth periods are normally observed at points along an axon's growth pathway when decisions have to be made, such as making a turn or reaching the particular target area (Goldberg and Grabham, 1999). The idea that changes in $[Ca^{2+}]_i$ could be responsible for both growth and inhibition of growth was accommodated by the set-point theory put forth by Kater and colleagues (Kater and Mills, 1991). The theory suggests that there is an optimal range for $[Ca^{2+}]_i$, and that a large deviation from this optimal concentration in either direction could inhibit or slow neurite growth. The theory itself makes sense since high or low $[Ca^{2+}]_i$ are cytotoxic,

making a tight regulation of $[Ca^{2+}]_i$ important for cell survival (Goldberg and Grabham, 1999).

Activation of both GABA-A and GABA-B receptors have been shown to alter $[Ca^{2+}]_i$ levels in a variety of developing neurons, including retinal neurons. Thus, one possible explanation for the different effects of GABA in the *in vitro* and *in vivo* could involve changes in $[Ca^{2+}]_i$ within and outside the optimal range. *In vitro* activation of GABA receptors could bring $[Ca^{2+}]_i$ into the optimal range for neurite extension and result in enhanced RGC neurite outgrowth. In contrast, the *in vivo* result of a shortened optic projection may be explained by activation of GABA receptors resulting in $[Ca^{2+}]_i$ out of the optimal range. Deviation from an optimal concentration could result in a pre-mature stalling. It is interesting to note also that the areas of highest GABA expression within the embryonic *Xenopus* optic tract are found in the 1) mid-optic tract, an area where RGC axons turn caudally, and 2) within the optic tectum, the target destination for these axons. As mentioned earlier, these two areas are points along the pathway where decisions are made, thus growth is slower or stalled. Perhaps then RGC axons may use an extrinsic GABA signal in these areas to assist in slowing growth. In summary, calcium regulation is one possible mechanism by which GABA and its receptor agonists could alter RGC neurite outgrowth *in vitro* and optic projection length *in vivo*. Specific experiments to test this idea could involve measuring internal calcium levels of RGCs in response to the application of GABA or its agonists. Presumably baclofen and muscimol would have different effects on internal calcium levels since their effects on neurite extension were different. To determine if the effects of GABA are mediated through receptor activation of calcium channels, GABA receptor agonists and specific calcium

channel blockers could be added to RGC cultures and neurite growth assessed. These are just a couple of experiments that may result in support for a calcium regulatory mechanism for GABA-induced effects on RGCs.

Neurotransmitters such as GABA are released at the growth cone (Hume et al., 1983, Young and Poo, 1983, Lockerbie et al., 1985; Taylor et al., 1991,Yao et al., 2000, Gao and van den Pol, 2000), where they are thought to assist in axon elongation and targeting, as well as synapse formation (Erskine and McCaig, 1995). Recent data put into question the idea that neurotransmitter release is involved in neural connectivity and CNS assembly (Verhage et al., 2000). This study identified the *munc18-1* gene as essential for all components of vesicular neurotransmitter release throughout the brain. The Munc18-1 protein was found to be part of the Sec-1-family of membrane-trafficking proteins that may be involved in secretion at synapses. A *munc18-1* knock-out mouse was generated through homologous recombination. The null-mutant mice are paralyzed and die shortly after birth. Upon analysis of the mice, the mutation appeared to cause a complete loss of neurotransmitter secretion from vesicles. The group claimed that the apparent loss of synaptic vesicle secretion of neurotransmitters had no affect on proper neural assembly, that is, formation of appropriate layers, fiber pathways and synapses in the brains of mutants developed normally. This study further showed that shortly following completion of CNS assembly, massive neuronal apoptosis occurs, leading to widespread neurodegeneration. From their analysis, the researchers concluded that neural connectivity does not depend on neurotransmitter release through vesicles, but that the maintenance of synapses requires vesicular secretion. This claim challenges the idea set

forth in this thesis, as well as in previous reports, that neurotransmitters like GABA are important for early stages of neuronal connectivity in development.

Although convincing evidence supporting this group's conclusion was presented, a few caveats arise from this study that weaken their claim. 1) Although they claim there were no differences between the null and control mice, examination of the figures from this study appear to show differences. For example, staining for growth cones, fibre bundles, and synaptic layers appeared less organized, lighter in some areas, and more spread out in others. Presynaptic marker staining was also reduced and less organized in the null mice when compared to controls. 2) It is important to realize that early in development, neurotransmitters can be released through Ca^{2+} -independent non-vesicular means (Taylor and Gordon-Weeks, 1988, 1989, Taylor et al., 1990). Previous studies with isolated GABAergic neurons from neonatal rat forebrain demonstrated that uptake and release of [^{3}H] GABA was K^+ stimulated and Ca^{2+} -independent (Taylor and Gordon-Weeks, 1989). The release of [^{3}H] GABA remained Ca^{2+} -independent up to post-natal day 5 (P5), whereupon, a Ca^{2+} -dependent component of [^{3}H] GABA release appeared. The onset of the Ca^{2+} -dependent component of [^{3}H] GABA release correlates with the appearance of synaptic vesicles in these isolated neurons (Taylor and Gordon-Weeks, 1988, 1989). Additional studies by Taylor et al. (1990) demonstrated that cultured GABAergic neurons from P3 rat forebrain could release both endogenous as well as [^{3}H] GABA from their growth cones. This GABA release was not due to vesicular release as antibody staining for vesicular component proteins p65 and synaptophysin was undetected in these neuronal cell bodies, axons and dendrites. Synaptophysin and p65 are vesicle-specific antigens that are found at all synapses (see Taylor et al., 1990 for

references). The results from this study demonstrated that GABA could be released from developing neurons before the onset of synaptic vesicles.

In addition, high levels of GABA immunoreactivity were found in neonatal rat optic nerve astrocytes and axons (Sakatani et al., 1992, Rogers and Pow, 1995). The abundant glial and axonal sources of GABA is puzzling since there is a lack of vesicular means of neurotransmitter release in axonal tracts (Sun and Chiu, 1999). A suggested release mechanism for GABA in the optic nerve, as well as the non-vesicular release in isolated neurons, is the reverse operation of neurotransmitter transporters (Chiu and Kriegler, 1993, Haycock et al., 1978, Levi and Raiteri, 1978, Schwartz, 1987). Generally speaking, neurotransmitter transporters function in the uptake of neurotransmitters, thus halting their actions and restoring their supply in nerve terminals (Levitan and Kaczmarek, 1997). GABA transporters are driven by Na^+ gradients and are electrogenic (i.e. they can change membrane voltage) (Levitan and Kaczmarek, 1997). Thus the normal movement of GABA into terminals is coupled to the movement of Na^+ ions through the membrane. During periods of activity, shifts in ionic gradients coupled to depolarizations could possibly drive GABA transporters in the optic nerve to release GABA (Sun and Chiu, 1999). Within neonatal rat optic nerves, three subtypes of the GABA transporter are differentially expressed. (Howd et al., 1997). Further support of this mechanism of non-vesicular GABA release comes from the studies of isolated GABAergic neurons from neonatal rat forebrain (Taylor and Gordon-Weeks ,1988).

Since non-vesicular release of GABA occurs prior to established neural connectivity, it is possible that not all neurotransmitter secretion is lost in the *munc18-1* knockout mice. If this is true, the assumption that neurotransmitter secretion is not

necessary for normal brain assembly may not be accurate. Experiments to resolve the extent to which non-vesicular release contributes to neurotransmitter levels early in development are necessary.

The possibility also exists that the role of neurotransmitters in early neural development and connectivity is a redundant one. Even though exogenous application of neurotransmitters such as GABA can affect neural development, removal of endogenous neurotransmitter activity early in development may not affect neural connectivity if additional extrinsic factors are involved. Finally, species difference between *Xenopus* and mouse may be one final explanation for the results seen here and those observed by Verhage et al. (2000).

Although the studies presented here focus on GABA-A and GABA-B receptors as mediators of GABA activity, the GABA-C receptor, which is a ligand-gated Cl^- channel, has also been shown to be prominently expressed in the vertebrate retina (Bormann, 2000). These channels are composed of ρ subunits and have a distinct pharmacology (Johnston, 1994, 1996, Bormann and Feigenspan, 1995, Bormann, 2000). The GABA-C receptor can be activated selectively by CACA and antagonized selectively by TPMA (Johnston, 1996, Bormann, 2000). Localization studies of GABA-C receptors in the early post-natal rat retina showed that they are restricted to axons of bipolar cells in the INL (Koulen et al., 1998). Few other localization studies in the developing vertebrate eye exist. In the adult vertebrate eye, many retinal neurons including bipolar cells (Feigenspan et al.,1993, Qian and Dowling, 1995, Qian et al., 1997, Lukasiewicz et al., 1994, Lukaisiewicz and Wong, 1997, Pan and Lipton, 1995, Nelson et al., 1999), horizontal cells (Dong et al, 1994, Kaneda et al., 1997), cone photoreceptors (Picaud et

al, 1998), and RGCs (Zhang and Slaughter, 1995) express GABA-C receptors. At this point, it is not known if developing RGC axons and growth cones express GABA-C receptors. No commercial antibodies are available for GABA-C receptors. Researchers that have studied GABA-C receptor expression developed their own antibodies that react to the ρ subunits that form the receptor channel. Thus the possibility exists to use these antibodies for immuncytochemical detection of GABA-C receptors on RGC growth cones. In addition, if GABA-C receptors were expressed on RGC growth cones, exposed brain experiments with medium containing the selective GABA-C receptor antagonist TPMA could be used to block endogenous GABA signaling through this receptor. Addition of all three receptor antagonists should block all GABA signaling through its receptors and thus reveal if RGC axons require an endogenous GABA signal for proper development within the optic tract.

In conclusion, my data suggest that GABA acts as an extrinsic signal for developing RGCs. One caveat is that these experiments tested the effects due to exogenous application of GABA. To further support GABA's involvement, additional experiments that investigate the endogenous function of GABA upon developing RGCs are required. A number of specific GABA receptor antagonists are available that act to block receptor signaling, thus reducing or eliminating the actions produced when endogenous GABA binds to its receptors. Exposed brain experiments could be performed where developing RGC axons are exposed to GABA receptor antagonists like bicuculline (GABA-A receptor antagonist) or phaclofen (GABA-B receptor antagonist). These experiments would remove the endogenous activity of GABA signaling through its receptors. Comparing the developing optic projections from embryos exposed to GABA

receptor antagonists with sister controls, would assist in determining if endogenous GABA within the optic tract is necessary for proper RGC axonal development.

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