Motion Processing in the Larval Zebrafish Tectum

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Motion processing in the larval zebrafish tectum

A dissertation presented
by
Abhinav Satish Grama
to
The Department of Molecular and Cellular Biology
in partial fulfillment of the requirements
for the degree of
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Motion processing in the larval zebrafish tectum

ABSTRACT

Larval zebrafish are highly visual animals that display a diverse repertoire of visually guided behaviors. Five days after their birth, they start tracking and hunting moving prey, a behavior that likely requires underlying neural circuits to analyze motion. The optic tectum, the largest structure in the zebrafish brain, is known to be involved in prey capture behavior. The specific role of this structure in motion processing is still an open question.

The larval tectum receives processed inputs from direction selective retinal ganglion cells (DSRGCs). How do these inputs influence the responses of tectal neurons? Do local tectal circuits further affect tectal responses to motion? To study this, we performed in vivo two-photon calcium imaging on populations of tectal neurons and in vivo whole cell recordings while presenting larvae with moving stimuli. We show that a substantial fraction of tectal neurons are sensitive to the direction and speed of moving stimuli. Direction selectivity (DS) in these neurons is weakly correlated with RGC inputs and strongly correlated with local inhibition. The inhibition comes from the null direction of the recorded neurons and appears to be mediated by direction selective inhibitory neurons. Our data demonstrates the presence of a
tectal circuit for computing the direction of motion, whose motif resembles the DS circuit in the vertebrate retina.

What roles do excitatory and inhibitory tectal neurons have in motion processing? To explore this, we recorded motion responses from labeled glutamatergic and GABAergic neurons in conjunction with a newly generated pan-neuronal transgenic line expressing the genetically encoded calcium indicator GCaMP6s. We show that excitatory and inhibitory tectal neurons display a matching degree of selectivity to motion. DS inhibitory neurons seem to cluster into two populations preferring head-directed or tail-directed motion. In contrast, DS excitatory neurons form three overlapping clusters. The preferred directions of these clusters appear to be phase shifted with respect to those of DSRGC inputs.

Our results show that rather than being a simple relay center for processed retinal inputs, the tectum builds direction selective responses by employing a network of highly selective interneurons. This processing appears to transform the representation of motion direction by RGCs into distinct representations by subpopulations of tectal neurons.
Table of Contents

Preface

Abstract ................................................................ iii

List of Figures ........................................................................ vi

Acknowledgements .................................................................... viii

Chapter One

Introduction ........................................................................ 1

Chapter Two

Direction selectivity in the larval zebrafish tectum is mediated by asymmetric inhibition.................... 25

Chapter Three

Distinct representations of motion by excitatory and inhibitory neurons in the larval zebrafish tectum ........................................................................ 56

Chapter Four

Conclusion ........................................................................ 95
List of Figures

Chapter One

Figure 1.1 Photomicrograph of the larval zebrafish................................. 3
Figure 1.2 Retinotectal projections in larval zebrafish............................... 4
Figure 1.3 Cytoarchitecture of the fish tectum......................................... 9
Figure 1.4 Distribution of preferred directions of direction selective
retinal ganglion cells in the rabbit retina........................................... 15
Figure 1.5 Models for direction selectivity............................................. 18

Chapter Two

Figure 2.1 Two-photon calcium imaging of tectal responses to
moving bars................................................................. 30
Figure 2.2 Population responses to bars moving at different
speeds and directions..................................................... 33
Figure 2.3 Inhibitory currents are biased towards the null direction
of motion................................................................. 36
Figure 2.4 Inhibition precedes excitation in the null direction and
and follows it in the preferred direction................................. 40
Figure 2.5 Sharpening of membrane potential DS by the spike
generating mechanism..................................................... 42
Figure 2.6 The proposed model for the direction selective circuit in
the larval zebrafish tectum............................................. 45

Chapter Three
Figure 3.1  GABAergic and glutamatergic neurons are spatially localized in the tectum.......................... 63

Figure 3.2  New pan-neuronal transgenic expressing GCaMP6s faithfully reports spiking activity for both GABAergic and glutamatergic neurons.................................................. 66

Figure 3.3  GABAergic and glutamatergic neurons have a broad range of receptive field sizes.................................................. 69

Figure 3.4  Example responses of glutamatergic and GABAergic neurons to drifting bars.................................................. 72

Figure 3.5  Glutamatergic and GABAergic neurons show differences in DSI but not 1-Circular Variance.......................... 75

Figure 3.6  Preferred directions (PDs) of direction selective GABAergic and glutamatergic neurons fall into distinct clusters................................................................. 78

Figure 3.7  GABAergic and glutamatergic neurons show significant distance dependent noise correlations.......... 81

Chapter Four

Figure 4.1  Direction selective responses are restricted to the superficial layers of the tectal neuropil......................... 97

Figure 4.2  Model outlining possible mechanism of direction selectivity in the larval zebrafish tectum....................... 100
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CHAPTER ONE

Introduction
“What The Frog’s Eye Tells The Frog’s Brain” goes the title of a classic neuroscience paper by Jerome Lettvin and colleagues (Lettvin et al., 1959). This paper pioneered the idea that the neural tissue in the eye isn’t just a simple luminance detector, but a parallel processor of visual scenes. They showed that the retina extracts visual information salient for an organism’s survival and transmits this to the primary visual structure in the frog’s brain, the optic tectum. What the brain does with this filtered information is still an open question in systems neuroscience. I have attempted to investigate one aspect of this question, namely, selectivity to moving visual stimuli, by studying the tectum in larval zebrafish.

Zebrafish, a ray-finned fish from the flood plains of India (Spence et al., 2008), have been one of the bastions of developmental biology owing to their rapid development, malleability to genetic manipulations and the transparent, optically accessible bodies of their embryos and larvae. These very same reasons combined with new imaging and electrophysiological techniques make them a powerful tool for neuroscience as well.

THE VISUAL SYSTEM IN LARVAL ZEBRAFISH

Retina

Larval zebrafish are highly visual organisms. This is evident by the range of visual behaviors they display and the predominance of neural hardware underlying vision (Easter and Nicola, 1996; Portugues and Engert, 2009). The first stage of the larval zebrafish visual system, the eyes, reside on either side of the larvae’s head and share a minimal binocular overlap (Figure 1.1). The larval zebrafish retina develops rapidly and builds its three cellular laminae in an inside-out fashion by first generating the output layer, the retinal ganglion cell (RGC) layer,
Figure 1.1 Photomicrograph of the larval zebrafish: Photomicrograph of a dorsal view of a fish viewed in the dissecting microscope. The lines indicate the body axis and the planes of the two pupils. The clockwise (CW) and counterclockwise (CCW) direct ions are indicated. Adapted from (Easter and Nicola, 1996)
Figure 1.1 (continued)
which is composed of glutamatergic (excitatory) projection neurons. The first RGCs are born at 27 hours post fertilization (hpf). The ganglion cells get added as an unfolding paper-fan to give a central patch of cells by ~36 hpf. The middle layer, inner nuclear layer, gets added by 38 hpf and the finally the input layer, photoreceptor layer, by 48 hpf (Easter and Nicola, 1996; Hu and Easter, 1999). This primary wave of neurogenesis is followed by post-embryonic neurogenesis that continues throughout the life of the animal. The new retinal neurons are generated by stem cells at the periphery of the retina in the ciliary marginal zone (CMZ) and are added as concentric rings to the existing retinal disc (Cerveny et al., 2012). Even in the larval stage, the zebrafish retina, like other vertebrate retinas, possesses enormous neuronal diversity. This was recently demonstrated by a study, which used dendritic morphology and axonal projection patterns to describe over 50 different types of RGCs (Figure 1.2) (Robles et al., 2014). This diverse set of RGCs cross the optic chiasm at 32 hpf and project exclusively to the contralateral side of the brain and terminate in 10 different areas called the arborization fields (AFs) (Figure 1.2C, (Burrill and Easter, 1994)). Studies characterizing the visual response properties of RGCs in the larval zebrafish retina, though few in number, tell us that they have receptive field (RF) diameters of about 10° visual angle and can show a range of visual responses, for e.g., motion selective responses, ON-OFF responses etc. (Emran et al., 2007; Sajovic and Levinthal, 1982; Zhang et al., 2011).

The retinotectal projection
Figure 1.2 Retinotectal projections in larval zebrafish: (A) Wiring diagram of the larval zebrafish optic tract with 20 stereotyped projection classes represented by horizontal lines. Line width represents relative frequency, and black circles indicate axonal branch points. Innervation site coloring is based on input divergence index (IDI) values. (B) Schematic summary of 14 RGC classes defined by IPL stratification. The scale bar represents 12.5 mm. Adapted from (Robles et al., 2014). (C) Schematic diagram of the retinofugal arborization fields (AFs) present at 6-7 days. (lateral views: rostral, left; dorsal, up). Adapted from (Burrill and Easter, 1994)
Figure 1.2 (continued)
The retinotectal pathway has been the poster child for investigations into topographic brain wiring, i.e., mapping from an input layer of neurons to an output layer that preserves the spatial relationship of the neurons (Flanagan, 2006). In the case of the retinotectal pathway, this wiring scheme helps recast the image formed on the retina at the level of the tectum. Retinotectal mapping was the focus of, what are now, a classical series of experiments by Roger Sperry that led to the establishment of the chemoaffinity hypothesis. This hypothesis argues that gradients of molecules, which are controlled genetically, are expressed in the membranes of efferent and afferent neurites and act as beacons for axons expressing the right receptors to terminate, so that an accurate mapping is maintained (SPERRY, 1963). This hypothesis has been validated in studies done since, with quite a few ligands and receptors that are expressed in gradients having been discovered (Flanagan, 2006). In addition to genetically predetermined targeting, neural activity is also important for refining and maintaining the retinotectal map (Gnuegge et al., 2001).

In larval zebrafish, the developing RGC axons reach the tectum by about 44 hours, close to when the photoreceptor layer finishes developing, and begin forming arbors around 60 hpf (Stuermer, 1988). Shortly after this (~3dpf), the first visually evoked responses are seen (Easter and Nicola, 1996). The RGC inputs to the optic tectum in zebrafish (and other teleosts) are exclusively from the contralateral side (Burrill and Easter, 1994). This is in contrast to the homologous retino-collicular projections in mammals with binocular vision where there is a decussation at the optic chiasm and ipsilateral projection of axons from RGCs which fall within the binocular field of view (Knudsen, 2011). In addition to the RGC axons mapping in a highly specified topographic manner, they also project to very specific laminae in the neuropil region.
Figure 1.3 Cytoarchitecture of the fish tectum: (A) Cells described from classical Golgi studies in the adult goldfish tectum. Fourteen types of neuron were identified on the basis of cell body position and morphology. Adapted from (Meek and Schellart, 1978). (B) A sampling of neuron morphologies observed in the larval zebrafish tectum using ‘genetic golgi’ methods. These include: radial glia (RG), periventricular projection neurons (PVPNs), periventricular interneurons (PVINs) and superficial interneurons (SINs). Retinorecipient laminae in the tectum are indicated by shading. Note the diverse dendrite morphologies of both projection neurons and interneurons in the tectum. In particular, PVINs have been observed containing arbors that are non-stratified (nsPVINs), mono-stratified (msPVINs) or bi-stratified (bsPVINs). Adapted from (Nevin et al., 2010).
Figure 1.3 (continued)

A  Cell types in adult goldfish tectum

B  Cell types in larval zebrafish tectum
of the optic tectum (Robles et al., 2014). The neuropil region of the tectum in larval zebrafish is a mesh of RGC axons, tectal dendrites and axons, and a sparse subset of neuropil restricted cells. The retino-recipient layers of the neuropil are the stratum opticum (SO), the stratum fibrosum et griseum superficiale (SFGS), stratum griseum central (SGC) and the stratum album central (SAC) (Figure 1.3), with the largest layer SFGS, possessing many sub-laminae. Lettvin and colleagues were one of the first ones to highlight the functional specialization of the many laminae in the tectum. That would imply that each sub-lamina represented a parallel channel conveying a filtered version of the visual scene. In the case of larval zebrafish, recent studies showed that this was indeed true for motion selective RGCs, which target superficial sub-laminae in the tectum (Lowe et al., 2013; Nikolaou et al., 2012).

The optic tectum: architecture and functional properties

The optic tectum or better known as the superior colliculus in mammals, is a multi-layered structure that integrates information from different sensory modalities such as, vision, and somatosensation (Deeg et al., 2009; Knudsen, 2011), thus forming a brain area with a coherent sensory representation of the outside world. In larval zebrafish, the tectum is the largest part of the brain and is the primary target of all retinal afferents (Burrill and Easter, 1994). Golgi stain anatomical studies in a closely related teleost, the goldfish, showed that there are at least 14 different cell types in the fish tectum (Figure 1.3) (Meek and Schellart, 1978). The first thirteen cell classes are sparse subsets of cells found in the neuropil layer. The majority cell class, type XVI, is comprised of neurons found in the cell body layer (stratum periventriculare or SPV), and which make up 95% of cells in the tectum (Niell and Smith, 2005). The SPV is the deepest layer in the tectum and is comprised of unipolar neurons that that
extend radial dendrites into the neuropil layer. This primary dendrite gives off collaterals and these may terminate in different sub-laminae of the neuropil, thereby accessing inputs from RGCs that encode for specific properties about the visual scene (Figure 1.3). Axon collaterals sprout from the dendritic arbor and may terminate locally or target a variety of brain regions including the contralateral tectum, hindbrain, etc. (Sato et al., 2007; Scott and Baier, 2009). The majority of the SPV neurons are either excitatory (glutamatergic) or inhibitory (GABAergic) with a sparse subset of cholinergic cells also reported (Higashijima et al., 2004; Mueller et al., 2004).

A rich history of physiological and behavioral studies has shown the important role that the tectum/superior colliculus plays in combining information from different sensory modalities, selecting for salient features, and then issuing commands to mediate goal directed behaviors (Knudsen, 2011). In larval zebrafish, this is done via glutamatergic neurons which send projections out of the tectum to downstream motor areas to coordinate eye movements and orienting turns (Bianco et al., 2011; Herrero et al., 1998; Sato et al., 2007).

A majority of our current knowledge about the responses properties of tectal neurons comes from recent experiments employing multi-photon microscopy (Grama and Engert, 2012; Niell and Smith, 2005; Ramdya and Engert, 2008). This non-invasive imaging technique utilizes a pulsed, near infrared laser to excite a small volume of fluorophores at its focal point. This point is raster-scanned over a sample to generate an optical section of the tissue. This allows for the non-invasive monitoring of fluorescence from deep structures in the brain. In combination with fluorescent dyes (organic or genetically expressed) that report the intracellular calcium concentration, a direct correlate of spiking neural activity, two-photon (2P) microscopy is a powerful tool that permits the recording of activity from large populations of neurons (Svoboda
and Yasuda, 2006). Cristopher Niell and colleagues performed one of the first 2P calcium imaging experiments in the optically translucent larval zebrafish tectum. They extensively mapped the response properties of its neurons in animals of different ages and demonstrated that SPV neurons start showing visual response properties soon after the RGC axons enter the tectum. They classified the recorded neurons into four clusters which showed a diverse set of response properties such as selectivity for motion (especially the direction of motion), looming responses etc. These clusters were developmentally stable and correspond well with the response profiles of adult tectal neurons (Niell and Smith, 2005; Sajovic and Levinthal, 1982).

VISUAL BEHAVIORS IN LARVAL ZEBRAFISH:

The past decade or so has seen a tremendous rise in the number visual behavioral paradigms being used to investigate the neural basis of behavior in larval zebrafish (Portugues and Engert, 2009). These can broadly be put into two classes based on the involvement of the optic tectum. Two examples of behaviors where the tectum doesn’t seem to be crucial are the optomotor response (OMR) and the optokinetic reflex (OKR) (Roeser and Baier, 2003). The optomotor response results when fish are presented with whole field motion and they respond to this by swimming in the perceived direction of motion (Orger et al., 2008). The optokinetic reflex is a robust behavior which results when visual stimuli moving across the visual field elicit rotational movements of the eye which take the form of smooth pursuits and saccades (Portugues et al., 2014). These two behaviors are some of the more thoroughly investigated in larval zebrafish literature, both at the level of quantification of behavioral descriptors and at the neural level. Of the tectal mediated behaviors, prey capture is perhaps the most baroque
Larval zebrafish are born with an innate reservoir of food, the yolk, that is almost completely depleted by 5 d.p.f. Continued survival of the animal relies on its ability to hunt and feed itself and it does so by preying on small microorganisms such as paramecia (McElligott and O’malley, 2005). This involves tracking prey with orienting eye and body movements, followed by stereotyped correcting turns to place the prey in the appropriate field of view, and a terminal lunging and swallowing of the prey (Bianco et al., 2011). Another example of a tectal mediated behavior is the avoidance of large, looming objects that might resemble predators (T.W. Dunn, unpublished observation). Similar to the tectal/collicular role in other species, the larval zebrafish tectum seems to be involved in selecting for behaviorally relevant stimuli and being a crucial part of circuits executing goal directed behaviors. These behaviors, like the visual response properties, persist into adulthood (Portugues and Engert, 2009).

**MOTION PROCESSING IN THE VISUAL SYSTEM**

Most of the visual behaviors highlighted above necessitate the computation of the direction of motion of the stimulus. If this was absent, i.e., if every part of the brain responds equally well to stimuli moving in all directions then the animal would be unable to register specific stimuli such as, a paramecium moving to the right, and react appropriately by performing cognate eye movements and body turns. This important computation of selectively responding to visual stimuli moving in a particular direction happens at the very first sensory stage, the retina. This was demonstrated nearly 50 years ago by Barlow and colleagues in a series of classic papers where they recorded from RGCs of rabbit retinae
Figure 1.4 Distribution of preferred directions of direction selective retinal ganglion cells in the rabbit retina: (A) Distribution of the preferred directions of 79 'on-off' direction-selective units. The axes are the horizontal and vertical in the visual field. The mean ± std is indicated for each group. (B) Preferred directions of 23 'on' type direction-selective units. Other details same as (A). Adapted from (Oyster and Barlow, 1967).
Figure 1.4 (continued)
(Barlow and Hill, 1963). Furthermore, they showed that the direction selective RGCs (DS-RGCs), rather than having preferred directions (PDs) for all possible directions, form clusters of cells with specific PDs (Figure 1.4) (Oyster and Barlow, 1967). The ON-OFF DSRGCs seemed to form four clusters that aligned remarkably well with the axes of innervation of ocular muscles, while the ON DSRGCs formed three clusters that seemed to align with the axes represented by the semicircular canals. In larval zebrafish, the DSRGCs projecting to the tectum show a similar trend by forming three equally spaced clusters. Moving up the hierarchy in the visual system, functional studies have encountered DS responses in wide variety of areas such as, V1, area MT, superior colliculus, etc., (Feinberg and Meister, 2014; Maunsell and Van Essen, 1983; Priebe and Ferster, 2005).

The mechanism behind direction selectivity in the different areas mentioned above continues to be an active area of research in systems neuroscience. The most prominent of DS models are the Barlow and Levick model for the vertebrate retina, and the Hassenstein-Reichardt model for the invertebrate visual system (Figure 1.5) (Borst and Euler, 2011). Both these models have an output node which compare the light intensities at two input photoreceptors with one (or both in the case of the Hassenstein-Reichardt model) of the photoreceptors sending its signals via a delay line. One of the main differences between these models arises at the level of comparison of the input lines. In the Hassenstein-Reichardt model, this takes the form of a correlation. In the Barlow and Levick model, the comparison is implemented as a veto (AND-NOT) gate, which, at the neuronal level, takes the form of inhibition from the null direction. Extensive work over the past two decades in vertebrate ON-OFF DSRGCs has shown the validity of the Barlow and
**Figure 1.5 Models for direction selectivity:** (A) The Reichardt detector calculates the direction of image motion by multiplying (M) the brightness values at two adjacent image points after one of them has passed a low-pass filter with a time constant \( \tau \). This is done in two mirror-symmetrical subunits, the outputs of which are subtracted from one another (-). (B) The Barlow-Levick detector calculates the direction of image velocity by processing the brightness values at two adjacent image points through a logical AND-NOT gate after one of them is delayed by \( \varepsilon \) ms. Adapted from (Borst and Euler, 2011).
Figure 1.5 (continued)
Levick model with notable additions: both the input excitatory and inhibitory currents are direction selective with opposite preferences. In the preferred direction of motion, the input excitatory currents are larger than the currents in the null direction (diagonally opposite to the preferred). The input inhibitory currents are larger in the null than the preferred direction. The primary source of this asymmetry has been traced to the starburst amacrine cell (SAC), which shows selectivity for centrifugal motion. In addition to the biased magnitude of the input currents, their relative timing plays an important role. In the preferred direction, the excitatory currents arrive before the inhibitory currents and vice-versa for the null direction. This difference in latencies arises from the fact that the SAC supplying inhibition is topographically shifted to the null direction of the DSRGC. The Hassenstein-Reichartdt model, though popular for its predictive power for neuronal and behavioral responses to motion, has not been conclusively demonstrated at the neuronal level (Borst and Euler, 2011). Different motion sensitive areas of the brain, both in vision and other senses, appear to compute direction of stimulus motion by employing a diverse array of mechanisms (Chacron and Fortune, 2010; Kuo and Wu, 2012; Priebe and Ferster, 2005). In the larval zebrafish tectum, a large fraction of neurons show robust direction selective responses (Grama and Engert, 2012; Niell and Smith, 2005; Ramdya and Engert, 2008). A previous study has implicated the role of local inhibition in maintaining direction selectivity in the tectum (Ramdya and Engert, 2008). Thus, rather than simply transmitting selective RGC signals, the tectum appears to be exerting its processing power on these inputs. In the following chapters, I examine the nature of this processing by employing tools from genetic engineering, single-cell electrophysiology, and population calcium imaging.
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CHAPTER TWO

Direction selectivity in the larval zebrafish tectum is mediated by asymmetric inhibition

The following chapter was originally published in Front Neural Circuits. 2012 Sep 4; 6:59. doi: 10.3389/fncir.2012.00059. I designed the study, carried out all the experiments, and analyzed all the data with helpful guidance from Florian Engert.
SUMMARY

The extraction of the direction of motion is an important computation performed by many sensory systems and in particular, the mechanism by which direction selective ganglion cells (DS-RGCs) in the retina acquire their selective properties, has been studied extensively. However, whether DS-RGCs simply relay this information to downstream areas or whether additional and potentially de-novo processing occurs in these recipient structures is a matter of great interest. Neurons in the larval zebrafish tectum, the largest retino-recipient area in this animal, show direction selective responses to moving visual stimuli but how these properties are acquired is still unknown. In order to study this, we first used two-photon calcium imaging to classify the population responses of tectal cells to bars moving at different speeds and in different directions. Subsequently, we performed in-vivo whole cell electrophysiology on these direction selective tectal neurons and we found that their inhibitory inputs were strongly biased towards the null direction of motion, whereas the excitatory inputs showed little selectivity. In addition, we found that excitatory currents evoked by a stimulus moving in the preferred direction occurred before the inhibitory currents whereas a stimulus moving in the null direction evoked currents in the reverse temporal order. The membrane potential modulations resulting from these currents were enhanced by the spike generation mechanism to generate amplified direction selectivity in the spike output. Thus our results implicate a local inhibitory circuit in generating direction selectivity in tectal neurons.
INTRODUCTION

The ability to encode the direction of motion of a stimulus is an important feature extraction from the dynamic world we live in. Specialized neurons across many sensory systems serve exactly this purpose (Fried et al., 2002; Priebe and Ferster, 2005; Wilent and Contreras, 2005, Chacron and Fortune, 2010; Wang et al., 2010; Ye et al., 2010). The optic tectum, better known as the superior colliculus in mammals, is a multi-layered structure that integrates information from different sensory modalities (Stein et al., 2009; Deeg et al., 2010) and has neurons that are direction selective (DS) for moving visual stimuli (Rhoades and Chalupa, 1976; Engert et al., 2002; Niell and Smith, 2005; Wang et al., 2010). DS neurons have also been found in larval zebrafish tectum (Niell and Smith, 2005; Ramdya and Engert, 2008), and this property is a likely mediator for the visual goal-directed behaviors that this animal performs.

The mechanism underlying direction selectivity has received a lot of attention with a specific focus on the retina across different invertebrates and vertebrates (Barlow and Levick, 1965; Kim et al., 2008; Joesch et al., 2008; see Borst and Euler, 2011 for review). Rather than one conserved motif, a variety of mechanisms have been reported by studies so far. The best understood direction selective circuit is that of vertebrate retinal ganglion cells (RGCs) (Barlow and Levick, 1965; Fried et al., 2002; Briggman et al., 2011). It was shown for On-Off direction selective RGCs (DS-RGCs) that inhibition is biased towards the null direction of motion and excitation biased towards the preferred. Also, inhibition arrives before the excitation for the null direction, thus preventing spiking, whereas the reverse happens for the preferred (Fried et al., 2002). This is mediated by a starburst amacrine cell that feeds inhibition to the On-Off DS-RGCs from the null direction. A different mechanism was reported in the visual and the auditory
cortex, where both excitation and inhibition were biased towards the preferred direction, but the latency relationship was similar to that in the retina (Zhang et al., 2003; Priebe and Ferster, 2005). In the auditory inferior colliculus and the barrel cortex, only the latency relationship was shown to be responsible for direction selectivity, with the magnitudes of excitatory and inhibitory inputs being the same for both directions (Kuo et al., 2012; Wilent and Contreras, 2005).

In the larval zebrafish tectum, the precise mechanism of direction selectivity still remains unknown. The fact that DS-RGC responses were recorded from axon terminals in a closely related teleost, adult goldfish (Maximov et al., 2005), and that DS-RGCs have also been shown to project to the tectum in other vertebrates (Kim et al., 2008; Huberman et al., 2009) suggests that tectal cells can already receive pre-processed DS input directly from the retina. Thus, the most parsimonious way to explain direction selectivity in tectal neurons, or for that matter in all retino-recipient areas across model systems, would be a direct relay from DS-RGCs to their specific downstream targets. However, in the mammalian visual system in particular there is accumulative evidence that the direction selective properties of higher order neurons, like those found in the visual cortex, are not explained by a simple feed-forward circuit from DS-RGCs, but are rather the product of local and intra-cortical processing (Priebe and Ferster, 2005; Priebe et al., 2010). Similarly, in the larval zebrafish, a previous study reported that it is possible for the tectum to extract direction selectivity independent of DS-RGCs, with a local blockade of inhibitory transmission causing a drop in selectivity (Ramdya and Engert, 2008). However, the details of how this information is extracted are still unclear.
Here we use a combination of two-photon calcium imaging and in-vivo whole cell patch-clamping to address this question. We found that many cells showed strong direction selectivity as well as a preference for speed. Surprisingly, we found that the input excitatory currents were only weakly tuned to the direction of motion, whereas, the inhibitory currents were strongly biased towards the null direction. When we examined the latency between the excitatory and inhibitory currents, we found that inhibition tended to precede excitation in the null direction and the reverse was true for the preferred direction. The membrane potential change resulting from the interaction of the excitatory and inhibitory inputs was further amplified by the spike generation mechanism to generate a stronger direction selective spike output. Thus our results point towards an inhibitory model of direction selectivity, a motif that seems similar to that found in the retina (Fried et al., 2002).

RESULTS

Two-photon calcium imaging of responses to moving bars

We first characterized the responses of tectal cells to moving bars by loading these cells with a synthetic, membrane permeable calcium indicator, Oregon green bapta-1-AM (OGB1-AM). This allowed us to record calcium signals from hundreds of neurons simultaneously, which have been shown to be a reasonable proxy for measuring action potentials in this preparation (Niell and Smith, 2005). To immobilize fish for imaging purposes, animals were restrained in agarose and visual stimuli were presented - to one eye - while the contralateral tectum was imaged on a custom two-photon microscope (Figure 2.1A).
Figure 2.1: Two-photon calcium imaging of tectal responses to moving bars. (A) Schematic of the experimental setup. Larval zebrafish were placed in a custom chamber (chamber not shown) and presented bars moving caudo-rostrally (CR) or rostro-caudally (RC) to the left eye. Calcium imaging and electrophysiological recordings were performed on the right tectum. (B) (left) Average image of a two-photon stack with three neurons highlighted as ROIs. Scale bar represents 20 μm (r-rostral, c-caudal, m-medial, l-lateral, 85% of active cells in this example fish were sensitive to motion). (right) Delta f/f (df/f) traces of the highlighted neurons. Red lines indicate stimulus moving in the CR direction and blue lines indicate RC direction at 60 deg/s. The cell in magenta was direction selective (DS) for the CR direction, the cell in cyan was non-DS and the cell in green was insensitive to motion. (C & D) In C, Average df/f responses (n = 3 trials) of two cells to different speeds are shown. Red indicates motion in the CR direction and blue motion in the RC direction. Darker colors imply slower speeds and lighter colors faster speeds. The responses to slowly moving bars are delayed with respect to the onset of the stimulus as the time taken for the bar to enter the receptive fields of the neurons is longer. This is seen in the second cell’s (bottom) response to slower stimuli. In D, the single trial peak df/f responses (filled circles) of the same two cells to bars moving in the CR (red) or RC (blue) directions at different speeds are shown. The responses were fit with a smoothing spline (light blue or red lines) and the preferred speed was estimated. The cell on top shows band-pass tuning, while the cell on the bottom shows low pass tuning.
Figure 2.1 (continued)
Stimuli consisted of 3º vertical bars moving caudo-rostrally (CR) or rostro-caudally (RC) at different speeds (10-100 deg/s). Responses of motion sensitive cells (Figure 2.1B) were analyzed for the different speeds and directions presented. A fraction of the active cells (31 ± 6% of n=327 cells from 5 fish) were found to be modulated by the speed of the moving bars (Figures 2.1C, D and 2.2A). To find the preferred speeds of these cells, the responses were fit with a smoothing spline and the speed that gave the maximum response was estimated (Figure 1D). There seemed to be a preference for low speeds for both the CR and RC directions of motion (Figure 2.2C).

To quantify the responses of the tectal cells to either direction, a direction selectivity index (DSI) was calculated based on the peak calcium responses. With this formulation, highly CR selective neurons would have a DSI of +1, highly RC selective neurons would have a DSI of -1 and non-selective neurons would have a DSI of 0. The DSIs of tectal cells spanned the entire range from -1 to 1 for all the speeds presented (Figure 2.2B). At 60 deg/s (the speed used for electrophysiological recordings), about 44 ± 10% of the active cells, or more than half the number of motion sensitive cells, were found to have a |DSI|>0.33 (Figure 2.2A). This implies that a cell is twice as responsive to motion in one direction as the other. This finding is in agreement with that of a previous study, which measured responses of tectal cells to moving spots (Niell and Smith, 2005).

**Direction selectivity of excitation and inhibition**

With quite a large fraction of tectal cells showing direction selectivity, one of the more pertinent questions that follow concerns the mechanism that leads to this property being
Figure 2.2: Population responses to bars moving at different speeds and directions. (A) The percentage of active cells that are motion sensitive, that are direction selective at a speed of 60 deg/s, and that show responses modulated by speed (mean ± st.dev). (B) Direction selectivity indices (DSIs) of individual tectal cells at different speeds are shown as raster ticks. Cells to the right of the red dashed line are CR selective and cells to the left of the blue line are RC selective. The green lines show the dependence of DSI on the speed of the stimulus for three cells. The cells shown have low (st.dev = 0.04), medium (st.dev = 0.12), and high (st.dev = 0.44) variability of DSIs at different stimulus speeds. (C) The histogram of preferred speeds for all speed tuned cells in the CR direction (red, n = 61 cells) and RC direction (blue, n = 73 cells).
Figure 2.2 (continued)
established. To address this, we performed in-vivo whole cell patch-clamp recordings of cells in
the stratum periventriculare (SPV) of the tectum, the region where over 95% of all tectal cells
reside (Niell and Smith, 2005) (Figure 2.3A). SPV cells are unipolar in morphology; they send
dendrites into the tectal neuropil where they synapse with RGC axons (Scott and Baier, 2009).
SPV cells in the right tectum were patched under infrared illumination while vertical bars
moving in either the CR or RC direction were presented to the left eye. Given the limited
recording time for electrophysiology, we didn’t measure the preferred speed of the neurons
and used a fixed speed (60 deg/s) while probing direction selective responses for all cells. There
is a possibility that the profile of the synaptic currents might vary at different speeds for
individual neurons and ideally one would like to characterize DSI tuning for all possible speeds
for each neuron. However, the population data from calcium imaging suggests that there isn’t a
strong dependence of DSI values on speed (the average standard deviation of DSIs for all cells
with responses to at least 3 speeds was 0.2) and therefore these effects are probably not
substantial. We isolated the excitatory and inhibitory currents by clamping the cells at -70 mV
and 0 mV respectively (for a description of the physiological properties of tectal cells see: Smear
et al., 2007; Zhang et al., 2011). Subsequently, we switched to current clamp mode and
recorded the spiking output of these cells. In response to the moving bars, the tectal cells
showed excitatory and inhibitory currents that were time locked to the spikes and membrane
potential changes (Figure 2.3B). We recorded from 17 neurons (from 15 fish) whose responses
spanned the range from highly CR selective to highly RC selective.
To look at the contribution of excitatory and inhibitory currents to the spiking output of the
cells in response to moving bars, excitatory (Exci), inhibitory (Inhi) and Spike DSIs were
Figure 2.3: Inhibitory currents are biased towards the null direction of motion. (A) Two-photon fluorescence image of a tectal neuron filled with dye (Alexa 594) from the recording electrode. Tectal neurons project dendritic arbors into a neuropil where they receive retinal ganglion cell axonal input from the contralateral eye. Scale bar represents 10 μm. (B) Voltage clamp (Exci and Inhi) and current clamp (Spike) recordings of a tectal cell’s response (for the duration the stimulus was presented) to bars moving in the CR direction (red) and the RC direction (blue). For the currents, the mean trace (thicker red/blue line) is shown superimposed over 5 trials (lighter red/blue lines). This cell is RC selective and has strong inhibition from the null CR direction. (C&D) Spike-DSI for all cells (n=17) plotted vs Exci-DSI and Inhi-DSI respectively.
Figure 2.3 (continued)
calculated. When we compared the Exci-DSI with the Spike-DSI (Figure 2.3C), we were surprised to find that the excitatory currents were not well tuned to the direction of motion \( R^2 = 0.32, p=0.02, n=17 \). For most of the cells the Exci-DSI did not exceed 0.33. Thus our data suggests that excitatory currents don’t play a major role in generating direction selectivity in most tectal cells.

In contrast, when the relationship between the inhibitory currents and the spike output was examined, a strong anti-correlation was found (Figure 2.3D, \( R^2 = 0.76, p<0.00001, n=17 \)). This means that for cells with a Spike-DSI close to 1, i.e., CR selective, the Inhi-DSI was close to -1, i.e., the inhibitory currents were heavily biased in the null RC direction. This finding establishes a mechanism for observations made in a previous study (Ramdya and Engert, 2008), where abolishing inhibitory transmission through \( \text{GABA}_A \) receptors resulted in a great reduction in direction selectivity. This arrangement of inhibition coming from the null direction of motion is also seen in the retina, where DS-RGCs receive inhibition in the null direction from starburst amacrine cells (Fried et al., 2002). Our results strongly implicate a local tectal computation of direction selectivity via inhibition.

**Timing of excitation and inhibition**

In DS-RGCs, apart from the magnitude of inhibition being biased towards the null direction, there is also an asymmetry in the timing of the inhibitory currents compared to the excitatory currents (Fried et al., 2002). To test for any such relationship for DS tectal cells, the temporal order of the excitatory and inhibitory currents (\( |\text{DSI}| > 0.33, n=11 \) cells) was examined. In the null direction, the inhibitory currents preceded the excitatory currents (Figure 2.4,
Figure 2.4: Inhibition precedes excitation in the null direction and follows it in the preferred direction. (A) Average excitatory (black) and inhibitory (gray) current profiles from a DS cell to bars in the preferred and the null directions are shown after normalization to illustrate the temporal relationship between them. (B) The latency between excitatory and inhibitory currents for preferred and null directions for all the DS cells (n = 6 preferred, n = 11 null, see text). Negative values mean inhibition precedes excitation.
Figure 2.4 (continued)
p<0.05, Wilcoxon’s signed rank test, median value=39 ms, n=11 cells). Thus, the bias in the magnitude of tectal inhibition and its temporal relationship to excitation ensured that there was very little spiking in the null direction. In the preferred direction, the reverse relationship was observed, where the excitatory currents were leading the inhibitory currents, thereby causing the cells to spike (Fig 2.4, p<0.05, Wilcoxon’s signed rank test, median value=157 ms, n=6 cells). The difference in the onset time of excitatory currents between the preferred and null direction is due to the bar entering the receptive field of the neuron at different times after the stimulus onset, i.e. the time when the bar first appears on the screen. The same reasoning applies to the inhibitory currents. The number of cells examined for latency in the preferred direction (n=6 cells) was less than that for the null direction (n=11 cells) since in quite a few cells there wasn’t any appreciable inhibition in the preferred direction (Figure 2.3B).

**Direction selectivity of membrane potential**

To examine the modulation of membrane potential in response to the CR and RC moving bars, we filtered out the spikes (Figure 2.5A) and calculated a membrane (Memb) DSI. When we compared the Memb-DSI with the Spike-DSI, we found almost a linear relationship (Figure 2.5B, R²=0.80 p<0.00001, n=17 cells) between the two. There seemed to be an enhancement of the direction selectivity in the spikes when compared to the membrane potential. This phenomenon is also seen in the visual and auditory cortex (Priebe and Ferster, 2005; Ye et al., 2010). The amplification can be attributed to a non-linear effect of the spike threshold (Priebe and Ferster, 2008), which enhances small differences of membrane potential
Figure 2.5: Sharpening of membrane potential DS by the spike generation mechanism. (A)

Membrane potential recordings in current clamp mode of the same cell as 3(B) to bars moving in CR (red) and RC (blue) directions. The spikes were filtered out by using a median filter. The mean trace (dark) is superimposed over traces from $n = 5$ individual trials (light). (B) Spike-DSI plotted vs Memb-DSI for all the recorded cells ($n = 17$).
Figure 2.5 (continued)
over the threshold to large differences in spike output. Thus, direction selectivity undergoes amplification in tectal cells through the spike generation mechanism.

**Discussion**

Our experiments in characterizing the population responses of larval zebrafish tectum confirmed earlier findings about the proportion of direction selective neurons (Niell and Smith, 2005). Tectal cells showed DSIs spanning the range from -1 to 1 at all speeds presented. In addition, some cells showed responses that were modulated by the speed of the moving bar. Speed tuning has been reported in the mammalian superior colliculus and other visual areas across species such as the area MT and V1 (Rhoades and Chalupa, 1976; Maunsell and Van Essen, 1983; Razak and Pallas, 2005; Priebe et al., 2006), with Razak and Pallas showing an inhibition mediated mechanism for speed tuning in the hamster superior colliculus. Speed encoding cells could be useful feature extractors for moving visual stimuli. In fact, the range of preferred speeds seen in this study matches the speeds at which the image of a paramecium moves on the retina of a hunting zebrafish larva, when it is located at the characteristic striking distance of 1.5 mm away from the fish (Bonini et al., 1986; Bianco et al., 2011). Thus speed encoding cells could aid in goal-directed behaviors such as prey-capture (Ewert et al., 2001; Gahtan et al., 2005).

Another critical feature that needs to be extracted from moving objects is their direction. This is performed across different sensory modalities (Fried et al., 2002; Wilent and Contreras, 2005; Chacron and Fortune, 2010; Ye et al., 2010) and, in the case of vision, even at different stages of
Figure 2.6: The proposed model for the direction selective circuit in the larval zebrafish tectum. The DS tectal neuron receives excitatory inputs from RGCs (open circles). This neuron also receives an inhibitory input (filled circle) from an interneuron that is topographically shifted towards the null direction of the DS tectal neuron. The interneuron is itself direction selective but for the opposite direction to that of the DS tectal neuron being considered. Thus, if a bar were to move in the preferred direction of the DS tectal neuron (orange arrow), the excitatory currents from the RGC terminals would arrive before the input from the Inhibitory DS interneuron. Also, the inhibition from the interneuron would be low. In the null direction for the DS tectal neuron, higher inhibition from the Inhibitory DS interneuron would arrive before the excitatory inputs from the RGC terminals.
Figure 2.6 (continued)
the same modality (Fried et al., 2002; Priebe and Ferster, 2005). Models to explain how
direction selectivity arises have been in existence for over half a century now (Barlow and
Levick, 1965; Reichardt, 1987). Rather than one conserved motif acting across brain areas,
there are now many different mechanisms that have been reported. Ranging from biases in
excitation and inhibition to just temporal asymmetries (Fried et al., 2002; Priebe and Ferster,
2005; Zhang et al., 2003; Kuo et al., 2012), these mechanisms have served to demonstrate the
range of evolutionary answers to a fundamental computational problem of sensory processing.

Tectal cells show robust direction selective responses but the mechanism underlying
this computation is so far not completely understood. Ablation studies in the mammalian
superior colliculus pointed to a role of cortico-tectal connectivity in this process (Wickelgren
and Sterling, 1969; Rosenquist and Palmer, 1971), but this is unlikely in larval zebrafish as no
structure equivalent to the visual cortex has been reported here. DS-RGCs projecting to the
tectum (Maximov et al., 2005) appeared to be the most likely source for direction selectivity. To
address their contribution, we performed in-vivo whole cell patch experiments in tectal
neurons and examined their input excitatory currents, a majority of which have been shown to
be retinal in origin (Zhang et al., 2011). To our surprise, there was only a weak correlation of the
direction selectivity of the spiking output with that of the excitatory currents, with only a few
cells showing a strong bias of excitatory currents towards the preferred direction. This calls to
question the role DS-RGCs play in this circuit. When we examined the inhibitory currents, we
found that they were biased towards the null direction of motion. The timing differences
between the excitatory and inhibitory currents revealed another level of detail in the direction
selective circuit. We found that the inhibition tended to precede excitation in the null direction whereas the reverse was true for the preferred direction. Thus asymmetries in the magnitude and timing of inhibition seem to underlie the generation of direction selectivity in the larval zebrafish tectum. This is most likely mediated by a direction selective tectal inhibitory interneuron that is topographically shifted towards the null side (Figure 2.6). This arrangement ensures that greater inhibition arrives before excitation in the null direction and lesser inhibition after excitation in the preferred direction. Although the individual elements are different, the logic behind this circuit is similar to that seen in the vertebrate retina (Fried et al., 2002). Thus there seems to be a conserved motif operating in the retina and the zebrafish tectum.

Our results raise interesting questions for future studies on this circuit. The first question would be that of the seeming redundancy of the DS computation in a part of the brain that already receives a pre-computed input. Given the architecture of the tectum, we would like to argue that the presence of a local computation can be useful. The tectum receives inputs from different sensory modalities that are in topographic register with each other (Deeg et al., 2009). This feature enables a coherent map of the world surrounding the fish to be represented internally. Thus the detection of motion in one sensory dimension could be corroborated with that in the other sensory dimensions owing to such local computations. The second question concerns the presence of direction selective tectal inhibitory neurons. How do these inhibitory neurons achieve their direction selectivity? Could they be the targets of the DS-RGCs? This study was agnostic about the neurotransmitter phenotype of the recorded cells but this could be addressed in future studies which would lead to further dissection of the direction selective
circuit in the zebrafish tectum. The third question concerns the projection of DS-RGC inputs in the tectum. In fish where the RGC axons from the ipsilateral eye, which normally project to the contralateral tectum, were made to project to the ipsilateral tectum, it was found that direction selective responses could be elicited by flashing spots of light in sequence to one eye and then the other (Ramdya and Engert, 2008). This experiment showed that direction selectivity in some SPV cells did not need the input from DS-RGCs. Our results show that the input from the retina is only weakly correlated with the DS property of the tectal cells. In the visual cortex, a pioneering study showed that neurons with highly tuned outputs sampled from a broad range of tuned inputs (Jia et al., 2010), the vector sum of which wasn’t highly tuned. If the sampling of retinal inputs by tectal cells in anyway resembles the sampling in the visual cortex, then this would account for the weak preference of direction we see in the excitatory currents.

MATERIALS AND METHODS

Zebrfish rearing conditions

*Nacre (-/-)* zebrafish used in this study were raised at 28ºC on a 14 h on/10 h off light cycle in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4). All experiments were approved by Harvard University’s Standing Committee on the Use of Animals in Research and Training.

*In vivo* calcium imaging

Calcium imaging experiments were carried out at 6–8 days post fertilization (dpf). For injections, zebrafish were anaesthetized using 0.02% MS222 and mounted in 1.5% low-melting agarose. 1 mM Oregon Green BAPTA-1 AM (Molecular Probes) Ester dissolved in DMSO with
20% pluronic acid (vol/vol) as well as E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4) containing 100 mM Alexa Fluor 594 (Molecular Probes) was bolus injected into the tectal neuropil of the right tectum with 10-50 ms pulses at 1 psi using a PV820 Pneumatic PicoPump (World Precision Instruments). Fish were freed and allowed to recover for at least 1 hour before imaging. For imaging, zebrafish were re-mounted in agarose on a custom-built cylinder shaped acrylic chamber. Calcium imaging experiments were carried out using a custom-built two-photon microscope coupled to a Mai Tai (Spectra-Physics) mode locked Ti:Sapphire laser (950 nm) and a 20x water-immersion objective with a 0.95 numerical aperture (Olympus). Images were acquired at 1 Hz.

**In vivo electrophysiology**

For recordings, larval zebrafish at 6-8dpf were paralyzed in alpha-Bungarotoxin (Invitrogen) at 1mg/ml in zebrafish external solution (NaCl 134 mM; KCl 2.9 mM; CaCl2 2.1 mM; MgCl2 1.2 mM; glucose 10mM; HEPES 10 mM; pH 7.8, 290 mOsm) with 0.02% MS222 for 10 minutes. Following this, the anaesthetized and paralyzed fish were mounted on a custom chamber using insect pins. To access the right tectum, the overlying skin was removed using a sharpened tungsten needle. Electrophysiological recordings were performed at room temperature using an Axopatch 200B (Axon Instruments) patch-clamp amplifier. Data were sampled at 5 kHz and filtered at 2 kHz with a National Instruments data acquisition board. Borosilicate glass capillary micropipettes (World Precision Instruments) were pulled for a resistance of 12-15 MΩ and were filled with a K-gluconate internal solution (K-gluconate 115 mM; KCl 15 mM; MgCl2 2 mM; HEPES 10 mM; EGTA 10 mM; Na2ATP 4 mM; pH 7.2, 280 mOsm). Excitatory post-synaptic currents (EPSCs) were recorded in whole-cell configuration
with voltage-clamp at -70 mV. Inhibitory currents (IPSCs) were recorded at a holding potential of 0 mV. The membrane potential was subsequently recorded in current clamp mode with the resting potential set to -50 mV.

**Visual stimulation**

Visual stimuli were presented using a DLP projector (Optoma) filtered by a #29 Wratten filter (Kodak). Images were passed through a 0.42X wide-angle lens (Kenko) and bottom-projected onto a screen encompassing 120° of visual angle. High contrast 3° width vertical bars were presented on a dark background in all experiments and moved caudo-rostrally (CR) or rostro-caudally (RC). For calcium imaging, the speed of the bar was varied from 10-100 deg/s in 10 degree increments and in a pseudo-random fashion, with 3 repeats for each speed and direction. For electrophysiological experiments, bars moved in the CR and RC directions at 60 deg/s (5 repeats in each direction, pseudo-randomly interleaved).

**Data analysis**

Regions of interest (ROIs) over cells were manually chosen from an average image of the movies and the fluorescence signals from these cells were converted to a (delta f)/f (df/f) value. Only neurons that showed calcium activity (spontaneous or stimulus locked) were further analyzed. The peak df/f in a window following the stimulus presentation (stimulus presentation time + 5 s) was chosen as the response of a neuron to the stimulus. To test for response to motion, a t-test was performed between the peak df/f of the baseline and that of the response periods and only neurons that showed a significant difference (p<0.005) were considered motion-responsive. The responses of neurons to different speeds were assessed with ANOVA and only cells with p<0.05 were considered as modulated by speed. The responses to different
speeds were fit with a smoothing spline and the maximum of this fit gave the preferred speed. Direction selectivity was quantified by a ratiometric direction selectivity index (DSI), where \( \text{Resp}_{\text{CR}} \) indicates response (either df/f or electrophysiological) in the CR direction, similarly for others.

\[
\text{DSI} = \frac{\text{Resp}_{\text{CR}} - \text{Resp}_{\text{RC}}}{\text{Resp}_{\text{CR}} + \text{Resp}_{\text{RC}}}
\]

The responses in voltage clamp experiments were calculated as the integrated charge of the mean neuronal discharge in response to the stimulus. For current clamp experiments, the spikes were first counted and then filtered out to give the membrane potential modulation. The membrane potential response was taken to be the maximum of the baseline subtracted mean trace in the response period. To calculate the latency between the excitatory and inhibitory currents, we fit the smoothed average currents with a double exponential function (Naumann et al., 2010) and computed the latency as the half maximal point of the fit.
REFERENCES


In the following chapter I report a manuscript in preparation. I designed the study, carried out all the experiments, and analyzed all the data. The GCaMP6s entry vector was generated by David Hildebrand.
SUMMARY

In order to better understand the workings of a neural circuit, it is essential to know the roles played by its neural components. The larval zebrafish tectum, the largest retino-recipient brain area in the fish, is known to be primarily composed of glutamatergic (excitatory) and GABAergic (inhibitory) neurons and has been the subject of many recent studies investigating sensory representation and visuo-motor transformations. A considerable number of these studies have focused on motion processing by characterizing the direction selective retinal ganglion cell (DSRGC) inputs into the tectum and the tectal circuitry that enacts its processing upon them. However, the roles of the two major subclasses of tectal neurons in visual processing are still unknown. We investigated the functional roles of these neurons by using a newly generated and functionally characterized pan-neuronal transgenic line expressing the genetically encoded calcium indicator, GCaMP6s, in combination with lines that label either all the glutamatergic or all the GABAergic neurons in the brain. We first show that inhibitory neurons possess slightly larger receptive fields than excitatory neurons. When probed with moving stimuli, both excitatory and inhibitory neurons showed robust responses and a matching degree of selectivity. When we examined the profile of preferred directions of these selective neurons, inhibitory neurons displayed two broad overlapping clusters that preferred stimuli moving either towards the anterior or the posterior end of the fish. In contrast, excitatory neurons, which form the output layer of the tectum, displayed three overlapping clusters with peaks at 40°, 185°, and 315° (polar coordinates). In addition, we find significant, distance dependent, pairwise noise correlations for both excitatory and inhibitory neurons, with neurons close by showing higher coupling than neurons far away, suggesting the presence of local subnetworks.
In summary, our results describe distinct representations of direction space in excitatory and inhibitory tectal neurons, which appear to be transformed from the DSRGC inputs.
INTRODUCTION

The visual system (of vertebrates and invertebrates) has been the focus of intense research in systems neuroscience for the past decades. This is likely due to the fine control over the stimulus parameters, the ease of accessibility of the first stage of processing, the retina, and to the fact that as highly visual animals, we are curious about how processing works in this sensory modality. The retina, the input stage of the visual system, performs a wide range of parallel processing of the visual scene, such as selectively responding to specific directions of motion, and sends highly filtered signals to different parts of the brain (Gollisch and Meister, 2008). In one of the earliest and seminal demonstrations of this feature, Lettvin and colleagues showed that the frog’s eye conveyed filtered, behaviorally significant information to the central visual structure in the frog’s brain, the optic tectum (Lettvin et al., 1959). In the larval zebrafish visual system, the direction selective retinal ganglion cells (DSRGCs) send inputs into the tectum which terminate in its superficial layers. The pattern of this projection appears to be stereotyped across fish (Lowe et al., 2013; Nikolaou et al., 2012). The DSRGC inputs form three equally spaced functional clusters based on their preferred directions (PDs), i.e., they partition the direction space in a triangular fashion, with a preponderance of inputs selective for tail-to-head motion. Clustering of DSRGC populations preferring specific directions was first described in rabbit retinae by Oyster and Barlow (Oyster and Barlow, 1967), with ON-OFF DSRGCs partitioning the direction space in cardinal directions and ON DSRGCs displaying triangular partitioning. How this information is used and transformed by the tectum, or other retinorecipient areas, is still an open question.
The optic tectum is a multilayered, multisensory structure known to be involved in mediating goal directed behaviors (Bianco and Engert, 2015; Bianco et al., 2011; Deeg et al., 2009; Gahtan et al., 2005). In larval zebrafish, the tectum can be subdivided into the neuropil layer and the cell body layer (stratum periventriculare or SPV). The SPV neurons are primarily either glutamatergic or GABAergic (Higashijima et al., 2004). They display diverse dendritic morphologies, which allow them to sample from different subtypes of RGC inputs, and local tectal inputs through the different neuropil layers (Nevin et al., 2010). In accordance with the tectum’s role in goal directed behaviors such as capturing moving prey, tectal neurons have been shown to display a diverse array of ethologically relevant visual responses (Bianco and Engert, 2015; Grama and Engert, 2012; Niell and Smith, 2005; Ramdya and Engert, 2008). These range from highly motion selective SPV neurons to those that represent complex combinations of stimulus features such as size, contrast and direction. In our previous study we investigated how tectal neurons combine RGC and local inputs to generate direction selectivity. We found evidence for a weak influence of RGC inputs and strong influence of local inhibition on the tuning of DS tectal neurons. Our results suggested the presence of a network of DS inhibitory neurons mediating direction selectivity in the tectum (Grama and Engert, 2012). Evidence for this was shown by a study which reported two transgenics labeling sparse populations of inhibitory neurons preferring opposing directions of motion (Gabriel et al., 2012). Thus, rather than being a relay station for filtered, selective RGC inputs, the tectum appears to process these inputs and generate behaviorally relevant sensory representations that show stability over the animal’s growth (Grama and Engert, 2012; Hunter et al., 2013; Niell and Smith, 2005).
How are these visual representations subdivided amongst the two major subclasses of tectal neurons? A recent study suggested that tectal neurons partition the direction space into cardinal directions, but did not specify the neurotransmitter phenotype of the recorded neurons (Hunter et al., 2013). Studies of cortical circuits have outlined connectivity motifs in the excitatory and inhibitory populations that appear to be shared across sensory modalities (Harris and Mrsic-Flogel, 2013). The principal/excitatory cells show highly tuned properties that appear to be a result of sampling from selective inputs and amplification from recurrent connectivity. In contrast, the inhibitory neurons in the network pool inputs from neighboring principal/excitatory cells and display visual responses that are averages of local network activity (Bock et al., 2011; Hofer et al., 2011). Thus, for carnivoran visual cortex, where the principal cells are organized into columns of similarly tuned neurons, the inhibitory neurons show highly selective responses (Cardin et al., 2007). For mice, which lack any such spatial organization, inhibitory neurons are broadly tuned (Hofer et al., 2011; Kerlin et al., 2010). Previous studies demonstrated a lack of spatial clustering of similarly tuned neurons in the larval zebrafish tectum (Niell and Smith, 2005). If this structure followed a similar wiring scheme to the cortex, one would expect tectal inhibitory neurons to be broadly tuned. Were the two, previously described, sparse populations of DS inhibitory neurons (Gabriel et al., 2012) the scant few that were selective in an otherwise unselective pool? How does their selectivity compare to that of the excitatory population?

In this study, we attempt to answer these questions by performing two-photon calcium imaging of identified populations of excitatory and inhibitory tectal neurons in awake animals. For this purpose, we first generated and functionally characterized a pan-neuronal transgenic
fish line expressing the genetically encoded calcium indicator (GECI), GCaMP6s (Chen et al., 2013). We show that inhibitory and excitatory neurons display a matching degree of selectivity to moving stimuli. DS inhibitory neurons fall into two overlapping clusters that prefer stimuli moving anteriorly or posteriorly, whereas DS excitatory neurons triangularly partition the direction space. In addition, by analyzing pairwise noise correlations, we found evidence for local subnetworks among excitatory and inhibitory neurons. The output of the tectum seems to preserve the triangular partitioning of the direction space displayed by the RGC inputs, but with distinct differences.

RESULTS

Spatial distribution of glutamatergic and GABAergic neurons in the tectum

The architecture of the larval zebrafish tectum has been characterized extensively by past studies (for a review see Nevin et al., 2010). The input RGC axons terminate in a retinotopic order in the four retino-recipient layers of the neuropil. Furthermore, these axonal terminations are restricted to specific sub-laminae in the retino-recipient layers (Robles et al., 2014). SPV neurons extend unipolar dendrites of diverse morphologies into the neuropil, which allow them to precisely sample inputs from the retino-recipient laminae (Gabriel et al., 2012). Previous studies have shown that the cell body layer in the tectum is primarily composed of glutamatergic and GABAergic neurons (Higashijima et al., 2004). Whether there is any spatial organization to these subclasses of neurons is still unknown. To investigate this we used two recently published BAC-transgenic zebrafish lines that label glutamatergic and GABAergic neurons (Bae et al., 2009; Satou et al., 2013). We crossed Tg:Gad1b:EGFP with
Figure 3.1: GABAergic and glutamatergic neurons are spatially localized in the tectum. (A)

Maximum intensity projection of a confocal stack of a double transgenic larva
(Gad1b:EGFP/VGlut2a:DSRed) labeling inhibitory neurons in green and excitatory neurons in red. Scale bar represents 50 microns. Ob – olfactory bulb; Ha – habenula; TeO – optic tectum;
Cb – cerebellum; Hb – hindbrain; S – spinal cord. (B) Optical slice of the left optic tectum from the same double transgenic as (A). Scale bar represents 10 microns. a – a’ represents the area used for the fluorescense intensity profile in (D). SPV – stratum periventriculare (or cell body layer); Np – tectal neuropil. (C) Shown are the xy, yz, zx (in a clockwise order from the larger image on top) slices of a confocal volume computed by averaging registered volumes (see Methods). (D) The mean fluorescence intensity profile for the excitatory (red) and inhibitory (green) neurons from all the imaged fish (n=6) along the path (a – a’) shown in (B). Shaded areas represent s.e.m.
Figure 3.1 (continued)
Tg:VGlut2a:DSRed to generate double transgenic larvae and imaged the tecta of these larvae on a confocal microscope (Figure 3.1A,B). When we examined the cell body layer, we found that both excitatory and inhibitory neurons were uniformly distributed along the rostrocaudal and dorsomedial axes (Figure 3.1B). However, in the radial direction, i.e. along the path (a-a’), shown in Figure 3.1B, we found that the glutamatergic and GABAergic neurons were spatially segregated. The glutamatergic neurons occupied a space closer to the neuropil-cellbody layer boundary whereas the GABAergic neurons formed an outer shell around them. To visualize this clearly, we morphed the tectal volumes of all the imaged fish (n=6) onto a reference tectal volume (Portugues et al., 2014). The average of all the volumes in this standard space is shown in Figure 3.1C, where the spatial localization of the two populations of SPV neurons can be clearly distinguished. This is also confirmed by the profile of fluorescence intensities in this standard space along the radial dimension (Figure 3.1D). Given the retinotopy in the tectum and the radial projection of neuronal dendrites into the neuropil, this arrangement of inner shell of glutamatergic neurons and outer shell of GABAergic neurons allows the sampling and analysis of the same patch of visual space by these two subpopulations.

**Functional characterization of new transgenic, Tg:elavl3:GCaMP6s.**

In order to image the functional responses of neurons in the tectum, we created a new stable transgenic line expressing the ultrasensitive GECI, GCaMP6s (Chen et al., 2013) under the control of the pan-neuronal transgene elavl3 (Higashijima et al., 2003). We did this by taking advantage of the efficient transgenesis of the Tol2 transposon system (Kwan et al., 2007)
Figure 3.2: New pan-neuronal transgenic expressing GCaMP6s faithfully reports spiking activity for both GABAergic and glutamatergic neurons. (A) Maximum intensity projection of the new Tg: elavl3:GCaMP6s. Scale bar indicates 50 microns. (B) Two-photon image of a successfully patched glutamatergic cell in a double transgenic (elavl3:GCaMP6s/VGlut2a:DSRed). The cell was patched under two-photon guidance (see Methods) using a fluorescently labeled pipette (arrowhead). This configuration was used to record electrical extracellular signals and optical calcium signal simultaneously. Scale bar indicates 10 microns. (C) Sample, simultaneously recorded, calcium trace (top) and extracellular spike recordings (bottom). Vertical scale bar: 25% deltaF/F; horizontal scale bar: 5 sec. (D) The black trace is the calcium kernel extracted from performing regression of time shifted versions of the spike trace with the calcium trace. The pink trace is the fit to the kernel, which has an exponential rising followed by exponential decaying phase. (E) In black is the actual calcium trace recorded from the cell. In pink is the predicted trace resulting from the convolution of the spike trace with the kernel fit. (F) The mean ± std of the correlation coefficient between the predicted trace and data for all the recorded GABAergic (green, n = 5 neurons) and glutamatergic (red, n = 4 neurons) neurons are 0.78 ± 0.07 and 0.85 ± 0.08 respectively.
Figure 3.2 (continued)

A

elavl3:
GCaMP6s
Vglut2a:
DSRed

B

C

25% dff
L
5 s

D

E

F

data
model

correlation
Gaba
Glut
We found a bright founder (Figure 3.2A) that allowed us to faithfully follow the calcium activity of neurons in animals heterozygous for the transgene.

To characterize how well calcium responses reported by this transgenic reflect membrane potential dynamics, we performed *in-vivo* two-photon guided extracellular recordings (Figure 3.2B). We simultaneously recorded the calcium signals reported by the patched neurons by performing two-photon calcium imaging on a custom designed rig (Grama and Engert, 2012). Furthermore, it is unknown whether the relationship between spiking rate and calcium fluctuations varies across genetically distinct populations of neurons. Therefore we used double transgenics Tg:elavl3:GCaMP6s/VGlut2a:DSRed (Figure 3.4B) and Tg:elavl3:GCaMP6s/Gad1b:DSRed (Figure 3.4A) to record specifically from glutamateergic and GABAergic neurons, respectively. A sample trace of the recorded spikes and calcium signal is shown in (Figure 3.2C). To find the correspondence between the recorded calcium signals and the spikes, we extracted the calcium kernel (Figure 3.2D) by using time-shifted versions of the spike trace as regressors and performing linear regression of these with the calcium trace. We then fit the resulting kernel with function consisting of an exponential rising phase followed by a decaying phase (Figure 3.2D). This fitted kernel was then convolved with the spike trace and the resulting model calcium trace showed high correspondence with the actual data (Figure 3.2E). This correspondence demonstrates the linear relationship between the spikes and the calcium traces. Both the GABAergic and glutamateergic neurons showed high correspondences (Figure 3.2F, corr. coefficient for GABAergic neurons = 0.78 ± 0.07 (mean ± std, n = 5 cells); corr. coefficient for glutamateergic neurons = 0.85 ± 0.08 (mean ± std, n = 4 cells)). Thus the new transgenic, elavl3:GCaMP6s faithfully reports the underlying spiking activity of tectal neurons.
Figure 3.3: GABAergic and glutamatergic neurons have a broad range of receptive field sizes. 

(A) Sample mean calcium trace (black) of a cells response to spot flashed sequentially (blue dashes, 5 s inter-stimulus interval) in different parts of its visual field. Note the bleeding of the response of one spot into the next spots presentation. The gray lines are the single trial responses. (B) The colored lines are the family of regressors used to estimate the contribution of each spot to a cell’s response. (C) The black trace is the same mean trace from (A). The pink trace is the regression fit. (D) Receptive field (RF) of the neuron from A. The units shown are z-scores of the regression coefficients of the fit from (C). (E) The left panel shows the distribution of RF diameters for GABAergic neurons. The right panel shows the same for glutamatergic neurons. GABAergic neurons (mean RF diameter ± std = 28 ± 15°) had slightly larger receptive fields than excitatory neurons (mean RF diameter ± std = 27 ± 16° p = 0.05, Wilcoxon’s ranksum test).
**GABAergic and glutamatergic neurons display a diverse range of receptive field sizes.**

Previous studies characterizing the receptive fields (RF) of neurons in the larval zebrafish tectum were agnostic to the differences between the inhibitory and excitatory neurons (Niell and Smith, 2005; Sajovic and Levinthal, 1982; Zhang et al., 2011). To probe this, we recorded calcium responses of glutamatergic and GABAergic tectal neurons. The animals were immobilized in agarose and mounted dorsal side up in a custom designed chamber. During the recordings, larvae were presented with 5° spots (0.5s duration) in different parts of the visual field. The stimuli were presented to the left eye of the fish while the contralateral tectum was imaged with a two-photon microscope. The calcium traces from different trials from a sample neuron are shown in Figure 3.3A along with the time of presentation of spots (blue vertical dashes). Since the inter-stimulus-interval was short (~5s), the responses from one stimulus presentation often bled into the next (Figure 3.3A). In order to correct for this, we adopted a strategy similar to that used by Portugues and colleagues (Portugues et al., 2014). We defined a neuron’s response to spots as the convolution of an impulse response with our previously estimated calcium kernel (Figure 3.2D). We created a family of regressors for all the spots with the amplitude of each response as the coefficient to be estimated (Figure 3.3B). We estimated the coefficients by performing linear regression of these regressors with the actual calcium trace (Figure 3.3C). The resulting coefficients correspond to the contribution of each spot to the calcium trace, i.e., the receptive field. Figure 3.3D shows the estimated receptive field of the neuron from Figure 3.3A. Thus, this strategy of designing regressors should be useful when probing responses using a slow functional indicator to rapidly fluctuating stimuli. When we
Figure 3.4: Example responses of glutamatergic and GABAergic neurons to drifting bars. (A)

Two-photon fluorescence image of tectum of a double transgenic larva (Tg: elavl3:GCaMP6s/Gad1b:DSRed), showing GCaMP6s+ cells in grey and GABAergic cells in green.

(B) Two-photon fluorescence image of tectum of a double transgenic larva (Tg: elavl3:GCaMP6s/VGlut2a:DSRed), showing GCaMP6s+ cells in grey and glutamatergic cells in red.

(C) Cartoon to help calibrate angles of motion of the drifting bars with respect to the axis of the fish. (D) Example mean (green) and single-trial calcium traces (left) of 5 neurons to bars drifting in 45° increments. In light green are the single trial responses. The right panel shows the tuning curves of the neurons. (E) Same as (D) but for glutamatergic neurons. Left panel: Red - mean calcium trace; light red - single trial responses. Right panel: Red - tuning curve.
Figure 3.4 (continued)

A

B

elav13:
GCaMP6s
Vglut2a:
DSRed
Gad1b:
DSRed

C

D

E

% diff

0 45 90 135 180 225 270 315

Angle of drifting bar (deg)

Gaba

0 45 90 135 180 225 270 315

Angle of drifting bar (deg)

Glut
examined the RFs of both GABAergic neurons and glutamatergic we found them to span a large range of sizes (Figure 3.3E, RF diameter for GABAergic neurons = 28 ± 15° (mean ± std); RF diameter for glutamatergic neurons = 27 ± 16° (mean ± std)). GABAergic neurons had slightly, but significantly larger RF diameters than glutamatergic neurons (p = 0.05, Wilcoxon’s ranksum test; median RF diameter GABAergic neurons = 25.1°; median RF diameter glutamatergic neurons = 21.5°). The RF sizes we measured agree well with previously reported RF sizes in adult zebrafish (~30°) (Sajovic and Levinthal, 1982).

Glutamatergic and GABAergic neurons show matching degree of selectivity to moving stimuli

A large fraction of SPV neurons are responsive to motion, with a substantial number of them selective for the direction of motion (Grama and Engert, 2012; Niell and Smith, 2005; Ramdya and Engert, 2008). How does this selectivity manifest itself in the inhibitory and excitatory SPV neurons? To answer this, we probed the calcium responses of identified glutamatergic and GABAergic neurons (Figures 3.4A & B) while the animals were presented with bars moving in 8 different directions. Figures 3.4D & 3.4E show the responses (mean and single trials) from sample GABAergic and glutamatergic cells respectively. Note the diversity in the tuning curves for both populations. To quantify selectivity of neurons, we chose two different metrics. The first is the more traditional direction selective index (DSI), which only takes into account responses in the preferred direction and the diagonally opposite direction. The second metric, 1-circular variance (1-CV) takes into account the responses of the neuron to all eight directions (see (Ringach et al., 2002) for a detailed exposition). Therefore, it is a better indicator of the degree of tuning of a neuron. These metrics run on a scale from 0 (non-...
Figure 3.5: Glutamatergic and GABAergic neurons show differences in DSI but not 1-Circular Variance. **(A)** The upper and lower panels show the distribution of direction selectivity indices (DSIs) for glutamatergic and GABAergic neurons. **(B)** Cumulative distribution functions (cdfs) of the DSIs of glutamatergic and GABAergic neurons. Excitatory neurons (mean ± std = 0.3 ± 0.2) had slightly, and significantly (p < 0.0001, Wilcoxon’s ranksum test), higher DSI values than GABAergic neurons (mean ± std = 0.25 ± 0.18). **(C)** Same as (A) for 1-Circular Variance (1-CV). **(D)** Same as (B) for 1-CV. There was no significant difference (p = 0.39, Wilcoxon’s ranksum test) between the 1-CVs for glutamatergic (mean ± std = 0.2 ± 0.11) and GABAergic neurons (mean ± std = 0.19 ± 0.1).
Figure 3.5 (continued)

A

Gaba

# cells

0 0.5 1

Glut

0 0.5 1

DSI

B

0 0.5 1

p 0.5

DSI

C

Gaba

# cells

0 0.5 1

Glut

0 0.5 1

1-Circular Variance

D

0 0.5 1

p 0.5

1-Circular Variance
selective) to 1 (highly selective). Both inhibitory and excitatory neurons displayed a range of DSI values (Figures 3.5A & B), with a substantial fraction of them having values greater than 0.4 (for inhibitory neurons: 20.5%, n = 779 neurons; for excitatory neurons: 28.4%, n = 1322 neurons). Thus, the tectum possesses a robust network of DS inhibitory and excitatory neurons. The DSI values of inhibitory neurons (mean ± std = 0.25 ± 0.18) were slightly but significantly (p < 0.001, Wilcoxon’s ranksum test) lower than those of excitatory neurons (mean ± std = 0.3 ± 0.2). When we compared 1-CV values (Figures 3.5C & D), we found that there was no statistical difference (p = 0.39, Wilcoxon’s ranksum test) between the two populations (for inhibitory neurons: mean ± std = 0.19 ± 0.1; for excitatory neurons: mean ± std = 0.2 ± 0.1). Thus, when the responses to all directions of motion are taken into account (1-circular variance), inhibitory and excitatory neurons show similar selectivity.

Distinctive clustering of preferred directions of motion in glutamatergic and GABAergic neurons

Given their matching degree of selectivity, do the glutamatergic and GABAergic neurons share similar preferred directions (PDs) of motion? In an enhancer-trap screen, Gabriel and colleagues found two transgenic lines, each of which labeled sparse populations of inhibitory neurons (Gabriel et al., 2012). These were direction selective and showed preferences for stimuli moving either anteriorly or posteriorly, respectively. Are these the only two DS inhibitory populations or are there other populations that prefer other directions? Do the excitatory neurons have similar subpopulations of direction selective neurons? To explore these questions we examined the distributions of preferred directions of all the recorded direction
Figure 3.6: Preferred directions (PDs) of direction selective GABAergic and glutamatergic neurons fall into distinct clusters. (A) Histogram of preferred directions of direction selective (DSI > 0.4) GABAergic neurons. This was fit with a sum of two von-Mises distrubtions (magenta and blue $R^2 = 0.62$)to estimate peaks of the two clusters. Magenta cluster peak - 188°; blue cluster peak – 318.7°. (B) The same as (A) for glutamatergic neurons. This was fit with three von-Mises distributions (yellow, cyan and black; $R^2 = 0.68$). Yellow cluster peak - 40°; cyan cluster peak - 185°; black cluster peak - 317°. (C) The mean ± std tuning curves of all the neurons that fell into the full width at half maximum (FWHM) of the magenta and blue von-Mises distributions from A for DS inhibitory neurons. Note the tuning curves show a preference for stimuli moving anteriorly (magenta) or posteriorly (blue). (D) Same as (C) for the von-Mises distributions in B for DS excitatory neurons. The mean tuning curves tile the direction space in a triangular way.
Figure 3.6 (continued)

A

Gaba

B

Glut

C

D

Angle of drifting bar (deg)

Angle of drifting bar (deg)

# cells

# cells
selective cells (DSI>0.4) from the excitatory and inhibitory populations. We found that the inhibitory neurons fell into two broad clusters that represented motion moving either caudally or rostrally (Figures 3.6A & C). These clusters had peaks at 188° and 318.7°, which were estimated by fitting the distribution with a sum of two von-Mises functions ($R^2 = 0.62$). In fact, these clusters seem to have a close correspondence to the two opposing clusters reported by Gabriel et al (Gabriel et al., 2012). Thus, the lack of additional subpopulations of DS inhibitory neurons lead us to conclude that the two transgenics reported by Gabriel et al., likely label the complete population of DS inhibitory neurons. We found that the glutamatergic neurons, which form the output layer of the tectum, have DS cells whose preferred directions seem to form three overlapping clusters (Figure 3.6B & D; $R^2=0.68$, peaks at 40°, 185°, 317°). While the glutamatergic neurons maintain the triangular partitioning of direction space the DSRGC inputs into the tectum exhibit (Lowe et al., 2013; Nikolaou et al., 2012), they show two notable differences. The DSRGCs have one cluster that has a roughly five-fold overrepresentation compared to the other two clusters (Lowe et al., 2013). The clusters in the glutamatergic population do not display such a bias and show roughly equal membership (Figure 3.6B). Furthermore, the preferred directions of the three clusters in the glutamatergic population appear to be phase shifted compared to those displayed by the retinal inputs.

**Noise correlations of E and I neurons**

The advantage of simultaneously recording from a large population of neurons is that we can get estimates of how functionally connected neurons are by examining their pairwise noise correlations (Hofer et al., 2011; Ko et al., 2011; Rothschild et al., 2010). Noise correlations
Figure 3.7: GABAergic and glutamatergic neurons show significant distance dependent noise correlations. (A) Histogram of pairwise noise correlations of GABAergic neurons. These are significantly higher than shuffled trial controls (black trace, $p < 10^{-10}$ K-S test). (B) Same as (A) for Glutamatergic neurons. The noise correlations are significantly higher than shuffled trial controls (black trace, $p < 10^{-10}$ K-S test). (C) Pairwise noise correlations of GABAergic neurons as a function of distance between them. Black line shows a linear fit ($R^2 = 0.11$). Dashed grey line denotes the 50 micron mark. (D) Same as (C) for Glutamatergic neurons. Black line - linear fit ($R^2 = 0.12$).
Figure 3.7 (continued)

A

B

C

D

# pairs

# pairs

# pairs

noise correlation

noise correlation

noise correlation

noise correlation

distance (microns)

distance (microns)

distance (microns)

Gaba

Gaba

Glut

Glut

shuffled

shuffled
are calculated by finding the correlation coefficient of the same trial, mean-subtracted, responses of neurons to repeated presentation of a stimulus. This estimate of correlated variability of neurons reflects either a pool of shared common inputs or mutual connectivity (Hofer et al., 2011). A noise correlation value closer to +1 or -1 reflects facilitatory or antagonistic coupling respectively, whereas values closer to 0 reflect no underlying functional connectivity. When we examined the noise correlations between all the pairs of glutamatergic neurons (n=38526 pairs), we found them to be variable and have significantly higher correlations (0.0738 ± 0.1041) compared to shuffled trial controls (Figure 3.7B, paired t-test, p < 10^{-10}). Inhibitory neurons showed a similar relationship in their pairwise noise correlations (Figure 3.7A, paired t-test, p < 10^{-10}, n=11921 pairs, mean ± s.d. = 0.0781 ± 0.0949).

Is the degree of noise correlation related to the distance between the pair of neurons? To investigate this, we plotted noise correlations as a function of pairwise neuronal distance for both excitatory and inhibitory neurons (Figures 3.7C & D). The noise correlations for both populations showed a distance dependency, where neurons closer to each other had higher noise correlations than neurons further away (for excitatory neurons: R^2 of fit = 0.12, slope = 8.11 x 10^{-4}, 95% CI (-8.328 x 10^{-4}, -7.89 x 10^{-4}); for inhibitory neurons: R^2 of fit = 0.11, slope = 6.99 x 10^{-4}, 95% CI (-7.34 x 10^{-4}, 6.65 x 10^{-4})). At all the distances the noise correlations were highly variable. To examine differences in the amount of local functional connectivity between the two subpopulations of neurons, we compared the noise correlations of pairs of glutamatergic and GABAergic neurons separated by less than 50 microns (~8-10 cell diameters). We found that glutamatergic neurons (mean = 0.113 ± 0.1104, n = 15241 pairs) had higher noise correlations than GABAergic neurons (mean = 0.111 ± 0.0954, n = 5248 pairs, p = 0.002,
Kolmogorov-Smirnov test). Notably, both these populations had long tails with high noise correlation values (> 0.3), representing 5.62% (n = 857 pairs) of the examined pairs for glutamatergic neurons, and 3.16% (n = 166 pairs) for GABAergic neurons. Thus, glutamatergic neurons display higher local functional connectivity than GABAergic neurons.

DISCUSSION

The goal of this study was to characterize the response properties of the two major classes of tectal neurons. We did this by first generating a new pan-neuronal transgenic fish expressing the highly sensitive GECI, GCaMP6s (Chen et al., 2013), and characterizing its fidelity. This line should prove to be a valuable tool in the growing arsenal of transgenic zebrafish lines to investigate neural circuits. We used this line in combination with lines labeling excitatory and inhibitory neurons (Bae et al., 2009; Satou et al., 2013) to perform functional imaging of the tectum while probing the fish with different visual stimuli. When we mapped the spatial RFs, we found that both excitatory and inhibitory neurons displayed a broad range of RF sizes with the mean sizes being on the larger size (~27-28° diameter). Taking into account RGC diameters (~10°) (Sajovic and Levinthal, 1982), this would imply a convergence factor of 3 going from RGCs to tectal neurons.

When we looked at tectal responses to moving stimuli, we found that inhibitory neurons matched the excitatory neurons in their degree of selectivity. Unlike the mammalian superior colliculus (Feinberg and Meister, 2014), the larval zebrafish tectum does not display any spatial clustering of neurons with similar responses to moving visual stimuli (Niell and Smith, 2005). Given this, the presence of highly selective inhibitory neurons would imply that rather than
pooling responses from nearby neurons and reflecting average local network activity, inhibitory neurons in the tectum form very specific synapses with selective inputs. This notion is corroborated by electrophysiological recordings from a previous study where DS inhibitory neurons had highly selective excitatory and inhibitory inputs (Gabriel et al., 2012). This represents a different motif of circuit connectivity than that of cortical circuits (Harris and Mrsic-Flogel, 2013).

A previous study suggested that the direction space in the tectum is split along cardinal directions (Hunter et al., 2013). We show that when taking the neurotransmitter phenotype into account, the direction space is represented very differently. We find that inhibitory neurons form two broad clusters representing forward and backward motion. Glutamatergic neurons, on the other hand, maintain the triangular representation of the retinal inputs but with different preferred angles and roughly equal membership of the clusters. Thus, tectal processing seems to normalize biased DS retinal inputs and generate more equal representation of preferred directions of motion. Given the central role of the tectum in goal directed behaviors such as prey capture (Bianco and Engert, 2015; Gahtan et al., 2005), estimating the trajectory of moving prey, such as paramecia, could have high value. Doing so from a highly skewed distribution of motion preference, as found in the retina, could prove to be difficult. Whereas the more normalized representation of direction space at the output layer of the tectum might be conducive for accurate tracking of moving stimuli.

By examining noise correlations between pairs of glutamatergic and GABAergic neurons, we found evidence for enhanced functional connectivity between pairs of tectal neurons located in close proximity. This implies that groups of spatially clustered neurons display
concerted activity. This could arise as a result of interconnectivity or common sources of input.

A recent study looking at tectal activity during a prey capture assay showed tightly clustered groups of neurons responding right before the larva showed a prey capture response (Bianco and Engert, 2015). Thus, this enhanced local functional connectivity might underlie a behaviorally relevant dynamic of tectal neurons.

The distinct representations of direction space across RGC inputs and tectal neurons raise interesting questions. Does the profile of PDs in the tectum reflect any aspect of the sensory statistics of larvae? In the cat visual cortex, it was shown that neurons clustered into preferred orientations along cardinal directions (Li et al., 2003). This is thought to reflect a bias in the visual world towards horizontal and vertical contours (Coppola et al., 1998). Does the visual world of the larval zebrafish show moving stimuli that show a matching bias to that of tectal neurons? Answering this would involve careful ethological studies that look into the visual statistics of the natural environments of zebrafish.

Do the different DSRGC clusters target specific tectal neurons? DS inhibitory neurons appear to receive directionally biased inputs from DSRGCs but the nature of these inputs remains poorly described (Gabriel et al., 2012). Future work focusing on generating transgenic lines labeling specific DSRGC populations should be able to unravel this by selectively modulating the activity of these subpopulations while monitoring its effects on tectal tuning profiles.

Previous studies have stressed the role of local DS inhibition in maintaining direction selectivity in tectal neurons (Gabriel et al., 2012; Grama and Engert, 2012). Our results bolster this notion by showing the presence of a substantial population of highly tuned inhibitory
neurons. In addition, we find two DS inhibitory neuron clusters, which seem to be captured by two established transgenic lines. Future work can use these to manipulate the activity of the two clusters and test their importance in the tectal DS circuit.

MATERIALS AND METHODS

Transgenic Zebrafish Lines

The Tg(elavl3:GCaMP6s) fish line was generated by first PCR amplifying the GCaMP6s (Chen et al., 2013) open reading frame (Addgene plasmid #40753) with forward primer ataACTAGTgccaccATGGGTTCTCATCATCAT and reverse ataCCGCGGcTCACCTCGCTGTCATCATTTGTAC (restriction site and coding sequences in upper case, respectively). This fragment was cloned into a plasmid with Tol2 arms flanking an upstream attR1-R2 cassette and the insertion site using restriction enzymes SpeI and SacII. Previously described elavl3 (HuC) cis-regulatory elements (Higashijima et al., 2003) were placed upstream via LR recombination (Invitrogen) with an attL flanked elavl3 entry clone. The resulting plasmid was then co-injected into 1-cell stage embryos at a concentration of 30 ng/μL with Tol2 transposase mRNA at a concentration of 30 ng/μL. A single founder was selected based on bright and spatially broad expression. Previously published lines, Tg:Vglut2a:DSRed, Tg:Vglut2a:EGFP, Tg:Gad1b:DSRed, and Tg:Gad1b:EGFP were kindly gifted to us by S. I. Higashijima. All fish used in this study were raised at 28ºC on a 14 h on/10 h off light cycle in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4). All experiments were approved by Harvard University’s Standing Committee on the Use of Animals in Research and Training.
**In vivo calcium imaging**

Calcium imaging experiments were carried out at 7–8 days post fertilization (dpf) as described previously (Grama and Engert, 2012). Briefly, larval zebrafish were anaesthetized using 0.02% MS222 and then paralysed in alpha-Bungarotoxin (Invitrogen). They were then mounted in 1.5% low-melting agarose on a custom-built chamber. Imaging was carried out on a custom-built two-photon microscope at 920 nm. The frame times were 930 ms for the population imaging experiments and 490 ms for the simultaneous calcium imaging-electrophysiology recordings.

**In vivo electrophysiology**

For recordings, larval zebrafish at 7-8dpf were first anaesthetized using 0.02% MS222 and then paralysed in alpha-Bungarotoxin (Invitrogen) at 1mg/ml in zebrafish external solution (NaCl 134 mM; KCl 2.9 mM; CaCl2 2.1 mM; MgCl2 1.2 mM; glucose 10mM; HEPES 10 mM; pH 7.8, 290 mOsm) for 1 minute. This was usually enough to get the fish completely paralysed. Following this, fish were mounted on a custom recording chamber using insect pins. To access the right tectum, the overlying skin was removed using a sharpened tungsten needle. Electrophysiological recordings were performed at room temperature using an Axopatch 200B (Axon Instruments) patch-clamp amplifier. Data were sampled at 5 kHz and filtered at 2 kHz with a National Instruments data acquisition board. Borosilicate glass capillary micropipettes (World Precision Instruments) were pulled for a resistance of 6-8 MΩ and were filled with external solution. To visualize the micropipettes under the two photon microscope we used the technique described by (Ishikawa et al., 2010). Briefly, the micropipettes were dipped in a solution of BSA-Alexa 594 (Catalog #: A13101, Life Technologies) for ~10s and allowed to dry for
~5s. The pipetted tips were guided under two-photon imaging at 790nm to DSRed positive cells and gentle suction was used to get loose seals (100-240 MOhm). Following this, we simultaneously recorded the patched neuron’s calcium responses (at 920 nm excitation wavelength) and extracellular spikes. The recordings were done using custom VIs in LabView.

Visual stimulation

Visual stimuli were presented using a mini projector (Dell M109S) that was modified to allow external control over the red LED with the other color LEDs turned off. The left wall of the recording chamber had a diffusive filter (#3026, Rosco, Inc.) bonded to it and acted as the screen for the projector. The screen spanned a visual angle of 120° X 60° (w X h). High contrast square spots (5° edge) and moving bars (5° width, 30°/s) were presented on a dark background. The spots were flashed for 0.5s followed by a 5s response period. They were spaced 10° from each other. The moving bars were presented pseudorandomly in 8 orientations spaced equally. Five repeats of every stimulus were presented.

Data analysis

For analyzing the joint calcium-electrical recordings, a region of interest (ROI) over the patched cell was manually chosen. The spikes from the electrical recordings were identified using published spike sorting routines (Quiroga et al., 2004). Time shifted versions of the spike trace were used to build a regressor matrix. We then estimated the contributions of these time shifted spike traces to the calcium trace by performing linear regression (Portugues et al., 2014). This gave us the calcium kernel. This kernel was fit by the function of the form $a*(1-exp(-x/b))*exp(-x/c)+d$, where $a$ is the amplitude; $b$ – rise time; $c$ – decay time; $d$ – DC offset. This fit
was then convolved with the spike trace and the correspondence between this model trace and the actual data was measured by calculating the correlation coefficient.

For analyzing the population calcium imaging data, ROIs were manually chosen over identified cells. The fluorescence signals from these cells were converted to a \( \delta f/f \) (df/f) values. Only traces that showed any calcium activity (Ahrens et al., 2013) were further analysed. The traces were highpass filtered to remove slow signal fluctuations that might result from bleaching. To analyze the responses to flashed spots, we modeled the response trace for each spot by convolving the previously estimated calcium kernel (rise time = 1.56 s; decay time = 2.56 s) with a delta function centered at the time of presentation of the spot. This sample trace was scaled with a coefficient to be estimated. We constructed a regressor matrix by using all the model response traces for the spots presented. The coefficients of all the model traces were estimated by performing linear regression of the regressor matrix with the calcium response to the spot presentation. This process was repeated 1000 times by bootstrapping the single trial calcium responses. This allowed us to discard coefficients whose value was not significantly \( (p < 0.025) \) different from 0. The resulting significant coefficients represented the receptive field of the neuron. This was fit by a 2-d Gaussian to estimate the size of the receptive field. Since the size of the presented spots were 5°, we only considered neurons with receptive fields with radii>5°. For analyzing responses to moving stimuli, we measured the peak \( df/f \) value in the period (~10s) following the start of stimulus presentation. Only neurons that showed a peak \( df/f > 25\% \) were further analyzed as this was a useful heuristic in eliminating noisy traces. In addition, the peak \( df/f \) responses in the response period were required to be significantly different from the baseline period (Wilcoxon’s ranksum test, \( p < 0.05 \)). The peak \( df/f \) response
to all eight drifting bars gave the tuning curve of the neuron. This was fit by a sum of two von-Mises functions which allowed the estimation of preferred direction (PD) of the neuron. DSI and 1-circular variance values were calculated as described by previously (Niell and Stryker, 2008). The PD distributions of glutamatergic and GABAergic neurons were fit by a sum of three and two von-Mises functions, respectively, to find the peak PD of the clusters. The noise correlation were calculated as described by Mizrahi et al., 2010.
REFERENCES


CHAPTER FOUR

Conclusion
The results I presented in the preceding chapters demonstrate that the tectum processes inputs from the RGCs and transforms these into very specific direction selective representations at the level of the output of the tectum. Given the tectum’s role in mediating goal directed behaviors, our results suggest that tectal processing is extracting visual information that might be relevant for such behaviors. In the following chapter, I will attempt to address some of the questions that are bound to crop up when considering our results in the light of current literature. I will also suggest some avenues that future studies might take to obtain a fuller picture of tectal processing.

**Open questions**

Previous work from Martin Meyer’s group showed that RGCs send direction selective inputs to the superficial layers of the tectal neuropil (Figure 4.1, Nikolaou et al., 2012). These inputs are excitatory in nature (Smear et al., 2007). From our electrophysiological recordings of DS tectal neurons, we showed that there was no bias in the input excitatory currents (Grama and Engert, 2012). This suggests that the DSRGC inputs are either not being used by the population of neurons we recorded from or they are being sampled in such a way that the average of all the tuned inputs is untuned (Jia et al., 2010). If the DSRGC inputs don’t define the tuning of DS tectal neurons, why then do the RGCs send such tuned inputs into the tectum? Gabriel et al’s study provides some clues in this regard (Gabriel et al., 2012). They performed electrophysiological recordings from two sparse populations of inhibitory neurons that showed preference for either caudorostral or rostrocaudal motion. The input excitatory currents to
Figure 4.1 Direction selective responses are restricted to the superficial layers of the tectal neuropil: (A) Distribution of vector angles for all direction selective voxels (23 optical sections from 9 larvae). Fitted von-Mises distributions confirm three populations of direction selective voxels centered at 30°, 164°, and 265°. (B) Parametric map of a single larva illustrating the three populations of direction selective responses superimposed onto the mean fluorescence image of SyGCaMP3-expressing axons. Direction selective responses occur in a superficial layer of SFGS. White arrow indicates skin autofluorescence. (C) Preferred angles of direction selective responses relative to the larval body axis. Arrows are scaled to reflect the relative proportion of voxels in each population.
Figure 4.1 (continued)
these neurons were biased to the preferred direction of motion. When they examined the dendritic arbors of these neurons, they found them to be localized to the area of neuropil receiving DS inputs from RGCs. Taken together these results suggest that the two DS inhibitory populations are targets of DSRGCs. From our data spanning all the inhibitory neurons in the cells body layer of the tectum, we didn’t find any additional direction selective populations of inhibitory neurons. Thus the three DSRGC populations (Figure 4.1) seem to be sampled and recombined by tectal DS inhibitory neurons to give rise to two distinct populations preferring anterior and posterior motion.

If DS inhibitory neurons in the tectum receive biased excitatory inputs from DSRGCs then what kind of neurons are the ones we recorded from? The most likely candidate class is that of excitatory neurons. There are two lines of evidence to support such a conjecture. First, there are more excitatory neurons with a higher degree of DS (~29%, DSI>0.4) than inhibitory neurons (~20%, DSI>0.4). Since we performed electrophysiological recordings from a random subset of neurons, it is likely that we encountered more DS excitatory neurons than inhibitory neurons. Second, excitatory neurons are spatially localized to the cellbody-neuropil boundary. Neurons occupying this region were shown to have dendritic arborizations restricted to the deeper neuropil layers (type XIII neurons, Meek and Schellart, 1978; Nevin et al., 2010). Since the DSRGC inputs are localized to the most superficial neuropil layer (Figure 4.1), excitatory neurons might not have access to these inputs. How then do these neurons become direction selective? If the random subset of tectal neurons we recorded from were indeed dominated by excitatory neurons, it would appear that DS excitatory tectal neurons inherit their direction
Figure 4.2 Model outlining possible mechanism of direction selectivity in the larval zebrafish tectum: DS inhibitory tectal neurons (green) get direction selective inputs from DSRGCs, the sum of which are biased to the preferred direction of the neuron (green arrow). These neurons also receive direction selective inhibition from the null direction. DS excitatory tectal neurons (red) may receive untuned excitatory inputs from the retina. Their preferred direction is indicated by the red arrow. They receive biased direction selective inhibitory inputs from their null direction from a local DS inhibitory tectal neuron.
Figure 4.2 (continued)
selective property from local DS inhibitory neurons. Inhibitory inputs biased to the null direction were also seen in the two DS inhibitory neuron populations Gabriel et al., recorded from. Thus, biased null direction inhibition may be a common denominator for all direction selective neurons in the tectum. The circuit scheme summarizing the ideas presented above is shown in Figure 4.2. If this arrangement of DSRGCs conveying direction selectivity to inhibitory tectal neurons, which in turn confer direction selectivity to excitatory neurons is true, it still begs the question why. The known structure of the DSRGC inputs into the tectum (Figure 4.1) and our population imaging data from excitatory and inhibitory tectal neurons (Chapter 3) suggests a few possible reasons. DSRGCs form three clusters which represent motion along three equally spaced directions, with a majority of neurons preferring stimuli moving from tail to head of the larva. This representation is drastically transformed at the level of inhibitory and excitatory DS tectal neurons. The observed subsets of tectal inhibitory and excitatory neurons display roughly equal membership and do not align with the preferred directions of the retinal clusters. The normalization of cluster membership and the transformation of preferred directions from DSRGCs to the DS excitatory and inhibitory tectal neurons might be the product of the way direction selectivity is built up in the tectum from retinal inputs. Such a representation of motion might have a relevance to behaviors the tectum is known to mediate (for example: prey capture). This notion and the circuit scheme presented in Figure 4.2 is speculative and will require extensive work spanning behavioral, physiological, anatomical and computational levels of analysis to put it to test.

**Future experiments**
The functional dissection of the motion selective circuitry in the retinotectal system will require the establishment of different transgenic lines labeling specific subsets of DSRGCs and specific subsets of motion selective tectal neurons. Given the repertoire of mouse transgenic lines labeling different DSRGC subpopulations (Huberman et al., 2009; Kim et al., 2008), a feasible starting point for generating similar transgenic fish lines would be to analyze and replicate the effects promoters in mice which target specific DSRGCs. For targeting specific cell types, the most versatile system in fish currently is that of Gal4-UAS (Scott et al., 2007). By expressing Gal4 in neurons of choice, it is possible to use UAS sequences to drive the expression of any reporter, such as genetically encoded calcium indicators, optogenetic proteins enabling the optical manipulation of circuits, etc. Using such lines future work will be able to turn on/off different DSRGCs populations, and investigate their contribution to different cell types in the tectum. These lines can also be used in combination with viral tracing technologies currently being developed in the Engert lab to trace mono-synaptically connected DSRGC-tectal neuron pairs. Doing so will allow the testing of the idea that DSRGCs connect to DS inhibitory tectal neurons. An alternative approach would be to perform electron microscopic tracing of labeled DSRGC axons and identify their synaptic partners in the tectum. Additionally, having access to specific DSRGC subpopulations will be useful in investigating the behavioral relevance of representing motion along the three observed directions (Figure 4.1). Specifically, why is there a need for an overrepresentation of the caudorostral direction.

In parallel to developing lines labeling specific DSRGC populations, future work should focus on developing lines that label distinct subsets of motion selective tectal neurons. Two such lines labeling the DS inhibitory tectal neuron populations were reported by Gabriel et al.,
from an enhancer trap screen. By following a similar strategy, it should be possible to identify lines labeling different DS excitatory populations. We are currently using the two DS inhibitory tectal lines to test the validity of our model for motion selectivity, which invokes biased inhibition from the null direction. Since these lines express the Gal4 protein in the labeled neurons, we can turn on/off their activity and see if we observe changes in the activity of other DS tectal neurons in a way our model would predict. If direction selectivity in the tectum is indeed dependent on DS inhibitory neurons, we could optically or chemically ablate the neurons labeled by two DS inhibitory neuron lines and engineer tecta with no direction selectivity. This could put us in the favorable position of investigating the behavioral relevance of direction selectivity in the larval zebrafish tectum.
References


