# Neuronal firing of Retinal Ganglion Cell Axons instructs Growth and Connectivity in the developing Visual System

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# Abstract

The phenomenon of neuronal circuit formation has been intensively studied dating back to the remarkable early descriptions of circuit complexity by Santiago Ramon y Cajal over a century-and-a-half ago. From axon guidance to the refinement of the circuit, the field has since made considerable progress in describing relevant phenomenology and in revealing the underlying mechanisms. Almost as old as the field itself is the discussion of whether neural activity is important in the formation of neuronal circuits. An influential hypothesis to explain how firing could instruct circuit plasticity was first developed by Donald Hebb for the field of learning and memory and later adapted by numerous others to explain activity-dependent circuit formation. This thesis describes the development of a new approach for investigating Hebbian forms of circuit formation. Using the *Xenopus laevis* visual system to pair visual stimulation with live observations of axonal growth, branching and remodelling in the awake animal at high temporal resolution and over extended periods of time, we tested whether the Hebbian model of circuit formation could explain our observations. For the first time, Hebbian events in circuit formation could be directly observed in a living animal at single cell resolution. However, we also discovered related mechanisms that were not predicted as part of Hebb's postulate. Axons regulate their growth, branching and remodelling depending on their firing rate and level of arbour complexity. The experiments described here provide a detailed explanation of how activity helps guide circuit formation and can help reconcile seemingly conflicting views in the field.

# Résumé

Depuis la description initiale de la complexité des circuits neuronaux par Santiago Ramon y Cajal il y plus d'un siècle et demi, le thème de la formation des circuits neuronaux a été au cœur de nombreuses études scientifiques. Du guidage des axones jusqu'au raffinement des circuits, ce domaine a fait des progrès considérables en décrivant en détails la phénoménologie pertinente et en révélant progressivement les mécanismes sous-jacents de ces phénomènes. Cependant, il persiste une question presqu'aussi vieille que le domaine d'études lui-même : l'activité neuronale est-elle importante pour la formation des circuits neuronaux? Une des hypothèses les plus influentes à ce jour pour expliquer comment l'activité neuronale peut instruire la plasticité du circuit a été formulée pour la première fois par Donald Hebb dans le contexte de l'apprentissage et de la mémoire, et a par la suite été adaptée par plusieurs autres chercheurs pour expliquer le rôle de l'activité neuronale pour la formation des circuits. Dans ce contexte, cette thèse décrit le développement d'une nouvelle approche expérimentale pour investiguer les formes hebbiennes de formation des circuits neuronaux. En utilisant le système visuel de la grenouille *Xenopus laevis* afin de pairer différentes stimulations visuelles et l'observation en temps réel de la croissance, de l'arborisation et du remodelage axonals dans l'animal eveillé à une résolution temporelle élevée et sur des périodes prolongées, nous avons testé si le modèle hebbien pouvait expliquer nos observations. Pour la première fois, les évènements prédits par Hebb au sujet de la formation des circuits ont pu être observés et confirmés directement au niveau cellulaire dans l'animal vivant. Cependant, nous avons également découvert d'autres mécanismes associés qui n'avaient pas été prédits par le postulat de Hebb. En effet, les axones régulent aussi leur croissance, leur arborisation et leur remodelage selon leur niveau d'activité et selon la complexité de l'arborisation. Les travaux décrits ici présentent ainsi une explication détaillée du rôle que l'activité neuronale joue et comment elle guide la formation des circuits, et espèrent réconcilier certaines vues qui peuvent paraître contradictoires dans le domaine.

# Acknowledgments

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Für meine  Schwester Simone, Phil, Fini und Titus

Für meinen Bruder Jürgen, Steffi, Henri und Toni

Für meinen Vater und meine Mutter

For Alex

Mama und Robert ich vermiss euch unendlich.

# List of abbreviations

**RGC**: retinal ganglion cell
**DSCAM:** Down syndrome cell adhesion molecule
**EphA:** Ephrin receptors A
**TTX:** tetrodotoxin
**NI:** nucleus isthmi
**SC:** superior colliculus
**cAMP:** Cyclic adenosine monophosphate
**LGN:** lateral geniculate nucleus
**dLGN:** dorsal lateral geniculate nucleus
**β2-nAChR:** nicotinic acetylcholine receptor
**8-CPT-cAMP:** 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate
**LTP:** long-term potentiation
**LTD:** long-term depression
**AMPARs:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
**NMDARs:** N-methyl-D-aspartate receptors
**AP5:** (2R)-amino-5-phosphonopentanoate
**CaMK II:** Ca2+/calmodulin-dependent protein kinase
**VAMP2:** vesicle-associated membrane protein 2 (synaptobrevin 2)
**dn VAMP2:** dominant negative VAMP2
**TeNT:** tetanus toxin light chain
**blu:** blumenkohl gene
**VGLUT2** Vesicular Glutamate Transporter
**BDNF:** Brain-derived neurotrophic factor
**TeNT-Lc:** tetanus neurotoxin light chain
**PA-GFP:** photoactivatable green fluorescent protein
**EGFP:** Enhanced Green Fluorescent Protein
**DAS:** Dark-Sync-Async
**DSA:** Dark-Async-Sync
**MK-801:** Dizocilpine
**PMSG:** Pregnant mare's serum gonadotropin
**MS-222:** Tricaine methanesulfonate
**MBSH:** Modified Barth's Saline
**CSCs:** Light-evoked compound synaptic currents
**GABAA:** y-aminobutyric acid type A

## Author Contributions

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## Preface to chapter 1: Introduction

The introduction is a literature review of the field of activity-dependent circuit formation with a focus on the amphibian (primarily *Xenopus laevis*) retinotectal system. However, the isthmotectal system of the frog and the mammalian retinofugal pathways are also reviewed.

# Chapter 1: Introduction

The formation of a neuronal circuit requires many decisions. For example in the visual system, retinal ganglion cell (RGC) axons grow from the eye to central targets including the optic tectum where they synapse onto tectal neurons. During this developmental process, RGC axons have to navigate out of the eye, travel along the optic nerve, decide which way to turn at the optic chiasm and finally wire up correctly in the optic tectum. Many of these decisions are based on genetically encoded programs, but some could potentially be instructed by neuronal firing. For example, when RGC axons are wiring to postsynaptic tectal neurons they could be influenced by genetically encoded gradients of EphAs and ephrin As (McLaughlin and O'Leary, 2005), by previously determined genetic tags targeting specific partners (Schmucker, 2007) or they could be influenced by the firing of the neuron. It is important to mention that none of these mechanisms necessarily exclude one another. There is good evidence that genetically encoded gradients are important for axons to find the appropriate target region. Further, it is very likely that genetic tags, like DSCAM or protocadherins, are a major determinant of some aspects the structure of neuronal circuits. However, there are also many examples where neuronal firing instructs the formation neuronal circuits. Processes that are instructed by neuronal firing are known as "activity-dependent", because it is thought that neuronal firing or "activity" ultimately determines how the cell reacts. Here, I will give an introduction of the retinotectal system, I will overview the current literature on activity dependent growth and review possible activity dependent mechanisms and how such mechanisms can help neuronal circuit formation.

## 1.1 The Retinotectal system and its development

One highly studied model for neuronal circuit formation is the projection from the eye to the brain in the frog (Ruthazer and Cline, 2004). The output neurons of the eye are the retinal ganglion cells (RGCs), whose axons exit the eye in the optic nerve, cross the midline at the optic chiasm, and innervate central brain structures. This axonal tract has proven ideal for studies of axonal development and regeneration due to the accessibility of the eye and optic nerve for discrete lesion, the compartmentalization of projection and target neuronal populations for restricted pharmacological treatment, and the highly ordered pattern of RGC axonal terminations (Fujisawa et al., 1982; Gaze and Jacobson, 1963; Sperry, 1943). The capacity of lower vertebrates to readily regenerate the optic nerve makes them valuable model systems to examine and compare patterned afferent synaptogenesis during development and rewiring following axonal damage.

The pattern of connections between RGCs and their major target in the brain, the optic tectum in lower vertebrates, is similar to many afferent projections in that it maintains the topographic properties of information in the afferent input to the retinorecipient target (Gaze, 1958). This order is accomplished by reconstituting the same spatial relationships between the positions of the soma of RGCs in the retina with the tectal neurons with which the RGCs form synapses. Maintaining such near-neighbor connections creates a topographic representation of the retina, and therefore, visual space in the tectum. Physically, the retina is mapped onto the tectum by axons of RGCs in the dorsal retina terminating in the ventral tectum, ventral retina RGCs projecting to the dorsal tectum, nasal RGCs projecting to the caudal tectum, and temporal RGC axons

terminating in the rostral tectum (Godement and Bonhoeffer, 1989; Roskies and O'Leary, 1994; Sperry, 1963; Vielmetter and Stuermer, 1989; Walter et al., 1987a, 1987b).

During early development of fish and frogs, the first RGC axons enter the optic tectum as simple projections that terminate in retinotopically appropriate regions (Holt, 1984; Holt and Harris, 1983; Sakaguchi and Murphey, 1985b). Thus, the retinotectal map is established at the earliest stages of RGC innervation. After reaching their target zones, RGC axons extend branches to form arborizations, which, especially in the frog, grow to cover relatively large regions of the tectum (Sakaguchi and Murphey, 1985b). While the tectum continues to grow, RGC axonal arbours do not expand their tangential coverage area in the tectum to the same extent. Therefore, the relative size of RGC axonal arbours compared to the entire tectum decreases throughout development (Gaze et al., 1974; Sakaguchi and Murphey, 1985b; Sretavan and Shatz, 1984) (Figure 1.1). This shift in the relative size of axon arbours compared to the tectal field is supported by electrophysiological recordings of tectal neurons demonstrating a progressive decrease in the area of tectum responding to a region of visual space with maturation. In the frog, both nasal and temporal RGC axons initially extensively co-arborize in the optic tectum but only the nasal RGCs continue to extend caudally as the tectum increases in size (O'Rourke and Fraser, 1990; Sakaguchi and Murphey, 1985b). This shifting of arbours corresponds to the emergence of more precise receptive fields measured electrophysiologically (Gaze et al., 1974). Thus, in normal development the retinotopic map goes through stages of initial ordered RGC axonal termination, followed by extensive axonal arborisation, and subsequent refinement of the map through an increase in tectum size and a decrease in receptive field size.

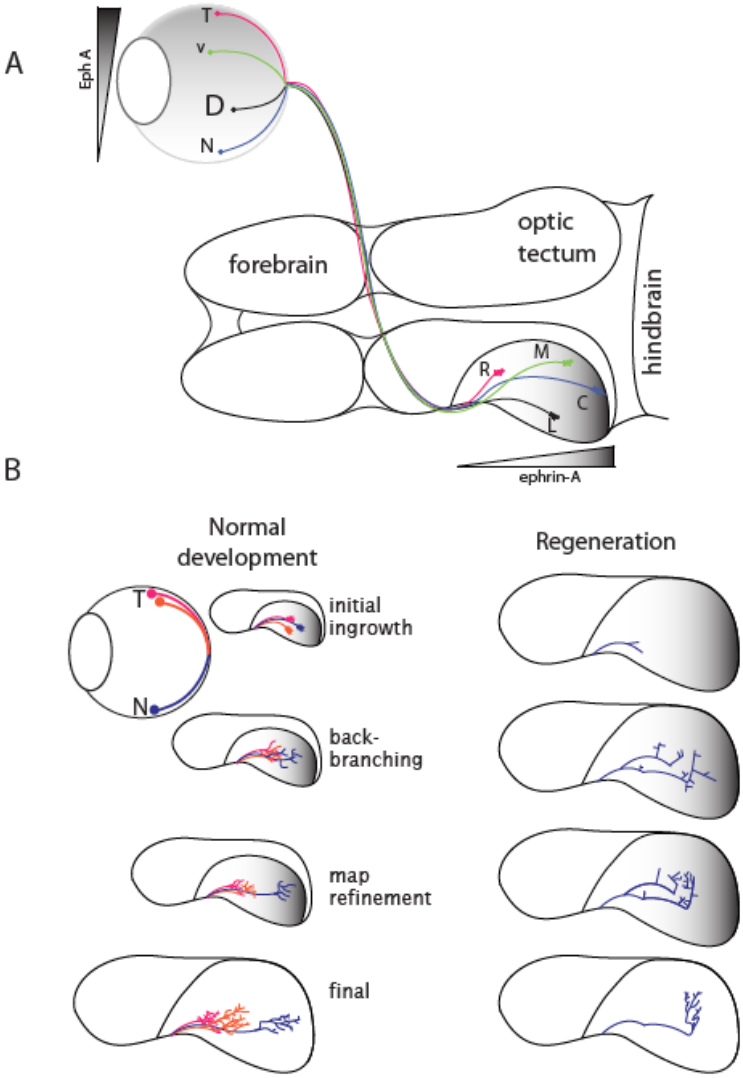


**Figure 1.1:** Development of retinotectal topographic projection in the visual system of Xenopus laevis. A) Retinal ganglion cells in the retina express EphA in a gradient of high temporal and low nasal expression. A repulsive response to the graded expression of the ligand Ephrin-A in the tectum mediates rostrocaudal mapping of axonal afferents. Other cues mediate dorsoventral mapping, which in large part may occur through pretarget sorting of axons within the optic tract. B) As development proceeds the optic tectum grows by adding new neurons at its caudomedial proliferative zone, while axons simultaneously undergo dynamic remodelling in several stages. After initial ingrowth, axons undergo extensive back-branching to elaborate arbours. Over time and with tectal growth, the temporal and nasal axons sort out in a topographic manner ultimately leading to a precise final map. In the regenerating retinotectal projection, Ephrin cues are either absent or weakly re-expressed explaining the circuitous route that axons make, as they rely heavily on activity cues to refine their final arbours.

## 1.2 Activity dependent target selection of RGC axons

Experiments suggest that the retinotectal circuit is set up through two types of processes. First, RGCs project axons from the eye to the target area in the brain. This step involves molecular guidance cues, and leads to a crude specificity of connections between RGCs and cells in the brain. Second, these crude connections are refined in an activity-dependent manner, hypothesized to be based on the existence of greater correlation in the firing of neighbouring RGCs than among those RGCs that come from separated retinal locations. This correlation-based mechanism for activity-dependent refinement was first proposed by the Canadian psychologist Donald O. Hebb in 1949 for the process of learning and memory (Bi and Poo, 2001; Hebb, 1949) and later adapted to the field of circuit formation (Changeux and Danchin, 1976; Shatz, 1990; Stent, 1973). Hence, it is referred to as Hebbian plasticity. Hebbian refinement of connections is especially important in cases where neurons need to find appropriate synaptic partners.

In the past, the visual system has been used as a common model system to unravel the mechanisms underlying Hebbian plasticity. Starting with the work of Hubel and Wiesel, visual activity (Hubel and Wiesel, 1965) and patterned spontaneous activity (McLaughlin et al., 2003a; Sretavan et al., 1988a; Triplett et al., 2009) have been thought to be involved in the choice of synaptic partners by detecting correlated activity. Hubel and Wiesel's initial experiments showed that the binocular map of a kitten is already in place shortly after birth (Hubel and Wiesel, 1963). However, they also reported a time period after birth during which this initial map can be changed dramatically, a period of time called the critical period (Wiesel and Hubel, 1965). In these experiments, a disruption of the binocular map was observed in kittens in which one eye was surgically closed, and

led to the possible conclusion that environmental cues could impact the development of the brain.

Because of the accessibility of the visual circuit in the frog, subsequent experiments using the frog as a model system could help to understand how activity can influence the formation of a neuronal circuit. Neuronal activity and guidance cues are thought to interact during circuit formation, thus it is challenging to experimentally isolate the involvement of neuronal firing in circuit formation. Moreover, more than 30 years ago, Martha Constantine-Paton showed that frogs that have a third eye implanted develop artificial ocular dominance bands in the dually innervated tectal lobe (Constantine-Paton and Law, 1978). These ocular dominance bands can be abolished by the application of tetrodotoxin (TTX) (Reh and Constantine-Paton, 1985). A similar experiment grafted duplicate portions of retina to one eye to create double-nasal or double-temporal retinae (Coletti et al., 1990; Ide et al., 1983). Yet another approach was to surgically force a tadpole's two natural eyes to both innervate a single tectal lobe (Higenell et al., 2012; Law and Constantine-Paton, 1980; Ruthazer et al., 2003). In each of these cases, duplicate populations of RGCs expressing the same guidance molecules innervated the optic tectum. Therefore, axons from pairs of spatially separated RGCs, receiving stimulation from different regions of visual space, were directed to the same tectal locations by identical chemoaffinity cues. If chemoaffinity cues were solely responsible for establishing the retinotopic map, it would be expected that the RGC axonal terminal arborizations from ectopic and endogenous populations would completely overlap. In general, chemoaffinity rules are followed and the RGC axons from both endogenous and ectopic populations terminate in the tectum according to a crude topography. However, in

the case of dually innervated tecta the RGC axonal terminal arborisations of the two eyes do not extensively intermingle, but segregate into eye-specific termination zones, or ocular dominance bands. These similar results from three separate types of surgical manipulations demonstrate that RGC axons segregate from other RGC axons programmed to recognize identical tectal targets when the RGC somata are not physically located next to each other. It seems unlikely that this segregation could be due to additional chemoaffinity cues since ephrin-A and EphA gradients do not change in dually innervated tectal lobes (Higenell et al., 2012) These results suggest that a force in addition to chemoaffinity must exist.

Silencing activity in the supernumerary eye of a three eyed frog with TTX resulted in degraded ocular dominance bands (Reh and Constantine-Paton, 1985). Interestingly, exposing goldfish tectum to TTX after crushing their optic nerve led to only minor differences compared to animals that were not exposed to TTX. TTX treated animals showed longer latencies only in the group of animals tested 28 to 31 days after surgery, indicating that neuronal activity is important in the maturation of the regenerating axon, but not in the reestablishment of the connections (Schmidt et al., 1983 but see Meyer, 1983). While these experiments show that neuronal activity can impact circuit formation they do not show that vision impacts circuit formation. To truly determine if vision helps the formation of the circuit a set of experiments was conducted in which three eyed frogs or goldfish were raised in darkness (Cline, 1991; Cook and Becker, 1990). These experiments determined that ocular dominance bands form in the absence of vision. One explanation is that spontaneous activity in the eye may provide enough information for

the segregation of the eyes (Cook and Becker, 1990; Demas et al., 2012). One way to avoid this possible confound is to provide the eyes with visual stimuli that contain almost no structure and activate all photoreceptors simultaneously. A way to achieve this is to provide the eyes only with short stroboscopic flashes of light. Indeed, with strobe rearing, the regenerating retinotectal system of the goldfish measured with subsequent retrograde labeling of axons with wheat germ agglutinin conjugated to horseradish peroxidase, or by field recordings did not fully refine (Cook and Rankin, 1986; Eisele and Schmidt, 1988).

## 1.3 The isthmotectal system

Some of the questions that were difficult to answer in the retinotectal system have been addressed in the isthmotectal system owing to the enormous capability of remodeling in the isthmotectal system when the eye is surgically rotated. The developing tadpole RGCs from the eye extend axons to the contralateral optic tectum and establish topographically organized connections with tectal cells. Each tectum receives a purely monocular input from the contralateral retina. However, much later in development, as the eyes begin to migrate to more frontal positions in the head during metamorphosis, binocular visual information does indirectly converge in the tectum as axons from the nucleus isthmi (NI), which receives direct topographic visual inputs from its ipsilateral tectal lobe, project across the midline to the contralateral tectum (Figure 1.2). This so-called isthmotectal projection carries information to the optic tectum representing the ipsilateral eye by way of the opposite tectum. It has been previously shown that the growth and targeting of axons from the nucleus isthmi are influenced by the activity of the RGCs (Udin and Grant, 1999).

Influential pioneering work by Prof. R.M. Gaze and later by Prof. Susan Udin characterized the normal development of the NI and the isthmotectal projection (Grant and Keating, 1989a). Importantly they demonstrated that surgical rotation of one eye during a critical period in early postmetamorphic frogs results in a dramatic activity-dependent remapping of the isthmotectal axons so that they remap their projection based not on matching retinal position, but instead on matching visual fields (Grant and Keating, 1989b; Guo and Udin, 2000). Interestingly, this remodelling underlies a critical period that is indefinitely prolonged when animals are reared in darkness from early stages on (Grant and Keating, 1992). While the axonal arbours first fail to develop normally, showing diffuse axonal growth, they refine as soon as they are exposed to visual stimuli, matching their axonal growth to the visual map of the rotated eye. These experiments nicely illustrate the tremendous degree to which a neuronal circuit can remodel in response to activity-based cues.

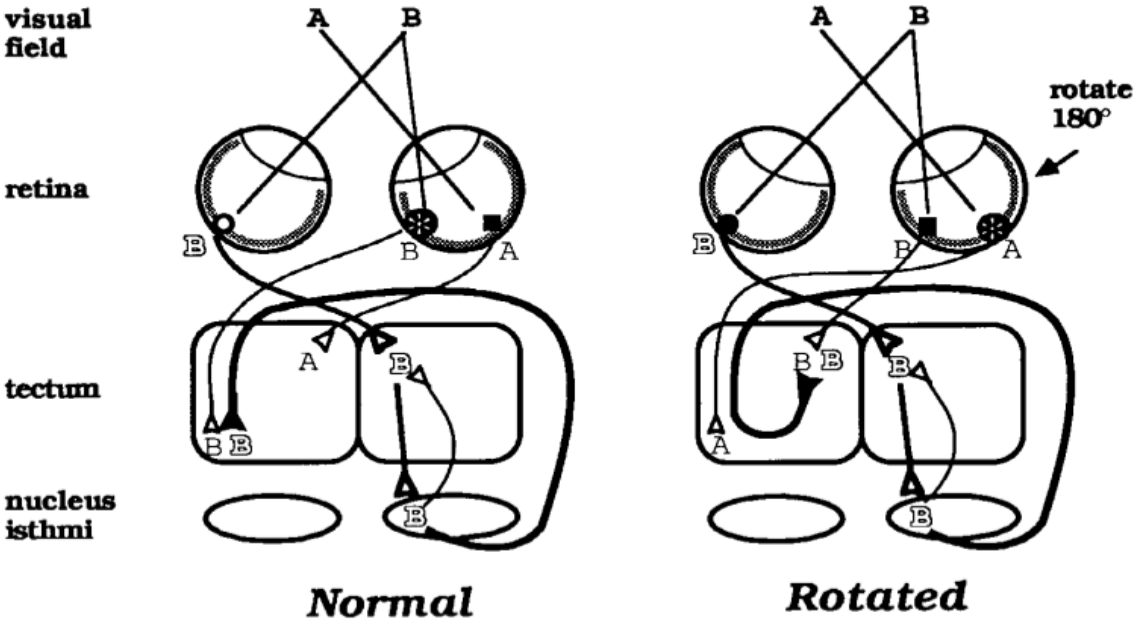


**Figure 1.2 - Wiring diagram of retinotectal and isthmotectal projections:** Neurons from the eye (RGCs) project to the tectum, where they synapse with cells projecting to the NI. Cells from the NI project to the contralateral tectum. Following an eye rotation (*right)*, the isthmotectal projection contralateral to the rotated eye realigns itself to reflect the altered mapping of visual receptive fields in the tectum. *From Udin & Grant, 1999.*

## 1.4 Superior collicular development in mammals

The mammalian equivalent to the amphibian optic tectum is the superior colliculus (SC). Unless otherwise mentioned, I will use the mouse retinocollicular system to explain the circuit and the experimental findings, because many studies were performed in mice due to the availability of genetic tools. Like the optic tectum the SC is also innervated by RGCs from the eye. In contrast to the optic tectum of the frog, the SC receives direct inputs from both eyes (Petros et al., 2008). This is referred to as eye-specific maps, and differs in mammals depending on the amount of visual overlap. In the mouse 3 to 5 % of the RGC axons run ipsilaterally. Over the first and second postnatal weeks in the mouse, RGC axons from the two eyes, sort out and refine to a final topographic map (Triplett, 2014). Ipsilaterally projecting axons retract from the caudal and superficial parts of the superior colliculus and end up in the deep layers of the rostral SC (Figure 1.3) (Assali et al., 2014). The absence of an apparent effect due to injection of TTX into the eye seemed to suggest that activity does not play a role in eye-specificity of the SC in the Syrian hamster (Thompson and Holt, 1989). However, a more recent study forced synchronized activity before RGCs receive visual input, by driving cells with optogenetic tools (Zhang et al., 2012). Here, synchronizing the activity of the two eyes led to an invasion of ipsilateral projecting RGCs into the contralateral eye regions of the SC. Moreover, Zhang et al., 2012 showed that differences in RGC firing between the two eyes of about 100 ms represent the threshold for the circuit to treat inputs as synchronized. This is much longer than the 5–20 ms spike-timing-dependent-plasticity time window observed at retinotectal synapses in frogs (Mu and Poo, 2006). However, theses differences likely represent differences in the experimental design and the statistical power.

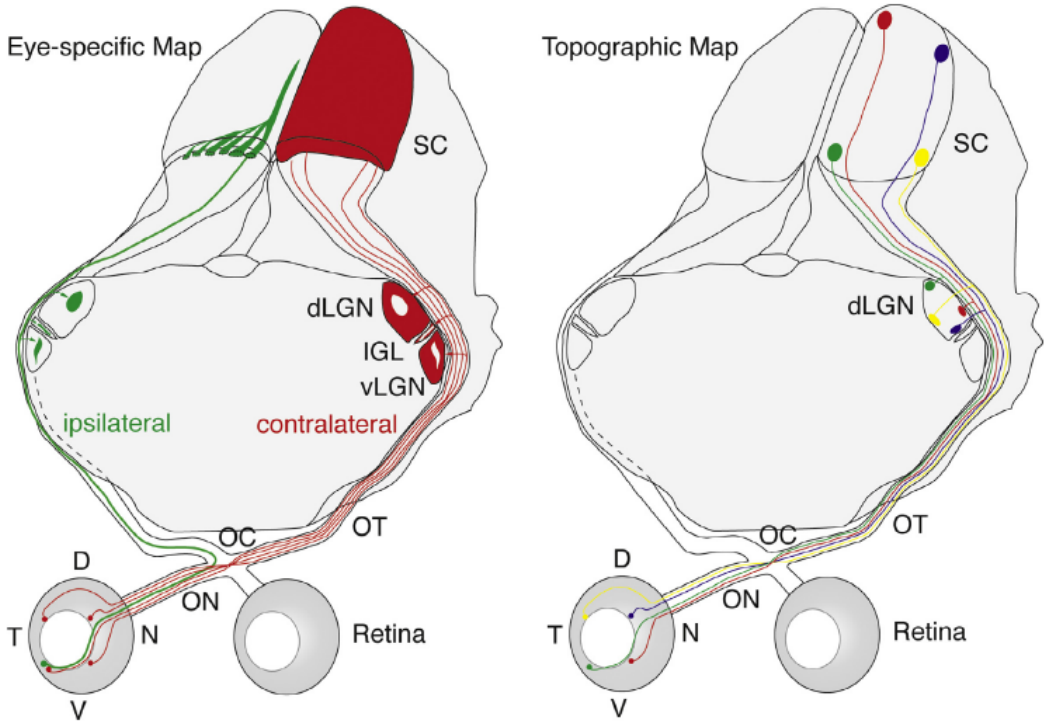


**Figure 1.3:** The retinal projections from the eye to the brain. Eye specific and topographic maps in the mouse visual system. ON: optic nerve, OC: optic chiasm, OT: optic tract, vLGN: ventral lateral geniculate nucleus, dLGN: dorsal lateral geniculate nucleus, IGL: intergeniculate leaflet; SC: superior colliculus, D: dorsal, N:nasal, V:ventral, T:temporal. Assali et al., 2014

How do RGC axons change their growth when their activity becomes synchronous or asynchronous with that of their neighbors? This question was answered recently in a study that we performed in the frog optic tectum (Munz et al., 2014). We found that axons increase their branch motility when they are asynchronously activated compared to their surrounding axons and decrease their branch motility when they fire synchronously with surrounding axons. This change in their growth behavior happens surprisingly fast. We showed that axons that go from inactive to asynchronously active, can increase their branch motility within 20 min. Axons experiencing synchronous stimulation are stabilized within 30 min. While in the frog, vision drives activity-dependent structural changes, in mouse the eyes only open about 2 weeks after birth. How can activity drive structural changes in the mouse visual system before eye opening then? The answer is that spontaneous activity generated in the eyes substitutes for vision and provides the information needed to create the visual circuit.

## 1. 5 Spontaneous calcium waves in the visual system drive axonal refinement

Before the onset of vision, RGCs generate action potentials in waves throughout the retina such that RGCs that are next to each other are likely to fire at the same time and RGCs that are far apart fire at different time points (Figure 1.4) (Meister et al., 1991). These so-called retinal waves have been shown with calcium imaging, multielectrode array, and paired patch-clamp recordings (Torborg and Feller, 2005), and have been shown in different species (Ackman and Crair, 2014) and different neuronal circuits (Blankenship and Feller, 2010). Furthermore, making retinal waves larger, faster, and

more frequent in one of the eyes by applying cAMP increases the size of that eye's layer within the lateral geniculate nucleus (LGN) (Stellwagen and Shatz, 2002). These experiments show that retinal waves instruct circuit formation in the visual system. On the contrary, Huberman et al. have described the function of retinal waves differently. The depletion of starburst amacrine cells using immunotoxin leaves the overall amount of activity intact but disrupts retinal waves (Huberman et al., 2003). Using this approach the authors found left and right eye inputs segregated normally in the dorsal LGN (dLGN) and only blocking all spontaneous activity was sufficient to block eye specific segregation in the dLGN. This latter work thus implied that retinal ganglion cell-bursting activity may be necessary for circuit refinement, but not patterned retinal waves.

To further understand the role of retinal waves during visual circuit development and address the above-mentioned contradictory experiments, a knock-out model was generated in which the β2 subunit of the nicotinic acetylcholine receptor (β2-nAChR) is ablated. Initial reports showed that β2 knockout mice ($\beta2^{-/-}$) completely lack retinal waves (Bansal et al., 2000). Subsequently, it was shown that these mice have altered neuronal circuits of the SC (Chandrasekaran et al., 2005; McLaughlin et al., 2003a; Mrsic-Flogel et al., 2005; Rossi et al., 2001), the dLGN (Grubb and Thompson, 2004) and the visual cortex (Cang et al., 2005). For example, focal injection of DiI into the retina between P1 and P8 reveals a refinement process in the SC in which RGC axons are first diffusely spread throughout the SC but refine to a focal point by P8 (Figure1. 4 C-E). In $\beta2^{-/-}$ mice axons fail to refine to a focal point (Figure 1.4 F-H).

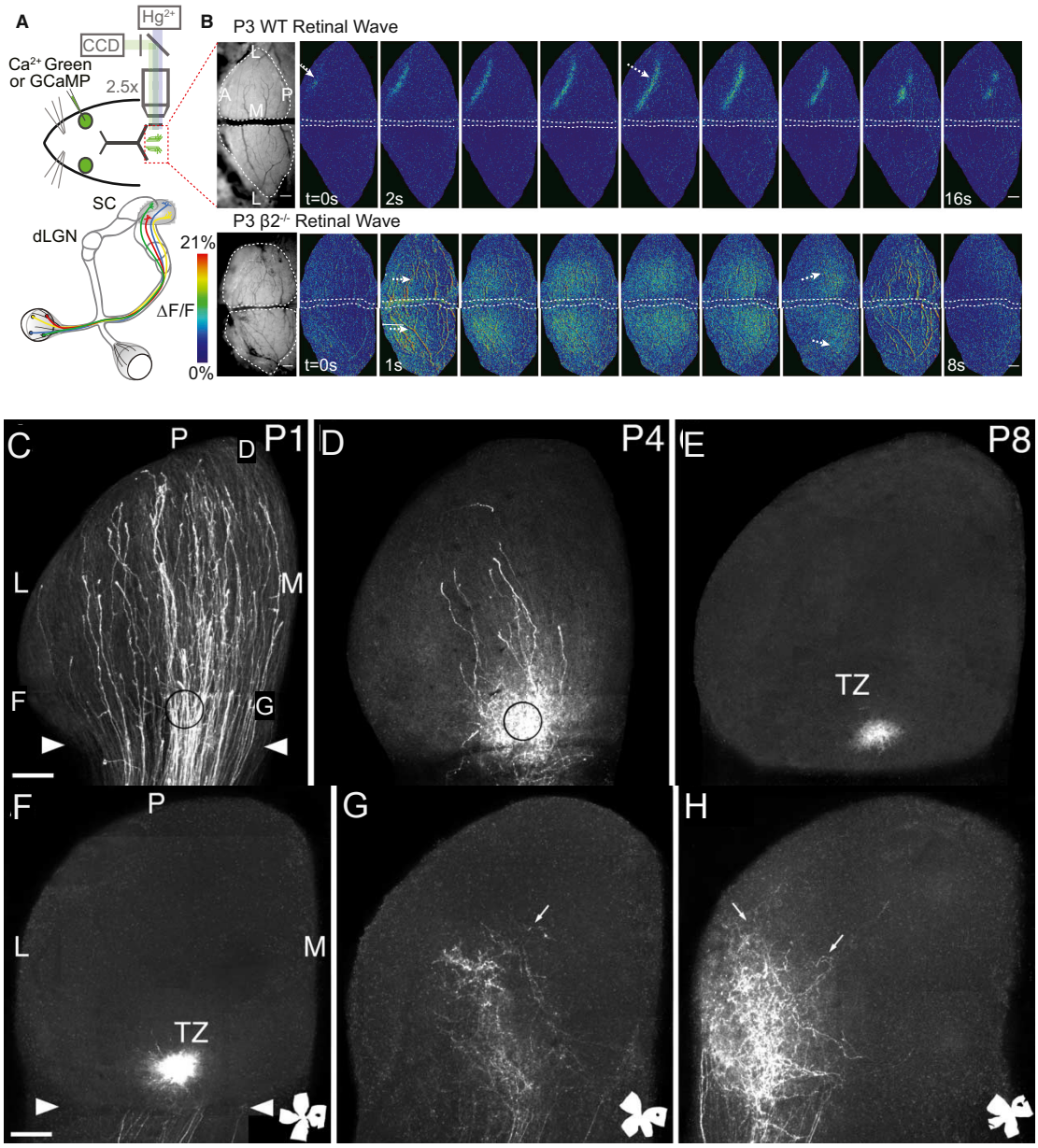


Figure 1.4: A Imaging of retinal waves in SC. B retinal waves in wild type (top row) and in β2-/- mice (bottom row) C-E DiI injection in the retina shows normal developmental refinement of RGC axons in the SC between P1 and 8. F wild type mouse injected with DiI at P7 in temporal retina. G&H β2-/- mice injected with DiI at P8 in temporal retina. Burbridge et al., 2014 (A&B) and McLaughlin et al., 2003 (C-H)

Under some experimental conditions, however, $\beta2^{-/-}$ mice showed normal retinal waves (Sun et al., 2008a, 2008b), which led to a debate about retinal waves and if they are important in the formation of the visual circuit (Chalupa, 2009; Feller, 2009). Additionally, $\beta2^{-/-}$ mice have less dendritic spines regardless of retinal waves, because they are full knockout for the β2 receptor (Lozada et al., 2012). These experiments created some doubt about the necessity of retinal waves in the development of the visual circuit.

However, two beautiful *in vivo* studies from the Crair lab resolved many of these issues, debates, and open questions. In the first study, Ackman et al., (2012) record retinal waves by calcium imaging throughout the entire visual system before eye opening. They, show that retinal waves are generated in the eyes and that retinal waves generate matched activity patterns in the midbrain and cortex. Interestingly, retinal waves are biased to originate in the ventral-temporal retina and propagate towards the dorsal-nasal retina, generating SC waves directionally biased along the rostral-caudal axis, something that had not been anticipated from previous *in vitro* studies. In their second study, Burbridge et al., (2014) examined retinal waves in $\beta2^{-/-}$ mice and in three novel conditional β2-nAChRs mutant mice *in vivo* before eye opening. They showed that conventional $\beta2^{-/-}$ mice have retinal waves that are more spatiotemporally diffuse and travel slower. Mice that have β2 nAChRs deleted only from the retina show defects in retinal waves and circuit morphology that are very similar to those seen in full $\beta2^{-/-}$ mice. However, mice that have β2- nAChRs deleted from the SC neurons have no impairments in retinal waves and circuit morphology. Mice that have β2- nAChRs deleted only from the temporal and nasal extremes of the eye, but leave expression intact along the central dorsal-ventral

axis, show retinal waves that propagate in the dorsal-ventral region but show no retinal waves in the nasal or temporal parts of the retina. Correspondingly, the caudal and rostral parts of the SC – the regions of the SC in which retinal waves are disrupted – show defects in axonal morphology. Finally, when retinal waves were partially rescued in $\beta2^{-/-}$ mice by the application of CPT-cAMP (Stellwagen and Shatz, 2002), the morphological circuit defects could be partially rescued as well. Taken together, these experiments show a strong correlation between retinal waves and circuit formation. Interestingly, $\beta2^{-/-}$ mice show waves in the SC that are independent from the remaining retinal waves seen in these mice. This indicates that retinal waves drive proper circuit refinement only if retinal waves drive SC activity reliably. SC waves by themselves might even enhance the morphological circuit alterations of the RGC axons detected in B2-/- mice.

In light of these studies, how can we explain single cell morphology in RGC axons reconstructed from the SC in $\beta2^{-/-}$ mice? Dhande et al., (2011a) show that RGC axons in $\beta2^{-/-}$ mice are more diffuse, larger and are elongated particularly along the rostral-caudal axis. This phenotype argues for a Hebbian mechanism in which axons grow larger in an attempt to find an appropriate postsynaptic partner, and because of asynchronous activity from the eye, axons fail to refine to a focal area. To understand this kind of axon morphology one would have to investigate the growth of single axonal arbors in $\beta2^{-/-}$ and wild type mice. In the *Xenopus laevis* imaging of single axons shows that neuronal activity of one axon that is out of synchrony with its surrounding axons leads to an increased growth that results in a massively increased arbor size (Munz et al., 2014). This increased growth behavior could explain the morphology of RGC axons seen

in the SC of the $\beta2^{-/-}$ mice. Finally, it is possible that persistent postsynaptic activity of the SC leads to the strong morphological changes of the RGC axons. The mechanism underlying these morphological changes could be plasticity of various forms, such as long-term potentiation (LTP) and depression (LTD) which in turn are influenced by metaplasticity (Bear, 2003) and homeostatic plasticity (Pozo and Goda, 2010).

## 1.6 Mechanisms underlying axonal morphology

*Long-term potentiation (LTP) and depression (LTD)*

LTP and LTD are changes in synaptic receptor composition that make a synapse more or less efficient, respectively. LTP is a stable increase in synaptic strength (Bliss and Lømo, 1973). In the last 4 decades it was shown that the most common change underlying LTP is an increase in the number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) at the synapse (Chater and Goda, 2014). In short, activation of the postsynaptic cell shortly after the activation of the presynaptic terminal leads to an opening of postsynaptic N-methyl-D-aspartate receptors (NMDARs). Calcium influx through the NMDAR leads to a cascade of phosphorylation events that move additional AMPARs to the postsynaptic surface and trap them at the postsynaptic density. LTD is a process by which synaptic strength is weakened. Classical hippocampal LTD involves NMDAR activation as well (Dudek and Bear, 1992) ultimately leading to the removal of AMPAR from the postsynaptic surface. LTP and LTD might gradually shape the functional and structural organization of the visual system. However, the full complement of receptors and signaling pathways involved in detecting correlation between pre- and

postsynaptic cells, and how pre- and postsynaptic mechanisms are orchestrated, are not yet fully understood.

*Plasticity can impact structural changes in the visual system*

Cline et al. (1987) showed that the NMDAR blocker (2R)-amino-5-phosphonopentanoate (AP5) can abolish the formation of ocular dominance bands and can desegregate existing ocular dominance bands in the optic tectum of three-eyed frogs. These experiments showed an involvement of NMDAR in neuronal circuit development and raised questions about changes at a single cell level. In experiments in which animals were exposed to APV and single axons were followed over a 2 h period, dynamic axon arbour rearrangements were measured by counting new and lost branches between time points. Axonal arbour branching was increased in the presence of APV (Rajan et al., 1999). In sharp contrast, dendrites showed decreased branching upon APV administration into the rearing media. A similar axon phenotype was observed when zebrafish larvae were exposed to either APV or MK801, both of which block NMDAR (Schmidt et al., 2000).

These experiments had significant impact on understanding the role of NMDARs in axonal and dendritic development. First, neuronal circuit development of the optic tectum is not entirely hardwired into the brain but is instructed by neuronal activity. Second, both axons and dendrites are susceptible to changes in neuronal activity. Third, NMDARs appear to play an important role in the detection of correlation in neuronal firing.

Besides providing many insights into neuronal circuit development, the above-

mentioned experiments produced many new questions as well. For example, after 24 hours of APV treatment, axonal dynamics returns to baseline suggesting that some homeostatic or compensatory mechanism is induced after the initial increase in dynamics (Rajan et al., 1999). Also, these experiments demonstrate that the presynaptic cell receives an unknown retrograde signal from the postsynaptic cell (Rajan et al., 1999; Ruthazer et al., 2003). In support of this hypothesis postsynaptic expression of a constitutively active CaMKII leads to confined axonal arbour growth (Zou and Cline, 1996). Several molecules have been suggested, including neurotrophins, cell adhesion molecules, and nitric oxide (Cogen and Cohen-Cory, 2000; Regehr et al., 2009). Many of these molecules are also implicated in functional synaptic plasticity.

Taken together, this suggests a model in which, correlated retinal inputs activate NMDA receptors in the postsynaptic tectal cells, which leads to $Ca^{2+}$ influx through NMDA receptors. This might result in the local activation of CaMKII (Zou and Cline, 1996, 1999) followed by LTP which leads to the stabilization of these synapses and the dendritic branches in which they are located. Synapse stabilization on the postsynaptic side, might concomitantly participate in stabilizing presynaptic retinal axonal branches, making the axon less motile. Moreover, it decreases branching of the axon. On the other hand, retinal inputs that fail to drive postsynaptic currents do not activate NMDA receptors. CaMKII activity is not activated at these synapses, and fails to strengthen the connections between pre- and post-synaptic cells. Therefore, RGC axons and tectal cell dendrites continue grow and search for more suitable partners to form mature synapses. This search phase might be regulated by the amount of activity in the circuit.

*Competitive mechanisms of structural plasticity*

Over-expression of a dominant negative form of VAMP2/Synaptobrevin-2 (dnVAMP2) decreases vesicular release and results in a decreased axonal arbour size (Hua et al., 2005). On the other hand, the expression of tetanus toxin light chain (TeNT), which cleaves VAMP2 and abolishes vesicular release completely, leads to an increased arbour size. It is not clear why these to studies show different effects. It is possible that decreased, but not abolished, synaptic release due to the over expression of the dnVAMP2 would decrease the synaptic weights, leading to a decrease in axonal arbour size. On the other hand, axonal boutons where vesicular release was completely abolished by TeNT would not be able to stabilize and might grow more to find different synaptic partners (Ben Fredj et al., 2010). In both studies a competitive mechanism is likely, because, when the animals are exposed to either TTX (in the case of dnVAMP2) or to Mk801 (in case of TeNT) all neurons in the circuit become equally incapable of driving the postsynaptic neuron. By manipulating the circuit to make all neuron equally competitive the effect is lost and axons do not grow smaller or larger, respectively.

## 1.7 The importance of circuit formation for the animal

Finally, does a failure in refinement affect visual acuity? (Smear et al., 2007) used the zebrafish mutant *blumenkohl* (blu) in which a vesicular glutamate transporter, closely related to mammalian VGLUT2, is mutated. These fish show an increased RGC axonal arbour size and RGC synapses fatigue with high frequency stimulation. These phenotypes are accompanied by enlarged receptive fields of the tectal neurons and decreased spatial resolution leading to an impaired pray capture behavior. Thus, the *blu* mutants clearly

demonstrate that impairment in synaptic transmission leading to an increased axonal arbour size can impact visual acuity. In Xenopus laevis tadpoles, visual training can make tectal neurons more susceptible to both LTP and LTD (Schwartz et al., 2011). The same conditioning stimulus enhances visual spatial frequency sensitivity in conditioned tadpoles several hours after conditioning. Thus, these experiments show that visual conditioning primes LTP and LTD, which contributes to improved visual acuity measure, behaviourally. This, susceptibility is dependent on BDNF as an antisense morpholino oligonucleotide against *bdnf* blocks the metaplasticity induced by conditioning.

## Author Contributions

Chapter 2 has been published as: Munz, M., Gobert, D., Schohl, A., Poquérusse, J., Podgorski, K., Spratt, P., and Ruthazer, E.S. (2014). Rapid Hebbian axonal remodeling mediated by visual stimulation. *Science 344*, 904–909.

Together with my supervisor Edward Ruthazer, I designed all the imaging experiments. Edward Ruthazer, Delphine Gobert and I designed the electrophysiology experiments.

Figure 1: I performed experiments for figure 1 a to c. Figure 1d to j and S2 was performed in equal parts by Anne Schohl, Delphine Gobert and by me. Delphine Gobert performed electrophysiological recordings.

I performed experiments for figure 2, 3, 4 and S1. In figure 2 I had help from Jessie Poquérusse an undergraduate student in the lab.

Edward Ruthazer and I wrote the manuscript and Delphine Gobert helped writing the parts of the manuscript that concerned electrophysiology experiments.

## Preface to chapter 2: Rapid Hebbian Axonal Remodeling Mediated by Visual Stimulation

At the beginning of my PhD many of experiments that had been performed to look at how correlation impacts circuit formation silenced the cells of interest, or at least did not

systematically keep the overall amount of firing constant. We realized that misguided, ipsilateral projecting RGC axons could be used in experiments in which we changed correlation but kept the overall amount of activity constant. This makes misguided, ipilateral projecting RGC axons a perfect model system in which to test for Hebbian mechanisms. The *Xenopus laevis* retinotectal system gives us the advantage to look at a single axon and to follow this axon with high temporal resolution over a long period of time. Experiments examining postfixation morphology of optogenetically stimulated retinal axons in the mouse retinofugal pathway have supported our results (Zhang et al., 2012).

# Chapter 2: Rapid Hebbian Axonal Remodelling Mediated by Visual Stimulation

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## One Sentence Summary:

In vivo retinotectal imaging and whole cell recordings paired with patterned visual stimulus presentation confirm Hebb's prediction that coactivated retinotectal inputs are stabilized both functionally and structurally, and further extend this model by revealing that asynchronously driven axons exhibit rapid synaptic depression and enhanced exploratory branching.

Abstract: To understand how correlated firing controls axon remodelling at a mechanistic level, we performed in vivo time-lapse imaging and electrophysiological analysis of individual retinal ganglion cell (RGC) axons, visually stimulated either synchronously or asynchronously relative to neighbouring inputs in the Xenopus optic tectum. RGCs stimulated out of synchrony with neighbouring inputs rapidly lost the ability to drive their tectal postsynaptic partners, and their axons increased the dynamic elaboration of new branches. In contrast, synchronously activated RGCs produced much fewer new branches but these were more stable, exhibiting longer lifetimes. The effects of synchronous activation were prevented by expression of tetanus neurotoxin light chain (TeNT-Lc) to inhibit neurotransmitter release as well as by N-methyl-D-aspartate receptor (NMDAR) blockade, consistent with a role for homosynaptic NMDAR activation in the production of retrograde signals that stabilize axonal branches and suppress further exploratory branch addition.

## Main Text:

Neuronal activity and molecular cues cooperate to form precise neuronal circuits (Cang and Feldheim, 2013; Ruthazer and Cline, 2004). Experimental blockade of action potential firing or synaptic transmission (Ben Fredj et al., 2010; Hua et al., 2005; Sretavan et al., 1988b), particularly involving N-methyl-D-aspartate receptors (NMDARs)(Bear et al., 1990; Cline and Constantine-Paton, 1989; Rajan et al., 1999; Simon et al., 1992), degrades topographic and functional maps in the developing nervous system. The precise pattern of neuronal firing is thought to be important for instructing the refinement of connectivity, as disrupting the temporal correlation of firing between neighbouring neurons, while sparing overall activity levels, results in axons with diffuse terminal arbours (Dhande et al., 2011b; Zhang et al., 2012).

Hebbian plasticity, an appealing model for activity-dependent refinement of neuronal circuits, posits that synapses may be strengthened or stabilized when the presynaptic cell participates in causing its postsynaptic partner to fire (Hebb, 1949). Many convergent inputs firing synchronously would cooperatively excite the postsynaptic neuron to fire. Thus, in principle, Hebbian plasticity would tend to aggregate coactive inputs at the same postsynaptic target, and in this way contribute effectively to circuit refinement (Cline and Constantine-Paton, 1989; Cline et al., 1987). However, because most of the evidence supporting Hebbian axonal remodelling comes from postmortem studies, the detailed mechanisms by which such remodelling might occur remains poorly understood.

The developing retinotectal system of the translucent albino *Xenopus laevis* tadpole is amenable both to live imaging and in vivo electrophysiological characterization. Retinal ganglion cell (RGC) axons in *Xenopus* tadpoles normally project to the contralateral optic

tectum. However, occasionally a single mistargeted ipsilaterally-projecting RGC axon can be observed (**Fig. 2.1A,B**)(Sakaguchi and Murphey, 1985a). Using postmortem intraocular DiI injections to bulk label all RGCs (**Fig. 2.S1**) in stage 46-48 tadpoles when the retinotectal projection is established but still refining, we detected no ipsilateral RGC axon in the majority of cases (61%), but occasionally animals with just one (21%) or two (9%) ipsilaterally projecting axons were observed (**Fig. 2.1C**). These results suggest that ipsilaterally projecting axons are unlikely to represent a unique class of RGCs but rather reflect random pathfinding errors at the optic chiasm. We reasoned that animals with sparse ipsilaterally projecting RGCs might constitute an ideal model in which to test the role of correlated activity on axonal refinement, as it should be possible to independently stimulate the lone ipsilateral and surrounding contralateral RGCs through the two eyes.

We first set out to determine whether ipsilaterally projecting RGCs are indeed capable of forming functional synapses onto tectal neurons. To allow us to find potential postsynaptic partners of a single ipsilateral RGC (**Fig. 2.1D**), we made F0 transgenic tadpoles expressing photoactivatable green fluorescent protein (PA-GFP) under control of the neuronal beta-tubulin promoter and then electroporated plasmid encoding tdTomato into one eye, which labeled an ipsilaterally projecting axon in a subset of animals. Scanning 780nm light in a volume immediately surrounding the ipsilateral axon (**Fig. 2.1E**) resulted in photoactivated PA-GFP backfilling a small number of tectal cells from their dendrites (**Fig. 2.1F**). In vivo perforated patch clamp recordings of compound synaptic currents from these cells, made while presenting alternating light flashes to each eye through optical fibers (**Fig. 2.1D**), revealed that all visually responsive neurons could be driven through the contralateral eye (**Fig. 2.1G**). Most cells were unresponsive to

flashes presented to the ipsilateral eye, but some cells backfilled with PA-GFP also responded to stimulation of the ipsilateral eye (**Fig. 2.1H**).

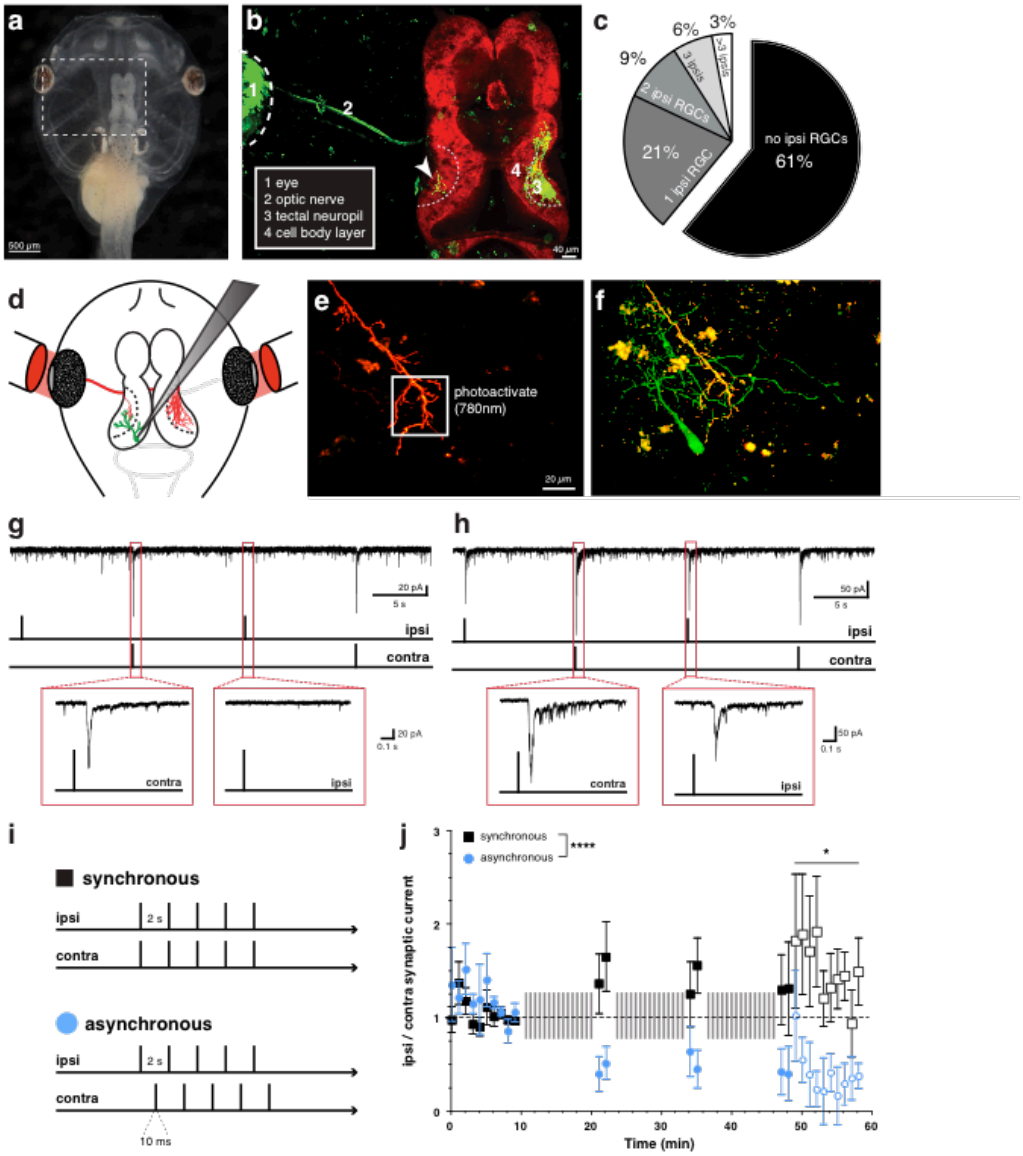

**Figure 2.1: Sporadic ipsilateral RGC axons can integrate into the retinotectal circuit**

(A) Albino *Xenopus laevis* tadpole. (B) Tadpole retinotectal projection visualized by retinal electroporation of EGFP plasmid. Brain (red) stained with BODIPY vital dye. Most RGC axons project to contralateral side. *Arrowhead*: single RGC ipsilaterally projecting axon. (C) Frequencies of tadpoles with ipsilaterally projecting RGC axons labeled by intravitreal DiI injection. (D) *In vivo* electrophysiology schematic showing targeted recordings with optical fibers to stimulate each eye. (E) Ipsilateral RGC axon (tdTomato-labeled) showing site to be targeted with 780nm photoactivation. (F) Photoactivation back-labels potential postsynaptic tectal neurons from dendrites adjacent to the ipsilateral RGC axon. (G, H) Examples of visually evoked synaptic currents in tectal cells that are (G) non-responsive or (H) responsive to light flashes in the ipsilateral eye. (I) Visual stimulation protocols used to induce synchronous or asynchronous activation of the ipsilateral RGC relative to the predominant contralateral inputs. A 10 ms flash was presented every 2 s to each eye, either in or out of phase. (J) Ratio of ipsilateral to contralateral eye evoked currents is depressed by 10 min of asynchronous visual stimulation (closed symbols, ****$P < 0.0001$, interaction by two-way ANOVA mixed design, n = 12 sync, 10 async) and this is subsequently maintained > 10 min (open symbols, *$P = 0.027$, main effect by two-way ANOVA mixed design, n = 7 sync, 5 async).

We next tested the Hebbian prediction that correlation in the firing of inputs would modify their synaptic strengths. Ten minutes of baseline light-evoked compound synaptic currents were measured by alternating a 10 ms light flash to each eye every 30 sec, holding the cells at -60 mV. Cells were then switched to current clamp mode to allow spiking, and one of two training paradigms was applied: **synchronous stimulation** in which both eyes were stimulated together every 2 sec, or **asynchronous stimulation** in which the eyes were stimulated 1 sec apart (**Fig. 2.1I**). Importantly, in all cases both eyes experienced light flashes every 2 sec, with only the relative timing between the two eyes differing across paradigms. After 10 min of training stimulation, light-evoked compound synaptic currents were again measured at -60mV to test the relative efficacy of the two eyes at driving the tectal neuron. This cycle of training and testing was repeated up to three times per cell, however it was already apparent after the first 10 min of training that asynchronous stimulation resulted in a dramatic loss of synaptic strength for the ipsilateral eye, whereas synchronous stimulation maintained the relative contribution of the ipsilateral eye at or above baseline levels (**Fig 2.1J, 2.S2**). These changes in synaptic efficacy stably persisted in cells held at -60 mV for at least 12 min after training stimulation (**Fig 2.1J, *open symbols***). Thus, simply presenting visual stimuli that reduce the degree of temporal correlation of one sensory input with respect to its neighbours, without otherwise altering stimulus intensity or frequency, is sufficient to profoundly weaken its synaptic strength.

We next sought to determine if correlated activity also regulates the growth and morphology of developing RGC axons. Following retinal electroporation of EGFP plasmid, we selected animals with single EGFP-labeled ipsilaterally or contralaterally

projecting axons. Using a video display directly beneath their rearing tanks, free-swimming tadpoles were continuously presented with either full-field stroboscopic flashes (0.5 Hz) to synchronize both eyes or large moving black dots to independently activate the two eyes (**Fig. 2.2A**). Dot-rearing leads to asynchronous stimulation of the ipsilateral RGC axon but synchronous activation of contralateral RGC axons relative to other contralateral eye inputs. RGC axon arbours in the optic tectum were imaged *in vivo* by 2-photon laser scanning microscopy once daily for 5 days (**Fig. 2.2B**). All three groups reared under synchronizing conditions exhibited comparable daily axon growth and branch elaboration (**Fig. 2.2C,D**). By comparison, dot-reared ipsilateral RGC axons, which experienced asynchronous stimulation relative to neighbouring inputs, grew much faster, with significantly larger and more diffuse arbours by the second day of stimulation. Together with the electrophysiology experiments, these results suggest that axonal inputs that are not coactive with neighbouring inputs are rapidly weakened by these sensory inputs and end up elaborating much broader arbours.

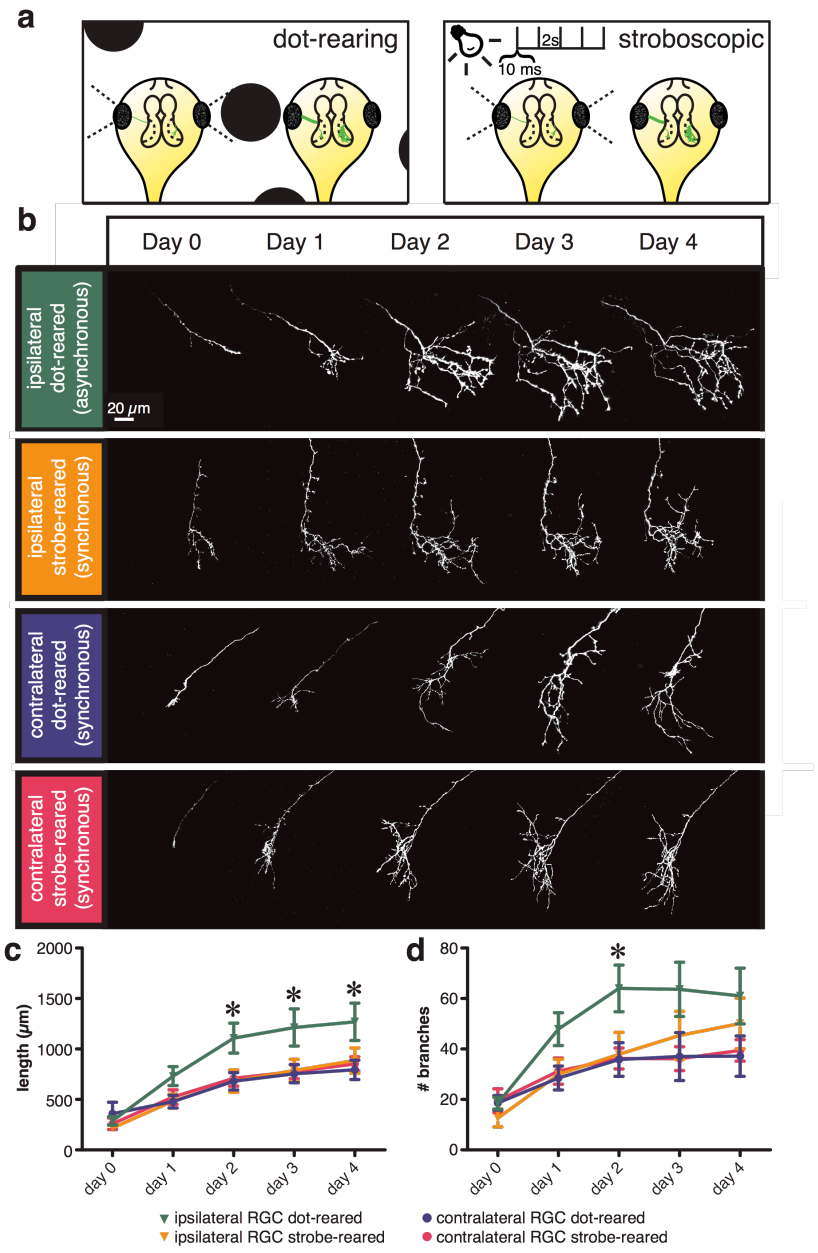
a
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**Figure 2.2: Rearing tadpoles with asynchronous binocular stimulation enlarges ipsilateral RGC axon arbours**. (A) Animals were reared for 4 days over a video monitor presenting visual stimuli to synchronously (stroboscopic) or asynchronously (dot-rearing) activate their two eyes. (B) Example ipsilaterally and contralaterally projecting RGC axons from animals reared under each experimental condition, imaged once daily. (C) Total arbour size over 4 days was dramatically larger for ipsilateral eye axons reared with moving dots to drive asynchronous activation relative to the contralateral inputs (D) Increased axon branching was also observed for this group (*$P < 0.05$, two-way ANOVA mixed design, Tukey's post-test, n = 8 ipsi strobe, 8 ipsi dots, 6 contra strobe, 12 contra dots)

To investigate the time course of the cellular events by which correlated activity regulates axonal growth and branching at higher temporal resolution we collected 2-photon time-lapse images of individual ipsilateral axon arbours in unanesthetized, agarose-immobilized tadpoles every 10 min for 5.5 h while simultaneously presenting stimuli to the eyes through a pair of optical fibers (**Fig. 2.3A-C**). One of two stimulation protocols was applied during imaging (**Fig. 2.3B**). In the first protocol (**D**ark-**A**sync-**S**ync), baseline images in darkness were collected for 90 min, after which each eye was stimulated at 0.5 Hz with a 5 ms light flash. **Asynchronous stimulation** ($180^{o}$ out of phase) was presented for the first 2 h followed by **synchronous stimulation** for the last 2 h. The second protocol (**D**ark-**S**ync-**A**sync) was identical except that the 2 h **asynchronous stimulation** followed the **synchronous stimulation**.

Applying the DAS protocol we observed that asynchronous visual stimulation produced a rapid and robust increase in axon branch dynamics compared with darkness (**Fig. 2.3D**). Within 20 min of asynchronous visual stimulation, the rate of new branch additions significantly increased (**Fig. 2.3E,F**). Branch tips were also eliminated more rapidly during asynchronous stimulation (**Fig 2.3G,H**), consistent with an overall increase in dynamic remodelling. Furthermore, asynchronous stimulation also significantly augmented the elongation lengths of branch tips compared to the growth rate in darkness (**Fig 2.3I,J**).

Remarkably, changing from asynchronous to synchronous stimulation, without altering stimulation intensity or frequency, significantly decreased the numbers of branches added (**Fig. 2.3E,F**) and lost (**Fig. 2.3G,H**), in addition to reducing branch elongation (**Fig. 2.3I,J**). The decrease in newly added branch tips was more gradual,

only reaching significance by 40 min after the onset of synchronized stimulation. Consistent with these observations, animals presented first with synchronous stimulation immediately following darkness using the DSA protocol, did not show a significant increase in branch additions (**Fig. 2.3E,F**) or elongation (**Fig. 2.3I,J**) until asynchronous stimulation was presented.

These data reveal that asynchronous visual stimulation makes axons grow and add more branches, whereas synchronous stimulation appears to suppress this increase. Although synchronized stimulation did elicit an increase in the rate of elimination of existing branches compared with darkness (**Fig 2.3H**), we found that new branches formed during synchronous stimulation were in fact more stable. Branches added during synchronous stimulation had longer lifetimes, with significantly more surviving longer than 30 min than those formed during asynchronous stimulation, using either the DAS (**Fig. 2.3K**) or DSA protocols (**Fig. 2.3L**). Thus, our live imaging data unequivocally support Hebb's postulate that correlated firing should stabilize connections, but also shed light on an unpredicted novel mechanism for the regulation of axonal branching and growth by correlated firing. In this model, activation of an RGC causes it to rapidly extend highly dynamic exploratory branches when its firing is mismatched to that of surrounding inputs, but to suppress this probing and consolidate stable contacts when its activity is well correlated with its neighbours.

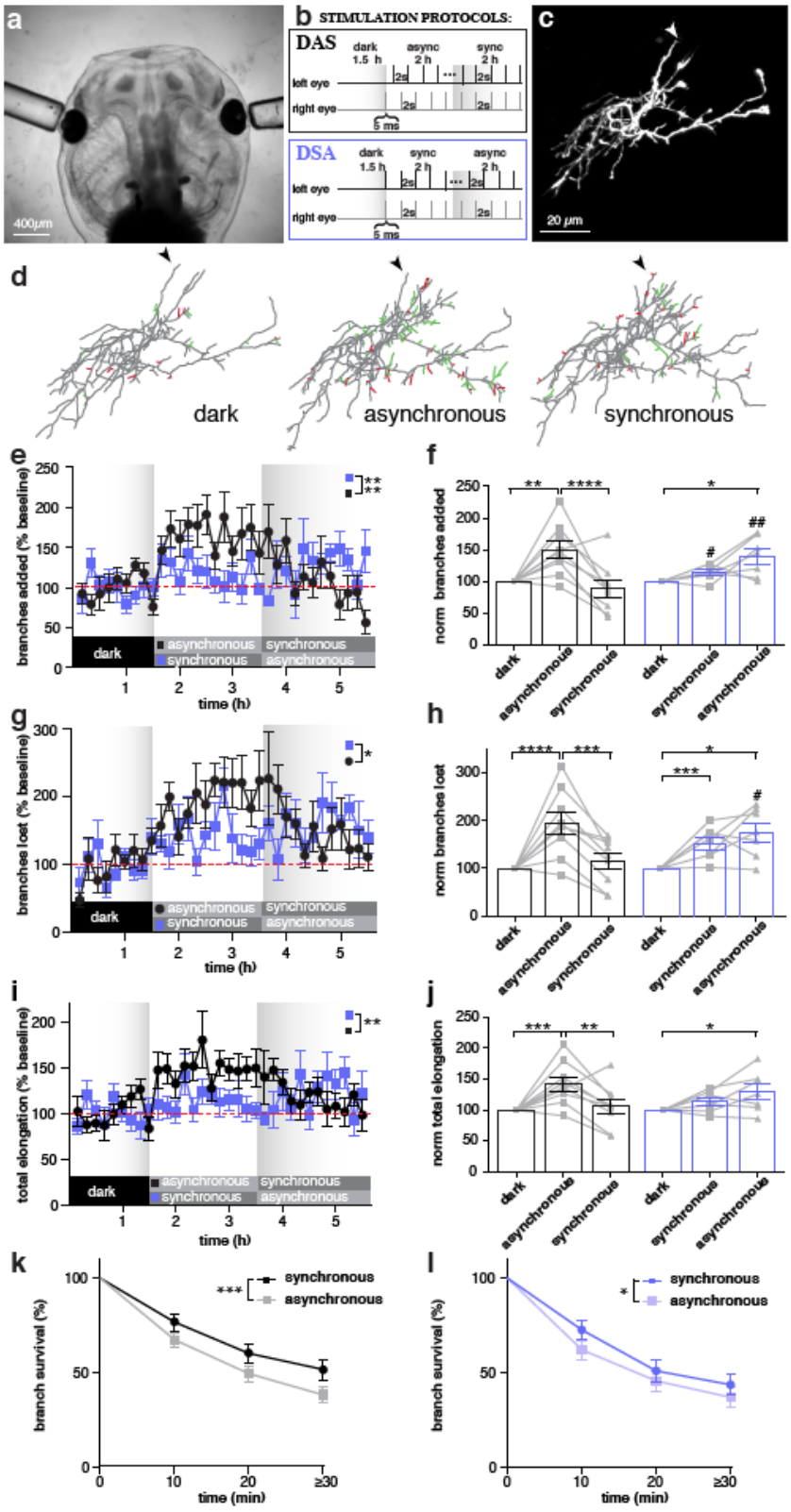
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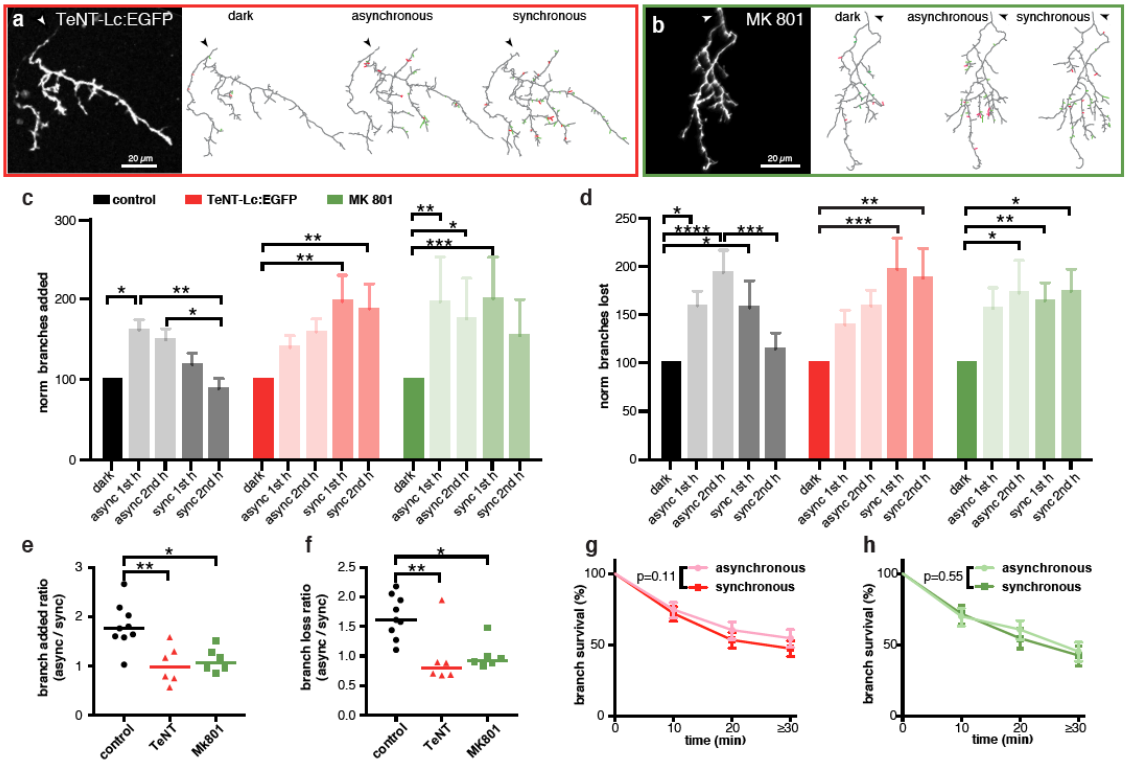
**Figure 2.3: Ipsilateral RGC axon growth and branch dynamics are differentially modulated by asynchronous and synchronous stimulation** (A) Tadpoles were immobilized in agarose for live imaging with optical fibers placed in front of the eyes. (B) One of two visual simulation protocols were used: Dark-Async-Sync consisted of 1.5 h darkness, 2 h asynchronous, followed by 2 h synchronous stimuli at 0.5Hz; and Dark-Sync-Async in which the order of stimulation was exchanged. (C) 2-photon z-projection of RGC axonal arbour in the optic tectum. Arrowhead marks axon stem. (D) Example reconstructed arbours show added (green) and lost (red) branches for individual time points during darkness, asynchronous or synchronous stimulation. Branches are most dynamic during asynchronous stimulation. (E) Numbers of branches added every 10 min normalized to baseline rate in darkness and (F) mean branch addition rates (10 min-1) for each stimulation condition. (G-J) Corresponding data for (G, H) branch elimination rates and (I, J) branch elongation lengths. DAS protocol data in black (n = 9) and DSA in blue (n = 7). (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way ANOVA for interaction in E, G, I, with Tukey's post-test comparing stimuli in F, H, J. #P < 0.05, ##P < 0.01, Sidak post-test comparing protocols.) (K, L) Branch survival fraction shows more stable branches (≥ 30 min) formed under synchronous than asynchronous stimulation, with either the (K) DAS or (L) DSA stimulation protocols. (K: n = 551 async and 326 sync branches from 9 cells. L: n = 321 async and 229 sync branches from 7 cells. *P < 0.05, *** P < 0.001 by log-rank test. Error bars indicate SEM (E-J) and 95% CI (K, L).

These findings support the idea that axonal growth may be controlled by retrograde cues from surrounding cells (Changeux and Danchin, 1976) and suggest several alternative mechanisms. One option is that RGCs upregulate dynamic growth in response to increased firing but correlated firing of the postsynaptic cell leads to branch-suppressing or stabilizing retrograde signals that inhibit this growth. Another possibility is that the asynchronous input may be specifically detected by postsynaptic neurons prompting them to deliver growth-promoting signals to the dissenting axon terminal. These two putative mechanisms predict opposite outcomes if retrograde signaling were blocked, with increased branching under the first model and reduced branching under the second.

Based on the assumption that the timing of synaptic activity determines the release of a retrograde signal from the postsynaptic cell, we tested the effects of blocking synaptic transmission, while still leaving presynaptic firing intact, by expressing tetanus neurotoxin light chain fused to EGFP (TeNT-Lc:EGFP) in ipsilaterally projecting RGC axons (**Fig. 2.4A**). TeNT-Lc:EGFP expression blocks glutamate release specifically in the axon being imaged (Ben Fredj et al., 2010). In addition, as synaptic NMDARs have been proposed to act as correlation detectors due to their voltage-dependent response to glutamate (Bear et al., 1990) and have previously been implicated in activity-dependent retinotectal map refinement (Cline et al., 1987; Rajan et al., 1999), we also tested the effects of general NMDAR blockade by treating animals with the blood-brain barrier permeant non-competitive NMDAR antagonist MK-801 (**Fig. 2.4B**). Because synchronized stimulation requires about 1 h to achieve its full effect on branch addition (**Fig. 2.3E**) and loss (**Fig. 2.3G**) rates, we separately analyzed dynamics during the first

and second hours of each stimulation period (**Fig. 2.4C,D**). Control ipsilateral axons exhibit a significant increase in dynamic branch additions (**Fig. 2.4C**) and losses (**Fig. 2.4D**) during the first hour of asynchronous stimulation, which returns to baseline levels by the second hour of synchronous stimulation. In contrast, both TeNT-Lc:EGFP-expressing and MK-801 treated axons actually showed their largest increases in branch additions and losses during synchronous stimulation. To further ascertain the relative responses of axons to asynchronous versus synchronous stimulation on a cell by cell basis, we divided the mean rate of branch addition or loss during the last 90 min of asynchronous stimulation by that during the last 90 min of synchronous stimulation to generate branch addition and branch loss ratios for each cell (**Fig. 2.4E,F**). Axonal expression of TeNT-Lc:EGFP and blockade of NMDARs both dramatically reduced these ratios to around 1, indicating that the axons no longer responded differentially to synchronous and asynchronous stimulation. We also examined stability of branches formed during asynchronous and synchronous stimulation from TeNT-Lc:EGFP-expressing (**Fig. 2.4G**) and MK-801-treated (**Fig. 2.4H**) ipsilateral RGC axons and found that branch survival times do not significantly differ between stimulation conditions, in clear contrast to control axons which form longer lasting branches under conditions of synchronous stimulation (**Fig 2.3K,L**). Taken together these findings suggest that NMDAR-mediated synaptic transmission leads to increased branch stability and a reduction in branch dynamics during synchronous activation through the action of a retrograde branch-suppressing signal.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asynchronous
synchronous
branch survival (%)
time (min)

**Figure 2.4: Homosynaptic neurotransmission and NMDARs mediate arbour stabilization during synchronous stimulation.** (A,B) Cells (A) expressing TeNT-Lc:EGFP or (B) bathed in MK-801, with example reconstructions under different stimulation conditions, as in Fig 3. (C,D) Transmitter release and NMDAR activation are both necessary to reduce branch dynamics during synchronous stimulation. (C) Branch addition rates for each h of stimulation normalized to darkness, for control (n = 9), TeNT-Lc:EGFP-expressing (n=6) and MK-801 treated (n=6) ipsilateral axons. (D) Corresponding data for branches lost. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way ANOVA mixed design with Tukey's post-test). (E) Ratios of numbers of branches added for each cell during asynchronous versus synchronous stimulation. (F) Ratios for branches lost. (*P < 0.05, **P < 0.01, Kruskal-Wallis test with Dunn's post-test) (G,H) Survival plots for branches formed during asynchronous and synchronous stimulation in (G) TeNT-Lc:EGFP-expressing or (H) MK-801-treated EGFP-expressing cells. Unlike controls (Fig. 3K,L), synchronous stimulation did not significantly enhance branch stability in these cells. (n.s., Log-rank test. n = 242 async and 300 sync branches from 6 TeNT-Lc:EGFP cells, n = 206 async and 191 sync branches from 6 MK-801-treated cells.). Error bars indicate SEM (C,D) and 95% CI (G,H).

These experiments present direct evidence in support of a mechanistic model for how correlated neural activity helps orchestrate the morphological remodelling of developing axons into precisely organized maps. Our data reveal that sensory stimulation promotes rapid exploratory branching and outgrowth of RGC axons within their target structure. Axons that may have extended into inappropriate territory where their firing patterns do not match those of nearby inputs would fail to maintain stable functional and structural contacts, and continue actively elaborating in search of appropriate partners. By contrast, axons that form synaptic contacts onto partners that receive other inputs from highly coactive axons, indicating their somata are likely to be proximate neighbours in the eye (Demas et al., 2012; McLaughlin et al., 2003a), will engage cooperative mechanisms to stabilize those contacts and the branches on which they reside. Our experiments blocking vesicular release and NMDARs confirm that synaptic activation of NMDARs, possibly serving as correlation detectors, is a critical first step in initiating a retrograde stabilizing signal, although the molecular identity of this signal is not yet known. Furthermore, as a single axon firing out of synchrony with numerous other synchronized inputs does not appear to benefit from the stabilization signals that these many coactive axons presumably receive, it seems that the retrograde signal must be very precisely spatially or temporally restricted, ruling out long-lived, diffusible molecules as plausible candidates.

Our observation that visual stimulation drives a rapid increase in branching and growth is consistent with earlier studies in the retinotectal projections of zebrafish and mouse in which suppression of RGC firing by expression of inward-rectifying potassium channels inhibited the dense elaboration of branches (Benjumeda et al., 2013; Hua et al.,

2005). Similarly, the enlarged arbours reported in zebrafish RGCs expressing TeNT-Lc match our findings that this treatment prevents the downregulation of branch formation during correlated activity (Fredj et al., 2010). These authors argued for a model based on activity-dependent competition between axons to explain their data, however our results suggest that correlation detection may offer an important alternative explanation for these findings. Activity-dependent competition is a useful model to explain pathological conditions like cortical amblyopia, but likely plays a much smaller role in normal retinotectal map development. A very similar correlated stimulation approach to the one we adopted was recently reported using transgenic mice expressing channelrhodopsin-2 in RGCs for stimulation during early postnatal development. Although it was not feasible to observe axonal remodelling in real time in that system, the consequences of synchronous and asynchronous stimulation were highly consistent with our findings, with synchronous stimulation leading to ectopic stabilization of ipsilateral eye projections in contralateral eye territory (Zhang et al., 2012).

Our findings represent an evolution of the classic view of Hebbian plasticity, which proposed that an input that participates in making its partner fire is strengthened or grows. The experiments presented here constitute the first real-time observations of Hebbian structural plasticity of axonal projections in the intact animal and provide a clear confirmation of the prediction that correlated inputs become stabilized (Ruthazer et al., 2003), however our use of rapid imaging has allowed additional fundamental mechanisms to be revealed. Most notably, we see a robust activity-dependent upregulation of exploratory growth, leading to a dramatic expansion of the axon arbour over days, under conditions where correlated firing is absent. Importantly this

exploratory growth is suppressed when the axon is coactive with other inputs. The rapidity with which physiological visual stimuli can drive such changes, greatly reducing the strength of synaptic currents evoked through the ipsilateral eye after just 10 min of asynchronous visual stimulation and significantly increasing the rate of new branch addition in under 20 min, was also unanticipated. The so far elusive goal of identifying the specific retrograde signals that mediate correlation-dependent structural plasticity will be greatly facilitated by exploiting the novel experimental protocol presented here.

**Methods Summary**

A detailed description of the methods is provided in Supplementary Information.

**Acknowledgements:**

We would like to thank Kasper Podgorski in the lab of Dr. Kurt Haas (UBC) for assistance with his MATLAB analysis tool Dynamo for dynamic morphometric analysis. We also thank Dr. Martin Meyer for providing us with the tetanus toxin light chain plasmid (5UAS-TeNT-EGFP) and David Freiheit for photography of tadpoles. We are grateful to Dr. Stephen Glasgow for advice on the statistical analysis and to Drs. Hollis Cline and Wayne Sossin for useful comments on our manuscript. This work was supported by grants from the Canadian Institutes for Health Research (CIHR) and the Natural Sciences and Engineering Research Council (NSERC) of Canada to ESR and by fellowships from the Deutscher Akademischer Austausch Dienst to MM, from the Fonds de la recherche en santé du Québec to DG, and from NSERC to PS.

The authors declare that they have no conflicts of interest.

**Author Contributions**

MM: Conceived of and performed all imaging experiments and data analysis. Participated in writing manuscript.

DG: Performed all electrophysiology experiments. Participated in writing manuscript.

AS: Generated transgenic animals.

JP: Assisted in daily imaging experiments and axon reconstructions.

PS: Performed reconstructions of axons in short-interval experiments, blind to experimental design.

ESR: Provided support and guidance. Participated in writing manuscript.

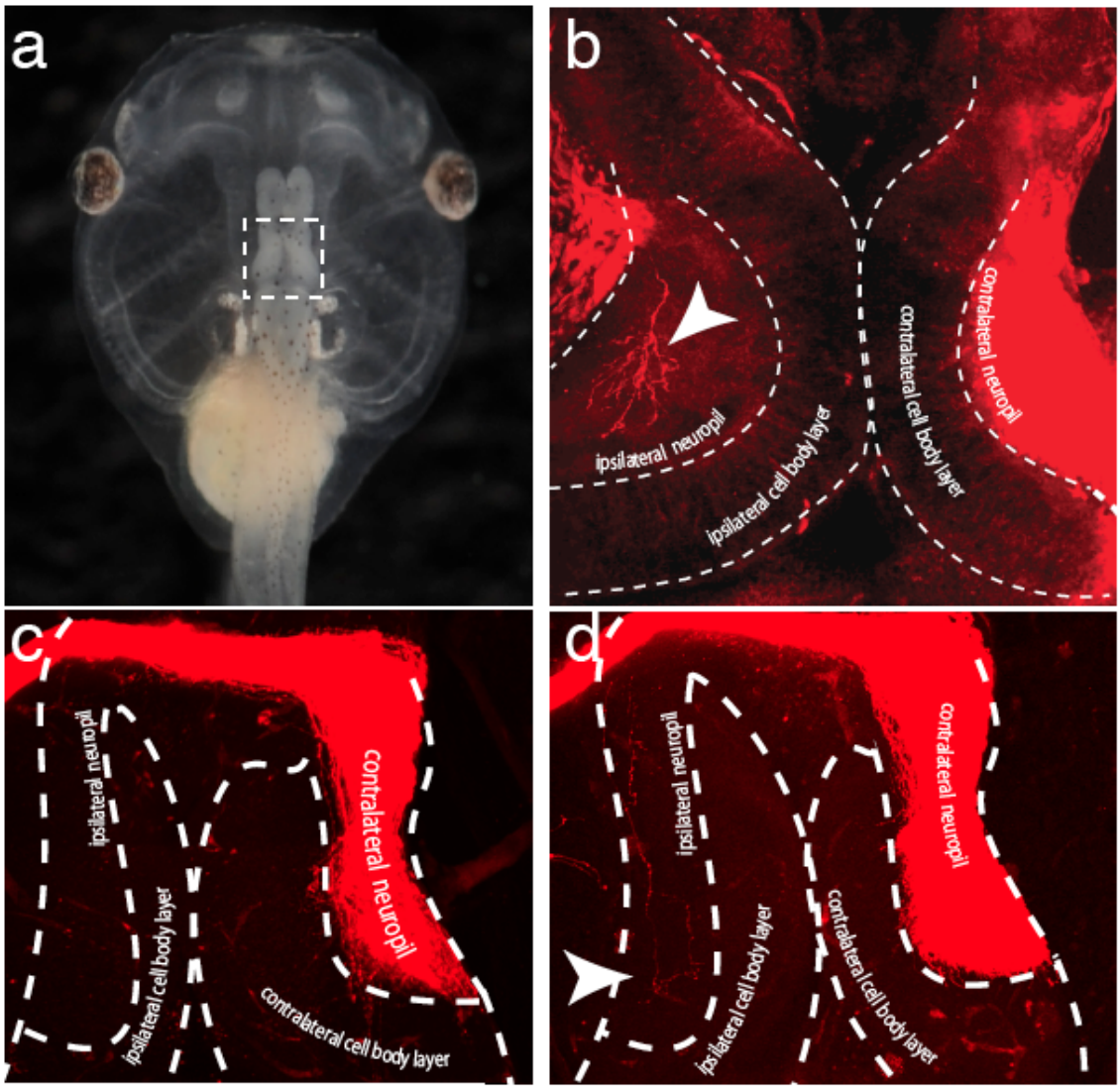
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**Fig. 2.S1. DiI labeling reveals ipsilaterally projecting RGC axons in a subset of *Xenopus laevis* tadpoles. (**A) *Xenopus laevis* tadpole. White box indicates the optic tectum. (B) Optic tectum of a paraformaldehyde-fixed tadpole in which RGC axons of the left eye were bulk labeled by intraocular DiI injection. Arrowhead indicates ipsilateral RGC axon. (C,D) Fixed and fructose-cleared optic tectum in which RGC axons of the left eye were bulk labeled with DiI. (C) Example of an optic tectum that contains only contralaterally projecting RGC axons. (D) Example of an optic tectum with a single ipsilaterally projecting RGC axon (indicated by arrowhead).

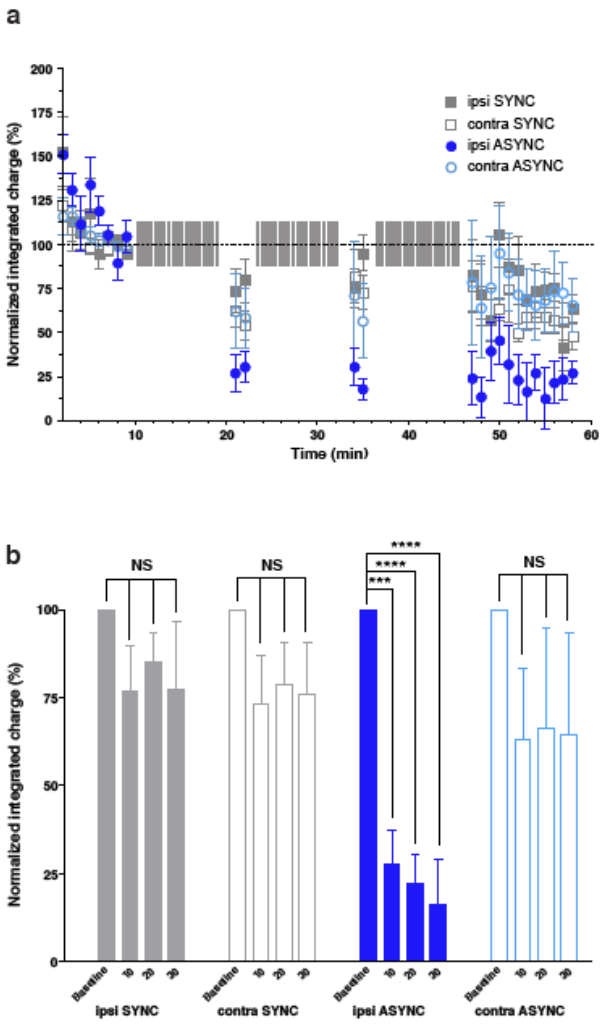
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**Fig. 2.S2. Ipsilaterally driven compound synaptic currents are depressed by asynchronous visual stimulation.** (A) Compound synaptic current test responses to 10 ms flashes presented to each eye at various times during the plasticity protocol, normalized to last 3 min of baseline. To induce plastic changes, three 10 min rounds of either asynchronous or synchronous stimulation were presented while cells were allowed to spike in current clamp mode (n = 8 for ipsi and contra ASYNC; n = 10 for ipsi and contra SYNC). (B) Normalized total integrated charge of compound synaptic currents for ipsilateral eye inputs was significantly reduced following asynchronous stimulation (blue bars; two-way ANOVA mixed design, followed by Dunnett's multiple comparison test; $P<0.001$ at 10 min, $P<0.0001$ at 20 and 30 min, as compared to baseline). In contrast, there was no significant reduction of the contralateral input following asynchronous stimulation (blue open bars) or of any inputs after synchronous stimulation (ipsi: gray bars, contra: grey open bars; two-way ANOVA mixed design, followed by Dunnett's multiple comparison test) (C) Ratios of ipsilateral to contralateral eye responses are significantly depressed by 10 min of asynchronous visual stimulation (two-way ANOVA mixed design; Interaction $P = 0.057$ and main effect $P < 0.05$).

## Materials and Methods:

*Animals*

All experiments were approved by the MNI Animal Care Committee in accordance with Canadian Council on Animal Care guidelines.

Female albino *Xenopus laevis* frogs from our in-house breeding colony were first primed by injection of 50 IU pregnant mare serum gonadotropin (PMSG). After 3 days, human chorionic gonadotropin (HCG) was injected into the dorsal lymph sacs of a male (150 IU) and the primed female (400IU) and the pair was placed together in an isolated tank for mating. Eggs were collected the next day and kept in standard Modified Barth's Saline-H (MBSH).

*I-SceI transgenesis*

Transgenic animals used for perforated patch recordings were obtained by *in vitro* fertilization. PMSG-primed female frogs that had been injected with HCG (400IU) the prior evening were squeezed to obtain eggs. For *in vitro* fertilization, a male frog was deeply anesthetized in 0.2% MS-222 in MBSH and testes were removed. A piece of testis was triturated in 1x MBSH solution and applied to the eggs, to which enough 0.1x MBSH to cover all the eggs, was added. After 5 min the dish was filled with 0.1x MBSH and the eggs were incubated for another 5 min. Fertilized eggs were then dejellied in 2% cysteine for 5 min and finally rinsed in 0.1x MBSH to remove the cysteine.

About 120 embryos at the 1-cell stage were injected with I-SceI treated vector DNA (20 pg / injection). Injections were completed within 25 min after fertilization. Animals were kept in 2% Ficoll/ 1x MBSH for 2-3h at 16C and then transferred to 1% Ficoll/ 0.1x

MBSH and kept at 16C overnight (animals were kept in petri dishes coated with 1% Agarose/ 0.1x MBSH). The next day, animals were transferred to 0.1x MBSH and kept at 21C until ready for electroporation (3 days). We followed the I-SceI transgenesis protocol of Ishibashi et al., 2012 (1).

Transgenesis vectors were constructed using the pTransgenesis system (2) (http://www.port.ac.uk/research/exrc/ptransgenesis/). The following vector plasmids were used: p1: γ-crystallin GFP, p2: NBT, p3: PA-GFP, p4: I-SceI SAR-CH4 Tol2 recombined with LR-Clonase II Plus. The p3 PA-GFP insert plasmid was constructed by replacing VenusGFP with PA-GFP. In brief, VenusGFP was cut out from p3 using BamHI/ SnaB1; PA-GFP was cut out with NotI/ blunted/ BamHI from the parental vector mito-PAGFP (Addgene 23348). In some cases Gal4-VP16-UAS-PA-GFP was used as the p3 insert for enhanced brightness. This was constructed from Gal4-VP16-UAS that had been PCR-amplified from α-act:Gal4-VP16-UAS-GFP (a generous gift from Holly Cline) using the following primers:

Gal4VP16UAS_SspI: 5'-ACGGAATATTCGCCCATGAAGCTACTGTCTTCTATCG-3'

Gal4VP16UAS_AgeI: 5'-ACGGACCGGTGCAAGCTCCTTGAATTTCGAGG-3'.

*DiI labeling*

Stage 45 to 47 tadpoles were anesthetized in MS-222 (0.02% in 0.1× MBSH) and fixed in 4% paraformaldehyde in 0.1M phosphate buffer. For each animal, one eye was injected with 0.2% DiI dissolved in absolute ethanol. We waited at least one week before screening animals for ipsilateral retinal ganglion cell (RGC) axons. In some cases, brains

were cleared using SeeDB to permit visualization of the entire optic tract down to the chiasm (3).

*Electroporation*

As shown in Fig. 1, only a subset of animals has ipsilaterally projecting RGC axons. EGFP or td-Tomato plasmid electroporations were performed as previously described (4). To increase the chances of finding an animal with an ipsilateral axon, we increased the duration of current pulses and used plasmid concentrations of up to 2 μg/μL. For the electroporation we used the following settings: 37 V, 3 ms, 2 pulses at reverse polarity 1 s apart. To obtain animals with single contralaterally projecting axons we used the following electroporation settings: 37 V, 1.6 ms, 2 pulses at reverse polarity 1 s apart. Axons with fewer than 10 branches were excluded.

*Electrophysiology*

I-SceI transgenic tadpoles expressing GFP in the lens under the control of the γ-crystallin promoter, used as a selection marker, were chosen for electroporation with tdTomato as described above. Two days after electroporation tadpoles (stage 45-47) were screened for the presence of ipsilaterally projecting RGC axons, anesthetized by immersion in 0.02% MS-222 in MBSH and placed in a Sylgard chamber under the two-photon microscope. A scanning volume was drawn immediately surrounding the ipsilateral RGC axon (visualized at 910nm) and PAGFP in the dendrites of tectal neurons was photoactivated by scanning at 780nm. This back-labeled one or more cell bodies which were further highlighted by photoactivating at their somata for easier subsequent visualization.

Animals were then paralyzed by intraperitoneal injection of d-tubocurarine hydrochloride pentahydrate (2.5mM) and kept at room temperature in an external solution that contained (in mM): 115 NaCl, 4 KCl, 5 HEPES, 10 glucose, 3 CaCl2 and 3 MgCl2, pH: 7.3, Osm: 250. In vivo perforated-patch recordings were performed as previously described (5). Briefly, the animal was held submerged in a custom-shaped Sylgard chamber using insect pins. The brain and overlying skin were then filleted along the midline and a broken patch pipette was used to carefully cut the ventricular surface of the optic tectum to expose the PA-GFP positive neuronal cell bodies. After perfusion with external solution to remove any remaining MS-222, cells were visualized for patching with a 60x 0.8NA water-immersion objective mounted on an upright microscope with a CCD camera. A BFL48-400 optical fiber was placed in front of each eye for presenting visual stimuli. Light flashes were generated with red luxeon LEDs controlled with a Master-8 stimulus generator.

Light-evoked compound synaptic currents (CSCs) were recorded using 9-12 MOhm borosilicate patch pipettes filled with an internal solution that contained (in mM); 100 K-gluconate, 8 KCl, 5 NaCl, 1.5 MgCl2, 20 HEPES, 10 EGTA, 2 ATP, 0.3 GTP, pH: 7.3, Osm: 250, supplemented with 240 mg/mL amphotericin B dissolved in DMSO and 0.4% Alexa Fluor 488 hydrazide to confirm the patch did not convert to whole-cell recording. Cells were voltage-clamped at -60mV to record CSCs, however, the recordings were switched to current clamp during presentation of visual stimuli to allow cells to freely spike (6).

Recordings were obtained using a MultiClamp 700B amplifier, digitized at 10 kHz and filtered at 2 kHz for offline analysis using pClamp10 software or Axograph X (John Clements).

*In vivo imaging*

Daily imaging: Two-photon imaging of RGC axons was performed using a confocal microscope custom-converted for multiphoton imaging with a 60x water immersion objective (1.1 NA). Excitation light was provided by a Ti:sapphire fs pulsed laser. Optical section z-series were acquired on a PC using Fluoview software (version 5.0). Stage 43 to 45 albino *Xenopus laevis* tadpoles were electroporated as described above to express EGFP. For this experiment, we chose animals that expressed EGFP in either 1 or 2 well-separated axons. We selected axons 48-72 h after electroporation that had grown into the tectum within the past 24 h. Animals were anesthetized by immersion in 0.02% MS-222 in 0.1x MBSH and immobilized in a Sylgard chamber carved to fit the tadpole's body and sealed with a cover glass. Image acquisition required less than 10 min, after which tadpoles were returned to 0.1x MBSH solution and recovered from anesthesia within several minutes. For continuous visual stimulation of freely moving tadpoles, animals were kept isolated in 6-well plates in a temperature-controlled environment while visually stimulated using a S243HL LCD monitor placed underneath the 6-well plates. The animals were imaged every day for 5 days in total. We applied 2 different visual stimulation paradigms: One was designed to desynchronize the 2 eyes by presenting randomly moving dots. The other was designed to synchronize the 2 eyes by delivering 10 ms full-field light flashes at 0.5Hz.

Short-interval imaging: Animals were electroporated with EGFP and selected for single ipsilaterally projecting RGC axons as described above. Animals with axons that had been present within the tectum for 1 to 3 days were selected to most closely match days 1-3 of the daily imaging experiments. Animals were paralyzed by intraperitoneal injection of d-tubocurarine hydrochloride pentahydrate (2.5mM) and mounted onto a cover slip by embedding in a drop of 2% [w/v] UltraPure™ Low Melting Point Agarose. The eyes, lips, tail and gills were freed of agarose to assure adequate oxygenation. Tadpoles were perfused with oxygenated 0.1× MBSH rearing solution for the duration of the experiment. A BFL48-400 optical fiber was placed in front of each eye for presenting visual stimuli. Light flashes were generated with red luxeon LEDs controlled by a Master-8 stimulus generator. Optical section z-series were collected at 1 μm intervals to capture the full terminal arbor every 10 min. For some experiments, neurotransmission was blocked by expressing tetanus neurotoxin light-chain fused to enhanced green fluorescent protein (TeNT-Lc:EGFP) in RGCs, a generous gift of Dr. Martin Meyer (7). To block NMDAR activity, animals were bathed in 10 μM MK-801 (Dizocilpine maleate) for 1.5 hr prior to imaging and throughout the imaging experiment. Images were collected using a 60x water immersion objective (1.1 NA) on a confocal microscope custom-converted for multiphoton imaging or an 20X (1.0 NA, water immersion) objective mounted on a commercial multiphoton microscope with resonant scanners. Both microscopes use a Ti:sapphire fs pulsed laser as the excitation source.

*Image Analysis*

All 2-photon image stacks were denoised using CANDLE non-local means denoising software implemented in MATLAB (8). For daily imaging data, cells were reconstructed

in 3D from z-series 2-photon stacks using the autodepth feature of Imaris. For short-interval imaging data, image stacks were reconstructed manually in 3D for dynamic morphometric analysis using Dynamo software, implemented in MATLAB (9).

*Statistics*

Statistical analysis was performed in GraphPad Prism 6. For electrophysiology, CSC total integrated charge (pC) was normalized to the last 3 minutes of baseline recordings and ipsi/contra response ratios were compared by two-way ANOVA mixed design followed by Dunnett's post-hoc tests. To study anatomical remodelling, branch additions, losses and elongations were analyzed by a two-way ANOVA mixed design followed by Tukey's post-hoc tests. For branch dynamics measurements, we only analyzed branches that obtained at least 1.5 μm in length at some point during their lifetimes. For branch elongations, we only considered changes greater than 1μm. In Fig. 3 E,G,I, we normalized to the entire baseline dark period. In Fig. 3 F,H,J and Fig. 4 C,D, we compared the last hour of darkness, asynchronous and synchronous stimulation periods and normalized everything to darkness. Branch survival was analyzed by performing a Log-rank (Mantel-Cox) test for branches that were born during stimulation at all time points 30 to 60 min into each stimulation period. In all figures error bars represent SEM, except for the branch survival analyses where they give 95% confidence intervals. Analyses of the branch addition and loss ratios during asynchronous versus synchronous stimulation were performed using a Kruskal-Wallis test.

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## Author Contributions

Together with my supervisor Edward Ruthazer, I designed all the experiments. Experiments were performed by me with the help of Tasnia Rahman, and undergraduate student in the lab.

## Preface to Chapter 3: Heterosynaptic modulation of axonal growth and branching

In Chapter 2 we showed that driving an axon and its surrounding inputs to fire out of synchrony leads to increased rates of branch dynamics and growth. In Chapter 3, we test whether the axon increases its growth cell-autonomously in response to its own firing or in response to the firing of surrounding cells.

# Chapter 3: Heterosynaptic modulation of axonal growth and branching


## Abstract

Neuronal firing instructs axonal morphology and connectivity. This implies that specific aspects of activity may be able to engage distinct downstream molecular mechanisms to drive the wiring of a circuit. However not much is known about the details of the mechanisms that are involved. This is because it has been difficult to manipulate activity in ways specifically that engage only a subset of the relevant molecular mechanisms. Here, we have taken advantage of the rare occurrence of individual retinal ganglion cell axons that project incorrectly to the ipsilateral tectal lobe in developing Xenopus tadpoles. We observed the effects on axon branch dynamics *in vivo* of specifically activating just the single ipsilateral axon or all its surrounding contralateral axons. We found that increased firing of the surrounding axons upregulates growth and branching of the single unactivated axon, while firing of the single axon itself modulates retraction of that axon. We further demonstrate that these mechanisms are developmentally regulated with more complex, highly branched axon arbours being less impacted by activity than simpler, immature axons.


## Introduction

There is a long-standing debate as to how neuronal firing instructs the formation of neuronal circuits (Crair, 1999). If neuronal firing is indeed instructive in this process, neurons would be predicted to change their growth behaviours in response to specific patterns of firing. One appealing model for instructive plasticity, initially proposed by Donald Hebb (Hebb, 1949), has been adapted to explain activity-dependent

developmental processes (Katz and Shatz, 1996; Okawa et al., 2014). In short, under so-called Hebbian plasticity the postsynaptic neuron makes the decision to maintain synaptic inputs based on whether their activity occurs at times such that they could have contributed to making the postsynaptic cell fire. Conversely, inputs might eventually be eliminated if they are inactive when the postsynaptic neuron is firing, or active when the postsynaptic cell is silent. Two recent studies, one in frogs and one in mice, confirm that retinal axons can change their growth behaviour depending on the timing, rather than just the total amount, of their firing (Munz et al., 2014; Zhang et al., 2012).

Retinal ganglion cells (RGCs), in the *Xenopus laevis* retinotectal system cross to the contralateral side of the optic tectum, where they make synapses onto tectal neurons (Ruthazer and Aizenman, 2010; Sakaguchi and Murphey, 1985b). We recently showed that in 39% of tadpoles one or very few RGC axons fails to cross and ends up on the ipsilateral side of the optic tectum (Figs. 2.1C, 3.1A,B). Ipsilateral RGC axons are a useful experimental model with which to address how the relative timing of RGC firing can direct axonal remodelling, because they can be visually stimulated independently from all the other contralateral RGC axons by simply presenting light flashes to each eye.

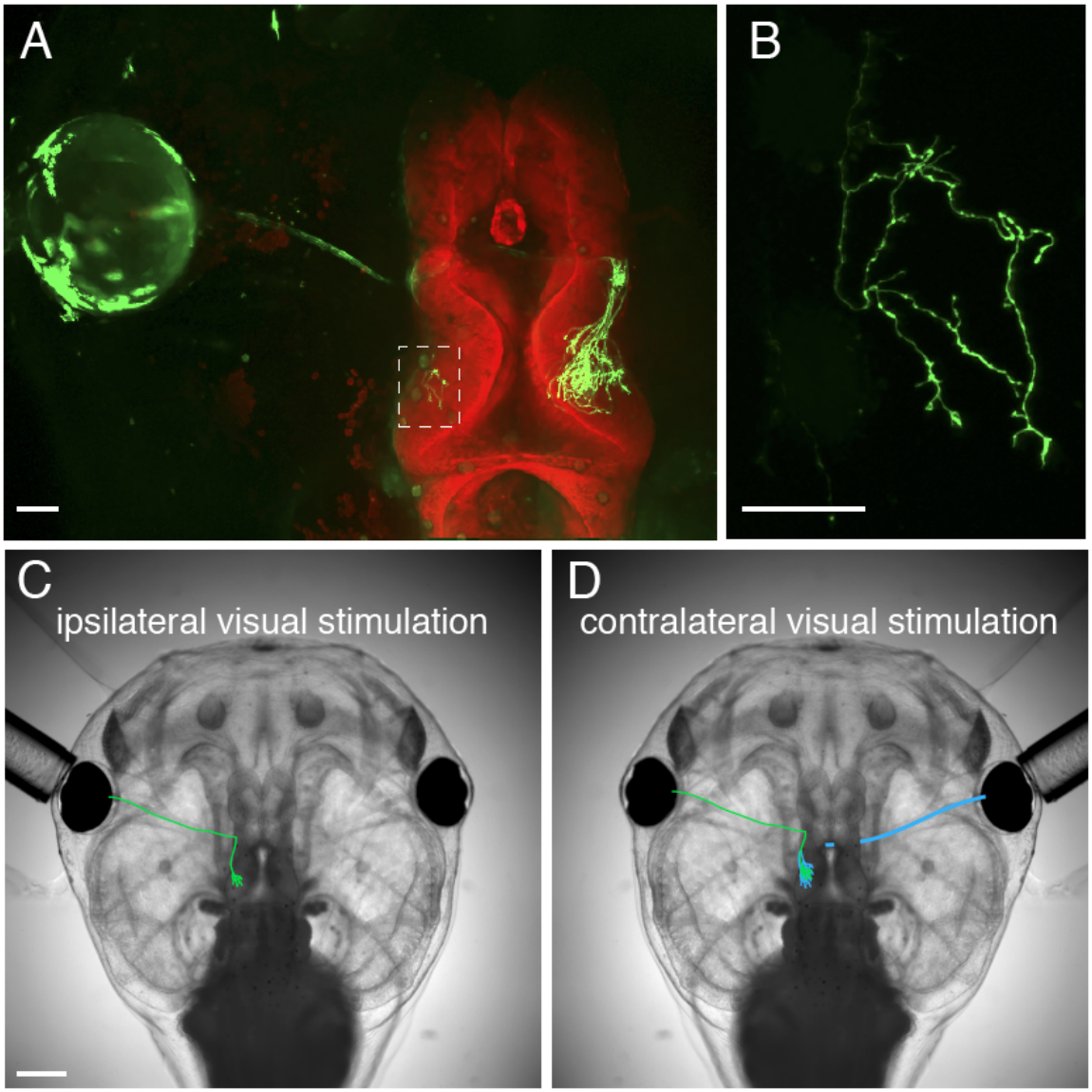



**Figure 3.1 Ipsilateral RGC axons.** A) Retinotectal projection of the *Xenopus laevis* tadpole stage 47. Green: EGFP electroporated into the eye reveal contralateral RGC axons and a single ipsilateral axon. Red: bodipy injection into the ventricle reveals brain structure. B) Ipsilateral RGC axon arbour from (A) C) Ipsilateral visual stimulation with an optical fibre leads to neuronal firing of the single ipsilateral axon D) Contralateral visual stimulation with an optical fibre leads to neuronal firing of all RGC axons in that tecal lobe other than the single ipsilateral axon.

Using this experimental approach combined with time-lapse 2-photon imaging, we found that axons that fire in synchrony with surrounding axons stabilize, while axons that fire out of synchrony with their neighbours destabilize. Overall levels of neuronal activity have been reported to change the patterns of growth as well (Munz et al., 2014; Sin et al., 2002). We further showed that in the case where a neuron and its surrounding inputs are activated out of synchrony with each other it increases its growth, by adding more branches and increasing branch lengths (Munz et al., 2014). This condition can be mimicked, even during synchronous stimulation, if the axon is prevented from activating postsynaptic NMDARs, either by pharmacological blockade or by expression of tetanus-toxin light chain (TeNT-LC:EGFP).

In fact, asynchronous stimulation consists of two components that could each affect axonal growth differently: the cell-autonomous effects on a cell that is firing without its neighbours and the non-cell-autonomous (or heterosynaptic) effects of the other inputs firing. Here we show that firing of the surrounding axons promotes a non-cell-autonomous increase in axonal growth and branching by the silent cell, while the firing of a neuron itself induces retraction. We further show that these phenomena are inversely correlated with arbour complexity. Thus simple axons with few branch tips increase branching, growth and retraction in response to activation of neighbouring inputs, while highly branched axons grow at a constant rate, independent of activity levels.

## Materials and Methods

*Animal breeding*

All animal experiments were approved by the MNI Animal Care Committee and are in agreement with Canadian Council on Animal Care guidelines. Animal breeding is performed in our in-house breeding colony. First, female albino Xenopus laevis frogs were primed by injection of 50 IU pregnant mare serum gonadotropin (PMSG) into the dorsal lymph sacs and kept separately for 3 to 5 days. Then, human chorionic gonadotropin (HCG) was injected into, both the primed females and a males, again into the dorsal lymph sacs (male: 150 IU and the primed female: 400IU. The pair was placed together in an isolated tank for mating. The eggs were collected the next day and the day after, and kept in standard Modified Barth's Saline with HEPES (MBSH). MBSH was replaced frequently to ensure the health of the eggs and tadpoles.

*Electroporation*

As shown in Munz et al., 2014, a subset of animals has an occasional ipsilaterally projecting RGC axon. To label ipsilaterally projecting RGC axons and to ensure that synapse stabilization can not happen we electroporated RGCs to express tetanus neurotoxin light - chain fused to enhanced green fluorescent protein, a generous gift of Dr. Martin Meyer (Ben Fredj et al., 2010). Electroporation was performed as previously described (Ruthazer et al., 2013). To optimize electroporation and to yield more tadpoles with an ipsilateral projecting RGC axon, we used plasmid concentrations of up to 2 μg/μL and increased the duration of current pulses. We used the following electroporation settings: 37 V, 3.5 ms, 2 pulses at normal and 2 pulses at reverse polarity 1 s apart.

*Short- interval in vivo imaging*

Tadpoles electroporated with TeNT-Lc:EGFP were selected for single, or double but well separated, ipsilaterally projecting RGC axons as described above. Animals with axons that had been present within the tectum for 1 to 3 days were selected. Next, animals were paralyzed by intraperitoneal injection of d-tubocurarine hydrochloride pentahydrate (2.5mM) and mounted onto a cover slip in a way that the dorsal side of the tadpole was close to the coverslip. Tadpoles were imbedded in 1.8% [w/v] UltraPure™ Low Melting Point Agarose. Next, agarose was removed from the lips, tail, eyes and gills to assure adequate oxygenation. We perfused tadpoles with oxygenated 0.1× MBSH rearing solution throughout the experiment. A BFL48 - 400 optical fiber was placed in front of the left or right eye presenting visual stimuli and to drive either the ipsilateral axon itself or to drive contralateral axons surrounding the ipsilateral axon. Light flashes were generated with red luxeon LEDs controlled by a STG4002 stimulus generator (multichannel systems) 10 ms flashes of light were delivered at 0.5 Hz. Optical section z-series were collected every 1 μm to capture the entire axon terminal, every 10 min. Images were collected using a 20X (1.0 NA, water immersion) objective mounted on a commercial multiphoton microscope (Thorlabs) with resonant scanners. The microscope uses a Maitai BB Ti:sapphire fs pulsed laser (Spectra Physics) as the excitation source.

*Image Analysis*

All 2-photon imaging data was denoised using CANDLE non-local means denoising software implemented in MATLAB (Coupé et al., 2012). 2-photon image stacks, were

reconstructed manually in 3D for dynamic morphometric analysis using Dynamo software, implemented in MATLAB (Hossain et al., 2012)

*Statistical Analysis*

Statistics were performed in GraphPad Prism 6. Anatomical remodelling, branch additions, losses elongations and retraction were analyzed by a two-way ANOVA mixed design followed by Bonferoni post-hoc test. For all axonal measurements, we only analyzed those branches that were at least 1.5 μm in length at some point during their lifetimes. For axonal measurements, except branch lifetimes, we normalized to the average of baseline (darkness). To analyze branch lifetime (survival) we performed a Log-rank (Mantel-Cox) test. We only considered branches that were born during stimulation. In all figures error bars represent SEM, except for the branch survival analyses where they give 95% confidence intervals.

## Results

We previously reported that synchronous visual stimulation of ipsilateral and contralateral RGCs in the developing *Xenopus laevis* tadpole stabilizes ipsilateral axon arbours consistent with Hebb's postulate (Munz et al., 2014). However, these experiments also revealed a novel, unexpected finding that asynchronous stimulation conversely leads to increased rates of dynamic remodelling and axon growth. Hebbian stabilization could be prevented by expressing TeNT-Lc:EGFP in the ipsilateral axons to prevent them from releasing neurotransmitter to activate postsynaptic partners. Under this condition, synchronous and asynchronous stimulation both increased axon branch growth and dynamics.

These experiments, however, did not determine whether the up-regulation in growth was due to a cell-autonomous mechanism activated by stimulation of the ipsilateral RGC itself or if it was due to an intercellular signal, generated as a result of the firing of the surrounding contralateral axons. To address this question, we performed *in vivo* time-lapse imaging of ipsilateral TeNT-LC:EGFP-expressing RGCs and stimulated either the ipsilateral (Fig 3.1C) or contralateral (Fig 3.1D) eye with light flashes every 2 sec (flash duration, 10 ms). TeNT-LC:EGFP was expressed in the ipsilateral axons being imaged to ensure that they did not drive any stabilizing postsynaptic activity in tectal neurons and that only the contralateral axons could release neurotransmitter. In this way it was possible to discriminate between inter- and intracellular mechanisms while excluding Hebbian synaptic stabilization by the ipsilateral axon. After mounting under the 2-photon objective, animals were imaged every 10 min in darkness for 60 min to establish baseline branch motility rates. We then continued imaging, while stimulating only the ipsilateral or contralateral eye for 2 hours. Contralateral eye stimulation led to a marked upregulation in branch additions of the ipsilateral axon within 20 min (Fig 3.2A, B). On the other hand, branch additions were indistinguishable from darkness baseline when the ipsilateral eye was stimulated alone. We therefore conclude that firing of the surrounding axons is able to promote non-cell-autonomous branching by an unstimulated ipsilateral axon, whilst direct stimulation of the ipsilateral axon failed to promote its own growth.

Branch elimination was also increased in ipsilateral axons by activation of the surrounding axons (Fig 3.2 C, D). Stimulation of the ipsilateral RGC axon alone did not produce a significant increase in branch losses, despite the appearance of a trend in the

plot (Fig. 3.2C), which can be entirely accounted for by a single outlier (Fig. 3.2D) that had very few initial branch tips. Next, we examined whether there was an effect on branch elongation and retraction, where elongation is the sum of all branch length increases and retraction is the sum of all branch length decreases throughout the axonal arbour from one time point to the next. Similar to branch tip additions, we found that axon arbour elongation is up-regulated compared to baseline when surrounding axons are stimulated, but is unaltered when the ipsilateral axon itself is activated (Fig 3.2E). Interestingly, branch tips retracted more compared to baseline during ipsilateral eye stimulation (Fig 3.2F). This was specific to stimulation of the ipsilateral axon, as stimulation of the surrounding contralateral axons did not increase retraction of the ipsilateral axon. Finally, changes in branch additions and losses cumulate in a significant difference in branch lifetimes with shorter lifetimes of ipsilateral axon branch tips when the surrounding contralateral axons were stimulated, possibly indicating a heterosynaptic destabilization of the branches (Fig 3.2G).

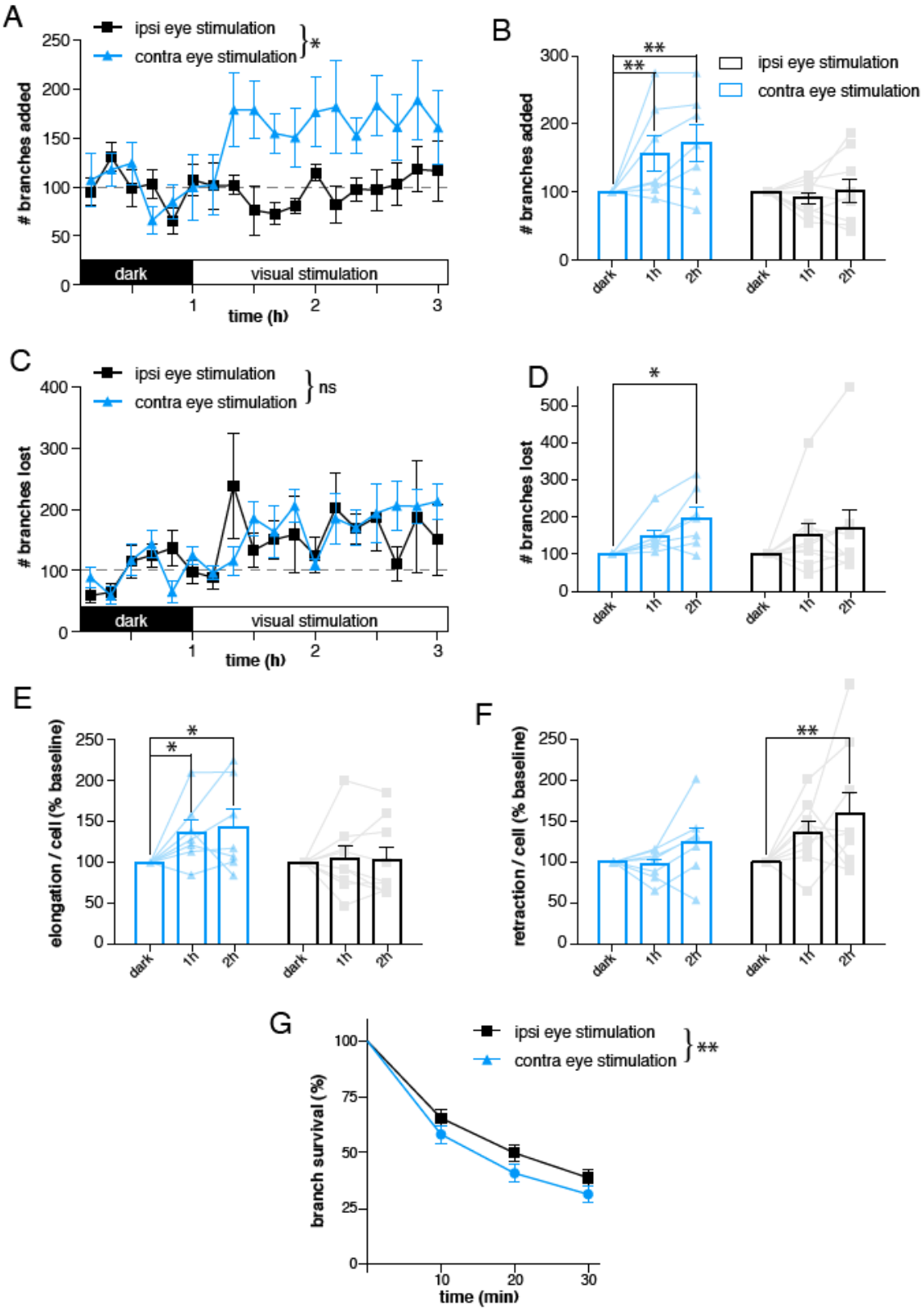

A
ipsi eye stimulation
contra eye stimulation
*
# branches added
dark
visual stimulation
time (h)
B
**
**
ipsi eye stimulation
contra eye stimulation
# branches added
dark
1h
2h
C
ipsi eye stimulation
contra eye stimulation
ns
# branches lost
dark
visual stimulation
time (h)
D
*
# branches lost
E
*
*
elongation / cell (% baseline)
F
**
retraction / cell (% baseline)
G
ipsi eye stimulation
contra eye stimulation
**
branch survival (%)
time (min)

**Figure 3.2 In TeNT expressing axons, firing of surrounding axons lead to increased growth and branching while firing of an axon itself leads to increased retraction** A) Branch additions over time normalized to first h of experiment (darkness) B) Analysis of upregulation in (A) divided into 1 h bins. C) Branch losses over time normalized to first h of experiment (darkness) D) Analysis of upregulation in (C) divided into 1 h bins. E) Elongation binned into 1 h bins F) Retraction binned into 1 h bins G) Survival analysis in % all branches over 30 min. two-way ANOVA mixed design followed by Bonferroni post-hoc test. To analyze branch survival we performed a Log-rank (Mantel-Cox) test. Error bars represent SEM, except for the branch survival analyses where they give 95% confidence intervals.

It has previously been shown that RGC axons normally attenuate their growth rates as they increase in size and complexity (Cantallops et al., 2000). We were interested to know if a cell's capability to control its growth and remodelling in response to neural activity was also regulated by complexity. To this end we compared the change in branch dynamic behaviours from baseline in darkness to the second hour of visual stimulation as a measure of sensitivity to stimulation. We found that axons with few branches at the beginning of the imaging session showed a higher increase in branching in response to contralateral eye visual stimulation compared to axons that had more branches from the outset of imaging (Fig 3.3). Thus, the susceptibility of an axon to activity-dependent control of branch additions appears to depend on initial axonal complexity. A similar trend can be seen for branch elimination in axons for which the surrounding contralateral inputs were stimulated (Fig 3.3B). As ipsilateral eye stimulation alone had no effect on branch additions or losses (Fig 3.2A-D), there was, not surprisingly, also no relationship of branch tip number to branch dynamics during ipsilateral eye stimulation (Fig 3.3C,D).

We also ascertained whether axon branch elongation and retraction were influenced by initial arbour complexity. We found that ipsilateral axon elongation in response to contralateral eye stimulation inversely correlates with axon arbour size (Fig 3.4A). This trend was less evident for ipsilateral axons when the ipsilateral eye was stimulated, but even here only cells with fewer then 40 branches showed an up-regulation in elongation compared to baseline (Fig. 3.4 B). There was no relationship of retraction to axon complexity observed in ipsilateral axons when the contralateral eye was stimulated (Fig 3.4C). On the other hand arbour retraction in response to activation of the ipsilateral axon does show a stronger effect for axons that initially have fewer branches (Fig 3.4D).

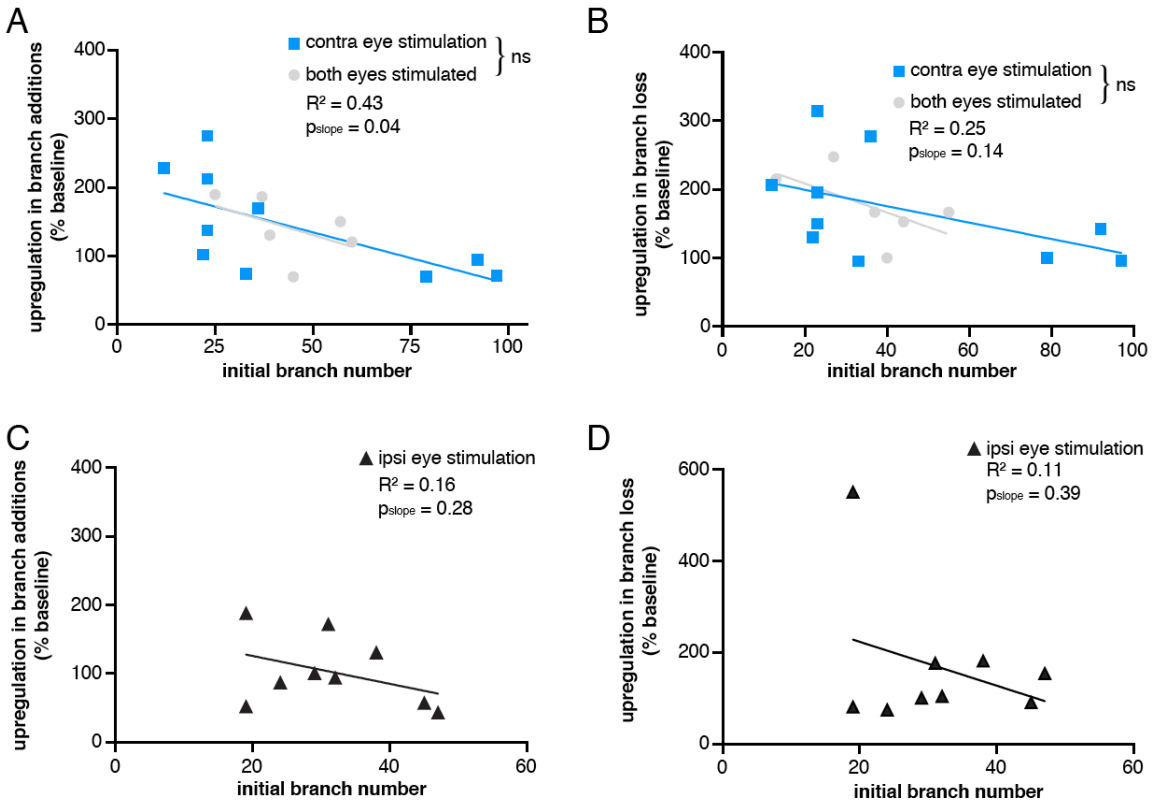



**Figure 3.3 Branching plasticity is regulated by complexity of the axonal arbour** A) upregulation of branch additions over initial branch number blue: contra eye stimulation gray: both eyes are stimulated (axons analyzed from Munz et al., 2014). B) upregulation of branch losses over initial branch number: contra eye stimulation gray: both eyes are stimulated (axons analyzed from Munz et al., 2014). C) upregulation of branch additions over initial branch number; ipsilateral eye is stimulated. D) upregulation of branch losses over initial branch number; ipsilateral eye is stimulated. Analysis of branch additions was performed on axons imaged previously (Figure 3.2) and an additional 3 axons with large axonal arbours (> 60 branches).

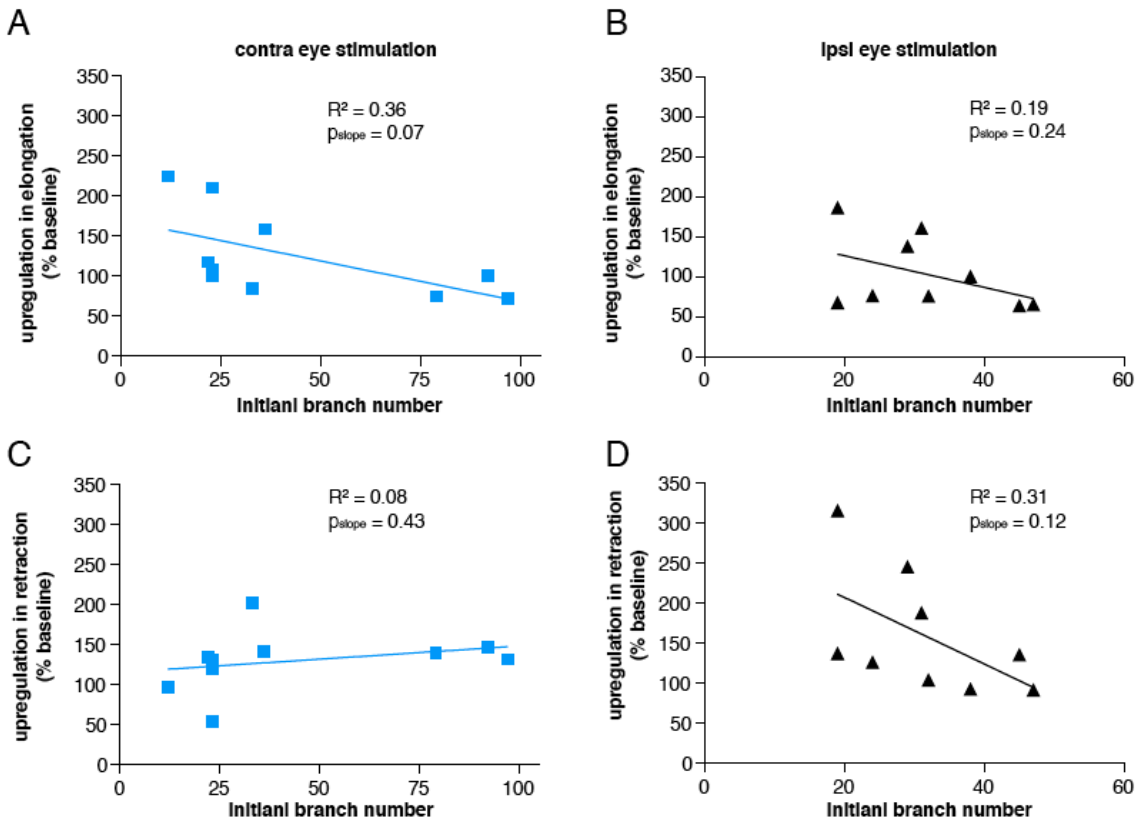



**Figure 3.4 Growth plasticity is regulated by complexity of the axonal arbour** A) upregulation of branch elongation over initial branch number; contralateral eye stimulation. B) upregulation of branch elongation over initial branch number; ipsilateral eye stimulation. C) upregulation of branch retraction over initial branch number; contralateral eye is stimulated. D) upregulation of retraction over initial branch number; ipsilateral eye is stimulated.

## Discussion

Here we show that axonal branch additions, elimination, elongation and retraction are regulated by visual stimulation of the retinotectal network. Interestingly, we found that stimulation of surrounding axons induces non-cell-autonomous increases in branch additions and axon elongation. On the other hand, stimulation of the ipsilateral axon itself leads to no reliable changes in branch addition rates or axon elongation, but up-regulates branch tip retractions. This dichotomy in which axons show increased growth in response to neural activity in neighbouring inputs but retraction as a consequence of their own activation, sheds important light onto how neuronal activity may regulate different aspects of circuit formation. We suspect that these two stimulation protocols probably engage distinct intra- and intercellular molecular mechanisms. We have previously shown that the timing of neuronal firing can be instructive through a Hebbian mechanism that is independent of the overall amount of firing. Here, we reveal additional mechanisms that may be more sensitive to the total amount of firing. Thus, much as Hebbian mechanisms can lead to either increased growth or stabilization, the mechanisms described here demonstrate that arbour elongation and branching appear to be driven by separate activity-dependent signals from those that result in branch tip retraction. One potential molecular candidate for a branch-promoting signal could be Netrin-DCC signalling (Lai Wing Sun et al., 2011). Signalling of Netrin via DCC has been shown to increase growth in Xenopus laevis RGC axons (Manitt et al., 2009).

In the future it will be interesting to see if the mechanisms described here require the participation of the postsynaptic neuron either by blocking AMPA mediated synaptic transmission in the tectum or by silencing tectal neurons specifically. In the developing

visual cortex of the cat, infusion of muscimol, a γ-aminobutyric acid type A (GABAA)-receptor agonist, to prevent postsynaptic neuronal firing while sparing presynaptic activity during the critical period for monocular deprivation, has been shown to shift ocular dominance in favour of the deprived eye. This finding suggests that some aspects of structural plasticity can occur independent of postsynaptic firing (Hata and Stryker, 1994; Reiter and Stryker, 1988). In light of our experimental results, it is possible to reinterpret those experiments. We would expect that the axons that are usually driven by the deprived eye might elaborate due to the activity of axons driven by the open eye. The axons of the open eye may retract because they are firing but fail to drive postsynaptic cells (Hata et al., 1999). In this case, firing but not being able to engage mechanisms for stabilization would lead both to increased retraction and to increased elongation and branching, but the retraction would ultimately outweigh elongation and branching. Axons usually driven by the deprived eye would not retract but instead only receive the signal to elongate and branch. A possible molecular mechanism could involve release of glutamate by the non-deprived eye, which would might bind to and activate metabotropic signalling by NMDARs that cannot pass current due to membrane hyperpolarization (Nabavi et al., 2013). The study by Nabavi et al. suggest that applying AP5 in our experimental design could address a possible metabotropic signalling by NMDARs. Subsequently, the postsynaptic neuron may signal to the presynaptic cells to branch and elongate. A possible candidate for such a retrograde signal could be nitric oxide which has been implicated in axonal branching and synaptic plasticity (Cogen and Cohen-Cory, 2000; Mu and Poo, 2006). However, only a few postsynaptic cells in the Xenopus laevis optic

tectum express the enzyme to produce nitric oxide (Peunova et al., 2001). Also we can not exclude a metabotrobic

An important conclusion from our study is the observation that an axon's susceptibility to activity-dependent growth is inversely correlated with arbour complexity. That is, less complex axons are more plastic in their growth while more complex axons grow at a more uniform rate. Our results support earlier reports by others suggesting that RGC axons may change their sensitivity to manipulations of neural activity once they reach a critical size. Massive overexpression of the activity-related, growth promoting gene CPG15 in postsynaptic tectal neurons has been shown to be effective in enhancing growth of large, complex RGC axons, to levels normally seen only in smaller, simple axons, suggesting that as axons grow and mature they may become insensitive to the normal levels of activity-regulated signals produced by experience (Cantallops et al., 2000). Here we used axon branch number as a proxy for arbour maturity because it is difficult to accurately determine the age of postmitotic electroporated RGCs which exhibit staggered growth into the optic tectum over a protracted developmental period. While it is simplest to conceive that this developmental regulation of growth plasticity by axonal maturity is regulated by a cell-autonomous signalling mechanism, it is also possible that a change in sensitivity to a retrograde signal originating with the post synaptic neuron regulates growth plasticity on the presynaptic side (Cantallops et al., 2000). We have presented evidence for cell-autonomous and non cell-autonomous mechanisms by which neuronal firing regulates axon growth and remodelling. Describing the precise function of such mechanisms will be helpful to tease apart the molecular underpinnings of activity dependent circuit development.

# Chapter 4: Discussion

Is neuronal firing instructive in the formation of neuronal circuits? We have provided strong evidence that neuronal firing can in fact instruct key aspects of circuit formation. It is becoming highly likely that different aspects of the firing activity engage different molecular mechanisms, which in turn push circuit formation in one direction or another. While we are still far away from fully understanding how firing directs circuit formation, approaches like the ones presented here can help to understand the potential roles of firing activity. We have controlled the firing of specific cells while applying genetic and pharmacological manipulations and observed the growth of single cells in real time and with high temporal precision. Figure 4.1 shows a possible model of how an axon decides to change its morphology and connectivity during circuit formation. I will go through this model step by step to discuss our and previous findings.

## Neuronal circuit formation is instructed by genetic cues

At all stages of circuit formation genetic cues most probably have a strong impact on the formation of the circuit. In many circuits it has been shown that gradients help axons find the appropriate location in that circuit (Drescher et al., 1997; Suetterlin et al., 2012; Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001) and branch and synapse formation are instructed by molecular cues (Craig and Kang, 2007; Craig et al., 2006; Schmucker, 2007; Siddiqui and Craig, 2011; Zipursky and Sanes, 2010). However, to make a neuronal circuit that is best suited for the environment in which the animal grows up, it is important for the circuit to be plastic and to incorporate sensory experience into the formation of the circuit. Because sensory experience is converted into neuronal firing

patterns it makes sense for the neurons in a given circuit to use neuronal firing to adjust their growth depending on the firing activity in that circuit.

## Neuronal firing and the lack thereof

For a given axon it only makes sense to engage activity dependent mechanisms when activity is actually present. One strategy that is described in detail in my introduction is that in the absence of visual input, spontaneous patterned activity is generated. In the visual circuit the retina creates waves of activity that co-activate RGCs that are in close proximity in the retina to ensure retinotopy throughout the visual system (Ackman et al., 2012; Assali et al., 2014; Feller, 2009; McLaughlin et al., 2003b). In species that have no retinal waves, like *Xenopus laevis* (Demas et al., 2012), and/or if there is no neuronal firing for other reasons, neurons would have to rely more on molecular guidance cues (McLaughlin et al., 2003b). For example, transplanting a single RGC into *lakritz* mutant zebra fishes that do not form their own RGCs still permits the axon terminal arbour to form in an appropriate location in the optic tectum (Gosse et al., 2008). During early normal development this might especially be of importance for wiring up cells that have not yet received afferent drive.

## Is the firing correlated with the postsynaptic neuron? Fire together wire together.

For a functional circuit, axons and dendrites need to make synapses and then to decide which synapses to keep and mature and which synapses to eliminate. One possible mechanism was first posited by Donald Hebb (Hebb, 1949). According to his hypothesis initial synapses are made and only sustained if they are able to activate the postsynaptic neuron. For the formation of an initial synapse the cell membranes of the axon and dendrite need to be in close proximity. While axonal filopodia have the machinery for vesicular release (Pinches and Cline, 1998), it appears that initially contact may be engaged by dendritic filopodia (Jontes and Smith, 2000; Jontes et al., 2000). This indicates that glutamate released from the axon may induce filopodia formation from the dendrite. Indeed, uncaging of glutamate or local stimulation of axon terminals in the cortex of mice induces formation of dendritic filopodia (Kwon and Sabatini, 2011; Maletic-Savatic et al., 1999). After initial synaptic contact between the axon and dendrite the decisions needs to be made if the synapse is stabilized and matured or if the synaptic contact is terminated. This decision is most probably determined on the postsynaptic side (Cantallops et al., 2000; Cline and Constantine-Paton, 1989; Cline and Haas, 2008; Haas et al., 2006; Rajan et al., 1999; Zou and Cline, 1996) and likely involves Hebbian mechanisms (Cline, 1991; Cline and Haas, 2008; Cline et al., 1987; Ruthazer, 2005; Ruthazer et al., 2003; Sin et al., 2002). Our results suggest that synchronous firing of presynaptic inputs drives firing of the postsynaptic neuron, leading to activation of NMDA receptors and thereby engages molecular mechanisms that instruct the

presynaptic axon to reduce branching and elongation, to stabilize branches. Although we did not specifically test for the maturation of synapses (e.g. by synaptic markers) it is likely that similar mechanisms to those implicated in hippocampal long-term potentiation also contribute to the maturation of synapses (Lisman et al., 2002, 2012). Indeed, interfering with AMPAR stabilization (Haas et al., 2006) or blocking calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) leads to exuberant growth of the postsynaptic neuron, whilst expression of a constitutively active CaMKIIa leads to confined axonal arbour growth (Zou and Cline, 1996). Furthermore, blocking NMDAR leads, at least transiently, to increased growth of axonal arbours (Munz et al., 2014; Rajan and Cline, 1998). These findings involve the same molecules as described in hippocampal LTP. Previously it has been shown that stabilization of synapses can lead to formation of new filopodia, referred to as synaptotropic growth (Cline and Haas, 2008; Vaughn et al., 1988; Ye and Jan, 2005). Synpatotropic growth is defined, as growth directed preferentially to regions that contain potential presynaptic elements (Vaughn et al., 1988).

Another possible scenario is that an axonal input to a postsynaptic cell, occasionally fires in correlation with its partner but is not strong enough to drive the firing of the postsynaptic neuron consistently. In such a case competitive mechanisms might be engaged, which could ensure that only those inputs persist that are consistently driving the postsynaptic neuron. Placing a cell at a competitive disadvantage by interfering with its firing or neurotransmission can lead either to axon retraction (Hua et al., 2005) or to exuberant axonal growth (Ben Fredj et al., 2010), however, in both cases, using inhibitors

of neural activity to equalize all neurons in the circuit leads to growth that is comparable to that of untreated control axons.

## Is the firing correlated with the postsynaptic neuron? Fire out of sync lose your link.

In our study we showed that, if the firing of a single axon is asynchronous to the firing of all surrounding axons, synapses specific to that axon rapidly show a long-term depression (LTD) phenotype. That is, it evokes smaller excitatory postsynaptic currents (EPSCs) in its postsynaptic partner. We show that with a similar time course, the axon starts to elongate and to make more branches that are not stable. We believe that this growth behaviour represents the axon trying to find an appropriate postsynaptic partners elsewhere. Interestingly, in contrast to stabilization of contacts, this increase in growth is not dependent on vesicular release from axon itself or on the activation of NMDARs. To determine if it is the firing of the axon itself or the firing of the surrounding axons that induces this activity-dependent structural plasticity we conducted an experiment in which we only stimulated that one axon or all surrounding axons. To our surprise we found that activity in the surrounding axons entices the silent axon to elongate more and add more branches that are not stable. However, it is not clear if this is due to a direct axo-axonal signal, if the postsynaptic cell provides a signal to the single axon to increase growth, or if both signals are used. In figure 4.1 this is indicated as “the postsynaptic neuron is firing but not the presynaptic”, though it would also include the hypothetical case of direct axo-axonal signalling. Interestingly, the firing of the single axon was found to promote axonal

retraction. These experiments indicate that separate mechanisms regulate branching and elongation and retraction.

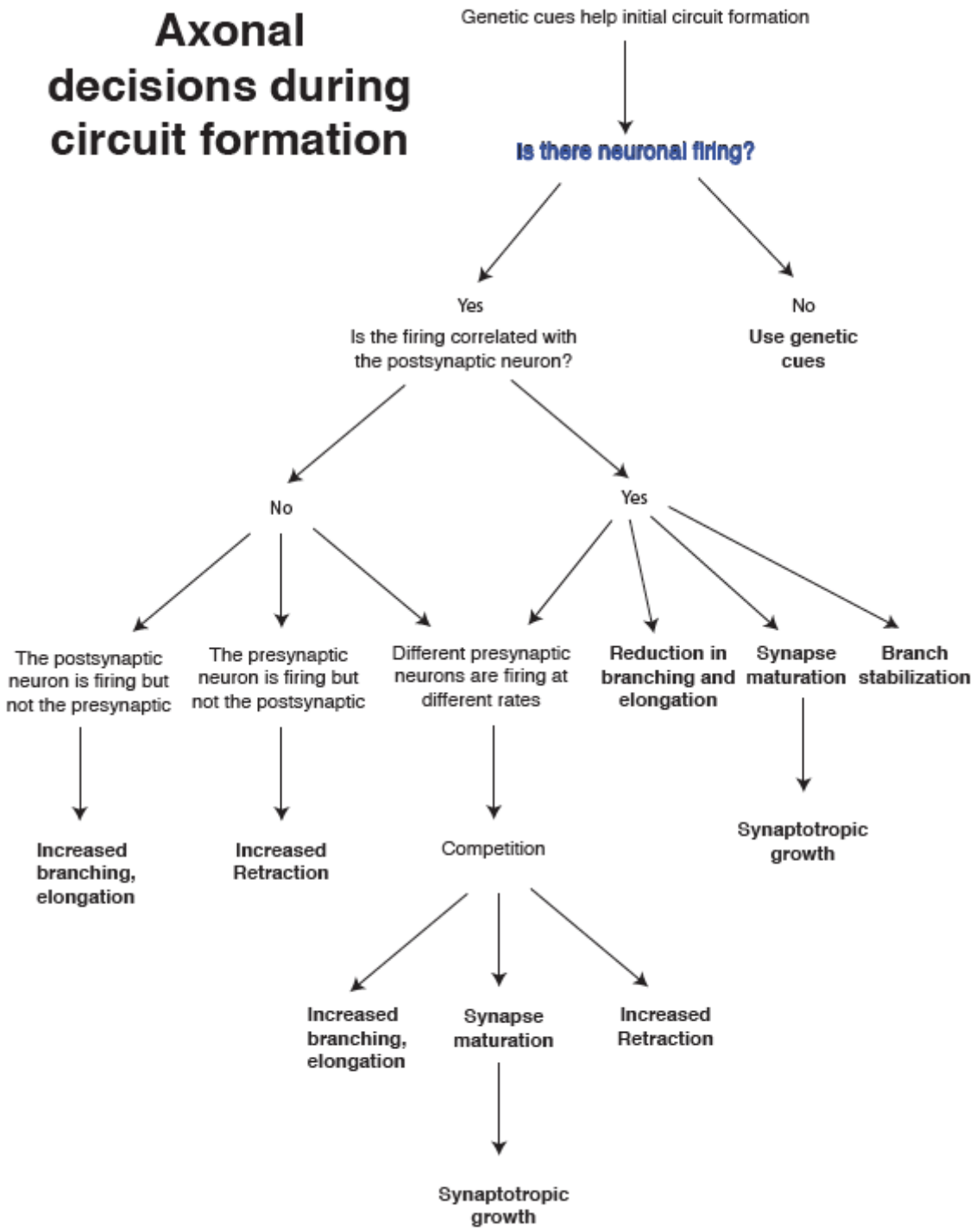
Competitive mechanisms might also play a role. For example, if a postsynaptic neuron has not yet accumulated the critical mass of inputs to fire reliably and thus is relatively silent, inputs might have a chance to compete for the postsynaptic neuron. A neuron that is firing more often would thus have a competitive advantage. A more competitive neuron can then stabilize its synapses, which in turn can lead to synaptotropic growth. For example, in the *lakritz* mutant zebrafish, in which all RGCs are missing a single transplanted RGC grows exuberantly larger than in wildtype animals (Gosse et al., 2008). In the regenerating retinotectal projection of the goldfish after nerve crush when the postsynaptic neurons receive little innervation axon initially grow up to five times larger than during normal development (Schmidt et al., 1988).

## Concluding remarks

In the field of circuit formation we often times look at simple measurements like growth and branching. However, many different mechanisms can induce growth and branching. On the other hand, one specific mechanism can induce different actions. For example, correlated firing might lead to synapse stabilization as well as synaptotropic growth. Here, I have tried to draw out a possible model of how axons make decisions to change their morphology and connectivity within a circuit, focusing on activity-dependent

mechanisms. However, it is important to mention that other mechanisms are also of importance. For example, homeostatic mechanisms can control the firing of a neuron and thus axonal growth (Pribiag and Stellwagen, 2014). Another such example is our finding that plasticity in growth is in turn regulated by the complexity of the axonal arbour itself.

In this thesis we provide evidence that neuronal firing helps the formation of neuronal circuits. Interestingly, the experiments provided here imply that different aspects of neuronal activity lead to different growth. This implies that different molecular mechanisms are involved. In the future, it will be interesting to determine these mechanisms and to see if other neuronal circuits are as plastic as the retinotectal projection, during development.


Axonal decisions during circuit formation
Genetic cues help initial circuit formation
Is there neuronal firing?
Yes
No
Use genetic cues
Is the firing correlated with the postsynaptic neuron?
No
Yes
The postsynaptic neuron is firing but not the presynaptic
The presynaptic neuron is firing but not the postsynaptic
Different presynaptic neurons are firing at different rates
Reduction in branching and elongation
Synapse maturation
Branch stabilization
Increased branching, elongation
Increased Retraction
Competition
Synaptotropic growth
Increased branching, elongation
Synapse maturation
Increased Retraction
Synaptotropic growth

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