Regulation of the functional development of vision, through an interaction between synaptic changes and experience-dependent regulation of plasticity.

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ABSTRACT

Early in development the visual circuitry in the brain is highly plastic. Factors that contribute to this plasticity and its expression are synaptic activity and the proteins expressed in the cell. As the system matures this plasticity is expressed and the system changes such that it becomes better at resolving finer details of the environment. Synaptic input from the retina participate in the initiation, progression, and once established the maintenance of visual function. Thus synaptic drive is key to the normal development of vision. However, the exact relationship between synaptic activity with the nature and distribution of proteins is not well established. It is not yet fully clear the extent to which synaptic activity directs the use of proteins in remodeling, modulates the levels of proteins in the cell, and how these changes regulate plasticity in the developing brain.

We propose that synaptic changes and experience-dependent changes in proteins expressed in the cell interact to regulate the functional development of vision. To test this we combined electrophysiology, imaging, and molecular biology to examine the effects of activity on the structure of dendritic arbors, and the properties of glutamatergic synapses, in the retinotectal projection system of the developing Xenopus tadpole. We observed that synaptic activity regulates the activity of the transcription factor Nuclear Factor of Activated T cells (NFAT). Furthermore, we found that distinct NFAT interactions lead to dissociable changes in α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAr) mediated synaptic strength, and dendritic remodeling. We also show that visual stimulation, sufficient to regulate NFAT activity, leads to increased expression of pro Brain Derived Neurotrophic Factor (proBDNF) in the tectum and that up-regulation of this protein leads to a facilitation of bi-directional plasticity.

Finally we show that the facilitation of bi-directional plasticity leads to an improvement in visual acuity.

Résumé

Au début du développement, les circuits visuels dans le cerveau sont très plastiques. Les facteurs qui contribuent à cette plasticité et son expression sont l'activité synaptique et les protéines exprimées dans la cellule. Avec la maturation du système, cette plasticité est exprimée et le système change tel qu'il peut mieux résoudre les détails les plus fins dans l'environnement. Les entrées synaptiques de la rétine participe à l'initiation, la progression, et une fois établi, le maintien de la fonction visuelle. Ainsi l'action synaptique est la clé du développement normal de la vision. Toutefois, la relation exacte entre l'activité synaptique à la nature et la répartition des protéines n'est pas bien établie, c'est à dire il n'est pas clair dans quelle mesure l'activité synaptique dirige l'utilisation de protéines dans le remodelage, et les niveaux des protéines dans la cellule, et l'effet de cette regulation sur la plasticité du développement du cerveau.

Nous proposons que les modifications synaptiques et les changements dépendants de l'expérience dans les protéines exprimées dans les cellules interagissent pour réglementer le développement fonctionnel de la vision. Pour tester cette hypothèse nous avons combiné, les methods de l'électrophysiologie, de l'imagerie et de la biologie moléculaire en étudiant les effets de l'activité sur la structure des arbres dendritiques, et les propriétés des synapses glutamatergiques, dans le système de projection rétino-tectale chez le têtard de Xenopus en développement. Nous avons observé que l'activité synaptique réglemente l'activité du facteur de transcription Nuclear Factor of Activated T-cells (NFAT). En outre, nous avons trouvé que les interactions distinctes de NFAT conduisent à des changements de la force synaptique médiée par

le récepteur α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPAr), et de la remodelage dendritique. Nous montrons également que la stimulation visuelle, suffisante pour réguler l'activité NFAT, conduit à une expression accrue de pro Brain Derived Neurotrophic Factor (proBDNF) dans le tectum et que l’élévation de cette protéine conduit à une facilitation de la plasticité bi-directionnelle. Enfin, nous montrons que cette facilitation de la plasticité bi-directionnel conduit à une amélioration de l'acuité visuelle.

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CONTRIBUTIONS TO PUBLICATIONS

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CONTRIBUTION TO ORIGINAL SCIENCE

The studies in this project imply that synaptic changes and experience-dependent regulation of transcription interact to regulate the functional development of vision. We propose this interaction based on observations made in two studies conducted in the intact, developing retinotectal circuit of the Xenopus tadpole.

In *the second chapter* we observed that synaptic activity increases the ability of a neuron to remodel through activation of NFAT. Once activated, the transcription factors NFAT and CBP interact to regulate transcription and increase the rate of dendritic remodeling. However, we observed that the increased susceptibility or ability of a neuron to remodel is not expressed unless there is concurrent synaptic activity. Thus ongoing synaptic activity may determine the location of dendritic remodeling.

For *the third chapter* we decided to investigate the manner in which gene products produced following NFAT activation affected plasticity. Based upon existing literature we selected BDNF as a candidate protein that would be regulated by NFAT, and that would regulate bi-directional plasticity. We observed that ProBDNF is up-regulated in response to visual stimulation. Upon up-regulation, proBDNF facilitated plasticity bidirectionally, but did not affect basal AMPAr mediated transmission. In a series of synaptic and behavioral assays we observed that bi-directional facilitation of plasticity led to functional refinement and an improvement in visual acuity.

I did the experiments, analysis, and figures in these studies with the ongoing, relentless guidance of Ed Ruthazer. Anne Schohl did the calcineurin knockdown experiments and many of the

western blots. Anne was also instrumental in guiding, and in some cases carrying out the subcloning of the plasmids in both studies, and provided outstanding technical support.

GENERAL INTRODUCTION

Refinement:

Developing sensory circuits become better suited to their environments through a process of activity-dependent refinement. Refinement of the functional properties of the circuit is likely based on synapse selection, with environmental input contributing to the concurrent strengthening of appropriate synapses and weakening of inappropriate synapses. It has been proposed that this process of activity-dependent synapse selection contributes to, and is accompanied by structural rearrangements (Vaughn; Katz & Shatz 1996, Cline and Haas, 2008). Thus remodeling during refinement likely improves the ability of sensory circuits to resolve the features in the environment (Greenough et al., 1996).

The ability of neurons and their circuits to exhibit these activity-dependent changes is developmentally regulated, and is most prominent during stages of major circuit refinement (Kirkwood et al., 1995; Kemp et al., 2001; Hensch 2005; Jo et al., 2006). Two major determinants of this ability are the nature of neuronal activity elicited by environmental stimuli, and the level and nature of proteins in the cell (Leinekugel et al., 1999; Hensch 2005 Yoo et al., 2009; MacDonald et al., 2009) . One of the major determinants of the nature of activity elicited by stimuli is the lack of inhibition observed in immature circuitry (Leinekugel et al., 1999; Akerman & Cline 2006; Hensch, 2005; Spitzer, 2010). In fact it has been shown that during early development, transmission mediated by GABA acts as a coincidence detector for potentiation of synapses (Kasyanov et al., 2004). Another contributor is the inability of immature neurons to limit heterosynaptic spread of plasticity (Tao et al., 2001). During this period these properties are especially important, given that the

concurrent genetic state of the neurons renders them particularly susceptible to activity dependent-modulation of transcription, and as such modulation of the level and nature of proteins in the cell (Cohen et al., 2008; Yoo et al., 2009; MacDonald et al., 2009). Together these observations imply that during refinement the neuron is primed to undergo environmentally-driven remodeling.

RATIONALE

The arborization pattern of a neuron is a determinant of its connections. As such the structure of a neuron is a determinant of the properties of the circuit in which it is embedded. The properties of the circuit in turn determine the abilities of the system. In this case of sensory systems, this is manifest as the ability to resolve features of the environment. (Lewis et al., 2005). Therefore a key component in the process of functional refinement must be the pruning of a neuron's inputs, in part manifest as remodeling of the dendritic tree (Niell et al., 2004, Lohmann & Wong 2005).

High levels of correlated activity and activity conducive to the induction of long-term potentiation (LTP) have been shown to stabilize dendritic structures (Sin et al., 2002; Jourdain et al., 2003; Zhouet al., 2003; Duch & Mentel 2004; Sorensen & Rubel 2006; Rex et al., 2007;). In contrast activity conducive to long-term depression (LTD) or depotentiation, both of which are frequently characterized by the activation of calcineurin is likely upstream of destabilization and retraction of inappropriate dendritic structures (Malleret et al., 2001; Kang-Park et al., 2003; Zhou et al., 2004; Shinoda et al., 2005; Bastrikova 2008).

Calcineurin is a serine/threonine phosphatase that has a high affinity for calcium. It is thus able to respond to small increments of this ion within the cell (Bito et al., 1996). There

are several indicators that calcineurin is important for remodeling during development. First calcineurin is likely to respond to the small increments of calcium that lead to pruning of inappropriate processes during development (Rusnak et al., 2000; Lohmann & Wong 2005). Second, calcineurin has been shown to reduce the conductance of and surface expression of ionotrpic glutamate receptors (Shi et al., 2000; Townsend et al., 2004; Morishita et al., 2005). This function may lead to synapse disassembly accompanied by morphological rearrangements (Bastrovika et al., 2008). Finally calcineurin can have more global effects including the regulation of transcription (Shi et al., 2000; Snyder et al., 2003, Townsend et al., 2004, Flavell et al., 2006; Shalizi et al., 2006). Thus calcineurin is well suited to regulate two major determinants of refinement namely, synaptic activity and available proteins in the cell.

A major downstream target of calcineurin is the transcription factor, NFAT. In hippocampal cultures NFAT activity has been shown to be regulated by the neurotrophin BDNF and by electrical stimulation. In turn NFAT has also been shown to regulate the expression of BDNF (Graef et al., 1999, Groth et al., 2003).

BDNF has been shown to be regulated developmentally, to play a role in the maturation of inhibitory circuity, to be transcribed and translated in an activity dependent manner, to regulate bi-directional plasticity of AMPAr containing synapses, and to participate in pruning of dendrites (Tao et al., 1998; Tropea et al., 2001; An et al., 2008; Hong et al., 2008; Greenberg et al., 2009). Thus BDNF seems to be a major effector of activity-dependent calcineurin/NFAT signaling.

In this project we have sought to determine how local changes at the synapse and experience-dependent regulation of transcription interact to affect the function of a developing visual circuit. To test this question we used the retinotectal projection of the Xenopus laevis tadpole as a model system. This system has proven to be an ideal *in vivo* model system for studying the cellular mechanisms underlying refinement that occur at this stage of development, as it is readily amenable to genetic manipulation, electrophysiological recording, and *in vivo* time lapse imaging (Zhang et al., 1998; Sin et al., 2002; Tao & Poo 2005;).

LITERATURE REVIEW

Activity-dependent dendritic remodeling

The "synaptotropic hypothesis" has been put forward as a mechanism by which synaptically driven remodeling of neuronal architecture may occur (Niell et al., 2004; Haas & Cline, 2006; Meyer & Smith, 2006; Ruthazer et al., 2006). Briefly this hypothesis postulates that dendritic structure is determined by 1) the intrinsic propensity of a neuron to branch and form synapses, and 2) the availability of pre-synaptic partners that may serve to both attract and stabilize these contacts (Vaughn 1989). This hypothesis draws a clear distinction between the propensity of a neuron to branch and form synapses, and the role of synaptic interactions in determining where this branching occurs. A number of reported findings indicate that this dichotomy is lax, and in fact that there is substantial overlap between the two processes.

The formation of a synapse requires the presence, recruitment, deposition, and functional organization of a number of different components. The first requirement determining the

propensity of the neuron to form synapses is that these components be present and available within the cell. Available components can either be newly *synthesized* or *recycled* from other synapses.

Synthesis: The presence of newly synthesized proteins within the cell is a function of the transcriptional state of the cell. The transcriptional state of the cell is developmentally regulated and is subject to activity-dependent control. For example, mutation of the Brahma-associated factor (BAF) chromatin-remodeling complex has been shown to impede activity dependent dendritic outgrowth (Yoo et al., 2009; Ho & Crabtree, 2010). Similarly, the level of proteins regulating DNA methylation, including methyl-CpG binding protein 2 (MeCP2), have been shown to be regulated developmentally (Kishi & Macklis, 2004). Furthermore, the activity of these proteins and, as a consequence, levels of methylation have been shown to be regulated by conditions during pregnancy and in response to activity. This regulation has been shown to affect the expression of proteins involved in remodeling including BDNF (Abdolmaleky et al., 2004, MacDonald et al., 2009; Cohen et al., 2008).

Once transcribed, message can either be translated in the ER or transported to synapses for local translation (Wang et al., 2010). Both the destination of the message and the rate of local translation and degradation, are regulated in an activity-dependent manner (Martin & Zukin, 2006; Corbin et al., 2009). For example, BDNF mRNA has been shown to be trafficked to, and translated by eukaryotic elongation factor 2 in dendrites in an activity-dependent manner (Tongiorgi et al., 2004, Verpelli et al., 2010). Interestingly, the activity of the ubiquitin proteasome degradation system has also been shown to be regulated by activity (West et al., 2002). In addition, post-translational modifications are regulated by activity (DiAntonio & Hicke, 2004). Thus, existing synapses utilize different mechanisms to

modulate the levels of, and the nature of synaptic components. Furthermore, these mechanisms are most apparent during the process of circuit formation (Schratt 2009; Manakov et al., 2009).

Recycling: For recycling to occur, materials can either be sequestered from a neighboring synapse, or they can be attracted once released from a disassembled synapse. As the nature of synaptic activity determines the relative strength of synapses as well as whether a synapse is disassembled or not, to date there is no clear way to distinguish between the two processes (Becker et al., 2008; Bastrikova et al., 2008). However, the recruitment and recycling of synaptic components have been shown to be subject to activity control. For example, it has been shown that inhibition of protein synthesis reveals competition between synapses for the materials required to maintain synaptic potentiation. Thus following a previous round of stimulation, this competition results in the weakening of stimulated synapses located in previously potentiated independent pathways, and the strengthening of synapses located in the stimulated pathway (Fonseca et al., 2004). It has also been shown that in the intact brain, larger spines that are expected to be more active capture and retain more of the post-synaptic density-95 (PSD-95) protein compared to smaller neighbors (Gray et al., 2006).
Once synthesized, proteins are stored in different compartments within the cell. These locations likely determine the ease and nature of stimulus required for their recruitment (Yoshii & Constantine–Paton 2007; Greger et al., 2007). Therefore, it seems likely that the occurrence of recycling will be inversely related to the level of synthesis and the availability of the particular protein.

Glutamatergic synapse plasticity

In the glutamatergic post-synaptic specialization there are two major ionotropic receptors that bind glutamate. The first is the NMDA receptor. This receptor is permeable to both monovalent and divalent cations including calcium. However, at hyperpolarized potentials it does not conduct due to a magnesium ion block of its pore. The second is the AMPA receptor. AMPA receptors lacking a post-translationally modified GluR2 subunit are permeable to both monovalent ions and calcium (Dingledine et al., 1999; Greger & Esteban 2007). This channel is not subject to a voltage dependent block, but, depending upon its component subunits, is inwardly rectifying at positive potentials. Therefore sufficient activity of AMPAr leads to a depolarization of the synapse and relief of the voltage dependent block of the NMDAr. Thus the NMDAr and activity downstream of its activation, are believed to act as synaptic integrators (Xia & Storm 2005). Both receptors are connected to the cytoskeleton through a number of adaptor proteins that also couple them to effector molecules within the cell (Sheng et al., 2007). The properties of both the receptors and the adaptor proteins are subject to regulation by activity, and the effects of neuromodulators. Examples of activity dependent receptor regulation include changes in phosphorylation state leading to modulation of the conductance of NMDAr, and trafficking of AMPAr (Levine et al., 1998; Shi et al., 2000;). Examples of activity dependent adaptor protein regulation include the ubiquitination, phosphorylation, and palmitoylation of the adaptor protein PSD-95, leading to changes in synaptic efficacy (Bruneau et al., 2009). Finally examples of regulation by neuromodulators, include modulation of AMPAr trafficking in response to norepinephrine and acetylcholine and, changes in conductance of NMDAr upon activation of

the p75 and TrKB neurotrophin receptors (Hu et al., 2007; Sandoval et al., 2007; Fernandez et al., 2008). Trafficking of these ionotropic glutamate receptors has been shown to be accompanied by structural and functional consequences at the synaptic and behavioral levels (Bastrovika et al., 2008; Becker et al., 2008; Kessels & Malinow 2009).

The trafficking of these receptors has been most extensively studied in the hippocampus. However, their trafficking in other brain regions including the visual cortex has been shown to be required for functional development and acquisition of different behaviors. It has therefore been proposed that receptor trafficking and persistent redistribution represent a cellular substrate for learning (Malenka & Bear 2004; Kessels & Malinow 2009). At the synaptic and cellular level a number of mechanisms have been proposed to underlie the persistent potentiation and depression of the activity of these synapses (Malenka & Bear 2004). It should be noted that in addition to postsynaptic receptor trafficking, pre-synaptic plasticity may underlie or accompany plasticity at glutamatergic synapses (Smirnova et al., 1992; Dolphin et al., 1983). Presynaptic depression has been reported to occur upon the pre-synaptic activation of cannabinoid type 1 or of NMDA receptors, and dephosphorylation of proteins mediating vesicle exocytosis (Leenders & Sheng 2005, Feldman 2009). Conversely, presynaptic potentiation can be induced by activation of presynpatic metabotropic glutamate receptors, presynaptic TrKB receptors, and phosphorylation of proteins mediating vesicle exocytosis (Herrero et al., 1992, Schinder et al., 2000; Leenders & Sheng 2005).

A current postulate regarding the manner in which glutamate receptor plasticity leads to structural remodeling is: “cells that fire together wire together”. This postulate can be considered to be an over-simplification of the hypothesis put forward by Hebb(1949). But is conceptually useful in re-interpreting the synaptotropic hypothesis put forward by Vaughn

(1989), such that synaptic activity may serve to both attract and stabilize contacts, and also to weaken and disassemble contacts.

Many of the models developed to investigate glutamate receptor plasticity have also been extended to investigate the structural rearrangements that accompany the receptor plasticity. For example a single round of LTD has been shown to be accompanied by reductions in dendritic spine size and presynaptic terminal retraction (Zhou et al., 2004, Bastrikova 2008). In accord with this it has also been shown that repeated inductions of LTD can induce spine retraction (Shinoda et al., 2005).

Further to these studies investigating structural changes accompanying long-term plasticity in isolated preparations are studies that have focused on the manner in which plasticity induced by environmental stimuli or environmental manipulations is dependent upon and regulated by these synaptic and structural changes (Hofer et al., 2008, 2009). Common elements shared by these studies are that high levels of correlated activity lead to high levels of calcium influx, synapse strengthening, and increased dendrite stability and complexity. In contrast, uncorrelated activity has been shown to lead to lower levels of intracellular calcium, activation of calcineurin and destabilization and retraction of inappropriate dendritic structures (Kang-Park et al., 2003; Morishita et al., 2005; Bastrikova 2008, Becker et al., 2008, Bourne et al., 2008).

Common to both potentiation and depression is the initiation of more protracted changes through the regulation of transcription or translation (Costa-Mattioli et al., 2009; Kandel, 2001; Linden, 1996; Manahan-Vaughan et al., 2000). As discussed above these processes determine the availability of materials required for synapse formation and restructuring. In

addition to the production of molecules used in the synapse, neuromodulators including neurotrophins, that regulate processes of plasticity may also be produced (Poo, 2001; Waterhouse 2009)

.*Activity-dependent refinement of the functional properties of the visual system*

Visual resolution acuity is a measure of the ability to resolve spatial details and is a reflection of the properties of the receptive fields neurons in the visual cortex. One method for measuring acuity is the Teller acuity test. This is a behavioral test in which it has been observed that preverbal infants will preferentially look at a grating that they can resolve, versus either a grey screen of comparable luminance, or a higher spatial frequency grating that they cannot resolve (Dobson & Teller, 1978). Thus, this test is based on the observation that gratings of different sizes can elicit different behaviors. Furthermore, cortical responses to gratings of different sizes can be extrapolated to determine the subject's acuity thresholds, with comparable results to the behavioral tests (Campbell & Maffei, 1970; Good, 2001). Interestingly, acuity is to a large extent dependent upon normal cortical development.

Cortical lesions and abnormal brain development have been shown to impair acuity (Weiskrantz et al., 1963). Interestingly, in the absence of visual input, receptive field properties develop normally but subsequently degrade. This degradation can be prevented by exogenously increasing the levels of BDNF (Gianfranceschi et al., 2003). These observations imply that visual input is required for the maintenance but not the initial development of receptive field properties and that BDNF plays a major role in this process (Crair et al., 1998, Gianfranceschi et al., 2003, Carrasco et al., 2005). It has also been shown that the acuity of different regions of the eye can be independently regulated. Not

surprisingly, under normal conditions input from the fovea has a better acuity than non-foveal inputs (Mayer et al., 1985; Allen et al., 1996; Maurer et al., 1999; Good, 2001).

Cells in the mammalian visual cortex receive binocular input. As discussed above, the functional properties of these inputs are subject to regulation by synaptic activity. Suturing one eye closed (monocular deprivation) is a common model used to reduce synaptic drive from one eye to these binocular cells in the visual cortex. Monocular deprivation results in a strengthening of the inputs from the open eye and a concurrent weakening of the inputs from the sutured eye (Wiesel & Hubel 1963). This functional change has been shown to be accompanied by activity of NMDAr, and trafficking of AMPAr (Heynen et al., 2003, Yoon et al., 2009). Reopening the eye is an extension of this model and is used to investigate the effects of increased synaptic drive (Hofer et al., 2006; McCoy et al., 2009). Monocular deprivation and eye-reopening have been shown to lead to bi-directional changes in acuity (Dews et al., 1970; Muir et al., 1973; Iny et al., 2006).

The retinotectal circuit of Xenopus laevis, a model system to study refinement

The optic tectum of Xenopus tadpoles receives monocular input from the contralateral eye. In this animal retinal ganglion cell (RGC) axons project from the contralateral eye to the optic tectum. In the tectum these axons form predominantly glutamatergic synapses on the dendrites of aspiny tectal cells a schematic is presented in Fig 1. A key advantage of this model organism is that it is relatively easy to repeatedly image RGC axon-tectal cell synapses and structure, as these structures are located superficially in the brain, beneath a translucent uncalcified skull. Importantly, both the *function* of these retinotectal synapses

and the ***structure*** of the axons and dendrites associated with them have been shown to be regulated by activity.

Activity dependent regulation of synaptic function

As the retino-tectal system matures the synaptic *ratio of AMPAr to NMDAr*, the *balance between excitatory and inhibitory inputs*, and the ability to limit the *spread of synaptically induced changes* have all been shown to develop in an activity-dependent manner.

As a neuron matures the synaptic *ratio of AMAPr to NMDAr* increases. Two of the changes that contribute to this increase are an increase in the number of synapses that contain both stable AMPAr and NMDAr, and an increase in the ratio of AMPAr to NMDAr mediated responses in the retinal inputs. Correlated activity and processes akin to LTP are believed to underlie both changes (Malenka 1997, Rumpel et al., 1998, Cline 2001). This maturation has been shown to occur at the retinotectal synapse, and to likely occur in an activity-dependent manner (Wu et al., 1996; Cline 2001).

As neurons that receive sensory input mature, *excitatory and inhibitory inputs* become more balanced. This process relies in part on heterosynaptic scaling of inhibitory inputs, and upon changes in expression of the KCC2 chloride transporter (Ackerman & Cline 2006). These changes have been shown to occur in Xenopus, and to result in an activity-dependent increase in overlap between excitatory and inhibitory receptive fields (Tao & Poo, 2005; Ackerman & Cline 2006; Liu et al., 2007).

Development results in a reduction of the *spread of plasticity* from the activated synapse to neighboring syanpses. As tectal neurons mature, calcium buffering improves, and the spread of plasticity decreases (Tao et al., 2001). Another factor that may also contribute to

this change is the reported activity dependent change in intrinsic excitability that has been reported to occur (Pratt & Aizenman 2007). In this system it has also been shown that visual function develops in a NMDAr-dependent manner in the first weeks after fertilization (Dong et al., 2009).

In addition to these activity-dependent developmental changes, the properties of retinotectal synapses are subject to regulation by a number of common plasticity inducing protocols including spike timing-dependent protocols, theta burst stimulation, and visual stimulation (Zhang et al., 1998; Zhang et al., 2000; Zhou et al., 2003). These properties have made the Xenopus tadpole a useful model for studying the role of activity in development of synaptic function.

Structure: In this developing system it has been shown that axons will segregate as a function of how well correlated their activity is with that of their neighbors (Ruthazer et al., 2003). In a series of different experiments it has also been shown that increased levels of activity will induce the formation of new synapses, regulate transcription, and induce dendritic growth (Sin et al., 2002; Van Keuren-Jensen & Cline, 2006; Aizenman & Cline, 2007;). Thus both structure and synaptic function are regulated by activity in this model system.

Specific molecules involved in refinement

Calcineurin:

Calcineurin is an evolutionarily conserved serine/threonine phosphatase that has a high affinity for calcium (Bito et al., 1996; Rusnak & Mertz 2000). According to the Human Genome Organization gene nomenclature committee the former name of calcineurin is PP2B

and the current name is PPP3CA. Calcineurin can be divided in to 5 functional domains. These include the N-terminal domain which controls calcineurin's substrate specificity, a phosphatase domain which is responsible for calcineurin's enzymatic activity, a calcium calmodulin binding domain which initiates the activation of calcineurin, a binding domain that interacts with both cytoskeletal proteins and transcription factors, and an autoinhibitory domain which under resting conditions shields the catalytic domain of calcineurin (Mansuy 2003; Groth et al., 2003; Xie et al., 2009).

In neurons the cytoskeletal protein, A-kinase anchoring protein 79 (AKAP79) binds to and controls the location of calcineurin (Dell'Acqua et al., 2002). AKAP79 is enriched post-synaptically, is associated with voltage gated L-type calcium channels and as such tethers calcineurin to areas of calcium entry into the cell (Oliveria et al., 2007). A rise in intracellular calcium results in the activation of calcium calmodulin. Calcineurin has a high affinity for calmodulin in contrast to many kinases, as such it is preferentially activated (Bito et al., 1996). Upon activation calcineurin can exert effects both *in the vicinity of the synapse* and more cell-wide or *globally* within the neuron.

In the vicinity of glutamatergic synapses, activated calcineurin curbs excitability and leads to structural destabilization. Synaptic excitability is curbed as dephosphorylation mediated by calcineurin has been shown to reduce the current conducted by NMDAr in neurons in the superior colliculus, and to induce the internalization of AMPAr in neurons in the hippocampus (Shi et al., 2000; Townsend et al., 2004; Morishita et al., 2005). Calcineurin also leads to structural destabilization through regulation of actin (Zhou et al., 2004). In addition to being activated at the synapse calcineurin can also be activated in the dendrites and somata of neurons (Yasuda et al., 2003).

More globally, activated calcineurin has been shown to depress the intrinsic excitability of pyramidal neurons (Zhang et al., 2004). A possible mechanism through which calcineurin does this is dephosphorylation of the voltage dependent Kv2.1 channel. Dephosphorylation of this channel has been shown to lead to its redistribution from storage clusters and a change in the voltage dependence of the channel (Misonou et al., 2004). Furthermore, and on a more protracted time course, calcineurin has also been shown to regulate a number of transcription factors including NFAT and MEF2 (Bito et al., 1996; Graef et al., 1999; Flavell et al., 2006; Oliveria et al., 2007).

In the visual cortex modulation of calcineurin signaling has been shown to prevent ocular dominance shifts upon monocular deprivation (Yang et al., 2005). This result is not surprising given that calcineurin has been shown to be required for homosynaptic LTD, which has been proposed to account for some of the changes upon monocular deprivation (Rittenhouse et al., 1999; Morishita et al., 2005; Yoon et al., 2009).

In the optic tectum of Xenopus the role of calcineurin has not been investigated. However, Xenopus calcineurin has been cloned and over-expression of the autoinhibitory domain has been shown to regulate neurite outgrowth in Xenopus spinal neurons (Lautermilch & Spitzer 2000).

NFAT:

In vertebrates there are 5 NFAT family members, 4 of these are regulated by activity (Hogan et al., 2003). A key feature of these transcription factors is that in their inactive state they are phosphorylated at multiple serine residues (Okamura et al., 2000). Once activated calcineurin binds to NFAT, mediates its dephosphorylation, and leads to the exposure of a

nuclear localization sequence (Crabtree 2002; Hogan et al., 2003; Wu et al., 2007). Exposure of the nuclear localization sequence leads to translocation of NFAT from the cell soma and dendrites to the cell nucleus (Graef et al., 1999; Aaron et al., 2006). NFAT is then actively transported across the nuclear membrane (Zhu et al., 1999). A number of kinases located in and surrounding the nucleus limit NFAT activation by re-phosphorylation (Aaron et al., 2006; Gwak et al., 2006). Thus NFAT activation is regulated by a balance of kinase and phosphatase activity and as such functions as a working memory of calcium signaling within the cell (Dolmetsch et al., 1998; Tomida et al; 2003).

NFAT has a weak affinity for DNA. Therefore it is thought that a major role of NFAT in the nucleus is to form complexes with a number of other transcription factors. Interestingly NFAT has been shown to both up- and down-regulate transcription within the cell depending on the nature of its binding partners (Wu et al., 2007; Hogan et al., 2003). In neurons a number of transcription factors in addition to NFAT have been shown to be regulated by activity. Of these NFAT has been shown to directly interact with MEF2 and with CREB binding protein (CBP) (Greer et al., 2008). MEF2 has been shown to directly regulate synaptic number, and CBP has been shown to directly interact with CREB which has been shown to affect both dendritic structure and synaptic function (Redmond & Gosh 2005; Flavell et al., 2006; Sato et al., 2006; Wayman et al., 2006). Modulation of NFAT therefore results in the modulation of the activity of a number of transcription factors. Notably deregulation of NFAT during development is believed to lead to a number of complex pathologies including schizophrenia and Down's syndrome (Sun et al., 2001; Park et al., 2009). One protein that has been shown to regulate and be regulated by NFAT, is BDNF (Groth et al., 2003).

In the visual cortex the role of NFAT signaling has not been investigated. However, it seems likely that NFAT will play a key role in this process given that calcineurin has been shown to be activated under a number of different circumstances during visual system development (Shi et al., 2000; Townsend et al., 2004;Yang et al., 2005). Furthermore NFAT has been shown to affect axon patterning in sensory systems (Graef et al., 2003; Nguyen & Giovani, 2008).

In Xenopus the NFAT family members have been identified based upon sequence homology (Borchers et al., 2006). One of the conserved regions in NFAT is the calcineurin binding domain. This homology renders Xenopus NFAT susceptible to inhibition by a synthetic peptide with a sequence of VIVIT that has a much higher affinity for calcineurin compared to NFAT (Aramburu et al., 1999). Furthermore, it has been shown that Xenopus NFAT lacking its regulatory domain is constitutively active (Saneyoshi et al., 2002).

BDNF

The expression of BDNF is tightly regulated at multiple levels. Multiple promoters have been shown to direct tissue specific expression (Aid et al., 2007; Koppel et al., 2009). Within the brain the BDNF exon IV promoter has been shown to be most sensitive to regulation by synaptic activity (Greenberg et al., 2009). Once transcribed, BDNF message is found in multiple forms. Transcripts with either a short or long 3' untranslated region (3'UTR) have been reported. Message with the short 3'UTR has been shown to be localized and translated in the somatic compartment. In contrast, message with the long 3'UTR has been shown to be localized and translated in the dendrites of the neuron, and to affect the developmental pruning of synapse (An et al., 2008). Translation of BDNF is also subject to

regulation by activity (Verpelli et al., 2010). BDNF is initially translated as proBDNF. If translation occurs in the endoplasmic reticulum then proBDNF will likely be enzymatically cleaved to its mature form mBDNF. The mature form will then enter the regulated secretory pathway to be released in an activity dependent manner. In contrast if BDNF is locally translated in the vicinity of the synapse then it will likely enter the constitutive secretory pathway and be secreted as proBDNF (Barker 2009). It should be noted that proBDNF can also be secreted in an activity-dependent manner likely due to activity-dependent regulation of local translation (Nagappan et al., 2009). Once secreted, proBDNF can be cleaved in an activity-dependent manner. Extracellular cleavage of proBDNF is largely dependent upon the activity-dependent recruitment of the activator of plasmin, tissue plasminogen activator (tPA) (Gualandris et al., 1996; Pang et al., 2004; Barker 2009; Nagappan et al., 2009). ProBDNF may also be cleaved extracellularly in an activity-dependent manner by other enzymes (Keifer et al., 2009).

Polymorphisms of the human BDNF gene have been shown to affect its intracellular cleavage and trafficking within the trans-golgi network, and to lead to increased proBDNF secretion. Increased extracellular proBDNF has been shown to reduce spine density and to lead to apoptosis (Koshimizu et al., 2009; Lebmann & Bridgadski, 2009). In a study by Egan et al. in 2003, humans with a point mutation of valine to methionine in the 5' pro-region, exhibited poorer episodic memory compared to controls. However, acute application of proBDNF to neurons does not affect basal AMPAr mediated synaptic transmission. In contrast, during development application of proBDNF or expression of a mutated cleavage resistant version has been shown to facilitate AMPAr dependent LTD. This facilitation has also been shown to be dependent upon binding of proBDNF to the p75 neurotrophin receptor

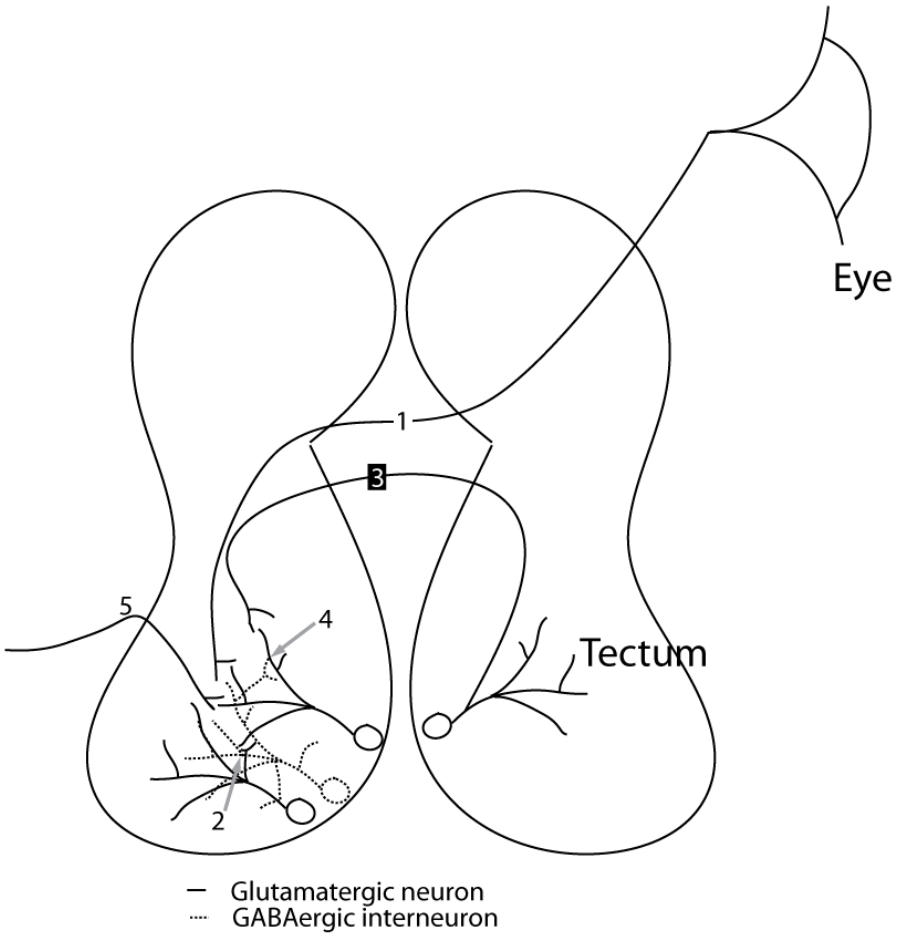
(Woo et al., 2005). In contrast extracellular cleavage of proBDNF and activation of the receptor for mBDNF, TrkB, has been shown to be required for the late phase of AMAPr dependent LTP (Korte et al., 1998; Pang et al., 2004). Together these findings indicate that BDNF is subject to stringent regulation, and is also a potent regulator of bi-directional plasticity of AMPAr containing synapses.

In addition to effects on AMPAr mediated transmission BDNF has been shown to have diverse effects pre- and post-synaptically. Like calcineurin, the diverse roles of BDNF can be divided into three categories. Those that are spatially restricted, those that regulate cell-wide changes, and those that regulate more protracted changes. The spatially restricted effects of BDNF are pre and post-synaptic. Pre-synaptically BDNF has been shown to regulate synapsin I and RIM1α, and to leading to increases in glutamate release (Jovanovic et al., 1996; Jovanovic et al., 2000; Simsek-Duran & Lonart, 2008). In addition, NMDAr have been shown in some cases to be located pre- and post-synaptically, and BDNF increases the open probability of these receptors (Levine et al., 1998; Lien et al., 2007). At a cell-wide level, local application of BDNF by microspheres has been shown to induce the cell-wide redistribution of PSD-95 from somatic to dendritic compartments (Yoshii & Constantine-Paton 2007). Furthermore, over a more protracted time course BDNF has been shown to regulate local translation of a number of proteins involved in remodeling, and to initiate CREB signaling in synaptically coupled neurons (Kuczewski et al., 2008; Waterhouse & Xu 2009). Based on these observations it is not surprising that BDNF has been shown to play an integral role during development in the regulation of dendritic arborization (Horch & Katz 2002; Liu et al., 2007).

In the visual cortex, some of the effects of light deprivation have been shown to be blocked by over expression of BDNF (Gianfranceschi et al., 2003). Recovery from monocular deprivation has also been shown to depend upon BDNF-TrkB signaling (Kaneko et al., 2008). Furthermore, during normal development BNDF has been shown to regulate acuity (Heimel et al., 2010). BDNF levels have also been shown to be regulated by activity and to be highest in early stages of development (Tropea et al., 2001). Interestingly the higher levels of BDNF observed early in development have been shown to inhibit LTD (Akaneya et al., 1996; Jiang et al., 2003).

In the Xenopus retinotectal system BDNF has been shown to be required for spike timing-dependent potentiation of the retinotectal synapses, and to lead to a presynaptic retrograde spread of LTP from the tectum to the retina (Mu et al., 2006, Du et al., 2009). BDNF from the tectum has also been shown to regulate the morphology of the RGC axons, and to determine the stability of PSD 95 puncta in tectal cells (Alsina et al, 2001; Sanchez et al., 2006). BDNF expression has also been shown to be regulated developmentally in the tectum (Cohen-Cory et al., 1996). BDNF transcription has been shown to be regulated by activity in Xenopus, and a homology comparison between the Xenopus BDNF promoters and the mammalian promoters has been published (Kidane et al., 2007, 2009). However, neither the role of activity in regulating expression in the tectum nor the functional consequences of this regulation have been investigated.

Figure 1



Glutamatergic tectal neurons receive 5 main inputs. The first is from retinal ganglion cells that project from the contralateral eye to the optic tectum. In the tectum these axons make predominantly glutamatergic synaptic contacts with both glutamatergic tectal neurons and gabaergic interneurons (1). Projections from glutamatergic neurons in the ipsi (2) and contra lateral (3) tectum compose the another set of inputs (Pratt et al 2008). The 4th is made of inputs from GABAergic interneurons in the tectal lobe (Lien et al 2006) . Finally tectal neurons also receive inputs from somatosensory structures (5)(Deeg et al 2009).

List of constructs

1) AIXCN – A CMV promoter driving expression of the auto inhibitory fragment of Xenopus calcineurin with a myc tag. The auto inhibitory fragment blocks the phosphatase activity of calcineurin.
2) VIVIT – A CMV promoter driving expression of the peptide VIVIT tagged with GFP. The VIVIT peptide has a high affinity for the binding site of calcineurin which controls its location and ability to bind to and activate the transcription factor NFAT. Expression of VIVIT is expected to affect the cell wide distribution of calcineurin by inhibition of binding to structural proteins including AKAP, as well as to inhibit NFAT signaling.
3) N VIVIT - A CMV promoter driving expression of the peptide VIVIT tagged with GFP and 3 copies of a nuclear localization sequence. This peptide is expected to be localized in the vicinity of the nucleus. Its major effect is expected to be inhibition of NFAT signaling.
4) NFAT-gfp - A CMV promoter driving expression of NFAT tagged with GFP.
5) NFATΔreg - A CMV promoter driving expression of Xenopus NFAT in which a large section of the regulatory domain had been removed. Removal of the regulatory domain renders NFAT constitutively active but also reduces the transcription factors that NFAT can interact with.
6) NV-NFAT-ST(2+5+8)- A CMV promoter driving expression of a constitutively active Xenopus NFAT in which serine residues were mutated to alanines to mimic constitutive dephosphorylation, the calcineurin binding site had been mutated to VIVIT, and three NLS sequences and CFP had been attached.
7) BDNF exon IV promoter-kaede – A 1500 base pair fragment of the BDNF exon IV promoter driving expression of photoconvertible Kaede. A basic pGL3 backbone was used for this construct.

CHAPTER 2

Neural activity regulates synaptic properties and dendritic structure *in vivo* through Calcineurin/NFAT signaling

Hypothesis and outline

We hypothesize that synaptic changes and experience-dependent changes in proteins expressed in the cell interact to regulate the development of dendritic structure in the developing visual system.

It has been shown that calcineurin can be activated by synaptic activity. Upon becoming active calcineurin activates NFAT. NFAT can then translocate from the cell soma and dendrites to the nucleus and participate in transcriptional complexes to regulate the proteins expressed in the cell. To test whether calcineurin and NFAT play a role in dendritic arborization we will inhibit their activity and monitor the effect on AMPAr mediated synaptic strength, and dendritic remodeling in tectal cells of the developing Xenopus tadpole. We will then test whether visual activity can regulate NFAT, by monitoring NFAT translocation in response to changes in levels of visual activity. We will then test if distinct NFAT interactions with other transcription factors lead to dissociable changes in AMPAr mediated synaptic strength, and dendritic remodeling.

Summary

The calcium-regulated protein phosphatase Calcineurin (CaN) participates in synaptic plasticity and the regulation of transcription factors, including Nuclear Factor of Activated T-cells (NFAT). To understand how CaN contributes to neuronal circuit development, whole-cell mEPSC recordings and multiphoton imaging were performed in the visual system of living *Xenopus laevis* tadpoles electroporated to express either a CaN phosphatase inhibitor or N-VIVIT, a nuclear localization sequence-tagged VIVIT peptide that blocks the binding of CaN to select substrates including NFAT. Both strategies increased mEPSC frequency and dendritic arbor complexity in tectal neurons over three days. Expression of either of two constitutively active Xenopus NFATs (CA-NFATs) restored normal synaptic properties in neurons expressing N-VIVIT. However, the morphological phenotype was only rescued by a CA-NFAT bearing an intact regulatory domain, implying that transcriptional control of morphological and electrophysiological properties of neurons are mediated by distinct NFAT interactions.

Introduction

As neurons pass through a series of developmental stages and differentiate into distinct neuronal cell types their diversity is reflected in their unique transcriptional profiles (Polleux et al., 2007; Spitzer, 2006). As development progresses, connectivity and consequently environmentally driven activity begin to play larger roles in influencing the transcriptional profile of a neuron. Refinement of connectivity and the concomitant sharpening of receptive fields should impact the activity-dependent transcriptional profiles of neurons and in turn their subsequent responses to patterns of activity. Activity-dependent regulation of transcription factors has been shown to produce dramatic effects on dendritic remodeling of neurons (Parrish et al., 2007; Redmond, 2008). To better understand how activity mediated by sensory experience

regulates transcription factors and how these transcriptional changes alter synaptically driven dendritic remodeling *in vivo*, we took advantage of the developing visual system of the *Xenopus laevis* tadpole in which single-cell gene transfection, time lapse imaging and electrophysiology can be performed in the intact, living animal.

The "synaptotropic hypothesis" has been put forward as a model to explain how synaptically driven remodeling of neuronal architecture may occur (Vaughn, 1989; Cline & Haas 2008). Briefly this hypothesis postulates that neurons possess an innate tendency to extend branched dendritic processes, and that synaptotropic interactions (i.e., between the dendrite and potential presynaptic partners) provide the extrinsic cues that help direct this dendritic growth into patterns that optimize synaptic interactions. Thus growth or branching is most likely to occur in regions of the arbor where there is a stabilized synapse. In contrast retraction is more likely to occur in regions where synapses fail to mature or become destabilized (Haas et al., 2006; Meyer and Smith, 2006; Niell et al., 2004; Ruthazer et al., 2006; Wu and Cline, 1998). An extension of this hypothesis is that patterns of synaptic activity and mechanisms underlying synaptic long-term potentiation (LTP) may stabilize the synapse, and conversely that mechanisms related to long-term depression (LTD) might destabilize the synapse. Importantly, while LTP and LTD are both induced and expressed locally at the synapse, there is a growing body of evidence that transcriptional or translational regulation is required for expression of long-term changes initiated by either cascade (Costa-Mattioli et al., 2009; Kandel, 2001; Linden, 1996; Manahan-Vaughan et al., 2000).

A growing number of calcium-responsive transcription factors has been implicated in the regulation of dendritic growth and in LTP (Bito et al., 1996; Impey et al., 1996; Silva et al., 1998; Wayman et al., 2006). Thus, a more thorough implementation of the synaptotropic

hypothesis should not only take into consideration the genetic program a neuron draws upon to grow its dendritic arbor and the local interactions with synaptic partners that further shape the arbor, but also how the neural activity resulting from synaptic interactions may impact the cell by further modifying its transcriptional profile.

Calcineurin (CaN) is a calcium/calmodulin-dependent serine/threonine phosphatase that is sensitive to small changes in intracellular calcium levels. The pattern of CaN expression in the brain and its activity have been shown to be regulated in an activity-dependent manner during development (Townsend et al., 2004; Nakazawa et al., 2001; Goto et al., 1993; Agbas et al., 2005). CaN is required for the expression of NMDAR-dependent LTD of AMPAR transmission (Morishita et al., 2005; Mulkey et al., 1994). Furthermore, it has been shown to regulate transcriptional programs that control synapse formation and function (Bito et al., 1996; Flavell et al., 2006; Shalizi et al., 2006). Thus, CaN is able to serve both as a synaptic and a cell-wide activity-dependent regulator of neuronal connectivity.

Nuclear Factor in Activated T-cells (NFAT) is a transcription factor that is activated and translocates to the nucleus in response to CaN-mediated dephosphorylation. NFAT is expressed in neurons and has been demonstrated to play an important role in axonal outgrowth and neuronal response to extrinsic cues involved in circuit development and refinement (Graef et al., 1999, 2003; Groth and Mermelstein, 2003). These findings argue for a potentially important role of CaN/NFAT signaling in activity-dependent developmental plasticity.

We therefore sought to examine in the context of an intact, developing neural circuit, the visual system of *Xenopus laevis* tadpoles, how CaN and NFAT signaling influences synaptic properties and dendritic structure. Pharmacological CaN inhibition and expression of cell-autonomous CaN inhibitors revealed that it is a potent regulator of dendritic complexity and

synaptic function in neurons in the visual system *in vivo*. Surprisingly, the same morphological and physiological effects could be induced with a nuclear localization sequence (NLS)-tagged inhibitor that spared CaN signaling at synapses. We further demonstrated that CaN mediates its effects on dendritic branching and synaptic function through its activation of NFAT transcription factors, the activity of which was regulated by visual stimulation.

Results

CaN inhibition alters excitatory synaptic connectivity

To understand how endogenous CaN activity contributes to the synaptic connectivity of neurons in the visual system, we examined the changes in miniature excitatory postsynaptic current (mEPSC) properties induced by expression of peptide inhibitors of CaN in individual optic tectal neurons in the living Xenopus tadpole. Three different genetically encoded inhibitors were used to block CaN within individual tectal cells in this study: the auto-inhibitory domain of the Xenopus CaN A subunit (AI-XCN, (Lautermilch and Spitzer, 2000)); an EGFP-tagged VIVIT peptide (seq: MAGPHPVIVITGPHEE) that competitively displaces CaN from a subset of its substrates by mimicking the highly conserved CaN docking sequence (PxIxIT) at 25-fold higher affinity (GFP-VIVIT, (Aramburu et al., 1999)); and a NLS-tagged version of GFP-VIVIT (N-VIVIT). Although VIVIT was originally created to be a selective blocker of NFAT activation by CaN (Aramburu et al., 1999), in recent years it has become clear that it can also disrupt the interaction of CaN with other proteins including some synaptic proteins (Czirjak and Enyedi, 2006; Dell'Acqua et al., 2002; Liu, 2003; Tavalin et al., 2002). We therefore developed N-VIVIT by adding a nuclear localization sequence to GFP-VIVIT to restrict its localization within the cell. This peptide was designed to act principally as an inhibitor of nuclear transcription downstream of CaN. In experiments presented below, N-VIVIT impairs activation

of the CaN-dependent transcription factor NFAT, but has no detectable effect on CaN at synapses in dendrites.

CaN is known to regulate the expression of BDNF, the IP3 receptor and the Kv2.1 channel (Amberg et al., 2004, Graef et al., 1999; Groth and Mermelstein 2003). Expression of either N-VIVIT or bath application of the selective CaN inhibitor FK506 resulted in similar reductions of mRNA levels for these three targets in Xenopus brain compared to controls as measured by relative quantification real-time PCR (Supplemental Fig S1). The three genetically encoded inhibitors therefore provide progressively refined substrate specificity, from cell-wide inhibition of the phosphatase activity of CaN (AI-XCN) to interference with the small subset of CaN targets that contain the PxIxIT binding sequence throughout the cell (GFP-VIVIT), and culminating with N-VIVIT.

To determine the physiological consequences of expression of each of the three inhibitors we recorded AMPAR mEPSCs *in vivo* from untransfected tectal neurons (n=15) and from neurons three days after transfection with EGFP (n=6), AI-XCN (n=9), GFP-VIVIT (n=15) or N-VIVIT (n=10). Because there was no difference between untransfected and EGFP-transfected controls, these control groups were pooled. The frequency of mEPSCs in cells expressing AI-XCN (2.13±0.60 Hz), GFP-VIVIT (2.09±0.41 Hz) or N-VIVIT (2.95±0.60 Hz) all increased dramatically compared to control cells (0.88±0.11 Hz), indicative of an increase in the total number or the proportion of AMPAR-bearing synapses (Fig 1 A,B). The distribution of mEPSC amplitudes in cells transfected with either AI-XCN or N-VIVIT also shifted toward significantly larger values relative to controls (Fig 1C,D). Surprisingly, mEPSC amplitudes of neurons transfected with GFP-VIVIT decreased relative to controls, perhaps reflecting the interaction of cytoplasmically expressed GFP-VIVIT with proteins in the synapse bearing the PxIxIT motif

(Dell'Acqua et al., 2002; Tavalin et al., 2002).

It was therefore important to demonstrate that the actions of N-VIVIT were restricted to the nuclear region. Co-expression of N-VIVIT with cell-filling mCherry or with a nuclear-localized mCherry-NLS by bulk electroporation in tectal neurons clearly demonstrated that even at very high levels of expression, N-VIVIT protein expression was tightly localized to the nucleus (Fig 1E). The presence of CaN in the nucleus of neurons has been somewhat controversial. Anthony and co-workers (1988) failed to detect phosphatase activity in nuclear fractions from chick forebrain neurons. On the other hand, there have been several reports that CaN is found in the nucleus in neurons (Pujol et al., 1993; Solà et al., 1999; Yang et al., 2005). We observed a low, but measurable, level of CaN immunostaining in the nuclei of Xenopus neurons that was confirmed using antisense Morpholino oligonucleotide knockdown to be specific for endogenous CaN (Fig 1F).

To confirm that N-VIVIT expression does not inhibit CaN signaling at synapses, we performed a chemLTD experiment. Amplitudes of mEPSCs were monitored before and after bath application of 20 μM NMDA to the optic tectum in an isolated brain preparation. Cells expressing GFP-VIVIT showed little or no reduction in mEPSC amplitude in response to the chemLTD protocol, indicating that CaN function was inhibited at synapses in neurons expressing untargeted GFP-VIVIT (Fig 1 G,H). In contrast, cells transfected with N-VIVIT as well as controls, both exhibited robust chemLTD similar to previous studies (Beattie et al., 2000). Furthermore, inclusion of FK506 in the whole cell patch pipette blocked chemLTD induction in N-VIVIT-expressing cells. The small change observed with both GFP-VIVIT and FK506 was presumably CaN-independent. These results demonstrate that N-VIVIT overexpression does not block CaN activity at the synapse.

The increase in mEPSC frequency and amplitude observed with dominant negative AI-XCN expression is potentially consistent with a net increase in synaptic AMPARs as a consequence of long-term inhibition of LTD, however it seems likely that homeostatic mechanisms like synaptic scaling would have curtailed this effect over three days (Turrigiano et al., 1998). In light of the observation that a comparable enhancement of mEPSC frequency and amplitude occurred with N-VIVIT expression, our data instead favors a critical role for CaN signaling in the regulation of nuclear transcriptional events that play a role in determining synapse number and efficacy.

Chronic inhibition of CaN increases dendritic branching

To examine the role of CaN signaling on structural remodeling we monitored the effects of the above inhibitors on the morphology of single tectal neurons expressing plasmids encoding EGFP (n=10) and, in the experimental cases, either AI-XCN (n=6), GFP-VIVIT (n=7) or N-VIVIT (n=6). Cells co-expressing the respective constructs were then imaged once daily by laser scanning two-photon microscopy in the intact tadpole over the next three days. Cells with relatively complex arbors were selected, as mature tectal cells have a larger number of active synaptic inputs (Rajan and Cline, 1998; Tao et al., 2001; Wu et al., 1996). All three treatments resulted in a gradual increase in the complexity of the dendritic arbor compared to EGFP-expressing controls (Fig 2A-D). Similarly, application of 1μM FK506 to the rearing solution led to a comparable increase in branch number (Fig 2E). This increase in complexity was not caused by accelerated dendritic outgrowth. There was no difference from control cells for cells in any group, even by the third day of imaging, in either total dendritic arbor length or in the fractional daily rate of arbor growth (Fig 2F,H). In contrast, the total number of, and percentage change in, number of dendritic branch points was greatly elevated in all three groups expressing CaN inhibitors by the third day of imaging (Fig 2G,I).

These results reveal that the density of dendritic branches is dramatically increased by chronic CaN blockade. Two lines of evidence suggest that this effect may be mediated by inhibition of CaN regulation of nuclear transcription rather than inhibition of CaN at synapses. First, the increase in branch density occurred regardless of whether inhibition was applied pharmacologically using FK506, cell autonomously throughout the cell as in the case of AI-XCN expression, or in a manner that spared synaptic CaN activation as in the case of N-VIVIT. Second, a similar increase in branch density was observed in GFP-VIVIT expressing neurons, despite the fact that AMPAR current amplitudes are decreased in these cells but increased in AI-XCN and N-VIVIT expressing cells.

The increased branch density in neurons in which CaN was chronically inhibited appears to be attributable in large part to a striking proliferation in the number of small branch tips (insets Fig 2A-E). Tectal cells at this stage of development are aspiny neurons that make synaptic contacts all along their dendritic arbors. The majority of new synapses on tectal neurons form on dynamic dendritic filopodia that are subsequently either stabilized or eliminated depending on the fate of the new synapse they bear (Niell et al., 2004). We observed that the mean length of branch tips was greater in controls than in AI-XCN or N-VIVIT expressing cells and increased progressively over three days of imaging as a fraction of these branches elongated. In stark contrast, the proportion of short branch tips in neurons expressing the inhibitors remained high at all time points, indicative of a sustained high rate of addition of new branch tips (Supplemental Fig S2).

Blocking CaN increases rates of dendritic branch dynamics.

To examine directly the rates of dynamic branch remodeling of tectal neurons *in vivo,* a short interval imaging protocol which consisted of acquiring 4 images at 40 minute intervals was

applied on day three after electroporation (Fig 3A). Neurons expressing either AI-XCN (n=5) or N-VIVIT (n=6) added many more branches compared to controls (n=5) during the two hours of imaging: Control cells added 21.2±3.5 new branches over 2 hours compared with 41.5±3.5 and 43.5±2.9 added branches for AI-XCN and N-VIVIT expressing neurons respectively (Fig 3B). Furthermore, rates of branch loss also were enhanced by CaN inhibition (Control:17±2.7; AI-XCN 27.5±1.8, N-VIVIT 28±3.6), but in the AI-XCN and N-VIVIT cells the rates of branch addition were significantly greater than the rates of branch elimination. Thus, for both manipulations we observed an acceleration of dendritic branch dynamics, with a bias favoring branch addition over branch loss. This bias may account for the increase in cumulative branch number observed by the day 3 time point in neurons in which CaN was inhibited. Given that new synapses have been reported to form on added branches (Niell et al., 2004), it is also consistent with the increase in mEPSC frequency observed which may reflect an increase in the number of AMPAR containing synapses.

CaN participates in the regulation of local remodeling of dendritic and synaptic structures during LTD (Zhou et al., 2004). However, under normal developmental conditions LTD is likely to be taking place at only a few synapses in the arbor. Based on our observations that cell-wide inhibition of CaN phosphatase activity which was able to block at least one form of LTD, did not decrease the rate of branch retractions and that targeted inhibition with N-VIVIT was as effective as AI-XCN at altering branch dynamics (Fig 3), our data suggest that the predominant means by which CaN activity regulates branch dynamics may be through control of the transcriptional state of the cell. While these experiments certainly do not exclude a contribution of LTD to dendritic remodeling, they reveal that the dynamic regulation of the transcriptional profile of neurons by sensory experience is indeed one of the principal mechanisms that modulates the ability of the

dendritic tree to remodel.

How could activity-dependent gene transcription exert its effects at distinct sites in the dendritic arbor? One possible mechanism would be for transcriptionally regulated changes to also require local synaptic signals for their manifestation. To test whether synaptic activity could modulate the increase in dendritic branch dynamics caused by N-VIVIT, we repeated the short interval time lapse imaging of neurons expressing N-VIVIT on day three, but this time acutely blocked synaptic transmission for the duration of the two hours of imaging. After acquisition of the first image, tadpoles were transferred into a solution containing 100µM AP5, 100µM GYKI and 100µM picrotoxin. We then took three subsequent images at 40 min intervals with the animals remaining under synaptic blockade for the full two hours. Synaptic blockade prevented the increase in branch additions observed in N-VIVIT expressing cells (27.8±2.3, n=6 vs. 43.5±3.6, n=6, Fig 4). Interestingly, there was no significant effect of blocking synaptic transmission on the rates of branch loss in these cells. Control cells were unaffected by synaptic block in accordance with previous reports (Rajan and Cline, 1998). These results indicate that while modulation of transcription regulates the propensity of the tree to remodel, this remodeling is rapidly gated by synaptic activity. Thus, the CaN-regulated transcriptional profile of a given neuron would define its overall propensity to add or lose branches and synapses, but the decisions about whether and perhaps where they should be added would be controlled by local synaptic events.

Visual stimulation activates CaN/NFAT

The VIVIT peptide used in our experiments was originally identified as a high affinity, selective inhibitor of the binding interaction between CaN and transcription factors of the NFAT family (Aramburu et al., 1999). NFAT is present and is regulated by extrinsic cues in neurons

(Graef et al., 2003; Groth and Mermelstein, 2003). When activated by Calmodulin, CaN binds to and dephosphorylates NFAT resulting in the exposure of nuclear localization sequences on NFAT that cause transport of the NFAT complex towards the nucleus (Hogan et al., 2003; Okamura et al., 2000). It is likely that the effects mediated by N-VIVIT expression in tectal neurons are in part attributable to blockade of NFAT, as N-VIVIT should interfere with the binding of CaN to NFAT and reduce its activation. We therefore sought to determine whether CaN regulates synaptic and morphological changes in tectal neurons through NFAT activation.

To determine if neuronal activity can control activation of NFAT by CaN in the Xenopus visual system, we monitored the translocation of NFAT tagged with EGFP (NFAT-GFP) in neurons in the optic tectum of living tadpoles. In some cases neurons were co-transfected with constructs encoding a cell filling td-tomato red fluorescent protein to reveal the full dendritic morphology. Three days after electroporation, animals were immobilized in low melting point agarose and the distribution of NFAT-GFP in these neurons was imaged before and after an intraventricular injection of 100µM AP5 to prevent calcium entry through NMDA receptors . Within 20 min we observed a clear decrease in somatonuclear intensity of NFAT-GFP fluorescence, (-31.5±11%, n=6) compared to controls (-4.3±3.7%, n=7) (Fig 5B). A similar decrease was observed with intraventricular injection of 1µM FK506 (-42±10%; n=8) confirming that CaN mediates the translocation of NFAT-GFP. These results suggest that resting levels of CaN/NFAT activation in the optic tectum are sustained by ongoing NMDAR-mediated synaptic transmission. The observed decrease in NFAT-GFP in the nucleus was not an artifact of intraventricular injection, as injection of 20µM NMDA led to an increase in somatonuclear NFAT-GFP (63±24%; n=5), that could be blocked by pre-incubation of the tadpoles in 1µm FK506 (53.3±18.6%; n=6, Supplemental Fig. S3)

Interestingly, 40 minutes of visual stimulation, using a low-frequency simulated motion stimulus previously demonstrated to induce NMDAR-dependent enhancements in dendritic growth rate and changes in intrinsic excitability in tectal neurons (Aizenman et al., 2002; Sin et al., 2002), was highly effective at driving the translocation of NFAT-GFP to the somatonuclear compartment (57.8±21.6%; n=7, Fig 5). This visually induced translocation of NFAT-GFP could be blocked by preincubation of the animals in either FK506 (-48±11.2%; n=8) or AP5 (-30.9±6.9%; n=8). The specific pattern and timing of visual stimulation appeared to be critical, as stimulation at different frequencies or of animals that were freely swimming did not show significant changes in NFAT-GFP localization (data not shown).

Although it was easier and more sensitive to measure translocation from the dendrites into the entire somatonuclear compartment, the relative changes of NFAT-GFP intensity in the soma were clearly accompanied by similar changes in the nucleus (Supplemental Fig S4). The presence of NFAT protein in the dendritic processes, while possibly a consequence of overexpression in this case, is not without precedent. For example, neuronal processes in mice exhibit intense immunoreactivity for endogenous NFAT (Bradley et al., 2005; Graef et al., 1999; Ho et al., 1994).

Our data suggest that CaN/NFAT has a significant basal level of activation in developing tectal neurons but its level of activity can be further upregulated by visual stimulation *in vivo* in an NMDAR-dependent manner. If expression of exogenous NFAT had resulted in either excessive NFAT activation or the overwhelming of normal CaN signaling, bidirectional regulation of NFAT-GFP translocation would not have been observed. The fact that NFAT activation is dynamically regulated by visual experience makes it an appealing candidate for mediating the CaN-dependent regulation of dendritic morphogenesis and synapse maturation that

we have observed in tectal cells.

To confirm that N-VIVIT expression indeed inhibits NFAT activation in tectal neurons, we compared the nuclear-to-cytoplasmic ratio of NFAT-GFP in neurons expressing N-VIVIT-mCherry, a modified N-VIVIT in which the GFP was replaced with mCherry, and control neurons expressing mCherry-NLS (Fig 5C,D.). We observed a significantly lower resting nuclear-to-cytoplasmic ratio for NFAT-GFP fluorescence in neurons expressing N-VIVIT-mCherry compared to controls (Fig 5D). Furthermore, N-VIVIT-mCherry expressing cells also showed a much smaller increase in nuclear-to-cytoplasmic ratio in response to visual stimulation (Fig 5E). Thus, expression of N-VIVIT, due to its nuclear targeting, did not affect CaN at the synapse (Fig 1) but clearly inhibited NFAT activation at the nucleus.

NFAT regulates mEPSC properties of tectal neurons

We attempted to rescue the effects of N-VIVIT expression on synaptic properties by expression of a constitutively active form of Xenopus NFATc3 (CA-NFAT) (Fig 6A). This CA-NFAT, NFATΔreg lacks most of its regulatory domain and therefore no longer requires binding and dephosphorylation by CaN to become active (Saneyoshi et al., 2002). To determine whether NFATΔreg is able to rescue the physiological consequences of N-VIVIT expression, we recorded mEPSCs from control cells (n=15), cells expressing N-VIVIT (n=10), and cells co-expressing N-VIVIT with NFATΔreg (n=9, Fig 6B). Co-expression of N-VIVIT with NFATΔreg rescued mEPSC frequency (0.94±0.32 Hz), restoring it to the same levels as controls (1.04±0.14 Hz, Fig 6D). The amplitudes of mEPSCs were also reduced toward control levels (Fig. 6C). This result suggests that the effects of N-VIVIT on synaptic properties are mediated at least in part by its inhibition of NFAT-dependent transcriptional activation in the nucleus.

The regulatory domain of NFAT is required for modulation of dendritic morphology

We next attempted to rescue the morphological phenotype of N-VIVIT expression by co-expresion of EGFP, N-VIVIT and NFATΔreg (n=5) in single cells. Neurons co-expressing these three constructs exhibited an increase in the number of branches by day 3 (65±13.5%) that was similar to cells co-transfected with N-VIVIT and EGFP only (82±26%, Fig 6E-H). Thus NFATΔreg, although capable of reversing the change in mEPSC frequency and amplitude, did not prevent the morphological changes induced by N-VIVIT.

The cAMP Response Element-Binding Protein (CREB) is an important mediator of transcriptional control of morphogenesis (Deisseroth et al., 2003; Polleux et al., 2007; Wayman et al., 2006). The NFATΔreg construct lacks the CREB Binding Protein (CBP) binding domain, which is located within the regulatory domain of NFAT (Yang et al., 2001). Consequently NFATΔreg will activate only NFAT-dependent transcription that does not require interactions involving its regulatory domain and by extension possible interactions with CBP or other transcriptional coactivators (Hogan et al., 2003). The inability of NFATΔreg to reduce the enhancement in branch density caused by N-VIVIT expression implies a requirement for the regulatory domain in regulating the morphological, but not the synaptic, effects of NFAT signaling. We reasoned that a different CA-NFAT construct that contained the full regulatory domain should be able to rescue both the branch density and mEPSC phenotypes.

For this experiment, we generated nv-NFAT-st(2+5+8). This mutant mimicked an almost completely dephosphorylated, activated NFAT (Okamura et al., 2000), had its CaN binding domain mutated to VIVIT, and was tagged with three nuclear localization repeats (Fig 7A). This mutant NFAT also rescued the frequency of AMPAR mEPSCs, reducing it to control levels (control 1.04±0.14Hz, n=20; rescue 1.43±0.29pA, n=10; Fig 7B,D). The distribution of AMPAR mEPSC amplitudes was partially shifted toward values closer to those of control cells (Fig 7C).

Next we co-transfected single cells with EGFP, N-VIVIT, and nv-NFAT-st(2+5+8) for in vivo imaging over a three day period. Unlike NFATΔreg, expression of nv-NFAT-st(2+5+8) successfully reduced the rate of increase in branch density caused by N-VIVIT to a level comparable to that of controls by day three of imaging (controls 8.9±9.0%, n=10; mutant 15.5±13.4%, n=6; p=0.42). This differential ability of NFATΔreg and nv-NFAT-st(2+5+8) to rescue morphology was not due to a difference in the expression efficiency of the two constructs, as western blotting for myc-tagged versions of these two CA-NFATs showed that the construct that gave the more complete rescue (nv-NFAT-st(2+5+8)) was in fact expressed at slightly lower levels (Supplemental Fig S5).

These experiments confirm that CaN activates members of the NFAT family of transcription factors to negatively regulate synaptic development and dendritic branch addition. Furthermore, the transcriptional control of dendritic arbor morphology by NFAT appears to require interactions through its regulatory domain. Therefore NFAT modulation of synaptic properties is separable from its regulation of dendritic morphology.

Discussion

One of the principal signaling events mediated by CaN is the regulation of gene transcription. The NFAT family of transcription factors is strongly regulated by CaN activity. The experiments presented here show that nuclear transcription downstream of NFAT activation is a key regulator of the ability of neurons to undergo synaptic and dendritic structural changes in the developing visual system. Our results reveal that basal levels of CaN/NFAT activity in optic tectal neurons normally sit at intermediate levels, susceptible to reduction or elevation by changes in neural activity. In response to chronic inhibition of the CaN/NFAT interaction we observed a dramatic increase of AMPA mEPSC frequency and amplitude and a massive

proliferation of new dendritic branches. Furthermore, these effects on physiology and morphology appear to be regulated independently by distinct NFAT interactions in the nucleus. The expression of NFATΔreg, a CA-NFAT construct lacking its regulatory domain, was able to rescue only the physiological consequences of CaN/NFAT suppression, whereas rescue of the dendritic branching phenotype was achieved by expressing nv-NFAT-st(2+5+8), a CA-NFAT in which the regulatory domain is available to bind transcriptional coactivators. Importantly, the morphological changes downstream of NFAT-mediated gene transcription were found to be gated acutely by synaptic activity. Thus, sensory input plays a regulatory role both in the induction and in the ultimate expression of transcriptional events that modulate the synaptic and morphological properties of a neuron.

The ability of CA-NFAT to rescue the phenotype caused by overexpression of N-VIVIT implies that N-VIVIT exerts its effects in cells by interfering with the activation of NFAT by CaN. Despite this and other evidence for CaN-regulated nuclear transcription in neurons (Bito et al., 1996; Flavell et al., 2006; Shalizi et al., 2006), the subcellular compartments where CaN acts to regulate transcription remain unclear. There are reports of translocation of CaN from dendrites to neuronal somata (Yasuda et al., 2003). We also observed a striking translocation of NFAT-GFP from the dendrites to the cell soma and nucleus in response to neuronal activity, indicating that NFAT can be activated by synaptic, or at least dendritic, CaN. However, the fact that N-VIVIT, an NLS-tagged inhibitor of the CaN-NFAT interaction clearly reduced the nuclear-to-cytoplasmic ratio of NFAT-GFP, suggests that a critical regulatory interaction between NFAT and CaN also occurs at the nucleus. It seems unlikely that CaN and NFAT can translocate into the nucleus as a complex. There are two possible mechanisms by which CaN could regulate NFAT activity at the nucleus,: The first is that the small resident pool of CaN in

the nucleus (Fig 1F) may be regulated by nuclear calcium or calmodulin signaling (Deisseroth et al., 1998; Saha & Dudek 2008). The second possibility is that activated CaN accumulates in the perinuclear compartment where it could rapidly reverse the effects of kinases like GSK3ß and DYRK1A that drive the nuclear export of NFAT.

Implications for the synaptrotropic hypothesis and its applicability to dendritic remodeling in the visual system

The synaptotropic hypothesis postulates that interactions at the synapse will play a role in the modulation of the innate tendency of neurons to branch by providing the extrinsic control capable of directing branching into patterns that optimize synaptic interactions (Vaughn, 1989). Our data extend this model by providing evidence that synaptic transmission, in addition to mediating local interactions, also carries information in the patterns of synaptic activity that, through NFAT activation, provide feedback to the nucleus to alter the propensity of the neuron to branch and develop synapses. Thus the transcriptional profile of the cell, referred to by Vaughn as "innate" properties, can be drastically altered by patterned synaptic activity. Furthermore, we also observed that acutely blocking synaptic activity could regulate the continued addition of new branches. These findings suggest that synaptic activity is able to both modulate the innate ability of the neuron to branch, and to direct branching into patterns that optimize connectivity.

Regulation of branching in the visual system

Another postulate of the synaptotropic hypothesis is that dendritic growth or branching is most likely to occur in regions of the arbor where there is a stabilized synapse. By this logic, retraction should occur in regions where synapses fail to mature or become destabilized. Studies in the retinotectal system have supported a relationship between the stabilization of dendritic

branches and synaptic maturation, characterized by the increase in AMPA/NMDA ratio that occurs as AMPARs are delivered to NMDAR-only "silent" synapses (Wu et al., 1996). Expression in tectal neurons of a constitutively-active form of calcium/calmodulin-dependent protein kinase II (CaMKII) leads to rapid un-silencing of synapses; the same manipulation results in stunted dendritic arbor elaboration, suggesting accelerated stabilization of existing branches (Wu and Cline, 1998). In contrast manipulations that reduce CaMKII activity or prevent the delivery of AMPARs to synapses lead to unfettered dendritic growth and abnormal branch elaboration (Haas et al., 2006; Zou and Cline, 1999).

Our result that inhibition of CaN prevents at least one form of AMPAR LTD in tectal neurons and leads to a concomitant increase in dendritic branch number is superficially consistent with this model. However, several lines of evidence suggest that a direct causal relationship may not exist in this particular case. First of all, short interval imaging revealed that the increase in branch number was attributable to an increase in the rate of branch additions, rather than to a decrease in the rate of branch retractions that might be expected from blockade of LTD. Secondly, in contrast to AI-XCN and N-VIVIT expression, diffusible GFP-VIVIT decreased AMPAR mEPSC amplitudes, yet all three manipulations increased branch densities. Finally, the rescue of mEPSC frequencies and amplitudes through expression of NFATΔreg did not reverse the increase branching phenotype, which appeared to require the additional transcriptional interactions in nv-NFAT-st(2+5+8) to be restored to control levels. We therefore observed a clear dissociation between synaptic maturation and dendritic branching in this case. Our data does not rule out the possibility of an independent contribution of synaptic LTD-like mechanisms to dendritic remodeling, but simply reveals that activity-dependent regulation of CaN and NFAT signaling is able to exert potent, separable influences on morphology and

synaptic development through transcriptional control.

Coordination between transcription factors for control of dendritic morphology

The enhanced branching phenotype caused by N-VIVIT could be almost entirely rescued by co-transfection of nv-NFAT-st(2+5+8), a CA-NFAT that contains an intact regulatory domain capable of interacting with other transcriptional co-factors. It is believed that NFAT requires independently regulated transcriptional co-activators, referred to as NFATn, to bind DNA (Belfield et al., 2006; Flavell et al., 2006; Ho et al., 1994; Wu et al., 2007). The identities of the putative co-activators responsible for the morphological and synaptic phenotypes we observed are not yet known, but several transcription factors important in neuronal plasticity, including CREB and MEF2, are known to interact with NFAT. Although CREB does not appear to bind to NFAT, both interact directly with CBP (Blaeser et al., 2000; Garcia-Rodriguez and Rao, 1998; Yang et al., 2001). The binding of CBP to NFAT greatly potentiates NFAT signaling. NFAT and CREB may also further cooperate to activate specific sets of genes that differ from those activated by either transcription factor alone (Sato et al., 2006). In contrast, direct co-immunoprecipitation of MEF2A and MEF2D with NFATc2 has been demonstrated in T lymphocytes, where CaN-dependent activation of MEF2 requires NFAT (Blaeser et al., 2000). In neurons CaN is also able to dephosphorylate MEF2A and MEF2D and thus directly regulate synaptogenesis (Flavell et al., 2006; Shalizi et al., 2006). Given the ability of CA-NFAT to rescue the effects of N-VIVIT and the absence of an NFAT-like CaN-binding consensus sequence in MEF2, we consider it unlikely that the morphological plasticity caused by N-VIVIT is due to any direct inhibition of MEF2 dephosphorylation, but could potentially reflect the cooperation of MEF2 with NFAT. It will be interesting in the future to explore the consequences for neuronal development of interactions of NFAT and its co-activators.

These observations begin to delineate a hierarchy of CaN function in the control of neuronal development. Basal levels of neuronal CaN activity are governed by activity-dependent activation of calcium influx. In addition to inducing a rapid, local reduction in synaptic efficacy through LTD, CaN signaling also exerts control over gene transcription in the nucleus through its regulation of transcription factors including NFAT. These transcriptional programs then regulate, directly or indirectly, the rates of dendritic branch dynamics across the dendritic tree as well as the potential of the cell to change its number and maturity of AMPAR containing synapses.

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Figure Legends

Figure 1 AMPAR mEPSC frequency and amplitude are increased by N-VIVIT, a non-synaptic inhibitor of CaN. A. Sample recording epochs from tectal cells transfected with EGFP, AI-XCN, GFP-VIVIT, or N-VIVIT. **B.** Chronic CaN inhibition increases mEPSC frequency. **C.** Sample AMPAR mEPSCs from transfected tectal cells (50 events averaged). **D.** Cumulative probability plots of mEPSC amplitudes, (n= 15, 10, 12, 10 cells, 100 events per cell, each condition, $p<0.01$ vs. control for all conditions by K-S test). **E.** Tectal cells bulk electroporated to express N-VIVIT and cell filling mCherry sequestered N-VIVIT in the nucleus (top panel) where it colocalizes with mCherry-NLS (bottom panel). **F**.(i) Western blot of Xenopus brain using anti-

CaN antibody. (ii) Injection at the four-cell stage of fluorescently tagged antisense Morpholino oligonucleotides (MO) against CaN produces knockdown in part of each animal. Sections through the CNS of a stage 27 tadpole immunostained for CaN and the nuclear marker ToPro3. (iii) CaN antisense MOs reduced nuclear immunofluorescence while control MOs had no effect (N=50 cells, each condition, paired t-test ***,$p<0.005$). **G**. N-VIVIT does not prevent CaN-dependent synaptic plasticity. ChemLTD was blocked in cells expressing GFP-VIVIT, but not in N-VIVIT expressing cells. Inclusion of 1μM FK506 in the patch pipette prevented LTD in N-VIVIT expressing cells. Insets: traces for one representative cell before and after chemLTD, each point averaged over 1 min **H.** Change in AMPAR mEPSC amplitudes following chemLTD induction. *$p<0.05$, **$p<0.01$, ANOVA. Scale bars are 10μm in all figures.

Figure 2 - Inhibition of CaN by AI-XCN, GFP-VIVIT, N-VIVIT or FK506 all drive increases in dendritic complexity. **A-E**. Two-photon z-projections and dendritic arbor reconstructions of EGFP-expressing neurons imaged once daily over a three day period. Individual neurons were electroporated with EGFP only (**A**) or co-transfected with AI-XCN (**B**), GFP-VIVIT (**C**), N-VIVIT (**D**) or given bath application of FK506 for 3 days (**E**). Reconstructions have been rotated in 3D to align dendritic arbors over time. Insets: higher magnification projections of the main dendrite reveals fine branch tip elaboration in CaN-inhibited neurons. Z-projections have been cropped to exclude unlabeled fields. Axons (arrowheads) were not included in the analysis. **F**. Dendritic arbor length was not affected by CaN inhibition. **G.** All forms of CaN inhibition similarly increased the number of dendritic branch points from day 1 to day 3 (n=6 to 10 per group, * $p<0.05$, ANOVA). All groups had similar total lengths and numbers of branch points on day 1. **H**. Percent change in total length compared to day 1. **I.** Percent change in the number of branch points normalized to day 1. **

p<0.01 ANOVA with Dunn's post-test.

Figure 3 - Blocking CaN signaling with either AI-XCN or N-VIVIT increases dendritic branch dynamics. **A.** Three dimensional reconstructions of cells imaged at 40 minute intervals over 2 hours on day 3 after transfection. Cells expressing either AI-XCN (n=5) or N-VIVIT (n=6) exhibited more dynamic branch behaviors compared to controls (n=5). **B.** Quantification of the number of branches added and lost per neuron over the 2 hour imaging interval. * p<0.05, **p<0.01, ANOVA.

Figure 4 - Synaptic activity gates the enhanced branch addition induced by N-VIVIT. A. Three dimensional reconstructions of cells expressing EGFP with or without N-VIVIT imaged at 40 minute intervals for 2 hours on day 3 after transfection. **B.** Blockade of synaptic input immediately after the first image by addition of AP5, GYKI and PTX to the rearing solution reduced the rate of branch additions in N-VIVIT expressing cells to control levels (n=6, all conditions, ** p<0.01 ANOVA). There was no effect of synaptic blockade on branch dynamics in control cells. **C.** Initial and final number of branch tips after 2 hours for cells expressing N-VIVIT with normal synaptic activity or under synaptic blockade. Mean values plotted in gray. ** p<0.01 paired t-test.

Figure 5 - Visually driven NMDAR activity activates CaN/NFAT in vivo.

NFAT-GFP translocates towards the nucleus upon dephosphorylation by activated CaN**. A**. Representative tectal cell co-transfected with cell-filling td-tomato and NFAT-GFP. A resting level of NFAT-GFP is present in the dendrites, cell soma and nucleus. Following 40 min of repeated visual stimulation translocation of NFAT-GFP toward the soma and nucleus from the dendrites was observed. *Lower panel* shows single optical sections in three planes through the cell soma of NFAT-GFP fluorescence following visual stimulation. **B.** Pre-incubation of the

animals in 1 µM FK506 (N=8) or 100 uM AP5 (N=6) reduced the amount of somatonuclear NFAT-GFP and blocked the visual stimulation-induced increase (* $p<0.05$, ANOVA). **C-E.** N-VIVIT inhibits nuclear translocation of NFAT-GFP. **C.** NFAT-GFP expressing neuron, co-expressing mCherry-NLS to mark the nucleus, shows robust nuclear translocation of NFAT-GFP at 10min following visual stimulation that returns to baseline within 4hr. In neurons expressing N-VIVIT-mCherry (arrowhead), NFAT-GFP translocation is greatly reduced. **D**. N-VIVIT expression reduces the baseline nuclear-to-cytoplasmic ratio of NFAT-GFP fluorescence (** $p<0.001$, t-test). **E.** N-VIVIT reduces the increase in nuclear-to-cytoplasmic ratio of NFAT-GFP induced by visual stimulation.

Figure 6 -NFATΔreg rescues electrophysiological effects of N-VIVIT expression. A. Schematic of full-length NFAT and NFATΔreg mutant lacking regions of the regulatory domain (Saneyoshi et al., 2002) **B**. Sample recording epochs from tectal cells transfected with EGFP alone, or cotransfected with N-VIVIT or N-VIVIT plus NFATΔreg. **C.** Cumulative probability plots of mEPSC amplitudes shows a shift down toward control values with NFATΔreg expression (n=15 control, 10 N-VIVIT, and 9 N-VIVIT+NFATΔreg, 100 events per cell, each condition). **D.** mEPSC frequencies are restored to control values by NFATΔreg. **E-F.** Reconstructions of dendritic arbors from EGFP-expressing tectal neurons coexpressing N-VIVIT or N-VIVIT plus NFATΔreg on day 1 and day 3 of imaging. **G.** number of branch points in NFATΔreg expressing cells is not different from N-VIVIT cells. **H.** Change in number of branch points normalized to day 1 branch number shows no rescue of morphology by NFATΔreg (n=5 to 10 per group) *$p<0.05$, **$p<0.01$, ANOVA.

Figure 7 -NFAT regulatory domain interactions rescue dendritic morphology. A. Schematic of NV-NFAT-st(2+5+8) construct with intact regulatory domain mutated to mimic dephosphorylation. **B.** Sample recording epochs from tectal cells transfected with EGFP alone, cotransfected with N-VIVIT or with N-VIVIT plus nv-NFAT-st(2+5+8). **C.** Cumulative probability plots for mEPSC amplitudes shows a shift down toward control values with nv-NFAT-st(2+5+8) expression (n= 15, 10, 10 cells, 100 events per cell, each condition). **D.** mEPSCs frequencies are restored to control values by nv-NFAT-st(2+5+8)**. D-E.** Reconstructions of dendritic arbors from EGFP-expressing N-VIVIT and nv-NFAT-st(2+5+8)-rescued neurons on day 1 and day 3 **F.** The number of branch points is reduced to control levels in nv-NFAT-st(2+5+8)-rescued neurons compared to cells expressing N-VIVIT (*$p<0.05$, **$p<0.01$, ANOVA), **G.** Change in the number of branch points normalized to day 1 branch number is rescued by nv-NFAT-st(2+5+8) (n=6 to 10 per group). **$p<0.01$ N-VIVIT vs. control, † $p<0.05$ N-VIVIT vs. nv-NFAT-st(2+5+8), ANOVA.

Supplemental materials

Constructs and reagents

N-VIVIT was generated by isolating the nuclear localization sequences from the Clontech pECFP-Nuc vector with BsrGI and Afl II and inserting them into the GFP-VIVIT plasmid with the same cut sites.

V-NFAT-st(2+5+8) was generated by mutating amino acids 101 to 106 of XNFAT from SIQIT to VIVIT and by mutation of 9 serines located at positions 171,177,216,220,224,268,272,276,280 to alanines to mimic an almost completely dephosphorylated NFAT (Okamura et al., 2000). These mutations were performed by PCR using the QuikChange Multisite-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Primers used for this kit were designed based upon the design tool on the Stratagene website http://www.stratagene.com/sdmdesigner/default.aspx. Following these mutations of XNFAT, PCR primers 5'ATCTTGTACATAGAATACAAGCTACTTGTT3' and

5'CCTGGCCTCGAGCGTGAGACATGTC 3' were used to amplify a PCR fragment of v-nfatst(2+5+8) with BsrGI and XhoI cut sites. This fragment was then digested with the respective enzymes and inserted into Clontech pECFP-Nuc vector to form the nv-NFAT-st(2+5+8).
To generate N-VIVIT-mCherry, first an EGFP-NLS was made by inserting the NLS (see above) using the BsrGI and Afl II sites into the pEGFP-N1 vector (Clontech). EGFP was then replaced with mCherry using the HindIII-BsrGI sites. N-VIVIT was PCR amplified from the VIVIT plasmid with primers 5'tataaagcttGCTCAAGCTTCGAATTCTGC3' and 5'atatggtaccCTCACCATGCTAGCGACAGG3' and then inserted at HindIII-KpnI sites into mcherry-NLS.

RT-PCR

Primers used were BDNF IV: forward CACAGCTCCAGAGGGCTGATCA, reverse AATGGCTCCATTTGATCTTGTT, IP3r: forward TGTGTTGGCGGAAGATACAA, reverse CAGGCTCACAAAGGTGTCAA, Kv2.1: forward GACGGGTGGTGCAGATATTC, reverse TCTCAAGGTGAAACCCAAGG, GAPDH forward GCTCCTCTCGCAAAGGTCAT, reverse GGGCCATCCACTGTCTTCTG. The run was performed on a 7300 Applied Biosystems realtime
PCR cycler and consisted of 30min at 48oC, 10min at 95oC, 40 x (15sec at 95oC, 1min at 60oC), well volume 20 ul. After completion, the product was run on a 2% agarose gel to confirm the specificity of the primers.

Whole brain transfections

All animal experiments were approved by the McGill University Animal Care Committee. Tadpoles were bred by HCG induced mating of albino Xenopus laevis frogs in the Montreal Neurological Institute Animal Care Facility. Embryos were then reared in bowls containing Modified Barth Solution-H.

Whole brain electroporation was used to transfect multiple neurons within the optic tectum for electrophysiology and translocation experiments. Briefly the plasmids of interest, suspended in water or Tris acetate buffer at a concentration of 1 to 8ug/uL were injected into the brain ventricles of stage 42-43 animals (Nieuwkoop and Faber, 1956) anesthetized with 0.02%MS-222. As described in Ruthazer et al (2005), multiple cells within the tectum were electroporated by application of a 1.5ms pulse of 30 to 40mV that was delivered across the tectum by means of a pair of platinum plate electrodes positioned on the skin overlying the the lateral sides of each

tectal lobe using a SD9 Grass stimulator with a 3μF capacitor placed in parallel to generate an exponentially decaying pulse. Animals were returned to their rearing tanks for one to three days to allow expression of the constructs before imaging or electrophysiology experiments.

Single cell transfections

Single neurons in the optic tecta of MS-222 anesthetized tadpoles were electroporated with plasmids of interest as described by Haas et al. (2001). Briefly a glass pipette with an approximate tip diameter of 1 μm containing plasmids dissolved in water or Tris acetate buffer at a concentration of 1 to 8ug/uL was positioned in the superficial tectum under microscope guidance using a Narashige micromanipulator , a 0.5s 200 Hz burst of 1ms squarewave pulses was then applied using a grass SD9 stimulator. One day later the first images of single neurons expressing the constructs were collected. Unless otherwise indicated, farnesylated EGFP was expressed to visualize complete dendritic morphology.

Live imaging

A custom built two-photon microscope was used for all live imaging experiments. The microscope consisted of a converted Fluoview FV300 confocal microscope mounted on a BX61WI base (Olympus, Japan) with external R3896 multialkali PMTs (Hamamatsu, Japan) for detection of emission signal. Red and green emission light was simultaneously collected after passing through a 565DCLPXR beamsplitter with HQ525/50 and HQ607/45 filters specially blocked for two-photon excitation (Chroma Technology, Brattleboro, VT). Excitation was provided by a Maitai-BB Ti:Sapphire femtosecond pulsed IR laser at 910nm or 990nm (Spectra Physics, Mountain View, CA). Optical z-series were collected at 1μm intervals using a 60x 1.1NA water immersion objective (Olympus).

Quantification for translocation experiments

To determine the amount of somatic translocation of NFAT-GFP, average intensity projections of z-series containing a set number of optical sections through the somata of identified neurons were generated using ImageJ software. Two areas were then defined: The first consisted of a rectangular area that included the soma and a small surrounding area; the second consisted of a rectangular area in the background. Change in fluorescence intensity was measured by comparing the change in somatic intensity after correcting for background.

To determine the amount of nuclear translocation in cells expressing NLS-mCherrry

constructs, average intensity projections of z-series containing a set number of optical sections through the nucleus were generated using ImageJ software. The perimeter of the nucleus as defined by the mcherry was traced by hand. This perimeter was then collapsed by 1um, thereby selecting a region in the middle of the nucleus. A band surrounding this region was generated to include the soma and exclude the nucleus. The ratio of nuclear-to-somatic intensity was then calculated.

Western blots

Brains were extracted in a HEPES buffered extraction buffer containing protease inhibitor SetV (Calbiochem, San Diego) Samples were separated on acrylamide gel, transferred to PVDF membrane and stained with either mouse anti-human calcineurin antibody (BD Transduction laboratories, C19220, diluted 1:10000) or mouse anti-myc antibody (Invitrogen R950-25, 1:20000) and rabbit anti-β-tubulin (Santa Cruz, sc-9104, 1:20000) as a loading control.

Morpholino antisense

10ng of fluorescent lissamine-tagged Morpholino antisense oligonucleotide (MO) MOXCn, (sequence CTTCATTGGCCTTGTGCTCGGACAT) from Gene Tools (Oregon) targeting Xenopus calcineurin A (Genbank: AF019569) was injected into one dorsal animal blastomer in Xenopus embryos at the four-cell stage to target one-half of the spinal cord. As a control, 40 ng of nonsense sequence (MOCtrl) CCTCTTACCTCAGTTACAATTTATA was injected. Embryos were allowed to develop until stage 27 and were then fixed in PFA followed by Dent's fixation. Animals were sectioned coronally and then immunostained with mouse antihuman

calcineurin antibody (BD Transduction laboratories, C19220, diluted 1:500) , nuclei were stained with ToPro3 (Invitrogen).

Using ImageJ software nuclear calcineurin signal was quantified in confocal optical sections, collected on a Zeiss Meta 510 by measuring immunofluorescence in 3μm diameter regions-of-interest (centered in the nuclei) from pairs of cells symmetrically located in the MO+ and MO- halves of each spinal cord. 50 pairs of cells in each group were selected blind to the calcineurin staining pattern. Levels of expression were compared for matched pairs in each section and between MoXCn and MOctrl groups.

Electrophysiology

The procedure for recording AMPAR mEPSCs was performed as described by Haas et al.

(2006). Brains were dissected out and placed into external ACSF solution containing 115 mM NaCl, 2 mM KCl, 3 mM CaCl2, 3 mM MgCl2, 5 mM HEPES, 10 mM glucose, 0.1 mM picrotoxin, 1 uM TTX [pH 7.2; osm, 255]. Patch pipettes containing 90 mM Csmethane sulfonate , 5 mM MgCl2, 20 mM TEA, 10 mM EGTA, 20 mM HEPES, 2 mM ATP, 0.3 mM GTP [pH 7.2; osm, 255] were used to obtain whole cell access to cells visualized with an Olympus BX51WI with a 60X 0.9 NA water immersion objective. Transfected neurons were identified by epifluorescence excitation of EGFP. AMPAR mediated mEPSCs were then isolated by clamping the cell at -60 mV. A minimum of 100 events were recorded per cell. Only cells that met the following criteria were selected for analysis, a holding current of <25 pA for -60 mV, Rm ~1 Gohms, access resistance <80 Mohms. Minis were dectected and analyzed with Synaptosoft mini-analysis software (Fort Lee, NJ). To induce chemLTD cells were held at -35 mV during the 3 minute bath application of 20 μm NMDA.

Statistics

All numbers are presented as mean ± SEM. A p-value less than 0.05 was considered significant. Unless otherwise indicated, a two-tailed Student's t-test was used for comparisons of two groups and ANOVA with Newman-Keuls post-test was used to compare more than two conditions. The Kolmogorov-Smirnov test was used to test for normality and to compare cumulative probability distributions.

Figure 1

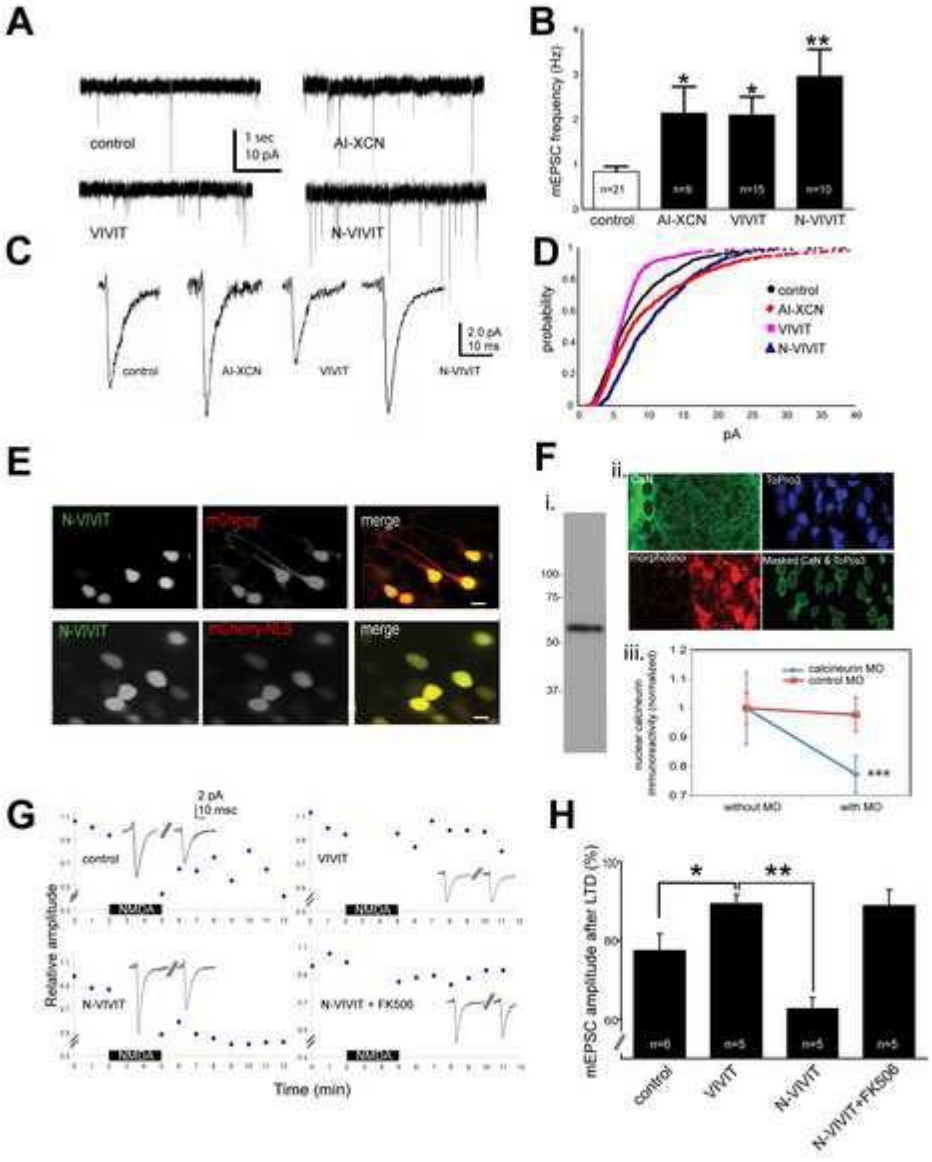

A
control
1 sec
10 pA
AI-XCN
VIVIT
N-VIVIT
B
mEPSC frequency (Hz)
control
AI-XCN
VIVIT
N-VIVIT
C
control
AI-XCN
VIVIT
N-VIVIT
2.0 pA
10 ms
D
probability
control
AI-XCN
VIVIT
N-VIVIT
pA
E
N-VIVIT
merge
F
i.
ii.
iii.
calcineurin MO
control MO
without MO
with MO
G
2 pA
10 msec
control
VIVIT
N-VIVIT
N-VIVIT + FK506
NMDA
Relative amplitude
Time (min)
H
mEPSC amplitude after LTD (%)
control
VIVIT
N-VIVIT
N-VIVIT+FK506

Figure 2

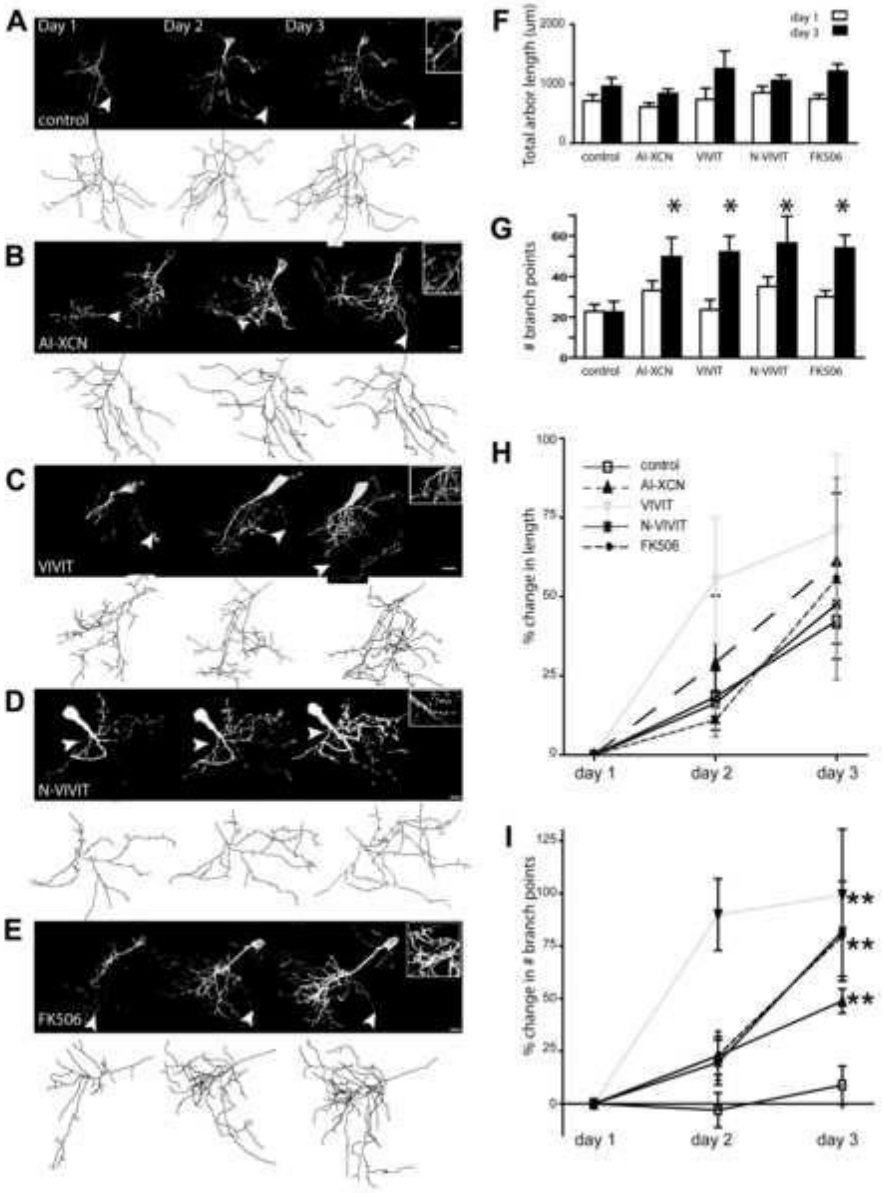

A
Day 1
Day 2
Day 3
control
B
AI-XCN
C
VIVIT
D
N-VIVIT
E
FK506
F
Total arbor length (um)
day 1
day 3
control
AI-XCN
VIVIT
N-VIVIT
FK506
G
branch points
H
% change in length
control
AI-XCN
VIVIT
N-VIVIT
FK506
day 1
day 2
day 3
I
% change in # branch points
day 1
day 2
day 3

Figure 3

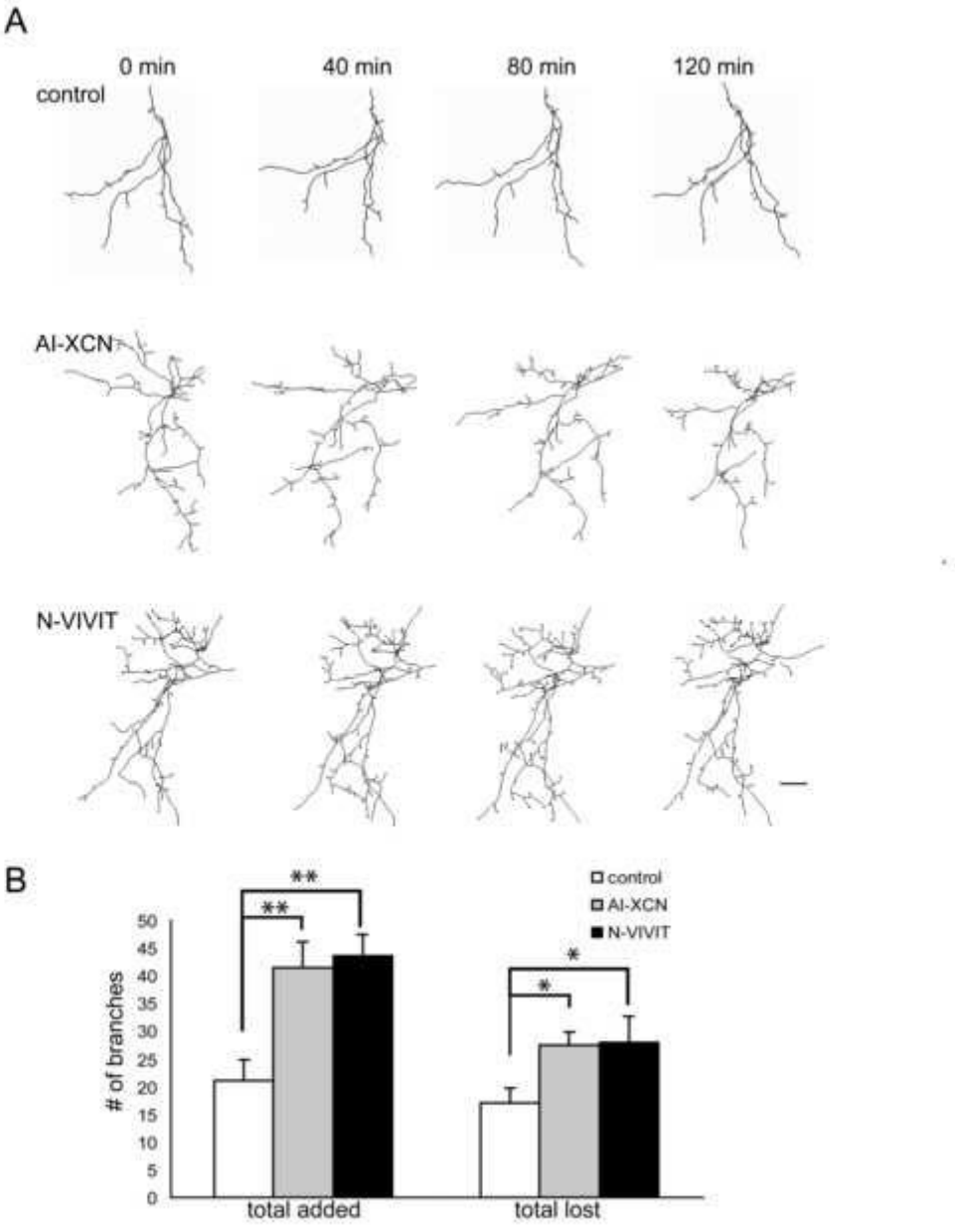

A
0 min
40 min
80 min
120 min
control
AI-XCN
N-VIVIT
B
control
AI-XCN
N-VIVIT
# of branches
50
45
40
35
30
25
20
15
10
5
0
**
**
*
*
total added
total lost

Figure 4

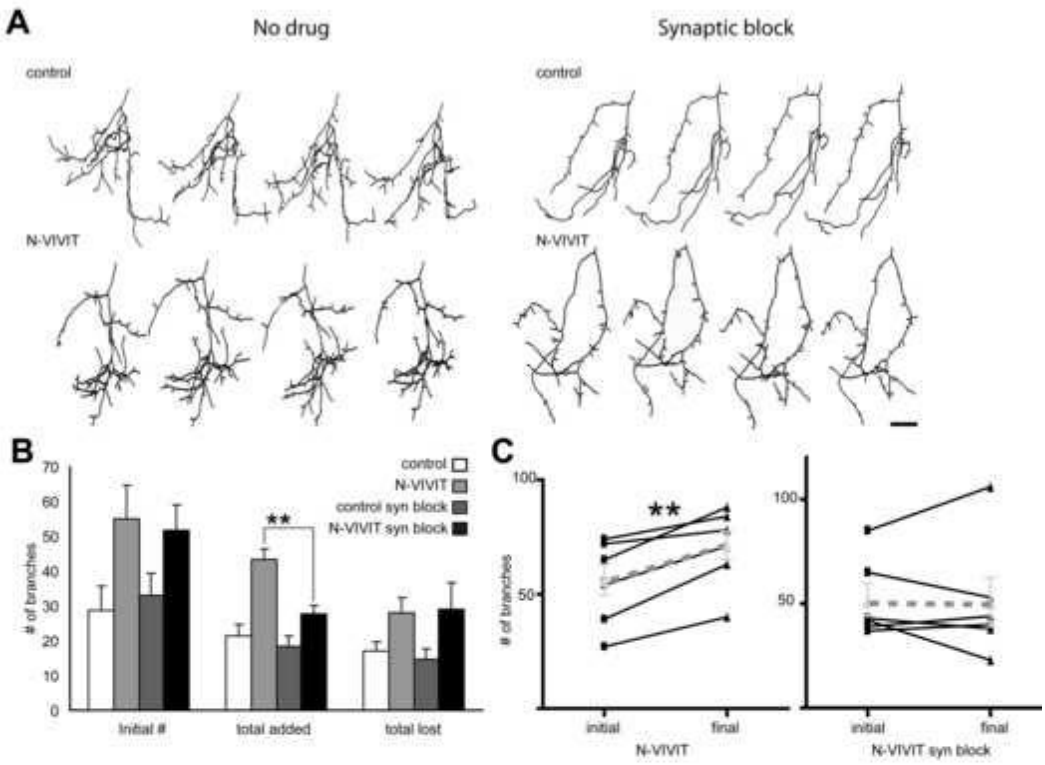

A
No drug
Synaptic block
control
N-VIVIT
control
N-VIVIT
B
control
N-VIVIT
control syn block
N-VIVIT syn block
# of branches
70
60
50
40
30
20
10
0
**
Initial #
total added
total lost
C
# of branches
100
50
**
initial
final
N-VIVIT
initial
final
N-VIVIT syn block

Figure 5

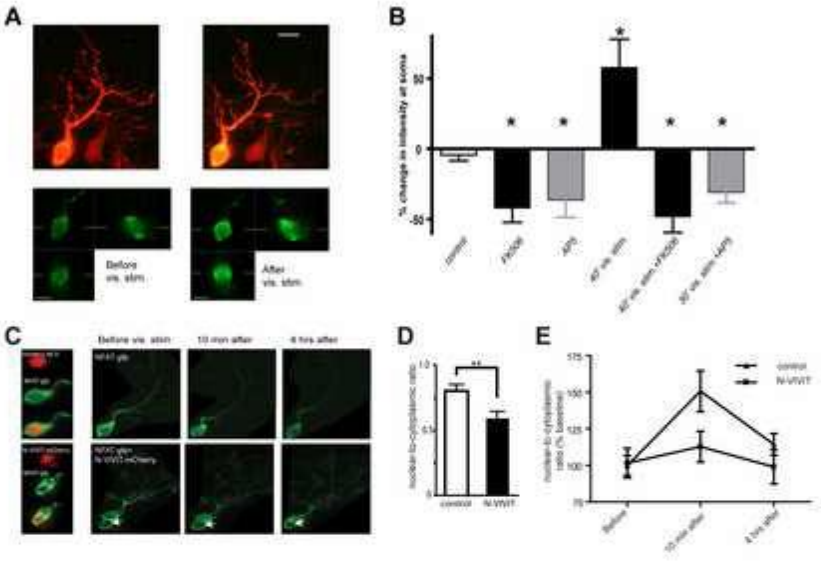

A
Before vns. stim.
After vns. stim.
B
% change on intensity at soma
control
FK506
APV
40' vns. stim.
40' vns. stim.+FK506
20' vns. stim.+APV
C
Before vns. stim.
10 min after
4 hrs after
D
control
N-VIVIT
E
control
N-VIVIT
Before
10 min after
4 hrs after

Figure 6

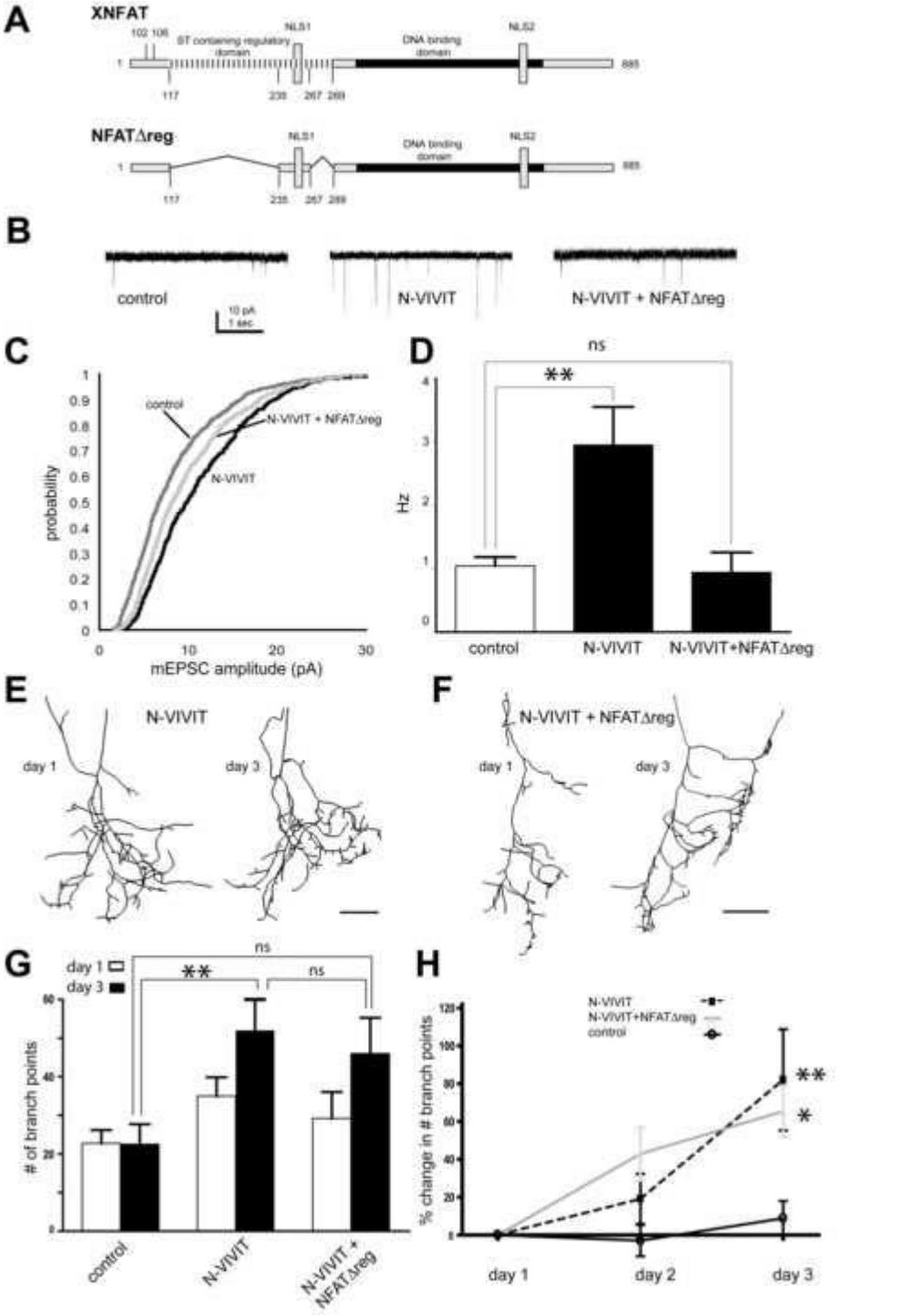
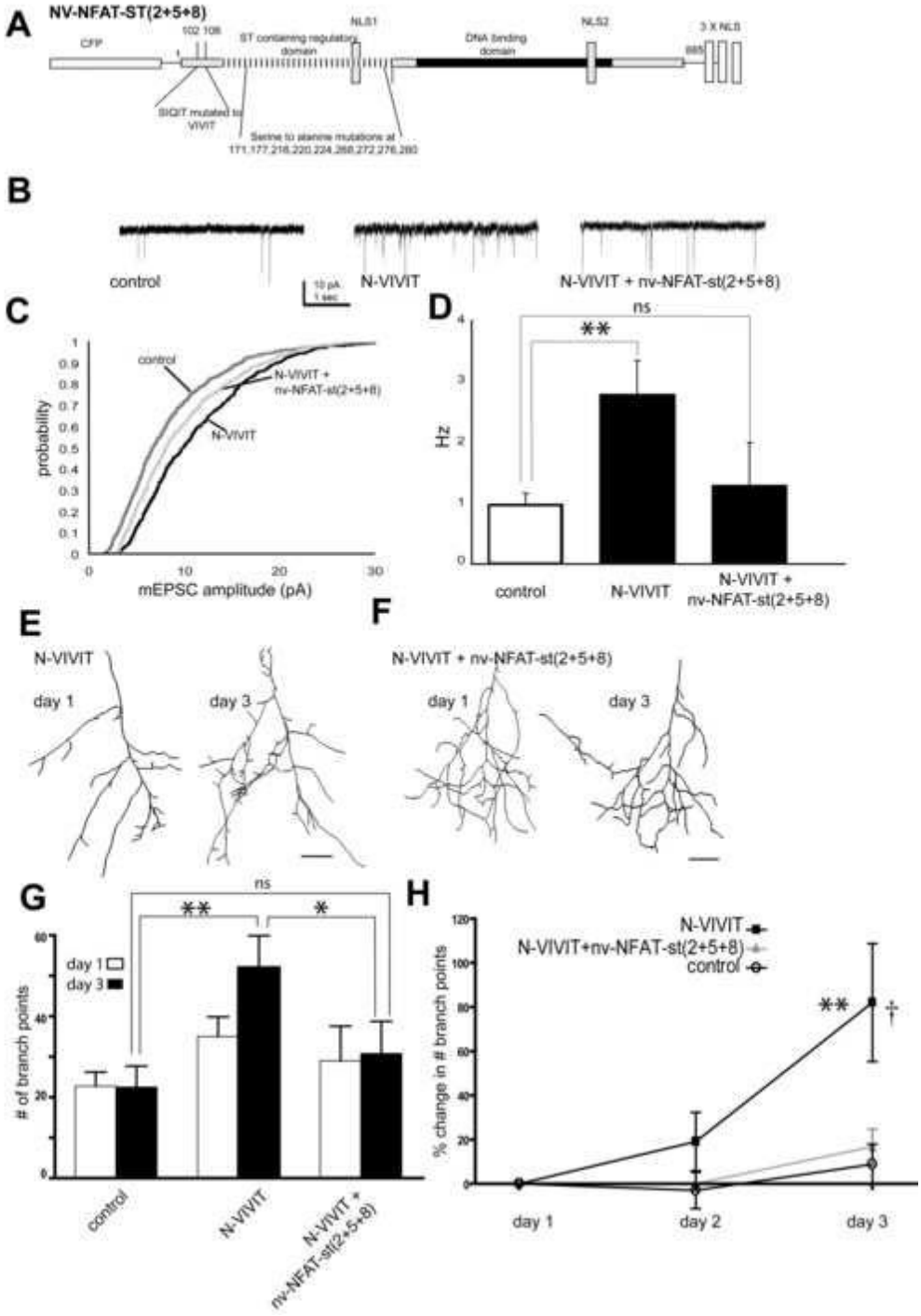
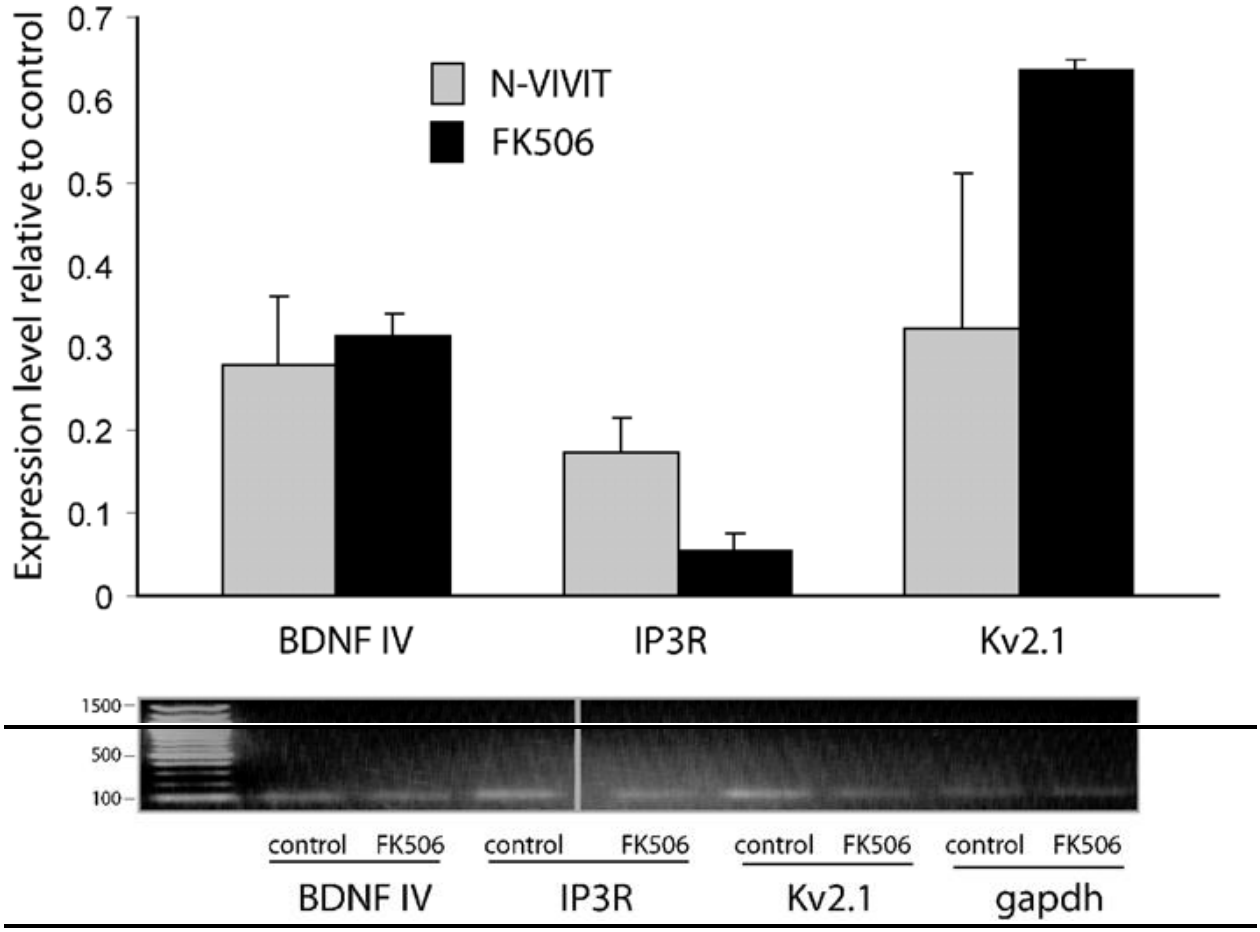

A
XNFAT
NFATΔreg
NLS1
NLS2
DNA binding domain
B
control
N-VIVIT
N-VIVIT + NFATΔreg
C
probability
mEPSC amplitude (pA)
D
Hz
ns
**
control
N-VIVIT
N-VIVIT+NFATΔreg
E
N-VIVIT
day 1
day 3
F
N-VIVIT + NFATΔreg
G
# of branch points
day 1
day 3
H
% change in # branch points
day 1
day 2
day 3

Figure 7

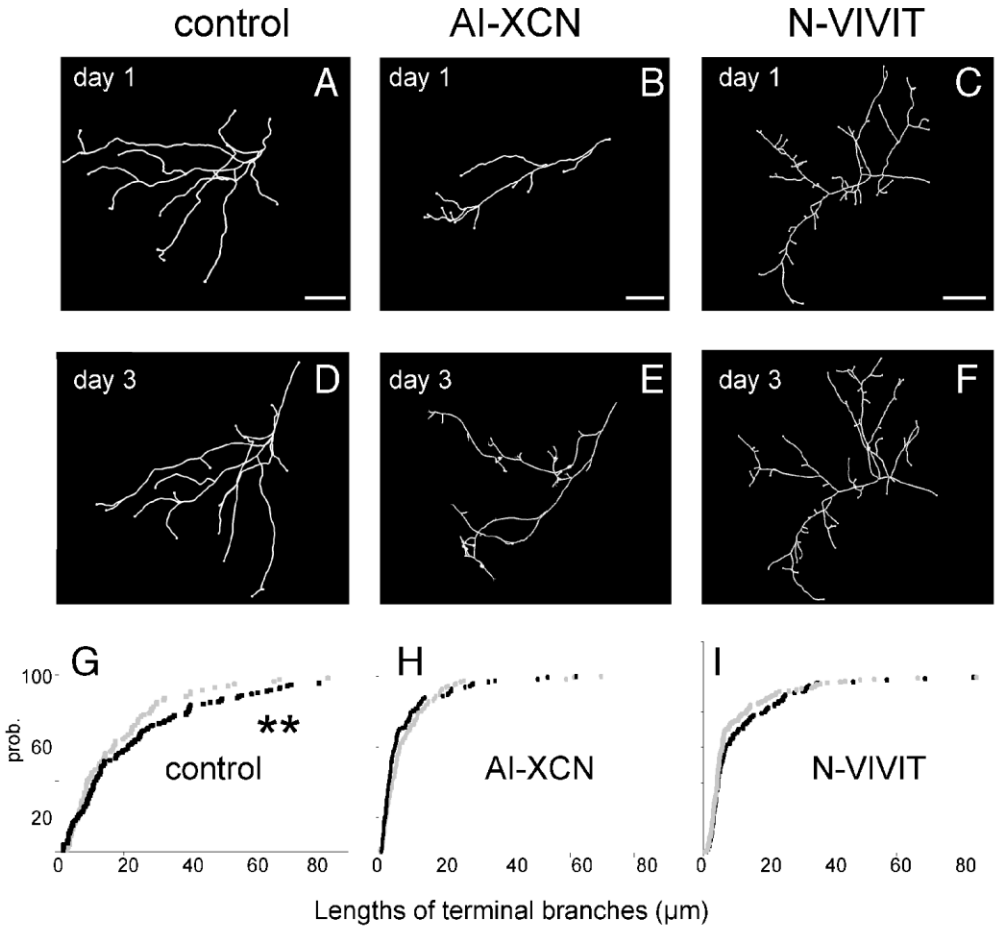

A
NV-NFAT-ST(2+5+8)
CFP
102 108
ST containing regulatory domain
NLS1
DNA binding domain
NLS2
3 X NLS
SIQIT mutated to VIVIT
Serine to alanine mutations at 171,177,216,220,224,268,272,276,280
B
control
10 pA
1 sec
N-VIVIT
N-VIVIT + nv-NFAT-st(2+5+8)
C
probability
control
N-VIVIT + nv-NFAT-st(2+5+8)
N-VIVIT
mEPSC amplitude (pA)
D
ns
**
Hz
control
N-VIVIT
N-VIVIT + nv-NFAT-st(2+5+8)
E
N-VIVIT
day 1
day 3
F
N-VIVIT + nv-NFAT-st(2+5+8)
day 1
day 3
G
ns
**
*
day 1
day 3
# of branch points
control
N-VIVIT
N-VIVIT + nv-NFAT-st(2+5+8)
H
N-VIVIT
N-VIVIT+nv-NFAT-st(2+5+8)
control
**
†
% change in # branch points
day 1
day 2
day 3

Supplemantal Figure 1



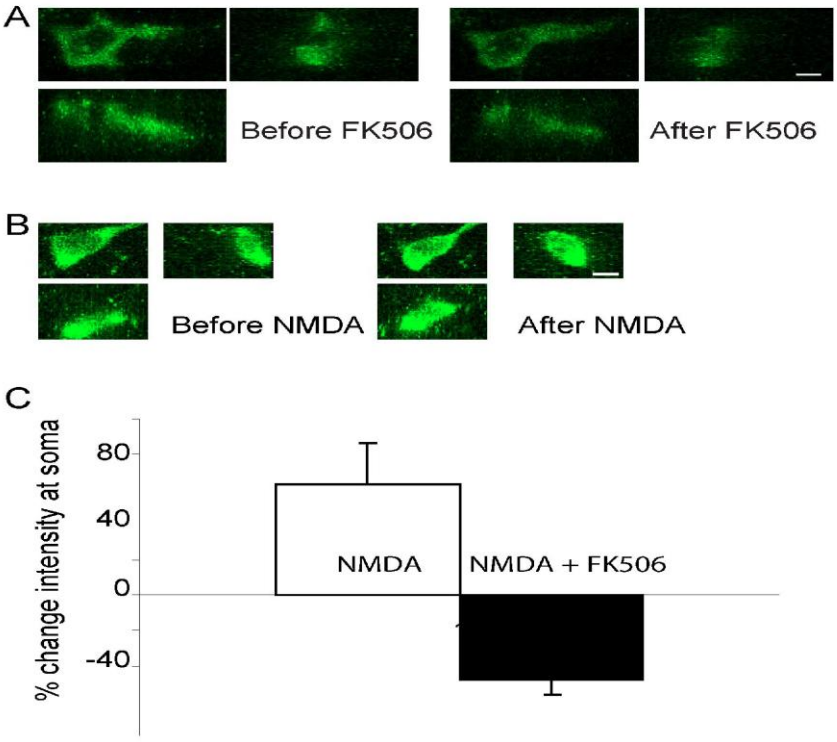
Supplemental Figure S1 Inhibition of CaN for 3 days by FK506 or N-VIVIT expression reduces mRNA levels of BDNF IV, IP3 receptor and Kv2.1 as measured by relative quantification RT-PCR. mRNA levels were normalized to GAPDH levels and to controls in the same plate using the ddCT method. Results are presented as mean±sem for 3 independent experiments. *Below*, unique PCR product after RT-PCR run of 40 cycles demonstrates the specificity of the primers.

Supplemantal Figure 2



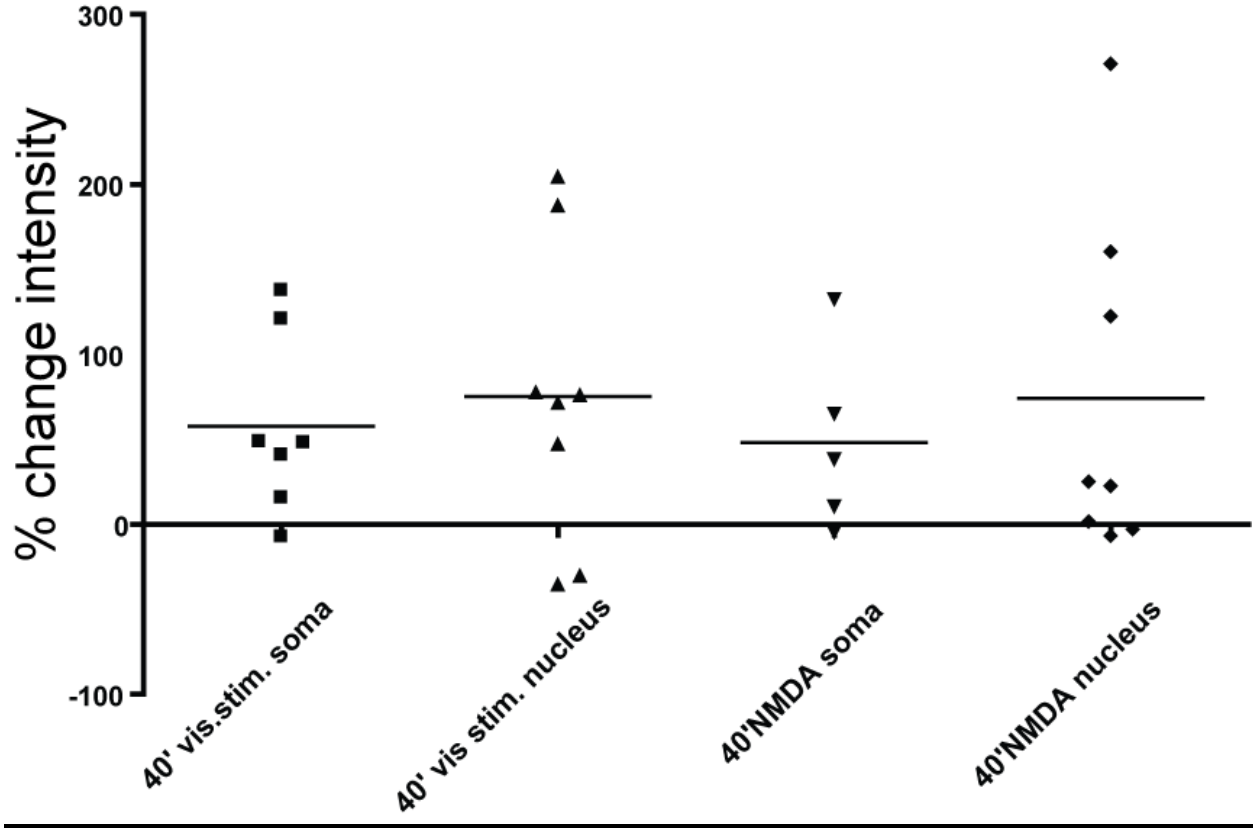
Supplemental Figure S2 - CaN inhibition results in accumulation of fine branch tips. Reconstructions of cells on day 1 (**A**-**C**) and day 3 (**D**-**E**) with corresponding cumulative probability plots **(G-I)** for the lengths of all branch tips at both time points. Note that in contrast to control cells, there is no net increase in mean terminal branch tip length with time in the treatment groups (day 1 vs. day 3, control,* $p<0.001$, K-S test).

Supplemantal Figure 3



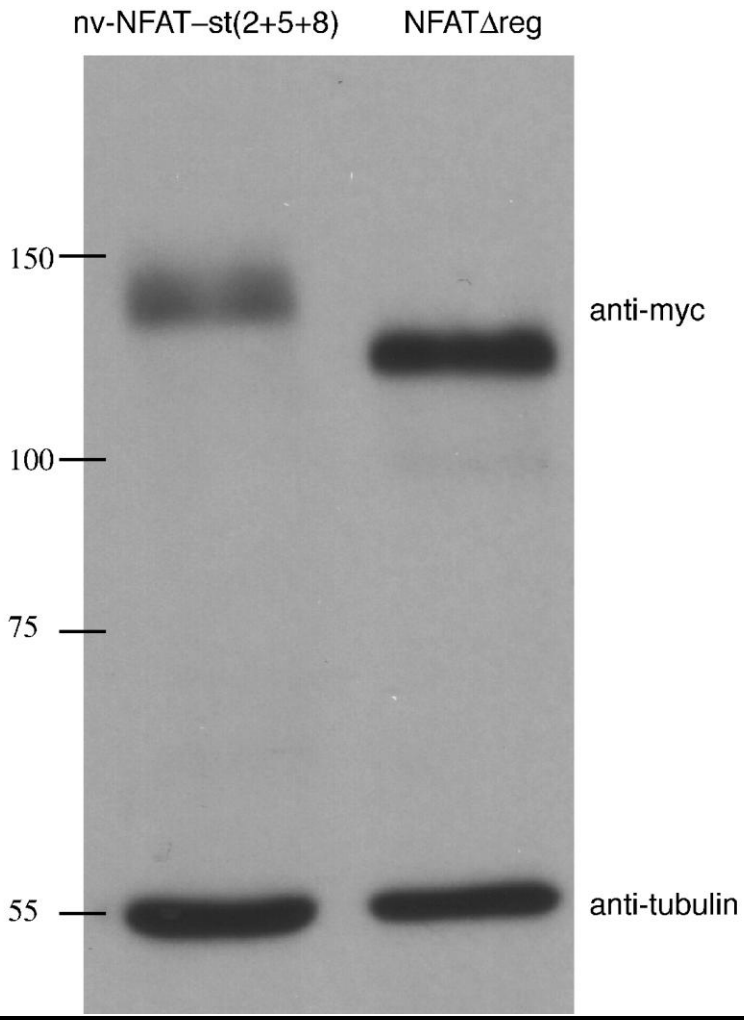
Supplemental Figure S3 - NMDAR activity regulates CaN/NFAT *in vivo*. NFAT translocates towards the nucleus upon dephosphorylation by active calcineurin**.** Neurons in the optic tectum were electroporated with NFAT-GFP and its intensity in the somatonuclear compartment was quantified. **A**. Maximum intensity projections of the cell soma in xy, yz and xz projection planes through 10μm of a representative tectal cell expressing NFAT-GFP before and 20 min after bath application of the calcineurin inhibitor FK506 (1μM). FK506 reduces the amount of somatic NFAT-GFP. **B.** Maximum intensity xy, yz and xz projections through a tectal cell expressing NFAT-GFP before and after bath application of 20 μM NMDA. Bath application of NMDA increases the amount of NFAT-GFP in the cell soma and nucleus. **C.** Quantification of percent change in fluorescence intensity at the soma 20 min after bath application of NMDA or FK506 followed by NMDA. The NMDA induced somatic translocation of NFAT-GFP was blocked by application of FK506.

Supplemantal Figure 4



Supplemental Figure S4 - Comparable somatic and nuclear changes in NFAT-GFP fluorescence Both visual stimulation and bath application of NMDA induce similar percent increases of NFAT-GFP fluorescence in the cell soma and in the nucleus.

Supplemental Figure 5



Supplemental Figure S5 – Expression levels of CA-NFAT constructs in optic tectum. Western blot of myc-tagged versions of nv-NFAT-st(2+5+8) and NFATΔreg proteins probed using an anti-myc antibody (and anti-tubulin loading control). The CA-NFAT constructs were each independently co-electroporated together with N-VIVIT into the optic tectum and allowed to express for two days. The protein equivalent of one brain was loaded in each lane. Slightly less nv-NFAT-st(2+5+8) was expressed, despite the fact that this construct produced a more complete rescue.

CHAPTER 3

Activity dependent transcription of BDNF enhances visual acuity during development

In chapter 2, we observed that visual stimulation regulated NFAT activity and that this lead to changes in AMPAr mediated transmission and dendritic arborization. For this chapter we will investigate the manner in which gene products produced following NFAT activation affect plasticity. Based upon existing literature we selected BDNF as a candidate protein that would be regulated by NFAT, and that would regulate bi-directional plasticity.

We monitored the activity of the BDNF exon IV promoter using a reporter construct, and measured proBDNF and mature BDNF protein levels. BDNF was up-regulated in response to visual stimulation. Upon up-regulation, proBDNF we observed facilitation of bidirectional plasticity of AMPAr mediated transmission. In a series of synaptic and behavioral assays we also observed that bi-directional facilitation of plasticity led to functional refinement and an improvement in visual acuity.

Acknowledgements: We thank Peter O'Connor for help with MATLAB, Alexendra Fletcher for help with the behavioral experiments, and Paul Patterson for the 1500 BP BDNF IV promoter plasmid. We also thank Phil Barker for technical advice and Carlos Aizenman for critically reading the manuscript. Funding provided to E.S.R. from CIHR and EJLB Foundation. Funding provided to N.S. by Scottish Rite Charitable Foundation and by Jeanne-Timmins Costello Fellowship.

Abstract

In the developing Xenopus tadpole, 20 min of visual conditioning leads to increased proBDNF protein levels in the optic tectum within 4 hours. At this later time point, plasticity of direction selective visual responses in tectal neurons, as well as retinotectal LTP and LTD, was facilitated in conditioned animals. This facilitation of plasticity could be blocked by Morpholino antisense knockdown of BDNF expression in tectal neurons. Tectal neurons in animals that had been conditioned and then received normal visual input for 7 to 11 hours exhibited lower spatial frequency response thresholds to counterphasing gratings than non-conditioned controls. An improvement in visual acuity was confirmed in behavioral tests demonstrating enhanced sensitivity to counterphasing gratings. These results indicate that brief sensory stimulation, leading to the initiation of nuclear transcription and *de novo* protein synthesis of BDNF, can enhance plasticity and facilitate the refinement of visual acuity.

Introduction

Developing neural circuits adapt to their environments through a process of activity-dependent refinement in which sensory input contributes to the concurrent strengthening of appropriate synapses and weakening of inappropriate synapses (Maurer and Lewis, 2001; Fox et al., 2010). This developmental process of synapse selection is thought to utilize plasticity mechanisms akin to long-term potentiation (LTP) and depression (LTD) (Katz and Shatz, 1996; Feldman and Knudsen, 1998; Zhang and Poo, 2001). In addition to its participation in the ongoing process of developmental refinement, rapid synaptic plasticity also occurs in response to strong or salient environmental stimuli (Engert et al., 2002; Malenka and Bear, 2004; Feldman, 2009; Smith et al., 2009). Plasticity inducing stimuli can further initiate the production of different neuromodulators, including neurotrophins. In turn, plasticity mechanisms are themselves subject to regulation by neurotrophins (Poo, 2001; Cohen and Greenberg, 2008; Lu et al., 2008). Thus, gene products synthesized in response to a strong, brief stimulus can play a dual role by directly inducing changes related to that stimulus, and by modulating the ongoing process of circuit refinement.

The neurotrophin Brain Derived Neurotrophic Factor (BDNF) can be synthesized in an activity-dependent manner primarily through regulation of the BDNF exon IV promoter (Greenberg et al., 2009). Its immature form proBDNF has been shown to play a role in LTD, through activation of the p75 neurotrophin receptor (Wu et al., 1996; Rosch et al.,2005). ProBDNF can either be cleaved intracellularly to form the mature protein mBDNF, or extracellularly in response to LTP inducing stimuli through tissue plasminogen activator (tPA) mediated activation of plasmin. Activation of the TrkB receptor by mBDNF participates in LTP (Pang et al., 2004; An et al., 2008; Barker, 2009; Lessmann and Brigadski, 2009; Nagappan et

al., 2009). Thus, proBDNF and mBDNF are both regulated by activity, but are thought to regulate LTD and LTP respectively. As circuit refinement is a process of concurrently strengthening appropriate synapses and weakening inappropriate synapses, BDNF is positioned to regulate both arms of this process and thereby improve circuit function.

To test if up-regulation of BDNF synthesis in response to an acute visual stimulus facilitates ongoing synaptic plasticity and functional refinement during development, we examined its role in the developing visual system of Xenopus tadpoles. Previously, we observed in this system that a conditioning visual stimulation up-regulated the activity of the transcriptional regulator Nuclear Factor of Activated T cells (NFAT), and that inhibition of NFAT led to a decrease in the levels of BDNF transcript (Schwartz et al., 2009). BDNF signaling has been linked to LTP in the developing Xenopus visual system (Mu and Poo, 2006; Du et al., 2009). In the current study we have found that prior presentation of a conditioning visual stimulus can up-regulate levels of proBDNF protein thereby facilitating not only retinotectal LTP, but also LTD, and plasticity of stimulus direction selectivity. We further examined whether visually conditioning animals to induce an up-regulation in BDNF levels affected subsequent functional refinement driven by normal sensory input. Significantly, we found that visual acuity was improved in conditioned animals compared to controls. As acuity is a measure of visual system function (Maurer et al., 1999; Sale et al.,2009), these results imply that elevated neurotrophin levels induced by earlier visual conditioning facilitated subsequent functional circuit refinement.

Results

Repeating visual stimulation up-regulates the activity of the BDNF exon IV promoter and expression of proBDNF protein in the optic tectum.

BDNF is transcribed in response to neuronal activity primarily through regulation of the BDNF exon IV promoter (Greenberg et al., 2009). Thus to determine if a brief period of intensive visual stimulation could regulate the activity of this promoter, neurons in the optic tectum, the principal visual nucleus in the Xenopus brain, were electroporated with a pGL3 basic plasmid (Promega) in which a 1500 bp fragment of the BDNF exon IV promoter drove expression of the green-red photoconvertible fluorescent protein Kaede. In non-conditioned animals, the basal level of promoter activity produced sufficient Kaede protein to allow visualization of the dendritic arbor by two-photon microscopy (Figure 1A). To determine the effect of visual conditioning on promoter activity, the amount of Kaede produced in the 4 hr after exposing animals to 20 min of a low-frequency simulated motion sequence was compared to the amount produced in the 4 hr before conditioning. A similar visual stimulation paradigm has been shown to activate the transcriptional regulator NFAT through the activation of N-methyl D-aspartate type glutamate receptors (NMDAr), as well as to induce NMDAr-mediated changes in dendritic growth (Schwartz et al., 2009; Sin et al., 2002). *De novo* protein synthesis was assessed by photoconverting the Kaede to red at the beginning of each 4 hr period and then measuring the amount of green fluorescence produced by newly synthesized Kaede at the end of the period. In the 4 hours after 20 minutes of visual conditioning, more new Kaede was produced compared to the preceding 4 hr period (166±20%, n=14), or to the amount produced in non-stimulated animals experiencing normal visual conditions (106.6±7.9%, n=13). Incubating animals in the blood-brain-barrier-permeant NMDAr antagonist 3-(2-Carboxypiperazin-4-yl)propyl-1-

phosphonic acid (CPP, 20 μM) starting 25 to 30 min before conditioning prevented the increase in Kaede (52.2±21% n=6). These results demonstrate that 20 min of visual conditioning is sufficient to up-regulate the activity of the BDNF exon IV promoter in an NMDAr dependent manner in tectal neurons in the intact animal (Figure 1B-C).

Next we tested whether this enhanced BDNF exon IV promoter activity in the tectal neurons led to a change in BDNF protein levels in the tectum. At 5 hours after visual conditioning, midbrains, including the optic tectum, from stimulated and control tadpoles were surgically isolated and homogenized for western blotting. Blots were probed with an antibody that recognizes both the immature and mature forms of BDNF. Visual conditioning led to an increase in the ratio of proBDNF to mBDNF (control 0.04±0.01, conditioned 0.26±0.04; Figure 1D,E, n=3 repeats, 5 animals each experiment). Because our antibody gave several non-specific bands, we performed an experiment to confirm the identity of the BDNF bands by introducing a BDNF antisense Morpholino oligonucleotide (BDNF MO). At 5 hrs post conditioning, brains that been electroporated with the BDNF MO showed reduced expression of pro-BDNF in contrast to brains electroporated with a scrambled MO or conditioned animals without MO treatment (Figure 1F, n=2 experiments, 4-5 animals each experiment). Thus, visual conditioning up-regulated proBDNF protein levels, most likely through regulation of the BDNF exon IV promoter.

Enhanced levels of proBDNF facilitate visual system plasticity

In addition to being regulated in an activity-dependent manner, BDNF can also modulate the susceptibility of synapses to undergo plasticity. In the hippocampus, proBDNF has been shown to facilitate LTD and in Xenopus mBDNF is thought to be required for retinotectal LTP

(Du et al., 2009; Mu and Poo, 2006; Woo et al., 2005). To determine if the proBDNF synthesized in response to visual conditioning affected retinotectal plasticity, we first examined the effects of visual conditioning on visual direction selectivity training, a protocol that is believed to engage both LTP and LTD at tectal cell synapses (Mu and Poo, 2006; Zhou et al., 2003).

To increase endogenous proBDNF levels, animals were visually conditioned and then returned to their normal visual environment for 4 to 6 hr. At 4 to 6 hr post-conditioning, animals received 3 bouts of training by repeated presentation of a bar sweeping in one direction across the retina. The bouts were delivered at 4 min intervals. This spaced training protocol is designed to induce direction selectivity in tectal neurons as previously described by Poo and colleagues (Engert et al., 2002; Zhou et al., 2003). (n.b., throughout this article, the term "conditioning" refers to the visual stimulation used to up-regulate BDNF expression whereas the term "training" refers to the visual stimulation protocol used to rapidly shift receptive field properties.) Thirty minutes to 2 hr after training, the compound synaptic current (CSC) elicited by a bar moving in each of 4 orthogonal directions was measured in whole cell voltage clamp recordings of tectal neurons. The response to each direction was normalized to the average response across all four directions. A schematic of the experimental timeline is shown in Figure 2A. Only cells (n=14) from conditioned animals developed a significant preference for the bar moving in the trained direction (trained direction (141.7±15.4%), + 90^{O} (83.2±10.4%), +180^{O} (87.2±11%), +270^{O} (88.8±10.1%)), . Cells (n=11) from the group that had not been conditioned (trained direction (105.4±9.4 %), + 90^{O} (94.0±12%), +180^{O} (108.3±9.8%), +270^{O} (92.2±9.5%)), or conditioned cells with MO knockdown of BDNF (n=11) (trained direction (92.8±8.1 %), + 90^{O} (104.4±14.5%), +180^{O} (96.9±16.4%), +270^{O} (105.8±12.6%) did not exhibit significant direction

training (Figure 2B,C). There was no significant difference between cells from animals that had not been electroporated (n=7), and cells from animals that had been electroporated with the scrambled MO (n=4). These groups were therefore combined. These results indicate that the up-regulation of proBDNF induced by prior visual conditioning facilitated a change in direction selectivity in tectal neurons. The limited malleability of direction selectivity in our non-conditioned animals is noteworthy, as a similar spaced training protocol was sufficient to induce a persistent modification of direction sensitivity in a subset of tectal neurons at an earlier stage of development (Zhou et al., 2003). These observations suggest that elevation of BDNF may restore the levels of plasticity found in younger tadpoles. As plasticity of direction sensitivity in these neurons is thought to involve the induction of LTD and LTP (Mu and Poo, 2006), we next examined how conditioning modified these forms of retinotectal synaptic plasticity.

Although spike timing-dependent LTP and LTD have been proposed as possible mechanisms underlying the induction of direction selectivity at the retinotectal synapse (Engert et al., 2002; Mu and Poo, 2006), we instead used a synaptic pairing protocol (holding -32mV, 300 pulses at 1 Hz) to induce LTD in this study. This protocol was selected because the sensitivity of the retinotectal synapse to spike-timing protocols is substantially reduced by the stage of development used in this study (Tsui et al., 2010). In non-conditioned animals, the pairing protocol induced a transient depression that recovered around 20 min after stimulation (100.6±5.8%). In stark contrast, in animals with elevated proBDNF levels due to visual conditioning, we observed a robust LTD (44±10.6%, $p<0.05$ vs. non-conditioned) (Figure 3A,B). The induced depression was stable for as long as recordings were made, in some cases up to 1 hr after induction (Figure 3A). In animals that had been bathed in the transcription inhibitor actimomycin D (50 μM) for 90 min around the time of conditioning, starting 30 min before, to

prevent the visually induced synthesis of proBDNF message, the facilitation of LTD was not present (105±10.5%, p=.002 vs. conditioned) (Figure 3Bii). Similarly, conditioning failed to facilitate LTD in cells with MO knockdown of BDNF (85±10.2%, p=.03 vs. conditioned; Figure 3A,Biii). In the hippocampus, acute exogenous application of proBDNF without an accompanying plasticity stimulus does not affect evoked α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutatmate receptor (AMPAr) events (Woo et al., 2005). Similarly, in the retinotectal system following exogenous co-application of proBDNF with tPA-stop, an inhibitor of tissue plasminogen activator (tPA), a key regulatory enzyme in the proBDNF cleavage cascade, we observed no effect on evoked AMPAr EPSCs (Figure S1). In contrast, application of proBDNF (2ng/ml) together with tPA-stop, in conjunction with the LTD induction protocol, reproduced the facilitation of LTD observed following visual conditioning (56±5%, p=0.03 vs. non-conditioned) (Figure 3Biv.). Curiously, higher doses of proBDNF did not facilitate LTD (data not shown), implying that endogenous proBDNF levels must be likely tightly regulated by cells. These results suggest that the increased levels of proBDNF protein that resulted from earlier visual conditioning facilitated the induction of LTD at the retinotectal synapse.

ProBDNF can be cleaved to mBDNF intracellularly by various convertases or extracellularly by plasmin (Barker, 2009). tPA, which activates plasmin, is one of the principal enzymes in the activity-dependent extracellular cascade responsible for the cleavage of proBDNF to mBDNF (Nagappan et al., 2009; Pang et al., 2004). Like BDNF, tPA is also subject to regulation by activity, and is activated by LTP-inducing stimuli (Baranes et al., 1998; Gualandris et al., 1996; Salles and Strickland, 2002). mBDNF has a well-established role in the potentiation of synaptic transmission in many systems, including the retinotectal synapse of

Xenopus (Du and Poo, 2004; Mu and Poo, 2006; Du et al., 2009). Thus we next tested the effects of visual conditioning on retinotectal LTP.

To induce LTP we used a spaced induction protocol. One repetition of the protocol only induced a transient synaptic facilitation (Figure S2). In contrast, two repetitions stably increased the amplitude of the EPSC to 128±6.5% of baseline in animals that had not undergone visual conditioning. In animals that had been conditioned, this increase was facilitated, resulting in a stable potentiation of 204±8.5% ($p<0.05$ compared to non-conditioned) (Figure 4A,Bi). Next we blocked tPA activity to determine if extracellular cleavage of proBDNF to mBNDF was required for the visually induced facilitation. Bath application of the inhibitor tPA-stop blocked LTP induction (80±7.1%, $p=0.01$ vs. conditioned) (Figure 4Bii). As tPA can be involved in cascades other than the extracellular cleavage of proBDNF, we next tested LTP induction in cells with MO knockdown of BDNF. MO knockdown of BDNF blocked the facilitation induced by conditioning (125±5.3%, $p<0.05$ vs. conditioned)(Figure 4Biii). In the visually conditioned group there was no difference between cells from animals that had not been electroporated (n=6) and cells from animals that had been electroporated with the scrambled oligonucleotide (n=3). These groups were therefore combined. These findings imply that proBDNF synthesized in response to visual conditioning was cleaved in a tPA-dependent manner in response to the LTP protocol, and that the mBDNF facilitated LTP. Because MO knockdown had blocked the synthesis of proBDNF following conditioning, but reduced the levels of mBDNF in the brain only modestly (Figure 1F), it is likely that BDNF MO knockdown would block the conditioning-dependent synthesis of proBDNF, leaving mBDNF levels sufficiently intact for LTP to be induced in a manner comparable to that in non-conditioned animals. To test this idea we blocked kinase signaling downstream of BDNF using K252a in conditioned animals. This treatment

entirely blocked LTP induction (97±3.8%, $p<0.05$ vs. no drug; Figure 4iv) in agreement with previous reports (Mu and Poo, 2006; Du et al., 2009).

Next we determined whether a weaker pairing protocol that failed to induce LTP under control conditions could be facilitated to produce long-lasting potentiation. In controls, the weak protocol produced inconsistent long term plasticity which tended to slight depression. In contrast, conditioning facilitated the induction of LTP by this weaker pairing protocol (147±13%) and MO knockdown blocked the facilitation (110±19%) (Figure S3).

Together, our findings imply that the BDNF synthesized in response to 20 min of robust visual conditioning, can facilitate bidirectional plasticity at the retinotectal synapse hours later. As developmental circuit refinement is thought to rely upon environmentally driven strengthening of appropriate, and weakening of inappropriate, synapses through mechanisms like LTP and LTD (Katz and Shatz, 1996; Zhang and Poo, 2001), we next tested whether visual conditioning affected the ongoing process of circuit refinement.

Animals exhibit improved visual acuity 7 to 11 hr after conditioning.

Visual resolution acuity is a measure of the ability to resolve spatial details. One method for measuring acuity in humans is the Teller acuity test (Dobson and Teller, 1978). This is a behavioral test in which it has been observed that preverbal infants will preferentially look at a grating that they can resolve versus either a grey screen of comparable luminance or a higher spatial frequency grating that they cannot resolve. Furthermore, cortical responses to gratings of different sizes determined by measuring transcranial visually evoked potentials can be extrapolated to determine a subject's acuity thresholds, producing comparable results to the behavioral tests (Campbell and Maffei, 1970; Good, 2001).

To determine if the proBDNF produced in response to visual conditioning affects the ongoing process of circuit refinement, leading to a change in acuity, animals were conditioned and then returned to their normal rearing environment for 7 to 11 hr. This period permitted at least 3 to 7 hr of visual experience during the time of enhanced LTD and LTP, as described above. We used pipettes containing CsFl to measure compound synaptic current responses of tectal cells to full-field flash stimulation at -70 mV, and at +40 mV in the presence of an AMPAr antagonist. As Fl inhibits chloride flux, recordings at -70 mV were predominantly mediated by AMPAr and recordings at +40mV were primarily mediated by NMDAr. The events are likely a mixture of monosynaptic input from the retina, and polysynaptic input from tecto-tectal connections. However, as recordings conditions were the same for each group the ratio is representative of the AMPA response normalized to the NMDA response. This ratio has been shown to correlate with synapse maturity and synaptic potentiation (Wu et al., 1996). Interestingly, the AMPA/NMDA ratio of responses to full-field OFF stimuli, but not ON stimuli, was greater in conditioned animals (0.85±0.23) compared to controls (0.35±0.23; $p<0.05$ ANOVA). This increase in AMPA/NMDA ratio was prevented by MO knockdown of BDNF (0.48±0.13) (Figure S4). In the non-conditioned group there was no difference between cells that had not been electroporated (n=8) and those that had been electroporated with the scrambled MO (n=4). This was also true for visually conditioned animals (n=3 and n=3 respectively). These respective groups were therefore combined. Tectal cells receive 3 classes of retinal ganglion cell input, namely ON, OFF and ON/OFF (Edwards and Cline, 1999). Thus the selective change in the OFF ratio, suggests that only specific inputs were affected. A likely reason for this selectivity is that OFF responses are generally larger in tectal cells, and thus these synapses may have been more robustly activated (Figure S4A; Zhang et al., 2000). These

findings indicate that by 7 to 11 hours after conditioning, there was a stimulus-specific change in glutamatergic transmission in the tectal cells consistent with synaptic plasticity having occurred.

To determine whether the synaptic changes detected contributed to an improvement in stimulus sensitivity by the visual system, we measured the responses of tectal cells to counterphasing square wave gratings of a range of spatial frequencies projected onto the retina. Tectal cells predominantly responded in a graded fashion to bars of decreasing size (Figure 5A,B), with the full field OFF stimulus eliciting the largest compound AMPAR current in 18 of 20 cells from controls, in 21 of 21 cells from the conditioned group, and in 20 of 21 cells from the BDNF MO group. For measurements of visual sensitivity, responses were considered only from cells where the full-field OFF response was largest. We normalized all other responses including the ON response to the full-field OFF response for each cell. The sensitivity threshold for each cell was calculated as the X-intercept value in a linear extrapolation of spatial frequency versus response plots (Figure 5C). Cells from animals that had been conditioned and returned to their normal rearing environment for 7 to 11 hr had lower thresholds than non-conditioned control that had remained in a normal rearing environment, or conditioned cells with MO knockdown of BDNF (Figure 5D) ($p<0.05$ t-test, threshold spatial frequency, conditioned: 0.076 ± 0.009 cycles μm^{-1}, non-conditioned: 0.052 ± 0.003 cycles μm^{-1}, conditioned BDNF MO: 0.064 ± 0.005 cycles μm^{-1}). Thus, normal visual experience during the time period when plasticity was facilitated led to a BDNF-dependent improvement in the spatial resolution sensitivity of tectal neuron AMPAR mediated responses to visual stimuli.

Previous behavioral experiments in Xenopus tadpoles have shown that as tectal circuitry matures, animals develop improved visual avoidance behaviors (Dong et al., 2009). It has also been reported that the kinematics of Xenopus tadpoles has evolved such that they are better

adapted to bursts of rapid maneuvering, rather than to sustained high-speed swimming (Wassersug, 1989). For example, increasing intensity thermal stimuli elicit progressively more frequent and briefer bouts of swimming by tadpoles, with a concurrent reduction in the duration of the bouts (Sillar and Robertson, 2009). Thus we anticipated that more salient stimuli might elicit more erratic swimming dominated by frequent changes in acceleration.

To test if the improved tectal spatial sensitivity described in Figure 5A-D, led to a modification in the animals' behavior, we measured the responses of freely swimming tadpoles to counterphasing gratings. One animal was placed into each well of a six-well dish. Swimming behavior was then monitored by video in response to the onset of counterphasing of background gratings of 3 different spatial frequencies (SF), namely 4, 2, and 1.4 cycles per mm, corresponding to bars that were 1, 2, and 3 pixels wide. Background grating counterphasing at 0.2 Hz immediately caused swimming patterns to become more erratic (Figure 5 E,F). We measured the frequency of behavioral responses elicited by two counterphases following a 2 min adaptation period during which a stable grating was presented without counterphasing. A response was defined as a change in absolute acceleration greater than one standard deviation from the mean during the 10 sec period immediately preceding the first counterphase. At the three highest spatial frequencies tested, control non-conditioned animals (n=36 animals, 3 experiments) showed modest, but comparable, probabilities of responding to all test stimuli (0.55±0.05 for SF 4; 0.39±0.04 for SF 2, 0.47±0.03 for SF 1.4). In contrast, in conditioned animals (n=36 animals, 3 experiments), there was an increased likelihood of a counterphase eliciting a response as spatial frequency decreased (0.61±0.02 for SF 4, 0.72±0.08 for SF 2, 0.81±0.05 for SF 1.4). These results suggest that visual conditioning had improved the ability of the animals to detect counterphasing of all but the very finest gratings tested. To determine if the

enhanced BDNF signaling present at 4 to 6 hrs after conditioning played a role in this improvement, we injected K252a twice into the tectal ventricle at 3.5 hr and 4.5 hr after conditioning, corresponding to the period when plasticity is facilitated. Animals were then tested at 8 and 10 hr after conditioning (n=24 animals, 2 experiments). Inhibition of TrkB prevented the effects of conditioning on visual acuity (Figure 5G). Injection of vehicle had no effect. Thus, BDNF signaling during the period of facilitated plasticity, is required for visual conditioning to improve acuity.

To confirm that the observed change in swimming acceleration in response to visual stimuli involves retinotectal transmission, we thermally lesioned the optic tract just anterior to the optic tectum using the two-photon microscope with the infrared laser set at high intensity (800 mW at 810 nm) (Figure S5). At 5 hr after lesioning, we repeated the behavioral test. Although animals that had undergone optic tract lesions still exhibited startle responses to full-screen ON stimuli, their responses to counterphasing were impaired (Figure S5). This finding is in accordance with previous studies attributing the visual acuity of behavioral responses to sensory processing in the optic tectum (Yolen and Hodos, 1976). These findings demonstrate that BDNF-dependent facilitation of plasticity leads to a rapid, behaviorally relevant improvement in the animal's visual acuity, resulting in better discrimination of fine stimuli.

Discussion

We previously reported that a repeating visual stimulus was able to up-regulate plasticity-related gene transcription in the Xenopus optic tectum (Schwartz et al., 2009). Here we have extended this finding by showing that sensory stimulation-mediated activation of the BDNF exon IV promoter, and subsequent synthesis of proBDNF, can modulate synaptic plasticity in the developing visual system. These increased levels of proBDNF, as well as an accompanying enhancement of signaling downstream of mBDNF, did not appear to induce synaptic changes on their own, but rather facilitated ongoing plasticity mechanisms. Importantly, enhanced BDNF signaling contributed to a behaviorally detectable improvement in visual acuity. In summary, our findings reveal that the BDNF synthesized in response to 20 minutes of visual conditioning can facilitate bidirectional plasticity at the retinotectal synapse with direct behavioral consequences for the developing animal. A summary is presented in Figure 6.

BDNF regulation of synaptic plasticity

Recent studies, carried out mainly in the CA1 area of mouse hippocampus, have revealed key roles for BDNF signaling and processing in synaptic LTP and LTD. Late-phase LTP (L-LTP) in CA1 is largely absent in transgenic mice lacking BDNF, and early-phase LTP is also substantially reduced in these animals (Korte et al., 1995; Patterson et al., 1996). Neurons are able to release both the precursor and mature forms of BDNF, however the site of release may be a critical determinant of what form the released protein takes (Matsuda et al., 2009; Yang et al., 2009). As the protein synthesis machinery present in most dendrites lacks the Golgi-like organelles that process constitutively secreted proteins (Horton et al., 2005), it is likely that dendritically synthesized BDNF is secreted in its precursor form (An et al., 2008). Secreted

proBDNF at synapses would then be cleaved to mBDNF by plasmin, activated from plasminogen by the activity of tPA, consistent with reports that tPA is also required for L-LTP (Pang et al., 2004). Our findings in the retinotectal system suggest a similar requirement for the synaptic release and cleavage of proBDNF, as acute inhibition of tPA activity reduced retinotectal LTP to the same degree as pharmacological inhibition of TrkB signaling. Furthermore, the knockdown of BDNF by MO antisense electroporation into tectal neurons reveals that BDNF from the postsynaptic cell is required for LTP.

On the other hand, the activation of the p75NTR by proBDNF has been reported to facilitate hippocampal LTD (Woo et al., 2005). Our retinotectal data confirmed the facilitation of LTD by recently synthesized proBDNF, and demonstrated that this could be mimicked by exogenous application of proBDNF if tPA activity is inhibited. In light of these findings, it is interesting to consider how the regulation of the rate of proBDNF cleavage could regulate not only the efficacy but also the direction of synaptic plasticity (Nagappan et al., 2009). For a developing neural circuit, excessive strengthening or weakening of synapses would be detrimental. Future investigations into the mechanisms that regulate the balance between these opposing changes will be important for fully elucidating the contributions of neurotrophins to circuit development.

Locus of action of BDNF in the retinotectal system

Application of exogenous BDNF to the optic tectum rapidly and profoundly impacts the retinotectal circuit. In vivo imaging of puncta of the synaptic vesicle protein GFP-synaptobrevin (GFP-syb) in Xenopus retinotectal axons reveals a rapid up-regulation of axonal branching and presynaptic punctum number within minutes to hours of BDNF application (Alsina et al., 2001; Hu et al., 2005). A BDNF-mediated increase in the number of PSD95-GFP positive postsynaptic

specializations appears to occur subsequent to presynaptic changes, becoming evident only many hours after neurotrophin application (Sanchez et al., 2006). Functionally, a rapid increase in mEPSC frequency, but not amplitude, has been reported in response to application of BDNF to the tectum (Du and Poo, 2004). Our experimental protocol differed from these approaches in two important ways. First, the elevation of BDNF levels in our experiments relied on activity-dependent synthesis and release of endogenous protein rather than application of exogenous neurotrophin. Secondly, the BDNF-mediated changes that we described occurred only in response to specific LTP- and LTD-inducing electrical and visual stimulation protocols. Thus the specific timing and location of neurotrophin delivery may determine its effects on the circuit. This is consistent with the report that the human BDNF val66met polymorphism which impairs dendritic trafficking and activity-dependent, but not constitutive secretion of BDNF results in abnormal hippocampal function (Egan et al., 2003).

While our own experiments do not distinguish between pre- and postsynaptic sites of action of the BDNF synthesized in response to visual conditioning stimuli, the efficacy with which MO knockdown of tectal BDNF synthesis fully prevented facilitation of both LTP and LTD clearly points to the postsynaptic cell as the source of newly synthesized BDNF. Retinotectal LTP experiments by Du and colleagues (2009) using MO antisense against TrkB targeted to presynaptic retinal or postsynaptic tectal neurons suggested that BDNF signaling onto both synaptic partners contributed to BDNF-dependent LTP expression. Quite remarkably, this same study also observed a retrograde change in synaptic transmission back in the retina within minutes of BDNF applied exclusively to the tectum. It is clear from our optic chiasm stimulation experiments that endogenous BDNF directly facilitated plasticity at the retinotectal synapse. However, we cannot exclude the additional possibility that the newly synthesized BDNF may

also have had retrograde effects in the retina that could have contributed to the refinement of visually evoked and behavioral responses that we observed.

Other signaling pathways activated by visual conditioning

It should be noted that modulation of glutamatergic synaptic transmission by *de novo* BDNF synthesis, is only one of many elements that contribute to the changes induced by visual conditioning. Diverse protocols using visual stimulation of Xenopus tadpoles have been shown to regulate the expression of Homer 1a, the synthesis of polyamines which modulates ion channel properties, and the activity of small GTPases which regulate cytoskeletal growth (Aizenman et al., 2002; Sin et al., 2002; Van Keuren-Jensen and Cline, 2006). Thus, BDNF modulation of glutamatergic synaptic transmission, is only one of a number of cascades initiated in response to visual activity that contributes to visual system refinement. However, the unique feature of BDNF we report here is its ability to bi-directionally facilitate plasticity in its cleaved and uncleaved forms (Woo et al., 2005). Because of this bi-directional facilitation, experiments that disrupt BDNF signaling are likely to have a more profound effect on refinement compared to manipulations that modulate plasticity in only one direction.

Experience-dependent and experience-expectant developmental plasticity

Early sensory activity can influence circuit development both permissively and instructively. Greenough et al. (1986) provided an insightful framework for considering these influences by categorizing developmental plasticity as either "experience-expectant" or "experience-dependent". The former represents those processes that have evolved to be part of normal development through generations of interactions between the developing brain and a predictable sensory landscape, whereas the latter constitutes a mechanism for adaptation to the different

forms of sensory information each unique organism receives. A classic example of experience-dependent plasticity would be the ocular dominance shift observed in response to monocular occlusion. Recent experiments have revealed that while TrkB signaling appears to be dispensable for the deprivation-induced loss of responsiveness to the deprived eye, it is required to mediate the recovery of binocular responses following re-opening of the deprived eye (Kaneko et al., 2008).

There are also many experience-expectant aspects of response selectivity development, including receptive field refinement in the superior colliculus, and the emergence of orientation selectivity in visual cortex, which occur to a remarkable extent even in naïve animals deprived of visual experience (Crair et al., 1998; Carrasco et al., 2005). Despite this initial developmental progress, long-term dark-rearing is not benign and eventually leads to the decline of these response properties. Remarkably, BDNF overexpression is able to rescue many detrimental effects of dark-rearing (Gianfranceschichi et al., 2003). The ability of BDNF overexpression to substitute for normal sensory experience has been proposed to reflect the acceleration of GABAergic circuit maturation downstream of BDNF signaling (Hanover et al., 1999; Huang et al., 1999).

Our experiments offer an alternative role for BDNF in the control of circuit development. We found that robust sensory stimulation led to the delayed up-regulation of BDNF protein, resulting in a facilitation of both synaptic LTP and LTD. Under this condition of bidirectional elevated synaptic plasticity, experience-dependent direction selectivity training, as well as experience-expectant visual acuity refinement, was readily enhanced. Interestingly, we observed a preferential effect on OFF stimuli. Given that tectal cells receive 3 classes of retinal ganglion cell inputs, namely ON, OFF and ON/OFF (Edwards and Cline, 1999), this result implies that

BDNF preferentially modulated a specific subset of functional synaptic inputs in this case, and argues against it having exerted its action via non-specific, homeostatic mechanisms or a general enhancement of GABAergic transmission.

What might be the benefit of the several hour delay between the conditioning stimulus and the elevation in BDNF expression levels? Given that BDNF expression bidirectionally facilitates ongoing experience-expectant developmental plasticity, it may serve as a kind of "gain control", setting the kinetics of baseline circuit refinement. Immediately after an intensive sensory stimulus that might have rapidly driven a disproportionate plastic change in the circuit (Tsui et al., 2010), such a mechanism would help to normalize the response properties of the cell by temporarily making it more sensitive to ambient sensory inputs, thus efficiently resetting its basal synaptic input strengths in accord with the sensory environment.

Materials and methods

ELECTROPORATION: Cells in the optic tectum were electroporated as described previously (Falk et al.; Haas et al.). Sequences for the BDNF morpholino and scrambled oligonucleotides have been previously published (Yang et al.,2009). For the reporter plasmid the luciferase encoding region of the pGL3 basic plasmid (Promega) was replaced with Kaede using cut sites HindIII and XbaI. These cut sites were introduced onto the Kaede fragement by PCR using primers, 5': CGCGAAGCTTATGAGTCTGATTAAACCAG, and 3': ATCGTCTAGATTACTTGACGTTGTCCGGCA. After insertion of the Kaede into the reporter plasmid, a 1500 base pair fragment of the BDNF exon IV promoter isolated with restriction cuts at SacI and Hind III, from the reporter plasmid used in Tao et al., (Tao et al.,1998) was inserted at these sites. After 48 hr, cells that were fluorecently labeled were used for experiments. ImageJ software (NIH) was used to analyze changes induced in fluorescence intensity. Average intensity projections were used for quantification in fixed volumes as described previously (Schwartz et al., 2009).

WESTERN BLOTS: Brains were extracted in a HEPES buffered extraction buffer containing protease inhibitor SetV (Calbiochem, San Diego). Samples were separated on acrylamide gel, transferred to PVDF membrane and stained with either rabbit anti BDNF sc-546 (1:1000) or with rabbit anti beta-tubulin sc-9104 (1:20000) as a loading control. Both antibodies were from SantaCruz, the anti-BDNF antibody has been used previously in Xenopus (Calle et al., 2006; Yang et al., 2009). To confirm the specificity of the BDNF antibody two additional controls, in addition to the MO knockdown were done. First, we obtained the cDNA encoding full length Xenopus BDNF from Open Biosystems, tagged this sequence with GFP and expressed it under the control of a CMV promoter in intact animals. After confirming expression of the GFP, these brains were used for western blots. The gel was stained with anti-GFP(Invitrogen A6455) antibody. The gel was then stripped and re-stained with the rabbit anti-BDNF antibody. The rabbit anti-BDNF antibody recognized the GFP-labeled bands and the endogenous protein at their appropriate molecular weights (data not shown). Second, we probed blots with chicken anti-BDNF (Chemicon AB9042). Although this antibody also recognized bands at the appropriate molecular weight the rabbit antibody was selected for experiments.

ELECTROPHYSIOLOGY: Whole cell patch clamp recordings were conducted in the tectum in stage 47-48 tadpoles. Visual stimuli were projected directly onto the retina as described previously (Engert et al.). Electrical stimuli (100 μsec) were generated with an ISO-flex stimulus isolation unit (AMPI, Israel), delivered through a custom bent 25 μm cluster electrode (FHC, Maine) to the optic chiasm. The external solution consisted (in mM) of 115 NaCl, 2 KCl, 1.5 CaCl2, 3 MgCl2, 5 HEPES and 10 glucose- ph 7.4 and osmolarity 255. For plasticity experiments 3mM CaCl2 was used. Recordings were made from the same area within the tectum to reduce cell variability. For recording electrically evoked responses, pipettes (5 to 9 MΩ) were filled with (in mM): 120 K gluconate, 5 NaCl, 1.5 MgCl2, 1 EGTA, 20 Hepes, 2 NaATP and 0.3 Na2GTP. LTD was induced with a pairing protocol similar to that used in the hippocampus: depolarization to -35 mV, 300 pulses at 1 Hz (Daw et al., 2002). The single LTP protocol consisted of depolarization to -12 mV, then 3X(40 pulses at 10Hz) with 20 sec rest between each set. As this protocol did not induce robust LTP (supplemental figure 2), we repeated it at a 3 min interval. During the interval cells were held at -40 mV. For the weak induction protocol cells were depolarized to -12mV, and 3X(60 pulses at 3Hz) with 20 sec rest

between each set. To isolate AMPA events evoked by visual stimuli cells were held at -70mV, and pipettes were filled with (in mM): 114 CsFl, 1.5 MgCl2, 20 TEACl, 10 EGTA, 20 HEPES, 2 NaATP and 0.3 Na2GTP . The CsFl, effectively blocked chloride flux as previously described (Marchionni and Maccaferri, 2009). Events and responses were selected for analysis based upon published criteria, series resistance was monitored throughout all experiments, cells with changes >20% were not included (Schwartz et al., 2009). Comparisons between groups were done based on responses recorded between 24 to 30 min after the plasticity stimulus.

Visual stimuli were generated using ImageJ macros, and analysis was done with programs written in house in MATLAB. For the direction selectivity experiments the integrated current was considered for the first 50 ms of the response, when it is dominated by retinotectal inputs, as previously described (Mu and Poo, 2006). Stimulus presentation and the spaced training protocol have been previously described (Zhou et al., 2003). Full screen and gratings stimuli were presented in a pseudorandom order. A minimum interval of 20 sec was used between full screen stimuli. A minimum interval of 8 sec was used between grating stimuli. Gratings were presented in groups that started and ended with a full screen stimulus. At the start of the group, a full screen stimulus was presented, 20 sec later this was switched to the first grating and 10 sec later this grating was counterphased. The same grating was then counterphased 2-3 times at 8-12 sec intervals. The next sized grating was then presented for 10 sec until its counterphase. This sequence was repeated and ended with a full-field stimulus. There was a 20 to 30 sec rest until the start of the next group. For quantification the average of a minimum of 4 responses was used. To determine AMPA/NMDA ratios, the first 100ms and 200 ms of the respective responses were quantified. To isolate visually evoked NMDA events a holding potential of +40 mV was used. In addition 100μm GYKI52466 (Tocris) was also picospritzed onto the tectum with a patch pipette. K252a (200nM, Calbiochem), tPA-stop (4.5μM + 0.1% BSA; American Diagnostics), proBDNF (2ng/ml + 0.1% BSA, Alomone Labs), were applied 20 to 30 min before induction protocols unless otherwise stated. CPP (20 μM) was obtained from Tocris.

BEHAVIOR- One animal was placed in each well of a six-well dish. The dish was then placed onto a flat screen monitor (LG Flatron model #L17NT-A, screen resolution 1280X1024). ImageJ was used to generate full screen square wave grating stimuli. Stimuli were delivered in pseudorandom order. At the start of the test the screen was black for 4 min, and then the first grating was presented for 2 min until the counterphase. The grating was then counterphased at 0.2 or 0.22 HZ for 1 min. Afterwards, the screen was held black for 4 min and then the next size grating was presented in a similar manner. Animals were recorded at 15 frames per sec. Videos were then re-sampled at 5 frames per sec and analyzed using ImageJ and programs written in house in MATLAB.

Figure legends

Fig 1. **ProBDNF levels are up-regulated by 4 hrs after a visual conditioning stimulus.**

(A-C) Visual conditioning up-regulates the activity of the BDNF exon IV promoter

A) Maximum intensity projection of tectal neurons electroporated to express a plasmid with the 1500 bp BDNF exon IV promoter driving photoconvertible Kaede. Box indicates region of interest in B.

B) Average intensity projections (10 optical sections at 1µm steps in ROI from A.) top panel Kaede protein converted to red, bottom green Kaede protein; (i.) projection immediately after 1st photoconversion 4 hours before conditioning; (ii.) 2nd image immediately before conditioning. (iii.) image 30 min after visual conditioning and another photoconversion (iv.) image 4 hrs after conditioning. Arrows indicate cells that show higher promoter activity after conditioning compared to before.

C) Kaede produced in the second 4 hour period as a percentage of amount produced in the preceding 4 hours. Visual conditioning up-regulates the activity of the BDNF exon IV promoter, inhibition of NMDA receptors blocks the visually induced increase (*$p<0.5$, **$p<0.01$)

D,E)The ratio of ProBDNF protein to mBDNF protein is increased by 4 hrs after conditioning. (mean +/- STDEV, **$p=0.007$)

F) MO. knockdown of BDNF blocks the increase in proBDNF protein produced in response to conditioning. Scrambled MO has no effect on induced proBDNF levels compared to controls.

Fig 2. **In the presence of higher levels of visually induced BDNF, tectal neurons are more susceptible to directional training with a moving bar**

A) Timeline of experiment: Animals were conditioned and returned to their rearing bowls. Four to 6 hours later, tectal cells were trained by repeatedly moving the image of a bar across the retina in the same direction. Thirty minutes to 2 hours after training, the first 50 msec of the compound synaptic current (CSC) elicited by a bar moving in each of the 4 orthogonal directions was measured.

B) Black traces are 3-4 representative examples of CSC elicited by a bar moving in each of the 4 listed directions. Red traces are the average of the underlying black traces. Grey boxes indicate the direction trained in each animal (i) (scale 75pA, 100 msec), and (ii) from a cell electroporated with BDNF MO. (scale 50 pA, 100 msec)

C) Cells from conditioned animals exhibit a larger response to the trained direction compared to each of the untrained directions. This effect was blocked by MO. knockdown of BDNF (*$p<0.05$ ANOVA, Bonferroni post hoc test).

Fig 3. **In the presence of higher levels of visually induced proBDNF, tectal neurons are more susceptible to LTD**

A) Representative AMPAR EPSCs recorded in response to electrical stimulation at the optic chiasm (10 events averaged), numbers (1,2) on the corresponding scatter plot indicate the time of points averaged for each trace. Arrow indicates the time of the LTD inducing stimulus.
B) Averaged EPSC amplitude as a percentage of baseline, Arrow indicates time that LTD stimulus was delivered (0 min). (i.) Conditioning facilitates LTD; (ii.iii.) Facilitation induced by conditioning is blocked by the transcription inhibitor actinomycin and by MO knockdown of BDNF. (iv.) Pre-incubation of animals in exogenous proBDNF with tPA-stop for 30 min before LTD induction facilitates LTD.

Fig 4. **In the presence of higher levels of BDNF, tectal neurons are more susceptible to LTP**

A) Representative AMPAR EPSCs recorded in response to electrical stimulation of the optic chiasm (10 events averaged), numbers (1,2) on the corresponding scatter plot indicate the time of points averaged for each trace. Arrow indicates the time of the LTP inducing stimulus.
B) Averaged EPSC amplitude as a percentage of baseline, LTP stimulus was delivered at time 0 min. (i.) Conditioning facilitates LTP; Facilitation induced by conditioning is blocked by (ii.) inhibiting cleavage of proBDNF with tPA-stop, (iii.) MO. knockdown of BDNF, and (iv.) inhibiting kinase activity with K252A.

Fig 5. **Animals have better visual acuity at 7 to 11 hrs post-conditioning**

A) Example images of gratings projected onto the retina. Below the line is the corresponding counterphased grating. Below are the estimated bar sizes that make up the gratings projected onto the retina.
B) Black traces are 3-5 representative examples of AMPAr mediated synaptic currents elicited by counterphasing the grating images. Red traces are the average of the underlying black traces. Grey boxes indicate the time period quantified.
C) Linear regressions for the average responses elicited by counterphasing gratings shown in B normalized to the averaged full screen off response of the same cell.
D) Mean visual acuity shown as spatial frequency threshold determined by linear regression. Conditioning led to improved acuity (*$p<0.05$, ANOVA with Dunnett's post-test)
E) Example of a single tadpole in one well of a six well dish with gratings of spatial frequency 4 mm^{-1}, 2 mm^{-1}, and 1.4 mm^{-1} presented. White traces are the tadpoles' tracked positions during the 10 sec before counterphasing and for the 15 sec after the onset of counterphasing.
F) Plots of absolute acceleration corresponding to the data in E. Counterphasing increases the number of changes in acceleration that tadpoles exhibit.
G) Mean probability that the tadpole exhibits a change of acceleration greater than one standard deviation of absolute acceleration before the onset of counterphasing. Only conditioned animals were observed to exhibit graded responses to the bar sizes tested.

Fig 6. **Summary:** At time 0, animals are conditioned with a repeating visual stimulus (1). In response to conditioning, activity of the BDNF exon IV promoter is up-regulated (2). Four to 6

hours later, proBDNF levels are increased in the tectum. (3). In the presence of higher levels of BDNF, bidirectional plasticity of tectal neurons is facilitated (4). Acuity measured at 7-11 hrs postconditioning is improved in a BDNF-dependent manner (5).

Supplemental 1.

Evoked AMPA EPSCs are not modulated by exogenous application of proBDNF in the presence of an inhibitor of its primary extracellular cleavage activator tPA. The black bar indicates the period during which proBDNF was applied.

Supplemental 2.

At 4 to 6 hrs after visual conditioning the potentiation induced by depolarization to -12 mV with 3X(40 pulses at 10Hz) at 20 sec intervals is weakly facilitated (control 93±23%, conditioned 118±23%).

Supplemental 3.

In the presence of higher levels of visually induced BDNF, tectal neurons are more susceptible to potentiation by a weak pairing protocol. Averaged EPSC amplitude as a fraction of baseline, pairing protocol was applied at time 0 min. A) Conditioning facilitates potentiation; B) Facilitation is blocked by tPA-stop, and C)by MO knockdown of BDNF.

Supplemental 4.

The AMPA/MNDA ratios of responses elicited by a full-screen OFF visual stimulus, are higher in conditioned animals.that are returned to their normal rearing environment for 7 to 11 hours.

A) Grey traces are 3-4 representative examples of synaptic currents elicited by full-screen OFF or ON stimuli. Red traces are the average of the underlying grey traces. Top pharmacologically isolated NMDAr current at +40 mV, bottom AMPAr mediated current at -70 mV. The vertical scale bar corresponds to 40pA (control), 45pA (8 hrs post-conditioning) and 50pA (+BDNF MO). The horizontal scale bar corresponds to 100msec for all traces.
B) Grouped data. Only the AMAP/NMDA ratio of OFF responses is modulated in animals that have been conditioned. (*$p<0.05$ ANOVA, Dunnets post-test)

Supplemental 5.

Counter phasing gratings do not affect swimming behavior in animals with lesions to the optic tract. A) Maximum intensity projection of mCherry-expressing retinal ganglion cell axons and their projection into both tecta, before (top) and after (bottom) lesioning by repeated slow scanning with a 2-photon microscope. C) 5 hr after lesioning, the response to counterphasing gratings was reduced.

Figure 1

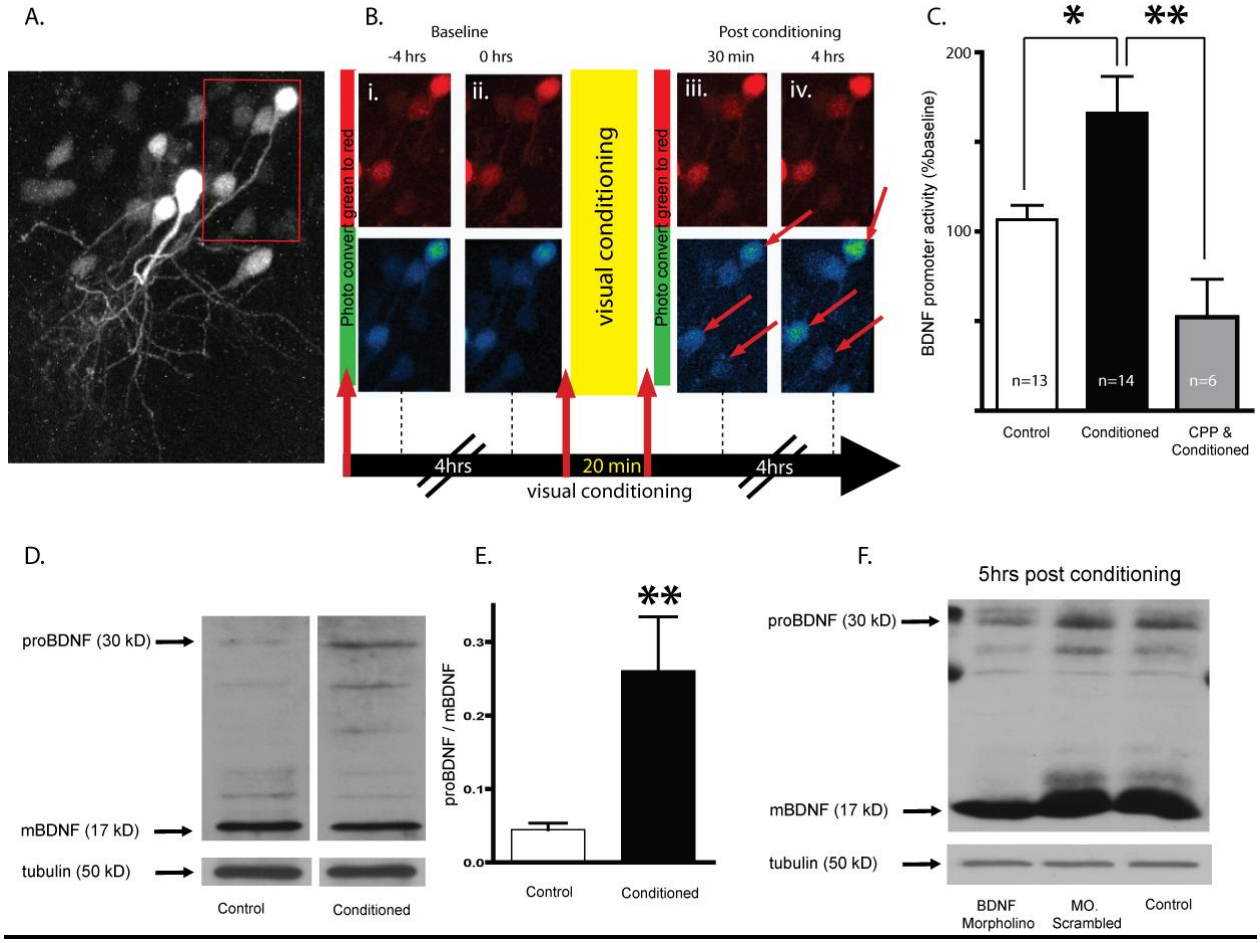

A.
B.
Baseline
-4 hrs
0 hrs
Post conditioning
30 min
4 hrs
i.
ii.
iii.
iv.
Photo convert green to red
visual conditioning
Photo convert green to red
4hrs
20 min
4hrs
visual conditioning
C.
*
**
200
100
BDNF promoter activity (%baseline)
n=13
n=14
n=6
Control
Conditioned
CPP & Conditioned
D.
proBDNF (30 kD)
mBDNF (17 kD)
tubulin (50 kD)
Control
Conditioned
E.
**
proBDNF / mBDNF
0.3
0.2
0.1
0.0
Control
Conditioned
F.
5hrs post conditioning
proBDNF (30 kD)
mBDNF (17 kD)
tubulin (50 kD)
BDNF Morpholino
MO. Scrambled
Control

Figure 2

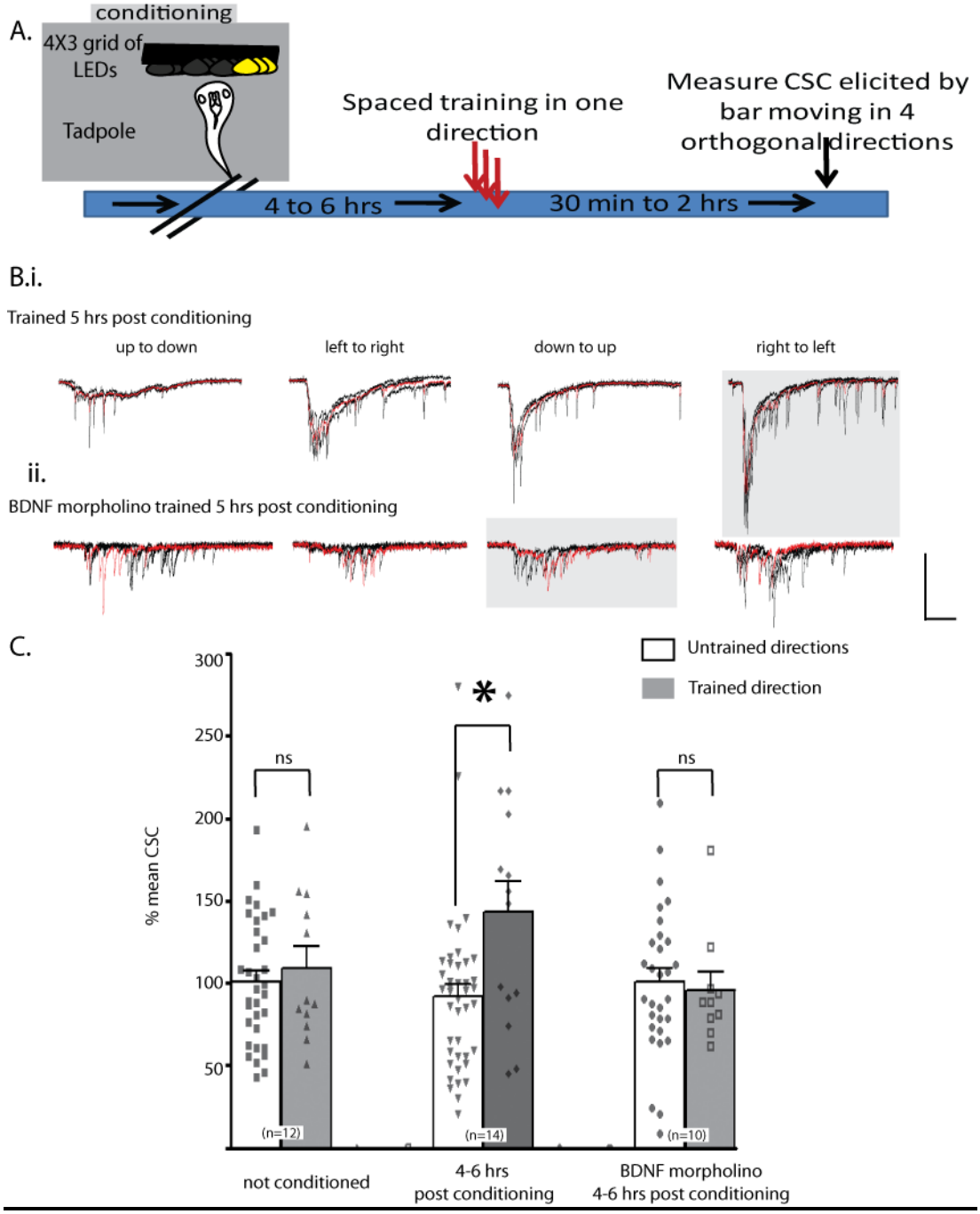

A.
conditioning
4X3 grid of LEDs
Tadpole
Spaced training in one direction
Measure CSC elicited by bar moving in 4 orthogonal directions
4 to 6 hrs
30 min to 2 hrs
B.i.
Trained 5 hrs post conditioning
up to down
left to right
down to up
right to left
ii.
BDNF morpholino trained 5 hrs post conditioning
C.
Untrained directions
Trained direction
% mean CSC
300
250
200
150
100
50
ns
*
ns
(n=12)
(n=14)
(n=10)
not conditioned
4-6 hrs post conditioning
BDNF morpholino 4-6 hrs post conditioning

Figure 3

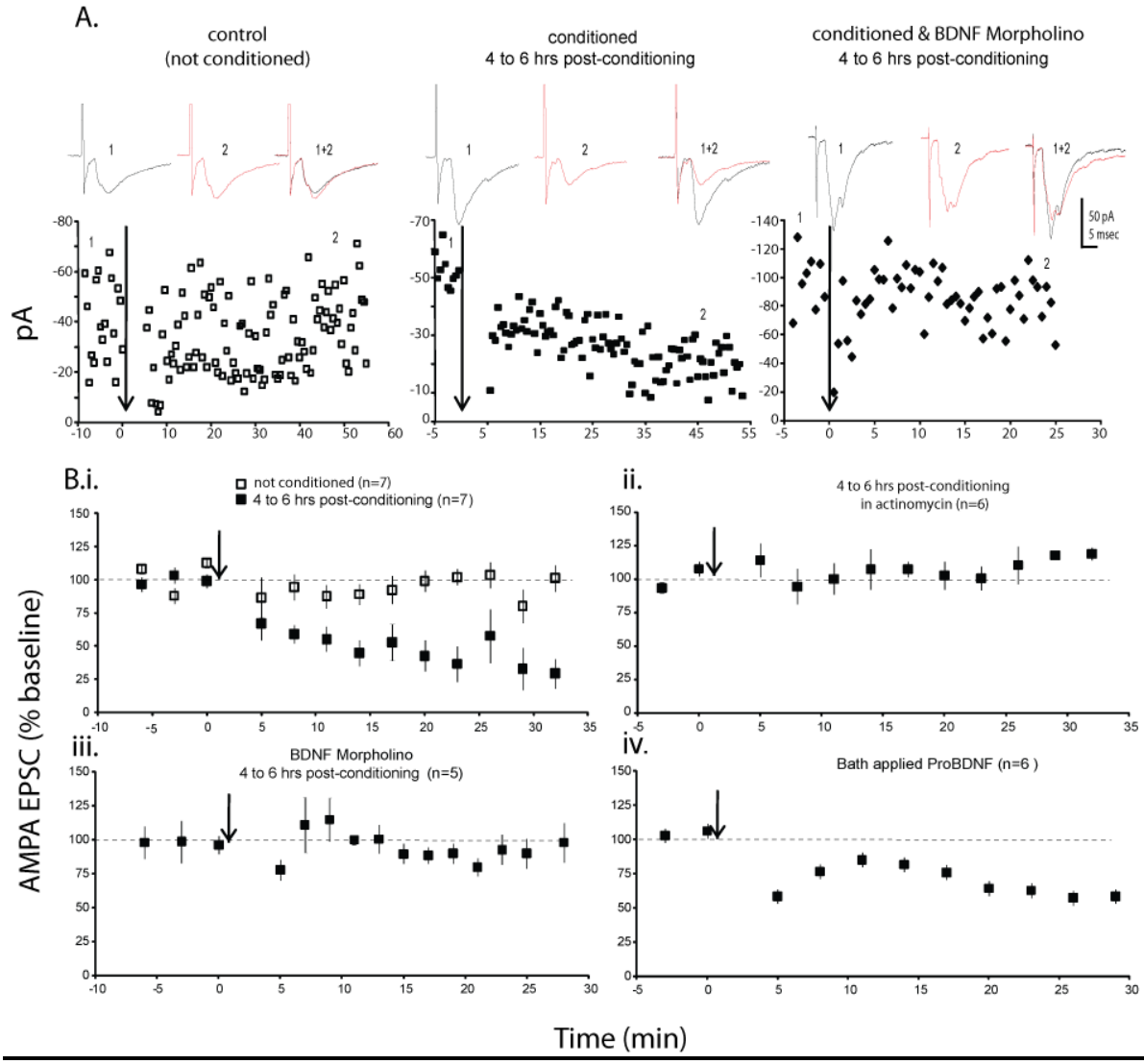

A.
control
(not conditioned)
conditioned
4 to 6 hrs post-conditioning
conditioned & BDNF Morpholino
4 to 6 hrs post-conditioning
1
2
1+2
50 pA
5 msec
pA
B.i.
not conditioned (n=7)
4 to 6 hrs post-conditioning (n=7)
ii.
4 to 6 hrs post-conditioning
in actinomycin (n=6)
iii.
BDNF Morpholino
4 to 6 hrs post-conditioning (n=5)
iv.
Bath applied ProBDNF (n=6)
AMPA EPSC (% baseline)
Time (min)

Figure 4

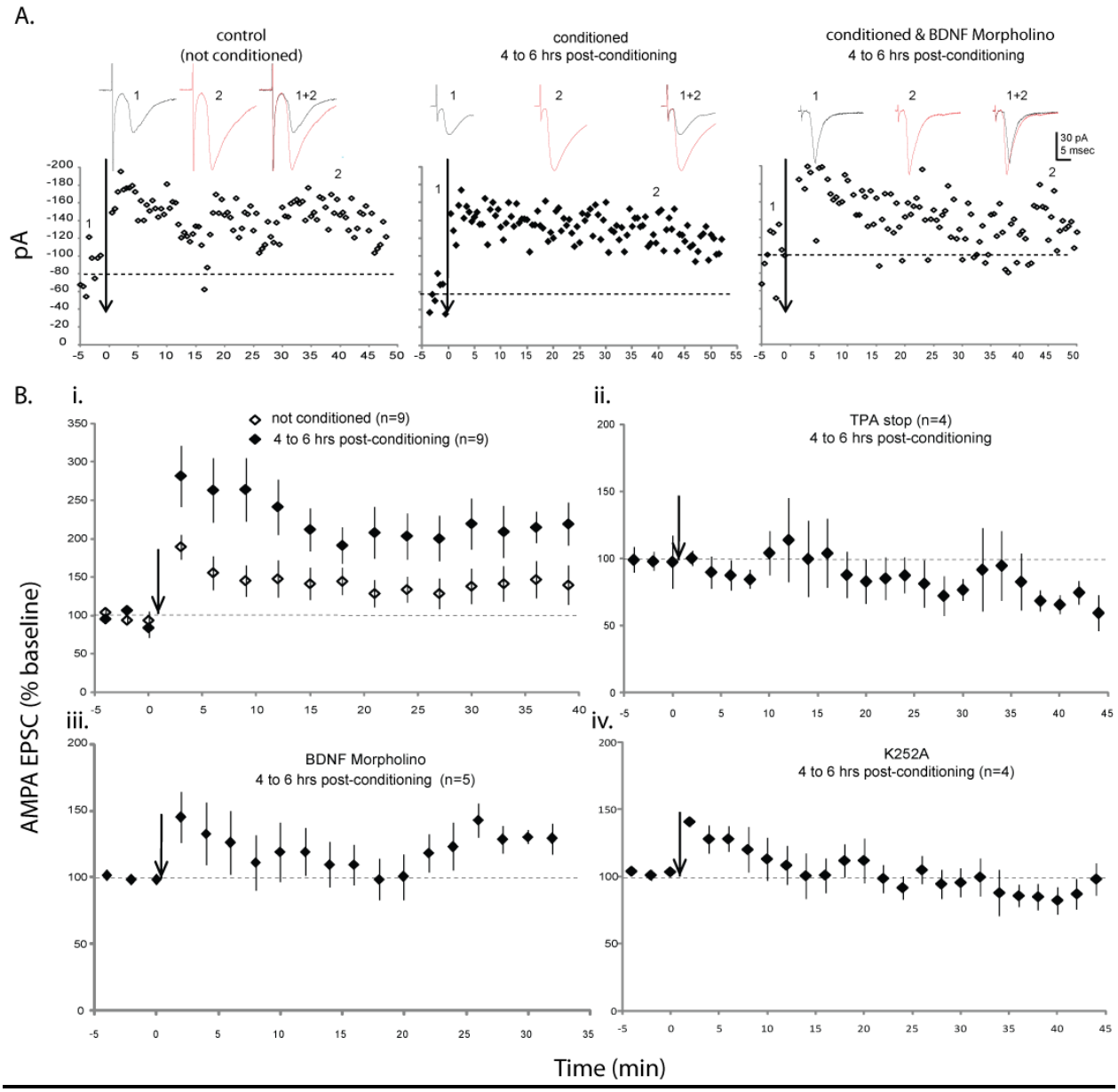

A.
control
(not conditioned)
conditioned
4 to 6 hrs post-conditioning
conditioned & BDNF Morpholino
4 to 6 hrs post-conditioning
1
2
1+2
30 pA
5 msec
pA
B.
i.
not conditioned (n=9)
4 to 6 hrs post-conditioning (n=9)
ii.
TPA stop (n=4)
4 to 6 hrs post-conditioning
iii.
BDNF Morpholino
4 to 6 hrs post-conditioning (n=5)
iv.
K252A
4 to 6 hrs post-conditioning (n=4)
AMPA EPSC (% baseline)
Time (min)

Figure 5

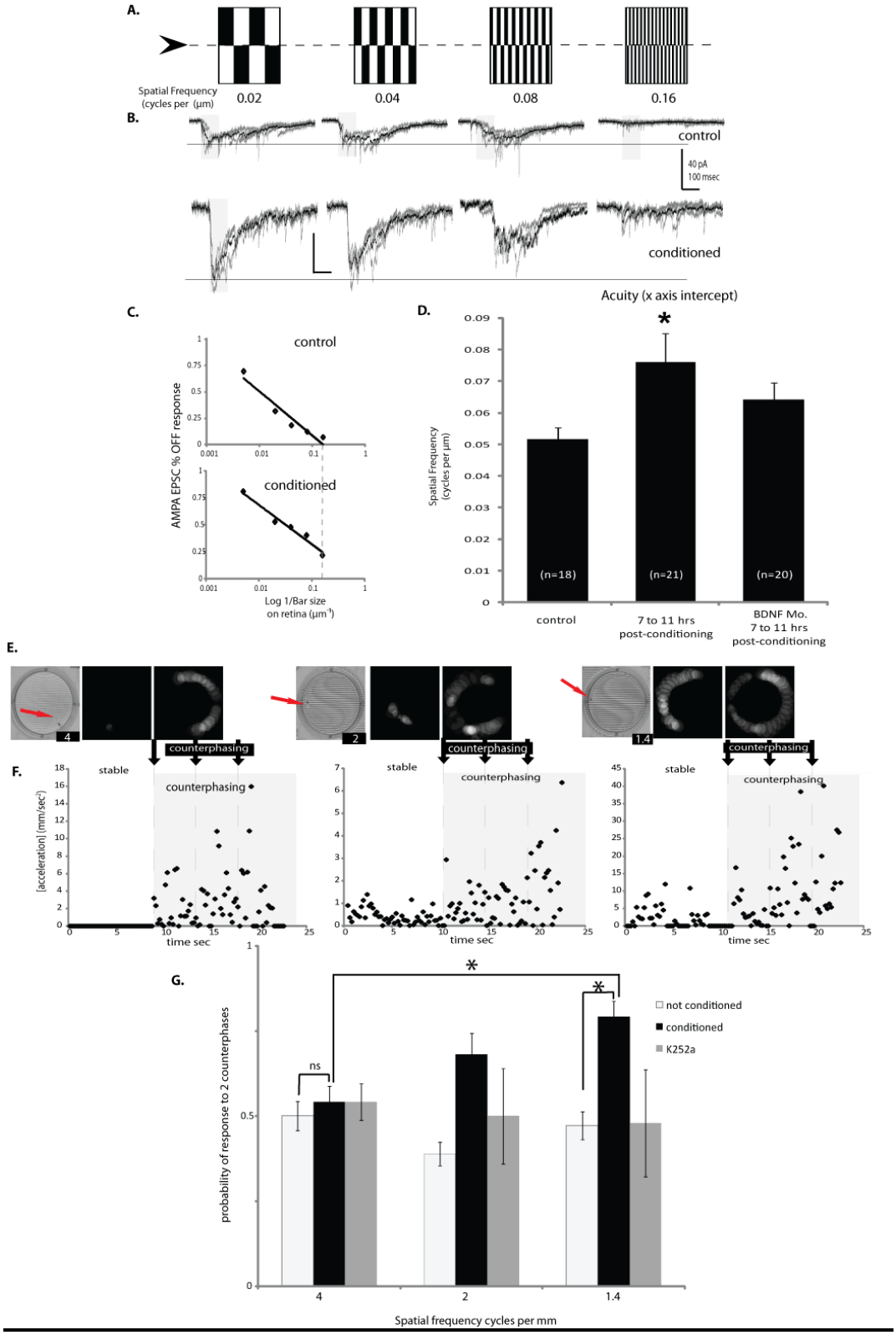
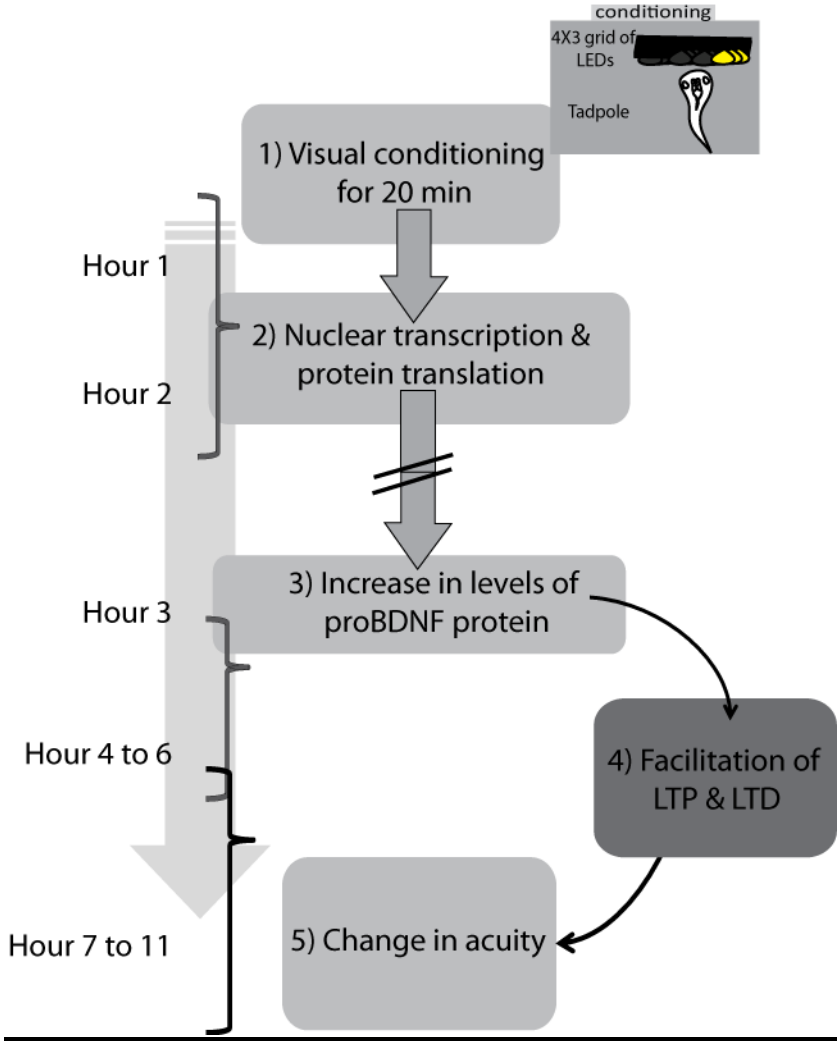
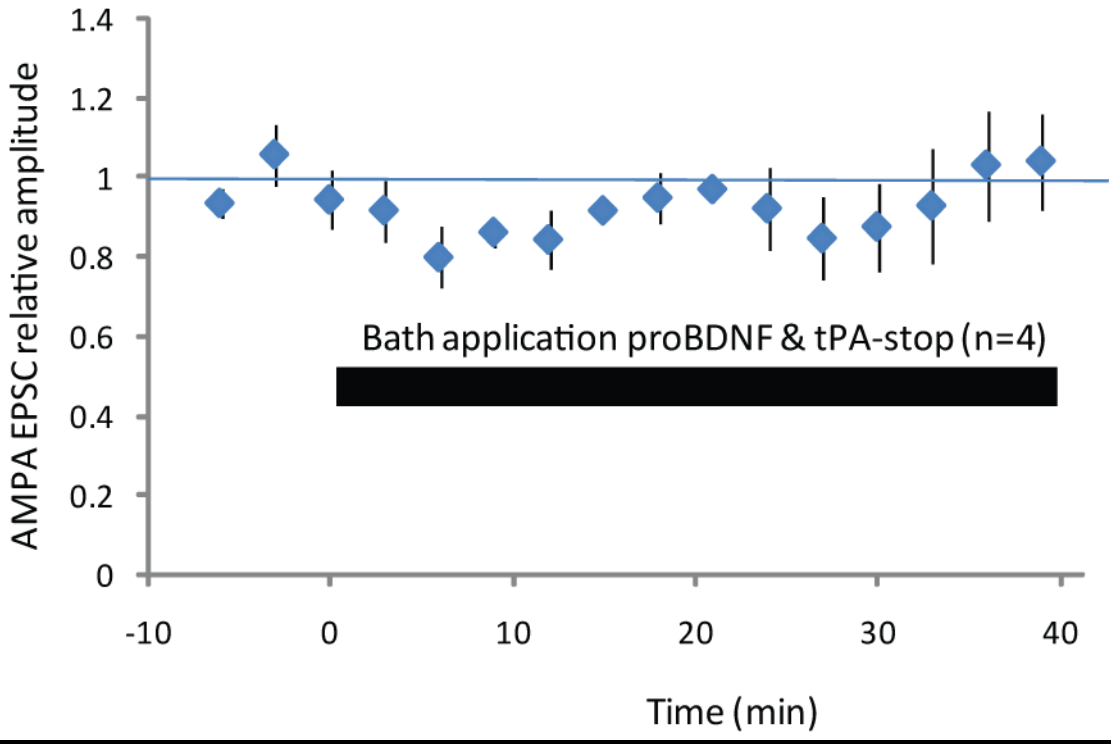

A.
Spatial Frequency (cycles per (µm)
0.02
0.04
0.08
0.16
B.
control
40 pA
100 msec
conditioned
Acuity (x axis intercept)
C.
control
conditioned
AMPA EPSC % OFF response
Log 1/Bar size on retina (µm⁻¹)
D.
Spatial Frequency (cycles per µm)
(n=18)
(n=21)
(n=20)
control
7 to 11 hrs post-conditioning
BDNF Mo. 7 to 11 hrs post-conditioning
E.
4
2
1.4
counterphasing
F.
stable
counterphasing
[acceleration] (mm/sec²)
time sec
G.
probability of response to 2 counterphases
ns
not conditioned
conditioned
K252a
Spatial frequency cycles per mm

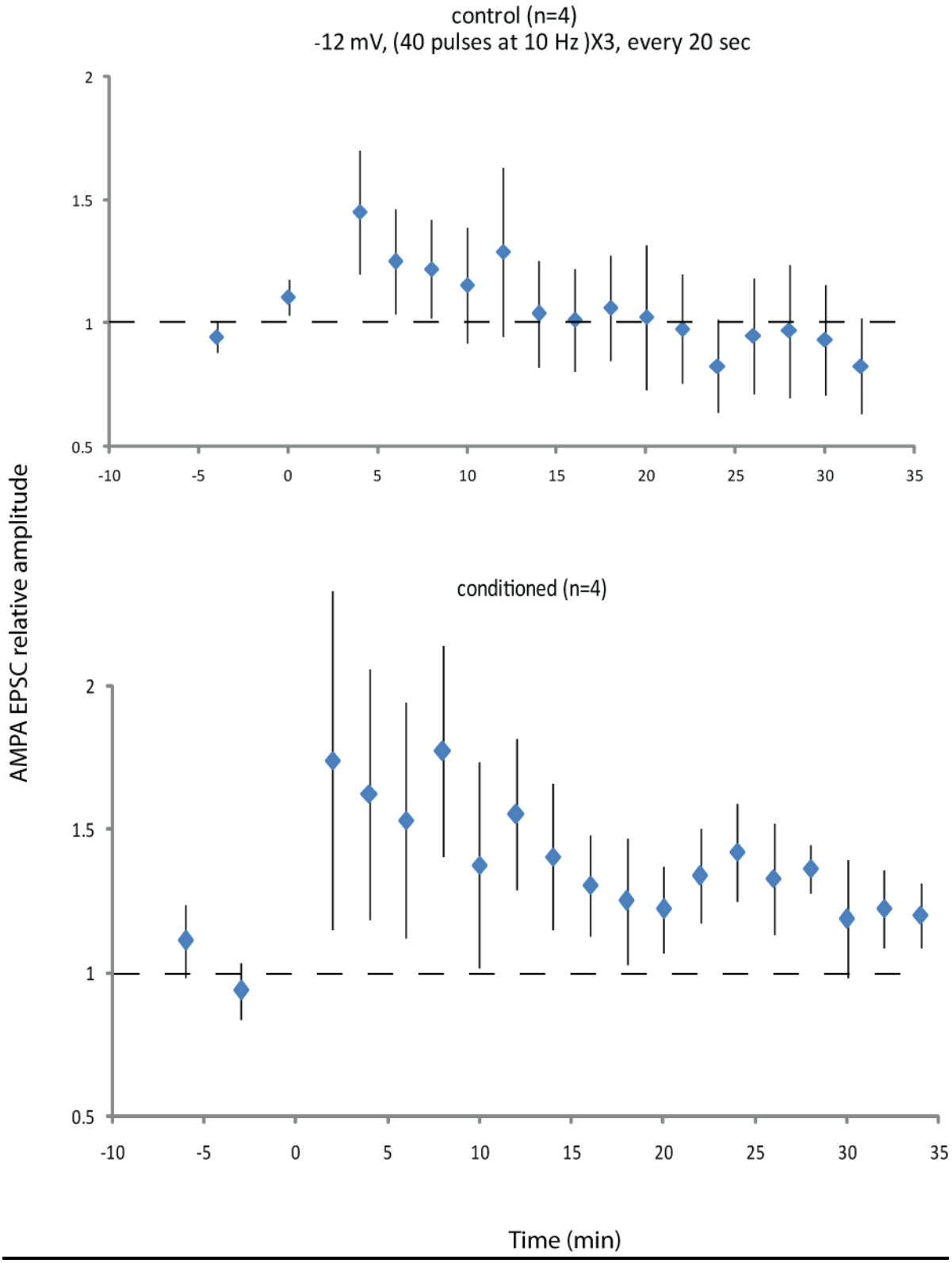
Figure 6


conditioning
4X3 grid of LEDs
Tadpole
1) Visual conditioning for 20 min
Hour 1
2) Nuclear transcription & protein translation
Hour 2
3) Increase in levels of proBDNF protein
Hour 3
4) Facilitation of LTP & LTD
Hour 4 to 6
5) Change in acuity
Hour 7 to 11

Supplemental Figure 1

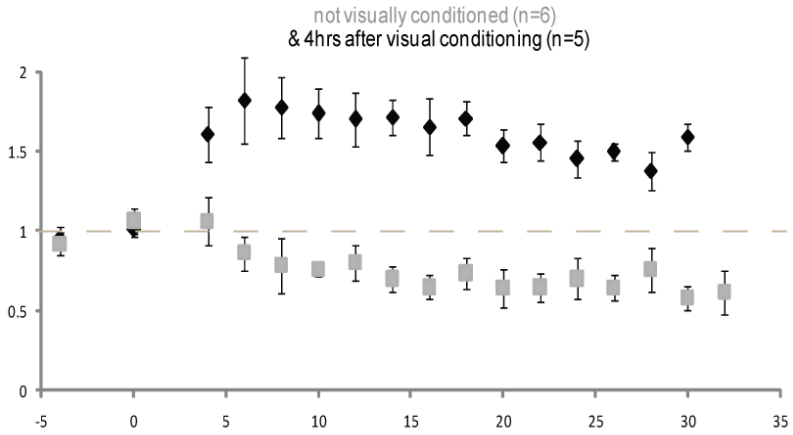


Supplemental Figure 2

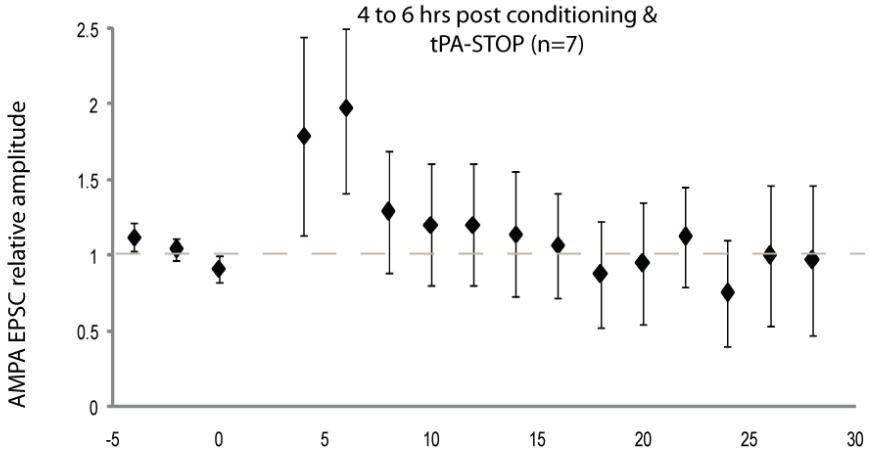


Supplemental Figure 3

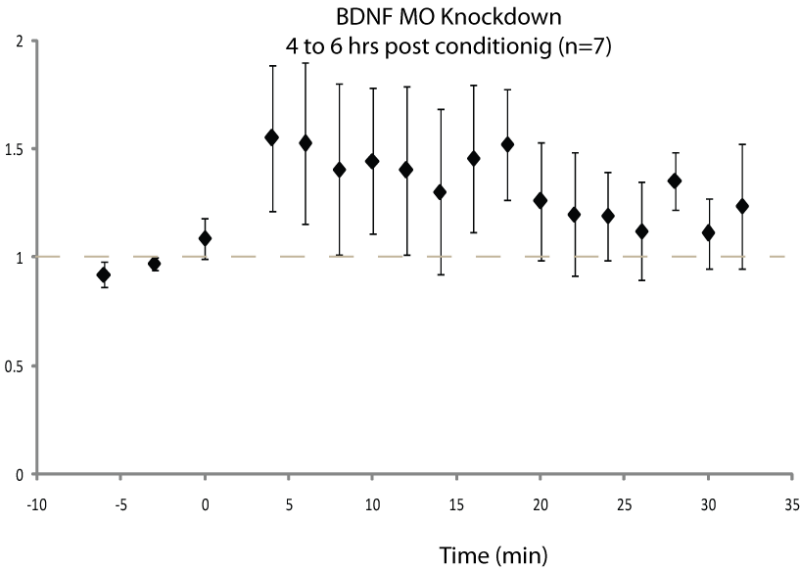
A.



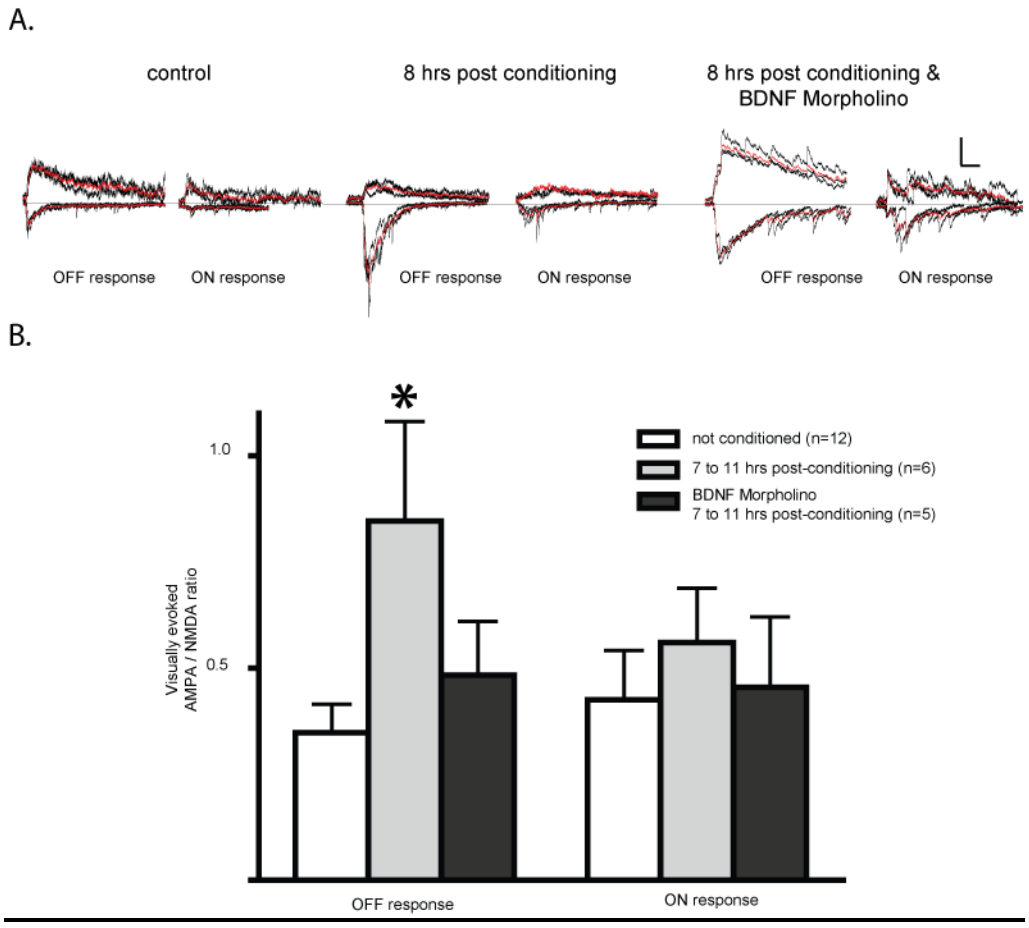
B.



C.

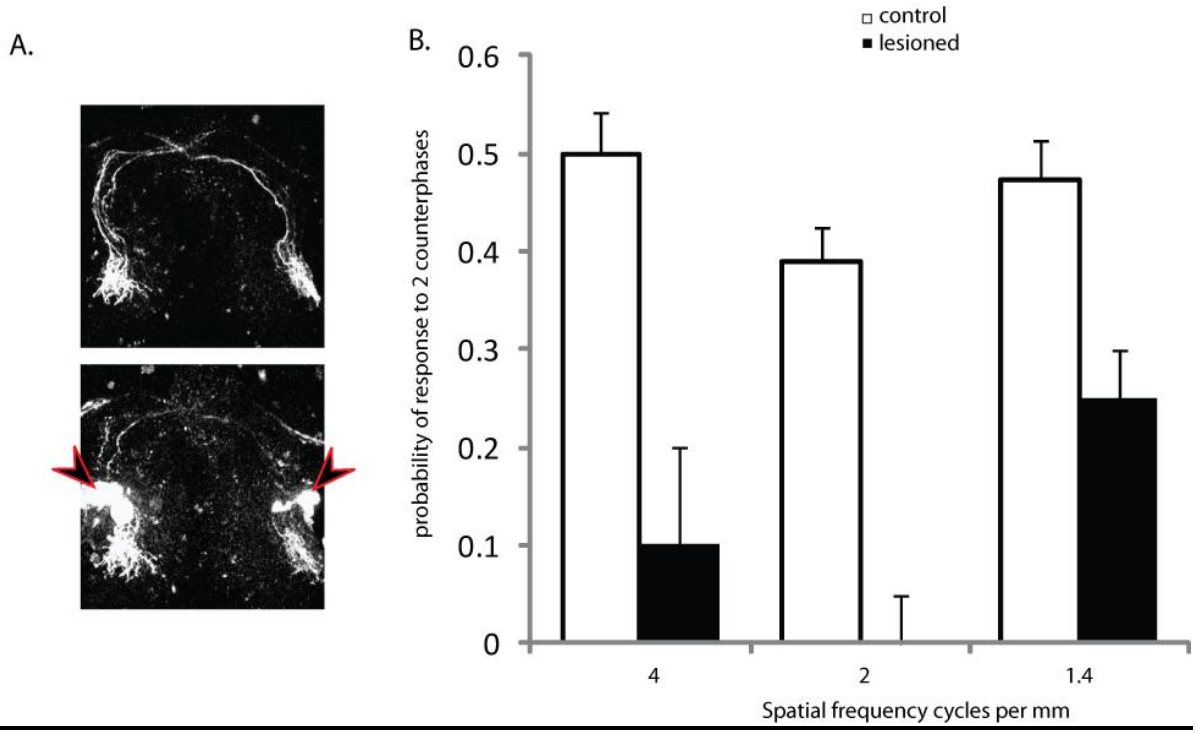


Supplemental Figure 4



Supplemental Figure 5

Supplemental Figure 4.



GENERAL DISCUSSION

Encoding of "experience-expectant" and "experience-dependent" information

Greenough et al. (1986) provided an insightful framework for classifying the nature of information stored in circuitry during development. In this hypothesis it is proposed that changes in circuitry can store information that is "experience-expectant" or "experience-dependent". The former represents the storage of ubiquitous properties of the environment in which the animal has evolved, and the latter constitutes a mechanism for adaptation to the unique experiences that the animal undergoes. In accordance with the interpretation of the data in this thesis the authors do note that these processes likely exhibit interactions.

Interestingly, in our initial experiments we observed that there was an intermediate level of ongoing NFAT activity, and that the level of this ongoing activity, could be up- or down-regulated dependent upon recent visual experience. We further observed that chronic inhibition of NFAT led to an increased rate of dendritic remodeling. Notably, the increased rate of remodeling was not present if ionotropic synaptic activity was inhibited. The implications of these observations are that synaptic activity regulates the level of NFAT activity in the cell; that the products transcribed and translated in response to experience-dependent modulation of NFAT, are a reflection of the recent synaptic history of the cell; that these products then affect the propensity of the cell to remodel; and finally that the expression of the NFAT-dependent propensity to remodel is expressed contingent on current synaptic activity. It is therefore tempting to speculate that the activation level of NFAT serves as a readout of the recent synaptic activity in the cell, and determines the cell's propensity to remodel contingent upon current synaptic input. Therefore the regulation of

NFAT fits well into a model in which experience-dependent processes regulate the efficiency with which experience-expectant processes are encoded. To test the nature of this interaction we performed our second study.

To assay function of the visual system in our second study we measured resolution acuity. The refinement of acuity is an ongoing process and likely represents the encoding of experience-expectant information. Intuitively, major reasons why this process is ongoing and classified as experience-expectant are that stimuli that regulate it are found in most visual scenes, including our conditioning (NFAT inducing) stimulus, and that it represents the normal developmental course that most animals will go through. Thus it is not surprising, that visual conditioning eventually lead to an improvement in resolution acuity.
One consideration is what is the organization of and interaction between events underlying this process? A possible scenario is that an episode of training, in this instance, the conditioning stimulus, would sequester plasticity mechanisms in the neuron, whilst the process of acuity-refinement would temporarily be impeded. However, if plasticity mechanisms are sequestered, then it is plausible to assume that the neuron would exhibit an improvement specific to the training, and an impairment of acuity-refinement. In this study we observed that there was an improvement in acuity. Thus, it seems likely that the process of acuity refinement is not impeded. Interestingly, the underlying mechanism, NFAT dependent up-regulation of BDNF, which eventually facilitated an improvement in acuity, occurred long after the initial induction and stabilization of plasticity induced by the conditioning stimulus (Tsui et al., 2010). It is tempting to speculate that this temporal staggering, ensures that neither the plasticity specific to the stimulus, nor the ongoing process

of acuity-refinement, impairs its counterpart. A representative example of an ability that depends upon plasticity induced during the relevant training experience is direction sensitivity (Engert et al., 2001, Li et al., 2008). Therefore, as acuity-refinement relies on ubiquitous properties of the visual environment, it occurs continuously, but there seem to be mechanisms in place to ensure that it does not impede the encoding of stimulus specific experiences during development. Further support for this postulate is the observation that resolution acuity is generally only impaired in cases of degraded input (Maurer et al., 2001).

Similarities to the effects of eye reopening, following monocular deprivation in visual cortex: In this study a repeating visual stimulus was used to acutely augment visual input to developing Xenopus tadpoles. In rodents, reopening the eye after monocular deprivation is commonly used to investigate the effects of increased synaptic drive in the mammalian visual system (Hofer et al., 2006; McCoy et al., 2009). Interestingly, in both cases BDNF is regulated (Rossi et al., 1999). Moreover, it is tempting to speculate that in both cases the functional plasticity facilitated by BDNF may be similar.

In rodents, reducing synaptic drive by monocular deprivation has been shown to decreases the expression of BDNF in the cortex (Rossi et al., 1999; Ichisaka et al., 2003). Therefore, BDNF levels are likely low upon eye reopening. Despite these reduced levels, binocular responses in the contralateral cortex are rapidly reinstated in a protein synthesis independent manner. However, more complete long term recovery, has been shown to depend upon BDNF-TrkB signaling (Krahe et al., 2005; Kaneko et al., 2008).

In this thesis we observed that by 4 hours after conditioning, there is a transcription-dependent up-regulation of BDNF protein. Here we observed that this newly transcribed

BDNF, present hours after the initial plasticity induction, facilitated ongoing refinement. A similar up-regulation is expected to occur in rodents after eye opening (Rossi et al., 1999; Ichisaka et al., 2003). If it does then in both models up-regulation of BDNF would serve to facilitate refinement of finer functional characteristics of the system, including acuity. In support of this, are the observations that in rodents, recovery is BDNF-TrkB dependent (Kaneko et al., 2005), and recovery of ipsilateral responses and acuity are protein synthesis dependent, and take much longer to occur (Krahe et al., 2005; Iny et al., 2006; Mitchell et al., 2001; He et al., 2007). This role for BDNF as a facilitator of the development of specific functional properties in the visual system is consistent with its effects during normal development (Heimel et al., 2010).

Conclusion

In this project we sought to determine how local changes at the synapse and experience-dependent regulation of transcription interact to affect the function of a developing visual circuit. We observed that in the developing visual system synaptic regulation of transcription led to a delayed facilitation of bi-directional plasticity, and that this facilitation leads to improved visual system function. Thus our results indicate that during development the encoding of specific episodes, facilitates the development of abilities to resolve ubiquitous features of the environment.

Future directions

In this thesis we showed that NFAT was regulated by the same visual stimulus that also regulated BDNF expression. However, although NFAT has been shown to regulate BDNF expression in other systems, we did not provide a direct demonstration that NFAT is required

for the up-regulation of BDNF in this paradigm (Groth et al., 2003). Thus the first future direction is to provide proof that the NFAT binding site in the BDNF exon IV promoter is required for the observed up-regulation of BDNF. We also observed synapse specific changes. Therefore it seems likely that the observed transcriptional response led to an up-regulation of message with a long 3'UTR. An interesting consequence of the up-regulation of this long form is that local translation in the dendrites resulted in the observed increase in proBDNF. Thus other future directions would be to determine with PCR if the long 3'UTR message is up-regulated, and if there is an accompanying up-regulation of local translation in the dendrites. In the retino-tectal system BDNF has been shown to mediate a retrograde spread of plasticity from the tectum to the retina, one possible mechanism is through the retrograde transport of BDNF along the rgc axons (Du et al., 2004 & 2009). A final direction would be to determine if the endogenously synthesized BDNF is transported retrogradely and its effect on retinal circuitry.

ABBREVIATIONS

A-kinase anchoring protein 79 (AKAP79)

α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAr)

auto-inhibitory domain of Xenopus calcineurin (AI-AXCN)

Brahma-associated factor (BAF)

Calcineurin (CaN)

Excitatory post synaptic potential (EPSC)

Green fluorescent protein (GFP)

Long term depression (LTD)

Long term potentiation (LTP)

messenger ribonucleic-acid mRNA

neurotrophic tyrosine kinase, receptor, type 2 (TrkB)

methyl-CpG binding protein 2 (MeCP2)

N-methyl D-aspartate (NMDAr)

Nuclear Factor of activated T cells (NFAT).

polymerase chain reaction (PCR)

post-synaptic density-95 (PSD-95)

pro Brain Derived Neurotrophic Factor (proBDNF)

retinal ganglion cell (RGC)

tissue plasminogen activator (tPA)

3’ untranslated region (3’UTR)

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