SENSORY EXPERIENCE DRIVEN NETWORK PLASTICITY IN THE AWAKE DEVELOPING BRAIN

by

DEREK JAMES DUNFIELD

B.Sc.H - SSP, Queen’s University, 2003
M.Sc., Queen’s University, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES (Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

September 2009

© Derek James Dunfield, 2009
ABSTRACT

During embryonic activity-dependent brain circuit refinement, neurons receiving the same natural sensory input may undergo either long-term potentiation (LTP) or depression (LTD). While the origin of variable plasticity in vivo is unknown, the type of plasticity induced plays a key role in shaping dynamic neural circuit synaptogenesis and growth. Here, we investigate the effects of natural visual stimuli on functional neuronal firing within the intact and awake developing brain using calcium imaging of 100s of central neurons in the *Xenopus* retinotectal system. We find that specific patterns of visual stimuli shift population responses towards either potentiation or depression in an N-methyl-D-aspartate receptor (NMDAR)-dependent manner. In agreement with the Bienenstock-Cooper-Munro (BCM) theory, our results show that functional potentiation or depression in individual neurons can be predicted by their specific receptive field properties and endogenous firing rates prior to plasticity induction. Enhancing pre-training activity shifts plasticity outcomes as predicted by BCM, and this induced metaplasticity is also NMDAR dependent. Furthermore, network analysis reveals an increase in correlated firing of neurons that undergo potentiation. These findings implicate metaplasticity as a natural property governing experience-dependent refinement of nascent embryonic brain circuits.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>x</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xi</td>
</tr>
<tr>
<td>COAUTHORSHIP STATEMENT</td>
<td>xii</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1. General introduction to developmental plasticity</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Long-term changes in synaptic transmission</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 “Traditional” long term potentiation and long term depression within the mature mammalian hippocampus</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Molecular mechanisms of LTP induction</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3 Long term depression</td>
<td>9</td>
</tr>
<tr>
<td>1.2.4 LTP / LTD induction protocols and spike time dependent plasticity</td>
<td>11</td>
</tr>
<tr>
<td>1.2.5 Evidence for LTP/LTD in vivo</td>
<td>12</td>
</tr>
<tr>
<td>1.2.6 LTP/LTD in the developing brain</td>
<td>15</td>
</tr>
<tr>
<td>1.3. Plasticity of intrinsic excitability</td>
<td>17</td>
</tr>
<tr>
<td>1.3.1 Neuronal excitability, ionic channels, and the input–output function</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2 NMDA receptor dependent EPSP-spike plasticity</td>
<td>18</td>
</tr>
<tr>
<td>1.4. Experience-driven plasticity in the Xenopus tadpole retinotectal system</td>
<td>20</td>
</tr>
<tr>
<td>1.4.1 Morphological plasticity</td>
<td>20</td>
</tr>
<tr>
<td>1.4.2 Visually-driven synaptic plasticity</td>
<td>22</td>
</tr>
</tbody>
</table>
1.4.3 Homeostatic regulation of intrinsic excitability .......................................................... 26

1.5. Metaplasticity .................................................................................................................. 27

1.5.1 The plasticity of plasticity .......................................................................................... 27

1.5.2 The Bienenstock, Cooper and Munro (BCM) computational model of synaptic plasticity .......................................................... 29

1.5.3 NMDA receptor-mediated metaplasticity ................................................................ 31

1.5.4 Metaplastic E-S plasticity: common learning rules? .................................................. 32

1.5.5 Metaplasticity induced by environmental stimuli ...................................................... 33

1.6. Research hypothesis and objectives ............................................................................ 36

1.7. References ..................................................................................................................... 39

2. Metaplasticity governs natural experience-driven plasticity of nascent embryonic brain circuits .................................................................................................................. 55

2.1. Introduction .................................................................................................................. 55

2.2. Results .......................................................................................................................... 57

2.2.1 Long lasting functional plasticity in central neuronal ensembles driven by natural sensory stimuli .......................................................... 58

2.2.2 Variable plasticity of individual central neurons to the same sensory training paradigm .................................................................................................................. 59

2.2.3 Synaptic mechanisms underlying visually induced tectal RF plasticity .................. 61

2.2.4 RF mapping across tectal circuit and specificity of plasticity to the characteristics of the training sensory stimuli .......................................................... 61

2.2.5 Metaplastic rules predict visually induced RF plasticity of individual neurons ...... 62

2.2.6 Experimentally increased pre-training activity shifts spaced training plasticity outcomes toward depression through an NMDAR dependent mechanism ..................... 64

2.2.7 Potentiation is associated with an increase in spontaneous correlated firing ...... 65

2.3. Discussion ..................................................................................................................... 66
2.4. Experimental procedures .................................................................................................................. 69
   2.4.1 Animal rearing conditions ............................................................................................................ 69
   2.4.2 Calcium indicator loading ............................................................................................................ 69
   2.4.3 In vivo two-photon calcium imaging of neuronal dynamics ........................................................ 70
   2.4.4 Visual stimulation ......................................................................................................................... 70
   2.4.5 Single cell excitability probing (SCEP) ....................................................................................... 71
   2.4.6 Visual training ............................................................................................................................... 71
   2.4.7 White noise .................................................................................................................................. 72
   2.4.8 Blockade of NMDAR ................................................................................................................... 72
   2.4.9 Analysis of imaging data .............................................................................................................. 72
   2.4.10 Evoked responses ....................................................................................................................... 73
   2.4.11 Spontaneous spiking ................................................................................................................... 73
   2.4.12 Inclusion criteria ........................................................................................................................ 73
   2.4.13 Plasticity criteria ........................................................................................................................ 74
   2.4.14 Spontaneous correlations ........................................................................................................... 74
   2.4.15 Clustering .................................................................................................................................... 75
2.5. Acknowledgements ............................................................................................................................ 75
2.6. Figures ................................................................................................................................................ 76
2.7. References .......................................................................................................................................... 96

3. In vivo single cell excitability probing of neuronal ensembles in the intact developing brain ................................................................. 101
   3.1. Introduction ..................................................................................................................................... 101
   3.2. Materials ........................................................................................................................................ 104
       3.2.1 Reagents ..................................................................................................................................... 104
3.2.2 Equipment.................................................................................................................. 105
3.2.3 Reagent setup .............................................................................................................. 106
3.2.4 Equipment setup ........................................................................................................ 107
3.3. Procedure ..................................................................................................................... 109
  3.3.1 Staining neurons with calcium-indicator dye ....................................................... 109
  3.3.2 Two-photon imaging and excitability probing .................................................... 111
3.4. Anticipated results ..................................................................................................... 114
3.5. Tables .......................................................................................................................... 116
3.6. Figures ........................................................................................................................ 118
3.7. References .................................................................................................................. 122
4. **Concluding chapter** ................................................................................................. 125
  4.1. Metaplasticy’s role in development ................................................................. 125
  4.2. Bridging the gap between cells and systems ..................................................... 128
    4.2.1 Population recordings with single cell resolution ............................................. 128
    4.2.2 Alternatives to SCEP ....................................................................................... 129
  4.3. Status of working hypotheses and contribution to the field ................................. 130
  4.4. Future directions: Linking functional and morphological plasticity ...................... 132
  4.5. Clinical relevance ..................................................................................................... 134
  4.6. References ................................................................................................................ 137
5. **Appendix A: Ethics board certificates** .................................................................... 142
LIST OF TABLES

Table 3.1  Training paradigms .......................................................................................... 116
Table 3.2  Troubleshooting table .................................................................................. 117
I would like to thank my committee members – including Dr. Brian MacVicar, Dr. Catharine Rankin, and Dr. Ann-Marie Craig – for their invaluable support of my work. Special thanks go to my supervisor, Dr. Kurt Haas, for providing me the freedom to develop my own research from the ground up. The insights I’ve gained from being involved in the front line of a start-up lab will undoubtedly colour my vision of research for years to come. Incentives in academia may not promote taking risks, but we did, and they paid off. Thank you to K. Podgorski for help with correlation and clustering analysis in Chapter 2; D. Hines for supportive insights on calcium imaging; the Haas lab for arduous, day-long meetings and advice; and T. Murphy, D. Allan, S. Bamji, and B. Chen, for revisions of my Chapter 2 manuscript. I would also like to thank my lab mates for input on the following pages: B. Duncan for p. 1 and 12; S. Chen for p. 12, 25, 15, 21, 18, and 20; S. Hossain for p. 8, 5, 19, and 9; X. Liu for p. 19, 1, 18, and 5; S. Hewapathirane for p. 2, 5, 12, 15, 14, 7, and 20; W. Yen for p. 15, and 21; and P. Edgecumbe for p. 19.

This work and was supported by the National Science and Engineering Council of Canada, the Canadian Institute of Health Research, the Michael Smith Foundation for Health Research, the Canadian Foundation for Innovation, the British Columbia Innovation Council, The EJLB Foundation, and the Human Early Learning Project. My full salary was funded by the Canadian Federal Government, and Ontario and British Columbian Provincial Governments. As such, I believe I have a great responsibility to contribute back to Canada. To the Canadian Tax Payer, I will not let you down. Finally, thank you to Dr. Heidi Gordon. You brought me to Vancouver and made every minute worth while.
DEDICATION

To Robert and Susan Dunfield, Ian and Barbara Browne, and Lois Dinwoodie:

Let’s hope this is the last summer I miss being in your company.
COAUTHORSHIP STATEMENT

Derek Dunfield conceived, designed, executed, and analyzed all research herein. Dunfield prepared all figures and manuscripts. Dr. Kurt Haas contributed valuable comments for the revision of the manuscripts presented in Chapters 2 and 3.
1. **INTRODUCTION**

1.1. **GENERAL INTRODUCTION TO DEVELOPMENTAL PLASTICITY**

One of the most complex tasks in all of biology is the formation of the human brain. Throughout early development, neural circuits form by an active process of trial and error where synaptic inputs are created and strengthened, or weakened and lost to optimize each neuron’s response to a specific set of afferent stimuli or receptive field. During critical developmental periods, receptive fields of central neurons undergo dramatic changes as they are shaped by sensory input (Hubel and Wiesel, 1963; Katz and Shatz, 1996; Pratt et al., 2008). The size and shape of a neuron’s structure also undergo dramatic changes throughout development as newly differentiated neurons grow from simple spheres to extension of elaborate dendritic tree-like structures (Jontes et al., 2000; Kaethner and Stuermer, 1997; Niell et al., 2004; Wu et al., 1999). Brain neuron structural growth is also highly dynamic coinciding with, and integrally dependent on, developmental synaptogenesis. Synapse strengthening and weakening confers morphological stabilization and extension, or destabilization and retraction to highly plastic growing axons (Meyer and Smith, 2006; Ruthazer et al., 2003; Ruthazer et al., 2006) and dendrites (Niell et al., 2004). Likewise, brain neuronal structural growth is also influenced by environmental experience (Cline and Haas, 2008; Greenough et al., 1989; Sin et al., 2002b).
Synapse plasticity, which is believed to mediate synaptotropic dendritic growth during critical periods of early brain development, has primarily been studied as long-term potentiation and depression (LTP/LTD) of glutamatergic synaptic transmission of Schaffer collateral-CA1 synapses in the mature rodent hippocampus, a brain region considered integral in memory formation (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). LTP is a persistent increase in synaptic strength in response to a strong training stimulus. It has been detected at excitatory synapses throughout the brain and can last from hours to months (Malenka and Bear, 2004). Experimentally, LTP is typically induced in hippocampal brain slices by strong electrical stimulation of large numbers of afferent inputs using large bipolar electrodes (Bliss and Collingridge, 1993; Rose and Dunwiddie, 1986). Long-term depression (LTD), a persistent decrease in synaptic strength, can be induced in Schaffer collateral-CA1 synapses by low frequency electrical simulation in the hippocampal brain slice preparation (Thiels et al., 1994).

The most commonly studied forms of LTP and LTD are N-methyl-D-aspartate receptor (NMDAR) dependent (Dudek and Bear, 1992; Mulkey and Malenka, 1992) and require Ca^{2+} influx for activation (Mulkey and Malenka, 1992). LTP and LTD have also been described in the developing brain (Crair and Malenka, 1995; Dudek and Friedlander, 1996; Kirkwood et al., 1995; Rittenhouse et al., 1999; Sermasi et al., 1999; Tao et al., 2001; Yasuda et al., 2003; Zhang et al., 1998).

One critical question in the field of LTP and LTD is whether it represents an endogenous mechanism used by the brain for learning and memory, or whether it is an experimental artefact. This question arises from the highly unnatural electrical stimuli typically employed for LTP and LTD induction. Large bipolar electrodes are used to drive synchronous axonal firing of
large numbers of afferent inputs in a manner unlikely to occur under physiological conditions. To address this concern, a number of studies in the adult nervous system have provided evidence that natural sensory stimuli, such as fear conditioning and motor training, may induce long-term synaptic modifications similar to electrical stimulation-induced LTP and LTD (Moser et al., 1998; Rioult-Pedotti et al., 1998; Rogan et al., 1997; Rumpel et al., 2005; Xu et al., 1998).

A central question in developmental neuroscience is whether the same molecular mechanisms underlying synapse plasticity in the mature brain associated with learning and memory are utilized during development for synaptogenesis and synaptotropic growth during experience-dependent brain circuit formation. In the mammalian brain, ability to experimentally induce LTP by electrical stimulation is increased during developmental periods of neural circuit formation (Crair and Malenka, 1995; Kirkwood et al., 1995), suggesting it may serve in activity-dependent synaptogenesis and competition-based synapse stabilization or pruning. In the *Xenopus laevis* tadpole optic tectum, during periods of active synaptogenesis, neuronal growth, and receptive field refinement, NMDAR dependent LTP and LTD can be induced by repetitive electric stimulation of afferent retinal ganglion cells in a similar fashion to LTP and LTD induction at mature rodent Schaffer collateral-CA1 hippocampal synapses (Tao et al., 2001; Zhang et al., 1998).

A wealth of evidence demonstrates that developmental neural circuit formation is strongly influenced by natural sensory stimuli during critical periods of brain development (Greenough et al., 1986; Greenough et al., 1989; Katz and Shatz, 1996; Rittenhouse et al., 1999). To support the hypothesis that LTP and LTD play a role in dendritic morphological
development, LTP and LTD plasticity must be shown to occur endogenously in response to natural experience. Importantly, a number of studies have demonstrated LTP and LTD in the developing brain induced by natural sensory stimuli (Engert et al., 2002; Tao et al., 2001; Xu et al., 1998; Zhang et al., 2000; Zhou et al., 2003). Electrophysiological evidence of visually-driven LTP \textit{in vivo} was demonstrated in the developing \textit{Xenopus laevis} tadpole retinotectal system using repetitive dimming light stimuli and moving bar stimuli applied to the retina (Engert et al., 2002; Zhang et al., 2000; Zhou et al., 2003). Visually-driven LTP induction resulted in persistent, NMDAR dependent, enhancement of RGC glutamatergic synaptic inputs on tectal neurons and exhibits similar characteristics to electrically-induced LTP in this system (Tao et al., 2001; Zhang et al., 2000).

**1.2. LONG-TERM CHANGES IN SYNAPTIC TRANSMISSION**

1.2.1 “Traditional” long term potentiation and long term depression within the mature mammalian hippocampus

In the adult nervous system, synaptic plasticity generally refers to modulation of synaptic strength caused by changes in postsynaptic currents and potentials in response to unvarying pre-synaptic input. With over 10000 publications, the most studied form of synaptic plasticity is NMDAR-dependent long-term potentiation (LTP) of glutamatergic synaptic transmission – a long term increase in synaptic strength that can last from hours to possibly months (Malenka and Bear, 2004). LTP has been identified at synapses throughout the mammalian brain, yet it is classically studied in area CA1 of the hippocampus, a brain region
considered an integral component of memory formation (Bliss and Collingridge, 1993). LTP induction is accomplished by electrically stimulating the Schaffer collateral-CA1 pathway. Schaffer collaterals are axon projections given off by CA3 pyramidal cells that project to area CA1. NMDAR-dependent LTP is characterized by four basic properties: cooperativity, associativity, input-selectivity, and NMDAR dependence. Cooperativity underlies the requirement of excitation of multiple afferents for LTP induction. ‘Weak’ electrical stimulation, activating few afferent fibres, does not trigger LTP (McNaughton et al., 1978). Similarly, the intensity and frequency of electrical stimulation is directly associated with the duration of the induced potentiation – ‘strong’, multiple afferent, stimulation engages long-term potentiation while stimulation with intensities between ‘strong’ and ‘weak’ induces a shorter termed potentiation (Lovingier and Routtenberg, 1988; Malenka, 1991). Associativity describes the ability of ‘weak’ stimulated afferents to become potentiated if active at the same time as secondary and separate ‘strong’ stimulated afferents (Levy and Steward, 1979; McNaughton et al., 1978). Input selectivity is considered a property of LTP because only synapses that are activated during stimulation are involved in potentiation (Andersen et al., 1977; Lynch et al., 1977). These three properties follow the rule that a synapse will undergo LTP only when both the synapse is active and the dendrite is sufficiently depolarized (Bliss and Collingridge, 1993). Such a rule is achieved molecularly by using the NMDA receptor as a coincidence detector within the post-synaptic site for pre-synaptic signals and strong post-synaptic depolarization, explaining the forth property – NMDAR-dependence.

By definition, NMDAR-dependent LTP requires activation of NMDARs during postsynaptic depolarization. This is because $\mathrm{Ca}^{2+}$ influx through the NMDAR channel into the
postsynaptic site is absolutely necessary to trigger LTP (Malenka et al., 1992). NMDA receptors play an essential role in detecting correlated pre- and postsynaptic activity; they are opened by glutamate release from pre-synaptic axonal terminals but only transmit ions when magnesium ions (Mg\(^{2+}\)) blocking their pores are removed by sufficient postsynaptic depolarization (Nowak et al., 1984). When two cells with overlapping axonal arbours terminate on the dendrite of a single neuron, correlated activity between these arbours will depolarize the post-synaptic cell dendrite to a greater extent than when one fires alone. Excitation from converging axons is more likely to release postsynaptic Mg\(^{2+}\) blockade of NMDA receptors, making them permeable to Ca\(^{2+}\) and activating second messenger cascades which mediate synaptic plasticity.

### 1.2.2 Molecular mechanisms of LTP induction

NMDAR-dependent LTP is typically classified in two stages – an early phase (E-LTP), lasting only a few hours (Frey and Morris, 1997), and a late phase (L-LTP), which is protein synthesis dependent and reported to last from hours to possibly months (Malenka and Bear, 2004). The molecular mechanisms governing the early phase of potentiation of the excitatory postsynaptic current (EPSC) and its maintenance into L-LTP are the focus of hundreds of labs. A few molecules have been pin-pointed as necessary for LTP’s induction and maintenance. Both the early and late phases of LTP require calcium influx through activated NMDAR channels during LTP induction, though a quantitative analysis of the necessary post-synaptic Ca\(^{2+}\) signal remains elusive. Ca\(^{2+}\) ions are rapidly bound by calmodulin which triggers adenylate cyclase production of cAMP as well as Ca\(^{2+}\)/calmodulin dependent kinase II (CaMKII) activation (Kennedy et al., 2005). It is believed that the activation of protein kinases such as CaMKII, which then phosphorylate proteins local to the site of calcium influx, underlies the expression of E-
LTP. Overall, CaMKII is a key component of the molecular machinery of LTP (Malenka and Nicoll, 1999). It is found in high concentrations at the post-synaptic density; inhibition or genetic deletion of CaMKII blocks LTP induction (Malenka et al., 1989; Malinow et al., 1989); and constitutively active CaMKII both enhances synaptic transmission and occludes LTP in CA1 cells of the hippocampus (Lledo et al., 1995; Pettit et al., 1994). CaMKII is not, however, associated with the maintenance of previously established E-LTP to the late L-LTP phase (Chen et al., 2001; Malenka et al., 1989; Malinow et al., 1988; Otmakhov et al., 1997). One important target of CaMKII is the AMPA-receptor (AMPA) subunit GluR1. AMPARs mediate the majority of the EPSC during synapse activation, and LTP has been shown to both enhance AMPAR single channel conductance by phosphorylation, as well as to increase the number of AMPARs inserted at the post-synaptic site (Malinow and Malenka, 2002). GluR1 phosphorylation by CaMKII has been show to directly enhance AMPAR single channel conductance (Andrasfalvy and Magee, 2004; Derkach et al., 1999; Poncer et al., 2002). Furthermore, CaMKII activation has been shown to promote insertion of GluR1 subunit-containing AMPA receptors to synapses to increase AMPAR number (Shi et al., 2001a).

L-LTP requires stronger stimulation for induction than E-LTP (Lovinger and Routtenberg, 1988; Malenka, 1991). An attractive theory suggests molecular mechanisms at the post-synaptic site, unused in E-LTP, are activated during the strong stimulation to establish L-LTP. Strong input triggers a higher threshold involving transmission of signals to the nucleus to initiate synthesis of plasticity associated proteins. The non-specific kinase inhibitor staurosporine blocks both E-LTP and L-LTP (Hanse and Gustafsson, 1994), indicating kinase activity at the site of calcium influx may be the molecular mechanism involved in the messenger
pathway that sends signals to the nucleus. A number of signalling molecules have been investigated including CaMKIV, cAMP-dependent protein kinase (PKA), and mitogen-activated protein kinase (MAPK), which in turn activate the key transcription factor CREB (cAMP-response element binding protein) as well as immediate early genes (IEGs) (Abraham and Williams, 2003; Lynch, 2004; Pittenger and Kandel, 2003; Silva et al., 1998). CREB’s downstream target, the Ca\(^{2+}/\)calmodulin and cAMP-dependent nuclear transcription factor CRE is selectively activated by a ‘strong’ L-LTP induction protocol but insensitive to a ‘weak’ E-LTP stimulus (Impey et al., 1996), suggesting CREB is indeed involved in L-LTP.

Along with enhancement of EPSCs, L-LTP is also associated with morphological structural changes which are believed to underlie the long term maintenance of the synaptic weight change. Morphological changes include growth of new dendritic spines, enlargement of pre-existing spines and their post-synaptic densities (PSDs), and possibly the splitting of single PSDs and spines into two functional synapses (Abraham and Williams, 2003; Yuste and Bonhoeffer, 2001), though this is controversial (Maletic-Savatic et al., 1999). Using two-photon microscopy and localized photolysis and release of caged glutamate, dendritic spine enlargement has been directly associated with LTP (Matsuzaki et al., 2004). A popular model explaining the growth of the PSD and dendritic spine, involves the insertion of “slot proteins” which act as placeholders for addition of AMPARs to the synapse and associated molecules to the PSD (Malinow and Malenka, 2002; Shi et al., 2001b). These slot proteins would allow a stable number of AMPARs to be maintained at the synapse since AMPARs appear to cycle rapidly in and out of the synaptic membrane (Luscher et al., 1999; Nishimune et al., 1998; Shi et al., 2001b; Song et al., 1998).
1.2.3 Long term depression

A key breakthrough in activity dependent synaptic modulation was the discovery of an electrical stimulation pattern of the Schaffer-collateral-CA1 pathway that elicited homosynaptic NMDAR-dependent long term depression (LTD) of basal synaptic response (Dudek and Bear, 1992). LTD is a phenomenon similar to LTP but causes a long term decrease in synaptic strength rather than long term increase. LTD is both NMDAR dependent (Dudek and Bear, 1992; Mulkey and Malenka, 1992) and requires Ca\(^{2+}\) influx for its activation (Mulkey and Malenka, 1992). While quantitative characterization of the amount of Ca\(^{2+}\) required for LTD induction remains to be determined, a model is emerging in which low levels of calcium elevation produces LTD and higher levels induce LTP. NMDAR-dependent LTD is widely expressed throughout the mammalian brain, but is typically studied in the CA1 region of the hippocampus.

In an analog to the proposed signalling pathway of LTP, which requires protein phosphorylation for its induction, LTD is correlated with dephosphorylation of postsynaptic substrates, specifically PKC and PKA (Hrabetova and Sacktor, 2001; Kameyama et al., 1998; Lee et al., 1998; van Dam et al., 2002). The strongest case for the dephosphorylation of substrates has been made for PKA, where postsynaptic inhibition of PKA, inducing displacement of PKA from intercellular anchoring proteins (AKAPs), causes a run-down in synaptic transmission similar to LTD and also occludes LTD. Post-synaptic activation of PKA can also abolish previously induced LTD without affecting the basal synaptic response (Kameyama et al., 1998). A curiosity with respect to the dephosphorylation model is the exclusion of CaMKII substrate phosphorylation, which doesn’t change with LTD. It has been suggested that a very precise recruitment of protein phosphatases, in particular protein phosphatase one (PP1), to selected
substrates such as PKA via the binding to specific post-synaptic targeting proteins may explain the observed selective dephosphorylation (Morishita et al., 2001). In fact, post-synaptic introduction of protein phosphatase inhibitors targeting calcineurin and PP1 prevent LTD (Kirkwood and Bear, 1994; Mulkey et al., 1994; Mulkey et al., 1993).

Like LTP, the expression of LTD is thought to be a consequence of changes in AMPAR phosphorylation. Dephosphorylation of ser-845 on the C-tail of the GluR1 subunit, a PKA substrate, decreases AMPAR open channel probability and occurs with LTD. Interestingly, LTD shows no change in phosphorylation of ser-831, the substrate of CaMKII implicated in LTP. Because ser-845 is similarly unaffected by LTP, phosphorylation of ser-845 and ser-831 can be used as markers for LTD and LTP in vivo (Shukla et al., 2007; Whitlock et al., 2006). In contrast to LTP, AMPARs are rapidly internalized in response to LTD (Malinow and Malenka, 2002). Precisely how Ca\(^{2+}\)-dependent phosphatase activity rapidly reduces surface expression of AMPARs remains unclear.

LTD can also be classified into two stages, an early phase (E-LTD) and a late phase (L-LTD) which requires protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003). However, in contrast to L-LTP, L-LTD seems to require only mRNA translation, not transcription, to uphold stable expression of LTD. However, little is known about which newly synthesized proteins are required to maintain LTD. It is likely that structural modification of synapses and dendritic spines are required to maintain a steady-state reduction over time. The theory of “slot proteins” suggests LTD could result from a net loss of slot proteins in contrast to the net recruitment required for LTP (Malinow and Malenka, 2002;
Shi et al., 2001b). Consistent with this theory, the over-expression of PSD-95 increases AMPAR number at the synapse (Schnell et al., 2002), whereas removal of PSD-95 from the synapse via depalmitoylation depletes synaptic AMPARs (El-Husseini Ael et al., 2002).

1.2.4 LTP / LTD induction protocols and spike time dependent plasticity

The most common form of LTP induction is tetanic stimulation of afferent fibres. A typical tetanus consists of pre-synaptic stimuli delivered at 100Hz over 1 second (Bliss and Collingridge, 1993). Tetanic stimulation causes large depolarization of the post-synaptic cell, in turn delivering the calcium influx required for potentiation by activation and unblocking NMDARs, activating voltage-sensitive calcium channels and calcium-induced release of intracellular calcium stores. Typically, 3-4 tetanic stimuli spaced 5 minutes apart are used for consistent induction of L-LTP. Slightly more physiological forms of burst stimulation, called ‘theta-burst stimulation’ (typically several sets of 4 stimuli at 100Hz delivered at 200ms intervals) and ‘primed-burst stimulation’ (Rose and Dunwiddie, 1986) (a single stimulus followed by a single set of 4 stimuli at 100Hz 200ms later) are also commonly used as induction protocols. These stimuli have the benefit of mimicking similar firing patterns that have been reported in the hippocampus during learning (Otto et al., 1991). Repetitive low frequency (0.1 - 1 Hz) pairing of single afferent stimuli with strong depolarization of the post-synaptic cell (0mV for 100ms) has also been show to induce LTP (Gustafsson et al., 1987). The paring is repeated 50-100 times.

In a similar paradigm, called spike-time dependent plasticity (STDP), the depolarizing pulse is shortened to cause a single post-synaptic action potential (AP). To induce LTP this single post-synaptic AP must back-propagate into the dendrites within a narrow time window (usually
less than 50ms) after the pre-synaptic stimuli produces an excitatory post-synaptic potential (EPSP) within the innervated cell. The degree of LTP diminishes as the interval between the pre- and post-synaptic spikes increases (Bi and Poo, 2001). A recent study by Vislay-Meltzer et al. demonstrated LTP induction by STDP requiring only a single pairing (Vislay-Meltzer et al., 2006).

LTD can be induced by low frequency electrical simulation (900 stimuli at 1Hz for 15 minutes) with either single or dual pulses respectively (Thiels et al., 1994). E-LTD alone can be induced in a similar fashion (900 stimuli massed at 5 Hz for 3 minutes) (Kauderer and Kandel, 2000). LTD can also be induced by pairing protocols where the pre-synaptic spike and post-synaptic cell depolarization are set out-of-phase (Debanne et al., 1999). STDP produces LTD when the post-synaptic AP is induced before the pre-synaptic spike (usually by less than 50ms), rather than afterward. Both LTP and LTD induction by electrical stimuli can be blocked by the application of the NMDA receptor antagonist D-amino-5-phosphonovalerate (APV). Blocking of the NMDAR channel stops local Ca²⁺ influx, which is thought to be the principal trigger for molecular cascades underlying synapse specific LTP and LTD (Cavazzini et al., 2005).

1.2.5 Evidence for LTP/LTD in vivo

The first full reports of LTP in the hippocampus were described in vivo (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). LTP was not reported in the in vitro slice preparation until years later (Andersen et al., 1977). In other brain areas, such as the neocortex, the complexity of the in vivo preparation has severely limited the number in vivo studies (Glazewski et al., 1998; Lee and Ebner, 1992; Sakamoto et al., 1987; Tamura et al., 1992), and advantages of the slice preparation have made in vitro work comparatively common (Artola and Singer, 1987; Komatsu et al., 1988). A significant benefit of in vivo LTP and LTD experiments is
the ability to assay the effect of long term synaptic modification on natural sensory responses and animal behaviour.

The influence of LTP induction on natural information processing has been demonstrated in the visual cortex: The functional consequences of NMDAR-dependent LTP were monitored in the adult rat visual cortex after induction via theta burst stimulation of the dorsal lateral geniculate nucleus. Visually evoked potentials to full-field flash were found to be significantly enhanced after LTP, as well as responses to grating stimuli across a range of spatial frequencies (Heynen and Bear, 2001). Similar findings were also demonstrated in the lateral amygdale: High frequency stimulation (HFS) of the medial geniculate body resulted in long-lasting potentiation of field potentials in the lateral amygdala elicited by a naturally transduced acoustic stimulus (Rogan and LeDoux, 1995).

In vivo LTP has also been demonstrated to affect learning and memory. Saturating induction of LTP was shown to occlude learning in the rat hippocampus (Moser et al., 1998). Rats implanted with a multielectrode stimulating array were repeatedly excited with tetanus pulses causing cumulative potentiation. Spatial learning was found to be disrupted in animals with no residual LTP (less than ten percent) but not in animals that were capable of further potentiation, suggesting saturation of hippocampal LTP impairs spatial learning (Moser et al., 1998). Moreover, reversal of previously induced LTP in the rat hippocampus was demonstrated following exposure to a new environment. In these experiments, exploration of a new, non-stressful environment rapidly caused a complete and persistent reversal of the expression of high-frequency stimulation-induced E-LTP in the CA1 area of the hippocampus (Xu et al., 1998).
The expression was not affected by exploration of a familiar environment. This novelty-induced reversal of LTP suggests decreases in synaptic efficacy may act in tandem with enhancements at selected synapses to facilitate the storage of new information by the hippocampus.

Even with this in vivo work, a strong criticism of traditional LTP and LTD paradigms is that the electrical activity used to evoke synaptic change does not resemble natural sensory input. In an effort to link the phenomena to natural activity within the brain, a number of correlative studies have provided evidence that natural sensory stimuli may induce long-term synaptic modification in adult nervous systems in the form of LTP (Moser et al., 1998; Rioult-Pedotti et al., 1998; Rogan et al., 1997; Xu et al., 1998). In the adult rat brain, sensory stimuli associated with fear conditioning have been demonstrated to induce LTP in the amygdale. Fear conditioning in the amygdala involves pairing of an amygdala-dependent innocuous conditioned stimulus (CS) with an aversive unconditioned stimulus (US). Auditory responses in the lateral amygdale show similar enhancement after conditioning as demonstrated with LTP induction. The changes parallel CS-elicited fear responses and are enduring. They do not occur if the CS and US remain unpaired (Rogan et al., 1997). Motor-skill learning has also been suggested as a source of LTP within the adult rat primary motor cortex (M1). Rats, trained for three or five days in a skilled reaching task with one forelimb, were found to have increased field potentials in the forelimb region of horizontal intracortical connections in layer II/III contralateral to the trained limb (Rioult-Pedotti et al., 1998). The ‘untrained’ hemisphere and hind-limb regions showed no change. Moreover, this strengthening was accompanied by a reduction in the extent of subsequent electrically induced LTP, suggesting the effect of training was possibly due to LTP-like mechanisms (Rioult-Pedotti et al., 1998).
While the fear conditioning and motor training show correlative evidence of experience-driven LTP induction, a recent study by Whitlock et al. (2006) was the first to show direct evidence that leaning, in the form of simple inhibitory avoidance training, induces LTP and LTD in CA1. LTP and LTD were assayed by measuring phosphorylation of ser-831 and ser-845 of the GluR1 subunit. The same phosphorylation of glutamate receptors found in HFS-LTP was demonstrated with one-trial inhibitory avoidance learning in rats. The training also caused spatially restricted increase in the amplitude of evoked synaptic transmission in CA1. Furthermore, the learning induced synaptic potentiation occluded subsequent HFS-induced LTP. A similar study using contextual fear conditioning – another hippocampus-dependent learning task – has also demonstrated learning associated LTP-like changes in ser-831 (Shukla et al., 2007).

1.2.6 LTP/LTD in the developing brain

Understanding the role of LTP and LTD in development is critically important since both phenomena are likely involved in the activity-dependent restructuring of neural circuits. Nonetheless, comparatively few studies of LTP and LTD have been performed in early development. The main reason for this is technical inconvenience – in utero electrophysiological experiments are complex and brain slices of foetal animals are difficult to perform. As such, developmental studies of LTP and LTD traditionally investigate critical periods in the postnatal mammalian brain (the exception being Xenopus Laevis, described in section 1.4). Overall, the mechanisms, expression and maintenance of NMDAR-dependent LTP and LTD during development appear similar to those found in adult animals, with the added complexities of developmental switches in neurotransmitter subunit expression and in the
molecular cascades associated with NMDAR Ca\(^{2+}\) influx at time points in development when CaMKII expression is low (Wu and Cline, 1998; Yasuda et al., 2003). Some of the first investigations of LTP in the developing brain demonstrated compelling evidence of its importance to the formation of cortical circuitry: This work was completed in both the rat somatosensory cortex (S1) (Crair and Malenka, 1995) and the visual cortex during ocular dominance plasticity (Kirkwood et al., 1995). In mammalian development, the refinement of topographical projections of the thalamus to form whisker barrels in the somatosensory cortex includes a critical period, early in development, when barrels can be modified by sensory input (Crair and Malenka, 1995). If normal activity is altered during this critical period, the topographical map is disrupted (Crair and Malenka, 1995). The ability to induce LTP in the barrel cortex was discovered to closely match this critical period and to be lost with age due to a simultaneous decrease in NMDA receptor-mediated synaptic currents (Crair and Malenka, 1995). Similar experiments in the visual cortex during ocular dominance plasticity also demonstrated the inability to induce LTP after the critical period (Kirkwood et al., 1995). LTP induction was attempted in cortical slices taken at different ages from either light-reared or dark-reared rats. Susceptibility to LTP was found to coincide with the critical period and, like the critical period, could be prolonged by rearing animals in darkness (Crair and Malenka, 1995; Kirkwood et al., 1995). These findings support the hypothesis that LTP reflects a normal mechanism of experience-dependent synaptic modification in the developing mammalian brain.

LTD has also been induced in developing visual cortex (Dudek and Friedlander, 1996; Rittenhouse et al., 1999; Sermasi et al., 1999). Unlike LTP however, the ability to induce LTD
does not seem to be limited to the critical period and can be elicited in the mature brain. Dark rearing from birth resulted in a reduction of electrically induced LTD amplitude while light deprivation from P17-P30 showed no effect.

1.3. **Plasticity of intrinsic excitability**

1.3.1 **Neuronal excitability, ionic channels, and the input–output function**

While synaptic plasticity is an appealing candidate for learning and memory, LTP and LTD are unlikely to be the whole story for neuronal computation. In fact, most network models consider neuronal output via action potential (AP) firing to be the fundamental unit used to decode information. As such, neuronal excitability – defined as the likelihood of a neuron to generate an output signal from some given presynaptic input – may be the most important component in neural computation. Synaptic potentiation makes it more likely for the postsynaptic neuron to fire an AP by increasing the postsynaptic response (excitatory postsynaptic potentials) to the same afferent input. Synaptic depression works in the opposite direction. However, while synaptic input plays a direct role in neuronal excitability, it is only one of many steps required to induce neuronal firing. Neuronal excitability is largely mediated by voltage-gated ion channels, located in dendritic and somatic membrane, that couple excitatory postsynaptic potentials to the AP. Modulation of ion channel properties or density distributions can dramatically alter intrinsic excitability, and in turn firing rates, in response to a given afferent input (Daoudal and Debanne, 2003; Debanne et al., 2003).
Functionally, all ion channels, whether they are located in the dendritic spine or shaft, or in the neuronal cell body, play a crucial role in AP generation. Ion channels can amplify (persistent Na⁺ current and T-type Ca²⁺ channels) or attenuate (A-type K⁺ current and H-type cationic current) the excitatory postsynaptic potentials amplitude (Reyes, 2001). Regulation of ion channels concentrated near the synapse (either in the postsynaptic spine or pre-synaptic bouton) may play a particularly important role if regulated at a local level.

1.3.2  NMDA receptor dependent EPSP-spike plasticity

When Bliss and Lomo first reported LTP induction in the hippocampus after high frequency stimulation (HFS) (Bliss and Lomo, 1973), they also recorded an enhanced probability of AP firing in postsynaptic neurons after excitatory input. This parallel phenomenon is an example of intrinsic excitability and has been labelled excitatory postsynaptic potential-to-spike potentiation (E-S potentiation). While E-S potentiation has been overshadowed by LTP, it is complementary to LTP, functionally important, and also long lasting. E-S plasticity has been demonstrated in hippocampal, neocortical, and cerebellar neurons and may be persistent throughout the brain (Debanne et al., 2003).

The most commonly studied E-S potentiation requires the activation of NMDARs for its induction (Daoudal et al., 2002; Jester et al., 1995; Wang et al., 2003). Mechanistically, at least part of this potentiation is linked to GABA₆ channels, as the potentiation is eliminated by the GABA₆ channel blocker picrotoxin (Abraham et al., 1987; Chaveznoriega et al., 1989; Tomasulo and Ramirez, 1993). A picrotoxin-resistant E-S component has also been confirmed (Asztely and Gustafsson, 1994; Daoudal et al., 2002; Jester et al., 1995). Recent studies have also demonstrated NMDAR dependent long lasting E-S depression (Daoudal et al., 2002).
plasticity is also partially dependent on the GABA\textsubscript{A} channel, with approximately 60% of E-S depression blocked by picrotoxin (Daoudal et al., 2002).

NMDAR dependent E-S potentiation is input specific (Daoudal et al., 2002; Jester et al., 1995) suggesting that modulation of intrinsic excitability may be restricted to a specific area of the dendrite. Theoretical studies suggest that input specific changes in excitability are possible when inputs are located on separate dendritic branches (Wathey et al., 1992). E-S depression is also input specific (Daoudal et al., 2002).

Dendritic integration, the summation of excitatory postsynaptic potentials across a neuron's dendritic tree, also plays a major role in E-S plasticity. Bidirectional enhancement and weakening of summation follows associative LTP and LTD induction respectively (Wang et al., 2003). As with E-S plasticity, changes in synaptic integration are input specific. Long lasting down regulation of K\textsuperscript{+} channels and up-regulation of NMDARs may account for enhanced summation of dendritic excitatory postsynaptic potentials (Schrader et al., 2002; Wang et al., 2003).

Similarities between E-S plasticity, bidirectional changes in dendritic integration, and synaptic plasticity – including NMDAR dependence and input selectivity – may suggest a common induction pathway for all plasticity types.
1.4. EXPERIENCE-DRIVEN PLASTICITY IN THE *Xenopus* TADPOLE RETINOTECTAL SYSTEM

1.4.1 Morphological plasticity

During early brain development, newly differentiated neurons begin as simple spheres but rapidly grow elaborate and extensive axonal and dendritic processes. These processes are responsible for making the appropriate pre- and postsynaptic connections that ultimately form functional brain circuits. Most of our knowledge of the mechanisms involved in establishing circuits between distant CNS neuronal populations comes from studies of axonal projection from the eye to central brain targets (Gaze, 1970; Horder and Martin, 1978; Jacobson, 1978; Sperry, 1943, 1963). The output neurons of the eye are the retinal ganglion cells (RGCs), whose axons exit the eye as the optic nerve, cross the midline at the optic chiasm, and innervate central brain structures. This axonal tract has proven ideal for studies of axonal development due to the accessibility of the optic nerve for discrete lesion, the compartmentalization of projection and target neuronal populations for restricted pharmacological treatment, and the highly ordered pattern of RGC axonal terminations (Fujisawa et al., 1982; Gaze and Jacobson, 1963; Sperry, 1943).

The pattern of connections between RGCs and their target tissue, the optic tectum in lower vertebrates, is similar to many afferent projections in that it maintains the spatial properties of information from one layer to the next (Gaze, 1958). This order is accomplished by preserving the same spatial relationships between the soma positions of RGCs in the retina.
and the tectal neurons with which they form synapses. Maintaining such near-neighbour connections creates a topographic representation of the retina, and therefore visual space, in the tectum. Physically, the retina is mapped onto the tectum by axons of RGCs in dorsal retina terminating in the ventral tectum, ventral retina RGCs projecting to dorsal tectum, nasal RGCs projecting to caudal tectum, and temporal retina RGC axons terminating in rostral tectum (Godement and Bonhoeffer, 1989; Roskies and O'Leary, 1994; Sperry, 1963; Vielmetter and Stuermer, 1989; Walter et al., 1987a; Walter et al., 1987b).

During early development, the first RGC axons enter the optic tectum as simple projections that terminate in retinotopically appropriate regions (Holt, 1984; Holt and Harris, 1983; Sakaguchi and Murphey, 1985). Thus the retinotectal map is established at the earliest stages of RGC innervation. After reaching their target zones, RGC axons extend branches to form arborisation, which grow to cover relatively large regions of the tectum (Sakaguchi and Murphey, 1985). While the tectum continues to grow, RGC axonal arbours grow little or condense. Therefore, the relative size of RGC axonal arbours compared to the entire tectum decreases throughout development (Gaze et al., 1974; Sakaguchi and Murphey, 1985; Sretavan and Shatz, 1984). This shift is supported by electrophysiological recordings of tectal neurons demonstrating a progressive decrease in the area of tectum responding to a region of visual space with maturation. Thus, in normal development, the retinotopic map goes through stages of initial highly ordered RGC axonal termination, followed by extensive axonal arborization, and subsequent refinement of the map through an increase in tectum size and pruning of RGC axonal arbours. Functionally, this produces a developmental progression towards a smaller...
proportion of the tectum responsive to a given area of visual field and associated increase in visual resolution.

1.4.2 Visually-driven synaptic plasticity

As a model system for the developing nervous system, *Xenopus laevis* tadpoles are particularly suited for studies of LTP and LTD. Unlike mammalian systems, where investigation of development typically includes only postnatal critical periods, investigation of the developing *Xenopus* retinotectal system allows study of the very onset of activity-dependent refinement. Moreover, the ease of preparation allows all experimental work to be done *in vivo*. Nearly all LTP and LTD work in this system has been carried out by the lab of Mu-ming Poo using whole-cell or perforated-patch clamp electrophysiology. The first study of developmental LTP and LTD in the retinotectal system was also the first paper to identify spike-time-dependent-plasticity (STDP) as a method of LTP and LTD induction (Zhang et al., 1998). STDP has since been demonstrated in nearly all traditional LTP preparations. In a similar fashion to induction of LTP and LTD in CA1 via the Schaffer collaterals, LTP and LTD in the tectum was induced by repetitive electric stimulation of innervating retinal ganglion cells. Both LTP and LTD were also shown to be NMDAR-dependent. Input specific LTP in tectal neurons via theta burst stimulation (TBS) was found to develop over time (Tao et al., 2001). The change from non-input specific to input specific LTP occurred between stages 42 and 43 in the developing tectum and corresponded to increased complexity of tectal neuron dendrites as well as a restricted distribution of Ca²⁺ influx during NMDAR activation.

The process of activity-dependent morphological plasticity in developing neural circuits through Hebbian competition may be related to LTP and LTD, as both are activity-dependent
modifications (Constantine-Paton et al., 1990; Malenka and Bear, 2004; Zhang and Poo, 2001). However, for LTP and LTD to be directly involved in activity-dependent axonal and dendritic refinement, natural sensory stimuli must be able to induce LTP and LTD during development. Direct evidence of experience-driven LTP in vivo in the developing retinotectal system was first demonstrated using repetitive dimming light stimuli applied to the contralateral eye (Zhang et al., 2000). The LTP induction protocol consisted of 80, 50ms dimming stimuli at 0.3 Hz. In patch-electrophysiological studies, endogenous dimming stimuli elicited bursting activity similar to electrically stimulated TBS (Tao et al., 2001). 50ms dimming stimuli were found to elicit only OFF responses, though both OFF and ON responses could be induced if the dimming time exceeded 300ms. Visually-driven LTP induction resulted in persistent enhancement of glutamatergic inputs, but not GABAergic or glycinergic inputs, on tectal neurons. Enhancement required spiking in postsynaptic cells and was NMDAR-dependent. It was both long-lasting and occluded further LTP of retinotectal synapses by direct electrical stimulation. Overall, visually-driven LTP is thought to have the same underlying cellular mechanisms as electrically-induced LTP.

These experiments also demonstrated the first direct single cell electrophysiological evidence of activity-driven LTP in vivo, as well as the first evidence of activity-driven LTP at such an early stage in development. In future studies, Poo and colleagues expanded their natural visual stimulation paradigm to include a moving bar stimulus (Engert et al., 2002; Zhou et al., 2003). Using a similar training pattern to dimming stimuli (60 sweeps at 0.2Hz or 240 sweeps at 0.5Hz) tectal neurons could be trained to become direction-sensitive within minutes after repetitive exposure to moving bars in a particular direction. Direction-sensitivity was dependent
on the speed of the moving bar, suggesting enhancement was constrained to a defined set of spatiotemporal patterns. Direction selectivity could not be induced by random visual stimuli. Direction selectivity was associated with activity-induced enhancement of glutamate-mediated inputs, required spiking of the tectal neuron, and was NMDAR-dependent.

Further investigation demonstrated that synaptic modifications induced by the moving bar stimuli were in fact due to spike-time dependent induction (Mu and Poo, 2006; Vislay-Meltzer et al., 2006). To prove moving bar stimuli were elicited by STDP, visual stimuli were first paired with postsynaptic depolarization to demonstrate long-term enhancement or reduction of light-evoked responses could be achieved using an STDP paradigm. Next, moving bar stimulation was mimicked using a precisely timed sequential three-bar stimulation pattern. The properties (size and width) of the bars were modified such that the middle bar consistently induced postsynaptic spiking. This three bar ‘moving bar’ stimulus reliably induced spike time dependent potentiation and depression of responses to the first and third bars respectively. At the same time, the spike train elicited by the moving bar stimulus after training exhibited the same direction selectivity shown in previous experiments. Therefore, spike timing-dependent LTP and LTD was shown to account for the asymmetric modification of the tectal cell response induced by moving bar (Mu and Poo, 2006). Further investigation of visually-driven LTP and LTD in the retinotectal system has identified brain-derived neurotrophic factor and nitric oxide as elements required for light-induced excitatory synaptic modification (Mu and Poo, 2006).

Though the work done in *Xenopus* demonstrates that natural sensory stimulation can evoke NMDAR-dependent LTP and LTD similar to electrically evoked LTP and LTD in CA1, the methods used to investigate synaptic modifications have had direct effect on the modifications
themselves. In nearly all electrophysiological recordings, tectal cells were voltage-clamped at a constant membrane potential pre- and post-training, preventing spontaneous neuronal spiking. Under natural conditions, however, postsynaptic neurons are likely to spike spontaneously, as a result of random sensory inputs due to ambient illumination (Tao et al., 2001) or inputs from other brain regions (Sin et al., 2002a; Udin and Grant, 1999; Weliky and Katz, 1999). To address the question of how synaptic modifications become stabilized in the constant presence of neuronal activity in vivo, Zhou et al. (2003) investigated the effect of spontaneous firing either via random visual stimulation or intermittently switching to current clamp.

Spontaneous burst firing was found to erase electrically induced LTP and LTD as well as moving-bar visually evoked LTP and LTD when training was massed. This reversal was dependent on NMDAR activation. Similar loss of potentiation and depression has also been demonstrated in the retinotectal system after STDP (Vislay-Meltzer et al., 2006). In contrast, spaced induction of repetitive electrical stimulation stabilized LTP and LTD in the presence of random neuronal activity, suggesting that repeated event training is necessary for synaptic modification in the developing brain. For the retinotectal system, an inter-TBS interval of five minutes was found to be optimal for stabilized potentiation (Zhou et al., 2003). If the inter-TBS interval was increased beyond ten minutes, no stabilization was observed. Increased spiking in favour of the direction of training was also found after spaced moving-bar stimulation (3 sets of 60 sweeps at 0.2Hz separated by five minute intervals); however, no work was done to show visually induced potentiation or depression for long term.

Finally, a separate visual stimulation assay, consisting of 4 h of enhanced visual stimulation to freely swimming Xenopus tadpoles, has been shown to enhance both dendritic
growth rate of optic tectal neurons (Sin et al., 2002b) as well as the number of nascent synapses (Aizenman and Cline, 2007). Synaptic recordings were based on the electrophysiological finding that paired-pulse facilitation is greater in immature retinotectal synapses compared with more mature synapses due to a lower release probability (possible because of a lack of complete synaptic machinery) (Aizenman and Cline, 2007). As such, paired pulses can be used to elicit synaptic responses selectively from nascent synapses. Enhanced visual stimulation caused a decrease in the AMPA/NMDA ratio in nascent synapses compared to control, suggesting the addition of new immature synapses.

1.4.3 Homeostatic regulation of intrinsic excitability

In general, changes in intrinsic excitability tend to occur synergistically with changes of excitatory synapses: if synapses potentiate, so does E-S coupling (Burrone et al., 2002; Desai et al., 1999a, b). While this type of synergistic plasticity is likely to also occur in the Xenopus optic tectum, it has not yet been observed. Alternatively, experience-dependent decreases in synaptic drive seem to enhance intrinsic excitability (Aizenman et al., 2003; Aizenman et al., 2002). During activity dependent refinement of the retinotectal map, four hours of enhanced visual experience has been shown to induce a polyamine synthesis-dependent reduction in Ca²⁺-permeable AMPAR-mediated synaptic drive of tectal neurons (Aizenman et al., 2002). This decrease in synaptic efficiency is correlated with an enhancement of voltage-gated Na⁺ currents (Aizenman et al., 2003). Enhancement of intrinsic excitability also requires polyamine synthesis and could be prevented by blocking polyamine synthesis during visual stimulation. Na⁺ currents were rescued when Ca²⁺-permeable AMPAR-mediated transmission was selectively reduced. This result suggests that a homeostatic mechanism may exist during development to maintain a
stable input-output relationship. Further evidence, experimentally manipulating intrinsic excitability by electroporation of a leak K+ channel gene or peptide that interferes with AMPA receptor trafficking, supports this theory, as both manipulations result in an enhancement of voltage-gated Na+ currents to compensate (Pratt and Aizenman, 2007). Regulation of intrinsic properties likely plays a crucial role during functional development and may serve to keep neuronal input-output relationships within a stable dynamic range.

1.5. **METAPLASTICITY**

1.5.1 The plasticity of plasticity

Long term modulation of synaptic transmission and intrinsic excitability plays a key role in storing information within neural networks (Martin et al., 2000; Neves et al., 2008). Saturation of LTP or LTD could compromise the ability of neural networks to decode stimuli and discriminate events by limiting information storage. Moreover, Hebbian positive-feedback (Hebb, 1949; Turrigiano and Nelson, 2000) – a process in which effective synapses undergo strengthening and further increase their transmission, and ineffective synapses undergo weakening and further decrease their transmission – will ultimately lead to destabilization of postsynaptic firing rates, reducing them to zero or increasing them to an intrinsic maximal level. Furthermore, excessive strengthening of glutamatergic synapses via LTP may lead to damaging amounts of glutamatergic transmission and excitotoxicity, producing cell damage or death (Rothman and Olney, 1987). As such, it is imperative that neurons employ mechanisms to
maintain synapses, dendritic integration, and resulting afferent-evoked activity within a useful
dynamic range (Abraham et al., 2001). The degree of LTP and LTD induction has, in turn, been
found to be regulated by various intercellular signalling molecules and neurotrophic factors
(Stoop and Poo, 1996). However, a persistent long lasting regulation mechanism also exists,
where a neuron’s activity in the recent past can affect its ability to undergo LTP, LTD, or intrinsic
plasticity when presented later with plasticity-inducing stimulus. Neurons with distinct initial
states can therefore respond differently to the same presynaptic stimulus. This form of
plasticity regulation is termed metaplasticity (Abraham and Bear, 1996), ‘Meta’ referring to a
higher-order of plasticity: the plasticity of synaptic and intrinsic plasticity.

Importantly, the metaplastic effects of an initial metaplasticity trigger outlast the
activity that causes the initial priming. This priming remains at least until a second activity
period where synaptic or intrinsic plasticity occurs. The long lasting regulation caused by the
metaplasticity trigger is a key feature distinguishing metaplasticity from other forms of
plasticity modulation, where the modulation events and primary plasticity events must overlap.
Long lasting regulation also makes metaplasticity extremely difficult to study, since multiple
metaplasticity triggers can take place over time before the primary plasticity event occurs, and
previous metaplasticity triggers (unknown to the researcher) could influence experimentally
induced priming. Nevertheless, a number of key experiments have demonstrated metaplasticity
as an important factor regulating neuronal activity in vivo: For comprehensive reviews of the
theory of metaplasticity and the current research in the field see Abraham (2008) and Abraham
and Tate (1997).
1.5.2 The Bienenstock, Cooper and Munro (BCM) computational model of synaptic plasticity

BCM theory (Bienenstock et al., 1982) refers to a computational model for synaptic plasticity proposed by Elie Bienenstock, Leon Cooper, and Paul Munro in 1982 to account for orientation selectivity and ocular dominance in the visual cortex. BCM theory has two main features:

First, it proposes that neurons have a plasticity modification threshold ($\theta_m$) which dictates whether a neuron’s activity at any given moment will lead to strengthening or weakening of its input synapses. Synaptic modification varies as a complex nonlinear parabolic function ($\Phi$) of a neuron’s postsynaptic activity ($c$). $\Phi$ changes sign from negative to positive as postsynaptic activity passes the modification threshold. $\Phi$ can be approximated by

$$\Phi(c(t), \theta_m(t)) = c(t)[c(t) - \theta_m(t)]$$

(1)

If $c(t) > \theta_m(t)$, the synaptic modification value for active synapses is positive and their synaptic efficiency increases (potentiation). If $0 < c(t) < \theta_m(t)$ the synaptic modification value for active synapses is negative and their synaptic efficacy weakens (depression). Hence, $\theta_m$ is the cross-over point from LTD to LTP.

Postsynaptic activity between the neuron’s excitation threshold and saturation can be reasonably approximated as a linear input-output function (Benuskova et al., 2001): the sum of the product between presynaptic activity ($x$) and synaptic efficiency ($w$) for all synapses

$$c(t) = \sum x(t)w(t)$$

(2)
Hence, the change in synaptic efficiency (or the amount of potentiation / depression) $\Delta w$ varies as a product of the synaptic modification ($\Phi$) and presynaptic input ($x$)

$$\Delta w = \eta \Phi x$$

where $\eta$ is the modification rate. If $x=0$, there will be no change in synaptic efficiency. As such, synaptic weight changes according to Hebb’s learning rule (Hebb, 1949), requiring correlate pre- and postsynaptic activity at the synapse.

Second, BCM theory states that the cell-wide modification threshold for LTP induction is metaplastic and varies as a function of the neuron’s historic firing rate. $\theta_m$ varies proportionally to the square of the neuron’s activity averaged over some recent past ($\tau$) (Benuskova et al., 2001)

$$\theta_m(t) \propto \left< c^2(t) \right>_{\tau}$$

$\theta_m$ depends only on average postsynaptic activity, and hence is heterosynaptic in nature. A history of high postsynaptic activity shifts the modification threshold to the right, increasing the postsynaptic activity required to induce potentiation and biasing active synapses to weaken. A history of low postsynaptic activity shifts the modification threshold to the left, making LTD more difficult and LTP easier to induce (Abbott and Nelson, 2000; Abraham and Tate, 1997). As such, BCM learning adjusts plasticity thresholds to keep the overall synaptic weightings of a neuron within a dynamic range that prohibits saturation and stabilizes Hebbian plasticity by negative-feedback.
A seminal paper by Abraham et al. (2001) confirmed, in an *in vivo* model, two key postulates of the BCM theory modification threshold: its heterosynaptic expression and its regulation by postsynaptic neural activity. Electrical stimulation of two distinct synaptic inputs converging on the dentate gyrus of awake, freely moving rats allowed researchers to apply experimental conditioning stimulation via one input path and later evoke heterosynaptic LTP via the neighbouring path. Conditioning stimuli raised the threshold for LTP for a 7- to 35-day period depending on the stimulation protocol.

Historic neuronal firing rates are the key computational unit of the BCM model; however, intercellular calcium levels may be the physiological metric underlying plasticity modification (Cho et al., 2001; Gold and Bear, 1994a). Ca\(^{2+}\) is a key component required for LTP and LTD induction (Bear et al., 1987; Gold and Bear, 1994b) and some of the pioneering work on metaplasticity has focused on metaplastic regulation of expression of NR2A and NR2B subunits of the NMDA receptor, which produce short and long calcium entry, respectively, during receptor activation (Erreger et al., 2005). Cooper and colleagues have expanded the theoretical BCM framework to construct wholly Ca\(^{2+}\)-based plasticity models, emphasizing the role of NMDARs in generating the relevant Ca\(^{2+}\) signals (Shouval et al., 2002a; Shouval et al., 2002b). Most importantly, these models also feature a form of homeostatic metaplasticity, generated by activity-dependent changes in the regulation of intracellular Ca\(^{2+}\) levels (Yeung et al., 2004).

1.5.3 **NMDA receptor-mediated metaplasticity**

NMDAR mediated metaplasticity follows general BCM learning rules, with historical cell firing rates replaced with NMDAR activity in the recent past. NMDAR priming 60-90 minutes
prior to tetanic stimulation inhibits LTP induction (Huang et al., 1992). The effect is restricted to primed and activated synapses and slowly decays with time. Increasing the intensity of the tetanic stimulus can recover LTP induction, suggesting that NMDAR priming stimulation increases the threshold for LTP rather than blocking it completely (Huang et al., 1992). Nonetheless, even when LTP is induced by strong or repeated tetanic stimulation, NMDAR priming can reduce the duration LTP (Fujii, 1996; Woo and Nguyen, 2002). NMDAR mediated metaplasticity should not be confused with LTP occlusion (O'Connor et al., 2005), where inhibition of LTP is explained by a simple saturation of potentiation, as it can occur even when the priming NMDAR activation does not induce any detectable changes in baseline synaptic transmission (Abraham and Huggett, 1997; Fujii, 1996; O'Connor et al., 2005).

NMDAR priming also enhances the later induction of LTD (Christie and Abraham, 1992; Wang et al., 1998) via associative stimulation or conventional low-frequency stimulation (LFS) protocols (Mockett et al., 2002). LTD enhancement can be generated by a low frequency priming stimulation (Christie and Abraham, 1992; Wang et al., 1998) and is restricted to the activated synapses for 60-90 minutes (Christie and Abraham, 1992). Similar to NMDAR mediated LTP inhibition, LTD priming stimulation enhances LTD without any datable change in synaptic transmission due to the priming stimulus.

1.5.4 Metaplastic E-S plasticity: common learning rules?

Phenomenologically, synaptic and intrinsic plasticity share the same learning rules proposed by the BCM curve (Daoudal and Debanne, 2003; Daoudal et al., 2002; Debanne et al., 2003; Wang et al., 2003). For example, in CA1 E-S potentiation is observed with both homosynaptic (Bliss and Lømo 1973; Abraham et al. 1987; Daoudal et al. 2002) and associative
LTP induction, and E-S depression occurs at the same time as synaptic depression or depotentiation (Daoudal et al. 2002; Wang et al. 2003). These intrinsic plasticity changes are NMDAR dependent similar to their synaptic counterparts. Plasticity of both synaptic efficiency as well as neuronal excitability may be reflected in long term changes in a neuron’s functional response properties.

In addition, shifts in the BCM plasticity threshold can also occur due to changes in intrinsic cell excitability. LTP induction by classical conditioning at convergent CA3-CA1 pyramidal neurons synapses causes a prolonged enhancement of slow afterhyperpolarization (sAHP) mediated by an increase in Ca\(^{2+}\)-activated K\(^+\) currents (Didier Le et al., 2004). This sAHP enhancement reduces cell excitability, decreasing the capacity of the neuron to fire prolonged bursts. Subsequent tetanization preferentially inhibits LTP on previously unpotentiated synapses. Here, sAHP enhancement shifts θ\(_m\) to the right. K\(^+\) channels in the postsynaptic membrane are good candidates for intrinsic metaplasticity induction in other cells types as well since they regulate the threshold for LTP induction in many neurons (Sah and Bekkers, 1996).

1.5.5 **Metaplasticity induced by environmental stimuli**

Natural stimuli, such as visual experience, stress, or environmental changes, can have significant effects on plasticity induction in the brain. Regulation of plasticity by these environmental stimuli may occur as a form of metaplasticity or as modulation of plasticity (via concurrent release of hormones or neurotrophic factors). In practice, it is difficult to separate metaplasticity from plasticity modulation *in vivo*. To account for this challenge, most natural stimuli experiments use *ex vivo* preparations, where the environmental stimuli is first presented to the living animal after which the brain is removed and plasticity induction is studied *in vitro*. 
Using this approach, enriched environments have been shown to enhance hippocampal LTP induction as well as LTP duration (Duffy et al., 2001; Van Praag et al., 1999), and stress caused by both tail shock and restraint has been shown to inhibit LTP and enhance LTD in the hippocampus for up to 24 hours after the initial stressor (Kim et al., 1996).

In one of the first experiments to demonstrate the BCM learning curve in vivo, monocular lid suture was shown to cause significantly greater depression of deprived-eye responses in kitten visual cortex than treatment with tetrodotoxin as assayed by shifts in ocular dominance columns (Rittenhouse et al., 1999). This is because lid suture leaves the retina spontaneously active, whereas tetrodotoxin eliminates all activity. Similarly, homosynaptic LTD caused by monocular deprivation is significantly greater than homosynaptic LTD caused by binocular deprivation (Kirkwood et al., 1996a) where cortical activity is lower (Blais et al., 1999).

Visual experience in the recent past has also been demonstrated to shift the BCM modification threshold. Dark rearing shifts \( \theta_m \) substantially to the left in visual cortical slices, consequently lowering the threshold for LTP threshold and reducing in the capacity for homosynaptic LTD (Kirkwood et al., 1996a) (Kirkwood et al., 1996b; Philpot et al., 2003). Light exposure for 2 days completely reverses the effects of visual deprivation (Kirkwood et al., 1996b). NMDAR subunit composition may be the underlying mechanism behind dark rearing induced metaplasticity. During regular development a maturational shift from the predominant slow \( Ca^{2+} \) influx NR2B subunit to the fast \( Ca^{2+} \) influx NR2A subunit occurs in the visual cortex (Quinlan et al., 1999a). This developmental shift is inhibited in dark reared animals but can be rescued by 2 hours of light exposure (Quinlan et al., 1999b). A rapid activity-dependent switch
in NMDAR subunit composition from NR2B to NR2A also occurs in neonatal hippocampus after tetanic stimulation (Bellone and Nicoll, 2007).

Recent experiments have demonstrated experience regulated metaplasticity in both the barrel cortex (Clem et al., 2008) and hypothalamus (Kuzmiski et al., 2009). NMDAR-dependent LTP can be induced in vivo between layer 4 and layer 2/3 neurons of the barrel cortex by single-whisker experience (SWE), where all whiskers but one is removed from the animal. Further induction of LTP with SWE is inhibited in an NMDAR dependent manner. However, if NMDAR channels are blocked, additional whisker stimulation can facilitate LTP through recruitment of mGluRs. Hence, a balance between NMDAR inhibition of LTP and mGluR enhancement of LTP is regulated by sensory experience. A behavioural tactile conditioning task pairing a non-aversive, habituating air-puff stimulus with whisker deflection demonstrated that the enhanced mGluR regulated LTP is correlated with increased learning behaviour. Metaplasticity mediated by mGluRs also occurs in the hypothalamus, though in the opposite direction. Haemorrhage induces a metaplastic enhancement of LTP through the down-regulation of mGluRs. Noradrenalin replicates this metaplasticity, which may underlie a homeostatic change in the network to combat acute physiological challenge.
1.6. Research Hypothesis and Objectives

I hypothesize: (1) that visual sensory input drives both synaptic plasticity and intrinsic excitability of retinotectal synapses undergoing receptive field refinement in the developing brain, and that these plasticity changes result in long lasting modification of evoked neuronal output; (2) that functional plasticity is both dependent on the pattern of visual training stimuli and regulated by activation of NMDA receptor mediated pathways; (3) that training induced functional plasticity follows BCM learning rules, including an activity dependent modification threshold and regulation of that threshold by historic postsynaptic neural activity.

AIM 1: To develop a non-invasive imaging protocol to assay long term changes in evoked neuronal firing of ensemble networks in vivo. To date, studies of experience-driven LTP and LTD in the developing retinotectal system have been performed using whole-cell or perforated patch electrophysiology, techniques that require an open-brain preparation. To assay changes in neuronal activity over time non-invasively, I used multi-cell-bolus-loading (MCBL) (Brustein et al., 2003; Stosiek et al., 2003) in combination with in vivo single cell excitability probing (SCEP) (Johenning and Holthoff, 2007). MCBL is a minimally invasive $\text{Ca}^{2+}$-indicator loading protocol that employs a membrane permeable AM-form $\text{Ca}^{2+}$-indictor to detect calcium changes within cells. MCBL allows cells to be monitored for hours without disturbing intercellular ion balance or washing out signalling molecules and proteins. This technique has specific advantages over electrophysiological work: it allows investigation of both single cell excitability (Johenning and Holthoff, 2007), as well as population responses (100’s of neurons) within the imaging field of view. Using the MCBL approach, I proposed to investigate
the functional responses of tectal neurons to visual stimuli over time, as well as how these responses are modified with visual training.

**AIM 2: To determine if visual stimulation induces long-term potentiation or depression of neuronal activity in the intact unanaesthetized developing brain.** To date, studies of experience-driven changes of visual receptive fields have focused on the plasticity of synaptic transmission rather than modifications of neuronal firing output. Here I focus on the receptive field properties encoded in neuronal firing and how these can be modified by brief visual experience. Using the *in vivo* SCEP technique developed in AIM 1, I proposed to measure changes in neuronal firing of neuronal ensembles after various visual stimulation protocols, including: high frequency repetitive OFF stimulation designed to elicit long lasting potentiation, invariant light stimulation designed to elicit long lasting depression, and a control probing stimulus – continuing SCEP throughout the training period. Moreover, I proposed to determine if plasticity changes follow the BCM learning curve, such that neurons posses a modification threshold that must be passed to undergo potentiation.

**AIM 3: To determine the effects of visual-stimulation induced training on network interactions.** *In vivo* SCEP may be compared to electrophysiological recording, such as patch clamp electrophysiology, which limits analysis to single cells, and field recordings, which do not allow investigation of large contiguous neuronal ensembles at the single neuron level. *In vivo* SCEP bridges the gap between single cell and population analysis, allowing measurement of neuronal activity of hundreds of neurons simultaneously at the single cell level within the intact brain. I proposed to utilize this technique to investigate changes in correlated neuronal firing
throughout the neuronal ensemble. The functional plasticity of individual neurons were then compared to changes in those neurons’ network interactions.

**AIM 4: To determine if previous experience regulates future visually-driven plasticity.**

BCM theory predicts that neuronal activity in the recent past will shift a neuron’s modification threshold. This threshold shift then regulates synaptic and intrinsic plasticity induction at a later point. I proposed that shifting of the modification threshold also effects functional plasticity. Using *in vivo* SCEP, I compared visually induced plasticity outcomes with historic neuronal firing rates. If BCM rules hold true, high pre-training firing rates will inhibit functional potentiation and facilitate functional depression. Low pre-training firing rates will act in the opposite manor. Furthermore, manipulating pre-training firing rates by enhancing visual experience should shift the plasticity outcomes of all neurons toward facilitation of LTD.

**AIM 5: To determine if BCM metaplasticity in the developing retinotectal system is regulated by NMDAR activity.** If pre-training activity does predict training-induced functional plasticity and modifying pre-training activity shifts the BCM modification threshold, I proposed that NMDAR blockade during modified pre-training activity would prohibit the shift.

The manuscripts reproduced in Chapters 2 and 3, address these aims. Chapter 2 demonstrates, for the first time, ensemble network analysis of natural-experience induced metaplasticity in the developing brain. Chapter 3 provides a detailed protocol for reproduction of the *in vivo* model system developed for the experiments performed in Chapter 2.
1.7. REFERENCES


Asztely, F., and Gustafsson, B. (1994). Dissociation between Long-Term Potentiation and Associated Changes in-Field Epsp Wave-Form in the Hippocampal Ca1 Region - an in-Vitro Study in Guinea-Pig Brain-Slices. Hippocampus 4, 148-156.


2. METAPLASTICITY GOVERNS NATURAL EXPERIENCE-DRIVEN PLASTICITY OF NASCENT EMBRYONIC BRAIN CIRCUITS

2.1. INTRODUCTION

During early periods of embryonic brain development, brief sensory experience plays a direct role in shaping neural circuit structure, connectivity, and function. Plasticity of neuronal firing during this critical period of development can have long lasting effects on network growth to influence normal and abnormal brain function later in life. Unlike mammalian embryos, frog and fish larvae provide an accessible developing brain circuit to study this stage of neuronal growth, in which afferent input can be driven by well-controlled visual stimuli for study of activity-dependent central circuit formation and refinement. In these systems, visual experience affects dendritic and axonal growth (Haas et al., 2006; Ramdya and Engert, 2008; Ruthazer et al., 2003; Sin et al., 2002), synaptic efficiency (Engert et al., 2002; Zhang et al., 2006).

1 A version of this chapter has been accepted for publication. Dunfield, D. and Haas, K. (2009) Metaplasticity governs natural experience-driven plasticity of nascent embryonic brain circuits. Neuron, in press.
and excitability (Aizenman et al., 2003; Aizenman et al., 2002) of central neurons. Single neuron recordings in open brain preparations have demonstrated NMDAR-dependent synaptic long-term potentiation (LTP) and long-term depression (LTD) at retinotectal synapses after electrical or visual stimulation (Engert et al., 2002; Vislay-Meltzer et al., 2006; Zhang et al., 1998; Zhang et al., 2000; Zhou et al., 2003). Interestingly, the same visual experience can induce either LTP or LTD (Zhou et al., 2003) simultaneously in different tectal cells. We hypothesize an explanation for such variable plasticity responses is provided by the Bienenstock-Cooper-Munro (BCM) (Bienenstock et al., 1982) theory which suggests that a neuron’s firing rate prior to plasticity induction may directly affect that cell’s ability to exhibit subsequent synaptic LTP or LTD. In this form of plasticity regulation, termed metaplasticity (Abraham, 2008), neurons with distinct initial states can respond differently to the same presynaptic stimulus. The BCM learning rule has been shown to be valid for plasticity induced changes in synaptic efficiency as well as neuronal excitability (Daoudal et al., 2002; Wang et al., 2003), both of which may be reflected in long term changes in a neuron’s functional response properties. It is presently unclear whether metaplastic rules govern synaptic plasticity of individual neurons during normal brain development or if metaplasticity occurs in the absence of experimental priming such as dark rearing or monocular deprivation. Proper testing of these theories requires simultaneous monitoring of activity in large populations of neurons within intact systems to determine whether individual neuronal pre-training firing rates predict variable plasticity results.

To measure endogenous activity and visual response properties of large neuronal ensembles, we utilized in vivo imaging of spontaneous and visually evoked calcium events. This
technique allows simultaneous probing of the visual properties of hundreds of tectal neurons in
the awake brain with single cell resolution. Visual receptive field (RF) responses were probed
both prior to, and up to one hour following visual training. Using population imaging and
network analysis of activity, we find experience-driven plasticity in the retinotectal system
causes variable functional plasticity of individual tectal neurons, driving functional RF responses
of neurons toward long lasting potentiation or depression. Plasticity is specific to the RF
properties evoked during training and shows evidence for BCM metaplasticity, in that pre-
training activity predicts plasticity outcome. Together, our results demonstrate that natural
sensory input plays a profound and lasting role in the functional development of intact brain
circuits, subject to each neuron’s previous history.

2.2. Results

To noninvasively investigate how sensory experience alters circuit function within the
intact and awake developing brain, we simultaneously monitored the activity of hundreds of
neurons within the optic tectum of unanaesthetized Stage 50 tadpoles (Nieuwkoop and Faber,
1967) in response to wide-field light ON or OFF stimuli (Gaze et al., 1974; Zhang et al., 1998)
using in vivo two-photon time-lapse single-cell-excitability-probing (SCEP) (Johenning and
Holthoff, 2007) of calcium dynamics (Brustein et al., 2003; Stosiek et al., 2003) (Fig. 2.1a).
Because the amplitudes of Ca^{2+} transients in individual neurons are correlated with action
potential firing (Brustein et al., 2003; Fetcho, 1998; Johenning and Holthoff, 2007; Niell and
Smith, 2005; Ramdya et al., 2006; Smetters et al., 1999; Sumbre et al., 2008; Yaksi, 2006), SCEP allows monitoring of the plasticity of functional RF responses within the intact circuit. We find that more than 45% of cells in a single optical section of the tectum demonstrate clear evoked calcium responses to 50ms OFF stimuli (Fig. 2.1b, Fig. 2.2, Fig 2.3). 50ms OFF stimuli trigger somatic action potentials in tectal neurons without residual ON responses (Tao et al., 2001; Zhang et al., 2000), allowing us to probe changes in a neuron’s OFF RF. Amplitudes of visually evoked wide-field responses remained stable over 1h 45min of probing at 60-second intervals in 69% of the visually responsive cells.

2.2.1 Long lasting functional plasticity in central neuronal ensembles driven by natural sensory stimuli

Can brief patterned visual training affect network activity and circuit RF plasticity in the intact and awake developing tadpole brain? While patterned input appears unnecessary for early RF refinement in the intact tectum of zebrafish (Niell and Smith, 2005), exposure to specific patterns of repeated visual stimuli can induce long lasting synaptic changes in single tectal neurons recorded using patch clamp electrophysiology in open brain preparations of developing Xenopus (Vislay-Meltzer et al., 2006; Zhou et al., 2003). To assess functional RF plasticity throughout the tectal circuit in response to visual training, we probed visually evoked calcium responses to 50ms OFF stimuli before and after a 25 minute ‘spaced training’ paradigm composed of repeated trains of high-frequency 50ms OFF stimuli (Fig. 2.4, Fig. 2.6a). High frequency stimulation significantly increases cell activity compared to pre-training probing (Fig. 2.5a). Average calcium transient amplitude is also greater than pre-training probing (28±6%, p<0.01 t-test). Spaced training induced long lasting potentiation of visual evoked responses to
OFF stimuli, evident from a significant increase in the ensemble average firing rate measured 30-60min post-spaced training (Fig. 2.1c).

If spaced training can cause mean potentiation of tectal neuron responses in the developing brain, is it possible for other patterns of brief visual experience to cause functional depression? Homosynaptic depression can be induced in vivo during weak afferent activity from the retina (Bear et al., 1987; Rittenhouse et al., 1999). BCM theory predicts this effect, postulating that low levels of presynaptic activity will depress active synapses (Bienenstock et al., 1982) and intrinsic excitability (Daoudal and Debanne, 2003), while high levels of presynaptic activity above a modification threshold, $\theta_m$, will lead to postsynaptic strengthening.

To reduce presynaptic activity, we presented an unchanging light stimulus to the immobilized eye. Because retinal ganglion cells (RGCs) respond most strongly to changes in the pattern of illumination, rather than to steady states of uniform illumination (Wade and Swanston, 2001), ‘invariant’ light stimulation elicits significantly less neuronal firing than baseline probing (Fig. 2.5a). There is no change, however, in average calcium transient amplitude compared to pre-training probing ($p=0.47$, t-test). Indeed, training with 25 minutes of invariant light stimulation induced significant long lasting depression of ensemble visually evoked calcium responses 30-60min post-training (Fig. 2.1c).

2.2.2 Variable plasticity of individual central neurons to the same sensory training paradigm

Single-cell excitability probing of visually evoked calcium responses allowed us to determine the long-term plasticity effects of natural visual stimulation on individual tectal neurons during both spaced and invariant training (Fig 2.6). The amplitudes of visually evoked
calcium responses in all visually responsive cells showed one of four types of plasticity: long-lasting potentiation (Fig. 2.7, a and e), short-term potentiation (Fig. 2.7, b and f), no change from pre-training levels (Fig. 2.7, c and g), and long-lasting depression (Fig. 2.7, d and h). Such variable RF plasticity is consistent with visually-driven synaptic plasticity previously observed in the retinotectal system (Zhou et al., 2003), and highlights the intrinsic complexity of natural plasticity induction in the intact developing brain. In response to spaced training, over 50% of neurons showed a short or long lasting potentiation to the probed stimulus, and 12% exhibited long lasting depression (Fig. 2.7k). Probing during spaced training demonstrates plasticity is additive over periods of high frequency stimulation (Fig. 2.5b). Similar to spaced training, invariant training induced varied plasticity in neurons throughout the tectal circuit (Fig. 2.7, f to h); however, the largest population of cells, 45%, exhibited long lasting depression (Fig. 2.7k). Among cells showing persistent depression, the amount of depression was significantly greater after invariant than following spaced training (invariant = -33±1%, spaced training = -26±1%, p=0.00001 t-test). Taken together, our results show that brief episodes of natural visual experience can cause long lasting functional changes within the intact, awake, developing brain, with specific patterns of stimulation favouring induction of either potentiation or depression. Training did not produce potentiation or depression in all cells; rather, a varied amount and type of plasticity was observed throughout the tectal circuit, with the specific training paradigm preferentially shifting the majority of cells towards potentiation or depression. Potentiation or depression of functional responses may reflect the plasticity of synaptic inputs (Debanne et al., 2003; Powers et al., 1992) or altered neuronal excitability (Aizenman et al., 2003; Campanac and Debanne, 2008; Daoudal and Debanne, 2003; Daoudal et al., 2002; Wang et al., 2003).
2.2.3 Synaptic mechanisms underlying visually induced tectal RF plasticity

Activation of N-methyl-D-aspartate receptors (NMDARs), a subtype of glutamate receptors, is required for induction of LTP and LTD at retinotectal synapses (Engert et al., 2002; Zhang et al., 1998; Zhang et al., 2000). We tested whether blockade of NMDARs by injection of D-aminophosphovalerate (D-APV) (50µM), a specific NMDAR antagonist, interferes with experience-induced plasticity of visually evoked tectal Ca\(^{2+}\) responses. NMDAR blockade significantly reduced visually-driven potentiation by spaced training (Fig. 2.7i) and depression after invariant training, albeit to a lesser degree (Fig. 2.7j). Residual depression may be due to other non-NMDAR dependent forms of LTD, such as mGluR mediated depression (Daoudal and Debanne, 2003). Mean evoked calcium responses 30-60 minutes post training demonstrated no significant difference between a continuous probing control and either spaced training + APV or invariant training + APV. APV injection did not affect visually evoked calcium response amplitudes directly (Fig. 2.8), suggesting that the elimination of training induced plasticity was not due to an APV-induced reduction of activity during the training period. Injection of vehicle control before training did not affect plasticity induction (Fig. 2.9). These results support an NMDAR dependent mechanism mediating induction of visually driven functional plasticity in the awake tadpole optic tectum.

2.2.4 RF mapping across tectal circuit and specificity of plasticity to the characteristics of the training sensory stimuli

Is spaced training induced plasticity specific to the properties of the training stimulus? Because 50ms OFF spaced training elicits only OFF responses, only OFF RFs should show plasticity if training specificity is true. Probing with 60s OFF stimuli allowed us to map the wide-
field OFF (stimulus onset) and ON (stimulus offset) RF properties of neurons throughout the tectum (Fig. 2.10). Approximately half of visually responsive tectal cells were purely OFF-dominated without detectable ON response, 5% were purely ON-dominated, and the remainder responded in varying degrees to both ON and OFF stimuli. Interestingly, analysis of network responses revealed significant anatomical clustering of cells with ON- and OFF-dominated RFs compared to random reassignments of RF values (Fig. 2.11, a and b). Clustering of ON- and OFF-center afferents is predicted in computational models (Miller, 1992, 1994) and has been demonstrated in mammalian primary visual cortex (Jin et al., 2008; Zahs and Stryker, 1988). By probing both ON and OFF responses pre- and post-spaced training, we found that plasticity induced by 50ms OFF spaced training is specific to OFF responses. 86% of cells demonstrate no change in ON response after spaced training (p < 10^{-10}, compared to OFF no change, chi-squared test). Moreover, the amplitude of OFF response plasticity increased with the degree of RF OFF-domination (Fig. 2.11c). These results clearly demonstrate specificity of plasticity to characteristics of the training stimuli as a defining characteristic of spaced training with wide-field ON and OFF stimuli in the intact tectum. Furthermore, we find that spontaneous activity of long lasting potentiated cells is reduced after spaced training (Fig. 2.12), suggesting spaced training induced potentiation is not due to a global increase in cell excitability.

2.2.5 Metaplastic rules predict visually induced RF plasticity of individual neurons

Do neurons within intact brain circuits exhibit metaplasticity, such that pre-training intrinsic properties of individual neurons influence their responses to training? A second feature of BCM theory is that the value of the modification threshold, $\theta_m$, which determines the degree and direction of synaptic efficiency (Bienenstock et al., 1982) and intrinsic excitability
(Daoudal and Debanne, 2003) changes, is not fixed but instead dependent on each neuron’s averaged postsynaptic firing rate during the recent past. If the averaged firing rate is high, \( \theta_m \) rises; if the averaged firing rate is low, \( \theta_m \) falls (Fig. 2.13a). The intact *Xenopus* retinotectal preparation in combination with uniform dye uptake after bulk loading (Fig. 2.14) (Garaschuk et al., 2006; Yasuda et al., 2004) allowed us to indirectly monitor the firing rate of individual tectal neurons during pre-training by measuring spontaneous calcium events driven by endogenous brain circuit activity (Zhou et al., 2003). Calcium transients reflect both slow cell firing rates through their frequency and burst firing rates through their amplitudes. Transient amplitudes have been shown to scale with the number of action potentials fired in a burst in multiple systems and organisms (Brustein et al., 2003; Fetcho, 1998; Johenning and Holthoff, 2007; Niell and Smith, 2005; Ramdya et al., 2006; Smetters et al., 1999; Sumbre et al., 2008; Yaksi, 2006). While we observed no significant differences in the frequency of pre-training spontaneous calcium transients between plasticity groups, the amplitudes of spontaneous calcium transients were significantly different (Fig. 2.13b). Cells that exhibited long lasting functional potentiation after spaced training had significantly lower pre-training spontaneous calcium transient amplitudes than other plasticity types, while cells demonstrating functional depression had significantly higher amplitudes. These results are predicted by BCM theory (Bear, 2003; Beggs, 2001; Daoudal and Debanne, 2003) (Fig. 2.13a). Moreover, potentiation and depression are unlikely to be caused by activity differences during the training period (Fig. 2.15). The BCM model is also supported by our findings that cells demonstrating depression following invariant training exhibited high pre-training calcium transient amplitudes. In this case, minimal presynaptic input during training prohibits neuronal activity from passing the modification
threshold, preventing potentiation. In turn, neurons with up-shifted $\theta_m$ values due to high levels of pre-training activity are more likely to undergo depression (Fig. 2.13a). Both examples provide evidence of metaplasticity within the intact developing brain, where intrinsic spontaneous firing predisposes expression of future plasticity. While these theories do not address induction of short-term plasticity, increased spontaneous firing has been shown to rapidly reduce synaptic plasticity in the tectum (Zhou et al., 2003). Here we find that cells that undergo short-term RF plasticity have significantly higher spontaneous firing rates post-training than those exhibiting long lasting plasticity, suggesting that enhanced spontaneous activity post-training reverses functional RF potentiation (Fig. 2.13c).

2.2.6 Experimentally increased pre-training activity shifts spaced training plasticity outcomes toward depression through an NMDAR dependent mechanism

Spontaneous activity in tectal neurons consists of random single spikes and bursts of spikes (Zhou et al., 2003). White noise light stimulation, such as flashes of randomly patterned ON and OFF checker boards (Zhou et al., 2003), or rapidly varying wide-field light intensities (used here; Ramdya et al., 2006) can readily induce enhanced firing rates (Fig. 2.5a) in tectal neurons with similar properties to endogenous spontaneous tectal activity (Zhou et al., 2003). We presented wide-field white noise stimuli at 5Hz to experimentally increase cell firing for 1 hour prior to SCEP imaging. After white noise stimulation, pre-training probing, spaced training, and post-training probing were presented the same as previous experiments.

Enhanced activity shifted plasticity outcomes toward depression (Fig. 2.16). BCM theory predicts this change in space training induced plasticity as a result of each cell’s historical
enhanced firing rate shifting the modification threshold (Fig. 2.13a). These results strongly support BCM metaplasticity as a plasticity regulator in the awake embryo.

Significantly, the shift in plasticity outcome due to enhanced activity can be abolished by injection of D-APV (50μM) directly before white noise priming during the metaplasticity phase (Fig. 2.16). Spaced training plasticity results after white noise + APV show no significant difference from spaced training alone without priming (p=0.1, chi-square test). The absence of reduction in potentiation compared to regular spaced training suggests that APV was washed out prior to training stimulation. Our results reveal NMDARs as an endogenous mechanism for metaplasticity in the awake developing brain.

2.2.7 Potentiation is associated with an increase in spontaneous correlated firing

Does spaced training induced plasticity alter intrinsic ensemble network activity? Analysis of spontaneous activity shows significant correlated firing between tectal cells (Fig. 2.17). Correlated firing is more common among nearest anatomical neighbours, suggesting shared afferent input or local interconnections (Tao et al., 2001) (Fig. 2.17a). Spaced training induced a significant increase in correlated spontaneous activity 0-10 min post training (Fig. 2.17b). Increased correlations of cells exhibiting long lasting potentiation were significantly greater than other plasticity types. Cells showing long lasting depression following spaced training showed no change in network correlation. Control tadpoles and tadpoles exposed to invariant training exhibited no change in network correlations.
2.3. DISCUSSION

Together, our results provide a circuit analysis of the effects of natural sensory experience on neural network function within the intact, awake, developing brain. Non-invasive functional imaging of sensory-induced plasticity expands upon previous electrophysiological studies (Engert et al., 2002; Pratt et al., 2008; Tao and Poo, 2005; Zhou et al., 2003) by simultaneously linking single cell and ensemble plasticity. We find that individual neurons within intact embryonic central circuits respond to plasticity-inducing sensory input in a complex manner, resulting in functional potentiation, depression, or no change toward a probed RF stimulus (Fig. 2.7). While individual neurons show variable plasticity after visual training, mean population responses to RF stimuli can be potentiated or depressed depending on the training paradigm.

Spaced training composed of repeated trains of high frequency OFF visual stimuli preferentially shifted cell response properties toward potentiation of the trained OFF responses. The absence of change in ON RFs following OFF spaced training, demonstrated clear specificity of RF plasticity to characteristics of the input training stimulus. Improved long-term neuronal performance restricted to characteristics of specific training stimuli has been demonstrated in primary visual and auditory cortex (Pantev et al., 1998; Schoups et al., 2001; Sengpiel et al., 1999; Zhang et al., 2001), though such acute effects at the embryonic stage where RFs are generally broad and unrefined are striking.
Here we find that spaced training is also associated with an increase in correlated spontaneous firing between potentiated neurons. Although enhancement of correlated circuit activity induced by spaced training is transient, these correlations may play a significant role in functional plasticity among neurons within select sub-networks by promoting transition to long lasting forms of potentiation (Voigt et al., 2005).

In contrast to spaced training, invariant light stimulation to the immobilized eye shifted the majority of ensemble response properties toward depression. Binocular deprivation in early postnatal development leads to similar effects, depressing synaptic transmission and rendering visual cortical neurons unresponsive to subsequent visual stimulation (Bear et al., 1987; Freeman et al., 1981; Prusky et al., 2000; Rittenhouse et al., 1999). In both invariant training and binocular deprivation, BCM theory predicts induction of depression due to decreased pre-synaptic activity which is insufficient to reach the threshold required for potentiation.

Variable long lasting functional plasticity outcomes induced by both spaced training and invariant training were accurately predicted by measuring the pre-training activity of individual cells. Neurons with high spontaneous firing rates during pre-training periods exhibited predisposition for training-induced depression, while neurons with low spontaneous firing rates demonstrated predisposition to potentiation. This metaplastic result follows the BCM learning rule, where the plasticity modification threshold depends on average pre-training activity and will increase or decrease with higher or lower firing rates.

We find strong support for BCM theory by increasing pre-training activity using white noise visual stimulation. We demonstrate that white noise dramatically shifts tectal neuronal
plasticity responses to spaced training towards depression. One explanation for these results is that the sliding threshold of BCM theory acts as a homeostatic mechanism to maintain synapses, dendritic integration, and resulting afferent-evoked neuronal activity within useful dynamic ranges (Abraham et al., 2001). Hence, this rule makes it more difficult for highly active neurons to potentiate further and easier for them to depress (Abbott and Nelson, 2000; Abraham, 2008; Abraham and Tate, 1997). Our findings that APV blocks the ability of white noise priming to shift plasticity outcomes implicate NMDAR-mediated transmission as an underlying mechanism of experience dependent metaplasticity in the awake developing brain.

The demonstration that brief sensory experience induces variable functional plasticity throughout nascent developing neuronal ensembles as a function of each individual neuron’s recent past activity and intrinsic RF properties has major implications for the functional development of neural networks. Neurons exhibit bias to refine their existing RF responses by strengthening if weak and weakening if strong, thereby maintaining responses within a functional dynamic range. Metaplasticity to restrict significant alteration in RF properties and training specificity to limit plasticity to distinct stimuli may elucidate evidence for stability in RF responses throughout maturation (Niell and Smith, 2005), as well as the ability for brief sensory stimuli to elicit substantial input specific plasticity. In this manner, environmental experience may drive discrete modification of developing central circuits to optimize performance within a relevant range.
2.4. **EXPERIMENTAL PROCEDURES**

2.4.1 Animal rearing conditions

Freely swimming albino *Xenopus laevis* tadpoles were reared and maintained in 10% Steinberg’s solution (1× Steinberg’s in mM: 10 HEPES, 58 NaCl, 0.67 KCl, 0.34 Ca(NO₃)₂, 0.83 MgSO₄, pH 7.4), and housed at 22°C on a 12-hour light/dark cycle. All experimental procedures were conducted on Stage 50 tadpoles (Nieuwkoop and Faber, 1967) according to the guidelines of the Canadian Council on Animal Care, and were approved by the Animal Care Committee of the University of British Columbia’s Faculty of Medicine.

2.4.2 Calcium indicator loading

The calcium-sensitive fluorescent indicator Oregon Green 488 BAPTA-1, AM (OGB1-AM, Molecular Probes, Eugene, OR) was bulk loaded into neurons within the tadpole brain (Brustein et al., 2003; Niell and Smith, 2005; Stosiek et al., 2003). OGB1-AM was prepared at a concentration of 10 mM in DMSO with 20% pluronic acid (Molecular Probes) and further diluted 10:1 in Ca²⁺-free Amphibian Ringers solution (in mM): 116 NaCl, 1.2 KCl, 2.7 NaHCO₃. Under visual guidance using an upright stereomicroscope, a sharp glass pipette loaded with OGB1-AM solution was inserted into the optic tectum of tadpoles anesthetized with 0.01% 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich, St. Louis, MO). Dye solution was slowly perfused into the brain using low-pressure (<10psi) on a Picospritzer III (General Valve Corporation, Fairfield, NJ). Tadpoles were subsequently returned to normal bath solution and allowed to recover from anaesthesia under dim light conditions (Brustein et al., 2003; Niell and Smith, 2005). One-hour following OGB1-AM loading, tadpoles were immobilized with 5 minute
bath application of 2mM pancuronium dibromide (PCD; Tocris, Ellisville, MO), embedded in 1% agarose, and placed in an imaging chamber continuously perfused with oxygenated 10% Steinberg’s solution. PCD is a reversible paralytic and typically wears off 2 hours post application at this dosage.

2.4.3 In vivo two-photon calcium imaging of neuronal dynamics

The imaging chamber was mounted on the stage of a custom-built two-photon laser-scanning microscope, constructed from an Olympus FV300 confocal microscope (Olympus, Center Valley, PA) and a Chameleon XR laser light source (Coherent, Santa Clara, CA). Optical sections through the optic tectum were captured using a 60X, 1.1 NA, water immersion objective (Olympus), and images were recorded and processed using Fluoview software (Olympus). The optic tectum was imaged at a resolution of 640 x 480 pixels and zoom factor of 1.5X, encompassing an area of 177 x133 μm, allowing simultaneous imaging of approximately 100-200 neurons in a single X-Y scan. Repeated X-Y scans of a single optical section were taken at a rate of 1.2s per frame using a wavelength of 910nm to excite OGB1-AM dye. The unique dye-loading pattern allows morphological corrections for drift over time without the need for a secondary morphological marker (confirmed by dual labelling with OGB1-AM and Red-fluorescent CellTracker Red CMTPX; Molecular Probes, Eugene, OR). Drift corrections (if necessary) were made every four minutes.

2.4.4 Visual stimulation

To apply light stimuli, a diode (590nm) was projected through the camera port of a trinocular eyepiece for whole-field illumination. A coloured Wratten Filter 32 (Kodak,
Rochester, NY) assured no bleed-through into the imaging channel. Illumination intensity, timing, and duration of light stimuli was varied with custom written software (Matlab, The Mathworks Inc., Natick, MA) synched to the microscope’s ‘ttl’ output for the onset of frame scanning. Step changes in whole-field light intensity from the background illumination were used as visual stimuli.

2.4.5 Single cell excitability probing (SCEP)

Evoked responses to visual stimuli were probed every 60 sec during 20 min pre-training and 60 min post-training (Fig. 2.4a). Two visual stimuli were used for SCEP probing: a 50ms OFF stimulus (Fig. 2.4a) and a 60 sec OFF stimulus (Fig. 2.4b). For control stimulation, 60 second probing was continued throughout the training window (Fig. 2.4a and c). Evoked OFF responses were recorded at each 50ms stimulus and at the beginning of each 60 sec stimulus. Evoked ON responses were recorded at the end of each 60 sec stimulus.

2.4.6 Visual training

To test RF plasticity after visual training, we utilized two separate training stimuli, each 25 minutes in duration (Fig. 2.4a and c). Invariant training consisted of 25 minutes ON light stimulation to the paralyzed eye. Spaced training consisted of three sets of 90, 50ms OFF stimuli at 0.3Hz, spaced by 5-minute intervals of ON stimulation. Previous studies have demonstrated persistent 5-minute spaced training with a moving bar stimulus can induce long term synaptic plasticity in the presence of endogenous spontaneous neuronal activity (Zhou et al., 2003).
2.4.7 White noise

Wide field white noise stimulation was accomplished by random variation of the diode voltage at 5Hz between empirically determined maximum and minimum intensity values within the diode’s linear range. White noise was presented to the paralyzed eye for one hour prior to pre-training SCEP probing.

2.4.8 Blockade of NMDAR

For NMDAR blockade of spaced training and invariant training induced plasticity, tadpoles were injected with 50 μM D-APV (Sigma Aldrich, St. Louis, MO) immediately before training. For NMDAR blockade during the metaplasticity phase, 50 μM D-APV was injected one hour prior to pre-training imaging, during which time white noise visual stimulation was presented to the tadpole.

2.4.9 Analysis of imaging data

Fluorescence data stacks were initially x-y aligned using Turboreg (Thévenaz et al., 1998) (ImageJ, NIH). Experiments that showed z-drift after alignment were discarded (approximately 1 in 4 cases). For each experiment, individual regions of interest (ROIs) were manually drawn over neuronal cell bodies in each optical section and analyzed using custom written software. For each neuron ROI the change in fluorescence intensity was calculated as \( \Delta F/F_0 = (F - F(t)_{\text{base}})/F(t)_{\text{base}} \), where \( F \) is the average intensity of the ROI in an image frame and \( F_{\text{base}} \) is a simple linear regression fit to image frames with fluorescence values one standard deviation from the minimum-recorded fluorescence intensity. This fitting served to eliminate the amplitudes of spontaneous spiking events from weighting the baseline fluorescence trace. Images frames
within 36 seconds (30 frames) of an evoked response were not included in the linear regression.

2.4.10 Evoked responses

SCEP records the evoked responses of single neurons over time. Response amplitudes were taken to be peak $\Delta F/F_0$ within three image frames after the visual stimulus. Only evoked responses with $\Delta F/F$ peak values >1.5 standard deviations (STD) above the mean baseline fluorescence trace were included in analysis (standard deviation of average baseline fluorescence trace, $\Delta F/F_0 = 0.09$). Somatic Ca$^{2+}$ spikes elicited by visual OFF stimuli are rapid (<100ms), long lasting (peak amplitudes of approximately 2sec), and reproducible (Fig. 2.2). ON stimuli evoke similarly long duration and consistent amplitude somatic calcium events, yet spike initiations are slower (approximately 900ms to peak) than OFF responses.

2.4.11 Spontaneous spiking

Fluorescence values were considered spikes if their $\Delta F/F_0 >$ STD above average baseline fluorescence trace and they showed characteristic fast onset. Coincident peak values > STD above the mean were taken as new spikes if $\Delta F/F_0$ values between events fell below the initial peak minus STD. To assure spontaneous spiking was not influenced by evoked responses, image frames within 48 seconds (40 frames) of an evoked response were not included in spontaneous activity analysis.

2.4.12 Inclusion criteria

For all analysis, only cells with >70% of probed evoked responses with $\Delta F/F_0$ peak values >1.5 STD above the mean were included. In addition, cells were required to have consistent
pre-training responses, where the mean evoked responses of 3 out of 4 five-minute epochs of SCEP pre-training (0-5, 5-10, 10-15, 15-20) could not differ significantly from the total mean evoked pre-training responses (0-20min) (unpaired heteroscedastic 2-tailed t-test). Cells with constantly drifting evoked responses were also excluded. These were determined by fitting pre-training responses with a linear regression and excluding all cells with slope values not equal to 0 within the 95% confidence interval. 92% of responding cells fit these criteria.

2.4.13 Plasticity criteria

For long-term changes, response properties were assayed by comparing responses during the 20 minutes of pre-training to both the responses during the first 20 minutes of post-training and the responses between 40-60 minutes post-training. Cells undergoing long lasting potentiation and long lasting depression showed significant changes both immediately and persistently after training ($t$-test $p < 0.05$). Cells undergoing short-term potentiation showed significant increase in responses during the first 20 minutes post-training but no significant change in responses 40-60 minutes post-training. Cells that showed no significant change in responses were categorized as ‘no change’.

2.4.14 Spontaneous correlations

Pair-wise correlations of spontaneous activity were calculated with the Pearson product-moment correlation coefficient (Rodgers and Nicewander, 1988). Correlations were calculated over 10-minute epochs.
2.4.15 Clustering

Numerical simulation was used to estimate the probability that observed levels of anatomical clustering of receptive fields and correlated activity arose under a null hypothesis of no spatial organization. To do this, the observed receptive field values or activity patterns were randomly reassigned among neurons within a given tadpole and clustering measures were calculated. This process was repeated 100,000 times. Reported p-values are the fraction of simulations that showed more clustering than was measured in the original data.

2.5. ACKNOWLEDGEMENTS

We thank K. Podgorski for help with correlation and clustering analysis, and D. Allan, S. Bamji, B. Chen, T. Murphy, and C. Rankin for comments on the manuscript. This work was supported by the National Science and Engineering Council of Canada, the Canadian Institute of Health Research, the Michael Smith Foundation for Health Research, the Canadian Foundation for Innovation, The EJLB Foundation, and the Human Early Learning Project.
Figure 2.1 | Visually-driven plasticity of evoked Ca$^{2+}$ events in the intact and awake brain.

**a**, Diagram of the experimental set-up: Visual stimuli are projected into the tadpole eye while performing two-photon imaging of contralateral optic tectum (red box, image area shown in b).

**b**, *Left*-Fluorescence optical section through tectum demonstrating neuropil (N), cell body region (C), and ventricle (V) (average of 5 images; scale bar = 140 μm); *Middle* – Ca$^{2+}$ responses immediately before visual stimulus; *Right* – Visually evoked calcium responses induced by brief, 50ms, OFF stimulus. Pseudo-color image using scale of fractional change in fluorescence intensity relative to average baseline levels. **c**, Mean SCEP recordings (± s.e.m.) of all cells after spaced training (*left*; n = 6 tadpoles, 168 cells; 14±1%, p < 10$^{-11}$, 40-60min post training, t-test) and invariant training (*right*; n = 5 tadpoles, 106 cells; -20±1%, p < 10$^{-12}$, 40-60min post training, t-test). Bar denotes training periods.
Figure 2.2 | Fluorescent calcium responses to visual stimuli. a, Line scan of single cell response to a brief, 50ms, OFF stimulus. The stimulus window is marked at one second. b, Fluorescence responses to 50ms OFF stimuli for four neurons located at different areas across the tectum. Stimuli were provided at 0.033Hz (indicated by black squares). Neurons respond consistently and robustly across trials.
Figure 2.3 | Characteristics of fluorescent calcium responses to visual OFF stimuli. Mean (±s.e.m.) SCEP recordings from tectal neurons to OFF stimuli of progressively longer duration at the maximal background illumination (grey, n=55 in two tadpoles) and progressively higher background intensities at the longest duration (black, n=36 in two tadpoles). The standard probing stimuli were chosen to be \((10^{-1}, 50\text{ms or 60sec})\). SCEP recordings were measured as peak \(\Delta F/F\) of the fluorescent calcium response. For each tadpole, stimulus series were randomly presented.
**Figure 2.4 | SCEP and training paradigms.** a, SCEP recording paradigm to probe visual OFF response properties (black bars, 50ms OFF stimuli presented every 60 seconds; 20 minutes pre-training, 60 minutes post-training). Training stimuli are illustrated between probing periods. **(Top)** Inv, Invariant ON light stimulation; **(Middle)** ST, spaced training with repetitive 50ms OFF stimuli; **(Bottom)** C, control stimulus (black bars, 50ms OFF stimuli). b, SCEP recording paradigm to probe both visual OFF and ON response properties (black bars, 60 second OFF stimuli presented every 120 seconds; ON response measure at end of OFF stimulus; 20 minutes pre-training, 60 minutes post-training). c, Expanded illustrations of training stimuli.
**Figure 2.5 | Activity during training.** a, Scatter plot of activity during high frequency (0.3Hz) spaced training (ST, green squares), invariant ON light stimulation (Inv, red squares), and white noise stimulation (see figure 2.16) (WN, dark blue squares) of individual cells versus activity during pre-training SCEP probing. Black line, pre-training and training (or WN) activity are equal. Mean activities are significantly different from pre-training: ST, 1.4±0.04 spikes/min (n=80 cells in two tadpoles); Inv, 0.8±0.02 spikes/min (n=80 cells in two tadpoles); WN, 1.14±0.05 spikes/min (n=33 cells in two tadpoles). b, Spaced training plasticity effects are additive with each presentation of high frequency OFF stimuli. Mean activity of different plasticity groups (± s.e.m.) (3 tadpoles): Long lasting potentiation (n=20, green circles), short term potentiation (n=8, grey circles), no change (n=48, blue circles), and long lasting depression (n=10, red circles).
Figure 2.6 | Natural visual stimuli induce variable functional plasticity in the embryonic brain.

a, SCEP recording paradigm to probe visual OFF response properties and training stimulation. SCEP is presented every 60 seconds: 20 minutes pre-training, 60 minutes post-training (black bars, 50ms OFF stimuli). Training stimulation: spaced training, three sets of high frequency (0.3Hz) repetitive 50ms OFF stimuli spaced by 5 minutes ON stimulation (top) and invariant ON light stimulation (bottom). Coloured boxes correspond to raster plots shown in b. b, Raster plot of the amplitude of Ca\textsuperscript{2+} transients for 100 randomly selected tectal neurons before and after spaced training (top) and invariant training (bottom). Each vertical section of the plot shows only 11 imaging frames; white lines separate time gaps. Green lines denote 50ms OFF stimuli. Means of all 50 Ca\textsuperscript{2+} transients, normalized to average pre-training peak values, are shown below raster plot.
Figure 2.7 | *In vivo* long lasting plasticity of single neurons and ensemble populations using visually evoked single cell excitability probing (SCEP). a-d, SCEP recordings from single tectal neurons exhibiting (a) long lasting potentiation (b) short term potentiation (c) no change and (d) long lasting depression to the probed, 50ms OFF stimulus after spaced training (ST). e-h, Mean SCEP recordings (± s.e.m.) of all cells exhibiting similar plasticity after ST (closed circles) and invariant training (Inv) (open circles); sample size same as k. i-j, Mean SCEP recordings (± s.e.m.) of all cells after (i) ST and (j) Inv. Plasticity is blocked by tectal injection of APV (red triangles). Mean amplitudes 40-60min post training are significant: i, n = 3 tadpoles, 79 cells, p < 10^{-18}; j, n = 3 tadpoles, 67 cells, p < 10^{-14} (t-tests). Bar denotes training periods and red arrow APV injection. k, Percentage of neurons exhibiting long lasting potentiation (ST 29%, Inv 1%**, Control 4%**, ST + APV 6%, Inv + APV 9**), short-term potentiation (ST 24%, Inv 13%, Control 1%**, ST + APV 14%, Inv + APV 16%), no change (ST 35%**, Inv 41%**, Control 69%, ST + APV 49%**, Inv + APV 45**) and long lasting depression (ST 12%**, Inv 45%, Control 26%**, ST + APV 39%, Inv + APV 30%) after various training. ST, n = 6 tadpoles, 168 cells; Inv, n = 5 tadpoles, 106 cells; Control, n = 4 tadpoles, 168 cells; ST + APV, n = 3 tadpoles, 79 cells; Inv + APV, n = 3 tadpoles, 67 cells. * p < 0.05, ** p<0.01, significant difference (chi-squared test) compared to underlined sample.
Figure 2.8 | NMDAR blockade does not affect visually evoked calcium response amplitudes.

Scatter plot of mean evoked calcium response amplitudes (±s.e.m.) pre- and post- APV injection (n=30 cells in 1 tadpole). Red line, linear regression fit of data (R²=0.95, slope=1.03±0.03).
Figure 2.9 | **Vehicle injection does not affect training.**  

**a,** Mean SCEP recordings (± s.e.m.) of all cells after spaced-training (black circles; n = 6 tadpoles, 168 cells) and spaced-training + vehicle injection control (blue squares; n = 3 tadpoles, 97 cells). Mean amplitudes 40-60min post training show no significant difference (t-test).  

**b,** Percentage of neurons exhibiting long lasting potentiation (28%), short-term potentiation (38%), no change (24%), and long lasting depression (10%), after spaced training + vehicle control injection. n = 3 tadpoles, 97 cells. Percentages show no significant difference from spaced training alone (p=0.09, chi-squared test).
Figure 2.10 | Line scan of single neuron OFF and ON response. **Top:** 60 second light stimulus; // time break. **Bottom:** Line scan of fluorescence response. Stimulus begins at 2 seconds and ends at 62 seconds.
Figure 2.11 | Tectal neurons cluster based on RF properties and RF plasticity is preferential to the trained stimulus. 

**a,** Purely ON and OFF-dominated RFs cluster anatomically. Coloured bars denote measured fraction of nearest anatomical neighbours with similar off-responsiveness; black bars and tails denote bootstrapped means and 95% confidence intervals for fraction of nearest neighbour pairs within similar off-responsiveness under random reassignment of observed receptive field values (see Methods); n = 3 tadpoles, 254 cells * p = 0.04, ** p = 0.001, significant difference (one-tailed t-test). 

**b,** Anatomical distribution of RFs in a single tadpole: neurons can be identified as purely OFF-dominated (red), purely ON-dominated (dark blue) or responsive to both ON and OFF stimuli (green) using 60sec OFF stimulus probing. Black circles highlight anatomical clusters. 

**c,** The induced plasticity correlates to strength of neuronal response to OFF vs. ON stimuli (mean ± s.e.m. of 10% bins, n = 3 tadpoles, 254 cells).
Figure 2.12 | Spontaneous activity of potentiated cells is unchanged after spaced training.

Scatter plot of mean spontaneous spike amplitudes (±s.e.m.) pre- and post- spaced training (n=20 cells in 3 tadpoles). Red line, linear regression fit of data ($R^2=0.65$, slope=$0.85±0.03$). Black line, pre-training and post-training activity are equal.
Figure 2.13 | Metaplasticity and stabilization of visually induced neuronal plasticity.

a, BMC theory schematic. Horizontal axis, neuronal activity during training: determined as the product of presynaptic activity and synaptic efficiency. Vertical axis, neuronal plasticity. A neuron’s modification threshold during training, $\theta_m$, is dependent on its average postsynaptic firing during pre-training. Neurons with high firing levels pre-training shift $\theta_m$ to the right, making potentiation more difficult and depression easier to induce. Neurons with low firing levels pre-training show the opposite effect. Reduced pre-synaptic activity during invariant training limits neuronal activity to below the modification threshold b, Mean amplitudes (± s.e.m.) of spontaneous Ca$^{2+}$ events predict plasticity outcomes (left). Spontaneous activity during pre-training is significantly greater in cells that undergo long lasting depression (red) regardless of the training paradigm. Cells that undergo long lasting potentiation (green) after spaced training show significantly less pre-training spontaneous activity than those of other plasticity types. Short-term potentiated cells (grey) and cells showing no plasticity change (blue) after spaced training show no significant difference; whereas, short-term potentiated cells after invariant training show no significant difference from depressed cells (* p < 0.05, ** < 0.01, t-tests). Spontaneous pre-training activity of individual neurons shows significant correlation to their plasticity outcomes (right) (spaced training; black line, linear regression $R^2$=0.55; slope=-0.07±0.019) c, Mean amplitudes (± s.e.m.) of spontaneous activity post-training is significantly lower in neurons exhibiting long lasting plasticity (* p < 0.05, ** < 0.01, t-test). n for all data sets in Fig. 2.13 are the same as in Fig. 2.7.
Figure 2.14 | Variations in bulk loading dye uptake are not correlated with calcium transient spike amplitudes. Scatter plot of pre-training calcium spike amplitudes (±s.e.m.) versus mean baseline fluorescence of individual cells. Raw fluorescence values correlate to the level of dye uptake by cells. ∆F/F fitting of baseline fluorescence allows for direct comparison of spike amplitudes to firing rates due to relatively uniform uptake of the dye. Red line, linear regression fit, slope = 0.008±0.015 (n=82 cells in 2 tadpoles).
Figure 2.15 | Training activity does not account for spaced training induced plasticity. Scatter plot of mean pre-training spontaneous calcium transient amplitudes (±s.e.m.) versus activity during spaced training for individual cells. Cells with high pre-training activity do not show significantly less activity during training and thus are unlikely to depress due to a reduction in input. Similarly cells with low pre-training, which potentiate, do not show significantly greater training activity. Red line, linear regression fit, slope=-0.0006±0.0012 (n=62 cells in 2 tadpoles).
Figure 2.16 | Increased activity prior to training shifts plasticity outcomes toward depression through an NMDAR dependent mechanism. a, Mean SCEP recordings (± s.e.m.) of all cells after spaced training (ST, black circles), white noise priming 1 hour prior to SCEP (WN, yellow circles), and WN priming + APV injection (WN+APV, red triangles). 40-60min post training: ST, 14±1%; WN, 13±1%; WN+APV, -26±1%. ST and WN+APV show no significant difference, p=0.44, t-test. Bar denotes training periods. b, This shift is blocked by APV injection during the metaplasticity phase. Percentage of neurons exhibiting long lasting potentiation (WN 9%, WN+APV 24%*), short-term potentiation (WN 6%, WN+APV 16%), no change (WN 26%, WN+APV 51%**), and long lasting depression (WN 58%, WN+APV 9%**), after various training. ST and WN+APV show no significant difference, p=0.1, chi-square test. ST, n = 6 tadpoles, 168 cells; WN, n = 3 tadpoles, 53 cells; WN+APV, n = 3 tadpoles, 70 cells. * p<0.05, ** p<0.01, significant difference (chi-squared test).
Figure 2.17 | Network analysis reveals increased correlated activity in neurons exhibiting potentiation. 

**a**, Correlated activity between neurons is significantly more common among nearest neighbours. Black bars denote fraction of nearest neighbour pairs with significant (p<0.05) correlations; grey bars and tails denote bootstrapped means and 95% confidence intervals for fraction of nearest neighbour pairs with significant correlations under random reassignment of neuronal activity patterns (* p < 0.05, ** < 0.01, one-tailed t-test). 

**b**, Correlated activity increases after spaced training. Average (± s.e.m.) fraction of significant (p<0.01) pair-wise correlations between all cells for each plasticity group (* p < 0.05, ** < 0.01, t-test). 

a and b data, pair-wise correlations calculated over 10-minute epochs; n for all data sets in Fig. 2.17 are the same as in Fig. 2.7.
2.7. REFERENCES


3. **IN VIVO SINGLE CELL EXCITABILITY PROBING OF NEURONAL ENSEMBLES IN THE INTACT DEVELOPING BRAIN**

3.1. **INTRODUCTION**

During embryonic development, brain neurons undergo extensive activity-dependent morphological (Cline and Haas, 2008; Haas et al., 2006; Ramdya and Engert, 2008; Ruthazer et al., 2003; Sin et al., 2002) and synaptic (Aizenman et al., 2003; Aizenman et al., 2002; Engert et al., 2002; Zhang et al., 2000) refinement. This experience-driven reorganization results in progressive persistent changes in the functional output of both single neurons and ensemble populations to influence normal and abnormal brain function later in life. In the developing embryonic retinotectal system of *Xenopus* tadpoles, tectal neurons receiving direct innervation from the optic nerve, exhibit functional changes reflected by both an age-dependent reduction in receptive field (RF) size (Gaze et al., 1974; Sakaguchi and Murphey, 1985; Sretavan and Shatz, 1984) as well as rapid potentiation or depression of firing rates induced by brief, RF-specific, visual experience (Dunfield and Haas, 2009; Engert et al., 2002; Vislay-Meltzer et al., 2006). A

\[\text{------------------}\]

\[2\] A version of this chapter has been submitted for publication. Dunfield, D. and Haas, K. *In vivo* single cell excitability probing of neuronal ensembles in the intact developing brain.
fundamental question of developmental neuroscience is how sensory experience modifies long-term firing behaviour and RF response properties of individual cells, as well as RF encoding of large neuronal ensembles in vivo.

Functional calcium imaging offers a non-invasive method to measure cell spiking in the awake and intact developing brain. Tectal neurons typically exhibit AP bursting in response to visual sensory input (Zhang et al., 2000; Zhou et al., 2003). Because calcium transients scale with the number of AP fired in a burst (Brustein et al., 2003; Fetcho, 1998; Johenning and Holthoff, 2007; Niell and Smith, 2005; Ramdya et al., 2006; Smetters et al., 1999; Sumbre et al., 2008; Yaksi, 2006), changes in calcium transient amplitudes can be used to map tectal RF response properties as well as functional plasticity over time (Brustein et al., 2003; Dunfield and Haas, 2009; Niell and Smith, 2005; Sumbre et al., 2008). Single cell excitability probing (SCEP) synchs stimulus probing with the scanning frames of time-lapse two-photon microscopy, allowing functional response mapping of large neuronal ensembles within a single optical section (100-200 neurons in the Xenopus optic tectum). In its simplest form, SCEP analysis records peak calcium transients in single cells directly after stimulus onset. By varying sensory stimuli, SCEP recordings can map cellular response properties of neuronal ensembles. SCEP can also record functional plasticity by probing before and after an induction stimulus. This technique has been used as a read out for synaptic plasticity in CA1 hippocampal slices after LTP induction (Johenning and Holthoff, 2007), as well as for RF mapping (Dunfield and Haas, 2009; Niell and Smith, 2005) and visually induced RF plasticity (Dunfield and Haas, 2009) in the awake and intact optic tectum. Both evoked and spontaneous neuronal activity can be assayed by recording calcium transients between probing stimuli (Dunfield and Haas, 2009).
During early embryonic development, *Xenopus* tectal neurons respond robustly to sharp changes in wide field illumination (ON or OFF stimuli) (Gaze et al., 1974; Zhang et al., 1998; Zhang et al., 2000). In this protocol, we outline *in vivo* SCEP paradigms for wide-field RF mapping in *Xenopus laevis* tadpoles as well as visual training paradigms that elicit both long term functional potentiation and long term functional depression of OFF responses in neuronal ensembles. A similar protocol may be suitable for localized spot stimulation (Tao and Poo, 2005; Vislay-Meltzer et al., 2006) or movement stimuli (Engert et al., 2002; Mu and Poo, 2006; Zhou et al., 2003). By imaging large neuronal populations with single cell resolution, the underlying contributions of individual cells to ensemble changes can be studied. This is particularly important in the developing brain, where visual experience has been shown to induce disparate synaptic (Vislay-Meltzer et al., 2006; Zhou et al., 2003) and RF plasticity (Dunfield and Haas, 2009) throughout the neural circuit.
3.2. MATERIALS

3.2.1 Reagents

• Albino or wild-type Xenopus laevis tadpoles (obtained from lab colony or Nasco, Fort Atkinson, WI). Here, we used stage 50 tadpoles (Nieuwkoop and Faber, 1967), although visually evoked responses may be elicited as early as stage 40 (Chen, 2001). For detailed instructions on acquiring and maintaining tadpoles, see ref. (Bhatt et al., 2004). Tadpoles used in excitability probing experiments were raised on a twelve hour light/dark cycle. ! CAUTION All experiments must be performed in accordance with the relevant authorities’ guidelines and regulations.

• 10% Steinberg’s solution (1× Steinberg’s in mM: 10 HEPES, 58 NaCl, 0.67 KCl, 0.34 Ca(NO₃)₂, 0.83 MgSO₄, pH 7.4).

• Anaesthetic agent: 0.01% MS222 (3-Aminobenzoic acid ethyl ester or Tricane; Sigma) diluted in 10% Steinberg’s Solution, adjusted to pH 7.4.

• Green fluorescent membrane-permeable calcium-indicator dye (e.g., Oregon Green 488 BAPTA-1 AM, OGB-1 AM, from Molecular Probes)

• 20% Pluronic F-127 in DMSO (e.g., 2 g Pluronic F-127 in 10 ml DMSO; Sigma)! CAUTION Strong detergent; skin, eye, and respiratory system irritant. Wear suitable protective clothing

• Standard pipette solution: Ca²⁺-free Amphibian Ringers solution (in mM): 116 NaCl, 1.2 KCl, 2.7 NaHCO₃.

• 2mM pancuronium dibromide (Tocris) dissolved in H₂O.
• Low melting point agarose (Invitrogen GmbH)

3.2.2 Equipment

• Electronics: BNC cable, insulated wire with banana clips, TTL output from scan mirrors (usually on microscope control unit), programmable stimulus generator (STG 2000, Multi Channel Systems).

• Two-photon laser-scanning microscope commercially available from several providers (e.g., Zeiss, Olympus, Nikon, Prairie, others)

• Red/green (565nm) dichroic (Chroma)

• Separate trinocular head (BX or BH series Olympus)

• Objective mount adaptors: Lens tube adaptor, tube coupler, and objective adaptor (SM1A2, SM1T1 SM1A3; Thorlabs)

• Two 60X long working distance water immersion lenses (e.g., Olympus LMPlanFl). One objective should be attached to the main imaging microscope and the other to the trinocular head used for visual stimulation.

• Light emitting diode, LED (590nm, 1.5V; Digi-Key, Thief River Falls, MN)

• Trinocular mount adaptors: Olympus C mount adaptor (Micro Tech Lab, Austria), and C mount to SM1 adaptor, tube coupler, and LED mount (SM1A10, SM1T1, S1LED; Thorlabs, Newton, NJ)
• Imaging chamber with central and side access openings: custom-made, see Figure 3.1b.

• Paintbrush and plastic transfer pipette to position and handle tadpoles.

• Dissection microscope equipped with fluorescence optics, to check for quality dye loading.

• Borosilicate capillary glass with filament: THIN GLASS: OD 1.0 mm, ID 0.78 mm, 75 mm and THICK GLASS: OD 1.5 mm, ID 0.86 mm, 75 mm (e.g. G150F-3; Warner Instruments, Hamden, CT).

• Pipette puller (e.g., P-97 equipped with either box or trough filament ~3 mm; Sutter Instruments, Novato, CA)

• Micromanipulator with coarse and fine control (e.g., MM-3; Narishige International, East Meadow, NY).

• Picospritzer III (General Valve Corporation, Fairfield, NJ)

• Millipore filter (Millipore, Billerica, MA)

• Unlubricated latex condoms

3.2.3 Reagent setup

**Tadpole bath solution:** Add 500 μL penicillin/streptomycin (5,000 U mL⁻¹/5,000 μg mL⁻¹, Invitrogen), to 10L of 10% Steinberg’s solution; pH to 7.4. Keep and use at room temperature (18–22 °C), oxygenate with 100% O₂ for perfusion.

**Calcium indicator:** Stock - dissolve membrane-permeable calcium-indicator dye at a concentration of 10mM in 20% Pluronic F-127/DMSO and store at -20 °C. Working solution -
dilute 10:1 in Millipore filtered (pore diameter, 0.45μm) Ca²⁺-free Amphibian Ringers solution and use immediately.

3.2.4 Equipment setup

Manipulator and pressure-application device To inject the staining solution into the brain we use the coarse Narashige Micromanipulator and Picospritzer III.

Two-photon laser-scanning microscope We use a custom-built microscope based on a mode-locked laser system operating at 700–960nm wavelength (Chameleon XR, Coherent) and a laser-scanning system (Fluoview, Olympus) coupled to an upright microscope (BX51WI, Olympus). Emission should pass through a filter cube containing a red/green dichroic. Such a custom-built system can be assembled following the instructions in refs. 18,19.

Imaging chamber A custom made perfusion chamber with both top and side openings to accommodate two microscope objectives is recommended for visual stimulation experiments. The side objective opening allows exact positioning of the visual stimulation apparatus aligned to the tadpole eye, assuring consistent illumination intensity and presentation of patterned stimuli across samples. The imaging objective is inserted into the top opening of the imaging chamber. A simple design for the imaging chamber requires only a lathing apparatus or drill press (with 30mm and 50mm drill bits), and a cylinder of hard plastic (60mm in diameter and 30mm in height; McMaster-Carr, Atlanta, GA). Begin construction by drilling a 30mm hole 20mm deep into the side of the plastic cylinder. Next drill the top opening with the 50mm bit to a depth of 15mm. Finish by rounding sharp edges by hand with sand paper. Perfusion tubes (Saint-Gobain PPL Corp., Courbevoie, France) can be fixed to the chamber with Instant Krazy
Glue (Elmer’s Products Canada), Figure 3.1. Sylgard (Dow Corning, Midland, MI) can be used to add extra height or depth to the chamber if necessary.

**Time synched visual stimulation** Visual stimulation must be synched precisely with the onset of scanning frames. This is achieved by triggering the stimulus generator (either hardware or software) with the frame TTL output of the scan mirrors. Typically frame scan TTL output begins with a pre-pulse ~300ms before the first imaging frame. Accordingly, an offset must be applied to the trigger to account for the lag time. Lag time can be measured empirically by imaging the light diode directly (in the red emission channel) and calculating the delay between the beginning of the first imaging frame and the onset of light stimulation (e.g., stimulation onset occurs ⅓ into the first imaging frame with 0.833 Hz frame rate and a 300ms lag time). Inter-stimulus intervals after the initial trigger can be calculated to synch with scan mirror frame rates (see Procedure 13). The STG 2000 series (Multi Channel Systems) programmable stimulus generator allows for freely programmable waveforms (mono- or biphasic, square pulses, ramps, sine wave) with individual time rates and duration of pulses. These stimulus protocols can be of unlimited duration and number of pulses, perfect for long-term stimulation. Stimulus programming timing is dependent on the complexity of the visual stimulation protocol (approx. 5-30 minutes). Output voltages should be set in the linear range of the LED.

**Equipment assembly** TIMING approximately 5 min:

Assemble the visual stimulus apparatus by attaching one of the 60X water immersion objectives to the separate trinocular head using the objective mount adaptors. Prepare the trinocular port for the LED by attaching the trinocular mount adaptors. In this configuration, the LED will
produce a wide-field stimulus. If patterned stimuli are desired (such as a multi-LED array), a lens tube should be inserted between the tube coupler and LED mount to house the necessary corrective optics (available at Thorlabs).

Connect the External Trigger of the scan mirror imaging system to the Additional Trigger Input of the programmable stimulus generator with a BNC cable. Connect the Output Voltage and Ground to the LED terminals with banana clips and fix the LED in the LED mount on the trinocular head. ! CAUTION As with all electrical equipment, familiarize yourself with the safety precautions of the equipment. During assembly, the equipment should be turned off.

3.3. PROCEDURE

3.3.1 Staining neurons with calcium-indicator dye

● TIMING ~ 15 min

1| Pull two micropipettes from borosilicate capillary glass, one THICK (the puncture micropipette) and one THIN (the staining micropipette; see equipment) using the pipette puller. Standard patch pipettes with a resistance of 6-9 MΩ filled with standard pipette solution work well. Back-fill the staining micropipette with the calcium-indicator dye working solution.

2| Anesthetize tadpoles in a Petri dish containing 0.01% MS222. Multiple tadpoles can be anesthetized simultaneously, but they should not be kept under anesthesia for over 30 min.

3| Place a tadpole under the dissecting microscope on a moist Kimwipe with a plastic transfer
pipette. Position the tadpole using a paintbrush perpendicular to the staining pipette with the dorsal side up, such that the brain is accessible.

4 | Adjust the light and contrast of the microscope to emphasize the tissue structure and cell outlines of the brain (see Figure 3.2).

5 | Insert the puncture micropipette into the micromanipulator. Pierce the skin adjacent to the tadpole brain with the puncture micropipette on the same side as the coarse micromanipulator and pressure set up. Be careful not to puncture the pia or disturb the brain. This small hole will be used to gain access to the brain without damaging the staining pipette. Remove the puncture pipette and discard.

6 | Insert the staining micropipette into the micromanipulator and pressure apparatus. While the pipette tip is suspended in air, test the pipette tip size by applying pressure and monitoring rate of solution extrusion. Dye solution should exit the tip of the pipette at pressures <20psi. If this is not the case, a new micropipette with a slightly wider tip opening should be pulled, filled, and re-tested.

7 | Focus on the target region and use the micromanipulator to carefully insert the staining pipette through the puncture hole in the skin. Insert the pipette into the brain at a low angle (~60-70°). Advance the pipette along its axis until it reaches the desired depth (Figure 3.2). Apply low pressure (1min, 10 psi) to eject ~400 femtoliters of staining solution. Infusion into the tadpole neuropil will typically allow for staining of the entire tectal lobe and thalamus to a depth of >300 μm with some auxiliary staining of the olfactory bulb. The area of dye loading can be quickly assayed using epi-fluorescence on the dissection scope. Remove the pipette.
Troubleshooting

■ Pause Point Place the tadpole in oxygenated bath solution and wait for ~1h to allow for recovery, and consistent and stable loading of tectal neurons (Stosiek et al., 2003).

3.3.2 Two-photon imaging and excitability probing

● Timing ~ 2-3 h

8 Paralysed the tadpole with 5 minute bath application of 2mM pancuronium dibromide (PCD). PCD is a reversible paralytic and typically wears off 2-3 hours post application at this dosage.

9 Prepare the imaging chamber by thoroughly rinsing an unlubricated latex condom and sheathing it over the imaging chamber, leaving the top access open. Latex condoms provide a water-tight barrier to encase the imaging chamber.

10 Place the tadpole in the imaging chamber with the eye contralateral to the dye-injected tectal lobe aligned with the side access port for visual stimulation. Cover the tadpole with a thin layer of liquid low melt agarose and let harden. Immediately perfuse the imaging chamber with oxygenated bath solution. ▲ Critical Step Agarose should be near room temperature before application.

11 Fix the imaging chamber to the microscope stage, inserting the objective into the top of the latex condom. Focus the microscope objective on to the tectal lobe with two-photon laser scanning, raising the excitation power carefully to avoid dye bleaching. Initial focusing should be done at high resolution (frame rate, >1Hz). At this stage in development, tectal neuron somata should be clearly visible, with a distinctive ring structure due to their large nuclei. Dark
areas corresponding to blood vessels are easily identifiable when focusing through the tissue and can be used as rough landmarks between tadpoles. For OGBAM-1, we use an excitation wavelength of 910nm. Refer to Ref (Xu, 2000) for the two-photon excitation spectra of different dyes.

**Troubleshooting**

**12** Before inserting the visual stimulation apparatus into the imaging chamber, make a small puncture hole in the latex condom at the center of the side access opening. The puncture hole assures optical clarity between the objective and the tadpole eye, and should be approximately 2mm in diameter – slightly larger than the front lens element of the objective, but small enough that pressure from the objective housing will prevent fluid from leaking out of the perfusion chamber. Insert the objective into the visual stimulus apparatus immediately after making the hole to prevent excessive leakage. Using the eyepieces on the trinocular head, align the visual stimulation apparatus on the eye. Fix the visual stimulation apparatus to the microscope stage and switch the trinocular to the camera port. Opening the trinocular port will project diffuse light from the LED directly into the tadpole eye. Slight realignment of the tectal lobe under the two-photon microscope may be necessary after this step.

**13** For SCEP set the frame rate such that the probe length plus inter-stimulus interval (ISI) divided by the 1/frame rate is an integer value (see Figure 3.3). Resolution and scan area should be set to assure near continuous scanning with minimal lag between frames. To begin SCEP, first focus at the depth of interest and then initialize the trigger on the programmable stimulus generator. At this point, when the user begins time-lapse two-photon scanning, the TTL trigger
output will synch the pre-programmed visual stimulus to the onset of the scan frame. Wide-field probing stimuli are typically set to correspond with frame onset (Figure 3.3). Because wide-field RF responses in the optic tectum have quick onset (~100ms) and long lasting peaks due to bursting behaviour (Dunfield and Haas, 2009; Tao et al., 2001; Zhang et al., 2000), functional responses of tectal cells can be measured by taking the peak calcium transient within 1-2 seconds of the stimulus frame. As such, minimum frame rates should be ~1 Hz, and two photon scanning durations should be set to a minimum of 5 seconds after the onset of the last presented stimulus. If presenting patterned visual stimuli, the user should determine minimum frame rates independently using the Nyquist criteria as a rule of thumb (frame rate 1/(2*peak time)). A simple wide-field SCEP protocol would be a single 1 sec ON stimulus with two photon scanning set to a frame rate of 1 Hz: Here the LED voltage should be initially set to 0V, and the stimulus generator programmed to output a 1V pulse for 1 second upon being triggered (then back to 0V). The time lapse movie would consist of 5 frames.

? TROUBLESHOOTING

14| To map wide-field RF properties, OFF and ON light stimuli with various durations and contrasts can be presented. When mapping response curves, stimuli should be presented in a pseudo-random sequence and averaged over multiple trials. ▲CRITICAL STEP When probing with wide-field dimming stimuli longer than 50ms (Tao et al., 2001; Zhang et al., 2000), some tectal cells will elicit both OFF (stimulus onset) and ON (stimulus offset) responses. If simply recording cellular responses as peak calcium transients, SCEP probing is unable to resolve ON and OFF responses in dual responding tectal cells for stimuli greater than 50ms and less than
~10 sec in duration. Other techniques may be used to resolve RF responses at these durations (Ramdya et al., 2006).

**15|** To probe changes in firing rates over time, SCEP of the same plane can be continued for 3+ hours (after which AM ester dyes may begin to leak from cells). Typically, RF responses are probed before and after visual training to study the effects of brief visual experience on the intact awake developing brain (Dunfield and Haas, 2009). Care must be taken to assure habituation to the visual response does not occur during probing. ISIs from 10-60 seconds are recommended. Possible visual training stimuli can be found in Table 3.1.

**? TROUBLESHOOTING**

Troubleshooting advice can be found in Table 3.2.

### 3.4. **ANTICIPATED RESULTS**

Figure 3.4a illustrates bulk AM ester calcium sensitive dye staining of a single lobe of the *Xenopus* tadpole optic tectum. Wide-field OFF stimulus probing elicits visually-evoked calcium transients in ~70% of the neurons within the imaging plane (Figure 3.4a). Transients are repeatable and consistent in amplitude (Figure 3.4b). 60s OFF stimuli evoke responses to both stimulus onset (OFF response) and offset (ON response) (Figure 3.4c). Wide-field RF properties of single cells can be assayed with respect to both duration and contrast sensitivity. For OFF RFs, functional response increases with both longer duration stimuli and higher contrast jumps in light intensity (Dunfield and Haas, 2009; Zhang et al., 2000). Single cell resolution allows anatomical localization of neurons in the tectum. Previous work using SCEP has demonstrated
local clustering of OFF and ON responding tectal neurons during development (Dunfield and Haas, 2009).

Visual experience can cause long lasting changes in a neuron’s functional response properties. As was shown by Dunfield and Haas, mean ensemble responses can be shifted toward either potentiation or depression to an evoked stimulus by brief periods of visual training (Dunfield and Haas, 2009). A spaced-training paradigm with high-frequency 50ms OFF stimuli (Table 3.1) causes preferential potentiation of OFF stimuli throughout the tectum. Plasticity induced by spaced-training is training stimulus specific (no change in ON responsiveness was shown after training). An invariant light stimulation into the paralyzed eye (Table 3.2) causes preferential depression to probed 50ms OFF stimuli. Visually-driven long lasting RF potentiation and depression in the tectum was shown to be N-methyl-D-aspartic acid (NMDA) receptor-dependent (Dunfield and Haas, 2009) and may be related to NMDAR dependent long-term synaptic plasticity (Engert et al., 2002; Zhang et al., 2000; Zhou et al., 2003). Correlation experiments highlight the power of SCEP: The functional plasticity of individual neurons could be compared to changes in those neurons’ network interactions. Correlated firing was found to increase in potentiated cells after spaced training. SCEP may be compared to electrophysiological recording, such as patch clamp electrophysiology, which limits analysis to single cells, and field recordings, which do not allow investigation of large contiguous neuronal ensembles at the single neuron level. SCEP, however, bridges the gap between single cell and population analysis, allowing researching to non-invasively measure neuronal activity of hundreds of neurons simultaneously at the single cell level within the intact brain.
3.5. **TABLES**

<table>
<thead>
<tr>
<th>TYPE</th>
<th>STIMULATION PROTOCOL</th>
<th>ANTICIPATED RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spaced training (potentiation)</td>
<td>Three sets of 90, 50ms OFF stimuli at 0.3Hz, spaced by 5-minute intervals of ON stimulation</td>
<td>29% long lasting potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24% short term potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35% no change,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12% long lasting depression</td>
</tr>
<tr>
<td>Invariant training (depression)</td>
<td>25 minutes ON light stimulation to the paralyzed eye</td>
<td>1% long lasting potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13% short term potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41% no change,</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>45% long lasting depression</strong></td>
</tr>
<tr>
<td>Continuous probing (no change)</td>
<td>50ms OFF stimuli presented every minute for 25 minutes with inter-stimulus ON light stimulation</td>
<td>4% long lasting potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% short term potentiation,</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>69% no change</strong>,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26% long lasting depression</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cells are not labelled</td>
<td>Failure to penetrate tissue</td>
<td>Re-puncture the skin beside the tectum using the thick-glass micropipette. Adjust angle of manipulator. Adjust light/contrast so that electrode tip can be visualized in the tissue. Make sure staining pipette is located in the target region.</td>
</tr>
<tr>
<td></td>
<td>Tip size too small / staining patch micropipette clogged</td>
<td>Dissolve Ca(^{2+})-indicator dyes immediately before use; filter the staining solution before use (e.g., with a Millipore filter; pore diameter, 0.45 (\mu)m). Increase pressure and/or modify puller program.</td>
</tr>
<tr>
<td></td>
<td>Pluronic F-127 crystallization</td>
<td>Re-make stock solution. Before adding pluronic F-127 and DMSO to stock solution heat to ~40°C and vortex.</td>
</tr>
<tr>
<td>Cells do not respond to visual stimulus</td>
<td>Imaging plane contains unresponsive cells</td>
<td>Depending on the visual stimulus, certain regions of the tectum may not be excited by visual input (see (Sperry, 1963) for a map of retinotopic innervations). Cells respond robustly to wide-field stimuli at depths of interest &gt; 100(\mu)m.</td>
</tr>
<tr>
<td></td>
<td>Insufficient calcium indicator loading</td>
<td>Increase time for pressure loading; increase concentration of working solutions to 1:5 (calcium indicator : Ringer’s solution). <em>CAUTION</em> Dye concentrations should be kept to a minimum. Excessive dye loading may cause calcium chelation.</td>
</tr>
<tr>
<td></td>
<td>Ca(^{2+}) stores released due to ischemic stroke</td>
<td>Assure agarose does not clog tadpole gills (located ventrally) when loading specimen into the imaging chamber. Increase perfusion rate of oxygenated 10% Steinberg’s solution.</td>
</tr>
<tr>
<td>Scanning triggered stimuli are off-time</td>
<td>Offset requires adjustment</td>
<td>Re-measure trigger offset by imaging LED directly; calculate trigger lag as (# lag scan lines / # scan lines in frame) / frame rate.</td>
</tr>
<tr>
<td></td>
<td>ISI is incorrect</td>
<td>1/(frame rate) should divide equally into the stimulus duration + ISI for each stimulus + ISI pair.</td>
</tr>
</tbody>
</table>
3.6. **FIGURES**

![Diagram of SCEP equipment setup]

**Figure 3.1** | **SCEP equipment setup.** (a) A custom dually-innervated imaging chamber is perfused with room temperature oxygenated Steinberg’s solution. Two-photon microscopy is used to image ensemble neuronal activity with a green calcium indicator, while red light of varying contrasts is projected onto the tadpole’s contralateral eye. (b) Schematic drawing of the imaging chamber (top) top view and (bottom) side view. Blue arrows indicate locations for fixation of perfusion tubes.
Figure 3.2 | Calcium indicator loading of the tadpole optic tectum. (a) Transparent albino *Xenopus laevis* tadpole. (b) Magnification of forebrain and optic tectum and placement of staining micropipette in neuropil of the optic tectum. The electrode is outlined in red. Arrow indicates skin puncture from the thick glass micropipette. (c) Fluorescence overlay of Ca2+ indicator loading. Scale bar, 1 mm.
Figure 3.3 | Scanning triggered visual stimuli. (top) Example of 50ms OFF stimuli with ON stimulation ISIs. The first OFF stimulus is triggered at the onset of laser scanning and a second OFF stimulus occurs 5 frames later. (bottom) Example of 5 frame OFF and 5 frame ON stimuli. The first OFF stimulus is set 2 imaging frames after laser scanning beings. Stimulus offset induces an ON response. A second 5 frame OFF stimulus occurs 5 frames later. (Red, ON light stimulation; BLACK, OFF light stimulation).
Figure 3.4 | In vivo SCEP of wide field visual stimuli in the awake developing brain. (a) (top) In vivo two-photon fluorescence image of OGB-AM calcium loading of an optical section of the *Xenopus laevis* tadpole optic tectum. Scale bar, 140 μm. (bottom) Visually evoked calcium responses induced by a brief, 50ms, OFF stimulus. Pseudo-color image using scale of fractional change in fluorescence intensity relative to average baseline levels. (b) Calcium transient evoked by 50ms OFF stimuli in two cells marked in panel (a); Transients are robust and long lasting. (c) OFF and ON calcium transients evoked by the onset and offset of a 60 second OFF stimulus. (bottom) 60 second light stimulus; // time break.
3.7. REFERENCES


4. CONCLUDING CHAPTER

4.1. METAPLASTICY’S ROLE IN DEVELOPMENT

Studies of metaplasticity during critical periods of activity-dependent development have mainly concentrated on the developmental shift on NMDAR subunits as a possible metaplastic mechanism. The NMDA receptor is a heteromer that contains an obligatory NR1 subunit along with one or more of the subunits NR2A – 2D (McBain and Mayer, 1994; Monyer et al., 1994). During early development, most NMDARs contain NR2B subunits (Monyer et al., 1994), which results in longer single-channel open times and significantly longer lasting Ca\(^{2+}\) inward currents. Because afferent activity is less synchronized in the early brain, NR2B subunits may help induce LTP by enhancing coincidence detection. NR2A subunits, which produce shorter duration Ca\(^{2+}\) currents, gradually increase in expression over development and reach a peak at the end of the critical period, while NR2B decreases (Flint et al., 1997; Roberts and Ramoa, 1999). This NR2B/NR2A switch is thought to be metaplastic because it is regulated by recent past history of visual experience. As an example, dark rearing prevents the switch from NR2B to A, and brief light exposure after dark rearing rescues NR2A expression (Philpot et al., 2001; Quinlan et al., 1999a; Quinlan et al., 1999b). Moreover, because NR2A subunits reduce Ca\(^{2+}\) entry into the cell (Erreger et al., 2005), an increase in NR2A is thought to facilitate LTD. Pharmacologically shortening NMDAR currents also enhances LTD (Philpot et al., 2003). Recent studies using NR2A-knockout mice have elegantly demonstrated that the NR2A/B ratio is critical for the induction of experience-dependent alterations in the plasticity threshold. NR2A-knockouts both
lose the facilitation of LTD with dark rearing (Philpot et al., 2007) as well as demonstrate an enhancement of LTP in non-deprived eye inputs (Cho et al., 2009). These studies suggest that NR2A/B regulation in the visual cortex may be one molecular mechanism underlying the sliding modification threshold of BCM theory in early development.

Our study complements the NMDAR subunit story by demonstrating that brief visual experience during embryonic development with white noise stimuli prior to visual training dramatically shifts the modification threshold in an NMDAR dependent manner. Further studies on NMDAR subunit expression pre/post white-noise stimulus are warranted to determine if the modification threshold shift is due to up-regulation of NR2A subunits. Recently, NR2A/B composition was demonstrated to regulate dendritic growth the Xenopus laevis optic tectum (Ewald et al., 2008) and may be the driving mechanism behind topographically organized sensitivity of the dendritic tree to patterned visual stimulation (Bollmann and Engert, 2009). Based on the complete sequences of X. laevis NR2A and NR2B cDNA, the amino acid sequence and all major functional regions and residues are conserved between Xenopus and rat NR2 subunits (Ewald and Cline, 2009).

Questions about the role of experience in development have been a primary focus of neuroscience since the coining of the phrase “Nature vs. Nurture”. Pioneering work by William Greenough has elegantly demonstrated that environmental experience significantly impacts brain development (Greenough et al., 1989; Jones et al., 1997). Our work provides significant insight into the role of visual experience-driven afferent activity in altering neuronal function in the embryonic brain. Previous studies have demonstrated that brief visual experience can cause
long lasting plasticity of tectal neuron synaptic transmission (Engert et al., 2002; Mu and Poo, 2006; Zhang et al., 2000; Zhou et al., 2003). Visually-driven synaptic plasticity in the tectum has been demonstrated to be spike-time-dependent (Mu and Poo, 2006; Vislay-Meltzer et al., 2006), suggesting that the precise timing of pre- and postsynaptic activity is critical in determining the direction of plasticity outcome. Under certain conditions, when neuronal firing rates conform to Poisson statistics, spike-time-dependent-plasticity may be the underlying mechanism of BCM metaplasticity (Izhikevich and Desai, 2003). Here, we expand on previous work by demonstrating that functional output of neurons also undergoes long lasting bidirectional plasticity after visual training. Moreover, we show that the threshold for plasticity switching from depression to potentiation can be shifted with prior visual experience.

If experience plays such a large role in regulating synaptic plasticity and neuronal output during development, it may come as a surprise that RFs in the zebrafish optic tectum develop rapidly (within 12h after the first evoked visual stimulus) and remain stable throughout life (Niell and Smith, 2005). BCM theory, however, may provide an answer for this phenomenon. The sliding threshold of BCM biases neurons to strengthen their output if weak and weakening if strong, thereby maintaining responses within a functional dynamic range. This homeostatic regulation may restrict significant alteration in RF properties in neurons after their initial development. Overall, our work, in combination with recent literature in the field, suggests that environmental experience may drive discrete modification of developing central circuits to optimize performance and prevent saturation of plasticity.
4.2. **BRIDGING THE GAP BETWEEN CELLS AND SYSTEMS**

4.2.1  **Population recordings with single cell resolution**

Historically, neuroscience research has been limited to the study of either the very small – molecular pathways, ions channels, and single cell morphology – or the very large – functional imaging of brain regions, and lesion studies. As such, our current understanding of neural network processing has relied on compiling statistics of single neuron recordings or bulk recordings of averaged neuronal responses. Functional magnetic resonance imaging, intrinsic optical signal imaging, and voltage-sensitive dye imaging have allowed study of activity across different brain regions to broadly assess changes in brain function; however, these techniques cannot capture network dynamics at the single cell level. This limitation is particularly important since the collective function of individual neuronal output is not linear – simply averaging response properties of individual cells overlooks the complex computational relationships of neural population coding, where firing rates of individual neurons in context with the full neuronal ensemble convey information about the input stimulus. Both multielectrode unit recording and functional multi-neuron calcium imaging allow us to bridge this gap between systems and cells (Wong, 1998).

Multielectrode unit recording allows simultaneous recording from dozens of neurons with high temporal resolution. Spike sorting algorithms are used to classify action potentials from individual neurons. Functional calcium imaging, in contrast, has low temporal resolution, but allows simultaneous imaging of large contiguous populations of neurons. The ability to identify the locations of all neurons in the population (include those that do not respond to a
probing stimulus) is the significant advantage of Ca\textsuperscript{2+} imaging (Takahashi et al., 2007). Currently the only fundamental limitation of multi-neuron analysis (either via electrode recording or Ca\textsuperscript{2+} imaging) is the lack of computation support to analyze large neuronal networks.

In Chapter 2, we show, for the first time, the effect of visual stimulus training on the correlated firing of a developing neural network. Correlated firing increases significantly in a subpopulation of functionally potentiated neurons after a high frequency visual training stimulus.

### 4.2.2 Alternatives to SCEP

Because SCEP is stimulus triggered, probed calcium fluctuations (measured as \(\Delta F/F\)) can be averaged to determine evoked RF responses. RF probing with various external stimuli permits RF mapping of large neuronal ensembles. These averages assume that stimulus interactions do not influence Ca\textsuperscript{2+} signals. Interactions between evoked responses can be limited by employing inter-stimulus-intervals of sufficient length. Alternatively, individual probed responses can be assayed over time to investigate changes in evoked response properties (as described in Chapter 3). In Chapter 2, a simple spike extraction algorithm requiring calcium fluctuations to cross a \(\Delta F/F\) threshold was also used to eliminate probed responses without evoked responses. This procedure is particularly important when one is interested in determining evoked transient amplitudes and when neurons throughout the ensemble respond with different levels of reliability. In Chapter 2, thresholding was also used to evaluate spontaneous cell firing between trigger stimuli. Thresholding Ca\textsuperscript{2+} responses or their derivatives is a relatively common approach (Cossart et al., 2003; Ikegaya et al., 2004).
Nevertheless, SCEP and thresholding techniques suffer from a number of limitations. First, SCEP requires relatively consistent and robust evoked responses to image large populations of neurons. Inconsistent, weak responses can also be assayed by stimulus triggers, but two-photon scanning speeds need to be increased to compensate for shifts in peak Ca$^{2+}$ response. Increasing scanning speeds means resolution must be decreased and magnification increased, defeating the ability to measure activity of large ensembles. Thresholding also suffers from semi-arbitrary threshold levels and sensitivity to optical noise.

Several methods have been developed to automatically reconstruct neuronal events from raw fluorescence signals to resolve these problems, including template-matching algorithms (Kerr et al., 2005), reverse correlation (Ramdya et al., 2006), a novel detection algorithm for rodent cortex in vivo (Greenberg et al., 2008), and a spike detection algorithm based on principal-component analysis (PCA) (Sasaki et al., 2008).

4.3. **STATUS OF WORKING HYPOTHESES AND CONTRIBUTION TO THE FIELD**

In Chapter 1, I proposed three working hypotheses: (1) that long lasting functional plasticity in the developing retinotectal system may be driven by visual sensory input via synaptic and intrinsic plasticity; (2) that this functional plasticity is NMDAR dependent and varies with the pattern of visual training; and (3) that training induced plasticity follows the
learning rules established in the Bienenstock, Cooper and Munro (BCM) computational model of synaptic plasticity (Bienenstock et al., 1982).

Consistent with hypotheses 1, research in Chapter 2 demonstrated that functional plasticity could be driven with wide-field visual stimulation, including high frequency wide-field OFF stimuli and invariant ON light stimulation. Functional plasticity was assayed using the SCEP protocol, outlined in Chapter 3, using low frequency wide-field OFF probing. A limitation of the SCEP protocol was our inability to determine the cause of plasticity in neuronal firing, though a number of pieces of evidence point to synaptically mediated regulation. In agreement with hypothesis 2, plasticity outcomes induced by different training protocols resulted in various ensemble plasticity outcomes – high-frequency OFF training promoting potentiation to the trained stimulus and invariant training promoting depression. Visually-driven functional plasticity was also demonstrated to be NMDAR dependent.

Metaplastic learning rules, according to hypothesis 3, were observed both with respect to individual endogenous neuronal firing rates as well as visually manipulated firing rates, where activity was enhanced during the pre-training period using white-noise stimulation. BCM learning rules were also evident in the existence of a plasticity threshold, such that weak presynaptic input resulted in ensemble depression and strong input results in ensemble potentiation.

The experiments in this thesis contribute to the field of developmental neuroscience by providing a number of firsts: The protocol in Chapter 3 is the first example of SCEP in vivo and outlines a novel developmental model system to study network plasticity in the intact,
developing brain. By making use of the albino *Xenopus laevis* tadpole, time-lapse two-photon imaging of calcium sensitive dyes, and imaging trigged visual stimulation, I demonstrate a powerful experimental model for studying functional plasticity of many neurons simultaneously at single cell resolution. My experiments are also the first to investigate changes in network dynamics after visually-driven plasticity. Previous studies of natural stimulus induced plasticity using patch or field electrophysiology have been unable to bridge this gap between single cell and network plasticity. I also expand upon previous work by demonstrating natural experience induced metaplasticity. While metaplasticity has been hypothesized to hold an important role in development, the brief durations required to induce metaplastic effects demonstrated here are striking. My experiments are also the first to examine metaplastic effects on neuronal ensembles in the intact, awake embryo. The results demonstrate that metaplastic rules hold at the single cell level even in a population that has been exposed to the same pre-synaptic stimulation. Overall, results from my experiments greatly enhance the understanding of sensory stimulation’s role the regulation of functional plasticity during brain development.

4.4. **FUTURE DIRECTIONS: LINKING FUNCTIONAL AND MORPHOLOGICAL PLASTICITY**

Because the size and shape of a neuron’s dendritic arbours dictate the number and type of synapses the neuron can form, it is believed that a growing neuron’s structural refinement is directly related to the functional plasticity of its RF. Dendritogenesis is an extremely dynamic
process which involves extension of branches via dendritic growth cones (Portera-Cailliau et al., 2003), the addition and retraction of motile interstitial filopodia from existing dendritic shafts, and the stabilization and extension of interstitial filopodia into branches (Niell et al., 2004). The high turnover and probing behaviour of dendritic filopodia suggest they may be involved in sampling the local environment for appropriate presynaptic contact sites. The ‘synaptotropic model’ of dendritic growth proposes that filopodial stabilization is mediated by correct axonal-dendritic contact and subsequent formation and maturation of synapses. The synaptotropic model is supported by direct evidence that synapse strengthening and weakening correlate with morphological dynamics (Konur and Yuste, 2004; Niell et al., 2004) and by time-lapse imaging studies of living neurons showing activity-dependent regulation of filopodial motility and stability (Haas et al., 2006; Konur and Yuste, 2004; Maletic-Savatic et al., 1999; Portera-Cailliau et al., 2003; Sin et al., 2002).

While brief periods enhanced visual stimulation (4h) applied to freely swimming tadpoles (Aizenman et al., 2002; Sin et al., 2002) can enhance both RGC axonal development (Ruthazer et al., 2006) as well as tectal neuron dendritic arbour growth (Sin et al., 2002) in the developing retinotectal system, to date there have been no direct imaging studies investigating the effects of visual stimulation paradigms eliciting potentiation or depression on early dendritic growth. Calcium imaging protocols outlined in Chapter 3 provided a perfect experimental backbone to explore this relationship.

To directly assess the effects of functional potentiation and depression on filopodial dynamics and dendritic growth, both the functional plasticity as well as morphological changes
must be measured simultaneously. This can be achieved by combining functional calcium imaging of green fluorescent dye with single-cell electroporation (SCE) (Dunfield and Haas, 2008; Haas et al., 2002; Haas et al., 2001) of a plasmid encoding red fluorescent protein. The morphology of SCE tagged neurons can be imaged in vivo at the very onset of activity-dependent plasticity with time-lapse multi-photon microscopy (Majewska et al., 2000; Svoboda and Yasuda, 2006). Combining morphological imaging with periods of SCEP functional imaging may provide important insight into how experience-dependent functional plasticity is linked to morphological changes within the intact developing brain during critical periods of activity-dependent growth.

4.5. CLINICAL RELEVANCE

Dysregulation of synaptic plasticity plays a major role in many neurological disorders including Fragile X-linked mental retardation syndrome (Huber et al., 2002), epilepsy (Goussakov et al., 2000), and traumatic brain injury (Albensi and Janigro, 2003; Kuzmiski et al., 2009; Schwarzbach et al., 2006). Understanding how regulatory mechanisms, such as metaplasticity, effect synaptic plasticity in the intact brain may play a crucial role in developing therapies to promote functional recovery.

For example, BCM theory proposes that neuronal firing shifts the modification threshold of cells to homeostatically regulate LTP and LTD and prevent the saturation of synaptic plasticity. A rightward shift in the modification threshold caused by high levels of recent activity
would inhibit future LTP and facilitate future LTD, thereby guarding against excitotoxicity by preventing Hebbian-driven positive feedback. Accordingly, enhanced activity may also prevent the formation of epileptic networks by inhibiting the formation of these positive-feedback loops. Indeed, both electrical and pharmacological stimulation of glutamate receptors during early kindling has been demonstrated to inhibit subsequent induction of epileptic seizures (Hesp et al., 2007; Ullal et al., 1989). Our studies suggest that experience-driven cell firing may also be sufficient to shift the plasticity threshold – offering new possibilities for potential behavioural therapy.

Metaplastic rules can also be employed to rescue morphological plasticity. Traditional dogma suggests that neurons in the mature brain largely lose their ability to exhibit the experience-dependent plasticity seen during development. In the cat visual cortex, the ability to induce ocular dominance plasticity was shown to diminish with age (Hubel and Wiesel, 1963). In the mature mouse barrel cortex, in vivo time-lapse imaging has demonstrated that whisker clipping produced no change in gross dendritic arbour structure (Trachtenberg et al., 2002). Because drastic arbour remodelling may be detrimental unless it is in response to severe alterations of sensory inputs such as direct brain lesions, the reduction of morphological plasticity over time may help ensure that any plasticity induced in the adult cortex would be reversible - the original pre-synaptic partners remain in place, as do the major dendritic branches.

Nevertheless, metaplastic rules have been used to completely rescue the capacity for ocular dominance plasticity in the adult visual cortex (He et al., 2006). Ocular dominance shifts occur during monocular deprivation if animals are initially primed with 7 days of dark exposure
(He et al., 2006). During monocular deprivation, the deprived eye receives minimal presynaptic input, causing its synapses to predominantly undergo LTD (Rittenhouse et al., 1999). While dark exposure is unlikely to effect this deprivation induced depression, dark priming shifts the modification threshold to the left, facilitating LTP in the open eye. Loss of visual acuity (amblyopia) associated with chronic monocular deprivation over long periods, can be nearly eliminated with a 3-10 day dark exposure prior to re-opening the deprived eye (He et al., 2007). Mechanistically, dark priming is thought to reduce LTP thresholds by up-regulating NR2B subunits of NMDARs (He et al., 2006). These data fit well with our experiments where visual experience was found to shift the metaplasticity threshold. Our work also suggests that enhanced visual activity after eye opening should increase LTP and, in turn, the therapeutic benefits to dark priming. Altogether, these experiments suggest a direct use for metaplastic priming in the treatment of adult amblyopia.
4.6. REFERENCES


5. **APPENDIX A: ETHICS BOARD CERTIFICATES**

**THE UNIVERSITY OF BRITISH COLUMBIA**

**ANIMAL CARE CERTIFICATE**
**BREEDING PROGRAMS**

**Application Number:** A09-0021

**Investigator or Course Director:** Kurt Haas

**Department:** Cellular & Physiological Sc.

**Animals:**

- Frogs Xenopus Laevis 60

**Approval Date:** February 12, 2009

**Funding Sources:**

- **Funding Agency:** Canadian Institutes of Health Research (CIHR)
  - **Funding Title:** In vivo imaging stimulation-evoked dendritic arbor growth and synaptogenesis in the CNS: role of synaptic transmission and downstream signalling in brain circuit development

- **Funding Agency:** Michael Smith Foundation for Health Research
  - **Funding Title:** Role of NMDA receptor-mediated transmission on dendritic growth in the intact brain - implications for the developmental origin of schizophrenia and epilepsy

- **Funding Agency:** Michael Smith Foundation for Health Research
  - **Funding Title:** An in vivo model of abnormal neuronal circuit information: the role of glutamergic synaptic transmission in dendritic arbor growth and synaptogenesis


<table>
<thead>
<tr>
<th>Agency:</th>
<th>Michael Smith Foundation for Health Research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding Title:</td>
<td>In vivo imaging of activity-dependent synaptogenic events between dynamic axonal and dendritic filopodia within the developing brain - connections to the development of schizophrenia and highly common neonatal seizures</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Michael Smith Foundation for Health Research</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Michael Smith Foundation for Health Research</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>EJLB Foundation</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>In vivo imaging of the formation of dysfunctional brain circuits: critical role of glutamatergic transmission in dendritic growth and synaptogenesis</td>
</tr>
</tbody>
</table>

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Animal review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
# ANIMAL CARE CERTIFICATE

**Application Number:** A07-0042  
**Investigator or Course Director:** Kurt Haas  
**Department:** Cellular & Physiological Sci.  
**Animals:**  

| Frogs Xenopus laevis tadpoles 20000 |

**Start Date:** February 17, 2007  
**Approval Date:** June 22, 2009

**Funding Sources:**

<table>
<thead>
<tr>
<th>Funding Agency:</th>
<th>Funding Title:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epilepsy Canada</td>
<td>Summertime Studentship/Simon Chen/Effects of excessive activity on dendritic arbor growth</td>
</tr>
<tr>
<td>UBC Human Early Learning Partnership (HELP)</td>
<td>Real-time Imaging of Brain Circuit Formation within the Developing Brain</td>
</tr>
<tr>
<td>British Columbia Ministry of Children and Family Development</td>
<td>HELP: Direct measurements of how environmental stimuli sculpt developing brain structure and function</td>
</tr>
<tr>
<td>EJLB Foundation</td>
<td>In vivo imaging of NMDA receptor-mediated control of dendritic growth and synaptogen</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>National Alliance for Research (US)</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>In vivo imaging of dendritic structural development A test of the glutamate hypofunction model of schizophrenia</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>UBC Faculty of Medicine</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>Start up grant</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Canadian Institutes of Health Research (CIHR)</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>Facility for imaging and recording of neuronal development and synaptogenesis</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Michael Smith Foundation for Health Research</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis</td>
</tr>
<tr>
<td>Funding Agency:</td>
<td>Michael Smith Foundation for Health Research</td>
</tr>
<tr>
<td>Funding Title:</td>
<td>An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis</td>
</tr>
</tbody>
</table>

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093