Investigating the Role of NRG1β/ErbB4 Signalling in LTP

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

Synaptic plasticity is the fundamental feature of the mammalian brain that enables learning and memory. One main form of activity-dependent, lasting increase in synaptic transmission termed LTP is observed at the hippocampal CA1 and mediated by the NMDARs. Schizophrenia susceptibility pathway NRG1β/ErbB4 has been implicated in physiological, as well as aberrant, regulation of NMDAR function. Prevention of LTP induction is the consensus phenomenology observed in studies involving exogenous application of NRG1β on acute hippocampal slice preparations. Recent efforts demonstrated that NRG1β-mediated suppression of LTP involves the inhibition of nonreceptor tyrosine kinase Src; however; the exact mechanism remains controversial.

After observing no effect of NRG1β application on LTP, I carried out a step-wise interrogation NRG1β/ErbB4 ligand-receptor pair. In this thesis, I present evidence for an experimental-condition-dependent downregulation of ErbB4 expression; this finding bears important implications for experimental design, in addition to broadening our understanding of the mechanisms governing NRG1β/ErbB4 signalling.
ACKNOWLEDGMENTS

I was extremely privileged to have had Dr. Michael W. Salter as my supervisor. Without his constant support and strong dedication to teaching, none of the work reported here could have been possible. I gratefully acknowledge the support and continued constructive feedback from Dr. Lu-Yang Wang and Dr. Zhengping Jia, members of my committee board. I would also like to thank Dr. Ameet Sengar for his expertise in the biochemical characterization of cellular dynamics, as well as sustained input in matters of experimental design. In addition, my appreciation goes to Dr. Avner Michaeli, talented electrophysiologist who provided a series of key insights for this project, who had also been working on hippocampal recordings, and who had been the source of vital input with regards to resolving methodological issues. Last but not least, I would like to specifically express my deepest gratitude to Dr. Graham M. Pitcher, dedicated and passionate researcher who has taught me all the aspects of the electrophysiological method and has served as a source of continued inspiration, guidance, and support.
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LIST OF ABBREVIATIONS

7-C1KY = 7-chlorokynurenate
AC = adenylyl cyclase
ACSF = artificial cerebrospinal fluid
AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR = AMPA receptor
ATPase = adenylpyrophosphatase
CA1 = Cornu Ammonis area 1
CA3 = Cornu Ammonis area 1
CAKβ = cell adhesion kinase β
CaM = calmodulin
CAMKII = Ca2+/CaM-dependent protein kinase II
cAMP = Cyclic adenosine monophosphate
CRE = cAMP-responsive element
CREB = CRE-binding protein
CNS = central nervous system
D-AP5 = D-2-amino-5-phosphonopentanoic acid
DG = dentate gyrus
EGTA = ethylene glycol tetraacetic acid
EGF = epidermal growth factor
E-LTP = early LTP
EPSP = excitatory post-synaptic potential
fEPSP = field EPSP
GABA = γ-aminobutyric acid
GABAR = GABA receptor
GluR = glutamate receptor
GPCR = G-protein-coupled receptor
iGluR = ionotropic GluR
IPSP = inhibitory post-synaptic potential
L-LTP = late LTP
LTP = long-term potentiation
mGluR = metabotropic GluR
NMDA = N-methyl-D-aspartate
NMDAR = NMDA receptor
NRG = neuregulin
PK = protein kinase
PKA = protein kinase C
PKC = protein kinase C
PPF = paired-pulse facilitation
PSD = post-synaptic density
PTP = post-tetanic potentiation
SC = Schaffer collaterals
SZ = schizophrenia
SEM = standard error of the mean
SNARE = soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

TARP = transmembrane AMPAR-binding protein

TBS = Θ-burst stimulation
1. INTRODUCTION

The capacity for activity-dependent alterations in synaptic strength is the remarkable property of the mammalian central nervous system (CNS), which enables it to adapt to the environment and form memories.

Long-term potentiation (LTP), a paradigmatic form of lasting, activity-dependent synaptic enhancement, is widely regarded as the probable cellular correlate of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Observed at the hippocampal cornu ammonis 1 (CA1) area, one main form of LTP (1) is expressed by an increase in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPAR) synaptic responses, and (2) is mediated by N-methyl-D-aspartate (NMDA) receptors (NMDARs). Despite that LTP has been the target of intense research in the four decades since its discovery, a number of important unknowns remains and will be addressed below.

NMDAR hypofunction model of schizophrenia (SZ), a prominent hypothesis based on noting the parallels between the symptoms of SZ and those induced by acute administration of NMDAR antagonists, in the past decade had been expanded to incorporate the evidence for aberrant signalling in the SZ susceptibility pathway NRG1/ErbB4 (Corfas et al., 2004), observed in rodents (Deakin et al., 2009), as well as human subjects (Law et
The major limitation of this model is its failure to account for the fact that NMDAR function is governed by a fine balance between phosphorylated and dephosphorylated states of its tyrosine residues, partly by the Src family of tyrosine kinases (Salter and Pitcher, 2012). Recent efforts revealed that NRG1β-mediated prevention of LTP induction occurs through the inhibition of Src-mediated enhancement of NMDAR activity (Pitcher et al., 2011). However, the major outstanding research question concerning the molecular nature of the interaction between NRG1β/ErbB4 and Src persists.

In this thesis, I set out to determine the molecular nature of the interaction between NRG1β/ErbB4 signalling and Src, using electrophysiological and biochemical methods. However, as my study progressed, I did not observe the elimination of LTP upon the exogenous application of nanomolar doses of NRG1β, the phenotype reported by arguably every laboratory to publish a study on the topic (Huang et al., 2000; Kwon et al., 2005; Bjarnadottir et al. 2007; Li et al., 2007; Iyengar and Mott, 2008; Pitcher et al., 2011). In order to determine why NRG1β had not had its expected effect under my experimental conditions, I had to generate a series of new hypotheses in a step-wise fashion. First, I asked whether the ligand used in the study was NRG1β, whether it possessed any biological activity, and whether ErbB4, the cognate receptor for NRG1β, was expressed in the
preparation. After obtaining positive answers to all three questions, I then hypothesized that the level of NRG1β/ErbB4 signalling might be experimental-condition-dependent. In this thesis, I present evidence in support of the contingency of ErbB4 expression on slicing media composition and incubation duration. In the next sections, I am going to provide a brief overview of the neurobiology of LTP, concluding with a detailed rationale for this study.

1.1. Basic neuroanatomy

Together with glia, neurons comprise the principal cells of the nervous system. Anatomically, a typical neuron is composed of a cell body, also known as soma, multiple dendrites, and one axon. Information input is generally relayed to the neuron through its dendrites, while the output is fed through the axon. Information encoded, transmitted, and decoded by neurons typically assumes the form of the action potential. When the action potential reaches the axon terminal, it is transferred to the target cell through a specialized connection between the presynaptic axon terminal and its target.

Synapses are classified as excitatory or inhibitory if synaptic transmission increases or decreases, respectively, the firing probability of the postsynaptic target. In terms of the modality of synaptic transmission, synapses are categorized as either electrical synapses or chemical synapses
(Furshpan and Potter, 1959). At the electrical synapse, the neurons are joined by gap junctions, which, by virtue of maintaining the continuity of the intracellular space across the neurons, allow a depolarizing impulse to traverse the connection directly. At the chemical synapse, the neurons are physically and electrically separated by a space called the synaptic cleft, thereby dictating which entails the conversion of signal from a wave of depolarization into a release of neurotransmitters.

The hippocampus, a cortical structure crucial for the formation and retrieval of new memories, is the principal anatomical structure investigated in the studies of synaptic plasticity. It is characterized by an orderly arrangement of its fibers, which form a circuit referred to as the trisynaptic loop (Figure 1). The first projection of the trisynaptic pathway is represented by the connections made onto the dendrites of the granule cells of the dentate gyrus (DG) by the axons stemming from the entorhinal cortex. The second element of the trisynaptic circuit is represented by the synapses formed between the projections from the granule cells of the dentate gyrus (DG) and the dendrites of the cornu ammonis area 3 (CA3) of the hippocampus. Projections sent by the CA3, contained in the Schaffer collateral-commissural (SC) pathway, make synapses with the dendrites of the cornu ammonis area 1 (CA1), which represents the third projection of the trisynaptic pathway.
Figure 1. Trisynaptic pathway of the hippocampus.
(A) Schematic representation of the hippocampal trisynaptic pathway. (B) Representative slice configuration.
1.2. Synaptic transmission at glutamatergic synapses

When an action potential reaches the distal termination of the axon, membrane depolarization causes voltage-gated Ca$^{2+}$ channels, which are abundantly expressed at the axon terminal, to undergo a conformation change, briefly permitting Ca$^{2+}$ ions to flow into the cell. Entry of Ca$^{2+}$ then recruits the SNARE protein complex (Söllner 1993), which initiates the exocytosis of previously docked vesicles containing neurotransmitter glutamate.

In the CNS, excitatory amino acid glutamate mediates the vast proportion of excitatory synaptic transmission, and plays an essential role in the expression of synaptic plasticity. Once exocytosed, glutamate diffuses across the synaptic cleft and binds to strategically localized postsynaptic glutamate receptors. Activation by glutamate induces a conformational change in ionotropic glutamate receptors, which leads to the opening of their channel pores and permits excitatory postsynaptic current (EPSC) into the dendrite. Sufficiently large excitatory postsynaptic potential (EPSP), which is a graded, temporary depolarization of postsynaptic membrane potential caused by the summation of EPSCs, has the potential of causing the postsynaptic neuron to fire an action potential. Owing to the features of glutamate receptors discussed below, postsynaptic responses at glutamatergic
synapses are characterized by a fast, AMPAR-mediated component, and a blocked, NMDAR-mediated component of the response.

1.3. Ionotropic glutamate receptors

Glutamate receptors (GluRs) mediate the vast majority of excitatory synaptic transmission in the mammalian brain, and, in terms of their mode of function, are classified into two chief categories (Sugiyama et al., 1989): ionotropic GluRs (iGluRs), which are ligand-gated cation channels, and metabotropic GluRs (mGluRs), which are G-protein-coupled receptors (GPCRs). iGluRs mediate rapid excitatory synaptic transmission, and are involved in a range of important physiological processes, including synaptic plasticity.

Three major classes of iGluRs are recognized based on their exclusive selectivity to glutamate analogues NMDA, AMPA, and kainate, as NMDARs, AMPARs, and kainate receptors, respectively (Collingridge et al., 2009). Amino acid sequence similarity across iGluRs confers to them a number of commonalities (Traynelis et al., 2010). Specifically, iGluRs are assembled as membrane-spanning tetramers with a channel pore formed by the four subunits. iGluRs possess the amino-terminal domain, the ligand-binding domain, the transmembrane segment, and the intracellular carboxy-terminal
domain. Differences in amino acid sequence determine the distinct biophysical properties of iGluRs, which will be discussed below for the two principal iGluRs, AMPARs and NMDARs.

AMPARs mediate the rapid component of excitatory synaptic transmission. Functional AMPARs are either homo- or heterotetramers comprised of four subunits GluA1-GluA4 (Dingledine et al., 1999; Hollmann and Heinemann, 1994). The presence of GluA2 in AMPARs renders them unable to flux Ca$^{2+}$, and since in the adult hippocampus, the predominant AMPAR subunit combinations are GluA1/GluA2 or GluA2/GluA3 heteromers (Lu et al., 2009), hippocampal AMPARs are primarily Ca$^{2+}$-impermeable. In terms of kinetics, the time course of AMPAR activation, desensitization, and deactivation assumes the scale of milliseconds (Hansen et al., 2007). In addition to the AMPAR proper, the AMPAR complex also includes transmembrane AMPAR-binding proteins (TARPS) which provide structural support and modulate the function of AMPARs (Nicoll et al., 2006).

NMDARs are involved in a broad spectrum of physiological, as well as aberrant processes (Kalia et al. 2008). Structurally, NMDARs are comprised of two obligatory GluN1 subunits and two regulatory subunits (GluN2A-D, or GluN3A-B). Unlike other GluRs, NMDAR activation requires the binding of its agonist, glutamate, to the GluN2 subunit, as well as the binding of its co-agonist, glycine, to the GluN1 subunit. Under resting conditions, NMDARs are
largely impermeable to $\text{Ca}^{2+}$ due to the suppression of ion flux by extracellular $\text{Mg}^{2+}$. During high-frequency glutamatergic signalling, depolarization events caused by AMPAR EPSCs overlap and depolarize the membrane potential, favoring the dissociation of $\text{Mg}^{2+}$ from the NMDAR channel pore, thereby permitting $\text{Ca}^{2+}$ into the dendritic spine.

1.4. Synaptic plasticity

Plasticity is the fundamental property of the mammalian CNS which allows it to adapt to the environment and encode new information. Synaptic plasticity assumes a wide variety of forms that differ on a number of levels. The timecourse of synaptoplastic phenomena can vary from fractions of a second to many days or even years. Mechanistically, synaptic plasticity can be expressed pre-synaptically, post-synaptically, or simultaneously at both synaptic compartments. Some examples of recognized forms of short-term synaptic plasticity include facilitation, which assumes a timescale of hundreds of milliseconds, augmentation, which lasts seconds, and post-tetanic potentiation, which spans tens of seconds (Zucker and Regehr 2002). The principal longer-lasting form of synaptic plasticity, LTP, will be discussed below.
1.5. NMDAR-dependent LTP at the CA1

First described almost 50 years ago (Lømo, 1966), long-term potentiation (LTP) is the phenomenological term used to refer to a lasting increase in synaptic efficacy, generally following high-frequency activation of a synapse. The most well-characterized form of LTP is expressed by an NMDAR-dependent upregulation of AMPAR EPSCs at excitatory synapses made by the SC fibers on the pyramidal cells of the CA1 area of the hippocampus. This form of LTP is considered the primary candidate for a cellular correlate of learning and memory (Malenka and Nicoll, 1999), owing to its three principal properties: it is (1) input-specific, meaning LTP is confined to the stimulated pathway, (2) cooperative, meaning there exists an intensity threshold for the induction of LTP, (3) associative, meaning that otherwise subthreshold stimulation can induce LTP, if it co-occurs with suprathreshold stimulation of an adjacent pathway (Bliss and Collingridge 1993). NMDAR-dependent LTP of AMPAR EPSCs at the CA1 was chosen to be the focus of this study, and will hereafter be referred to by the term LTP for brevity’s sake.

LTP is not a unitary phenomenon, and most models describe its mechanism using two chief categories: that of protein synthesis dependence and that of persistence (Park et al. 2013). LTP is thus discussed in terms of early-phase LTP (E-LTP), characterized by transient, protein-synthesis-
independent biochemical events that assume the timescale of 1-2 hours, and late-phase LTP (L-LTP), which involves de novo protein synthesis and can span hours or days. Secondly, within E-LTP as well as L-LTP, recognized are the induction, which is the term used to refer to the initial sequence of events that trigger synaptic enhancement, and the maintenance, which refers to the mechanisms that constitute the enhancement. It is important to note that E-LTP and L-LTP represent mechanistically different, but temporally overlapping forms of plasticity, rather than discrete events executed in a sequence (Park et al., 2013). Since the time frame of recordings in this thesis assumed the scale of hours, most attention in the sections to follow will be devoted to the discussion of E-LTP.

Finally, evidence exists for the role of both pre- and postsynaptic modifications in the expression of LTP. However, the primary locus of expression of the main form of LTP appears to be located in the postsynaptic compartment (Lüscher and Malenka, 2012); thus, discussion of the postsynaptic mechanisms underlying LTP will form the basis for the rest of this work.

1.5.1. Induction of the early-phase LTP

Within the large panel of pharmacological dissections carried out in the 1980s in an attempt to determine the required conditions for the induction of
E-LTP, of note are two influential lines of research, which made it apparent that the induction of E-LTP requires the activation of a range of protein kinases (PKs) by NMDAR-mediated Ca\(^{2+}\) currents.

In particular, investigations of E-LTP at the level of the NMDAR complex revealed that (a) the activation of the NMDAR by its two co-agonists, glutamate and glycine, as well as (b) Ca\(^{2+}\) influx through the NMDARs, are required for the induction of E-LTP. In a set of early studies, the glutamate site antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5; Collingridge et al. 1983), the glycine site antagonist 7-chlorokynurenate (7-C1KY; Bashir et al., 1990), and the pore blocker MK-801 (Coan et al. 1987) each were demonstrated to prevent the induction of E-LTP when applied to acute hippocampal slice preparations. Additionally, intracellular postsynaptic application of the Ca\(^{2+}\) chelator ethylene glycol tetraacetic acid (EGTA) prevented the induction of E-LTP (Lynch et al., 1983), whereas postsynaptic Ca\(^{2+}\) uncaging was shown to be sufficient to induce LTP (Malenka et al., 1988), revealing that NMDAR-mediated Ca\(^{2+}\) influx into the dendritic spine is required for the induction of LTP.

While the essential role of NMDAR activation in the induction of LTP was also being corroborated by a number of important behavioural studies that relied on LTP-dependent models of learning (Morris et al. 1986; Miserendino et al., 1990), the other influential line of research focused on
elucidating the intracellular interactome of Ca\(^{2+}\). These efforts led to the identification of the principal Ca\(^{2+}\)-dependent protein kinases (PKs) that are crucial for E-LTP maintenance. Among the large number of PKs, the major research interest was arguably attracted to Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII, Lisman et al., 2012). Highly enriched in synapses (Kennedy et al., 1983), CaMKII was demonstrated to be necessary and sufficient for the induction of LTP in acute hippocampal slice (Petit et al., 1994), as well as shown to be required for a variety of forms of learning in rodents (Giese et al., 1998; Elgersma et al., 2002; Lisman et al., 2012).

1.5.2. Maintenance of the early-phase LTP

Within the course of E-LTP, signalling cascades induced by Ca\(^{2+}\)-dependent PKs lead to the amplification of AMPAR EPSCs through the potentiation of AMPAR single-channel conductance, as well as through the upregulation of the number of AMPARs localized in the dendritic membrane.

Upon entering the dendritic spine, Ca\(^{2+}\) gets rapidly bound by its primary endogenous buffer calmodulin (CaM), and then serves as the substrate for PKs such as CaMKII. Once activated, CaMKII undergoes a conformational change that favors the autophosphorylation of its threonine 286 residue, which (a) increases CaMKII’s affinity for Ca\(^{2+}/\)CaM by orders of magnitude (Meyer et al., 1992), and (b) endows CaMKII with the capacity for
autophosphorylation, allowing CaMKII to remain persistently active on a
timescale that spans beyond the timescale of Ca$^{2+}$ spikes (Ohsako et al.,
1991; Fukunaga et al. 1993). Within the AMPAR complex, the catalytic
activity of CaMKII (McGlade-McCulloh et al., 1993), as well as that of other
PKs (Wang et al., 1994), converges on serine 831, the major phosphorylation
site of the AMPAR subunit GluA1. Phosphorylation of serine 831 causes
AMPARs to assume a higher single-channel conductance state, thereby
increasing AMPAR-mediated EPSCs (Lledo et al. 1995).

At basal state, synaptic AMPARs exist in a dynamic equilibrium with
the extrasynaptic and intracellular AMPAR pools. Signalling cascades
triggered by the activation of Ca$^{2+}$-sensitive PKs promote a shift in this
equilibrium towards a greater total number of synaptic AMPARs (Opazo and
Choquet, 2011). Compelling electrophysiological evidence made it apparent
that this increase occurs through the exocytosis of AMPAR-containing
vesicles stored in the dendrite (Collingridge et al., 2004; Hayashi et al., 2000;
Park et al., 2004), and is accompanied by the lateral diffusion of extrasynaptic
AMPARs, as demonstrated by more recent imaging studies (Triller and
Choquet, 2005). The probability of AMPARs remaining localized in the
dendritic membrane is further augmented through the TARP-mediated
reinforcement of the association between AMPARs and postsynaptic
scaffolding proteins (Petrini et al., 2009).
1.5.3. Induction and maintenance of the late-phase LTP

L-LTP represents the most persistent form of activity-dependent synaptic enhancement which requires de novo protein synthesis (Frey et al., 1988) and can last months in vivo and hours in vitro (Raymond, 2007). While the term L-LTP maintenance is used to refer to the expression of LTP effector proteins, the discussion of L-LTP induction assumes the focus on the biochemical events leading to the activation of nucleic (Luscher and Malenka, 2012) or dendritic (Sacktor, 2008) transcription factors.

Since its first description in 1987 (Montminy and Bilezikjian, 1987), cAMP-responsive-element-binding protein (CREB) has emerged as the primary nuclear transcription factor implicated in the upregulation of proteins necessary for the long-term preservation of plasticity at the synapse. CREB was demonstrated to undergo activation in the course of L-LTP (Deisseroth et al., 1996), and was shown to be required for the formation of a number of types of long-term memory in rodents (Bourtchuladze et al., 1994). In addition to the already mentioned PKs, postsynaptic Ca\(^{2+}\) spikes induce adenylyl cyclase (AC; Wong et al. 1999), producing an elevation in the levels of the major intracellular second messenger cyclic adenosine monophosphate (cAMP), which serves as the substrate for cAMP-dependent protein kinase A (PKA), major upstream mediator of CREB phosphorylation (Kandel, 2012). Genetic (Abel et al., 1997), as well as pharmacologic (Frey et al., 1993),
inhibition of PKA revealed that activation of the cAMP-PKA pathway is required for the induction of L-LTP. The binding of transcription factors such as CREB to their cognate activator sequences on the DNA promotes the expression of L-LTP effector proteins, which range from iGluR subunits such as GluA1 of AMPAR (Borges and Dingledine, 2001), to enzymes involved in neurotransmitter synthesis such as tyrosine hydroxylase (Kim et al., 1993), to hormones involved in promoting spine growth such as brain-derived neurotrophic factor (BDNF; Finkbeiner et al., 1997).

1.5.4. The role of Src family kinases in LTP

The gating of NMDAR activity is frequently described using the widely used and abused term coincidence detection, which refers to the twofold requirement for (1) ligand binding and (2) concurrent, sufficient membrane depolarization. However, regulation of NMDAR function is a more complex phenomenon, and is, in part, governed by the fine balance between tyrosine phosphorylation and dephosphorylation (Wang and Salter 1994).

Originally identified as a proto-oncogene and implicated in the context of cell differentiation (Stehelin et al., 1976), Src, together with the other four members of the Src family of nonreceptor tyrosine kinases Yes, Lck, Lyn, and Fyn, was subsequently discovered to be ubiquitously expressed in the developed CNS. Since then, it became increasingly apparent that one of the
major roles of Src in the adult CNS is regulation of ion channel function, such as enhancement of NMDAR activity (Kalia et al., 2004). Anchored to the NMDAR via nicotinamide adenine dinucleotide dehydrogenase subunit 2 (Gingrich et al. 2004), Src upregulates NMDAR EPSCs through direct phosphorylation of tyrosine residues on subunits GluN2A and GluN2B (Moon et al. 1994; Yang and Leonard 2001; Cheung and Gurd 2001).

Given the central role of NMDARs in the induction of LTP, and the ubiquitous expression of Src within the CNS, it comes as no surprise that further efforts to interrogate Src-NMDAR interactions uncovered a role for Src in LTP. Electrophysiological recordings from CA1 neurons revealed that Src-mediated enhancement of NMDAR function is necessary and sufficient for the induction of LTP (Lu et al. 1998). In terms of the mechanism, it has been subsequently demonstrated that during LTP induction, the early Ca$^{2+}$ influx into the postsynaptic cell recruits cell adhesion kinase β (CAKβ) which activates Src (Seabold et al., 2003), leading to the augmentation of NMDAR-mediated Ca$^{2+}$ currents in a positive feedback fashion. Furthermore, by acting as a point of convergence for a large number of signalling cascades such as GPCR pathways, Src acts as a molecular hub for the regulation of NMDAR activity (Salter and Kalia 2004).
1.6. Neuregulins, a family of EGF-like peptides

NRGs are a diverse family of related EGF-like signalling proteins that are crucial for the development and maintenance of a number of mammalian tissues, including the nervous system, the heart, and the breast (Falls 2003). At least four genes encoding NRG have been identified: NRG1 (Holmes et al., 1992), NRG2 (Carraway KL 3rd et al. 1997), NRG3 (Howard et al. 2005), and NRG4 (Harari et al., 1999). Each of the four genes, in turn, may additionally give rise to as many as 30 isoforms through alternative RNA splicing and promoter usage (Mei and Xiong 2008). Despite such heterogeneity, the extracellular EGF-like motif containing the three characteristic cysteine-cysteine pairs is present in all splice variants of NRG, and alone is necessary and sufficient to activate the cognate receptor for NRG, ErbB family of tyrosine kinase receptors (Holmes et al. 1992). On the basis of the divergence within the C-terminal portion of the EGF-like domain, NRG splice variants are commonly classified as type α or type β NRGs (Wen et al. 1994). While type β NRG isoforms are predominantly expressed in the nervous system, type α NRGs are thought to be more enriched in the mesenchymal tissue (Meyer and Birchmeier 1994). Approximately twenty years ago, several distinct growth factors were simultaneously discovered, and later recognized as NRG1 gene product. To date, NRG1 remains the most extensively characterized member of the NRG protein family. A large
body of research suggests a role for NRG1 in the context of regulation of nicotinic acetylcholine receptor expression at the neuromuscular junction (Falls et al. 1993), promoting Schwann cell proliferation (Marchionni et al. 1993), as well as activating ErbB receptors (Holmes et al. 1992).

1.7. ErbB tyrosine kinase receptor family

ErbB tyrosine kinase receptors are activated by a number of EGF-type proteins, and play a vital role during development as well as in the adult CNS (Olayioye et al. 2001). Originally comprised of gene ErbB1 that encodes the EGF receptor (Cohen 1965), the ErbB receptor family had been subsequently expanded to encompass proteins encoded by three additional genes: ErbB2 (Toyoshima et al. 1986), ErbB3 (Kraus et al. 1989), and ErbB4 (Plowman 1993). ErbB receptors are assembled as single-spanning transmembrane glycoproteins that possess an extracellular cysteine-rich ligand binding domain, and an intracellular tyrosine kinase catalytic domain, flanked by a carboxy-terminal tail containing a varying amount of tyrosine residues. On the cell surface, ErbB receptors exist as monomers that dimerize upon activation by their ligands. Dimerization induces a conformational change in the receptors that favors autophosphorylation of specific cytoplasmic tyrosine residues, which then recruits a panel of intracellular signalling molecules (Schlessinger 2000). Different ErbB receptors display distinct affinities for
their ligands. NRGs were demonstrated to preferentially bind to ErbB3 and ErbB4, but not EGF or ErbB2 receptors (Carraway and Cantley, 1994). Since ErbB3 and ErbB4 receptors are capable of forming heterodimers with EGF and ErbB2 receptors, NRGs are thus often said to have two direct receptors, EGFR and ErbB4, and two indirect receptors, EGF and ErbB2. Finally, by virtue of ErbB3 receptor’s tyrosine kinase domain catalytic deficiency, ErbB4 is effectively the only identified member of the ErbB receptor family that mediates NRG1 signalling. Together, the diverse array of NRG functions is largely determined by the marked heterogeneity on a number of levels, including NRG splice polymorphisms, ErbB receptor mode of activation, as well intracellular interactome (Burden and Yarden 1997).

1.8. NRG1β/ErbB4 signalling and LTP

Since the observation that bath-applied NRG1β prevents LTP induction without altering baseline synaptic responses was first described in the literature (Huang et al., 2000), unanimity has been reached regarding the effects of NRG1β on LTP. Specifically, it is accepted that exogenous application of nanomolar doses of NRG1β to acute hippocampal slice preparations suppresses LTP induction in a dose-dependent fashion, without affecting basal synaptic transmission (Kwon et al., 2005; Bjarnadottir et al. 2007; Li et al., 2007; Iyengar and Mott, 2008). Furthermore, genetic
inactivation of ErbB4 receptors expectedly permits enhanced LTP (Pitcher et al., 2008). Additionally, since larger doses of NRG1β can suppress LTP to the point of elimination (Huang et al., 2000), it is hypothesized that physiological levels of NRG1β/ErbB4 signalling are non-maximal (Salter and Pitcher, 2012).

Recent efforts have provided further insights into the molecular nature of NRG1β-mediated inhibition of LTP induction (Pitcher et al., 2011). In particular, (1) failure to enhance NMDAR activity by administering Src-activating peptide in the presence of NRG1β, as well as (2) prevention of LTP induction, but not maintenance, by NRG1β application, together suggest that NRG1β/ErbB4 signalling impedes LTP induction through the inhibition of Src, rather than direct suppression of NMDAR activity. Thus being said, the molecular mechanism by which NRG1β/ErbB4 signalling cascade affects Src remains elusive (Salter and Pitcher, 2012). Described model of LTP is summarized in Figure 1.

1.9. Rationale and course of thesis

Induction of the main form of LTP in the mammalian CNS, NMDAR-dependent LTP of AMPAR EPSCs at the hippocampal CA1, requires the activation of NMDARs, concurrently with Src-mediated enhancement of NMDAR currents. Gain-of-function aberrations in a SZ susceptibility pathway
Figure 2. Schematic cartoon depicting the key events surrounding LTP induction and NRG1β/ErbB4 signalling at glutamatergic synapses.
NRG1β/ErbB4 suppress LTP in a dose-dependent fashion through the inhibition of NMDAR upregulation by Src, without affecting basal synaptic transmission. However, the major outstanding question concerning the molecular basis of the interaction between NRG1/ErbB4 and Src remains.

Therefore, I set out to determine the biological mechanism underlying the suppression of Src-mediated enhancement of NMDAR function by NRG1β/ErbB4 signalling using electrophysiological, pharmacological, genetic, and biochemical tools. However, as I came along, I did not observe LTP suppression from exogenous application of nanomolar doses of NRG1β. The validity of this effect was corroborated by replicating NRG1β experiments on a different electrophysiological setup by an independent examiner. After I confirmed the biological activity of NRG1β and the expression of ErbB4 in acute hippocampal slice preparation, I generated a new hypothesis that the expression of ErbB4 is dependent on such experimental conditions as the concentration of Mg²⁺ in the dissection media, as well as the duration of recovery after animal sacrifice.
2 METHODS

2.1. Slice preparation

Unless stated otherwise, 3-6 week old CD1 mice were used for all experiments. All experiments were performed in strict adherence to the protocols approved by the Hospital for Sick Children Animal Care Committee and the Canadian Council on Animal Care. Mice were anesthetized and immobilized with 20% (w/v) urethane, administered intraperitoneally. Following sacrifice, the head of the animal was immediately submerged into a dissection bath filled with dissection artificial cerebrospinal fluid (dACSF) containing 1.25 mM NaH$_2$PO$_4$, 3.1 mM KCl, 7.7 mM MgCl$_2$, 19±1 mM NaHCO$_3$, 25 mM D-glucose, 210 mM sucrose, and 0.1 mM CaCl$_2$ (pH 7.2-7.4, 300–330 mOsm), saturated with carbogen gas (95% O$_2$ / 5% CO$_2$) and chilled to 0–4°C. In the dissection bath, the skull was rapidly cut along the sagittal suture and the brain was extracted. Then, the brain was placed onto a chilled petri dish platform filled with dACSF where, with a scalpel, it was cut into two hemispheres along the longitudinal fissure. After the dissection, each of the hemispheres was glued to a dissection platform along the midsagittal plane. Next, the platform was inserted into the dACSF-filled, carbogenated slicing chamber of the Leica Vibratome 1300TS, where, using a razor blade, the hemispheres were rapidly sliced in 300-μm parasagittal sections at the
speed of 0.2 mm per second. As soon as each hippocampal slice possessing desired anatomical landmarks (defined, but still elongated dentate gyrus; discernible apical dendritic layer of the CA1) was obtained, it was transferred into the incubation chamber filled with artificial cerebrospinal fluid (ACSF) containing 1.25 mM NaH$_2$PO$_4$, 3.1 mM KCl, 2 mM MgCl$_2$, 27±2 mM NaHCO$_3$, 25 mM D-glucose, 122+-6 mM NaCl, and 2 mM CaCl$_2$ (pH 7.2-7.4, 300–330 mOsm, saturated with carbogen gas (95% O2 / 5% CO2)), where it was allowed to recover at room temperature for at least 1.5 hours prior to the start of recording.

2.2. Perfusion setup

Perfusion ACSF (pACSF) used for recordings was supplemented with 10 µM bicuculline methochloride in order to control for the potential effects of GABA$_A$R-mediated inhibition, maintained at 28±1°C (as measured in chamber), carbogenated, and otherwise identical to the ACSF described above, unless stated otherwise. Temperature maintenance was achieved by thermally conditioning a segment of the perfusion line, monitoring the temperature online. Following incubation, a single slice was transferred directly to the recording chamber and immobilized using two horseshoe-shaped pieces of platinum wire. Throughout the entire duration of the recording, the slice had been continuously superfused with pACSF at a rate
of approximately 2 mL/min. During NRG1β experiments, a test slice was placed into the recording chamber, and if a stable baseline could be rapidly obtained, the ACSF was supplemented with 2 nM NRG1β, and the test slice was replaced with a new slice. The author was blind to whether he was supplementing the solution with 2 nM NRG1β dissolved in 1 mL purified water or 1 mL purified water. NRG1β stock was obtained from Genentech. All other chemicals were purchased from Sigma-Aldrich.

2.3 Recording configuration

In all protocols and all experiments, field excitatory post-synaptic potentials (fEPSPs) were evoked by delivering a 0.08 ms depolarizing pulse at the Schaffer collateral pathway (SC; approximately 50 μm away from the CA1 cell body layer) using a tungsten bipolar electrode. Evoked fEPSPs were recorded using a pACSF-filled glass micropipette inserted in the stratum radiatum (approximately 60 μm away from the cell body layer). Once a recording configuration that allowed for the recording of healthy response (see Figure 3) was obtained, electrode placement was kept constant for the length of any further electrophysiological recordings made from the given slice. TBS was used as the LTP-inducing stimulus. The protocol was comprised of 5 Hz bursts, each consisting of four 100 Hz pulses, for a total of 3 seconds (or 6 seconds, as described in section 3.2.)
Figure 3. Representative slice configuration.
survey synaptic transmission during baseline and after the administration of conditioning stimuli was delivered and recorded at 0.1 Hz in order to minimize the possibility of inducing undesired, confounding plasticity. Baseline stimulus intensity was set to approximately 30% of the maximal response, and stimulus intensity was kept constant throughout the entire LTP experiment. Input-output curves were obtained by evoking fEPSPs at the rate of 0.1 Hz using progressively increasing stimuli in 0.1 mV increments, beginning at 0.1 mV. Acquisition of input-output curves was aborted when the increase in the slope of the response increase started to plateau, or when excessive population spike contamination was observed. During paired-pulse facilitation experiments, pairs of fEPSPs were evoked at the rate of 0.1 Hz. Interstimulus interval was progressively decreasing with each consecutive fEPSP pair (in the order of execution, in milliseconds: 700, 600, 500, 400, 300, 200, 100, 50, 20). All recordings were done by Denis Osipov; Figures 10 and 11 were done by Avner Michaeli. Raw data were amplified using the Molecular Devices MultiClamp 700A amplifier and acquired using the Digidata 1322A acquisition system at a rate of 10 kHz. Axon Instruments pClamp 10.2 was used to record the data onto a Windows XP computer.
2.4 Biochemistry

In experiments addressing the expression of ErbB4 in hippocampal slices, slices were placed in 50 μL solution containing 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, protease inhibitors, and phosphatase inhibitors. Micro-dounce homogenizer was used in order to solubilize the slices (20 strokes were administered). Following solubilisation, slices were incubated on ice for 30 minutes, and then spun at 13,000 g for 30 minutes. Supernatant was saved, and then the lysates were probed using western blot. Primary antibodies used: anti-ErbB4 (obtained from Cell Signalling, catalogue number 4795) and anti-actin (obtained from Santa Cruz Biotechnology, catalogue number SC-1616). In breast cancer cell line experiments, pre-incubated T-47D cells were placed on ice, separated from media, washed with cold 1X PBS and mixed with a fresh lysis buffer. Following the harvesting of cells by scraping and pipetting, supernatant was collected and incubated on ice for 30 minutes. Then, it was spun at 13,000 g for 30 minutes and transferred into new microcentrifuge tubes. In SDS-Page gel experiments, equal amounts of protein not exceeding 15 μL were taken from each sample and placed in the microcentrifuge, and mixed with 15 μL 2X sample buffer each. While the samples were heated to 95°C for 6 minutes, running buffer and gel were set up. Following quick-spinning, samples were loaded into gel wells, and the gel was run for 30
minutes at 200 volts. For transfer, transfer sponges were soaked in transfer buffer, then the gel was removed from plates and soaked in transfer buffer for 10 minutes. Transfer sandwich was then prepared and placed in the transfer box. Then, freezer pack was added to the box, and the contents were run at 100 volts for 1 hour. Sandwich was removed and blot was placed in blocking buffer for at least 30 minutes at room temperature. Primary antibody was diluted in blocking buffer containing 0.1% Tween-20, and the blot was allowed to incubate overnight at 4°C with gentle rocking. After washing the blot with PBS containing 0.1% Tween-20 three times for 5 minutes, the secondary antibody was diluted in blocking buffer containing 0.1% Tween-20. The blot was allowed to incubate at room temperature for 1 hour with gentle rocking, then washed with PBS containing 0.1% Tween-20 three times for 5 minutes and scanned with the Li-Cor Odyssey system. Protein expression signal in acute slices was normalized to that observed in the standard preparation which involved the extraction of a whole hippocampus from a CD-1 mouse. All biochemical experiments and the standard preparation were performed by Ameet Sengar.

2.5 Data analysis

Since almost all experiments of the current project were carried out in the presence of the GABA<sub>A</sub>R antagonist bicuculline, response envelope was
particularly susceptible to being contaminated by epileptiform-like events. Hence, 10-60% of the fEPSP rising phase was chosen to quantify fEPSP magnitude as one of the least contamination-prone response envelope segment. In order to assess the level of LTP, I calculated the ratio of averaged synaptic efficacy (measured as described above) observed after the delivery of TBS to that recorded prior to the administration of TBS, unless stated otherwise. Data were analyzed using Axon Instruments Clampfit 10.2 and Microsoft Excel 2010. P=0.05 was chosen as the significance cut-off for all statistical comparisons.
3. RESULTS

3.1. Synaptic responses at CA1 vary as a function of stimulus intensity

Due to the fine temporal and spatial resolution it offers, electrophysiological recording remains the principal research method used to characterize the properties of excitable tissue. As far as preparations are concerned, the hippocampus is the most widely used experimental model for synaptic plasticity: it offers a convenient anatomical arrangement that facilitates experimentation, and possesses a high capacity for the expression of synaptic plasticity (Bortolotto et al. 2001). While intracellular recordings allow the experimenter to study elemental electrophysiological phenomena such as the action potential, the advantage of extracellular recordings is in its smaller susceptibility to selection bias, for they provide the experimenter with a read-out of the activity of populations of neurons. Evoked field EPSPs (fEPSPs) were used in this study as the read-out of AMPAR-mediated synaptic transmission.

Before proceeding to LTP experiments, I first set out to establish evoked synaptic responses and characterize basal synaptic transmission. I began with constructing the input-output curve, which is a frequently employed electrophysiological index used to describe the responsiveness of neural networks to stimuli of varying intensity (Leung and Au, 1994). Since
prolonged, submaximal stimulation may impede the induction of synaptic plasticity, as well as undermine the interpretability of the data (Bortolotto et al., 2001), the input-output curve was used to define stimulus intensity for LTP experiments.

In order to find out how fEPSP slope varies as a function of stimulus intensity, I evoked single fEPSPs at ten-second intervals starting at 0.1 mV stimulus intensity, increasing the intensity by 0.1 mV with each consecutive fEPSP. Then, I measured the slope of the fEPSP, as well as the fiber volley, for every response, and plotted each variable against stimulus intensity (Figures 3A and 3B, respectively; n=5-6). Relationship between fEPSP slope and stimulus intensity exhibited a linear profile, and this shape was also paralleled by the graph of fiber volley versus stimulus intensity (Figures 3A and 3B). In order to examine the variance between evoked responses, as well as to confirm that fEPSP envelope was consistent with the profile chosen for experiments in this thesis (defined, visible fiber volley preceded by the stimulus artifact, and followed by the fEPSP, comprised of a defined rising phase, and the relatively uncontaminated decay phase). In Figure 4C, I show population fEPSP averages, and their shape follows the expected profile. Together, these findings reveal that (a) the increase in fEPSP size was dominated by a progressive recruitment of afferent fibers, rather than an increase in neurotransmitter release, and (b) the both fEPSP slope size, as
well as the magnitude of the fiber volley, varied as a linear function of stimulus intensity. For subsequent LTP experiments, I chose 0.3 mV as the approximate fEPSP magnitude for it corresponds to approximately 40% of the maximal response and is therefore a reasonable compromise between a high signal-to-noise ratio and avoiding approaching the ceiling of the tissue’s response range which could impede the induction of LTP (Bortolotto et al., 2001).

3.2. CA1 is PPF-competent

Increase in the magnitude of the second response in a pair of fEPSPs evoked at a short interstimulus interval (ISI; tens of milliseconds) is commonly referred to as paired-pulse facilitation and represents an important form of short-term plasticity (Magleby 1987). Residual Ca$^{2+}$ hypothesis is a widely used explanation for paired-pulse facilitation (Fisher et al. 1997; Katz and Miledi 1968; Isaac et al. 1998); in brief, the crux of this premise presupposes that during repeated firing, delay between individual synaptic release events may become shorter than the time window for presynaptic Ca$^{2+}$ clearance, thus leading to the accumulation of Ca$^{2+}$ in the presynaptic compartment, which, in turn, results in to an increase in neurotransmitter release. In electrophysiological experiments, PPF is most often used as an aid in differentiating pre-synaptic effects from post-synaptic effects.
Figure 4. Input-output relationship at the CA1.
All panels represent experimental data obtained from the same sample (n=5-6 slices obtained from 3 animals).
(A) Slope of evoked fEPSPs plotted against stimulus intensity increments.
(B) Fiber volley magnitude plotted against stimulus intensity increments.
(C) Group average of fEPSPs evoked in the course of input-output experiments; solid line represents group average, shaded area represents standard error of mean.
However, evidence exists indicating that PPF may also be used as a predictor of the preparation’s LTP competence, which makes it a useful experimental tool. In an effort to further characterize basal synaptic transmission in the CA1 region of acute hippocampal slices obtained from CD1 mice, and determine whether the preparation under my experimental conditions was capable of expressing PPF, I tested evoked fEPSPs under the PPF stimulation paradigm briefly described below. Specifically, in a separate set of slices (n=3) and under room temperature, I evoked seven pairs of fEPSPs, each at a ten-second interval and the same stimulus intensity. I used the following ISIs in all slices (starting from first pair of responses, in ms): 500, 400, 300, 200, 100, 50, 20. I then measured the 10-60% slope of every evoked fEPSP, and for each pair, calculated the ratio of S2 slope to S1 slope. Then, I plotted the population slope ratio against ISI (Figure 5A). I observed the largest S2/S1 slope ratio (1.74±0.16; mean±SEM) at ISI=20 ms. In order to confirm that observed fEPSPs followed the profile expected of a healthy preparation, I plotted a population PPF fEPSP envelope at 20 ms (Figure 5B). Collectively, these results suggest that the preparation under the employed conditions was capable of expressing PPF.

3.3. TBS induces LTP at CA1

Then, I set out to evaluate the LTP competence of CA1 pyramidal cells in
Figure 5. Paired-pulse facilitation at the CA1.
All panels represent experimental data obtained from the same sample (n=3 obtained from 1 animal).
(A) Ratio of S2 fEPSP slope to S1 fEPSP slope plotted against interstimulus delay.
(B) Group average of fEPSPs evoked at the interstimulus delay that induced the highest facilitation (20 ms); solid line represents average, shaded area represents standard error of mean.
acute hippocampal slices obtained from adult CD1 mice. I administered TBS at the SC-CA1 synapses, and compared the post-TBS level of synaptic efficacy to that recorded prior to the delivery of TBS (as described in the Methods section). After the administration of TBS, the fEPSP slope was 33±6% (mean±SEM, n=7 slices) higher than it was prior to TBS (Figure 6A). Observed potentiation in the size of fEPSPs following the conditioning of SC-CA1 synapses exhibited a sharp rise immediately after the delivery of TBS, and it was followed by a sustained, higher-than-basal level of synaptic transmission.

Since the observed increase in fEPSP size might be attributed to an increase in the number of recruited afferents, I then attempted to find out whether this confounding factor had been present. In addition to recording fEPSPs, I also recorded the fiber volley, which represents the electric potential generated by the depolarization of afferents upon the administration of stimulus. I plotted peak afferent volley magnitude versus time (Figure 6B), and I observed no significant change over time: averaged standard deviation in mean fEPSP slope size recorded during each of the 7 experiments was found equal to 19.7%. Relative stability of the afferent volley during the course of the experiment suggests that the increase in fEPSP size following the administration of TBS was unlikely to be explained by inconsistencies in
Figure 6. LTP at the CA1. All panels represent experimental data obtained from the same set of slices (n=7 slices obtained from 4 animals). TBS was delivered at T=15 min.
(A) Timeplot of fEPSP slope (mean±SEM).
(B) Timeplot of normalized fiber volley magnitude (mean±SEM).
(C) Group average of fEPSPs sampled during the minute immediately prior to, immediately after, and one hour after the delivery of TBS, respectively; solid line represents group average, shaded area represents SEM.
the recording configuration. There exists evidence suggesting that under some experimental settings, observed LTP magnitude can vary as function of basal fEPSP size.

In order to determine whether the size of basal fEPSPs was kept consistent, as well as in order to find out whether any aberrant events (e.g., excessive population spike contamination) had taken place, I examined the population envelope of fEPSPs obtained immediately prior to TBS delivery, immediately after TBS delivery, and 60 minutes after TBS delivery, as shown in Figure 6C. Every response shown in Figure 6C represents a population average, each obtained from the 7 slices that were used in the experiments described in above. I observed the following peak amplitudes (mean + SEM): 0.31±0.03 mV (one minute immediately prior to TBS delivery), 0.50±0.06 mV (one minute immediately after TBS delivery), and 0.40±0.05 mV (minute 60 after TBS delivery), which suggest that between-slice variability in fEPSP size was consistently low throughout the experiments.

Based on the data discussed above, I conclude that administering three seconds of TBS at SC-CA1 synapses in acute hippocampal slices obtained from CD1 mice potentiates the size of fEPSPs by 33±6%, and this increase is unlikely to be explained by inconsistencies in recording conditions.
Figure 7. TBS response envelope.
Both panels represent experimental data obtained from the same sample (n=7 slices obtained from 4 animals).
(A) Area above the curve calculated for every train administered in the course of the TBS (mean±SEM), each data point comprising the average consists of four pulses.
(B) Group trace averages (mean+SEM) of the trains that induced minimal and maximal depolarization.
3.4. TBS-induced LTP was reaching saturation under employed experimental conditions

Production of interpretable and repeatable electrophysiological recordings imposes a high standard for stimulation fidelity; unaccounted for variability in the administration of conditioning protocols represents an important potential confounding factor. Hence, I then attempted to determine whether the LTP that I observed under my experimental conditions was correlated with potential inconsistencies in TBS delivery, namely, failure of the stimulating apparatus to deliver stimuli of appropriate intensity at appropriate time points. To investigate whether there had been failures in stimulus delivery during TBS, I performed an analysis of response envelopes obtained during TBS. Specifically, I measured the area above the curve for every one of the fifteen trains that comprise the TBS protocol used in the current study (excluding the stimulus artifact). Then, I have plotted the respective area values against the corresponding train serial numbers (Figure 7A).

Similarly as observed in basal and post-TBS recordings, fEPSPs evoked in the course of TBS exhibited low variability (averaged ratio of SEM to mean was estimated to be 14%). In terms of the change of its magnitude over time during the delivery of TBS, area under curve started to plateau at approximately 100 mV*s, after the delivery of the 9th train. The largest area
Figure 8. Effect of prolonged TBS on LTP. Timeplot of fEPSP slope (mean±SEM) for slices subjected to 3 s of TBS (n=7 slices obtained from 4 animals, blue) and slices subjected to 6 s of TBS (n=5 slices obtained from 2 animals, yellow) under the curve (mean±SEM: 332.3±37.2 mV*s) was observed during
the delivery of the 4th stimulus train, whereas the smallest area under the curve (mean±SEM: 111.5±17.6 mV*s) was observed during the delivery of the last, 15th stimulus train. Population response envelopes of the 4th and the 15th TBS trains are shown in Figure 7B. Across the slices examined, the area under the curve showed low variability, and reached a plateau after the delivery of the 9th train, which suggests that the responsiveness of the preparation to high-frequency stimulation started to decrease dramatically prior to the end of TBS, implying that the duration of stimulation was sufficient for the induction of LTP.

Next, in an attempt to determine whether I can increase the signal-to-noise ratio by augmenting the level of LTP, I attempted to examine the dynamic range for LTP. In particular, I sought to determine whether the LTP induced by TBS was reaching the ceiling of the preparation’s capacity for LTP. I hypothesized that a more aggressive stimulation protocol would lead to an augmentation of LTP, and I tested this hypothesis by administering TBS for 6 seconds (instead of 3 seconds, as described in the Methods section), and monitored the change in synaptic efficacy as described previously. I saw a 48±11% increase in fEPSP slope size following the administration of TBS (mean±SEM, n=5; Figure 8). The increase was not significantly different from that observed in the control group (see section 3.3), which was given 3 seconds of TBS (P>0.05, paired t-test).
Figure 9. Relationship between LTP magnitude and time elapsed after animal sacrifice. Scatterplot of LTP magnitude versus time after decapitation expressed in hours (n=17 from 11 animals, see text).
Together, these findings indicate that under my experimental conditions, the level of observed LTP was exhausting the tissue’s capacity for LTP.

3.5 LTP magnitude was not correlated with time elapsed after animal sacrifice

Finite lifetime of tissue preparations represents a potentially important confounding factor in the context of electrophysiological experiments, since deterioration of the preparation’s health during recording makes the interpretation of results ambiguous at best.

I carried out a set of LTP experiments in a different set of acute hippocampal slices obtained from CD-1 mice (n=16), and I then plotted the magnitude of observed change in fEPSP slope size after the delivery of TBS against time after animal sacrifice expressed in hours (Figure 9). Pearson’s product-moment correlation coefficient was determined to be equal to 0.172. This value is often cited as a non-significant correlation (Mukaka, 2012).

Since I have found no non-negligible correlation between LTP magnitude and time elapsed after animal sacrifice, I conclude that the level of LTP observed under my experimental conditions was not significantly affected by changes in the health of the preparation.
Figure 10. Effect of the application of 2 nM NRG1β on LTP. Timeplot of fEPSP slope (mean±SEM) for control slices that received TBS but were not exposed to NRG1β (n=7 slices obtained from 4 animals, blue), for slices that received no TBS and no NRG1β treatment (n=4 slices obtained from 4 animals, pale yellow) and for slices that received both TBS and NRG1β treatment (5 slices obtained from 5 animals, black).
3.6. Application of 2 nM NRG1β had no effect on LTP magnitude under employed experimental conditions

After characterizing basal synaptic transmission and the capacity for LTP of acute hippocampal slices prepared from CD1 mice, I then attempted to determine the effect of NRG1β on LTP. In particular, I sought to investigate the effect of the application of 2nM NRG1β on the increase in fEPSP slope size after the delivery of TBS. I expected to observe the same result as an overwhelming number of groups has, which is abolition of CA1 LTP in acute hippocampal slices exposed to nanomolar concentrations of NRG1β. In order to interrogate the effect of exogenous application of NRG1β on LTP, I performed LTP experiments in the same manner as described in the Methods section. In a blind manner, I supplemented the ACSF solution with either 2 nM NRG1β dissolved in 1 mL purified water, or 1 mL purified water. After performing the recordings, I compared the change in fEPSP slope size after TBS administration to the pre-TBS level of synaptic efficacy. The change was found to be equal to 54±18% (n=5; Figure 10) and the difference between this increase and that observed in control slices (see 3.3) failed to reach significance (P=0.34, paired t-test). I have also performed recording of synaptic efficacy in the absence of TBS and in the absence of NRG1β (n=4; Figure 10). The size of the fEPSP slope recorded in the course of these experiments was significantly different from that seen in the NRG1β + TBS
Figure 11. Replication of LTP experiments in the presence of 2 nM NRG1β. Timeplot of fEPSP (mean±SEM) for control slices that received TBS but were not exposed to NRG1β (n=6 slices obtained from 4 animals, purple), and for slices that received TBS and were exposed to 2 nM NRG1β (n=3 slices obtained from 1 animal)
group (P=0.70, paired t-test), as well as it was significantly different from the control group (P=0.70, paired t-test).

Together, these findings demonstrate that delivering TBS at SC-CA1 synapses in acute hippocampal slices that were obtained from CD1 mice and exposed to 2 nM NRG1β, leads to a sustained increase in fEPSP slope size. The magnitude of this increase is comparable to that seen in control slices that were not exposed to NRG1β. This potentiation is also unlikely to be explained by inconsistencies in recording conditions (see section 3.3), nor by a gradual, idiopathic change in fEPSP size in the absence of any conditioning stimuli.

3.7 Replication of NRG1β experiments by independent examiner supports lack of effect of 2 nM NRG1β on LTP

Contrary to the observations made by a very large number of other groups, 2 nM NRG1β did not affect LTP under employed experimental conditions. I then sought to further investigate the reason for this observation. It is conceivable that the results of electrophysiological experiments may be contingent upon the manner specific for any given electrophysiologist. So, I decided to compare the findings I have made to those made by a different electrophysiologist, who was conducting experiments independently from me within the same laboratory.
Figure 12. Effect of the application of 10 nM NRG1β on LTP. Timeplot of fEPSP (mean±SEM) for control slices that received TBS but were not exposed to NRG1β (n=6 slices obtained from 4 animals, purple), and for slices that received TBS and were exposed to 10 nM NRG1β (n=3 slices obtained from 3 animals)
Specifically, the same concentration of NRG1β, 2 nM, was tested on a
different recording rig. The observed increase in fEPSP slopee after the
delivery of LTP induction was determined to be 74±7% in the control group
(n=6 from 3 animals), and 55±8% in slices treated with 2 nM NRG1β (n=3
from 3 animals), summarized in Figure 11. Compared to control, the increase
in synaptic transmission after the administration of LTP-inducing stimulation
was not different in the 2 nM NRG1β group (paired t-test, P=0.14).

Together, the data shows that application of 2 nM NRG1β under the
conditions of two different recording rigs had no effect on the level of LTP.

3.8. Under employed experimental conditions, tenfold increase in
NRG1β concentration above dose demonstrated to almost completely
prevent LTP had no effect on LTP

It has been established that low nanomolar doses of NRG1β almost
completely eliminate LTP; in particular, 1 nM NRG1β was demonstrated to
decrease the level of LTP by approximately 80% (Huang et al., 2000). Hence,
I then attempted to determine the effect of 10 nM NRG1β, a ten times greater
dose than 1 nM, on LTP under the conditions described in the previous
section.

The observed increase in fEPSP size after the delivery of LTP-inducing
stimulation was determined to be 174±7% in the control group (n=6 from 4
Figure 13. Mass reconstruction of NRG1β.
Plot of ionic signal intensity (expressed in counts per second) versus mass expressed in tenths of daltons.
animals), and 178±13% in slices treated with 10 nM NRG1β (n=3 from 3 animals), summarized in Figure 12. Compared to control, the increase observed in the slices given 10 nM NRG1β was not significantly different (paired t-test, P=0.76). Lack of potentiation in fEPSP size in slices treated with 2 nM, as well as 10 nM NRG1β, suggests that at concentrations far exceeding those necessary for almost a complete elimination of LTP (Huang et al., 2000), NRG1β did not affect the level of LTP under the employed experimental conditions.

3.9. NRG1β used here had correct molecular mass

Next, I devised a framework for a step-wise investigation of the reason for the lack of an effect on LTP from NRG1β. I decided to investigate each constituent of the NRG1β/ErbB4 ligand-receptor pair. First, I set out to determine whether the peptide used in NRG1β experiments was actually NRG1β.

Specifically, I sought to determine whether the peptide mass was correct, and to answer this question, the special facilities at the Peter Gilgan Centre for Research and Learning carried out a mass spectrometry analysis. Mass reconstruction of NRG1β mass is shown in Figure 13. The band that elicited the dominant ionic signal intensity (expressed in counts per second)
corresponded to the mass equal to 78.77 kDa, followed by band representing the mass of 78.79 kDa, followed by band denoting the mass of 79.00 kDa. The majority of other signal peaks either fell into the range defined by the three biggest peaks, or lay close to it. This range of molecular mass corresponds to the expected mass of NRG1β supplied by Genentech, equal to approximately 78 kDa. Since the main mass came out as predicted, this eliminates the idea that the peptide used here was not NRG1β.

3.10 NRG1β used here was biologically active

The mass of NRG1β used here was correct, yet application of NRG1β had no effect on the level of LTP at concentrations (2 nM and 10 nM) far exceeding those required for an almost full elimination of LTP (1 nM; Huang et al., 2000). I then reasoned that the lack of the expected effect of NRG1β on LTP could be explained by a lack of biological activity.

To test whether NRG1β used here possess biological activity, I looked at the effect of two doses of NRG1β (2 nM and 20 nM) on the levels of autophosphorylated ErbB4 in an ErbB4-overexpressing human breast cancer cell line T-47D, measured by Western blot (Figure 14A). In this assay, level of ErbB4 phosphorylation was arbitrarily defined as the ratio of quantified band intensities emitted by phospho-specific primary and
Figure 14. Expression of phosphorylated ErbB4 in the ErbB4-overexpressing human breast cancer cell line T-47D upon the application of 2 nM and 20 nM NRG1β. Two doses of NRG were probed, 2 nM and 20 nM. ACSF and serum were used as the two control treatments. (A) Raw data. (B) Bar graph representing the ratio of the signal of phosphorylated Erbb4 to actin across the four treatments.
secondary antibodies for ErbB4 to the equivalent signal emitted by the pan-
specific ErbB4 antibody. This level was then divided by the signal emitted by
the pan-specific actin antibody. Protein expression quantification performed
on slices treated with NRG yielded indices of 0.05 and 0.23 when treated with
2 nM and 20 nM NRG, respectively (Figure 14B). The index obtained from
NRG-naïve slices exposed to ACSF was equal to zero, which was a true
result. Lastly, exposing the preparation to insulin- and estrogen-containing
media with bovine serum led to a marginal increase in Erbb4 expression
levels above zero (0.01).

These data reveal that exposing the human breast cancer cell line T-47D to a range of NRG1β concentrations induces a dose-dependent increase
in serine autophosphorylation of ErbB4 receptors. The ability of NRG1β to
activate its cognate receptor, ErbB4, in this assay suggests that NRG1β used
in electrophysiological recordings was biologically active.

3.11. ErbB4 was expressed in slice preparation used here

Next, I hypothesized that there might have been aberrant events at the
level of the receptor for NRG1β, which would explain why large doses of
NRG1β had no effect on LTP under the conditions of two different recording
rigs.
Figure 15. ErbB4 expression in the acute hippocampal slice preparation. Slices (n=2 from 1 animal) used for the analysis are denoted by 1,2; standard preparation is denoted by 3 (see Methods). (A) Level of ErbB4 expression normalized to the standard preparation; (B) Raw data obtained using Western blot.
Specifically, I determined to look at the level of ErbB4 expression in acute hippocampal slices using Western blot (Figure 15). Immediately after the slices had been acquired during the dissection, slices were frozen and then used for quantification. Western blot showed a strong signal in the two bands that correspond to ErbB4 lanes (264±44%, the ratio of ErbB4 signal was normalized to that of actin and subsequently normalized to the standard preparation, see Methods). Together, the evidence demonstrated so far suggests that NRG1β peptide used here had the correct mass, it possessed biological activity, and its cognate receptor, ErbB4, was expressed in the slice preparation.

3.12. ErbB4 expressed in acute slice is contingent upon dissection media composition

I then hypothesized that the expression of NRG1β receptor, ErbB4, was impaired during the preparation of slices. Indeed, there exists evidence for the contingency of protein expression levels on various aspects of the chosen slice preparation protocol.

I attempted to find out whether ErbB4 expression level in acute hippocampal slice was correlated with distinct slicing conditions. In particular, I attempted to see whether replacing dACSF (solution used for dissections,
Figure 16. ErbB4 expression in slices obtained under different dissection conditions.

(A) Each bar is a sample of two slices and represents the ratio of ErbB4 expression vs. actin expression in western blot experiments, normalized to the standard preparation; (+) sucrose denotes dACSF, (-) sucrose denotes pACSF, (+) rec denotes 3 hour recovery, (-) rec denotes no recovery.

(B) Raw data obtained using Western blot. Numbers 6,8 correspond to (-) sucrose (-) rec, numbers 2,4 correspond to (-) sucrose (+) rec, numbers 1,5 correspond to (+) sucrose (-) rec, numbers 3, 7 correspond to (+) sucrose (+) rec.
enriched in Mg\textsuperscript{2+} and sucrose, and low in Ca\textsuperscript{2+}) with pACSF (solution used for recordings, low in Mg\textsuperscript{2+}, high in Ca\textsuperscript{2+}, free of sucrose), and allowing or not allowing the slices to incubate for 3 hours at room temperature, could alter ErbB4 expression levels. Thus, I prepared two slices for each of the four possible conditions: prepared in pACSF and not allowed to incubate, prepared in pACSF and allowed to incubate for 3 hours, prepared in dACSF and not allowed to incubate, prepared in dACSF and allowed to incubate for 3 hours. ErbB4 expression levels detected by Western blot are summarized in Figure 16B (raw data) and Figure 16A (quantified). Highest level of ErbB4 expression was observed when slices were exposed to dACSF and not allowed to recover at room temperature in pACSF (264±45%, n=2; all values represented in this section are normalized to the standard preparation). Not allowing the slices to recover, but exposing them to pACSF, induces approximately a two-fold decrease in the signal (102±7%, n=2). Allowing the slices to recover almost entirely abolishes ErbB4 signal as detected by Western blot both in the pACSF (9±9%, n=2), as well as in the dACSF (5±5%, n=2).

Together, these findings indicate that preparing slices in pACSF instead of dACSF dramatically decreases the level of ErbB4 expression, and that allowing the slices to recover for three hours at room temperature, regardless of the solution identity, eliminates ErbB4 expression. These
results have a number of explanations. The most parsimonious explanation centers around the composition of the solutions: in particular, the four-fold difference in the amount of Mg\(^{2+}\) in dACSF compared to pACSF. It is conceivable that there exists an NMDAR-dependent mechanism for Erbb4 expression downregulation triggered by stress, which is inactivated by an excess of extracellular Mg\(^{2+}\). Partial loss of Erbb4 receptor expression in slices that were not allowed to incubate compared to slices that were incubated for three hours suggests a timescale of several hours for this process. This finding bears important implications for experimental design, because it proposes dissection protocol as an important consideration factor for electrophysiological studies.
4. DISCUSSION

4.1. Basal synaptic transmission and LTP

In this thesis, I demonstrated that delivering TBS at the SC-CA1 synapses in an acute hippocampal slice preparation obtained from adult CD-1 mice potentiates the size of evoked fEPSPs by a third of their basal magnitude. The timecourse of this increase (sharp rise in response immediately after TBS administration, followed by a sustained, higher-than-basal potentiation of synaptic efficacy) is characteristic and indicative of LTP at the CA1. The size of the afferent volley, which is an often-used index of the number of recruited afferents, remained relatively constant throughout LTP experiments, suggesting that inconsistencies in the recording configuration do not provide an adequate explanation for the detected potentiation in fEPSP size. Furthermore, the magnitude of the observed LTP was also unlikely to be a byproduct of unaccounted for variability in basal fEPSP size: starting (as well as post-TBS) fEPSP envelopes exhibited low variance.

Both the size of fEPSPs and the magnitude of the fiber volley, varied as a linear function of stimulus intensity. This indicates that the increase in fEPSP size was dominated by a progressive recruitment of new afferents, rather than an increase in neurotransmitter release efficiency. Moreover, the preparation’s capacity to produce fEPSPs of various magnitudes suggests
that potentiation in fEPSP size upon the delivery of conditioning stimulation likely represents a bona fide biological phenomenon, rather than a byproduct of unaccounted for variance in experimental protocol execution.

The slices were also capable of expressing certain forms of short-term plasticity at the CA1, in particular, paired-pulse facilitation: the size of the second fEPSP in a pair of fEPSPs evoked at a 20 ms interval was greater than the first response by approximately a half. In order to induce long-term potentiation, fifteen trains, each consisting of 40 ms of 100 Hz stimulation, were delivered at a frequency of 5 Hz at SC-CA1 synapses. In terms of its timecourse, the amount of post-synaptic depolarization measured as area above curve in the TBS response envelope peaked after 1 second of stimulation, and was followed by a decrease and a plateau prior to the end of the stimulation. This finding suggests that TBS exhausted the preparation’s responsiveness during this single episode of high-frequency stimulation. The observed levels of LTP did not vary as a function of time elapsed after animal sacrifice, indicating that change in the health of slices was unlikely to have had a significant influence on the observed results.

4.2. Effect of NRG1β on LTP

Originally, my goal was to elucidate the role of NRG1β in hippocampal long-term potentiation at the molecular level. A vast number of laboratories
reported that application of nanomolar doses of NRG1 abolishes CA1 LTP, so, I began my inquiry into the neurobiology of NRG1 with an attempt to replicate these experiments.

Specifically, I induced LTP at the SC-CA1 synapses in acute hippocampal slices obtained from adult CD1 mice, and compared its magnitude to that observed in slices exposed to 2 nM NRG1β and subjected to the same LTP-inducing stimulation. Expecting to see no significant potentiation in fEPSP slope size following the delivery of TBS in slices treated with NRG1β, I observed a result that was contrary to what many other laboratories report. Particularly, compared to control slices, nanomolar concentrations of NRG1β had no effect on the magnitude of LTP. Hesitant to expand on the finding without giving it a more careful examination, I decided to explore the observed result more closely. Namely, I reasoned that there could be a possibility for the observed result to be contingent upon the manner according to which electrophysiological experiments were carried out. Indeed, as quantifiable protocols can be made, a certain amount of room for subjective judgement remains. Hence, I asked an independent electrophysiologist from the same laboratory, Avner Michaeli, to examine the effect of NRG1β under his experimental conditions. He carried out LTP experiments in the presence of two doses of NRG1β, 2 nM and 10 nM. The magnitude of LTP observed in slices exposed to a range of nanomolar
NRG1β concentrations was not significantly different from that recorded in his controls. This suggests that failing to observe an effect of NRG1β on CA1 LTP potentially represents a real phenomenon, rather than a byproduct of inconsistent experimental protocol execution.

4.3. Confirmation of NRG1β mass and biological potency

Next, I reasoned that there was a possibility for the NRG1β stock to be defective. If the peptide was degraded or impure, applying it onto slices presumably would have had no effect on the activation ErbB4 receptors, and consequently, no effect on the physiological levels of Src-mediated upregulation of NMDA receptor function. Specifically, I sought to confirm that the amino acid sequence of NRG1β was structurally correct using mass spectrometry analysis. The results provided by the special facilities at the Peter Gilgan Centre for Research and Learning indicated that the strongest signal was emitted by the mass bands that corresponded to the expected mass range for NRG1β peptide (approximately 78 kDa). Lack of additional signals outside this range indicated that the structure of the peptide was correct.

Having observed no effect of nanomolar doses of NRG1β on the magnitude of LTP at the CA1 both myself and under the experimental conditions of an independent examiner, followed by having confirmed the
peptide's correct sequence, I then asked a more general question: does the NRG1β have any biological activity at all? I decided to test this in a cell culture assay that is frequently used in ErbB4 microbiology research. Specifically, I performed quantification of the levels of autophosphorylated ErbB4 in a human breast cancer cell line T-47D overexpressing ErbB4 using Western blot. Treating the breast cancer cell line with ACSF almost completely abolished the level of autophosphorylated ErbB4 as detected by the assay. Application of two doses of NRG1β, 2 nM and 20 nM, elicited a dose-dependent increase in the level of ErbB4 autophosphorylation. This important observation revealed that NRG1β peptide used for electrophysiological experiments possessed biological activity, and that the autophosphorylation of ErbB4 receptors is probably highly contingent on the extracellular environment.

4.4. Correlation between dissection solution composition and ErbB4 expression level

The last finding had been a crucial piece of the puzzle: the dramatic effect on ErbB4 autophosphorylation that the application of ACSF had prompted me to approach the problem from a different angle. I asked, what if the answer to the question why NRG1β had no effect on LTP is rooted in the neurobiology of the receptor ErbB4, and not its ligand NRG1β? Furthermore,
what if there exists a critical time window that directly affects the function of the receptor?

I then decided to investigate whether the expression of ErbB4 in acute slices is altered under different ACSF conditions during slice preparation, and whether this process exhibits any time dependence. With the aforementioned two dimensions in mind, I have constructed a 2x2 permutation matrix and prepared batches of slices that were or were not allowed to incubate for 3 hours, in addition to being exposed to either dACSF or pACSF. After the preparation of the samples, quantification of ErbB4 expression levels was done using Western blot. Slices prepared in dACSF elicited the strongest ErbB4 expression signal, followed by the signal of approximately 2 times smaller magnitude that corresponded to the slices exposed to pACSF and not dACSF during dissection. Importantly, regardless of the solution used as medium for dissection, ErbB4 expression was comparable to noise levels if the slices were allowed to incubate for 3 hours.

In the context of the current work, the last finding bears crucial importance. By drawing attention to the novel ways the design of experimental protocols can affect scientific observations, the discovery of a correlation between dissection solution composition and ErbB4 expression levels constitutes an important paradigm shift. In terms of its composition, the
dACSF used in this study differed from the pACSF in two regards. First, the dACSF contained four times the amount of Mg\(^{2+}\) dissolved in the pACSF. Indeed, the ability to suppress ion flow through the NMDA receptor conductance pathway is the property of Mg\(^{2+}\) that has been routinely used by many laboratories in order to minimize the impact of excitotoxic events triggered by stress through the suppression of the NMDA receptor function (Bortolotto et al. 2001). Secondly, the dACSF contained 210 mM sucrose, which was a high enough sucrose concentration to alter its physical properties to the extent of making its composition apparent to the naked eye. Addition of sucrose to the solution also is an often-employed measure used in order to minimize the amount of cell swelling in a preparation (Bortolotto et al., 2001). In vivo, there exists a physiological, tonic level of NRG1β-ErbB4 signalling (Pitcher and Salter, 2012). The loss of ErbB4 expression observed in the acute slice preparation is therefore likely to be at least partly a stress-related response, which assumes the timescale of several hours. Furthermore, this response seems to be contingent upon the permeability of the NMDA receptor channel pore. Presumably, one of the manifestations of excess excitotoxicity expected for a traumatic event such as slice preparation results in a downregulation of ErbB4 expression.

Since the design of the current study did not rely upon animal strains with null mutations, an outbred mouse strain CD1, which exhibits higher
between-animal variability than wild-type litter-mate control strains, was chosen for experiments. Animal model and ACSF Mg\textsuperscript{2+} concentration utilized in this thesis are compared to those used in works this study was conceived based upon (see Table 1).

Table 1. Comparison of select experimental models of NRG1 signalling. Magnesium concentration indicated in mM; NRG1β concentration indicated in nM.

<table>
<thead>
<tr>
<th>Original paper</th>
<th>Animal strain</th>
<th>Animal age</th>
<th>Direction</th>
<th>Magnitude</th>
<th>NRG1β</th>
<th>MgCl\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitcher et al., 2011</td>
<td>Src\textsuperscript{+/−} mice</td>
<td>21 days</td>
<td>↓</td>
<td>full inhibition</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pitcher et al., 2008</td>
<td>ErbB4\textsuperscript{+/−} mice</td>
<td>~20 weeks</td>
<td>↓</td>
<td>almost full inhibition</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Kwon et al., 2005</td>
<td>C57BL/6 mice</td>
<td>4-5 weeks</td>
<td>↓</td>
<td>full inhibition</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Kwon et al., 2008</td>
<td>C57BL/6 mice</td>
<td>4-6 weeks</td>
<td>↓</td>
<td>full inhibition</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Bjarnadottir et al., 2007</td>
<td>NRG1(ΔEGRF)\textsuperscript{+/−} mice</td>
<td>4-16 weeks</td>
<td>↓</td>
<td>full inhibition</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Shamir et al., 2012</td>
<td>C57BL/6J mice</td>
<td>3-6 weeks</td>
<td>no effect</td>
<td>0</td>
<td>2, 5, 10</td>
<td>2</td>
</tr>
</tbody>
</table>

4.4. Conclusion and future directions

Together, the findings presented in the current thesis broaden our understanding of NRG1β-ErbB4 signalling and its role in the expression of long-term synaptic plasticity. In particular, the positive correlation between low concentrations of magnesium, which is permissive of excitotoxicity, and
downregulation of ErbB4 receptors, provides us with novel insights about the way experimental protocol design can affect scientific observations. This finding, however, raises a substantial number of new questions, the most obvious ones concerning the nature of the ErbB4 expression downregulation. What happens to the existing ErbB4 receptors? Do they get degraded by proteases, or do they get internalized in an LTD-like fashion? Investigations of the mechanism underlying the conditional expression of ErbB4, potentially utilizing pharmacologic inhibition of protein synthesis and/or exocytosis inhibitors, could answer this question,

Further studies of ErbB4 expression dynamics, as well as the interaction between NRG1β/ErbB4 signalling and Src, will deepen our understanding of the physiology and pathophysiology of the NRG1β/ErbB4 signalling pathway, potentially providing us with new insights about the fundamental mechanisms governing the functioning of the mammalian CNS, as well as the progression of psychiatric disorders such as schizophrenia.
5. REFERENCES


