Investigating the role of Eph receptors and their molecular partners in anxiogenesis

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Abstract

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Psychological stress leads to the enhancement of anxiety and can trigger a variety of psychiatric disorders. The mechanisms by which stress regulates anxiety are unclear. Eph receptors and their ligands, Ephrins, are attractive candidates to consider due to their multifaceted functions and high expression in the limbic system. Eph/Ephrins have been shown to regulate both functional and structural neuronal plasticity as well as hippocampus-dependent behaviour in mice. Thus, the aim of this study was to investigate the roles of Eph/Ephrins in the hippocampus and the amygdala and their interaction with molecular partners upon stress.

First, I found that plasmin, a stress-related protease, cleaves EphA4 with high specificity. Mass spectrometry and bioinformatic analyses revealed the cleavage site is located within the fibronectin-like repeats of EphA4. EphA4, highly expressed in the hippocampus, interacts with EphrinB2. Following stress their interaction increases, as does the expression of EphrinB2. Studies in mice in which EphrinB2 was conditionally deleted in forebrain neurons demonstrated that EphrinB2 signalling is critical to the formation of anxiety-like behaviour. Furthermore, EphrinB2 mediates stress-related potentiation of contextual fear conditioning. These findings implicate the EphrinB2/EphA4/plasmin pathway as a new player in hippocampal regulation of anxiety.

Second, I found that in the amygdala an extracellular serine protease, neuropsin, cleaves EphB2 shortly after stress. This molecular event alters EphB2 membrane expression and promotes a dynamic interaction of EphB2 with NMDA receptors. Consistent with the role of EphB2 in the stress response, bilateral amygdala infusion of anti-EphB2 antibody before stress prevents the development of stress-induced anxiety. The anxiolytic phenotype of neuropsin-deficient mice is rescued by the infusion of neuropsin into the amygdala before stress, confirming the effect of neuropsin is acute and not developmental, and pointing to the amygdala as the locus of the neuropsin's effect. Taken together these findings implicate the acute, stress related cleavage of EphB2 by neuropsin in the amygdala as a key event in the development of anxiety-related behaviour.

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In memory of Pamela Lois Attwood.

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15mS	15 minutes restraint stress
18hR	18 Hours Recovery
5-HT	Serotonin
5mS	5 minutes restraint stress
6hR	6 Hours recovery
6hS	6 Hours restraint stress
A	Adenine
AC	Associational Commissural fibres
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
ANOVA	Analysis of Variance
Arg	Arginine
BDNF	Brain-derived Neurotrophic Factor
BLA	Baso-Lateral Amygdala
C	Cystosine
CA	Central amygdala
CA1-4	Cornu Ammonis 1-4
Ca2+	Calcium ions
CAMKII	Ca2+/calmodulin-dependent protein kinase II
cDNA	Complementary DNA
Cl2-	Chloride ion
CNS	Central Nervous System

CORT	Corticosterone
COS	CV-1 in Origin carrying the SV40 genetic material]
Cre +/-	mice carrying CAM kinase Cre insertion
CREB	cAMP Response Element-Binding
CRF	Corticotrophic-releasing hormone
CS	Conditioned stimulus
Cy-5	Cyanine 5
DG	Dentate Gyrus
DNA	Deoxyribo-Nucleic-Acid
DTT	Dithiothreitol
E-LTP	Early-LTP
ECM	Extracellular Matrix
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethyleneglycoltetraacetic acid
Eph proteins	Denotes both Eph receptors and Ephrin ligands
EphrinB2-CaMKII-Cre	Mice carrying floxed insertions at both EphrinB2 alleles and the CamKII-cre gene.
EphrinB2^{lx}	Carrying floxed insertions at one EphrinB2 allele
EphrinB2$^{lx/lx}$	Mice carrying floxed insertions at both EphrinB2 alleles
EPM	Elevated Plus Maze
ERK	Extracellular Signal-regulated Kinase
FKBP5	FK506 Binding Protein 5 gene
FKBP51	FK506 Binding Protein 51

G	Guanine
GABA	Gamma-Aminobutyric Acid
GAL2	Galanin Receptor 2
GC	Glucocorticoid
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GEF	Guanine Exchange Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GluRA	Metabotropic Glutamate Receptor A
Gly	Glycine
GPI	Glycosylphosphoinositol
Grb4	Growth factor Receptor Bound protein 4
GRIP	Glutamate receptor interacting protein 1
GTP	Guanosine Triphosphate
HEK293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	(buffer)
HPA	Hypothalamic Pituitary Adrenal
HRP	Horseradish Peroxidase
IEG	Immediate Early Gene
IgG	Immunoglobulin G
Ile	Isoleucine
JM	Juxtamembrane
JNK	c-Jun NH2 terminal kinase

L1CAM	L1 Cell Adhesion Molecule
LA	lateral amygdala
LBD	Ligand Binding Domain
LPS	Lipopolysaccharide
LRP1	Low Density Lipoprotein Receptor-related Protein 1
LSM5	Laser Scanning Microscope 5
LTD	Long Term Depression
LTP	Long Term Potentiation
LVDCC	L-type voltage dependent calcium channel
Lys	Lysine
MAP	Mitogen Activated Protein
MEM	Minimum Essential Medium
MF	Mossy Fibres
Mg2+	Magnesium ion
MMP	Matrix Metalloprotease
mPFC	Medial Prefrontal Cortex
Na3VO4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NCAM	Neural Cell Adhesion Molecule
NeuN	Neuronal Nuclei
NLS	Nuclear Localisation Sequence
NMDA	N-Methyl-D Aspartate
NP-/-	Neuropsin knockout mice
NP+/+	Wildtype mice

NR1, 2	NMDA receptor subunit 1, 2
OF	Open Field
P10	Post-natal Day 10
p75NGFR	p75 Nerve Growth Factor receptor
PAGE	Polyacrylamide Gel Electrophoresis
PAI-1	Plasminogen Activator Inhibitor-1
PAR-1	Protease-activated Receptor 1
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline - Tween
PDZ	Post-synaptic density-95/Drosophilia disc-large tumour suppressor/Zonula occludens 1
PKA	Protein Kinase A
PLC	Phospholipase c
PP	Perforant Pathway
PS1	Presenilin 1
PSD	Postsynaptic Density
PTSD	Post Traumatic Stress Disorder
PVN	Paraventricular Nucleus
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RAP	Ribosomal Acidic P proteins
RGS3	Regulator of G-protein Signalling 3
RHBDL2	Rhomboid like 2
RNA	Ribo-Nucleic-Acid

RT	Room temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAM	Sympathetic Adrenal Medullary
SAM	Sterile Alpha Motif
SB	Subiculum
SC	Shaffer Collateral fibres
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of mean
T	Thymine
Tiam1	T-cell lymphoma invasion and metastasis 1
TNF	Tumour Necrosis Factor
tPA	Tissue Plasminogen Activator
Tris	Tris(hydroxymethyl)aminomethane
uPA	Urokinase-type Plasminogen Activator
US	Unconditioned Stimulus

Chapter 1. Introduction

Stress and pathology

The term ‘stress’ was first used in a biological context by Hans Selye in 1926 when he was a medical student. Selye observed that, although many patients suffered from different ailments, they had a number of similar symptoms unrelated to their diagnosis. The symptoms were unspecific and included loss of appetite, decreased muscular strength and lack of ambition, which he called the ‘syndrome of just being sick’. During laboratory work ten years later, he published an article in *Nature* describing ‘a syndrome produced by diverse nocuous agents’ where he first used the term ‘stress’. This name originated from the physics nomenclature that described the result of strain applied to a system (Selye, 1956).

Broadly, stress can be divided into three different categories (Herman & Cullinan, 1997/2; Van de Kar & Blair, 1999/1):

Processive psychogenic- psychological stressors based on a learned response to the threat of an impending adverse condition (i.e. fear, anxiety, exposure to a novel or uncontrollable environment).

Processive neurogenic- stressors that consist of a physical stimulus and have a strong psychological component (i.e. pain, footshock).

Systemic- stressors which challenge cardiovascular homeostasis (i.e. haemorrhage, orthostatic stress/upright tilt, exercise, heat exposure).

The systemic stressors require an immediate allostatic response that is mediated by the paraventricular nucleus (PVN) of the hypothalamus. The other two forms of stress do not necessarily require an instantaneous response for survival and therefore higher processing is utilised before activation of the hypothalamus (Herman & Cullinan, 1997/2). This processing is performed by the limbic system.

The adaptation of the body to stress is termed ‘allostasis’, which "maintains homeostasis through change" (McEwen, 2000). The term ‘allostasis’ was first introduced by Sterling and Eyer, and characterizes the regulation of the vital body functions (blood pressure, respiratory rate and the glucocorticoid level etc.) after stress. These changes are beneficial and give the organism the capacity to meet and overcome stressful events. However, severe or prolonged stress may result in mismanagement of allostasis and failure of the coping mechanisms. The sum of the negative effects of stress on the body is called allostatic load (McEwen, 2000).

In the majority of cases, allostatic load is not the sole causative factor of an disease. However, it often contributes to the pathogenesis, prolongation or exacerbation. The mechanisms by which this occurs are better understood in some diseases than others. For example, the finding that stress is a risk factor for cardiovascular disease has been validated by an understanding of how stress affects the molecular mechanisms of the disease. In animal studies, the activation of the sympathetic – adrenal – medullary (SAM) system causes coronary artery disease, as the inflammatory, endothelial and coagulatory

pathways induced by the activation of the SAM system cause the pathology underlying the disease (Krantz & McCeney, 2002). In contrast, an illness such as chronic fatigue syndrome that has no identified pathology only has a correlative relationship with stress (Cohen *et al.*, 2007). Depression and anxiety also have a strong association with stress: approximately 25% of people who experience a major stress event develop depression and the severity of the stressful event predicts the duration of the depression (Cohen *et al.*, 2007). Although the molecular mechanisms of depression and anxiety are poorly understood compared to cardiovascular disease, there is mounting evidence that neuronal pathology in the limbic system underpins these illnesses.

It is known that the amygdala plays a role in formation of emotions, in particular fear and anxiety. Lesions to the amygdala in monkeys lead to the loss of fear and anger, increased exploration and hyperorality (Kluver-Bucy syndrome). In animal models the amygdala and the hippocampus have also been shown to regulate different anxiety states (Chotiwat & Harris, 2006; Davis, 1998).

The hippocampus

The hippocampus is a continuation of the cortex that has a well-defined laminar structure. The hippocampal formation contains the hippocampus proper, the dentate gyrus, the subiculum and the enterohinal cortex. The hippocampus proper can be further divided into four regions, CA1-CA4, with CA1 and CA3 being the largest. The laminar structure of the hippocampus

implicates the unidirectional nature of most connections. Information flow within the hippocampus is mediated by a trisynaptic excitatory circuit. First, input to the granular cells of the dentate gyrus comes via the perforant path from the enterohinal cortex. The perforant path also projects to the CA1 and CA3. Second, synapses are formed by the dentate gyrus connections to the pyramidal cells of the CA3 via the mossy fibres. Finally, the CA3 connects to the CA1 via the Schaffer collateral pathway. The output is directed to the subiculum and the contralateral hippocampus (Figure 1). This is a simplified view of hippocampal circuitry given the multiple interconnecting pathways of the subfields (Yeckel & Berger, 1990). There is also mounting evidence that the dorsal and ventral regions play distinct roles in both memory and the stress response (Reviewed in (Fanselow & Dong, 2010).

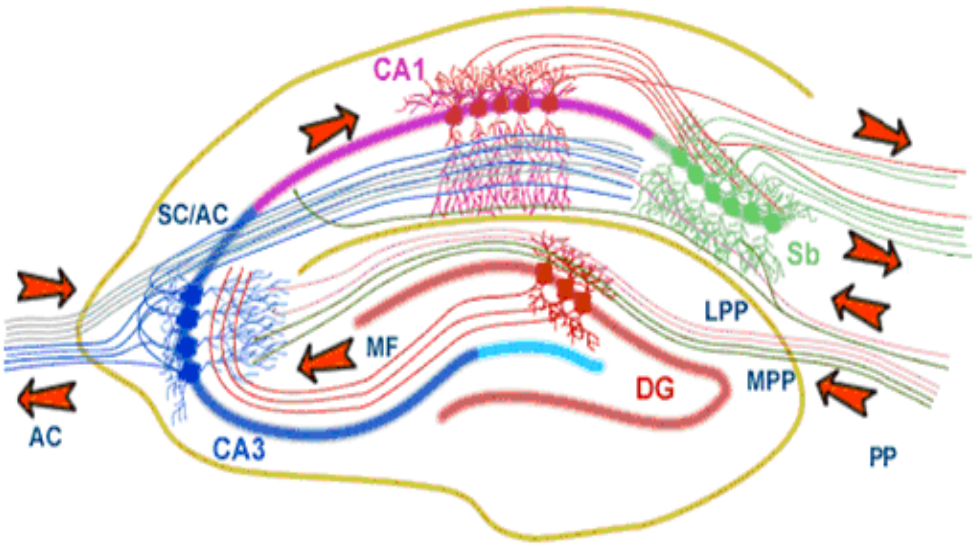


Figure 1. The trisynaptic excitatory circuit of the hippocampus

(Centre for Synaptic Plasticity: University of Bristol 2003).

PP – Perforant pathway (medial and lateral)

DG – Dentate gyrus

MF – Mossy fibres

AC – Associational commissural fibres

SC – Schaffer collateral fibres

SB – Subiculum

The amygdala

The amygdala (or amygdaloid complex) is a group of about 13 nuclei that are located in the mid-temporal lobe. It is a subcortical structure central to the function of the limbic system. It is possible to divide the nuclei into groups according to function, connections and cytoarchitecture. This most commonly results in four major groups; the basolateral, the central, the cortical and the medial (Figure 2). The afferent information comes to the amygdala from sensory (cortex), cognitive (cortex and hippocampus), autonomic (hypothalamus and brain stem) and behavioural systems. It receives information from all sensory modalities, mainly through the ipsilateral cortex (Sah *et al.*, 2003). The amygdala has substantial projections to the hippocampus and other limbic areas from the lateral nuclei. The stimulation of the lateral group enhances LTP in the dentate gyrus of the hippocampus (Akirav & Richter-Levin, 1999). The amygdala also has efferent connections to the cortex, hypothalamus and brainstem. The largest proportion of these is to the hypothalamus reflecting the control of physiological allostatic responses.

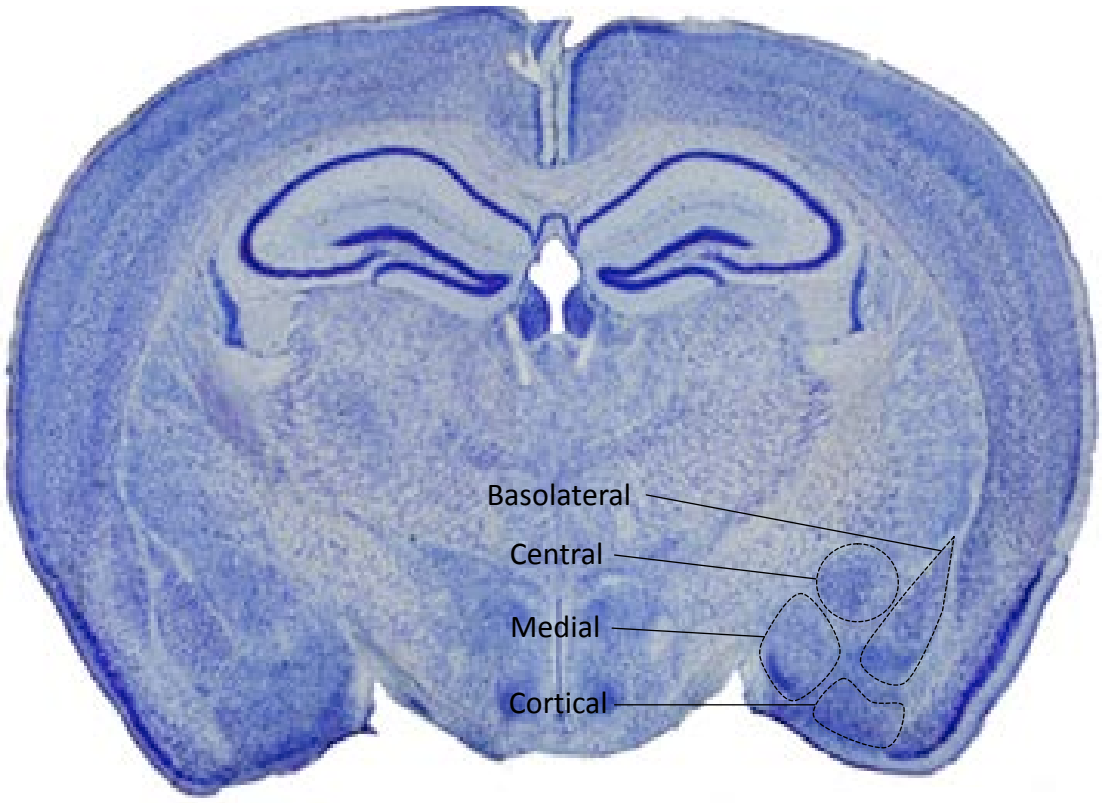


Figure 2. The functional groups of nuclei in the amygdaloid complex (DeFelipe, 2011)**.**

The relative positions of the functional groups of amygdaloid nuclei are highlighted on a coronal section of a mouse brain.

The limbic molecular mechanisms of the stress response

The basic communicative element of the brain is the synapse, whose modifications underlie learning and memory. The change in number, morphology and function of synapses in response to stimuli is called synaptic plasticity. In response to stress the hippocampus and the amygdala show alterations in synaptic plasticity. These changes are likely to underlie the behavioural change of the organism in response to stress. Stress has

consistently been shown to impair long term potentiation (LTP), the electrophysiological correlate of learning, in the hippocampus (Kim & Diamond 2002). The limbic structures contain the highest expression of glucocorticoid receptors in the brain, particularly in the hippocampus (Aronsson *et al.*, 1988; McEwen *et al.*, 1986; Diorio *et al.*, 1993). The effect of glucocorticoids are to impair LTP and this partially explains the effects of stress on LTP in the hippocampus (Kim & Diamond 2002). The deficit LTP also correlates with memory impairments in response to stress (Kim & Diamond 2002).

The alterations of neuronal plasticity during stress in the hippocampus and amygdala are also observed in morphological remodelling. The ability to regulate spine morphology and morphogenesis is an important component of synaptic plasticity. It is thought that the ability of spines to rapidly change morphology (in seconds to minutes) facilitates synaptic plasticity (Bonhoeffer & Yuste, 2002/9/12). Dendritic atrophy is seen following chronic immobilisation with a reduction in dendritic length and branch point in CA3 pyramidal neurons (Vyas *et al.*, 2002). It has also been shown that CA1 and dentate gyrus dendritic retraction is caused by prolonged chronic stress (4 weeks). Even acute stress (a single 5 hour stress protocol) results in a disruption of hippocampal spine integrity (Chen *et al.*, 2010). In contrast to the dendritic retraction described in the hippocampus and medial prefrontal cortex, chronic restraint stress causes an increase in length and branch points of basolateral amygdala pyramidal and stellate neurons (Vyas *et al.*, 2002).

Eph proteins, neuropsin and the tPA/plasminogen system and limbic stress related molecular mechanisms

The molecules investigated in this thesis have been targeted because of their involvement in the molecular mechanisms of the stress response in the limbic system. Eph proteins, neuropsin, tPA and plasmin are all expressed in the hippocampus and the amygdala. In the case of neuropsin it is striking for its limbic system expression (Chen *et al.*, 1995). Crucially these molecules regulate electrophysiological and morphological plasticity in the limbic system. They also have been shown to alter stress related behaviour or hippocampal dependent behaviour. Furthermore, in the case of neuropsin and the tPA/plasminogen system, their expression and function has been shown to be altered by stress. Eph proteins and the neuronal proteases of interest are reviewed below.

Eph Receptors and Ephrins

Erythropoietin-**P**roducing human **H**epatocellular (Eph) receptors were discovered in 1987 and constitute the largest known family of receptor tyrosine kinases (Hirai *et al.*, 1987). They have been the subjects of intense research since their discovery. Their widespread pattern of expression, involvement in a variety of important cellular phenomena and unique mode of action has stimulated interest across biological and medical domains. Recent advances suggest that Ephs play an important role in the development of brain pathologies.

Unlike most receptor tyrosine kinases, Eph receptors do not bind soluble ligands but ligands that are membrane bound. These are called Ephrins after '**Eph** family **r**eceptor **in**teracting proteins' or the Greek name Ephoros, meaning overseer or controller (Lemke, 1997). An exceptional property of the Eph proteins is that they signal bidirectionally: receptor / ligand binding leads to signalling events in both the cell expressing the receptor and the cell expressing the ligand.

In the mammal, the Eph protein family is made up of fourteen receptors and eight ligands. The receptors are divided into two classes, A and B, determined by their sequence conservation and binding affinity, which coincide (Lemke, 1997). There are nine A class receptors which bind to five A class ligands and five B class receptor which bind to three B class ligands (Pasquale, 2004). Each receptor binds promiscuously to the ligands in its class. In addition, EphA4 binds EphrinB2 and EphrinB3 and EphB2 binds to Ephrin A5 (Figure 3). This lack of specificity is commonly considered an indication of functional redundancy within the family. However, this is contested by binding studies and gene targeted mutations that provide evidence for specific receptor-ligand interactions effecting specific biological functions (Blits-Huizinga *et al.*, 2004; Haramis & Perrakis, 2006; Henkemeyer *et al.*, 2003).

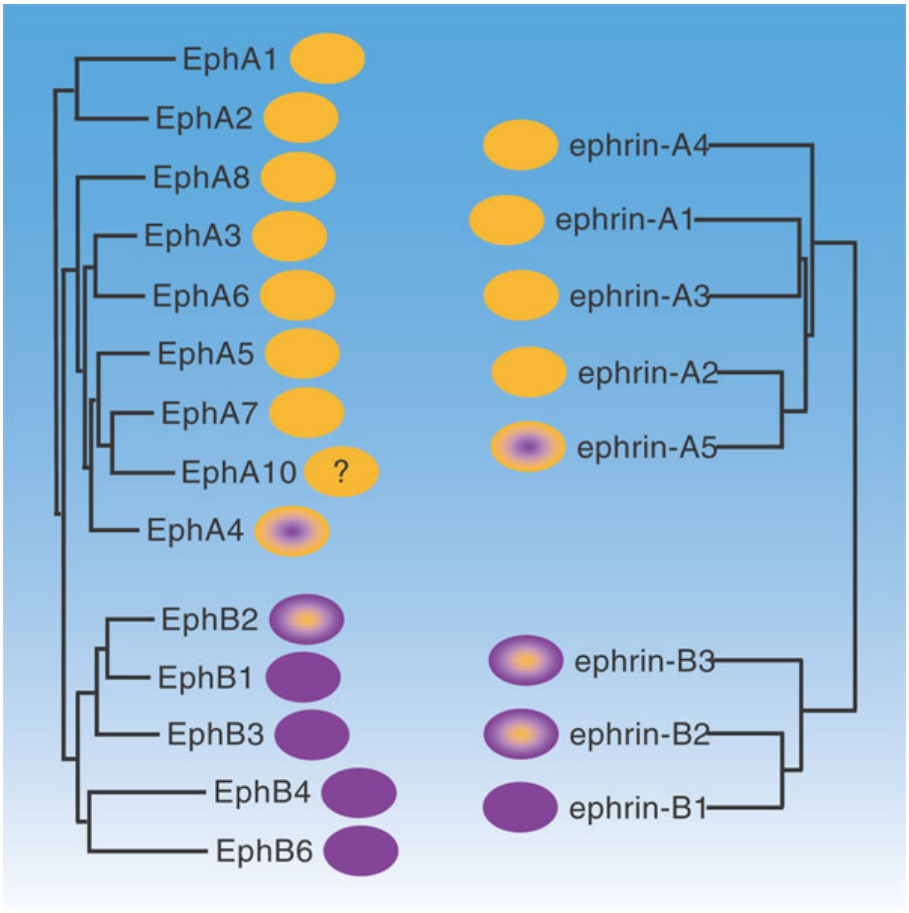


Figure 3. Dendrogram showing the binding preferences of the Eph receptors.

The yellow and purple indicate the binding preference of the Eph protein. A-class receptors bind A-class ligands; B-class receptors bind B-class ligands. In addition, EphA4 binds EphrinB2 and EphrinB3, and EphB2 binds EphrinA5. The lengths of the horizontal branches are proportional to sequence divergence between proteins and the arrangement of the branches indicates putative phylogenetic relationships (Pasquale, 2004).

Eph receptor structure

With the advent of new drug-design technologies, the molecular structure of the Eph proteins becomes increasingly important to instruct the design of therapeutic agents (Figure 4). The extracellular domain of Eph receptors includes two fibronectin repeats, a cysteine-rich region and a one hundred and eighty amino acid N-terminus sequence (Himanen *et al.*, 1998). The N-terminus is conserved within the class (A or B) and forms the ligand-binding domain (LBD) which determines the binding properties of the receptor. In particular, the H-I loop along with certain residues confer the class specificity and play the major role in ligand binding. The D-E and J-K loops show the largest conformational change when the ligand is bound, forming the ligand-binding channel (Himanen *et al.*, 2004; Himanen *et al.*, 2001).

The cysteine-rich domain contains a sushi (complement control protein) domain followed by an epidermal growth factor-like domain (Seiradake *et al.*, 2010). These regions and the fibronectin repeats play a role in receptor dimerization (Lackmann *et al.*, 1998), higher order clustering (Seiradake *et al.*, 2010), and interaction with NMDA receptors (Dalva *et al.*, 2000). They are also a common extracellular motif and allow the receptor to bind the ligand at a distance from the cell (Murai & Pasquale, 2004).

The intracellular domain of the receptor includes a juxtamembrane sequence (JM), a tyrosine kinase domain, a sterile-α-motif (SAM) and a binding site for proteins containing a ‘post-synaptic density-95/discs-large/zona occludens 1’ (PDZ) domain. The kinase active site is inhibited by the non-phosphorylated

JM domain, which structurally distorts the kinase domain. Upon auto-phosphorylation, this inhibition is relieved and the kinase becomes active. The phosphorylated JM domain also serves as a docking site for downstream signalling proteins (Himanen *et al.*, 2001; Himanen & Nikolov, 2003; Murai & Pasquale, 2003).

Ephrin structure

The Ephrin ligand's extracellular structure forms the receptor-binding domain in which various sequences are responsible for tetramerization, ligand-receptor docking and interaction with the Eph receptor ligand binding channel (Himanen *et al.*, 2001). The A class ligands are entirely extracellular and tethered to the membrane by a glycosylphosphoinositol (GPI) anchor (Figure 5). The B class ligands are transmembrane and have a cytoplasmic tail of eighty amino acids. This tail is highly conserved (approx. 95% homology across the class), contains a PDZ binding domain and five tyrosine residues, which become phosphorylated upon ligand binding (Bundesen *et al.*, 2003; Tanaka *et al.*, 2005; Gauthier & Robbins, 2003/12/5).

Eph-Ephrin binding

Prior to receptor-ligand binding, Eph receptors and Ephrins are associated with particular microdomains in the membrane that are rich in glycosphingo-lipids and cholesterol (lipid rafts) and form low affinity Eph-Eph or Ephrin-Ephrin dimers (Blits-Huizinga *et al.*, 2004). As cell-cell contact occurs, the receptor binding domain of the ligand interfaces with Eph receptors in a number of

ways. Principally it forms a dimer with one receptor. This involves the docking of the ligand to the receptor, relying on hydrogen bond networks for binding and recognition. Docking enables the hydrophobic binding loop of the ligand to become enveloped into the hydrophobic binding channel on the receptor in an action powered by van der Waals forces. Further to dimer formation, the ligand also interfaces with a second receptor to form a tetramer. This second interaction involves hydrogen bonds and van der Waals forces but has a much lower affinity (Himanen *et al.*, 2001). Not all receptor-ligand complexes are capable of forming tetramers as demonstrated by crystallography of the EphB2-EphrinA5 complex (Himanen *et al.*, 2004). However, it is likely that the formation of tetramers is critical to the development of Eph signalling (Smith *et al.*, 2004). A high concentration of receptor-ligand tetramers drives the formation of higher order clusters. The low affinity *cis* interactions prior to Ephrin bindings occur between the LBD and the sushi regions of the Eph receptors. Following Ephrin binding, the interaction between the *cis* Eph receptors alters to LBD-LBD and sushi-sushi interactions allowing the oligomerization to stabilise (Seiradake *et al.*, 2010). Once higher order clusters form, Eph receptors are recruited without further Ephrin binding (Wimmer-Kleikamp *et al.*, 2004). There is also evidence to suggest that the protease dependent degradation of the Eph receptors requires higher order clusters to form (Seiradake *et al.*, 2010).

Eph signalling: forward and reverse

The binding of the Ephrin causes conformational changes to the intracellular region of the Eph receptor. The catalytically repressed kinase domain of one

receptor phosphorylates inhibitory regions of the other receptor and the kinase becomes active. The phosphorylation of the juxtamembrane domain also allows for binding to other signalling proteins, such as those that contain SH2 domains (Zisch *et al.*, 2000). The forward signalling induced by Eph receptor activation includes important signalling pathways in the regulation of the cell cytoskeleton, activation of Rho family small GTPases, Ras-MAP kinases and Src family kinases, as well as other molecules involved in regulating experience driven plasticity in the adult brain (Murai & Pasquale, 2003; Vearing & Lackmann, 2005).

In keeping with the multifunctional properties of Eph receptors, forward signalling is regulated by a number of mechanisms in addition to direct ligand binding. For example, during neuronal development, the signalling of Eph receptors and Ephrins is regulated by their location in the cell membrane. Within the same axonal segment, Eph receptors and Ephrin ligands are separated to non-overlapping microdomains (Marquardt *et al.*, 2005). By regulating the microdomain specificity of Eph receptors and Ephrins, the cell can control the balance between *trans* and *cis* interactions (Kao & Kania, 2011). The *trans* interactions facilitate forward signalling and, depending on the identity of Eph receptors and Ephrins involved, serve as either repulsive or attractant cues for axonal guidance. The *cis* interactions attenuate the forward signalling, allowing the cell to precisely control the axonal trajectory (Hornberger *et al.*, 1999). The functional significance of the microdomain location and *cis* interaction of Eph receptors and Ephrins in adult neurons has yet to be determined.

The cellular expression levels of the Eph protein family also drive the signalling of the Eph-Ephrin interaction. For example, EphB1 mediated cell attachment during axonal guiding only occurs once the ligand density has surpassed a particular level (Huynh-Do *et al.*, 1999). The cell attachment in this study was dependent on EphB1 kinase signalling, indicating that the density of the ligand was critical in initiating downstream signalling rather than simple Eph-Ephrin binding. Furthermore, the density required to initiate signalling was reduced by pre-clustering the Ephrin molecules, indicating that the formation of higher order clustering determines the phosphorylation-dependent signalling of Eph receptors (Huynh-Do *et al.*, 1999). Indeed, *in vitro* stimulation of Eph receptors leading to signal transduction requires pre-clustering of the Ephrin ligand (Stein *et al.*, 1998), whilst monomeric Ephrin molecules act as antagonists (Lackmann *et al.*, 1998). The composition of the higher order Eph clusters also plays a role in Eph downstream signalling. Eph receptors of different classes can work together in the same cluster, independent of ligand binding. In EphA3 clusters, stimulated by exclusive EphA3 ligand binding, EphB2 receptors are recruited to the cluster and trans-phosphorylate EphA3 receptors (Janes *et al.*, 2011). The ability of Eph receptors to form clusters enables a much greater level of control over the Eph phosphorylation status and consequently, the signalling mechanisms (Janes *et al.*, 2012).

The Eph overall expression profile of the cell also determines the Eph signalling pathway and functional consequence of the Eph interaction. In a prostate cancer cell line, the invasive nature of the cell is determined by the

expression pattern of EphA receptors and EphB receptors. In these cells, the inhibition of invasion is mediated by the EphA-EphrinA interaction. However, this is lost due to the elevated expression of EphB receptors, which drive the invasive nature of the cell upon binding to EphrinB ligands. If the EphB expression level is reduced, the cells lose their invasive properties, indicating that the function of the Eph-Ephrin interaction is determined by expression pattern of the particular cell (Astin *et al.*, 2010).

Ephrin reverse signalling

The B class of Ephrins are transmembrane and can signal in two ways. First, they can bind PDZ domain proteins. A number of these have been identified as binding to EphrinBs, but the only one that has been shown to contain a catalytic domain is PDZ-RGS3 (Lu *et al.*, 2001) for review: (Schmucker & Zipursky, 2001). Reverse signalling has been shown to occur through the PDZ domain independently of Eph receptor binding. In isolated vascular endothelial cells, over-expression of EphrinB2 alone increases the motility of the cells without binding of Eph receptors (Bochenek *et al.*, 2010). The second mode of action is the phosphorylation of cytoplasmic residues. Tyrosine residues are phosphorylated by Src family kinases which results in recruitment of Grb4, a scaffolding protein that alters the actin cytoskeleton and regulates plasticity-related structural rearrangements (Cowan & Henkemeyer, 2001). A serine residue is also phosphorylated, which leads to regulation of AMPA receptors at hippocampal synapses (Essmann *et al.*, 2008).

Reverse signalling also occurs through Ephrin A molecules. Despite having no intracellular domain, the ligand communicates signals inside the cell and activates a Src family kinase for review (Aoto & Chen, ; Gauthier & Robbins, 2003/12/5). It is proposed that the GPI linkage of EphrinA to the cell membrane confers signalling specificity. Through association with particular lipid rafts, Ephrin A operates through messengers distinct to EphrinB signalling pathways. This is consistent with specific subpopulations of lipid rafts associating with different molecules involved with intracellular signalling. A common downstream effect of EphrinAs and B's activation is modulation of actin cytoskeleton with subsequent structural rearrangement of neurites (Gauthier & Robbins, 2003/12/5).

The Eph receptors and hippocampal synaptic plasticity

In the adult limbic system, the Eph receptors are involved in both adaptive and maladaptive mechanisms affecting neuronal plasticity. This includes regulating both synaptic and morphological plasticity.

In the hippocampus, EphB2 regulates LTP at the dentate gyrus, CA3 and CA1 synapses. At the mossy fibre – CA3 synapses, the disruption of EphB2 binding to pre-synaptic EphrinB2 inhibits LTP (Contractor *et al.*, 2002). This is dependent on the PDZ binding capabilities of EphB2 in the dendritic spine. Similarly, LTP in the dentate gyrus synapses and at the CA1 synapses is reduced in EphB2 knockout mice (Grunwald *et al.*, 2001; Henderson *et al.*, 2001). However, at these synapses, EphB2 function is independent of the kinase domain, suggesting that the extracellular portion of EphB2 and its

ligand-binding abilities drive the function (Grunwald *et al.*, 2001; Henderson *et al.*, 2001). In addition, EphB2 null mice show significant cognitive deficits in hippocampus-dependent memory tasks, which is consistent with the role of this receptor in synaptic plasticity, learning and memory (Grunwald *et al.*, 2001).

At the mossy fibre synapses, LTP is known to be NMDA-independent. Disrupting the PDZ-related downstream signalling of EphB2, or blocking the binding of EphB2 to EphrinB, inhibits mossy fibre LTP (Contractor *et al.*, 2002). Further studies reveal the importance of the reverse signalling in this form of LTP and a particularly critical role of EphrinB3 in this process. Experiments by Armstrong *et al*. demonstrate that replacing the C-terminal signalling domain of EphrinB3 with its truncated form significantly impairs LTP (Armstrong *et al.*, 2006). The importance of Ephrin-mediated reverse signalling in synaptic plasticity, LTP and LTD, is also observed in CA1 neurons, which unlike other regions express Ephrins postsynaptically (Grunwald *et al.*, 2004).

The EphA receptors also regulate neuronal plasticity in the hippocampus. Although EphA4 does not interact with NMDA receptors (Dalva *et al.*, 2000), it mediates early phases of LTP (Grunwald *et al.*, 2004). This is achieved through, EphA4 / EphrinA3 interaction modulating astrocytic modulation of LTP. Astrocytes regulate neuronal plasticity by controlling extracellular glutamate levels. In order to protect neurons from excitotoxicity, astrocytic glutamate transporters remove excess glutamate from the synapse. Astrocytic EphrinA3, activated by dendritic CA1 EphA4, reduces glutamate transporter

levels, regulating LTP at the CA1-CA3 synapse (Filosa *et al.*, 2009). EphA5 also regulates hippocampal LTP although the mechanism is not known. At the Shaffer collateral – CA1 synapses, the application of an EphA5 inhibitor to hippocampal slices impairs LTP and the application of an EphA5 agonist partially mimics it (Gerlai *et al.*, 1999; Gao *et al.*, 1998/8). To further investigate its role in plasticity, mice were infused with an EphA5 antagonist and subjected to behavioural tests sensitive to hippocampal function. The infusion of the agonist markedly impaired learning in both the T-maze and context dependent fear conditioning (Gerlai *et al.*, 1999). The effect of EphA5 on hippocampus-dependent memory was confirmed by inducing a retrograde amnesia in mice trained in a context-dependent fear conditioning. EphA5 agonists significantly ameliorated loss of memory in this learning paradigm (Gerlai & McNamara, 2000/3).

Eph receptors and morphological plasticity

Eph proteins are also important in morphological plasticity. Similarly to growth cone motility, the formation and maturation of spines requires cytoskeletal remodelling, implicating Eph proteins in the process. In fact, the formation and morphology of dendritic spines is dependent on Eph proteins; EphB1, EphB2 and EphB3 work in concert to form mature spines. The absence of one of the receptors does not affect spines, suggesting some functional redundancy, but loss of two of the receptors results in a dramatic reduction of spine maturation. Further studies reveal that the failure to form protrusions is caused by the inability of actin to accumulate in spines (Henkemeyer *et al.*, 2003). Although the full mechanism behind this phenomenon is not yet clear, it does involve

syndecan-2,as the phosphorylation of syndecan-2 by EphB2 is required for its clustering and the subsequent maturation of spines (Ethell *et al.*, 2001).

Multiple EphrinB molecules also regulate spine morphology through phosphorylation of their intracellular domains (Segura *et al.*, 2007). Rodenas-Ruano *et al* provided further evidence for the involvement of EphrinBs in plasticity when their 2006 study found that the intracellular signalling domain of EphrinB3 regulates synapse number without affecting LTP in the CA1 region.

Although EphA4 is not indispensable for spine formation, it does regulate spine morphology. EphA4 knockout mice show abnormal spine anatomy, which is dependent on the lack of EphA4 kinase activity (Murai *et al.*, 2003). It is also likely EphA4-EphrinA3 signalling between neuron and glial cells modulate spine morphology; similar spine abnormalities are seen in mice in which the gene encoding EphrinA3 has been disrupted (Carmona *et al.*, 2009). Furthermore, the addition of EphrinA3 to wild-type brain slices causes a reduction in spine length and density, which occurred through EphA4 interaction (Murai *et al.*, 2003).

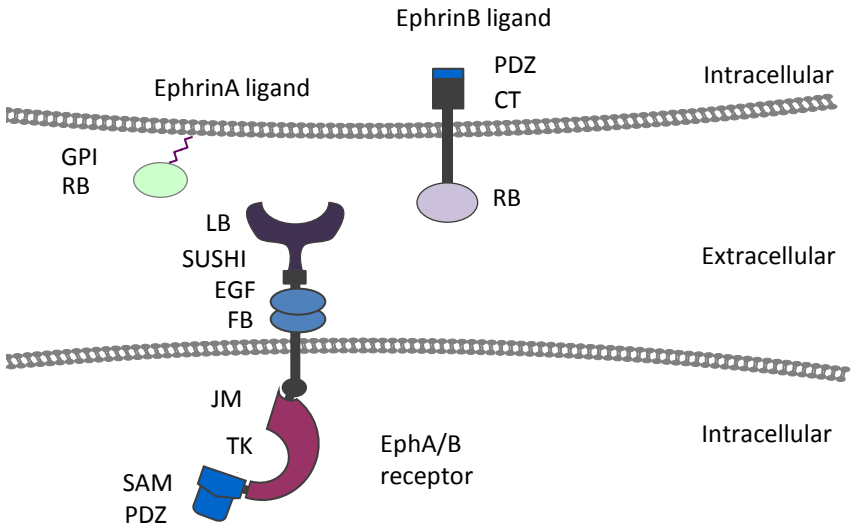


Figure 4. Eph receptor and Ephrin A and B class ligand structure. The Eph receptor's extracellular structure includes the ligand binding domain (LB), a SUSHI domain (SUSHI), a epidermal growth factor domain (EGF) and fibronectin repeats (FB). The intracellular structure contains a juxtamembrane domain (JM), the tyrosine kinase (TK), a sterile-α-motif and a postsynaptic density-95/Drosophila disc-large tumour suppressor/zona occludens 1 (PDZ) domain. EphrinA ligands contain a receptor binding domain (RB) and are tethered to the cell membrane by glycosylphosphoinositol (GPI) anchor. EphrinB ligands include an extracellular RB domain and a cytoplasmic tail (CT) which contains a PDZ domain.

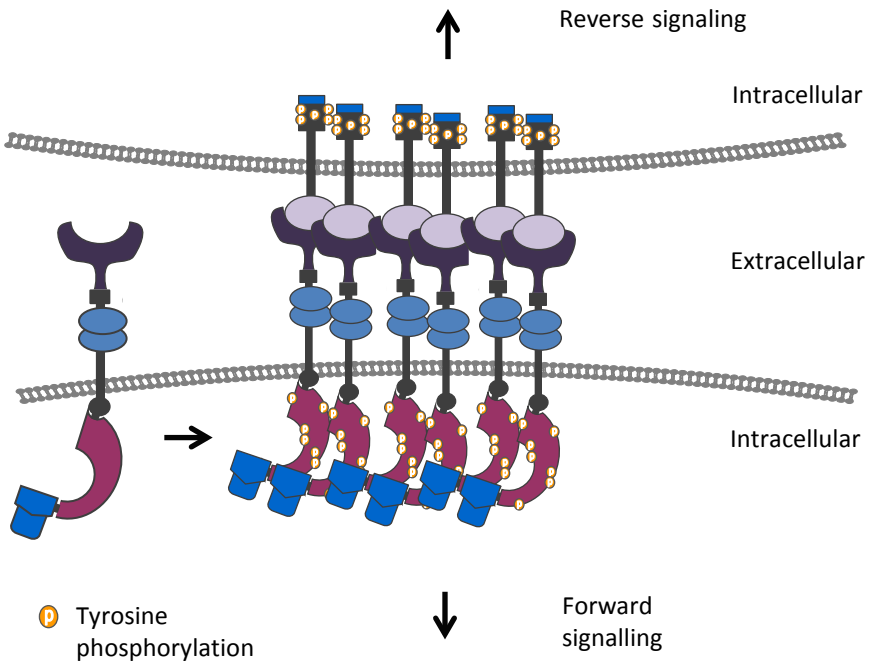


Figure 5. Eph receptor and Ephrin clustering and signal propagation

Eph/Ephrin binding results in the formation in the removal of tyrosine kinase auto-inhibition. The Eph-Ephrin dimers form a circular heterotetramer which leads to oligomerization which determines the overall signalling outcome. Tyrosine phosphorylation is initiated following conformational change and relief from auto-inhibition. The active tyrosine kinase domain trans-phosphorylates *cis* Eph receptors and downstream targets. This can also occur as Eph receptors join the oligomer without Ephrin binding. Conformational changes also propagate signalling through molecular interactions.

Neuropsin

Expression and activation

Neuropsin is an extracellular protease essential for various aspects of neuronal physiology, particularly learning and memory. It is uniquely positioned to affect learning and memory due to its expression in the limbic system. Its highest mRNA expression is found in the CA1 and CA3 regions of the hippocampus and the lateral amygdala (Chen *et al.*, 1995). Neuropsin is constitutively secreted as an inactive zymogen from neurites, enabling it to produce effects rapidly upon activation (Oka *et al.*, 2002). It is converted to its active form by cleavage of a Lys-Ile bond removing a four amino acid peptide from its N-terminus (Shimizu *et al.*, 1998). It is a serine protease with sequence homology to trypsin (Chen *et al.*, 1995). The tertiary structure of neuropsin also contains similarities to trypsin but forms unique loops which create lysine and Arginine specific pockets (Kishi *et al.*, 1999). *In vitro* experiments have confirmed that neuropsin cleaves lys-x and arg-x bonds (Shimizu *et al.*, 1998). So far, the physiological substrates discovered for neuropsin are extracellular matrix proteins - fibronectin and L1CAM (Shimizu *et al.*, 1998; Matsumoto-Miyai *et al.*, 2003).

Role in neuronal plasticity

Similarly to other proteases involved in neuronal plasticity, neuropsin gene expression increases following neuronal activity (Chen *et al.*, 1995). It is also altered following stress. Harada *et al* found that neuropsin mRNA was increased for at least 24 hours following 1 hour of restraint stress. Furthermore, the gene expression correlated temporally with CORT levels and

injection of CORT caused a similar rise in the neuropsin mRNA levels (Harada *et al.*, 2008). Consistent with this finding, stress related behaviour is altered in neuropsin knockout animals. Animals missing the neuropsin gene display increased anxiety as measured by the elevated plus maze (Horii *et al.*, 2008). Neuropsin deficient mice also display altered behaviour in memory related tasks. However, the results are not entirely clear as different groups found different results. Davies *et al* found no deficiency in neuropsin knockout animals performing the water maze (Davies *et al.*, 2001) whilst Tamura *et al* found impaired performance in the same test (Tamura *et al.*, 2006).

The neuropsin knockout animals also display altered synaptic plasticity. Whilst there were no deficits in late LTP, the animals did display significant impairments in early LTP (E-LTP) (Davies *et al.*, 2001; Tamura *et al.*, 2006). Not only is the gene expression of neuropsin regulated by neuronal activity, but the activation of non-active pro-neuropsin follows neuronal activity. This activity-dependent activation of neuropsin is NMDA receptor-mediated, although the exact mechanism of activation has yet to be elicited (Matsumoto-Miyai *et al.*, 2003). Although early LTP is both NMDA and neuropsin-dependent, neuropsin does not directly affect NMDA current in the hippocampus (Komai *et al.*, 2000). Instead, the search for the mechanism through which neuropsin regulates E-LTP has focussed on the alterations of the extracellular matrix (ECM). As LTP occurs, changes in the morphology of dendritic spines accompany it (Yang *et al.*, 2008). Furthermore, LTP is reduced by blocking interactions between the ECM and the synaptic membrane (Chun *et al.*, 2001; Luthl *et al.*, 1994). By proteolysis of fibronectin, neuropsin reduces

the interaction between fibronectin and α5β1 integrin and therefore allows cellular morphological changes to occur (Tani *et al.*, 2001). Neuropsin also acts to facilitate formation and maturation of hippocampal synaptic boutons through L1CAM (Nakamura *et al.*, 2006). Indeed L1CAM, which is known to modulate E-LTP, is cleaved by neuropsin during E-LTP (Luthl *et al.*, 1994; Matsumoto-Miyai *et al.*, 2003). Consistent with neuropsin regulating the ECM to affect neuronal morphology, neuropsin knockout animals have abnormalities in hippocampal synapses, with a decrease in asymmetric synapses being observed (Hirata *et al.*, 2001).

Recently, neuropsin has also been implicated in synaptic tagging. Through integrin/actin and subsequent L-type voltage dependent calcium channel (LVDCC) signalling, neuropsin permits late associative plasticity in the hippocampus (Ishikawa *et al.*, 2008). Furthermore, neuropsin acts to facilitate neuronal outside-in signalling resulting in phosphorylation of AMPA receptors and LVDCC signalling (Ishikawa *et al.*, 2008; Tamura *et al.*, 2006).

The evidence thus far points to a critical role for neuropsin in neuronal plasticity in the limbic system. Its location, temporal expression, effects on LTP and its target substrates make it a good candidate for further investigation in stress induced neuronal plasticity.

The tPA Plasminogen system

Expression and activation

The tPA/plasminogen proteolytic role has been well characterised for its fibrinolytic action during haemostasis (Figure 6).

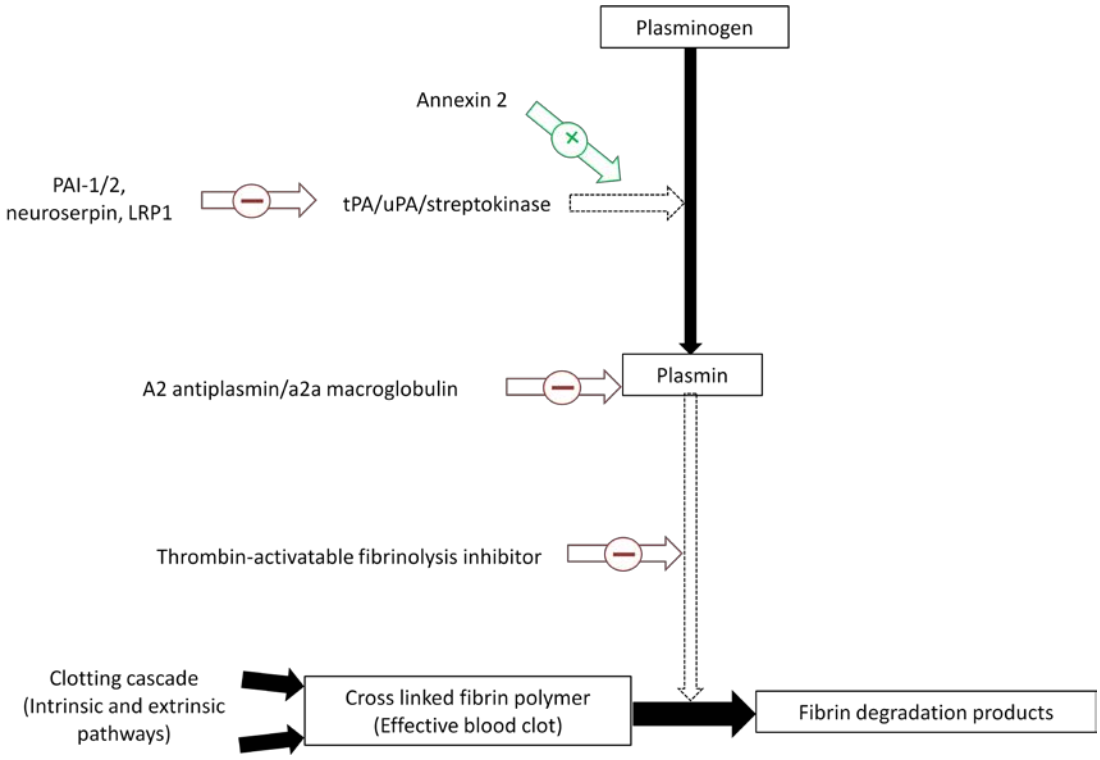


Figure 6. The tPA/plasminogen system. Diagram to show the activation of plasminogen to plasmin and its cleavage of fibrin, resulting in its anti-coagulation properties. The coagulation cascade results in a blood clot by forming fibrin polymers. Plasminogen is activated by a number of agents, including tPA and acts to cleave fibrin polymers. Anti-coagulatory cleavage is indicated by ⇢. Pro-coagulatory activity modulating the tPA/plasminogen system is indicated by ⊖➔. Molecular change is indicaed by ➡. Catalyst reaction indicated by ⊕➔ .(Coleman haemostasis and thrombosis, 2006; Osterwalder et al., 1996).

However, light has now been shed on its role in the central nervous system (CNS). Although tPA is more widely distributed throughout the brain than neuropsin, it also has a high expression in the limbic system. This includes the mossy fibre pathway of the hippocampus and the central and medial nuclei of the amygdala (Pawlak *et al.*, 2003; Salles & Strickland, 2002). Like neuropsin, tPA can be described as an immediate early gene, as the gene expression is upregulated rapidly following neuronal activity (Qian *et al.*, 1993). Using *in situ* zymography, it has been shown that the enzymatic activity of tPA also increases rapidly following neuronal activity, in the same anatomical location as the mRNA increases. This occurs in the hippocampus both after excitotoxic injury and glutamate stimulation but also in the amygdala following restraint stress (Salles & Strickland, 2002; Pawlak *et al.*, 2003; Shin *et al.*, 2004). Unlike neuropsin, tPA is not constitutively released to the extracellular milleu. Rather, it is stored in secretory granules and rapidly released into the synapse (Gualandris *et al.*, 1996). This occurs from both axon terminals and dendritic spines (Baranes *et al.*, 1998; Lochner *et al.*, 2006). Although plasminogen is not found in large quantities in the CNS, its mRNA expression is found primarily in the hippocampus, cortex and cerebellum (Basham & Seeds, 2001; Salles & Strickland, 2002). Its expression is also rapidly upregulated following neuronal activity (Shin *et al.*, 2004; Pawlak *et al.*, 2003).

Once in the extracellular space, tPA cleaves plasminogen to plasmin. Plasmin is a broad spectrum serine protease whilst tPA is more selective with relatively few substrates known (Ding *et al.*, 1995; Melchor & Strickland, 2005). Plasmin is activated when it is converted from its inactive zymogen, plasminogen, by

tPA or uPA (Rijken & Groeneveld, 1991). Plasmin is a powerful protease because of its broad spectrum and fast activation, which is partly due to a positive feedback mechanism. Once activated by tPA, plasmin activates other members within its proteolytic cascade. Not only does it covert plasminogen to plasmin more efficiently than tPA or uPA, but it also enhances tPA activity. Plasmin cleaves single chain tPA molecules into two chain molecules that possess increased plasmin-activating ability (Coleman haemostasis and thrombosis, 2006). The proteolytic action of tPA and plasminogen is tightly controlled by inhibitors, catalysers and scavengers. Serpin molecules - classic tPA and plasmin inhibitors - are expressed in the brain. Plasmin is inhibited by α-2 antiplasmin and tPA inhibited by plasminogen activator inhibitor-1. Furthermore, a neuronal specific tPA inhibitor, neuroserpin, has been described (Osterwalder *et al.*, 1996). Whilst the serpins inhibit the action of the tPA/Plasminogen system, the annexin II complex catalyses it. Association of tPA and plasminogen with this complex increases the conversion of plaminogen to plasmin. tPA activity is also attenuated by the LRP1 receptor which scavenges the protease from the extracellular space. Further to its enzymatic activity, tPA acts in the CNS to transmit signals in a non-proteolytic manner. It acts as a non-proteolytic ligand to maintain LTP and promote neuronal outgrowth, through different receptors (Lee *et al.*, 2007; Zhuo *et al.*, 2000). In this manner, tPA has neuroprotective, neurotrophic and microglial activating properties (Kim *et al.*, 1999; De Petro *et al.*, 1994; Rogove *et al.*, 1999).

Role in neuronal plasticity

The tPA/plasminogen has been implicated in neuronal plasticity through a number of different mechanisms. LTP and LTD are both modulated by the tPA/plasminogen system. In contrast to neuropsin, which affects the early phase of LTP, tPA and plasminogen act to promote late phase LTP (L-LTP) (Mizutani *et al.*, 1996). In the hippocampus, the tPA gene expression increases during L-LTP and animals deficient in tPA display normal E-LTP but fail to establish L-LTP (Qian *et al.*, 1993; Frey *et al.*, 1996; Huang *et al.*, 1996). The same effect is seen upon injection to the hippocampus of a tPA inhibitor, tPA STOP (Baranes *et al.*, 1998). Indeed, tPA acts not only to maintain LTP but to enhance it. Injection of tPA or the overexpression of tPA results in a higher level of LTP (Baranes *et al.*, 1998; Madani *et al.*, 1999). In line with these results, mice lacking the tPA gene show deficits in hippocampal related memory tests. Although the mice did not display deficits in the Barnes circular and Morris water maze, they were impaired in step down inhibitory avoidance paradigm (Baranes *et al.*, 1998; Pawlak *et al.*, 2002). The overexpression of tPA further revealed a role for tPA in hippocampal based memory formation as the animals performed better at the Morris water maze and another hippocampus-dependent test, the homing hole board test (Madani *et al.*, 1999). A key effector of tPA/plasminogens L-LTP effects is brain-derived neurotrophic factor (BDNF). The L-LTP not only requires protein synthesis but plasmin cleavage of proBDNF to BDNF is a critical step in this pathway (Pang *et al.*, 2004). However, the tPA/plasminogen system modulates neuronal plasticity in a number of ways. These include proteolytic events and non-proteolytic events that can also be independent of plasminogen activation.

Further analysis of the tPA knockout animals revealed that they were deficient in certain NMDA-dependent hippocampal tasks, indicating a mechanism through which tPA may affect neuronal plasticity (Horwood *et al.*, 2004). The evidence suggests that the tPA/plasminogen system acts through the NMDA receptor in a number of ways. Firstly, its interaction with the NMDA NR1 subunit has been suggested. There is evidence tPA interacts with and forms a complex with the NR1 subunit leading directly to cleavage of the amino terminal of NR1 by tPA (Fernandez-Monreal *et al.*, 2004). This causes a potentiation of NMDA-mediated calcium influx leading to an increase in neuronal cell death (Nicole *et al.*, 2001). Furthermore, interfering with the interaction between the tPA and NR1 *in vivo* led to decreased cleavage and behavioural deficits similar to tPA deficient animals (Benchenane *et al.*, 2007). However, this mechanism is controversial within the field. Whilst another group has demonstrated an interaction between tPA and NR1, other groups have been unable to show direct cleavage of NR1 by tPA (Kvajo *et al.*, 2004; Matys & Strickland, 2003; Liu *et al.*, 2004; Samson *et al.*, 2008).

As mentioned, tPA acts as a ligand for LRP1 which regulates tPA's concentration in the extracellular space by endocytosis. However this receptor-ligand interaction also influences LTP, as the enhancing effect of tPA on LTP is mediated by LRP1 signalling (Zhuo *et al.*, 2000). The stimulatory effects of tPA on NMDA signalling result in activation of the MAP kinase pathway through ERK1/2 phosphorylation (Medina *et al.*, 2005). Using LRP1 inhibitors and truncated PSD95 mutants, Martin *et al* discovered that the activation of

NMDA and downstream ERK phosphorylation relied on LRP1 interacting with NMDA. This occurred via PSD95, downstream from tPA binding LRP1 (Martin *et al.*, 2008). Further evidence suggests that the binding of tPA to LRP1 that causes NMDA potentiation requires tPA activity, and that tPA forms a complex with a serpin before engagement with LRP1 (Samson *et al.*, 2008). There is also evidence that astrocytic LRP1 also plays a role in tPA-NMDA LTP and so further discoveries in this pathway are still to be fully elicited (May *et al.*, 2004). tPA also mediates synaptic plasticity through LRP1 by upregulating MMP9, an extracellular protease (Wang *et al.*, 2003). MMP-9 is also indispensible for L-LTP and enhances LTP whilst acting concomitantly to modulate spine morphogenesis (Wang *et al.*, 2008; Nagy *et al.*, 2006). tPA also influences NMDA action in a nonproteolytic manner through the NR2B subunit. During ethanol dependence, an adaptive form of neuronal plasticity, tPA acts to upregulate the NR2B subunit. This is promoted by tPA through a nonproteolytic interaction with the subunit (Pawlak *et al.*, 2005).

An alternate mechanism through which tPA may mediate NMDA function is through direct subunit cleavage by plasmin. This has been shown to cause either partial or complete degradation of the subunit (Samson *et al.*, 2008; Matys & Strickland, 2003). The authors describing specific cleavage suggest that plasmin may regulate NR1 function directly although not through an increase in NMDA calcium influx (Samson *et al.*, 2008).

In contrast to the above, plasmin has been shown to potentiate NMDA current, although not through NR1 modulation. Firstly, it was demonstrated that

plasmin potentiates NMDA current through its activation of PAR-1. Plasmin-activated astrocytic PAR-1 leads to neuronal-astrocytic crosstalk which potentiates neuronal NMDA current (Mannaioni *et al.*, 2008). Secondly, plasmin can potentiate neuronal NMDA current through direct cleavage of the NMDA NR2A subunit. Cleavage at the N-terminus of the NR2A removed the zinc binding site and consequently the zinc inhibition (Yuan *et al.*, 2009).

The involvement of plasminogen in NMDA cleavage has also been implicated in stress. Following chronic restraint stress, hippocampal NMDA level was reduced, an effect mediated by plasmin, which led to hippocampal-associated memory deficits (Pawlak *et al.*, 2005). This effect was regulated by PAI-1 and illustrates the fine balance achieved by regulatory processes in the tPA/plasminogen system. Following acute restraint stress, tPA was inhibited by PAI-1, which led a decrease in NR2B phosphorylation resulting in NMDA internalisation. This change in plasticity results in hippocampal associated fear behaviour (Norris & Strickland, 2007). As described, the hippocampus and the amygdala act in concert during stress-induced plasticity. The tPA/plasminogen system also modulates stress-related plasticity and behaviour mediated by the amygdala, specifically the medial amygdala (Pawlak *et al.*, 2003). Corticosterone releasing factor (CRF), released by the PVN, causes an upregulation of tPA activity in the amygdala following stress. This alters both pre- and post-synaptic markers of plasticity as well as disturbing the circulating corticosterone levels during recovery from stress (Pawlak *et al.*, 2003; Matys *et al.*, 2004). Further to this, changes in spine morphology that accompany chronic stress in the medial amygdala are dependent on tPA activity (Bennur

et al., 2007/1/5). These plasticity related changes translate to a behavioural phenotype of reduced anxiety-like behaviour in mice deficient for tPA (Pawlak *et al.*, 2003).

The role of the tPA/plasminogen system in the CNS demonstrates the critical role that an extracellular protease can play in neuronal plasticity. Protease-mediated cleavage of or interaction with ECM proteins, transmembrane receptors and ion channels drive the neuron's ability to adapt to environmental stimuli.

Aims and objectives

The aim of the work presented in this thesis is to describe the molecular interactions of Eph proteins with neuronal proteases and their role in anxiogenesis. To achieve this a processive stress model, mouse restraint, will be used. The neuroanatomical focus will be the hippocampus and the amygdala. The *in vivo* model will be complemented by *in vitro* studies that will enable study of molecular interactions.

Objective 1: To investigate whether Eph proteins are susceptible to neuropsin, tPA or plasmin cleavage. This will be achieved using cellular lines that express Eph proteins and applying recombinant proteases to the culture medium. Cleavage events will be investigated further using Edman degradation, mass spectrometry, *in vitro* over-expression of Eph proteins and by using chimeric Eph proteins.

Objective 2: To investigate whether cleavage events described above occur *in vivo* during stress. Genetically modified mice, deficient for the protease of interest will undergo stress and the effect on Eph proteins susceptible to cleavage will be measured using Western blotting.

Objective 3: To investigate the Eph proteins and related molecular partners spatial expression in the hippocampus and amygdala using immunohistochemistry. Coronal mouse brain slices will be probed using appropriate antibodies and imaged using a confocal microscope.

Objective 4: To investigate the temporal expression in the hippocampus and amygdala of Eph proteins during stress. The gene and protein expression will be quantified, before and after restraint stress, in the hippocampus and amygdala. The gene expression will measured using qRT-PCR and the protein expression by Western blotting.

Objective 5: To investigate the effect of neuronal proteases on Eph - Ephrin interactions. Eph receptors will be over-expressed in cell culture and fluorescently tagged Ephrin chimeric proteins will be added to the medium so that receptor ligand binding can be visualised in real time using confocal microscopy. Neuronal proteases will be added to the medium to investigate their effect on the receptor – ligand interaction.

Objective 6: To investigate Eph protein molecular interactions during stress. Eph proteins will be immunoprecipitated from the hippocampus and amygdala. The interacting ligand that precipitates with the Eph proteins will then be quantified to analyse the effect of stress on the interaction. Immunoprecipitation of Eph proteins will also be used to investigate other, Eph interacting partners that are critical to neuronal plasticity. For example, EphB2 has been shown to immunopreciptate with NMDA receptors. Their interaction, before and after stress, could be investigated using immunopreciptation.

Objective 7: To demonstrate if Eph proteins and their interactions with neuronal proteases regulate anxiogenesis. Anxiety-like behaviour in mice that have been genetically modified to remove or under-express Eph proteins or

neuronal proteases will be measured. The effect of the stress on the behaviour of these mice will be also investigated. The behavioural tests used will include the open field or elevated plus maze. The time points at which behaviour will be measured will be dependent on the results from the molecular experiments described above. The direct effects of neuronal proteases on anxiety-like behaviour will be investigated using neuronal cannulae implanted to the mouse amygdala. The protease will be administered before stress and the anxiety-like behaviour measured as above.

Chapter 2. Materials and Methods

Cell Culture

SHSY-5Y cells (passage number <30) were incubated (37C, 5% CO_2) in medium (MEM + EARLES, 5% fetal calf serum, 5% new-born calf serum, 2mM L-glutamine, 1% fungizone, 1% fem strep) until 80-90% confluence. They were washed with PBS (+Ca^{2+}, +Mg^{2+}) three times before being incubated with PBS, PBS + neuropsin (50nM; R&D), PBS + tPA (Alteplase, Genentech; 1µg/ml) or PBS + tPA (Alteplase, Genentech; 1µg/ml) + plasminogen (R&D, 0.5/1.5/10µg/ml) for fifteen minutes after which the dishes were placed on ice and protease inhibitors (Complete, Roche) were added. The cells were collected using a cell scraper and homogenised (Tris 50mM, NaCl 150mM, EDTA 5mM, EGTA 5mM, Triton-100 1%, NP40 0.5%, pH7.5). The resulting protein sample was analysed by Western blotting as described below.

To further investigate the cleavage of EphB2 by neuropsin, SHSY-5Y cells and HEK293 cells were transfected with mouse EphB2-GFP (kindly donated by Dr. A. Kania, Montreal) using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions and the cells were incubated with PBS (+Ca^{2+}, +Mg^{2+}) or PBS (+Ca^{2+}, +Mg^{2+}) + neuropsin (300nM) for 15 or 45 minutes. The reaction was stopped, the supernatant collected, the cells homogenised and proteins analysed by Western blotting as described above for initial cell culture experiment.

For the imaging experiments, SH-SY-5Y cells were transfected with GFP, mouse EphB2-GFP and mouse EphA4-GFP (kindly donated by Dr. A. Kania, Montreal) using Lipofectamine 2000 (Invitrogen) and loaded with cell tracker

(Invitrogen). Images were taken with Zeiss LSM5 Exciter before and after 15-minute incubation with neuropsin (50nM, R&D), converted to grayscale and fluorescent signal intensity was quantified using Scion Image.

In a separate imaging experiment, SH-SY-5Y cells were transfected with GFP, mouse EphB2-GFP and mouse EphA4-GFP (kindly donated by Dr. A. Kania, Montreal) and loaded with cell tracker (Invitrogen). EphrinB2 Fc (R&D, 4µg/ml) was clustered with Cy-5 conjugated donkey anti-human IgG (Jackson ImmunoResearch, 0.75µg/ml) for one hour at room temperature. This was then incubated with transfected and non-transfected cells for 15 minutes (8µg/ml). The cells were washed with PBS (37°C) and images collected using Zeiss LSM5 Exciter confocal microscope

Western blotting, cell fractionation and immunoprecipitation

Control mice and mice subjected to restraint stress were anaesthetised using intraperitoneal sodium pentobarbital (50 mg/kg) and perfused transcardially with ice cold PBS. The brains were removed and hippocampi and amygdalae dissected from a slice, -0.58 to -2.3mm relative to Bregma using a brain matrix (Stoelting), frozen immediately on dry ice and stored at -80ºC.

Samples were homogenized in 0.1M Tris, 0.1% Triton X-100, pH 7.4, containing phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4) and protease inhibitors (Complete, Roche) and the protein concentration was adjusted to 2 mg/ml using the Bradford method (Pierce). Reduced (DTT) and denatured (100°C for 5 minutes) samples (40µg per lane) were subjected to SDS-PAGE

electrophoresis and transferred onto nitrocellulose membrane. After blocking (5% skim milk for 1h at RT) and washing with PBS-T (3x5mins), the membranes were probed with the following primary antibodies overnight at 4°C: goat anti- NCAM-L1 (SantaCruz Biotechnology, 1:300), goat anti-EphB2, anti-EphB6 and anti-EphrinB2 (R&D, 1:500, 1:500 and 1:300 respectively), mouse anti-EphA4 (Zymed, 1:1000, this was also used in blots to recognize the C-terminus of EphA4), EphA4-N-terminus (R&D, 1:500) rabbit anti pan-cadherin (Abcam, 1:2000), rabbit anti-p75NGF receptor (Chemicon, 1:1000), rabbit anti-NR1 (Upstate, 1:250), rabbit anti-neuropsin (Dr. Helena C. Castro, Niterói, Brazil), rabbit anti-plasminogen (Molecular Innovations, 1:2000). The membranes were then washed in PBS-T (3 x 8 mins) before incubation with a relevant HRP-conjugated secondary antibody as appropriate (Vector Labs, 1:1000, 1hr, RT). The signal was developed, after washing with PBS-T (6 x 8mins), using a Super Signal West Pico Chemiluminescent Substrate (Pierce). To normalise the results, the membranes were stripped using a stripping buffer (Pierce), blocked, washed as above and re-blotted using mouse anti-β-actin antibody (Sigma, 1:2500, 1hr, RT). Again, the membranes were washed with PBS-T (6x8mins) before incubation with an anti-mouse HRP-conjugated secondary antibody (Vector Labs, 1:1000, 1hr, RT) and developed as described above. To quantify the results, the band intensities were analysed using Scion Image software and normalized to the actin bands.

When indicated, cellular fractions of the amygdalar samples were separated using a cellular protein fractionation kit (PerkinElmer) as per the manufacturer's protocol, analysed using Western blotting and normalized to

pan-cadherin or EphrinB2 levels. The primary antibodies used to verify the purity of the subcellular fractions were rabbit anti-Calpain (Abcam, 1:500), rabbit anti-CREB (Cell signalling, 1:1000), mouse anti-EphA4 (Zymed 1:1000) and rabbit anti-Vimentin (Abcam 1:1000).

For immunoprecipitation, amygdala and hippocampal samples were homogenized using HO buffer (50 mM HEPES-NaOH, pH 7.5, 1% Triton X-100, 0.15 NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol) as previously described(Calo *et al.*, 2005), with protease and phosphatase inhibitors (Complete - Roche, 50mM NaF, 1mM Na_3VO_4), pre-cleared using goat or mouse IgG (Sigma, 1µg), appropriate to the precipitating antibody, before incubation with either goat anti-EphB2 antibody, goat anti-EphB6, goat anti-EphrinB2 (R&D, 2µg) or mouse anti-EphA4 (Zymed, 2µg) for 1 hour (4°C). The samples were then incubated with protein G-sepharose beads overnight before being washed with PBS four times and analysed by Western blotting.

Eph brain homogenate cleavage

Control mice were anaesthetised using intraperitoneal sodium pentobarbital (50 mg/kg) and perfused transcardially with ice cold PBS. The brains were removed and hippocampi and amygdalae dissected from a slice, -0.58 to -2.3mm relative to Bregma using a brain matrix (Stoelting), frozen immediately on dry ice and stored at -80ºC.

Samples were homogenized in buffer (0.1M Tris, 0.1% Triton X-100, pH 7.4), containing phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4). The

homogenate (100µl) was incubated with tPA (1mg/ml) alone, tPA (1mg/ml) + plasminogen (0.5/1.5/10mg/ml) or without proteases for 15 minutes. The homogenate was placed on ice and proteases inhibitors were added to stop the reaction. The samples were then analysed by Western blotting as described above.

EphA4 Fc cleavage

EphA4 Fc (1mg/ml) was incubated with tPA (1mg/ml), tPA (1mg/ml) + plasminogen (1.5/10/20mg/ml) or without proteases in a HEPES-Tween buffer (0.1M HEPES, 0.01%Tween, pH 7.4) as previously described (Quagraine *et al.*, 2005) for 15 minutes. The samples were placed on ice and protease inhibitors (Complete, Roche) were added to stop the reaction. The samples were then analysed by Western blotting as described above. For mass spectrometry analysis, the experiment was repeated but the samples were separated by SDS-PAGE and the gel prepared for mass spectrometry. The gel was washed for 30 seconds with deionized water, followed by (1x10minutes) under gentle agitation (50mg Coomassie Blue R250 in 50 ml Methanol, 50 ml di-Water). The membrane was then de-stained (3x10minutes) under gentle agitation (Acetic Acid, 200 ml di-Water, 250 ml Methanol) and dried. Individual bands were excised from the gel and analysed by the University of Leicester Protein and Nucleic Acid Chemistry Laboratory.

Animals

The experiments involving EphrinB2$^{lx/lx}$ and EphrinB2-CaMKII-Cre were performed on three-month-old mice. Generation of EphrinB2-CaMKII-Cre mice has been previously described(Grunwald *et al.*, 2004). EphrinB2^{lx} and CamKII–cre mice were the kind gift by Professor R. Klein (Essmann *et al.*, 2008). The EphrinB2^{lx} and CamKII–cre mice were bred to produce EphrinB2-CaMKII-Cre mice. EphrinB2$^{lx/lx}$-CamKcre mice were bred with EphrinB2$^{lx/lx}$ mice to produce EphrinB2$^{lx/lx}$ and EphrinB2-CaMKII-Cre littermates which were used for experiments. The EphrinB2$^{lx/lx}$ and EphrinB2-CaMKII-Cre were genotyped using the following method: DNA from mice tail samples was extracted by incubating the sample for 25mins in lysis buffer (30mM NaOH, 2mM EDTA) before adding neutralizing buffer (40mM Tris-HCl). The primers for the EphrinB2$^{lx/lx}$ genotype (forward 5' CTT CAG CAA TAT ACA CAG GAT G 3' and reverse 5' TGC TTG ATT GAA ACG AAG CCC GA 3') were bought from Invitrogen. The PCR program was as follows:

94°C	2 min	
94°C	15 sec	40x
61°C	45 sec	
72°C	45 sec	
72°C	10 min	
10°C	∞	

PCR products were separated on a 1.5% agarose gel, which produced a single band of 240bp for wildtype mice, a single band at 350bp for EphrinB2$^{lx/lx}$

mice and a band at both 240bp and 350bp for EphrinB2^{lx} mice (Figure 33). The primers for the CaMK-cre genotype (forward 5' GCC TGC ATT ACC GGT CGA TGC AAC GA 3' and reverse 5' GTG GCA GAT GGC GCG GCA ACA CCA TT 3') were bought from Invitrogen. The RT-PCR program was as follows:

94°C	5 min	
94°C	1 min	40x
67°C	1 min	
72°C	2 min	
72°C	5 min	
10°C	∞	

PCR products were separated on a 1.5% agarose gel and CaMK-cre positive mice produced a single band at 800bp. Wild-type mice did not produce a band (Figure 33).

Experiments using neuropsin deficient animals were performed on three-month old wild-type (C57/BL6) NP-/- mice backcrossed to C57/BL6 for 12 generations. To generate these animals, exons 1-3 of the neuropsin gene, including the protease active site, have been replaced by a neomycin resistance cassette, which resulted in a disruption of neuropsin proteolytic activity (Hirata *et al.*, 2001). A lack of full-length neuropsin transcript and proteolytic activity in the brain of these animals was confirmed by RT-PCR and amidolytic assay (Hirata *et al.*, 2001) respectively. NP-/- mice were genotyped as described (Matsumoto-Miyai *et al.*, 2003).

All animals were housed three to five per cage in a colony room with a 12 hour light/dark cycle (lights on at 7AM) with *ad libitum* access to commercial chow and tap water. The experiments were approved by the UK Home Office and the University of Leicester Ethics Committee.

Restraint stress

C57/BL6 J and NP-/- mice were kept undisturbed for at least one week in their home cages to become familiar with the environment. Restraint stress was performed during the light period of the circadian cycle. Mice were held in wire restrainers, secured at the head and tail end of the restrainer with clips, within their home cage for the required period of stress. Control animals were left undisturbed, and stressed animals were subjected to a single five minutes, fifteen minutes or six hours restraint stress in a separate room. In some cases, the animals were returned to their home cage for an eighteen-hour recovery period.

qRT-PCR

Primer design

Primers were designed for the following molecules: EphA4, EphB1, EphB2, EphB3, EphB4, EphB6, EphrinB1, EphrinB2, EphrinB3 (Table 2). Exon sequences (as determined in NCBI and ENSEMBLE databases) for each of the above molecules were used to design the primer pairs using Primer3 software, as previously described (Rozen, Skaletsky 2000). To avoid binding

to residual DNA, the primers spanned an exon-exon boundary. Specific parameters for the design of primers to be used in qRT-PCR were set in Primer3 as follows: the length of the primers between 70 and 120 base pairs, the melting temperature between 58 ºC and 60 ºC, the GC content between 45% and 58%, a maximum self-complementarity of 4 and a CG clamp of 1. Using Operon software (Operon Biotechnology 2007), each primer was first plotted against itself for its potential to form hairpins or primer-dimers and then against the second primer for the potential to form primer-dimers. Primer-dimers were defined as four consecutive bases that were complementary on two primers and hairpins were defined as two sets of four bases that were complementary on the same primer. The primers were then analysed for potential binding to other gene sequences in the mouse genome using nucleotide-nucleotide blast (NCBI Blast). The custom DNA primers were then ordered from Invitrogen. In order to quantify gene expression, the target genes were compared against the actin gene as previously described (Salter & Fern, 2005).

Primer	Gene	Forward sequence	Reverse sequence	Efficiency (%)
EphA4	NM_007936	ACCGAGGCTTTTTCAGAGC	TCCAAGTTCACCGATGTCTC	91.6
EphB1	NM_173447	AAGCCCCCTACCTCAAAGTGG	ATCAACCTTCCCCCAAAATC	103.6
EphB2	NM_010142	CTTCCTCATCGCTGTGGTC	ATGTGTCCGCTGGTGTAGTG	105.9
EphB3	NM_010143	GCCTAATGGAGTCATCTTGGAC	TCTGGCTGGTTACAGTGGAG	94.6
EphB4	NM_010144	TGCTTTAGAAGAGACCCTGTTG	CTGTGCTGTTCCTCATCCAG	100.9
EphB6	NM_007680	CTGAGCAAACAAATGGGAAC	ATGGTGAAGGAGTGGGATTC	101.7
EphrinB1	NM_010110	TGAGACTGTGAACCAGGAAGAG	CGAACAATGCTACCTTGGAG	93.6
EphrinB2	NM_010111	TCAAGTACCGCAGGAGACAC	CCATTGTTGTTGCCACCTC	94.3
EphrinB3	NM_007911	CTGGAGCCTGTCTACTGGAAC	TCTGAGGATAAAGCACGTAACC	96.5
Actin	NM_007393	TGCTCCTCCTGAGCGCAAGTACTC	CGGACTCATCGTACTCCTGCTTGC	99.1

Table 1. Table of primer pairs designed to amplify six Eph receptors mRNA and three Ephrins mRNA. Primer pairs complementary to the above nine Eph genes were designed and their efficiencies optimised. Forward and reverse primer sequences are shown along with their efficiencies. A primer efficiency between 90% and 110% is required for accurate quantification of the qRT-PCR. The actin primer pair was previously described (Salter, Fern 2005).

RNA extraction and conversion

Control and stressed mice were anaesthetised intraperitoneally using sodium pentobarbital and perfused transcardially with ice cold PBS. The brains were removed and dissected in ice-cold PBS using a vibrating microtome (Campden Instruments). To minimise RNA loss, equipment was treated with RNase erase (Biosystems). Hippocampi and amygdalae were dissected from a coronal slice -0.58 to -2.3mm relative to Bregma and were stored in RNA Later (QIAgen) at 4 ºC until processed. Samples were homogenised in QIAzol lysis reagent (QIAgen) and total RNA was isolated using Mini Spin Columns according to the manufacturers' instructions (RNeasy Lipid tissue mini kit, QIAgen). The RNA was treated with RNase-Free DNase (QIAgen) to remove any genomic DNA and RNA quantity was then measured using a spectrophotometer at absorption of 260nm. A total of 2µg of RNA from each sample was converted to cDNA using Superscript III (Invitrogen) and oligo (dT) primers according to manufacturer's instructions. To increase the sensitivity of the qRT-PCR, the cDNA was treated with RNase H (Invitrogen) to remove the RNA template.

qRT-PCR reaction

Triplicate wells contained 20µl of SYBR Green Master Mix (Applied Biosystems and BioRad), 250nM of the forward primer, 250nM of the reverse primer, 1µl of cDNA and nuclease-free water to a total of 40µl. The PCR was performed using Chromo4/PTC-200 thermal cycler (MJ research) under the following conditions:

95 ºC for 15 minutes

94 ºC for 15 seconds
55 ºC for 30 seconds } 40x

72 ºC for 30 seconds

10°C ∞

A melting curve analysis on the reaction was performed to assess specificity of the reaction. Control reactions were performed without DNA template and/or with unconverted RNA as the template. To calculate the reaction efficiency for each primer pair, the standard curve of the logarithm of the template concentration was plotted against the number of cycles necessary to reach the fluorescence threshold (Ct). Serial dilutions of mouse cDNA (amount 0.001ng, 0.01ng, 0.1ng and 1ng) were used. The reaction efficiency was then calculated using the following formula: $E = 10^{-1/slope}$. An efficiency of between 90% and 110% (-3.1 to -3.6 slope) was required for each primer pair. The products of the qRT-PCR were separated on a 0.8% agarose gel and the resulting band purified from the gel using DNA binding columns according to the manufacturers' instructions (QIAquick gel extraction kit, QIAgen). This DNA was then sent with the original primer pairs for sequencing. The results were analysed using Finch software (FinchTV).

Immunohistochemistry

Mice were anaesthetised using intraoeritoneal sodium pentobarbital and perfused transcardially with ice-cold phosphate buffered saline (PBS)

containing protease inhibitors (Complete, Roche) followed by ice-cold 3% paraformaldehyde (Sigma). The brains were dissected and fixed in 3% paraformaldehyde in PBS overnight at 4°C. The paraformaldehyde was washed out and 70µm thick coronal sections were collected on a vibrating blade microtome (Campden Instruments Vibroslice HA752) and stored at 4°C in PBS containing 0.002% sodium azide (Sigma). Before immunostaining, brain sections were pre-incubated in PBS-T (PBS solution containing 0.5% bovine serum albumin, 0.02% Triton X-100 and normal donkey serum at 1:500) for 5 hours at room temperature. Sections were then incubated with mouse anti-EphA4 (1:200, Zymed) and goat anti-EphrinB2 (1:200, R&D) overnight at 4°C in PBST. Next, the sections were washed for 8-10 hours with PBST and incubated overnight with secondary antibodies (1:500, Molecular Probes), donkey anti-mouse Alexa Fluor 594 and donkey anti-goat Alexa Fluor 488 in the same buffer. Control sections were processed as above but the primary antibodies were omitted. Finally, sections were then washed in PBS-T for 5 hours, mounted on glass slides using Vectamount medium (Vector Laboratories), and photographed using Zeiss LSM5 Exciter confocal microscope.

For triple Ephb2/neuropsin with NeuN and GFAP co-labelling, the same procedure above was followed except sections were incubated in PBST containing goat serum. Also, the primary antibodies used were goat anti-EphB2 (1:300, R&D) or rabbit anti-neuropsin antibodies (1:200, kindly donated by Dr. Helena Castro; the antibody was pre-absorbed on acetone powder prepared from NP-/- brain for 1 hour at RT prior to use), along with the mouse

anti-NeuN (1:200, Chemicon) and chicken anti-GFAP (1:1000, Abcam), whilst the secondary antibodies (1:500, Molecular Probes) were donkey anti-goat Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 488 (for EphB2 and neuropsin detection, respectively) along with goat anti-mouse Alexa Fluor 546 and goat anti-chicken Alexa Fluor 647 (for NeuN and GFAP detection, respectively) in the same buffer.

For double EphB2/neuropsin co-labelling, the same procedure as above was followed except sections were incubated in PBS-T containing goat and donkey serum. Also, the primary antibodies used were rabbit anti-neuropsin (1:200) and goat anti-EphB2 (R&D, 1:300) whilst the sequentially applied secondary antibodies were donkey anti-goat Alexa Fluor 546 as well as anti-rabbit Alexa Fluor 488. To visualise cell nuclei, 1nM TOTO-3 iodide (Molecular Probes) was applied.

Elevated Plus Maze

The elevated-plus maze test was performed as previously described (Pawlak *et al.*, 2003). The apparatus was made of four non-transparent black Plexiglas arms: two enclosed arms (50×10×30 cm) that formed a cross shape with the two open arms (50×10 cm) opposite each other. The maze was 55 cm above the floor and dimly illuminated. Wild-type and NP-/- mice were tested 18 hours after the restraint stress. Mice were placed individually on the central platform, facing an open arm, and allowed to explore the apparatus for 5 minutes. The behaviour was recorded with an overhead camera. The number of entries of the animal from the central platform (10×10 cm) to closed or open arms was

analysed with the ANY-MAZE software (Stoelting). The total number of entries into the four arms served as the indicators of total activity.

Open Field

Wild-type and NP-/- mice were placed in a 50x50x50 cm plexiglas box and were left free to move during 10 minutes. The box was cleaned with 70% alcohol after each session to avoid any odorant cues. A camera (Quickcam Sphere, Logitech) placed above the box recorded the session. Locomotor parameters were analysed with the ANY-MAZE software (Stoelting).

Fear Conditioning

EphrinB2$^{lx/lx}$ or EphrinB2-CaMKII-Cre mice were individually placed in the conditioning chamber (Coulbourn Instruments) for 2 minutes before they received three conditioned stimulus-unconditioned stimulus (CS-US) pairings. The last 2 seconds of the tone (CS, 30 seconds, 2.8 kHz, 85dB) were paired with the footshock (US, 2 seconds, 0.4mA) delivered through a grid floor. In control mice, the tone and footshock were delivered in a random manner. After training was completed, mice remained in the conditioning chamber for one more minute and were then moved to their home cage. The next day, the mice were placed back in the training chamber and freezing was monitored for 3 minutes to assess context-dependent learning. Cued-conditioning was evaluated 48 hours after training. The mouse was placed in a novel context (chamber with flat plastic floor and walls) for 2 minutes, after which the CS was

delivered (2 minutes, 2.8 kHz, 85dB) and freezing was monitored. Data was analysed using FreezeView software (Coulbourn Instruments).

Stereotaxic injections

Wild-type and NP-/- mice were anaesthetized with ketamine/xylazine (100 and 10 mg/kg respectively), placed in a stereotaxic apparatus and bilaterally implanted with stainless steel guide cannulae (26 gauge; Plastics One, Roanoke, VA) aimed above the basolateral complex of the amygdala (1.5 mm posterior to bregma, 3.0 lateral and 4.0 ventral from the surface of the scull). The cannulae were secured in place with dental cement. Dummy cannulae were inserted into all implanted cannulae to maintain patency. After one week, dummy cannulae were replaced with the injection cannulae (projecting 0.75 mm from the top of the guide cannulae to reach the basolateral complex of the amygdala) and the mice were injected with either an inhibitory anti-EphB2 antibody (R&D, 1 µl, 2 µg/ml) or recombinant neuropsin (R&D, 1 µl, 50nM) followed by 6-hour restraint stress in transparent plexiglass tubes. After the experiment, correct cannula placement was verified histologically by injecting 1.5 µl of PBS with bromophenol blue and analysing slices under the microscope.

Statistics

Student T-test (when two groups were compared) or analysis of variance (ANOVA) followed by Tukey's post-test were used as appropriate. P values of less than 0.05 were considered significant. The overall ANOVA p values are

reported in the text, the results of the post-test are indicated by the symbols of graphs.

Materials

Material	Company	Serial no
Acetic acid	Sigma Aldrich	A6283
Agarose	Sigma Aldrich	A9539
ANY-MAZE software	Stoelting	60000
Bovine serum albumin	Sigma Aldrich	A2153
Brain matrix	Stoelting	51380
Bromophenol blue	Sigma Aldrich	B3269
Camera - Quickcam sphere	Logitech	V-UCC22
Cell tracker	Invitrogen	E34250
Cellular protein fractionation kit	PerkinElmer	PRD101A001KT
Chicken anti-GFAP antibody	Abcam	ab4674
Chromo4/PTC-200 thermal cycler	MJ research	
Conditioning chamber	Coulbourn Instruments	
Coomassie Blue R250	Life Technologies	20278
Cy-5 conjugated donkey anti-human IgG	Jackson Immunoresearch	709-175-149
di-Water	Sigma-Aldrich	38796
Donkey anti-goat Alexa Fluor 488 antibody	Molecular probes	A11055
Donkey anti-goat Alexa Fluor 546 antibody	Molecular probes	A11056
Donkey anti-mouse Alexa Fluor 594 antibody	Molecular probes	A21203
Donkey serum	Jackson ImmunoResearch	017-000-121
Double guide cannulae	Plastics One	C313G(2)-G11/SP
DTT	Thermo-scientific	R0861
EDTA	Sigma-Aldrich	E6758
EGTA	Sigma-Aldrich	E3889
Fetal calf serum	Sigma-Aldrich	F0804
Finch software	Perkin Elmer	FinchTV
Fungizone	Life Technologies	15290-018
Freezeview software	Coulbourn Instruments	ACT-100A
Glycerol	Thermo Scientific	17904
Goat anti-chicken Alexa Fluor 647 antibody	Molecular probes	A-21449
Goat anti-EphA4 (N terminus) antibody	R&D	AF641
Goat anti-EphB2 antibody	R&D	AF467
Goat anti-EphB6 antibody	R&D	AF611
Goat anti-EphrinB2 antibody	R&D	AF496
Goat anti-NCAM-L1 antibody	SantaCruz biotechnology	SC-1508
Goat anti-mouse Alexa Fluor 546 antibody	Molecular probes	A11003
Goat anti-rabbit Alexa Fluor 488 antibody	Molecular probes	A11008
Goat IgG	Sigma	I 5256
Goat serum	Vector Laboratories	S-1000
HEPES	Sigma-Aldrich	H3375
HEPES NaOH	Sigma-Aldrich	H7006
HRP conjugated anti-mouse antibody	Vector-labs	PI-2000

HRP conjugated anti-goat antibody	Vector-labs	PI-9500
HRP conjugated anti-rabbit antibody	Vector-labs	PI-1000
Infusion dummy cannula	C313DC/1/SPC	C313DC/1/SPC
Infusion internal cannula	Plastics One	C313I/SPC
L-glutamine	Sigma-Aldrich	G3126
Lipofectamine 2000	Invitrogen	11668-019
MEM + Earles	Fisher Scientific	10454275
MgCl2	Sigma-Aldrich	M8266
Mouse anti-B-actin	Sigma	A5441
Mouse anti-EphA4 (C-terminus) antibody	Zymed	37-1600
Mouse EphA4Fc	R&D	641-A4
Mouse EphrinB2-FC	R&D	496-EB-200
Mouse anti-neuN antibody	Chemicon	MAB377
Mouse IgG	Thermo scientific	31903
NaCl	Sigma-Aldrich	S7653
NaF	Sigma-Aldrich	S7920
NaOH	Sigma-Aldrich	S8045
Na3VO4	Sigma-Aldrich	S6508
New born calf serum	Life Technologies	16010167
NP40	Life technologies	FNN0021
Nuclease free water	Life technologies	AM9916
Operon software	Operon biotechnology 2007	
Paraformaldehyde	Sigma-Aldrich	P6148
PBS	Sigma-Aldrich	P4417-50TAB
PBS (+Ca2+ + Mg2+)	Sigma-Aldrich	D1283-6X500ML
PCR primers	Invitrogen (custom)	
Penicillin-Streptomycin	Life Technologies	15070-063
Pierce Coomassie Bradford protein assay	Life technologies	23200
Plasminogen	R&D	1939-SE
Primer 3 software	Primer3	primer3.ut.ee
Protease inhibitors	Roche	1697498
QIAquick gel extraction kit	QIAgen	28704
QIAzol lysis reagent	QIAgen	79306
Rabbit anti pan-cadherin antibody	Abcam	ab16505
Rabbit anti-calpain	Abcam	ab28258
Rabbit anti-CREB antibody	Cell signalling	#9192
Rabbit anti-neuropsin antibody	Dr. Helena C. Castro, Niteroi, Brazil	
Rabbit anti-NR1 antibody	Upstate	AB9864
Rabbit anti-p75NGF antibody	Chemicon	AB1554
Rabbit anti-plasminogen antibody	Molecular innovations	IASMPLG-GF-HT
Rabbit anti-Vimentin	Abcam	ab45939
Recombinant Neuropsin	R&D	2025-SE
RNA Later	QIAgen	76104
RNase Erase	MP biomedicals	4821682

RNase H	Invitrogen	18021-014
RNase-Free DNase	QIAgen	79254
RNeasy lipid tissue mini kit	QIAgen	74804
Scion image	Scion image	
Skimmed milk	Marvel	
Sodium azide	Sigma	S2002
Stripping buffer	Pierce	21059
Super signal west pico chemiluminescent substrate	Pierce	34080
Superscript III and oligo (dT) primers	Invitrogen	18080-044
SYBR green Master mix	BioRad	179-8882
TOTO-3 iodide	Molecular probes	T3604
tPA (alteplase)	Abcam	ab92633
Tris	Sigma-Aldrich	T1503
Tris-HCL	Sigma-Aldrich	T5941
Triton X-100	Sigma-Aldrich	X100
Tween 20	Sigma-Aldrich	P1379
Vectamount medium	Vector laboratories	H-5000
Vibrating vibrotome (Vibraslice)	Campden instruments	HA752
Xylazine	Sigma-Aldrich	X1126
Zeiss confocal microscope	Zeiss	LSM5 exciter

Chapter 3. Eph Receptors and Neuronal Proteases

Introduction

Protease cleavage of Eph proteins

The *trans* interaction between the Eph receptor and the Ephrin ligand is just one of numerous interactions that occur between cells or at the cell/extracellular matrix interface. When changes in the cellular morphology occur, following stress for example, these interactions are often altered. Extracellular proteases are critical in reorganising the extracellular matrix and modulating molecular interactions. Not only do they act permissively to allow morphological adaptations, but their actions activate mechanisms that drive cellular responses.

An example of such protease-dependent regulation is seen in the Eph system. By offering attractant or repellent guidance cues, Eph receptors ensure correct axonal guidance during development. How Eph-Ephrin cell-to-cell binding can cause rapid retraction of an axon growth cone poses an interesting biological question (Lackmann *et al.*, 1997). One mechanism that allows this is endocytosis of the complete receptor-ligand complex by one cell (trans-endocytosis), allowing the cells to retract whilst the Eph-Ephrin interaction remains intact (Marston *et al.*, 2003; Zimmer *et al.*, 2003). A second mechanism, that utilizes proteolysis, involves cleavage of EphrinA2 from its membrane tether by a transmembrane metalloproteinase, ADAM10. Upon EphA3 binding EphrinA2, ADAM10 cleaves the ligand, which initiates axon withdrawal (Hattori *et al.*, 2000). ADAM10 also cleaves EphrinA5, an action

initiated by phosphorylation of the kinase domain of its binding partner, EphA3. The phosphorylation causes a conformational change in the EphA3 receptor as the kinase domain shifts away from the membrane, which in turn allows ADAM10 to engage more tightly with the EphA3-EphrinA5 complex (Janes *et al.*, 2009). The proteinase domain of ADAM10 now has access to a molecular recognition motif that has been formed by the binding of EphrinA5 to EphA3. Following cleavage, the complex is internalised and growth cone retraction occurs (Janes *et al.*, 2005). This mechanism ensures that cleavage of EphrinA5 is tightly regulated as ADAM10 only has access to the cleavage site upon receptor ligand binding.

There is also evidence that proteolytic cleavage is utilised during EphrinB mediated growth cone retraction. Upon EphB2 binding EphrinB1/2, metalloproteinases cleave the extracellular domain of both receptor and ligand (Georgakopoulos *et al.*, 2006; Litterst *et al.*, 2007).

Further evidence that EphrinBs were cleaved by proteases came from analysing presenilin 1 (PS1) knockout animals for potential substrates for γ-secretase cleavage. Within the knockout animals, an accumulation of EphrinB intracellular domain was found, which could be mimicked by inhibition of γ-secretase in wild-type cells (Georgakopoulos *et al.*, 2006; Tomita *et al.*, 2006). As γ -secretase is unable to cleave type 1 single span proteins in their full length form, it was evident that EphrinBs must undergo cleavage prior to the γ-secretase processing (Kopan & Ilagan, 2004). Using the same presenilin knockout system, it was shown that EphrinBs underwent cleavage by matrix

metalloproteinases (MMP) prior to the γ-secretase processing (Georgakopoulos *et al.*, 2006; Tomita *et al.*, 2006). Further study mapped the MMP cleavage site of EphrinB1 and MMP 8 was found to be the key protease cleaving this ligand (Tanaka *et al.*, 2007).

Y-secretase processing of EphrinB1 provides an exciting mechanism through which proteases may initiate cell signalling. Not only is the extracellular cleavage of EphrinB a mechanism for ectodomain shedding, but it leads to signal transduction beyond the usual Ephrin reverse signalling. Although the EphrinB intracellular domain does not contain a nuclear localisation signal (NLS), it does contain a basic amino acid structure which can act as an NLS and, when the intracellular domain was over-expressed, it was found to accumulate in the nucleus (Tomita *et al.*, 2006). Does it play any transcriptional roles? Although the EphrinB1 intracellular fragment has not been studied in this regard, other γ-secretase cleavage products both induce gene transcription and inhibit it (Kopan & Ilagan, 2004). Furthermore the γ-secretase cleavage of EphrinB2 leads to a direct interaction between the intracellular cleaved fragment and Src kinase. An EphrinB2 intracellular domain construct co-immunoprecipitates with Src kinase and acts to increase Src autophosphorylation. In turn, this acts as a negative feedback mechanism by causing EphrinB2 phosphorylation and thereby inhibiting its γ-secretase cleavage (Georgakopoulos *et al.*, 2006). The functional significance of γ-secretase cleavage of EphrinB2 is linked to alteration of cellular morphology through actin mobilisation. The EphrinB2 intracellular fragment induces the recruitment of Grb4 to EphrinB2 and the sprouting of endothelial cells.

However, Tomita *et al* found that the γ-secretase activity negatively regulated the protrusion of enriched F-actin processes from COS cells. Further evidence that the MMP cleavage of EphrinB alters cellular morphology has been shown in a cancer cell model. In pancreatic cancer cells, EphB2 binding causes EphrinB1 signalling to stimulate the release of MMP8 which in turn cleaves EphrinB1 along with degrading the extracellular matrix (Tanaka *et al.*, 2007). Furthermore, it is likely that other intramembrane proteases cleave Ephrins to either regulate their expression or initiate intracellular signalling. For example, a human rhomboid protease (RHBDL2) cleaves EphrinBs (EphrinB3 in particular), although the functional consequence of this event has not been elicited (Pascall & Brown, 2004/4/23).

Two studies have reported extracellular cleavage of EphB2 (Litterst *et al.*, 2007; Lin *et al.*, 2008). The studies identify two different stimulations that initiate the proteolytic processing of EphB2. Firstly, both groups investigated EphrinB2 binding dependent cleavage of EphB2. Lin *et al* found that EphrinB2 stimulation induces MMP-mediated cleavage of EphB2. Specifically, they demonstrate that MMP7 and MMP9 cleave EphB2 both *in vitro* and *in vivo.* This mechanism results in growth cone retraction and therefore describes an EphB2 process similar in function to the ADAM10 mediated EphrinA cleavage (Lin *et al.*, 2008). Similarly Litterst *et al* also found EphB2 proteolysis following EphrinB2 stimulation; however, the proteolytic cleavage was subsequent to endocytosis of the receptor. Following EphrinB2 binding, the EphB2 receptor was endocytosed and processed in the endosomal system. The cleavage was not MMP-dependent but was negatively regulated by a peptide inhibitor, ZVLL,

indicating activity of an unknown protease (Litterst *et al.*, 2007). It is possible that the proteolytic processing described by Litterst *et al* follows the alternative mechanism that allows EphB-EphrinB cell repulsion, where the receptor ligand complex is trans-endocytosed (Marston *et al.*, 2003; Zimmer *et al.*, 2003).

The second stimulation that induces extracellular proteolytic cleavage of EphB2 does not require ligand binding for the mechanism to occur, but is triggered by cellular calcium influx. The association of NMDA receptors and EphB2 receptors not only leads to NMDA regulation but proteolytic processing of EphB2 (Litterst *et al.*, 2007; Takasu *et al.*, 2002). NMDA-mediated calcium influx induces the ectodomain shedding of EphB2 by ADAM10. The cleaved extracellular portion is stable, and although it has yet to be investigated, has potential to act as a soluble ligand and bind cell tethered Ephrins. The ADAM10 cleavage of the EphB2 extracellular domain turns the remaining transmembrane EphB2 receptor into a substrate for γ-secretase activity (Litterst *et al.*, 2007). The presenilin processing of EphrinB produces an intracellular fragment that functions as a signalling peptide. The intracellular fragment released by γ-secretase cleavage still contains the kinase domain which was found to directly phosphorylate NMDA receptors and to increase their cell surface expression (Xu *et al.*, 2009).

Taken together, the literature shows that the extracellular cleavage of Eph proteins drives a number of processes that implicate this mechanism in neuronal plasticity. Through their cleavage the Eph proteins promote actin remodelling, extracellular matrix degradation, modulation of effector molecules

such as Src and regulation of NMDA receptors. This highlights the importance of proteolytic processing within the Eph/Ephrin system for facilitation of neuronal plasticity.

Figures

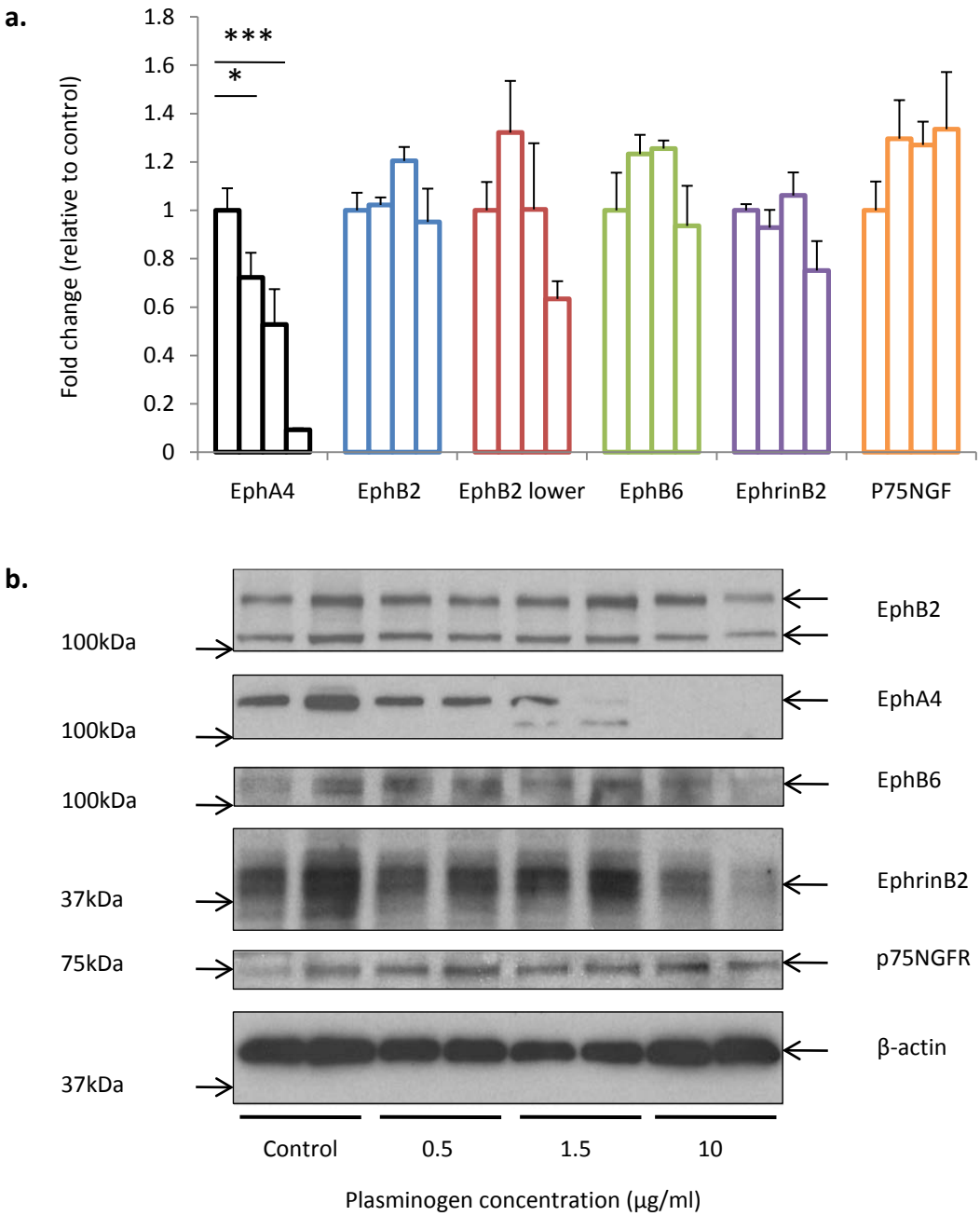


Figure 7. EphA4 is cleaved by plasmin in SHSY-5Y cells. tPA and plasminogen was added to SHSY-5Y cells for 15 minutes. Protease function was then inhibited, the cells homogenised and the protein expression analysed by Western blotting. The expression level of protein was normalised using β-actin and quantified. **(a).** The density of the EphA4 band showed a dose dependent decrease indicating a high sensitivity to plasmin ($F_{(3, 12)}$= 14.6; p<0.05 vehicle vs plasmin 1.5µg/ml, p<0.001 vehicle vs plasmin 10 µg/ml). No other Eph proteins showed a decrease at any concentrations of plasminogen. The trend towards a decrease seen at the highest concentration of plasminogen for EphrinB2 and the lower band of EphB2 represent unspecific cleavage. **(b).** Representative blots for Eph proteins, p75NGFR and β-actin. *= P<0.05 ***=P<0.001. n= 4 in each group.

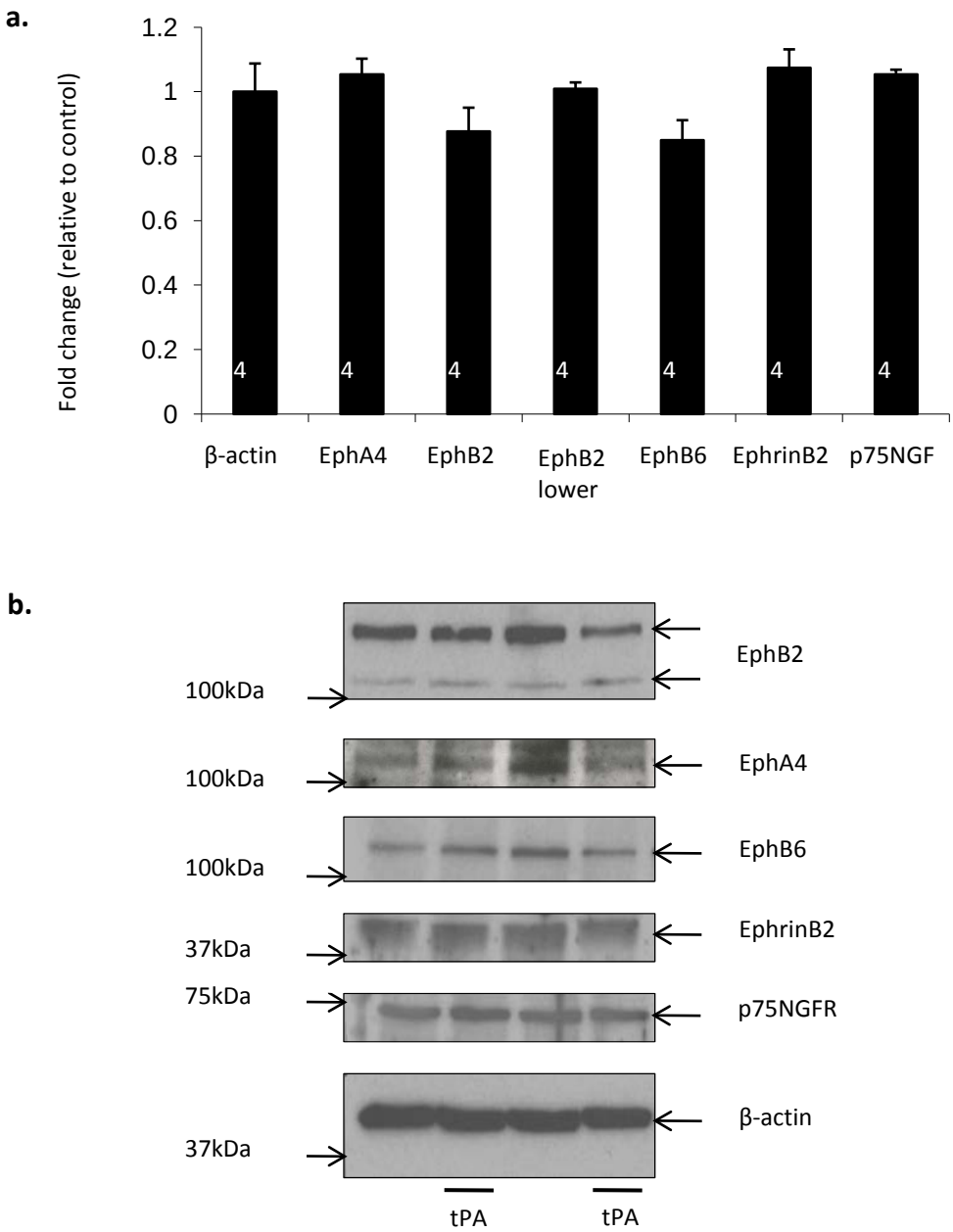


Figure 8. Eph receptors are not cleaved by tPA. tPA was added to SHSY-5Y cells for 15 minutes. Protease function was then inhibited, the cells homogenised and the protein expression analysed by Western blotting. The expression level of protein was normalised using β-actin and quantified. **(a).** The protease, tPA, did not cleave any of the proteins blotted for. **(b).** Representative blots for Eph proteins, p75NGFR and β-actin. Each lane represents one cell culture dish and alternate lanes are of those incubated with tPA.

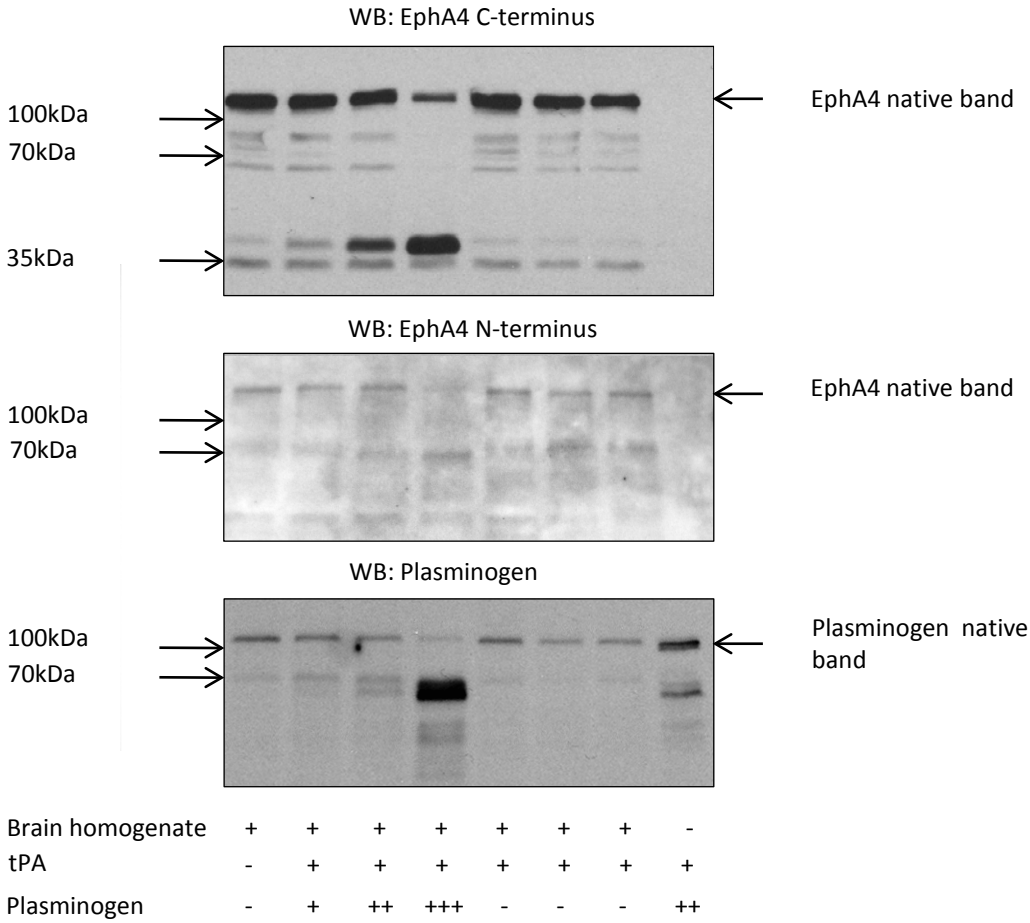


Figure 9. **Plasmin cleaves EphA4 in brain homogenate**. Wildtype hippocampi were homogenised and incubated with increasing concentrations of plasminogen and tPA , with tPA alone, or without proteases. Western blotting revealed that EphA4 was cleaved by plasmin in a concentration dependent manner. A decrease in the native EphA4 band using antibodies against the n-terminus and c-terminus of EphA4 was observed. Concomitant with the decrease in the native band an increase in a c-terminus fragment of approximately 40kDa and an increase in an n-terminus fragment of approximately 70kDa was also observed. The samples were also blotted for plasminogen which revealed native brain plasminogen. + tPA 1mg/ml plasminogen 0.5mg/ml. ++ tPA 1mg/ml plasminogen 1.5mg/ml. +++ tPA 1mg/ml plasminogen 10mg/ml . – no protease

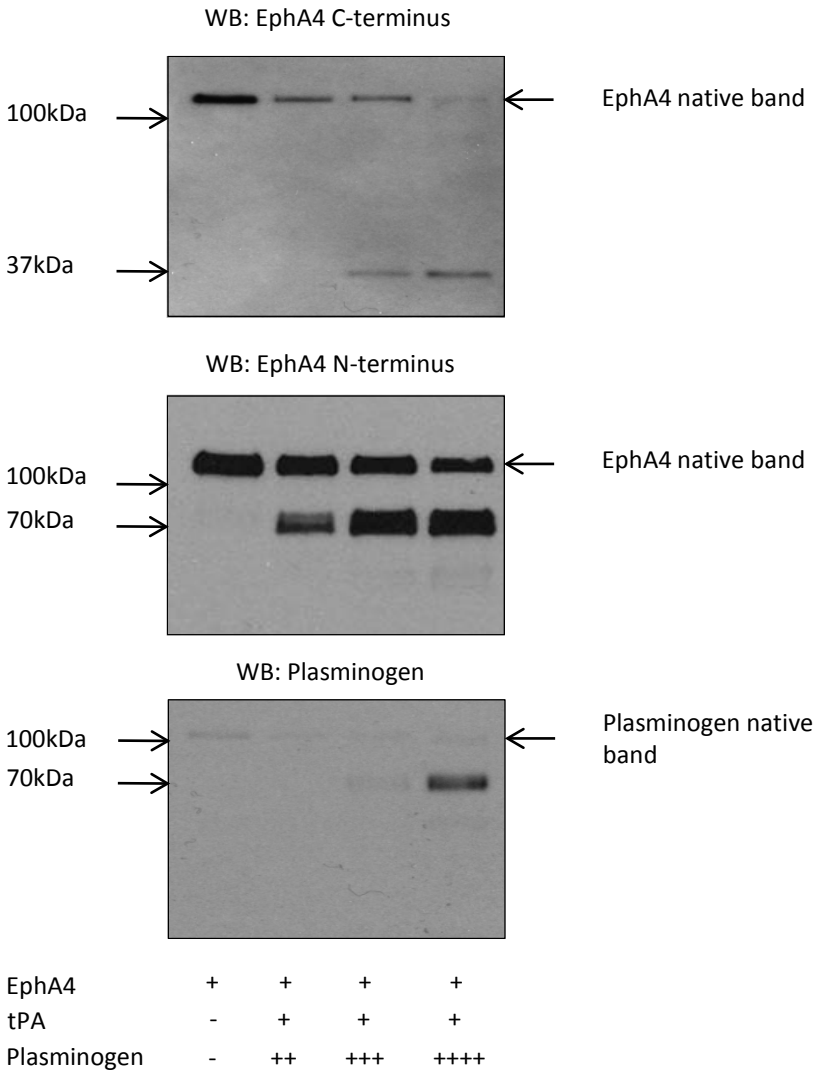
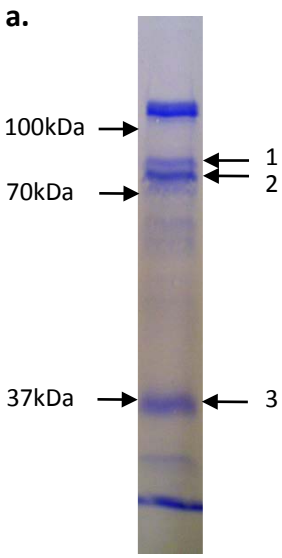


Figure 10. **Plasmin cleaves EphA4 Fc.** EphA4 Fc was incubated with increasing concentrations of plasminogen and tPA or without proteases in a hepes–tween buffer. A decrease in the native EphA4 band was accompanied with an increase of new bands. The strongest two bands are at approximately 70kDa and at approximately 37kDa. The smaller new band was recognised only by an antibody against the c-terminal portion of the Fc protein.



b.

VTGSRVYPAN EVTLLDSRSV QGELGWIASP LEGGWEEVSI
MDEKNTPIRT YQVCNVMEAS QNNWLRTDWI TREGAQRVYI
EIKFTLRDCN SLPGVMGTCK ETFNLYYYES DNDKERFIRE
SQFGKTDTIA ADESFTQVDI GDRIMKLNTE IRDVGPLSKK
GFYLAFQDVG ACIALVSVRV FYKKCPLTVR NLAQFPDTIT
GADTSSLVEV RGSCVNNSEE KDVPKMYCGA DGEWLVPIGN
CLCNAGHEEQ NGECQACKIG YYKALSTDAS CAKCPPHSYS
VWEGATSCTC DRGFFRADND AASMPCTRPP SAPLNLISNV
NETSVNLEWS SPQNTGGRQD ISYNVVCKKC GAGDPSKCRP
CGSGVHYTPQ QNGLKTTRVS ITDLLAHTNY TFEIWAVNGV
SKYNPSPDQS VSVTVTTNQA APSSIALVQA KEVTRYSVAL
AWLEPDRPNG VILEYEVKYY EKDQNERSYR IVRTAARNTD
IKGLNPLTSY VFHVRARTAA GYGDFSEPLE VTTNTVPSRT
IGDGANSTIE GRMDPKSCDK THTCPPCPAP ELLGGPSVFL
FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV
EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK
VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSRDELTKNQ
VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG
SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS
LSPGKHHHHH H

c.

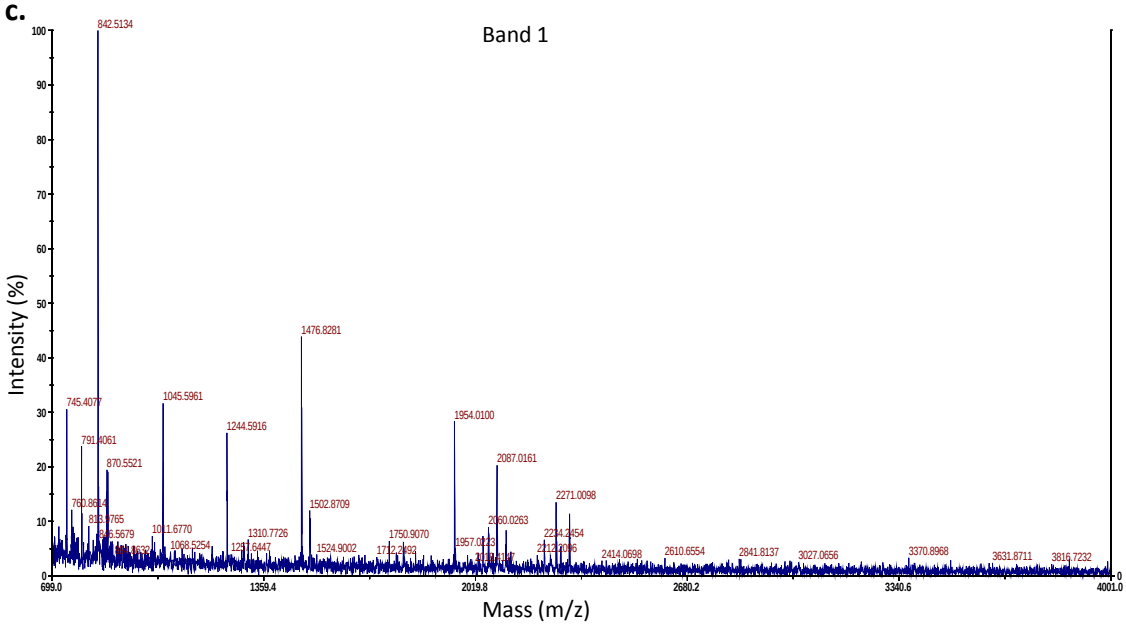


Figure 11 Mass spectrometry of EphA4 FC cleavage by plasmin. (a) The products from EphA4 Fc digestion by incubation with tPA and plasminogen were separated on an agarose gel and stained with Coomassie colloidal. Two bands at approximately 70kDa (1 and 2) and one at 37kDa (3) were analysed by mass spectometry. **(b).** Peptide mass fingerprinting of bands 1 and 2 revealed sequences, shown in red, from the extracellular domain of EphA4. The sequence closest to the carboxy-terminal was nine amino acids from the trans-membrane domain. Comparison with known plasmin cleavage sites indicated that Arg^{520}-Ile^{520} (indicated by the box) is the putative cleavage site. Band 3 consisted of sequences, shown in green, from the IgG sequence of EphA4 Fc. The linker sequence is shown in blue. **(c)** The peptide map for band 1**.** The peaks represent protein fragments produced by trypsin degradation of band 1. Each peak is analysed to identify its protein sequence.

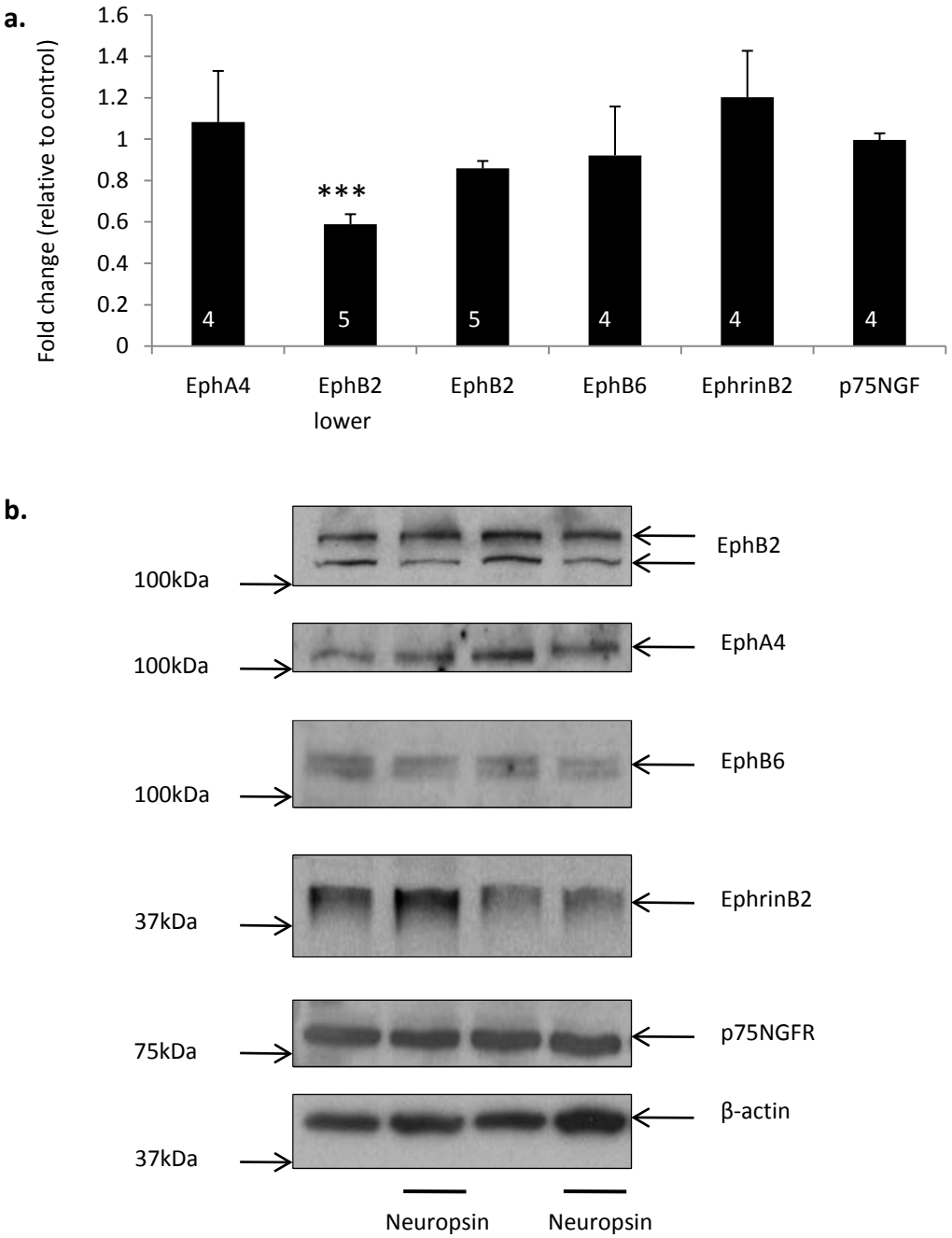


Figure 12. EphB2 is cleaved by neuropsin in SHSY-5Y cells. Neuropsin (50nM) was added to SHSY-5Y cells for 15 minutes. Protease function was then inhibited, the cells homogenised and the protein expression analysed by Western blotting. The membranes were incubated with antibodies against Eph proteins, p75NGFR and visualised using appropriate secondary antibodies. **(a).** The expression level was normalised using β-actin and quantified. Neuropsin treated groups relative to vehicle are plotted. Neuropsin caused a significant reduction in the lower native EphB2 band ($F_{(3, 18)}$= 11.24; p<0.001 EphB2 lower band vehicle vs neuropsin treated). **(b).** Representative blots for Eph proteins, p75NGFR and β-actin. *** = p<0.001. Numbers within bars represent n for each condition.

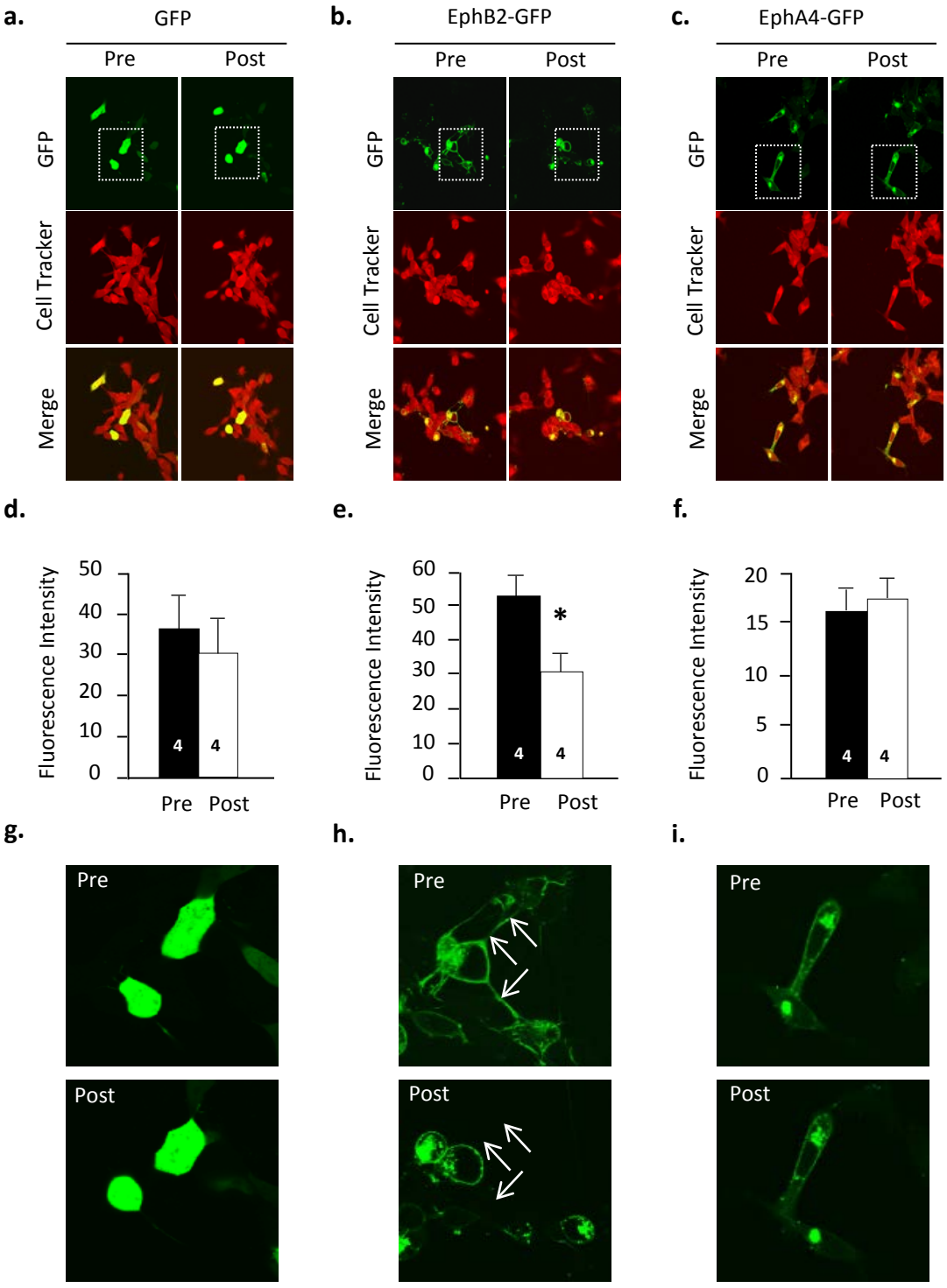
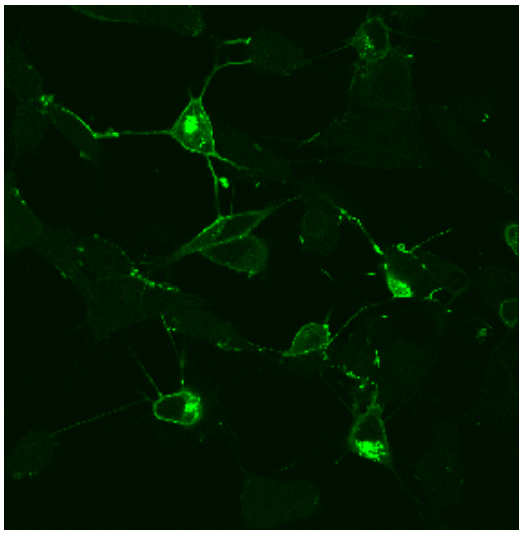


Figure 13. Neuropsin cleaves overexpressed EphB2-GFP . To confirm neuropsin-mediated cleavage of EphB2 we expressed free GFP (**a, d, g**) EphB2-GFP (**b, e, h**) or EphA4-GFP (**c, f, i**) in SH-SY-5Y cells, treated them with neuropsin for 15 minutes and analysed the intensity of membrane-associated GFP signal pre- and post-neuropsin. Cell tracker (red, Molecular Probes) was added to highlight cellular morphology. Neuropsin treatment resulted in a 45% loss of the membrane-associated EphB2 (T-test; $p<0.05$), while EphA4 and free GFP signals remained unchanged. High magnification of the framed areas for GFP, EphB2-GFP and EphA4-GFP (**g, h, i,** respectively). Results are shown as mean ±SEM. Digits inside columns indicate the number of observations. * = $p<0.05$

a.



b.

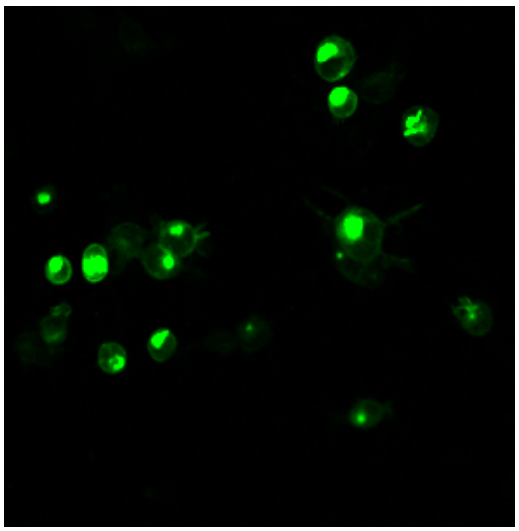


Figure 14. **Overexpression of EphB2-GFP in SHSY-5Y cells and HEK cells.** SHSY-5Y cells **(a)** and HEK cells **(b)** were transfected with EphB2-GFP vector and images were taken 24 hours post transfection. Note the distribution of the receptor both following the shape of the cellular membrane and also within intracellular stores

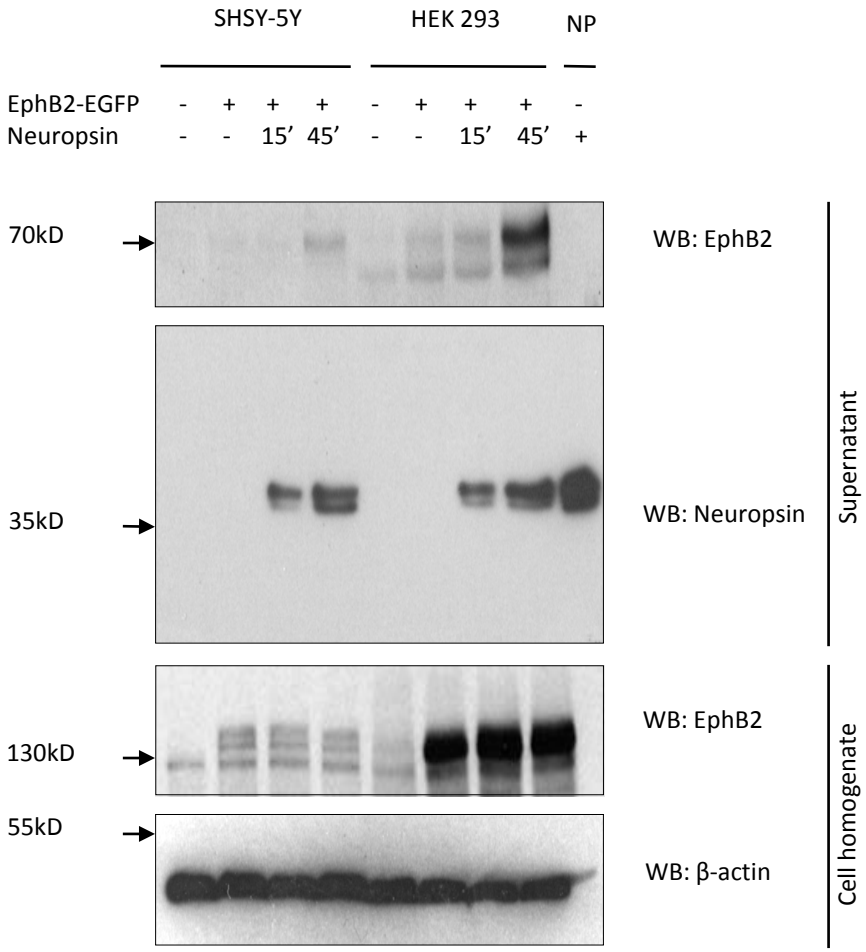


Figure 15. Cleavage of overexpressed EphB2-GFP reveals extracellular EphB2 fragment. SHSY-5Y and HEK293 cells were transfected with EphB2-GFP vector and incubated with neuropsin (300nM) for 15 minutes or 45 minutes. The proteins from the supernatant and cell homogenate were analysed by Western blotting. Extracellular domain fragments at approximately 70kD were detected in the buffer supernatant.

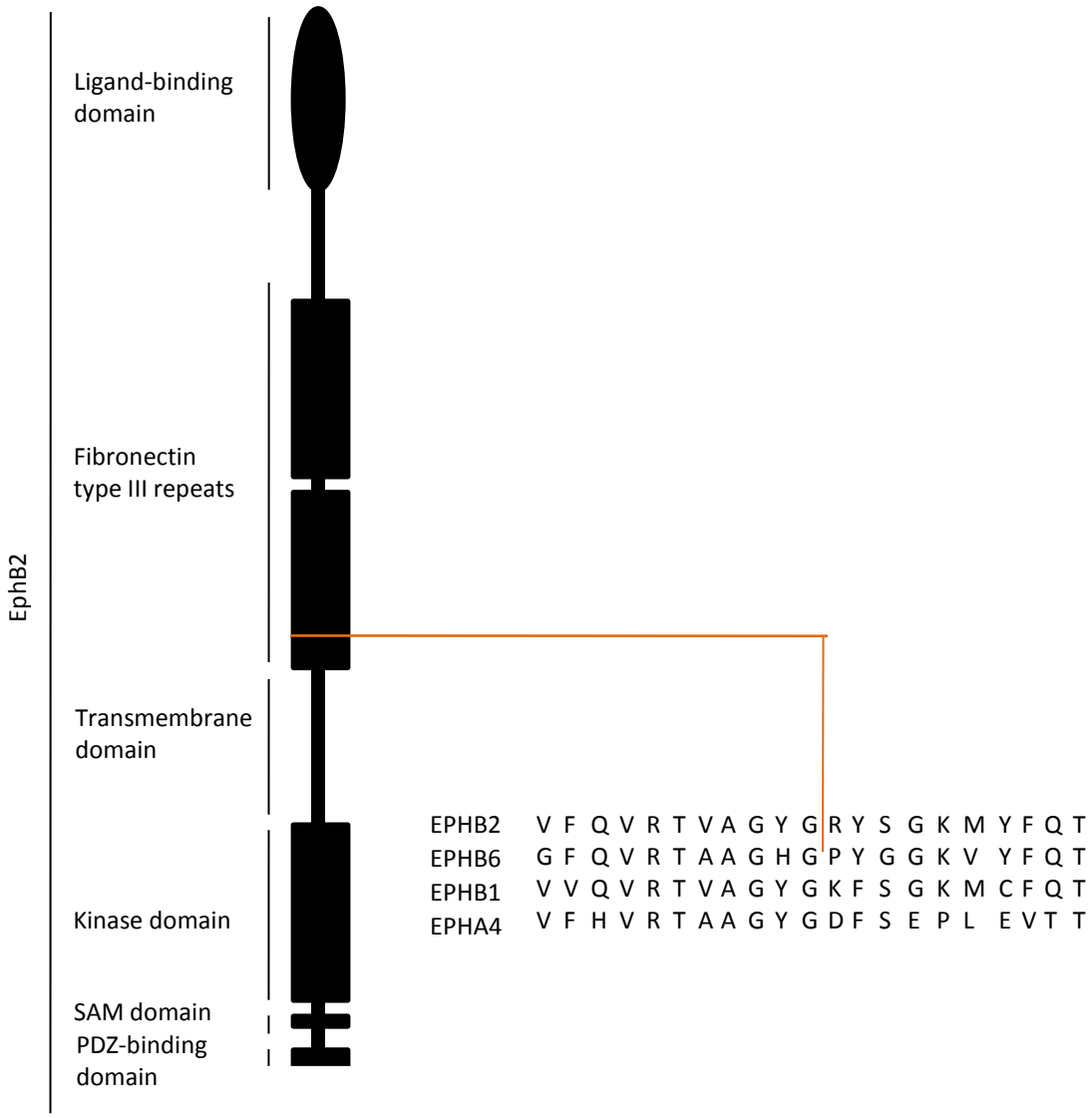


Figure 16. A Putative neuropsin cleavage site in EphB2. A scheme to show the structure of EphB2. It was previously demonstrated that neuropsin cleaves fibronectin thus making the fibronectin type III domain of EphB2 likely target for neuropsin-mediated proteolysis. Within this sequence we found a critical amino acid pair Gly-Arg at position 517 of EphB2 (orange box), consistent with the cleavage site found in fibronectin. Furthermore this sequence was not present in the fibronectin type III sequences of other Eph receptors, indicating specificity.

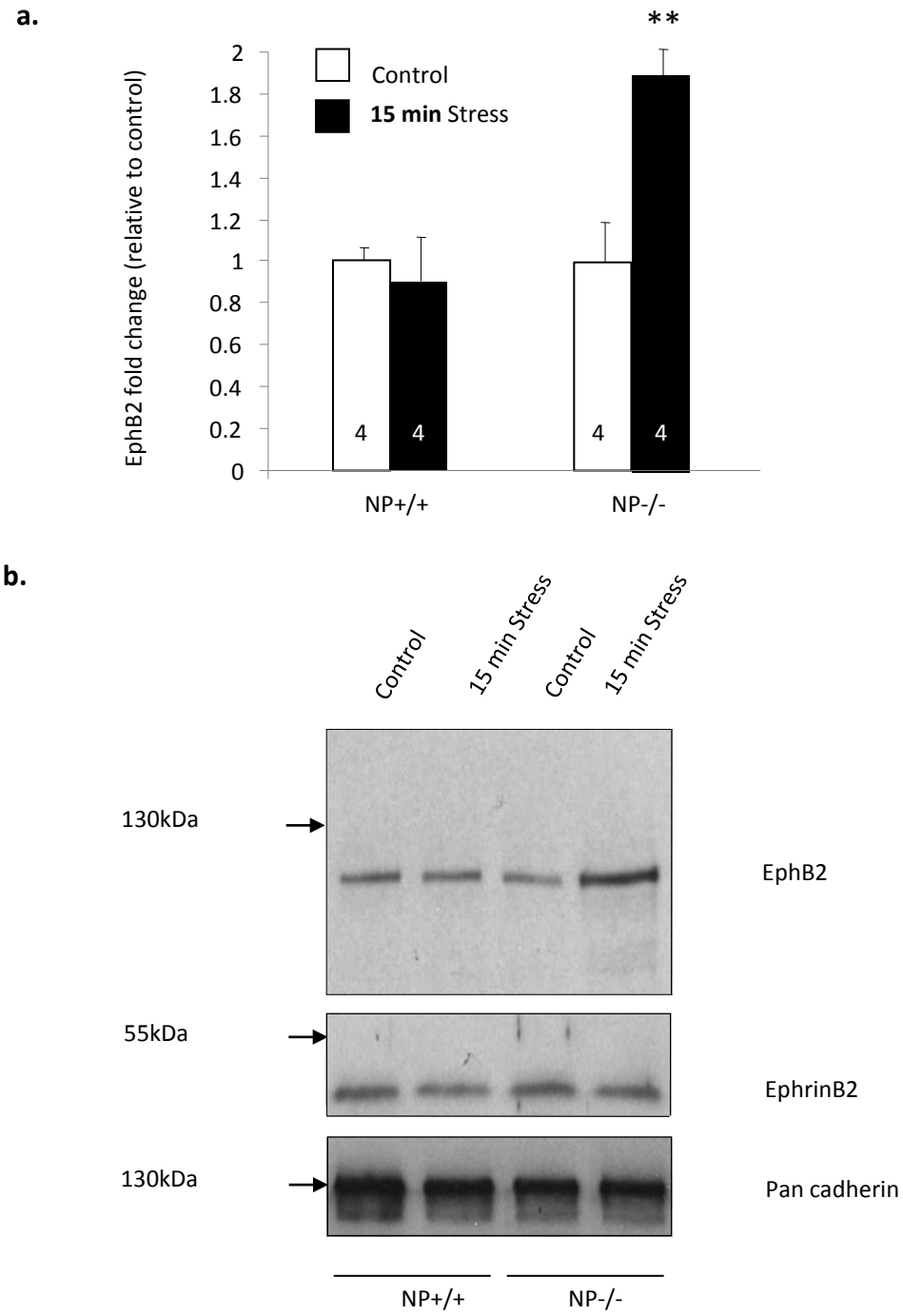


Figure 17. Membranous EphB2 increases shortly after stress, in the amygdala membrane of Neuropsin knockout mice. (a). Neuropsin+/+ and neuropsin-/- mice underwent 15minutes of stress, their amygdalae were dissected and the cellular fractions were separated. The membrane fraction was blotted for EphB2 and normalised used pan cadherin and Ephrin B2. An increase in membrane EphB2 was observed in neuropsin-/- mice but not in neuropsin+/+ mice ($F_{(3, 12)} = 6.40$; $p<0.01$ NP-/- control vs NP-/- stressed). **(b).** A representative blot against EphB2 in amygdala samples taken from neuropsin+/+ and neuropsin -/- mice. The EphB2 levels were normalised against levels of pan-cadherin and EphrinB2. ** = $p<0.01$

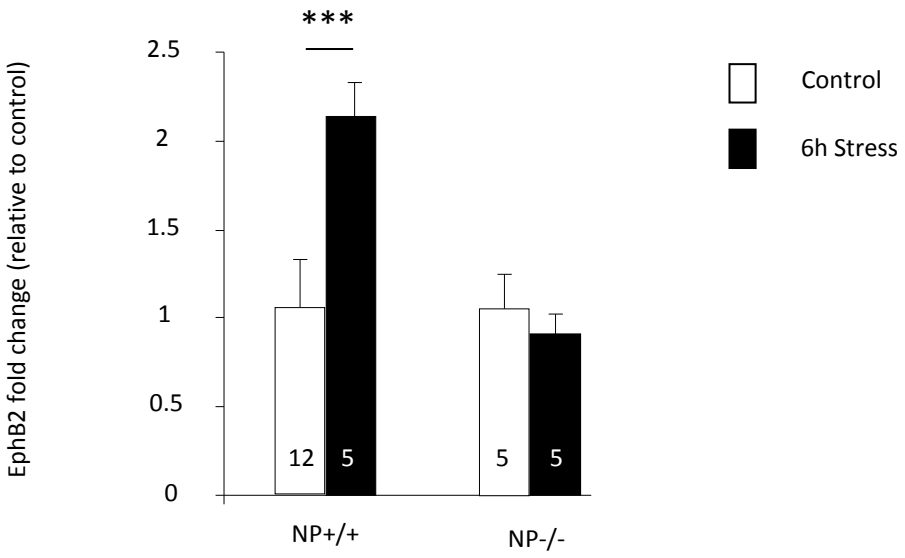


Figure 18. EphB2 gene expression is increased 6 hours following stress. Neuropsin+/+ and neuropsin-/- mice underwent 6 hours of stress and their amygdalae were dissected. mRNA was extracted, converted to cDNA and qRT-PCR was performed using EphB2 specific primers. A two fold increase in the EphB2 gene expression was observed in neuropsin+/+ mice ($F_{(3, 23)}$= 13.48; p<0.001 NP+/+ control vs NP+/+ stressed) not observed in neuropsin deficient animals. *** = p<0.001

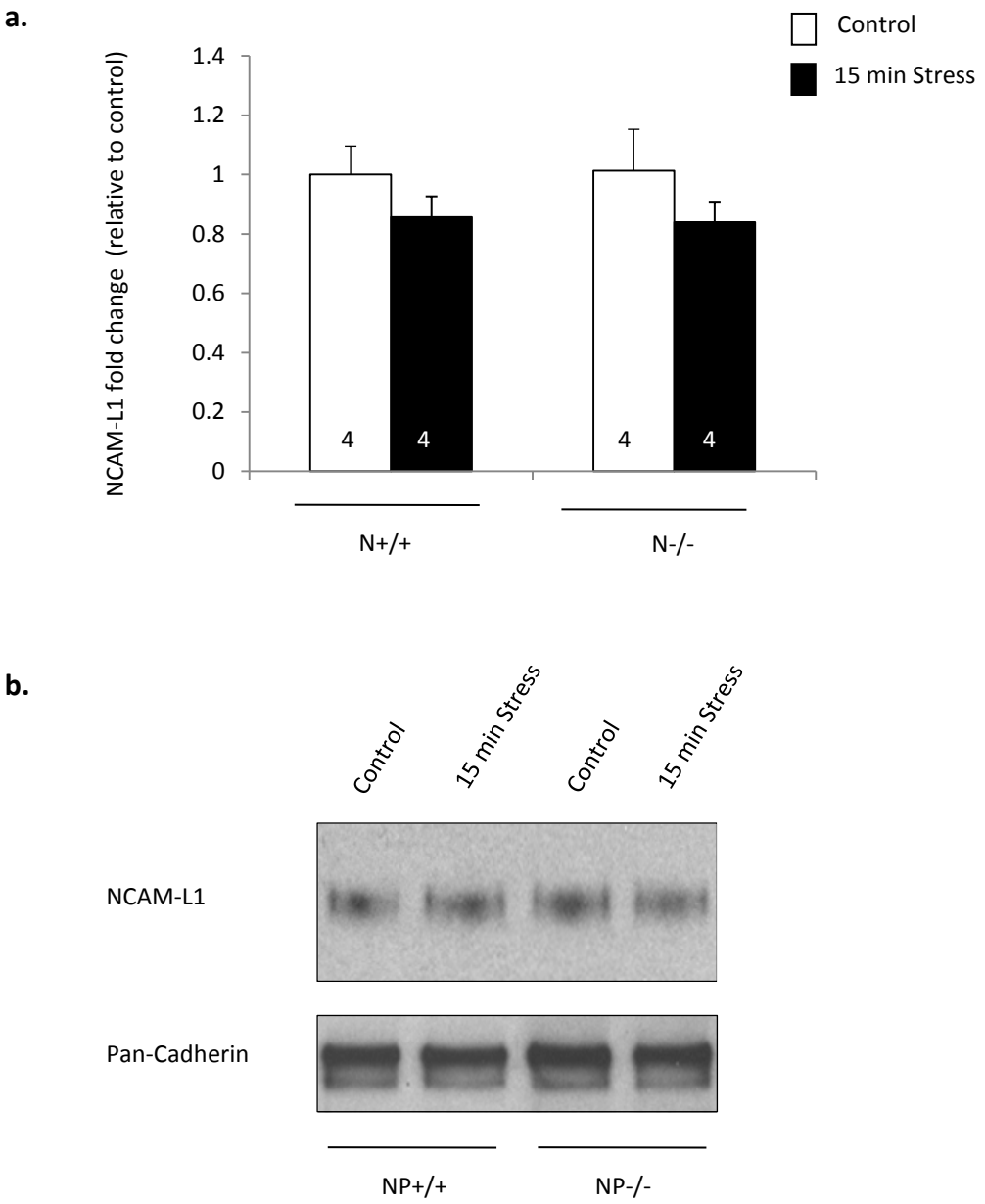


Figure 19. Neuropsin does not cleave L1CAM in the amygdala following stress. neuropsin+/+ and neuropsin-/- mice underwent 15minutes of stress and their amygdalae were dissected, and the cellular fractions were separated. The membrane fraction was blotted for NCAM-L1 and normalised used pan cadherin. **(a).** The levels of NCAM-L1, a known neuropsin substrate, did not change following stress in both neuropsin+/+ or neuropsin-/- mice. (**b)** A representative blot against NCAM-L1 in amygdala samples taken from neuropsin+/+ and neuropsin -/- mice. The NCAM-L1 levels were normalised against levels of pan-cadherin.

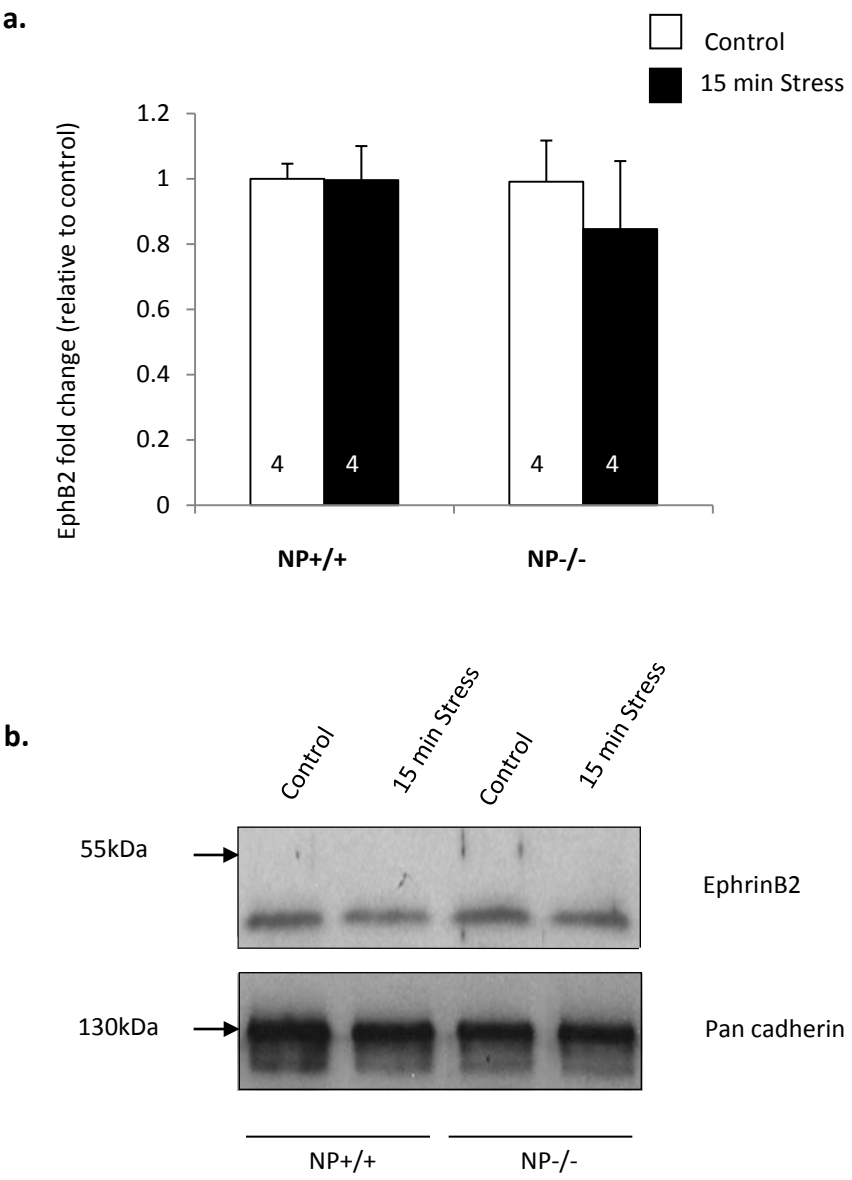


Figure 20. Neuropsin does not alter the membranous levels of EphrinB2 in the amygdala following stress. Neuropsin+/+ and neuropsin-/- mice underwent 15minutes of stress and their amygdalae were dissected, and the cellular fractions were separated. The membrane fraction was blotted for EphrinB2 and normalised used pan cadherin. **(a).** The levels of EphrinB2, did not change following stress in both neuropsin+/+ or neuropsin-/- mice. **(b).** A representative blot against EphrinB2 in amygdala samples taken from neuropsin+/+ and neuropsin -/- mice. The EphrinB2 levels were normalised against levels of pan-cadherin

Results

Eph receptors are cleaved by neuronal proteases

To examine if the tPA/plasmin system or neuropsin alter neuronal physiology through the Eph receptor system a number of Eph receptors were screened for sensitivity to tPA, plasmin or neuropsin cleavage. SHSY-5Y cells were incubated with either tPA alone, tPA and increasing doses of plasminogen or with recombinant neuropsin. After fifteen minutes of incubation, the protease activity was inhibited and the cells were homogenized. The homogenate was analysed by Western blotting with Eph receptor antibodies to reveal if the protease cleaved any of the receptors.

EphA4 is cleaved by plasmin

After incubation with tPA and increasing doses of plasminogen, the levels of EphA4 decreased dramatically indicating a strong sensitivity to cleavage by plasmin (Figure 7; $F_{(3, 12)}= 14.6$; $p<0.05$ vehicle vs. plasmin 1.5µg/ml, $p<0.001$ vehicle vs. plasmin 10 µg/ml). The decrease was dose-dependent and, at the highest concentration of plasminogen, the native EphA4 band was no longer present. In comparison, the levels of the other Eph molecules (Figure 7; EphB2 $F_{(3, 12)} = 1.70$; $p>0.05$, EphB2 lower; $F_{(3, 12)} = 2.26$; $p>0.05$, EphB6; $F_{(3, 12)} = 1.77$; $p>0.05$ and EphrinB2; $F_{(3, 12)} = 2.40$; $p>0.05$) did not decrease significantly with increasing concentrations of plasminogen, indicating that the cleavage of EphA4 was specific. The level of the p75-NGF, which was used as a control, was also unaltered (Figure 7**;** $F_{(3, 12)} = 0.89$; $p>0.05$). At the highest

concentration of plasminogen, the levels of the Eph receptors did show a trend towards a decrease, most likely due to unspecific cleavage by this broad-spectrum protease. Following incubation of the cells with tPA alone, the level of all the Eph receptors or p75-NGF were unaltered relative to the level of β-actin (Figure 8; EphB2 $F_{(3, 12)} = 5.52$; $p>0.05$, EphB2 lower; $F_{(3, 12)} = 5.52$; $p>0.05$, EphA4; T-test $p>0.05$, EphB6; T-test $p>0.05$ and EphrinB2; T-test $p>0.05$). Taken together, the screening experiment reveals a sensitivity of EphA4 to plasmin cleavage.

To confirm the sensitivity of EphA4 to plasmin cleavage, a similar experiment was performed using mouse brain tissue. Hippocampi were extracted from the brain of non-stressed wild-type mice and homogenized. They were then incubated with tPA and increasing doses of plasminogen. Following 15 minutes of incubation, the protease was inhibited and the protein analysed for EphA4 using Western blotting. Similarly to EphA4 in SHSY-5Y cells, the level of EphA4 decreased with increasing doses of plasminogen, confirming its sensitivity to plasmin cleavage (Figure 9).

To gain more information about the cleavage, the cleaved material was probed with one antibody recognizing an epitope at the c-terminus of the receptor (intracellular domain) and one recognizing an epitope towards the n-terminus of the receptor (extracellular domain). This revealed a single cleavage sites within EphA4, which generated a C-terminal fragment of approximately 40kDa and an N-terminal fragments of approximately 70kDa. This would correlate with an extracellular cleavage site close to the cell membrane. The blot was

also probed with an antibody against plasminogen, which showed that there was a small amount of native plasminogen present in the hippocampi homogenate. Incubating the hippocampi with tPA alone did not cause a decrease in the native EphA4 band confirming that tPA does not cleave EphA4 (Figure 9).

The plasmin cleavage site of EphA4 is close to the transmembrane domain

In order to characterize the plasmin cleavage site, EphA4 Fc was incubated with tPA and plasmin. EphA4 Fc is a recombinant protein containing the extracellular domain of EphA4 fused to the Fc fragment of human IgG. The cleavage pattern obtained following incubation with tPA and plasmin was very similar to that seen in hippocampal homogenate (Figure 10). The native EphA4 Fc band decreased with increasing plasminogen concentration, whilst a novel C-terminal band was identified at approximately 37kD. Closer inspection revealed two N-terminal bands of approximately 70kDa (Figure 10). The ability of plasmin to cleave EphA4 Fc allowed the sequences of three cleaved protein fragments to be identified by mass spectrometry. Following incubation with tPA and plasminogen, EphA4 Fc fragments were separated by electrophoresis and stained using Coomassie colloidal blue stain. Two bands identified at 70kDa and one at 37kDa were then excised from the gel and analysed by mass spectrometry (Figure 11, appendix 1 and appendix 2). Peptide mass fingerprinting of the band at 37kDa (Band 3) identified sequences from the human IgG portion of the chimeric protein, whilst the two bands at 70kDa identified sequences from the extracellular domain of EphA4. The fragment

closest to the transmembrane domain was just nine amino acids from the transmembrane domain of the EphA4 sequence, providing further evidence that the cleavage occurs close to the cell membrane.

EphB2 is cleaved by neuropsin

To investigate if neuropsin modulated Eph receptors the SHSY-5Y model was used again. The cells were incubated with recombinant neuropsin for fifteen minutes and Western blotting was used to analyse the Eph receptors. The density of each Eph band was normalized against the density of the actin band; controls (untreated samples) were compared to neuropsin-incubated samples. Whilst p75-NGF, EphrinB2, EphB6 and EphA4 showed no decrease in their levels following neuropsin incubation (Figure 12; T-test $p>0.05$), EphB2 receptor showed a significant decrease in the density of its native band indicating sensitivity to cleavage (Figure 12; $F_{(3,\ 18)}= 11.24$; $p<0.001$ EphB2 lower band control vs. neuropsin treated). The EphB2 receptor is expressed as two splice variants by SHSY-5Y cells. Following neuropsin incubation, the lower molecular weight splice variant (EphB2-S; Uniprot accession no. P29323-2) decreased in density levels by forty one percent.

To confirm the sensitivity of EphB2 to neuropsin cleavage, EphB2-EGFP, EphA4-EGFP or EGFP alone was expressed in SHSY-5Y cells and treated with recombinant neuropsin. The fluorescence intensity before and after neuropsin treatment was compared using confocal microscopy. This revealed a 45% decrease in the intensity of the GFP signal in cells that were expressing EphB2-EGFP (Figure 13; T-test $p<0.05$) but no change in the signal intensity

from the cells expressing either EphA4-EGFP (Figure 13; p>0.05 T-test) or EGFP (Figure 13; p>0.05 T-test) alone. The cellular morphology was highlighted by the use of cell tracker dye which showed the membranous location of the Eph receptor EGFP constructs and diffuse cytoplasmic distribution of the empty EGFP construct (Figure 13 and Figure 14). This distribution was also observed when HEK cells were transfected with the same EphB2 construct (Figure 14).

The neuropsin cleavage site of EphB2 is close to the transmembrane domain

When either SHSY-5Y or HEK293 cells that were expressing the EphB2-EGFP construct were treated with neuropsin, an extracellular fragment of approximately 70kDa was observed in the medium by Western blotting (Figure 15). Closer inspection revealed that there were two bands, one at 70kDa and one just smaller. Both fragments increased in density the longer the cells were incubated with neuropsin. The release of a 70kDa fragment to the medium would correlate with a neuropsin cleavage site in the EphB2 sequence close to the cell membrane within the fibronectin type III repeat sequences of the extracellular domain (Figure 4). Western blotting using the homogenate from the same cells revealed the presence of the EphB2-GFP construct 5-10kDa larger than the native EphB2 receptor (Figure 15).

It has been previously demonstrated that fibronectin is a cleavage substrate for neuropsin (Tani *et al.*, 2001) and so the known neuropsin cleavage sites were compared with the EphB2 fibronectin type III sequence for similarities. This

revealed a critical amino acid pair, Glycine - Arginine at position 517 of EphB2 (Figure 16). Comparison of this sequence with other Eph receptors showed that it is unique to EphB2 and explains the specificity of the cleavage. This indicates a putative cleavage site for neuropsin at Gly^{517}-Arg^{518} of EphB2.

In neuropsin deficient mice stress leads to an increased membrane EphB2 level in the amygdala

To Investigate if EphB2 is cleaved *in vivo* following stress, wild-type (NP+/+) and neuropsin-deficient (NP-/-) mice were subjected to fifteen minutes of restraint stress and their amygdalae dissected. The tissue was then separated into cellular fractions (Appendix 3 – courtesy of S. Patel) and the membrane fraction was analysed for the quantity of EphB2 by Western blotting (Figure 17). Without stress, there were no differences between membranous levels of EphB2 in wild-type and NP-/- mice. However, following stress, the levels of EphB2 in NP-/- mice increased, whilst in wild-type mice, they stayed constant (Figure 17; $F_{(3, 12)} = 6.40$; $p<0.01$ NP-/- control vs. NP-/- stressed).

To examine the Eph2 gene expression following stress, wild-type and NP-/- mice were subjected to six hours of restraint stress and the mRNA extracted from their amygdalae. Following conversion to cDNA, a qRT-PCR was performed using EphB2 specific primers. This revealed that the EphB2 gene was upregulated following stress (Figure 18; $F_{(3, 23)}= 13.48$; $p<0.001$ NP+/+ control vs. NP+/+ stressed), which was not observed in neuropsin deficient animals.

Previous data had shown that neuropsin cleaves L1CAM during E-LTP (Matsumoto-Miyai *et al.*, 2003). To investigate if neuropsin also cleaves L1CAM following stress, the levels of membranous L1CAM in NP+/+ and NP-/- mice were measured fifteen minutes after stress. The levels of L1CAM were not altered at this time point consistent with EphB2-specific neuropsin activity *in vivo* (Figure 19; $F_{(3, 12)} = 0.8754$; $p>0.05$). To examine other mechanisms that may influence the membranous levels of EphB2 following stress, the membranous levels of EphB2 binding partner, EphrinB2 were measured. However EphrinB2 levels were not altered following stress in either genotype (Figure 20; $F_{(3, 12)} = 0.31$; $p>0.05$)

Discussion

Summary

This chapter has described the identification of two previously unknown protease cleavage sites in Eph receptors implicated in stress-related neuronal plasticity. Firstly, plasmin cleaves EphA4 in a specific and dose-dependent manner. Secondly, neuropsin cleaves EphB2 *in vitro* and in response to stress *in vivo*. The Eph cleavage events share some common characteristics. They are both specific to particular Eph receptors as the proteases do not cleave other Eph receptors *in vitro*. Both are indicative of a process called shedding, as the cleavage sites are close to the cell membrane. This results in the release of a large proportion of the Eph receptor's extracellular domain. These cleavage events are likely to influence neuronal plasticity during stress, as both the protease and the cleavage target have been implicated in experience-dependent neuronal plasticity. Both plasmin and neuropsin have also been previously implicated in stress-related neuronal physiology.

Cleavage of EphA4 by plasmin

To my knowledge, the literature does not record any cleavage of EphA4 by a protease. The experiments presented here indicate that the extracellular domain of EphA4 is highly sensitive to plasmin cleavage. This is likely to occur in the hippocampus *in vivo* as hippocampal EphA4 is highly prone to plasmin cleavage *in vitro* (Figure 9). Both our work and other studies indicate that EphA4 is the most highly expressed Eph receptor in the hippocampus (Liebl *et*

al., 2003), preliminary data). EphA4 expression in the hippocampus spatially co-localises with the activity of the tPA-plasmin system (Appendix 4, collaborative data with Professor Wilczynski). The tPA activity within the brain is tightly restricted, but the hippocampus is one of the regions where its activity is prominent (Pawlak *et al.*, 2003; Sappino *et al.*, 1993).

Although tPA influences neuronal plasticity itself, our model suggests that activation of plasminogen is needed for EphA4 processing as tPA alone does not cleave this receptor. Plasminogen is mainly produced in the liver and exported to the blood to exert fibrinolytic activity. To play a role in neuronal physiology, it must either be transported across the blood brain barrier or be produced by neuronal tissue. Small amounts of plasminogen mRNA have been found in the hippocampus, suggesting that it may be produced by neuronal cells (Sappino *et al.*, 1993). Using a more accurate method of mRNA measurement, Tsirka *et a*l studied the neuronal tPA and plasminogen synthesis in detail (Tsirka *et al.*, 1997). Within the hippocampus, tPA mRNA was found along the neuronal cell layers but appeared to be expressed by microglia. Plasminogen mRNA was also found along the hippocampal cell layers but was only expressed by neurons. Furthermore the authors observed plasminogen mRNA in the dendrites, indicating a local post-synaptic production and release (Tsirka *et al.*, 1997). The presence of plasminogen protein in the hippocampus has also been confirmed by immunohistochemistry (Basham & Seeds, 2001). Our immunohistochemistry indicates that EphA4 is also predominantly expressed in post-synaptic neuronal processes (Chapter 4). Previous work has also shown that EphA4 is located to both dendritic

spines (Murai *et al.*, 2003) and astrocytic processes (Tremblay *et al.*, 2007). Taken together, EphA4 expression in the hippocampus spatially co-incides with tPA/plasmin system activity.

Functional studies have shown that plasminogen can be converted to plasmin in the hippocampus, most strongly in the CA1 region (Pawlak *et al.*, 2005). Importantly the tPA-plasminogen is upregulated in the hippocampus following chronic stress (Salles & Strickland, 2002; Pawlak *et al.*, 2005). The levels of plasminogen protein in the hippocampus have been observed to increase following excitotoxic injury. This increase was observed in the mossy fibre pathways and the hilus of the dentate gyrus. However, the authors could not exclude that this was due to plasminogen leakage from the bloodstream due to the nature of the injury (Salles & Strickland, 2002). Nevertheless, this line of evidence reiterates co-localization of the tPA-plasminogen activity in the hippocampus and EphA4 expression. Along with the cleavage studies I have performed, this suggests that stress is likely to induce modulation of EphA4 through direct cleavage by plasmin.

Plasmin-mediated cleavage of EphA4 and synaptic plasticity

EphA4 plays multiple roles in hippocampal neuronal functioning including the modulation of neuronal plasticity. EphA4 plays a crucial role in maintaining normal dendritic spine morphology. It is expressed on dendritic tips and interacts with astrocytic EphrinA3 to alter spine morphology (Murai *et al.*, 2003). Inhibiting the interaction between EphA4 and EphrinA3 resulted in spines with an irregular shape and with thinner spines heads (Murai *et al.*,

2003). Potentially, plasmin may provide a rapid, transient, activity-dependent mechanism of altering dendritic spine morphology by modulating the EphA4-EphrinA3 interaction.

This modulation may directly alter hippocampal plasticity. Following stress, the increase in plasmin activity in the hippocampus leads to a decrease in NMDA levels (Pawlak *et al.*, 2005). This modulation of NMDA levels could at least in part be due to an increase in synaptic glutamate levels following stress (Lowy *et al.*, 1995). Astrocytes are the main regulator of glutamate in the extracellular space. They contain glutamate transporters, which remove excess glutamate from the synapse in order to prevent glutamate excitotoxicity (Bergles & Jahr, 1998). The EphA4 – EphrinA3 interaction reduces the glutamate transporter current and thereby facilitates hippocampal plasticity (Filosa *et al.*, 2009). Thus, plasmin could alter NMDA levels and neuronal plasticity following stress by modulating the EphA4-EphrinA3 regulation of astrocytic glutamate transporters.

EphA4 may also mediate neuronal plasticity through its interaction with EphrinB2. The LTP mediated by EphrinB2 is dependent on the phosphorylation of its tyrosine residues (Bouzioukh *et al.*, 2007). The data from our studies suggests that cleavage of EphA4 by plasmin reduces the phosphorylation of EphrinB2 (Unpublished data from our lab). It is therefore possible that stress-induced plasmin activity could modulate the hippocampal stress response through cleavage of EphA4 to reduce EphrinB2 phosphorylation and therefore LTP. This mechanism is congruent with the

impairment in LTP that can occur in the hippocampus following stress (Wiegert *et al.*, 2006; Foy *et al.*, 1987). The relationship between stress, LTP and cognitive functions is not well understood. A number of factors including the type of LTP, level of sympathetic drive, area of the brain, the stage of stress and nature of the individual experiencing the stress all influence the level of LTP following stress (Joels & Krugers, 2007). Given this complexity and the range of neuronal mechanisms that Eph receptors regulate, it is likely that the modulation of EphA4 by a stress-induced protease will regulate the stress response in a number of different ways.

A putative plasmin cleavage site in EphA4

The experiments, of whose results are shown in figures nine, ten and eleven, were designed to gain more information about the cleavage of EphA4 by plasmin. Plasmin cleaved EphA4 in hippocampal homogenate producing bands at approximately 70kDa and 40kDa, whilst the native band decreased (Figure 9). This pattern is indicative of a single cleavage site in the extracellular domain of EphA4. In contrast the cleavage of EphA4 FC by plasmin indicated more than one cleavage site as two bands at approximately 70kDa were produced (Figure 10). Thre may be a number of reasons for this difference. It may be that the EphA4 splice variant sequence used for EphA4 FC production is different to that which is expressed in the hippocampus. Alternatively EphA4 from the hippocampus may have interacting partners that mask plasmin cleavage sites. It may also be that the Western blotting resolution did not allow identification of two bands at 70kDa.

To identify a putative cleavage site the two bands at approximately 70kDa and the band at approximately 40kDa were analysed by mass spectrometry. The peptide mass fingerprinting of the two bands at approximately 70kDa identified peptides in the extracellular domain of EphA4. The fragment closest to the transmembrane domain, identified in both bands, was nine amino acids from the transmembrane domain (Figure 11 and appendix 1). The inference from this is that the putative cleavage site would be between Arg^{520}-Thr^{529}. To identify a putative cleavage site this sequence was analysed for matching plasmin cleavage consensus sequences using the MEROPS and Cutdb databases. This revealed that Arg^{520}-Ile^{520} was the only matching cleavage site.

Cleavage of EphA4 by plasmin at this site is in keeping with other features characterizing plasmin activity, particularly cleavage at P1 Arginine sites. Arginine is the most common P1 amino acid (sixty five cleavage targets) in plasmin cleavage events (MEROPS database). The plasmin cleavage site in tPA is an Arginine – isoleucine bond that cleaves pro-tPA into two active tPA molecules (Pohl *et al.*, 1984). Plasmin is most known for its fibrinolytic action to maintain the haemostasis/fibrinolysis balance within blood circulation. This is achieved rapidly because plasminogen binds to fibrin at lysine and Arginine residues, and when activated to plasmin, cleaves the molecules C-terminally of these residues (Cesarman-Maus & Hajjar, 2005).

The putative cleavage site in EphA4 is found within a fibronectin domain N-terminal to the transmembrane domain. Our experimental data indicated that

the other Eph receptors tested were not sensitive to plasmin proteolysis. Analysis of the sequences of other Eph proteins revealed an absence of the Arginine-isoleucine bond in this domain, which is likely to explain why the other Eph receptors tested were not cleaved by plasmin (Figure 7).

Plasmin demonstrates specificity similar to trypsin, which shows preferential cleavage at arginyl bonds (Morris *et al.*, 1981). This highlights the limitations of this method to identify a cleavage site. The mass spectrometry employed in the experiments described above uses trypsin to cleave the initial protein fragment into smaller fragments which are subsequently analysed to identify the amino acid sequence. The modified trypsin used cleaves at the c-terminal side of Lysine and Arginine bonds, except when the following amino acid is Proline. Therefore it cannot be ruled out that the identified putative cleavage site is a result of trypsin cleavage rather than plasmin cleavage.

Apart from the 3 fragments analysed by mass spectrometry, the Comassie blue stain of the gel following plasmin cleavage of EphA4 Fc (Figure 10) reveals that there is likely to more than one plasmin sensitive cleavage site in EphA4. Therefore it cannot be excluded that plasmin cleaves EphA4 at other neighbouring putative cleavage sites (e.g. Arg ^{498}-Thr ^{499}).

A putative neuropsin cleavage site in EphB2

The extracellular cleavage site of EphA4 by plasmin is located close to the cell membrane. This phenomenon is also shared in the second novel proteolytic

event I report, namely that of EphB2 cleavage by neuropsin. As with EphA4, this was initially indicated by the size of the novel bands generated by incubation of cells with the protease. Similarly, the size of the EphB2 extracellular band found in the medium correlated with a cleavage site close to the cell membrane, approximately 70kD. One of the first neuropsin substrates described was fibronectin (Shimizu *et al.*, 1998). The extracellular domain closest to the cell membrane in the EphB2 sequence is the fibronectin repeat domain, indicating that it may be sensitive to neuropsin cleavage (Figure 4). Analysis of this region revealed a Gly-Arg sequence similar to a previously published neuropsin cleavage sequence (Shimizu *et al.*, 1998). Similarly to EphA4, this target sequence is absent from this domain in other Eph receptors.

Shedding of the extracellular receptor domain

In both Eph receptor cleavage events, the majority of the extracellular domain of the Eph receptor is released to the extracellular compartment. Shedding an extracellular fragment may result in an active molecule altering neuronal physiology. Our experimental data indicates that this would be more likely in the case of EphB2 than EphA4. In the medium an EphB2 extracellular fragment from cells treated with neuropsin was identified (Figure 15). However, in the case of EphA4, it appeared that plasmin caused further degradation of the released EphA4 fragment (Figure 9). The literature describes a number of mechanisms by which shedding alters molecular signalling. The release of the Eph extracellular fragment would contain the ligand binding domain and would signal in a distinct way to the intact transmembrane receptor. It may act as a competitive inhibitor of cognate Ephrin ligands, a phenomenon seen in

interleukin-1 and TNF signalling (Rose-John & Heinrich, 1994). This would also explain the use of Ephrin and Eph-Fc molecules to inhibit Eph receptors' function. Alternatively, the cleaved fragment may also act as an agonist, precipitating Ephrin-mediated reverse signalling. This has been observed in LPS signalling when the LPS receptor binds to cells and renders them sensitive to LPS signalling (Bazil, 1995).

The role of EphB2 and neuropsin in the early stress response

The ability of proteases to respond to rapidly changing physiological conditions has marked them as promising candidates for driving experience-dependent neuronal plasticity. Matsumoto-Miyai *et al* demonstrated that neuropsin is released in the hippocampus *in vivo* following LTP induction (Matsumoto-Miyai *et al.*, 2003). Following theta burst stimulation of the Shaffer collaterals, neuropsin was rapidly and transiently activated. The peak activity was at five to six minutes following stimulation (Matsumoto-Miyai *et al.*, 2003). Over a longer period stress upregulates neuropsin expression in the hippocampus. Following acute restraint stress, neuropsin mRNA is elevated for at least twenty-four hours following cessation of the stress (Harada *et al.*, 2008). The amygdala is a critical brain structure in co-ordinating the stress response and shows high neuronal activity following stressful events (Akirav *et al.*, 2001). In addition to the hippocampus, the highest expression of neuropsin is observed in the lateral amygdala (Chen *et al.*, 1995). Indeed, investigations in our laboratory have found that neuropsin protein is elevated in the amygdala following six hours of restraint stress (Attwood *et al.*, 2011). Taken together, the literature and the finding that neuropsin cleaves EphB2 *in vitro* indicates that this

cleavage event may occur on the amygdala over the minutes following stress. To test this hypothesis the membrane quantity of EphB2 was measured before and after stress in wildtype and neuropsin knockout mice. The predicted result would be a decrease in membrane EphB2 in wildtype mice but not in neuropsin knockout animals. However this was not observed (Figure 17). Rather, no differences in the membrane level of EphB2 was observed in the wildtype mice, whilst in mice deficient for neuropsin the level of membrane EphB2 increased. This indicates that neuropsin acts in the first 15 minutes following stress to regulate the membrane level of EphB2. This may be directly through cleavage of EphB2 or through indirect mechanisms.

If it were through direct cleavage it would infer that in the fifteen minutes following stress EphB2 receptors being trafficked to the cell membrane, whilst over the same time period neurospin cleaves a proportion of membranous EphB2. This would indicate the dynamism of neuronal physiology following stress and is consistent with the role of EphB2 being trafficked to the membrane to regulate synaptic plasticity. In the hippocampus, NMDA receptors are rapidly inserted in the postsynaptic membrane after LTP (Grosshans *et al*., 2002). Nolt *et al* have recently shown that EphB2 receptors regulate the amount of synaptic NMDA receptors and also NMDA Ca2+ desensitisation (Nolt *et al.,* 2011). Furthermore, cleavage of EphB2 by ADAM10 protease leads to an increase in membrane NMDA receptors (Xu *et al.,* 2009). Work from our laboratory shows that neursopsin knockout animals have decreased NMDA currents (Attwood *et al.,* 2011). The literature discussed and the result in Figure 17 could indicate this deficit is due to lack of

neurospin cleavage of EphB2 trafficked to the membrane. The interaction of EphB2 and NMDA receptors are explored further in the next chapter.

The trafficking of EphB2 to the membrane and regulation through direct cleavage could also play a role in stress-induced regulation of neuronal morphology. Following acute stress, amygdala neurons increase spine density (Vyas *et al.*, 2002). The increase in spine density is likely to be secondary to increased glucocorticoid levels in the amygdala (Mitra & Sapolsky, 2008). EphB2 may play a downstream role in the glucocorticoid pathway, modulating spine morphology through its interaction with Glutamate Receptor Interacting Protein 1 (GRIP1). This is a multi-PDZ domain scaffold protein that regulates the trafficking of AMPA receptors. It is likely to play a role in the glucocorticoid regulation of AMPA receptors following stress (Krugers & Hoogenraad, 2009). The trafficking of EphB2 from the Golgi apparatus to the cell membrane is regulated by GRIP1. When Hoogenrad *et al* inhibited GRIP1, they observed that dendritic morphology was not maintained and EphB2 accumulated in the Golgi membrane (Hoogenraad *et al.*, 2005). Thus, the possible stress-related trafficking of EphB2 to the membrane (Figure 17) could underlie the stress-induced morphological changes through its interaction with GRIP1.

Indirectly it may be that the Neuropsin deficient mice have a stress induced requirement for increased membranous EphB2 receptors. As discussed above neuropsin knockout mice have decreased NMDA currents in the amygdala and this may result in compensation after stress requiring an increase in membrane EphB2.

The lack of neuropsin may also cause an indirect upregulation of membrane EphB2 through previously described LTP regulation. Matsumoto Miyai *et al* discovered that neuropsin activation in the hippocampus resulted in the cleavage of the synaptic adhesion molecule L1CAM within ten minutes following NMDA stimulation (Matsumoto-Miyai *et al.*, 2003). As this synaptic adhesion molecule contributes to E-LTP, they reasoned that this was the mechanism through which neuropsin regulates LTP in the hippocampus (Komai *et al.*, 2000; Luthl *et al.*, 1994). This is less likely be the cause of the increased membrane EphB2 in nuropsin knockout mice. Whilst EphB2 regulates the late phase of LTP (L-LTP), neuropsin regulates the early phase of LTP. Furthermore, *in vitro* application of neuropsin did not alter the NMDA mediated current in the hippocampus. In contrast, the NMDA current was reduced in EphB2 knockout mice at the dentate granule neurons (Henderson *et al.*, 2001). Furthermore in the amygdala, the levels of L1CAM do not change after fifteen minutes of restraint stress in either wildtype or Neuropsin knockout mice (Figure 19).

The role of neuropsin in EphB2 gene upregulation

The differences in EphB2 regulation by stress beteen wildtype and neuropsin knockout mice are also reflected in the gene expression of EphB2 in the amygdala following stress. Following six hours of restraint stress, it was discovered that the EphB2 gene was upregulated whilst in neuropsin knockout mice it was unaltered (Figure 18). It is possible that this indicates that EphB2 receptors were cleaved by neuropsin in the early period (5-15minutes) following stress. Although there is no evidence that the intracellular domains of

EphB2, or indeed EphA4, could act as a transcription factor, an EphrinB1 fragment does demonstrate this potential. Following γ-secretase processing, an EphrinB1 intracellular fragment is released, which contains a sequence of basic amino acids which can act as an NLS explaining its accumulation in the nucleus (Tomita *et al.*, 2006). However the gene upregulation may be unrelated to a possible cleavage event and related to other factors involved in the neuropsin dependent neuronal stress response.

The role of neuropsin in modulating EphB2 - EphrinB2 interaction

A principle ligand for EphB2 is EphrinB2. Although this interaction has not been studied in the amygdala, both receptor and ligand are expressed in the amygdala (Liebl *et al.*, 2003). As the EphB2 receptor dynamism is increased during the first fifteen minutes of stress, it is possible that this may also regulate the membrane levels of EphrinB2. However, our data indicates that the membranous levels of EphrinB2 do not change in the first fifteen minutes of stress in either wild-type or neuropsin deficient mice (Figure 17). This is consistent with our *in vitro* studies, indicating that neuropsin specifically cleaves EphB2 and not other Eph receptors or EphrinB2. It also makes it less likely that the mechanisms that drive the increase in membrane EphB2 in the neuropsin knockout animals involve EphrinB2.

Chapter 4. Eph receptors, their binding partners and stress

Introduction

Eph receptor localization

The localisation of Eph proteins in the mature nervous system was studied by a number of investigators, including the author of this thesis. Information regarding the gene expression of Eph receptors can be found from projects targeted to describing the expression of all genes in the mouse brain (Lein *et al.*, 2007; Magdaleno *et al.*, 2006; Heintz, 2004). In addition, there have been studies dedicated specifically to the description of Eph receptors in both the mouse and the nonhuman primate (Liebl *et al.*, 2003; Xiao *et al.*, 2006).

The Eph receptors are essential for neuronal development and as such, their gene expression in the brain during embryonic stages is widespread, although they vary between the different receptors and ligands. The expression of EphB2, for instance, is vastly reduced during early postnatal period but re-appears by postnatal day 10 (P10), co-inciding with synaptogenesis (Henderson *et al.*, 2001). This is also accompanied by the change in subcellular location of EphB2 from axonal to dendritic compartments (Henderson *et al.*, 2001). Furthermore, as neurons mature, EphB2 becomes increasingly restricted to dendritic spines (Dalva *et al.*, 2000; Li & Sheng, 2003). This change in Eph receptor function throughout a lifespan is also demonstrated by EphA4; at P10, hippocampal EphA4 shows high levels of constitutive phosphorylation, whilst in adulthood, the levels are considerably lower (Murai *et al.*, 2003).

In the adult mouse, some brain areas lose Eph gene expression, including much of the midbrain and preoptic area, but in highly plastic areas, including the amygdala and hippocampus, the expression is enriched (Lein *et al.*, 2007; Liebl *et al.*, 2003; Magdaleno *et al.*, 2006). Similarly to the mouse, EphA4 and EphrinB2 showed enriched expression in highly plastic areas of the macaque brain (Xiao *et al.*, 2006). Studies of the Eph receptor expression in the adult mouse hippocampus has revealed that different Eph receptors and Ephrins have well defined expression patterns in the hippocampus and their expression often overlaps (Grunwald *et al.*, 2001; Henderson *et al.*, 2001; Martone *et al.*, 1997). For example, the EphA4 protein expression shows a distinct laminar distribution, reflecting hippocampal morphology. The pyramidal and granule cell layers containing neuronal cell bodies show very light staining whilst the layers containing neuronal projections show strong staining (Martone *et al.*, 1997). In contrast, EphrinB1 shows heavier staining in the pyramidal cell layer and little staining in the CA1 neuropil (Wang *et al.*, 2005). This signifies multiple Eph-Ephrin interactions as well as functional specificity.

The Eph-Ephrin interactions occur at many different sites. Within the hippocampus, Eph proteins have been localised at dendritic spines, axon tips, dendritic shafts, neuronal cell bodies and astrocytic tips (Buchert *et al.*, 1999; Murai *et al.*, 2003; Armstrong *et al.*, 2006; Aoto *et al.*, 2007). At the synapse, consistent with a traditional view of receptor-ligand functioning, it was believed that the Eph receptors would function post-synaptically and Ephrins would act as presynaptic ligands. This is the case at the perforant path-dentate granule synapses, the mossy fibre-CA3 synapses and the Shaffer collateral-CA1

synapses with EphB receptors localised post-synaptically and EphrinB ligands spanning the presynaptic membrane (Contractor *et al.*, 2002; Henderson *et al.*, 2001; Grunwald *et al.*, 2001). However, at both the mossy fibre-CA3 and Shaffer collateral-CA1 synapses, the Eph-Ephrin also functions in reverse orientation. Here, EphrinB ligands are found post-synaptically and EphB receptors are found pre-synaptically, meaning that EphB receptors and EphrinBs are simultaneously expressed both pre- and post-synaptically (Grunwald *et al.*, 2004; Armstrong *et al.*, 2006). The complexity of the system is further illustrated by the role that the EphA receptors play at the same synapses. At the CA1-CA3 synapses, EphA4 is expressed pre-synaptically and can therefore stimulate the post-synaptic EphrinB ligands (Grunwald *et al.*, 2004; Martone *et al.*, 1997). Concomitantly, EphA4 interacts with EphrinA ligands found on astrocytic tips (Murai *et al.*, 2003; Filosa *et al.*, 2009). EphA4 has also been found on dendritic spines in the CA1, CA3 and dentate gyrus (Tremblay *et al.*, 2007). The localization of Eph receptors in the adult hippocampus reflects their many roles in the central nervous system functioning.

Eph receptors and NMDA receptors

The post-synaptic membrane is a densely packed structure containing ion channels, structural proteins and transmembrane receptors. The many different components are highly organised and tightly regulated by both ECM proteins and transcellular scaffolding structures. The resulting formations must be able to respond rapidly, both morphologically and in composition to allow the organism to adapt to environmental stimuli. To enable this, various proteins

interact through common binding domains to organise the molecular composition of the synapse.

Eph receptors possess a number of domains, both intracellularly and extracellularly, that allow molecular interactions with their binding partners. One binding partner of EphB receptors that is crucial for learning and memory is the NMDA receptor. Using transfected 293T cells, Dalva *et al* immunoprecipitated EphB1-4 receptors with the NR1 subunit (Dalva *et al.*, 2000). In cortical cultured neurons, the association of the EphB receptors was demonstrated following stimulation by clustered EphrinB1. This association was specific to EphB receptors, as EphA4 was unable to immunoprecipitate NR1 in both transfected 293T cells and cultured neurons (Dalva *et al.*, 2000). The interaction has also been demonstrated *in vivo* by immunoprecipitation of EphB2 from hippocampal, cerebellum and cortical homogenates (Grunwald *et al.*, 2001).

The interaction domain between the two receptors has been identified using a series of mutated EphB2 and NR1 constructs. Neither the kinase activity nor any part of the intracellular EphB2 receptor is required. Rather, the extracellular domain of EphB2 acts with the EphrinB ligand to form a ternary complex with the NR1 extracellular domain (Dalva *et al.*, 2000). The dispensability of the kinase domain in this function was also confirmed *in vivo* using mutant knock-in mice expressing a truncated EphB2 lacking the kinase domain (Grunwald *et al.*, 2001). Although there is functional redundancy within the EphB receptor family, the EphB2 receptors' interaction with NR1 is

essential for a proper composition of EphB/NR1 complexes. In mixed hippocampal-cortical neuronal cultures from EphB2 -/- mice, the stimulation of clustered EphrinB1 Fc resulted in the formation of 20% less EphB clusters. This also resulted in a 20% decrease in the quantity of NR1 that co-clustered with the EphB receptors (Grunwald *et al.*, 2001). Furthermore, EphB receptors regulate the localisation and function of NR2B containing NMDA receptors in mature neurons (Nolt *et al.*, 2011).

The early work on EphB/NR1 interaction focused on the development of synapses. EphrinB-stimulated formation of EphB – NMDA clusters was hypothesised to serve as an early step in synapse formation. In line with this, the application of EphrinB or the overexpression of EphB2 in cultured neurons resulted in an increase of NMDA postsynaptic specialisations (Dalva *et al.*, 2000). Subsequently the role of EphB1, 2 and 3 was analysed using single, double or triple knock-out mice deficient for these receptors. These experiments confirmed that the receptors play a critical role in dendritic spine formation, morphogenesis and maturation (Henkemeyer *et al.*, 2003). It was also found that EphB2 also regulated the AMPA receptor localisation at excitatory synapses. This interaction occurs through the intracellular PDZ domain, distinct from its interaction with NMDA receptors (Kayser *et al.*, 2006). The ability of EphB receptors, in particular EphB2, to regulate the organisation of glutamatergic synapses indicates a crucial role for the receptor during experience-driven neuronal plasticity.

The role of EphB2 in hippocampal plasticity has been well established. At both the CA3/CA1 synapses and the perforant path/dentate granule synapses, LTP is impaired in EphB2-/- mice, as is LTD (Grunwald *et al.*, 2001; Henderson *et al.*, 2001). It was also found that, at the perforant path/dentate granule synapses, the NMDA current was reduced (Henderson *et al.*, 2001). Could EphB-NMDA mechanisms explain the plasticity deficits seen in these mice? Experiments thus far point towards either modulation of the NMDA receptor itself or activation of signalling pathways that alter plasticity-related gene expression and actin dynamics. Stimulation of EphB receptors results in the activation of Src kinases that are part of the NMDA complex. In turn, this activation results in the phosphorylation of NR2A and NR2B subunits (Grunwald *et al.*, 2001; Takasu *et al.*, 2002; Slack *et al.*, 2008). The phosphorylation of the NMDA subunits may result in altered regulation of Ca^{2+} influx and therefore altered plasticity. Indeed, stimulation of EphB receptors causes immature neurons to display NMDA mediated Ca^{2+} influx (Takasu *et al.*, 2002). Further to Src mediated phosphorylation of NMDA subunits, EphB2 can phosphorylate NMDA subunits independently. As discussed in chapter 3, γ-secretase cleavage of EphB2 results in the liberation of an EphB2 fragment to the cytosol that contains the Eph kinase domain. This fragment then phosphorylates NMDA subunits directly and increases the surface expression of NMDA receptors (Xu *et al.*, 2009). Additionally, EphB2 also recruits CamKII which is known to phosphorylate NMDA receptors, although this mechanism has yet to be demonstrated *in vivo* (Dalva *et al.*, 2000; Leonard *et al.*, 1999).

The interaction between EphB receptors and NMDA receptors also influences synaptic plasticity beyond direct modulation of the NMDA receptor. The activation of Src results in the phosphorylation of CREB, known to activate Ca^{2+} immediate early genes (IEG's). Whilst both EphrinB stimulation and calcium influx through NMDA receptors results in a modest increase in CREB phosphorylation, when neurons are stimulated, they act synergistically to cause a greater CREB activation (Takasu *et al.*, 2002). The potentiation of the glutamate-regulated gene expression by EphrinB upregulated specific IEG's. In particular they were c-fos, BDNF and cpg15 which are all implicated in the modulation of the synapse (Takasu *et al.*, 2002). Stimulation of EphB receptors also activates the ERK/MAP kinase pathway, which plays important functions during LTP. The glutamate-induced activation of the ERK/MAP kinase pathway was reduced by prior incubation of neurons with EphrinB Fc, showing the direct interaction between EphB and NMDA receptors affecting plasticity related signalling (Grunwald *et al.*, 2001). NMDA receptors also regulate spine morphology through the guanine exchange factor, Tiam1 (Tolias *et al.*, 2005). However, Tiam1 is also activated by EphB2, which results in the alteration of the actin cytoskeleton through Rac1 (Tolias *et al.*, 2007).

It is also worth noting that EphrinB ligands interact with NMDA subunits. Specifically, NR1 immunoprecipitated with EphrinB2 in the postnatal day 6-7 of the rat striatum, an interaction that is likely to be through a complex with group 1 metatropic glutamate receptors (Calo *et al.*, 2005). Furthermore, EphrinB ligands are located post-synaptically in CA1 dendrites in the hippocampus, where they facilitate NMDA-mediated long-term plasticity (Grunwald *et al.*,

2004). The investigations so far have uncovered a role for EphB receptors modulating synaptic plasticity through their interaction with NMDA receptors. Therefore, this mechanism may be utilised by the organism to facilitate neuronal adaptations during stress.

NMDA receptors

Glutamate signalling in the brain is mediated through metabotropic (slow) and iontropic (fast) receptors. The former are G-protein coupled receptors, the latter are ligand gated ion channels. Along with Kainate and AMPA receptors, NMDA receptors make up the iontropic glutamate receptors. The structure of the NMDA receptor channel is as complex as its function. The receptor is named after the selective, synthetic agonist N-methyl-D-aspartate (NMDA) but its principal endogenous agonist is glutamate. Furthermore, simultaneous binding of a co-agonist potentiates NMDA activation. Classically, glycine is considered the major co-agonist but D-Serine is a more potent co-agonist and may be of more importance *in vivo* (Johnson & Ascher, 1987; Matsui *et al.*, 1995; Shleper *et al.*, 2005). Due to its functional properties, the NMDA receptor is considered important in learning and memory (Riedel *et al.*, 2003). The electrophysiological correlate of learning and memory, long-term potentiation (LTP), requires postsynaptic calcium influx. It occurs when both pre- and post-synaptic neurons are active, resulting in Hebbian plasticity (Bliss & Collingridge, 1993). The NMDA receptor contains a binding site for Mg^{2+} that blocks the channel at the resting membrane potential. Therefore, for the channel to open, agonist binding and membrane depolarisation must simultaneously occur (Mayer *et al.*, 1984). Although the majority of calcium

influx into the post-synaptic membrane in response to glutamate is through voltage dependent Ca^{2+} channels, when glutamate release is paired with postsynaptic action potentials, the calcium influx is greatly potentiated by NMDA receptors (Schiller *et al.*, 1998). Furthermore, the NMDA receptor has slow gating properties, meaning that the calcium influx is prolonged resulting in a large accumulation of calcium in the dendritic spine (Muller & Connor, 1991). Consequently, the NMDA receptor functions as a coincidence detector facilitating neuronal plasticity. In addition, the receptor's function can be finely tuned to suit specific requirements by a number of modulatory agents. These include polyamines, phosphatases, kinases, redox agents and Zn^{2+} causing either an increase or decrease in the Ca^{2+} flow through the channel (Dingledine *et al.*, 1999).

The NMDA receptor is formed by a tetramer of membrane spanning subunits, consisting of the obligatory NR1 subunit along with NR2A-D subunits (Luo *et al.*, 1997; Laube *et al.*, 1998). Although less common, there are also NR3A-B subunits that replace an NR2 subunit (Chatterton *et al.*, 2002). The composition of the subunits allows for differential regulation of the NMDA receptor. For example, the NR1/2A receptors are very sensitive to Zn^{2+} inhibition, whilst the NR1/2B receptors are antagonised by ifenprodil (Williams, 1996). The NR3 subunit acts in a dominant negative fashion when incorporated into the receptor, by decreasing the Ca^{2+} current through the receptor (Sasaki *et al.*, 2002; Nishi *et al.*, 2001).

Despite the varied composition of subunits, all NMDA receptors share a similar membrane topology. The extracellular domain contains the ligand binding sites and a modulatory domain. There are three transmembrane loops (M1, 3-4) with an intramembrane pore loop (M2), contributing to the formation of the ion channel and the Mg^{2+} binding site. Finally, there is an intracellular domain that varies in size depending on the specific subunit. This intracellular loop is subject to modulation by phosphorylation and contains sites for protein - protein interactions (Laube *et al.*, 1998; Chatterton *et al.*, 2002).

NMDA receptors and stress

The NMDA receptor's functions and expression suggest its involvement in the neuronal stress response. It plays integral roles in both physiological and pathological mechanisms closely related to stress. For example, memory is closely related to the neuronal stress response and many patients with anxiety/stress disorders have learning and memory deficits (Mathews & MacLeod, 2005). The NMDA receptor is implicated in ischaemic and excitotoxic neuronal cell death, processes that result in similar damage to the hippocampus that severe stress causes (McEwen, 1999). As discussed, the stress response results in glucocorticoids acting on limbic brain areas, with the hippocampus being a major target for glucocorticoids (Chapter 1). The excitatory neurotransmitter system, and in particular the NMDA receptor, is also involved in the hippocampal morphological and functional changes seen during stress. Following stress, the hippocampal glucose metabolism is raised (De Bruin *et al.*, 1990). This increase is, at least in part, due to NMDA receptor activation, as the increase is attenuated by a NMDA receptor blocker

(Schasfoort *et al.*, 1988). Stress also increases excitatory glutamate transmission in the hippocampus, which is attenuated by adrenalectomy (Lowy *et al.*, 1993). The effects of stress on glutamate signalling in the hippocampus are not just regulated by glutamate levels. Stress also alters the NMDA/AMPA receptor ratio in the hippocampus. Twenty-four hours following immobilization stress (two and a half hours), an increase in the NMDA NR1 and NR2B subunits mRNA and a decrease in the AMPA GluRA subunit mRNA was observed (Bartanusz *et al.*, 1995). Subcutaneous implants of slow-release corticosteroid tablets for 10 days also resulted in a similar increase in the NR1 and NR2B, but not the NR2A subunit mRNA, although the AMPA subunit mRNA was not measured (Weiland *et al.*, 1997). The implant also resulted in an increase of the NMDA antagonist binding sites, indicating that the increased mRNA resulted in an increased NMDA receptor expression (Weiland *et al.*, 1997). Furthermore, dexamethasone (a synthetic glucocorticoid) increases the NMDA receptor activation, but not AMPA or kainate activation following interstriatal excitotoxin injection (Supko & Johnston, 1994).

Hippocampal neurons also show increased excitability following chronic corticosterone administration and its deleterious effects on hippocampal neurons during ischaemia or seizures are NMDA mediated (Beck *et al.*, 1994; Armanini *et al.*, 1990). The alterations in NMDA signalling lead to a number of hippocampal changes seen following stress. As discussed, a number of different stressors lead to hippocampal neuronal atrophy (Chapter 1). Following restraint stress or corticosterone treatment, rats showed significant atrophy of CA3 pyramidal neurons, which was blocked by phenytoin

(Watanabe *et al.*, 1992). Phenytoin prevents glutamate release and therefore its effects are consistent with attenuating the increase in glutamate release following stress. Further investigation revealed that an NMDA receptor antagonist, but not an AMPA receptor antagonist, also abolished the dendritic atrophy (Magarinos & McEwen, 1995). Consistent with this, the upstream activator of the NMDA mediated atrophy appears to be corticosterone, as inhibition of its synthesis blocked the atrophy (Magarinos & McEwen, 1995). Pharmacological studies have also implicated other monoaminergic transmitters in the neuronal remodelling. Tianeptine, a serotonin reuptake enhancer, blocks dendritic atrophy, whilst serotonin reuptake inhibitors do not block dendritic atrophy (Watanabe *et al.*, 1992; Magarinos *et al.*, 1999). Furthermore, stress paradigms reduce the binding of the inhibitory serotonin receptor 5-HT_{1A} and the serotonin transporter, whilst binding to and expression of 5-HT_2 receptor is increased (McKittrick *et al.*, 2000) As serotonin enhances NMDA receptor binding and activity, it is possible that the serotonin effects on the hippocampal morphology occur through the NMDA receptor (Rahman & Neuman, 1993).

The modulation of NMDA receptor in the hippocampus leads to electrophysiological changes as well as morphological ones. Following stress, hippocampal LTP is impaired in rats and LTD is enhanced (Kim *et al.*, 1996). This effect is blocked by a competitive NMDA antagonist administered before the stress. This preference towards a decrease in synaptic strength following stress fits with an increased excitability in the hippocampus during stress (McEwen, 1999). Corroborating the decrease in LTP formation in the

hippocampus is the impaired performance of stressed animals in hippocampal dependent behavioural tests (de Kloet *et al.*, 1999). The effect of glucocorticoids enhances glutamate signalling in the hippocampus during stress and regulates dentate gyrus neurogenesis through NMDA excitability (Gould *et al.*, 1997). An increase in stress and therefore glucocorticoids leads to a decrease in neurogenesis (Mirescu & Gould, 2006). This is important to consider in our studies as EphB receptors also regulate hippocampal neurogenesis. In particular, EphB1 and EphB2 double knockout mice had significantly less neural progenitor cells and a reduced dentate gyrus volume when compared to wild-type controls (Chumley *et al.*, 2007). Altered neurogenesis is an important factor in the development of stress-related disorders, including depression (reviewed by (Balu & Lucki, 2009).

The stress-induced neuronal remodelling of the hippocampus leads to a decrease in the NMDA receptor expression (Pawlak *et al.*, 2005). By examining the molecular interaction of NMDA receptors and extracellular proteins, Pawlak *et al* demonstrated that tPA-knockout mice and plasminogen-knockout mice did not show the same NMDA receptor decrease or dendritic atrophy. The potential molecular mechanism for regulating NMDA receptors in stress involves the direct cleavage of NMDA receptors by plasmin and possibly tPA (Pawlak *et al.*, 2005).

The NMDA receptor also plays a central role in the amygdala stress response. Glutamate turnover rate increases in the amygdala following footshock (Rainnie *et al.*, 1991; Miyauchi *et al.*, 1988). The increase in glutamatergic

signalling during stress is likely to be mediated, at least in part, by the NMDA receptor. Pharmacological inhibition of the NMDA receptor in the lateral/basolateral nuclei blocks stress-induced classical conditioning (Shors & Mathew, 1998). The blockade of NMDA receptors in the basolateral amygdala also inhibited the anxiety-like behaviour seen in the elevated plus maze by rats following exposure to a cat (Adamec *et al.*, 1999). In this study, the authors discovered that the NMDA-dependent initiation of different stress responses varied depending on which hemisphere the amygdala NMDA was inhibited (Adamec *et al.*, 1999).

The role of NMDA in the amygdala is closely associated with plasticity-like changes. LTP in the amygdala, dependent on NMDA functioning, underlies fear memory formation (reviewed by (Adamec, 1997). The crosstalk between different regions of the fear circuitry associated with the amygdala also depends on NMDA signalling during stress. For example, the LTP effects seen in the hippocampus depend on NMDA receptors in the amygdala (Ikegaya *et al.*, 1995). NMDA receptors have also been shown to be critical in the formation of spike firing in the amygdala during the acquisition phase of the memory (Goosens & Maren, 2004).

The amygdala and hippocampus display differing neuronal changes following stress. Unlike the hippocampus, which displays neuronal atrophy following stress, the amygdala displays an increase in dendritic branching (Mitra *et al.*, 2005). Both the hippocampus and amygdala effects have been linked to tPA signalling and modulation of NMDA receptors (Mitra *et al.*, 2005). However,

unlike the hippocampus, the mechanism through which this occurs does not appear to be plasminogen-dependent (reviewed by (Skrzypiec *et al.*, 2008).

Figures

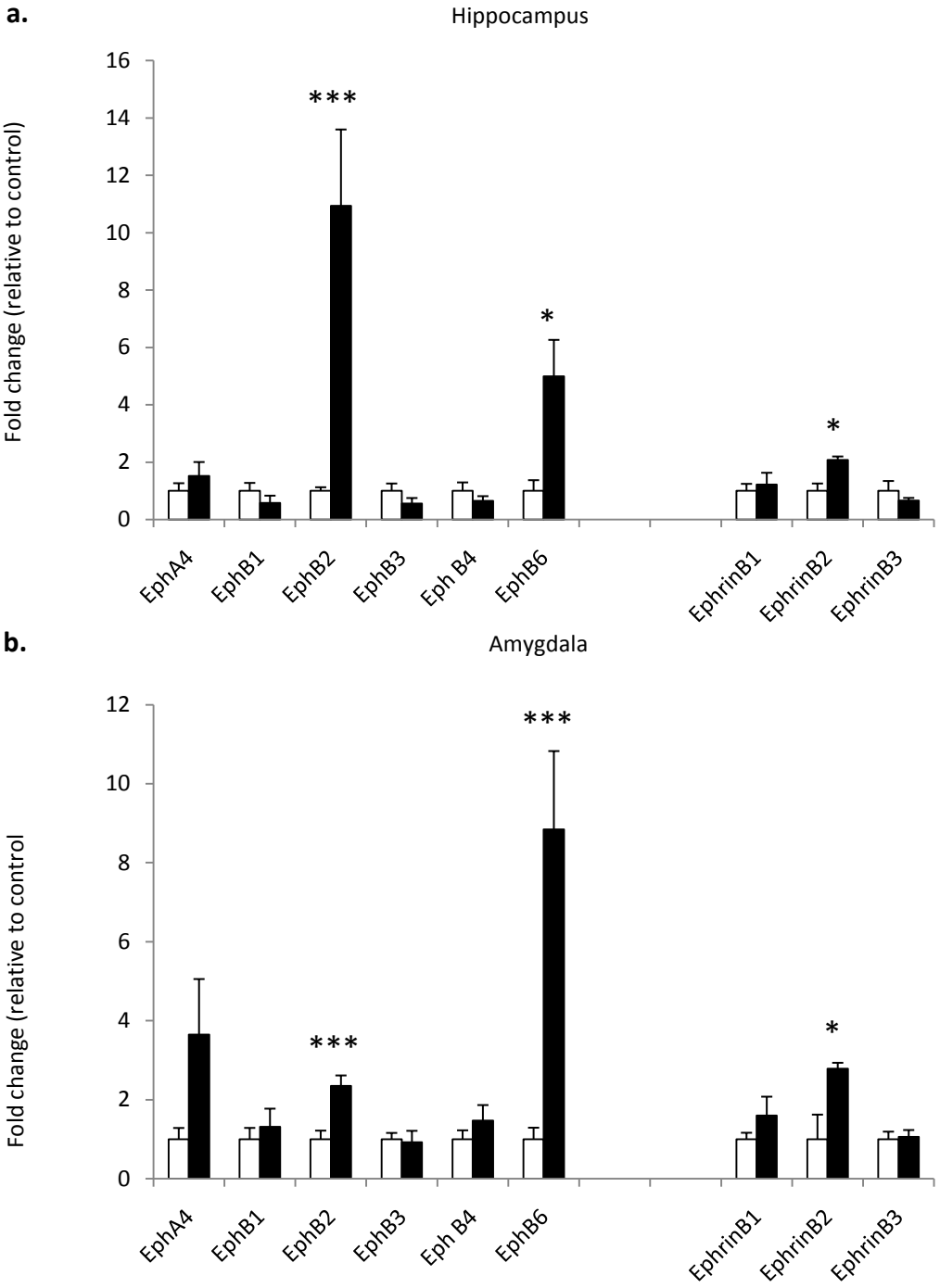


Figure 21. Eph gene expression in the hippocampus and amygdala following stress (preliminary data). Mice were subjected to 6 hours of restraint stress and the hippocampi and amygdala were dissected and homogenised. The RNA was extracted, converted to cDNA and qRT-PCR was performed. The gene expression after stress is shown relative to control gene expression. (**a and b).** After stress the Eph2 gene expression increased 11 and 2 fold (t-test $p<0.001$) in the hippocampus and amygdala, respectively. EphB6 gene expression increased 5 and 9 fold (t-test $p<0.05$, t-test $p<0.001$) in the hippocampus and amygdala respectively. EphrinB2 gene expression increased 2.1 and 2.8 fold (t-test $p<0.05$) in the hippocampus and amygdala, respectively. The other genes did not change expression after stress (t-test $p>0.05$). * = $p<0.05$ *** = $p<0.001$

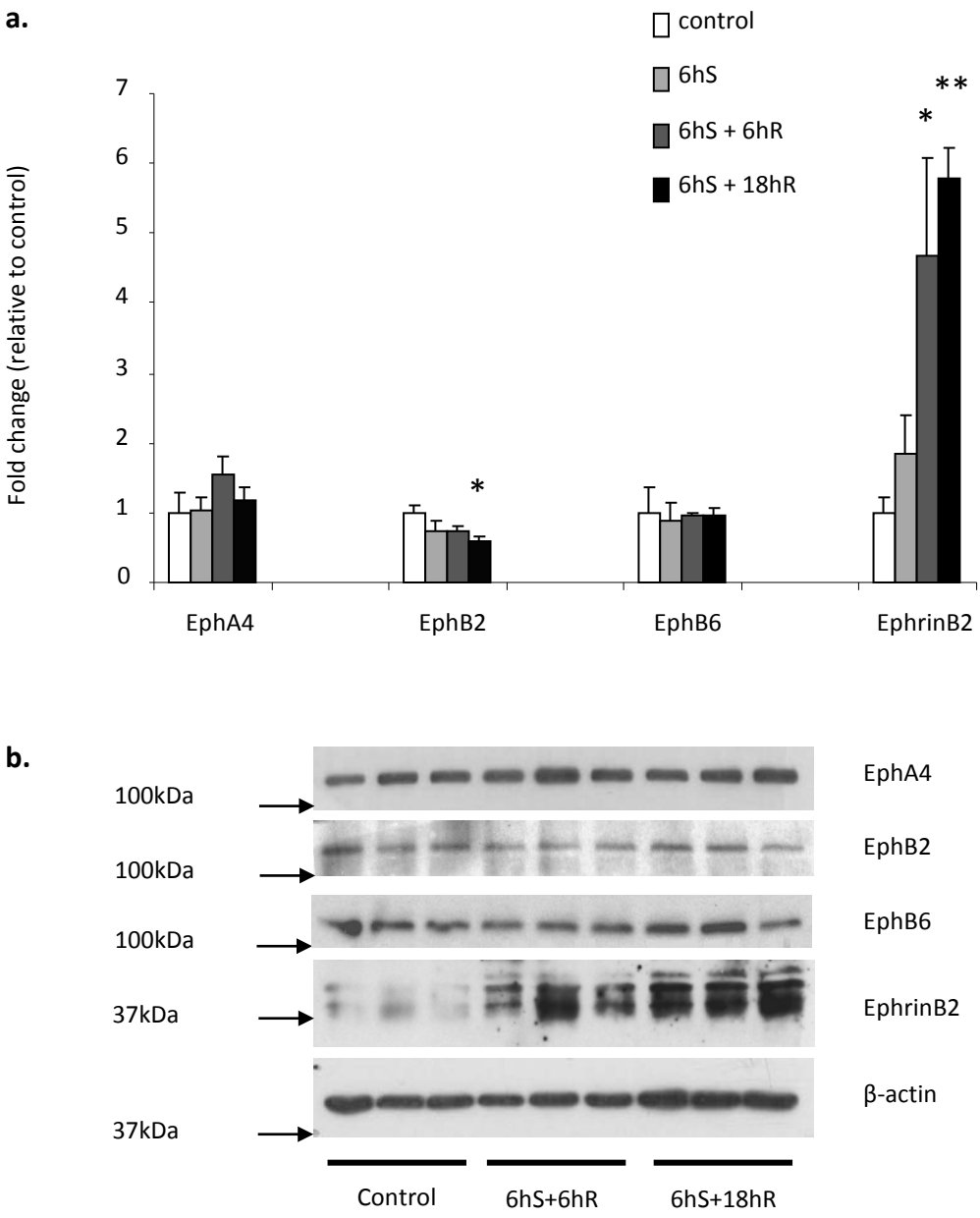


Figure 22. Hippocampal Eph protein expression following stress, with different recovery periods (preliminary data). (a). Western blotting for control samples, 6 hours of stress, 6 hours of stress plus 6 hours of recovery (6hs + 6hR) and 6 hours of stress plus 18 hours of recovery (6hS + 18hR) were quantified using Scion image. Each sample was normalised to β-actin and the protein expression of the stressed groups shown relative to the control protein expression. None of the Eph proteins showed any change after 6 hours of stress (F= p>0.05). EphrinB2 shows an increase of 4.7- and 5.8-fold after 6 and 18 hours of recovery, respectively (F = p<0.05 and p<0.01, respectively). EphB2 shows a decrease (F = p<0.05) after 18 hours recovery. **(b).** Representative Western blots showing protein expression from mouse hippocampal homogenates from control, 6hS+6hR and 6hS+18hR mice. * = p<0.05 ** = p<0.01

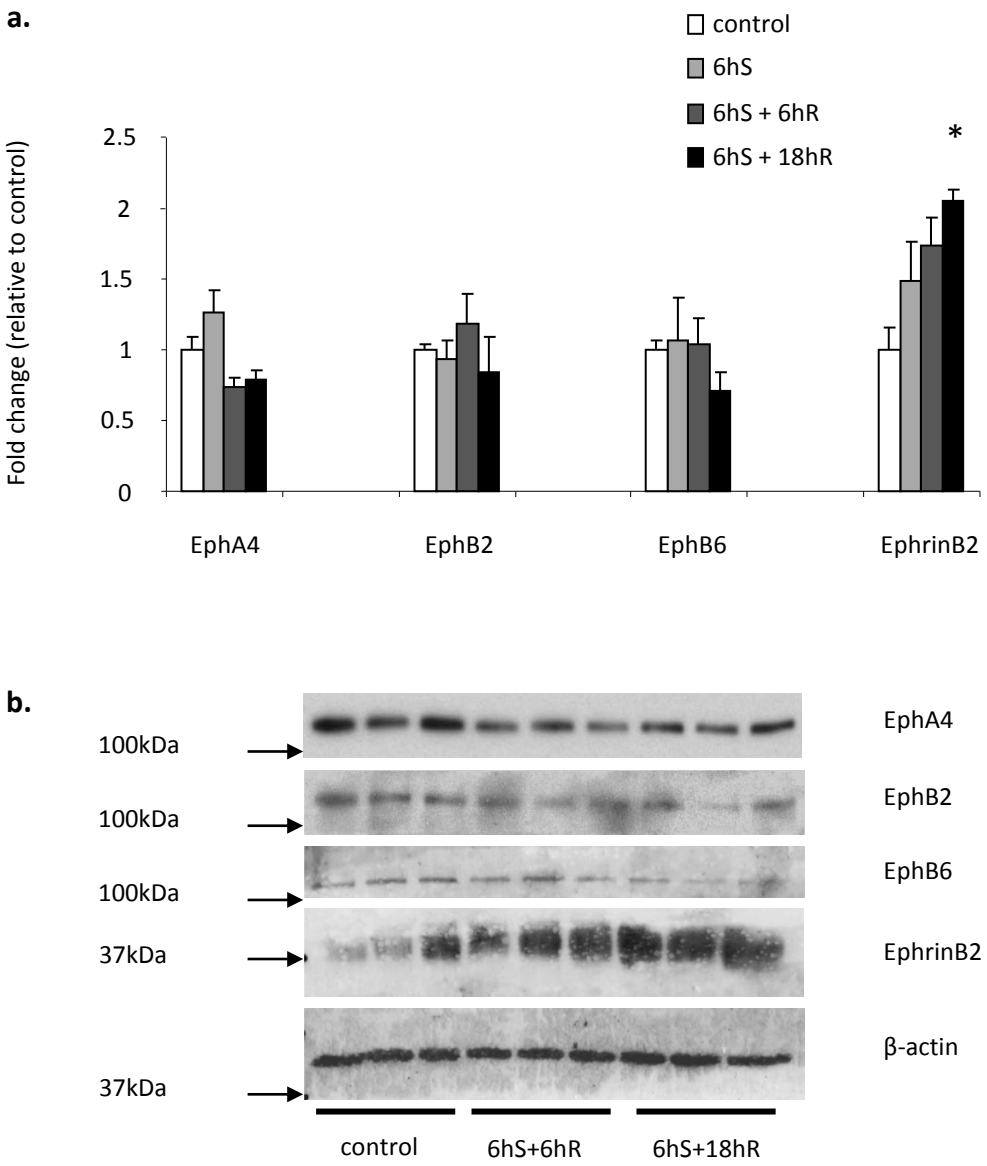


Figure 23. Amygdala Eph protein expression following stress, with different recovery periods (preliminary data). (a) Western blotting for control samples, 6 hours of stress, 6 hours of stress plus 6 hours of recovery (6hs + 6hR) and 6 hours of stress plus 18 hours of recovery (6hS + 18hR) were quantified using Scion image. Each sample was normalised to β-actin and the protein expression of the stressed groups shown relative to the control protein expression. None of the Eph proteins showed any change after 6 hours of stress ($F = p>0.05$). EphrinB2 shows an increase of 2.1 fold after 18 hours of recovery ($F = p<0.05$). (**b**) Representative Western blots showing protein expression from mouse amygdalae homogenates from control, 6hS+6hR and 6hS+18hR mice. * = $p<0.05$ ** = $p<0.01$

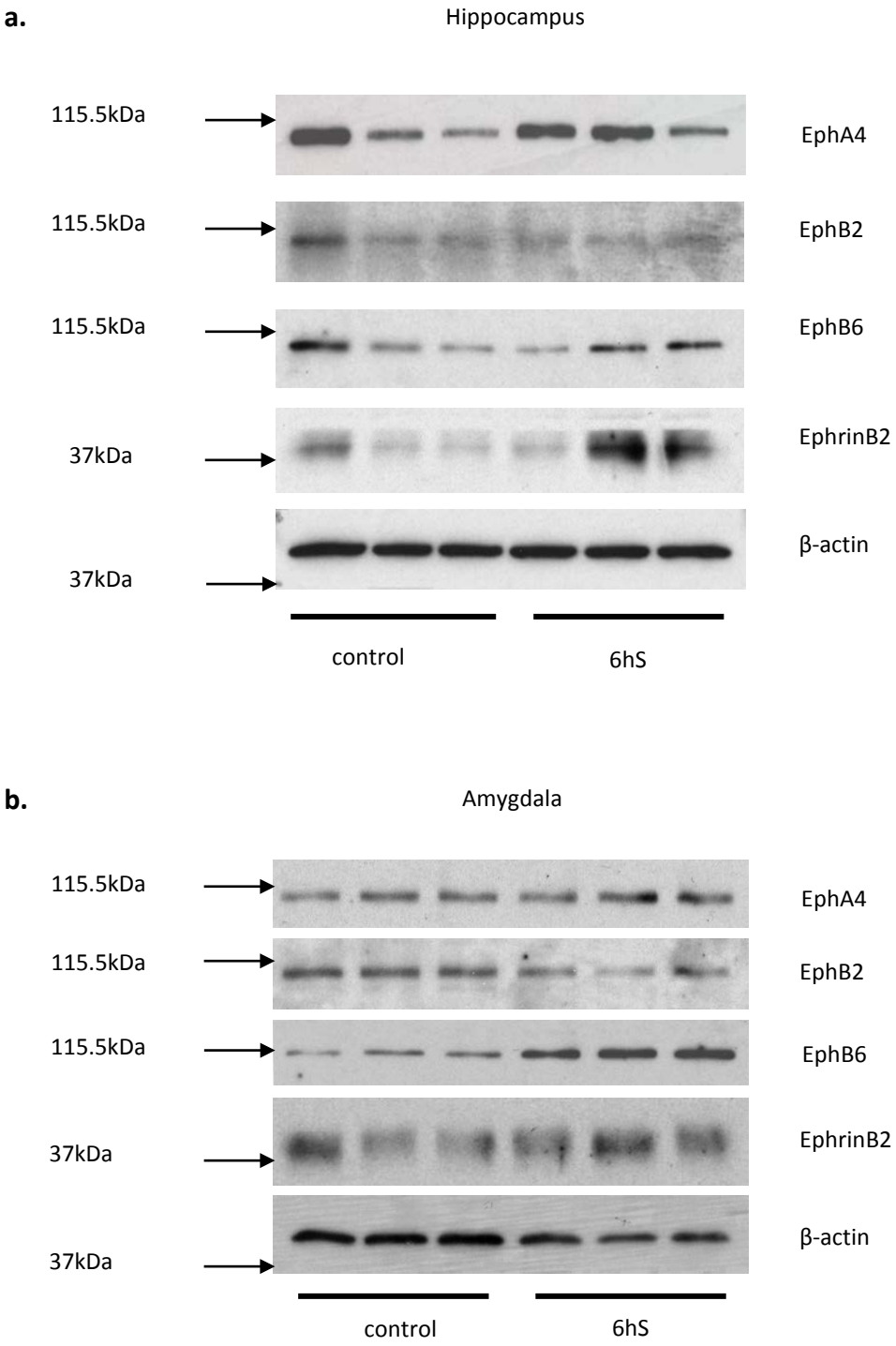


Figure 24. Eph protein expression in the hippocampus and amygdala following stress (preliminary data). Western blotting was performed on samples from mice that had not undergone stress (control) and mice that had undergone 6 hours of stress (6hS). (**a**) Representative Western blots for Eph proteins and actin from hippocampal samples (**b**) Representative Western blots for Eph proteins and actin from amygdalae samples. None of the Eph proteins showed any change after 6 hours of stress (figures 21 and 22).

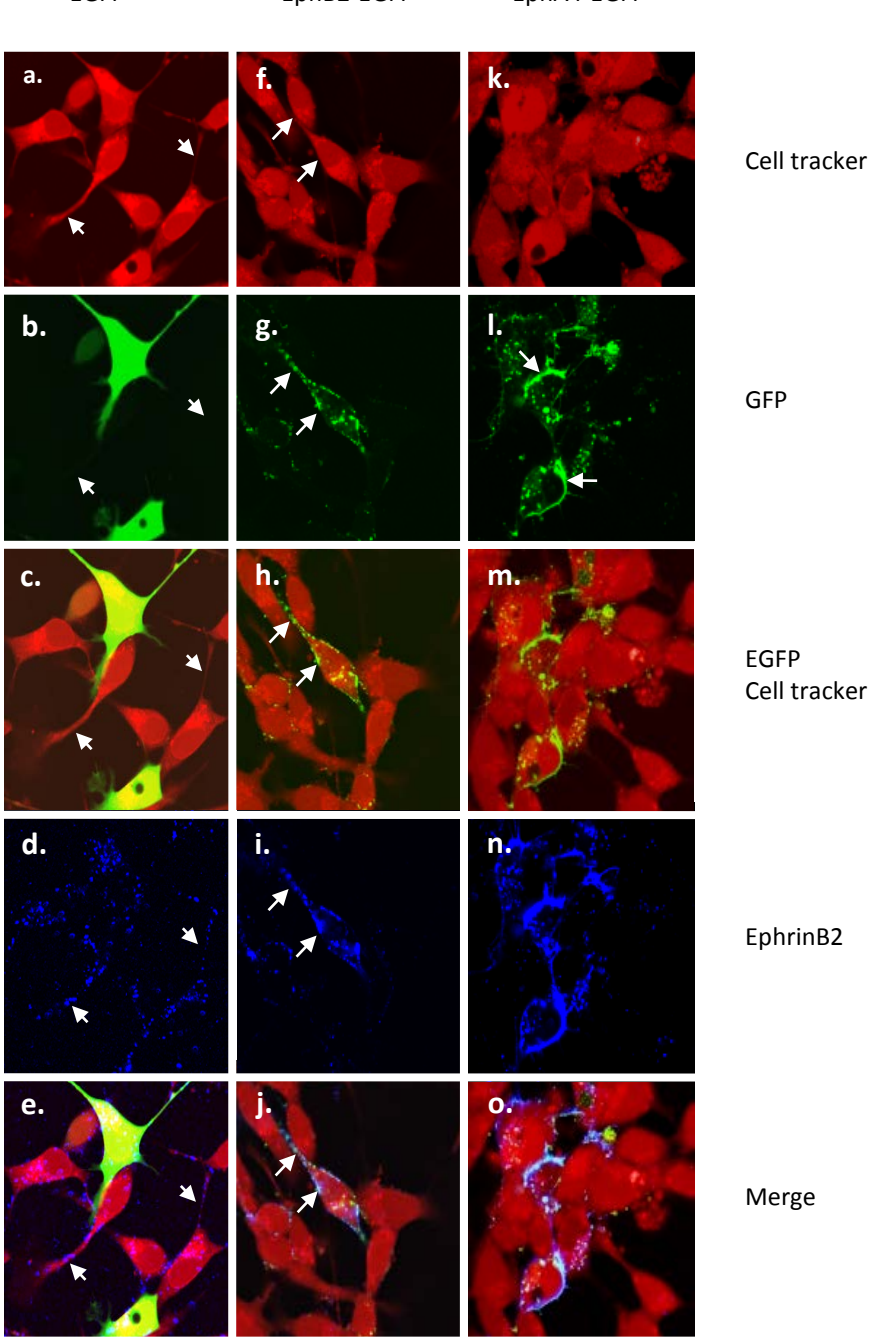


Figure 25. EphrinB2 binds to both EphA4 and EphB2. SHSY-5Y cells were transfected with either EGFP **(b)**, EphB2-EGFP **(g)** or EphA4-EGFP **(l)** constructs and the cell morphology was visualised using orange cell tracker **(a,f** and **k).** Whilst cells expressing EGFP show a diffuse cytoplasmic distribution of GFP **(c)**, those transfected with either EphB2-EGFP or EphA4-EGFP display a membranous and intracellular vesicular distribution **(h and m)**. EphrinB2-Fc was clustered using Cy-5 conjugated anti human IgG and incubated with the transfected cells **(d**, **j** and **n)**, (15 mins, 8µg/ml). Excess EphrinB2 complex was removed by washing and images were captured. Pre-clustered EphrinB2 locates to SHSY-5Y cells and preferentially co-localises with EphB2-EGFP **(j)** and EphA4-EGFP **(o)** compared to EGFP **(e)**.

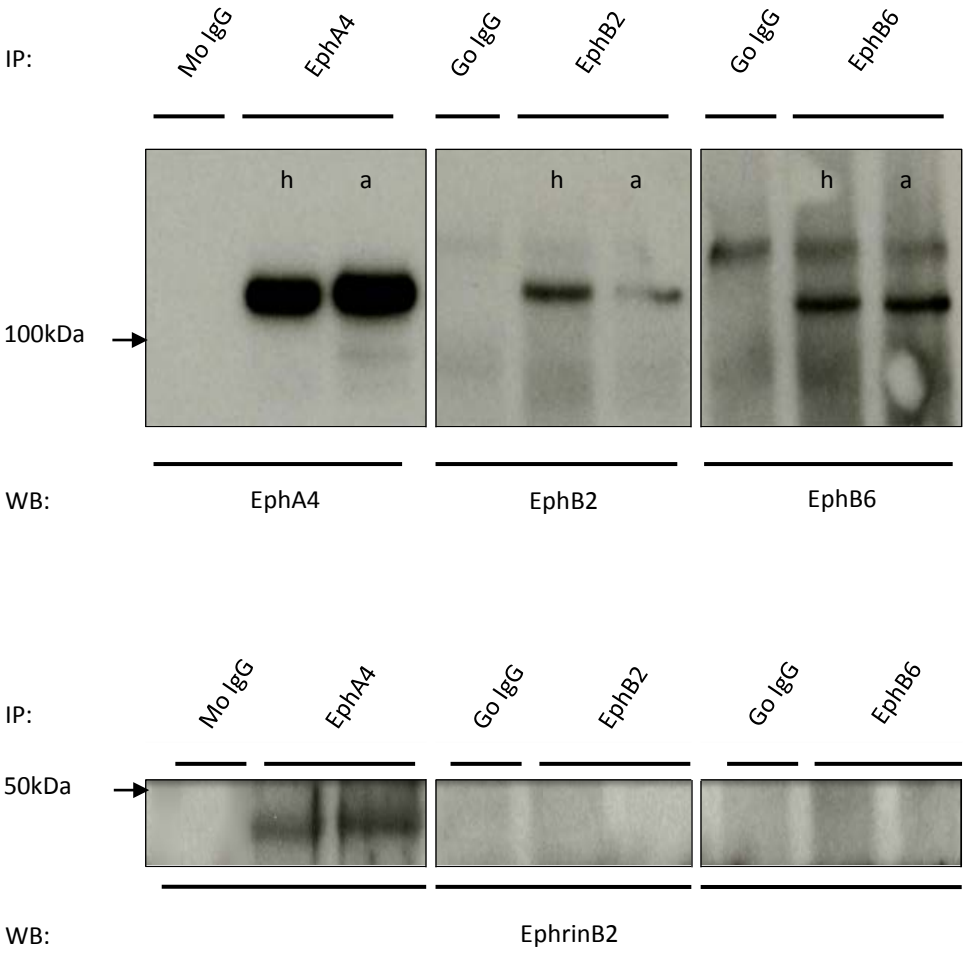
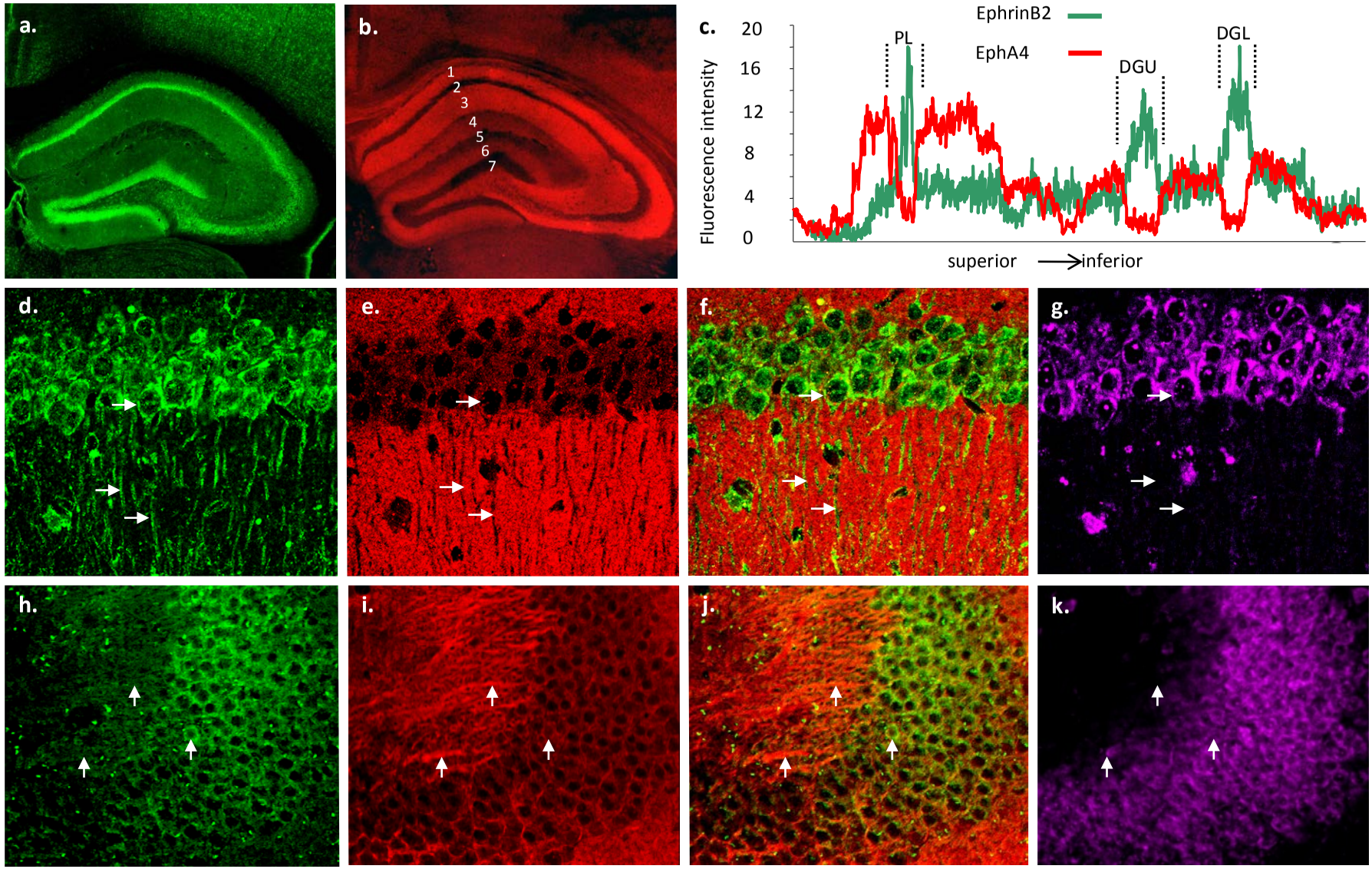
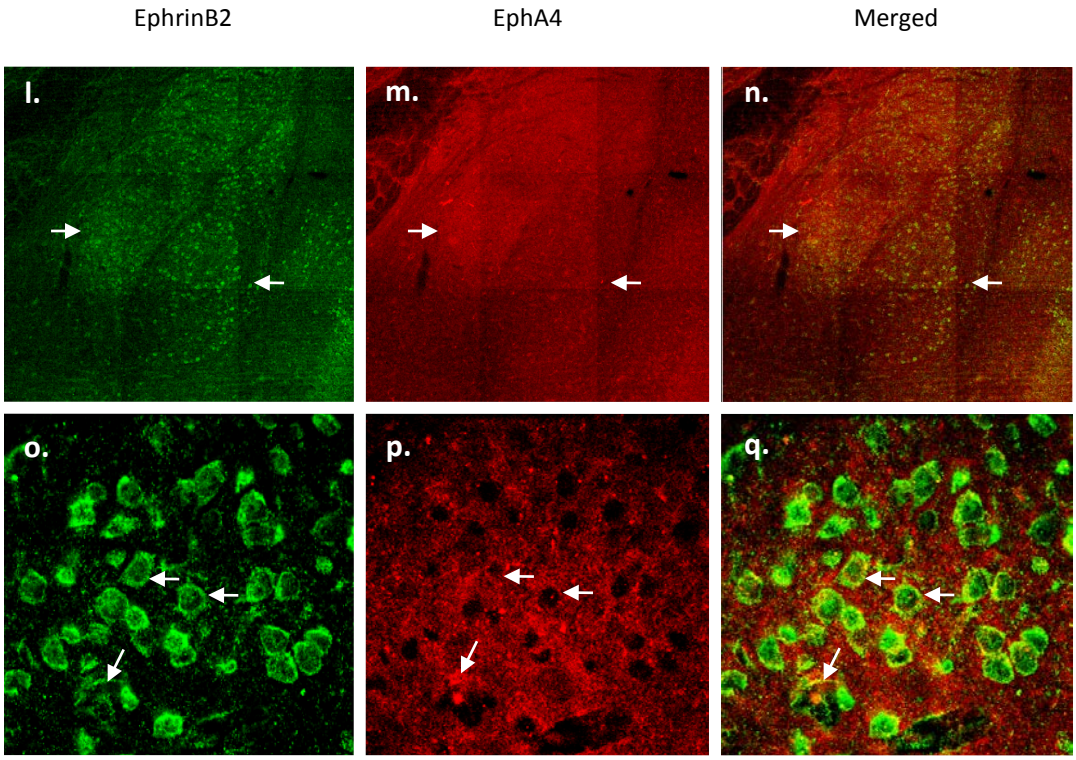


Figure 26. **EphrinB2 protein immunoprecipitates with EphA4 but not EphB2 or EphB6.** Hippocampal **(h)** and amygdalae **(a)** samples were homogenised and either EphA4, EphB2 or EphB6 was immunoprecipitated. The resulting precipitates were analysed by Western blotting for EphrinB2 as well as the original precipitating receptor. When the samples were stained for EphrinB2 a band corresponding to the correct size was only found in the EphA4 precipitates.


a.
b.
1
2
3
4
5
6
7
c.
20
16
12
8
4
0
Fluorescence intensity
EphrinB2
EphA4
PL
DGU
DGL
superior
inferior
d.
e.
f.
g.
h.
i.
j.
k.
EphrinB2
EphA4
EphA4
EphrinB2
Toto


EphrinB2
EphA4
Merged
l.
m.
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o.
p.
q.

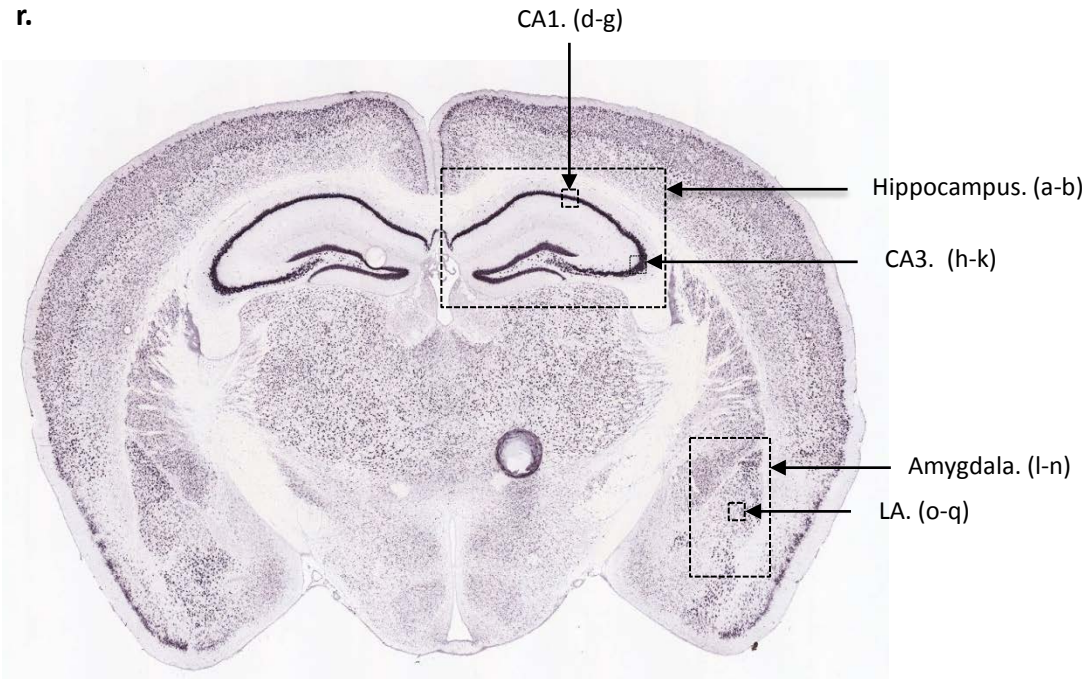

r.
CA1. (d-g)
Hippocampus. (a-b)
CA3. (h-k)
Amygdala. (l-n)
LA. (o-q)

Figure 27. EphrinB2 and EphA4 display reciprocal distribution in the hippocampus and amygdala. Whole mice brains were fixed in PFA and coronal slices of 70µm were cut. The slices were double stained for EphA4 and EphrinB2 and imaged using a confocal microscope**.** The staining of EphrinB2 revealed strong immunoreactivity within the stratum pyramidale and stratum granulosum, of the CA1/CA3 regions and dentate gyrus of the hippocampus **(a)**. Greater magnification revealed EphrinB2 to be located at the membrane of neuronal cell bodies **(arrows in d and h),** and on the dendrites extending to the soma, particularly in the CA1 region **(a and arrows in d)**. EphA4 staining revealed strong immunoreactivity that reflected the structure's laminar organisation **(b)**.

1. Stratum oriens
2. Stratum pyrimadale
3. Stratum radiatum
4. Stratum lacunosum - moleculare
5. Hippocampal sulcus
6. Stratum moleculare
7. Stratum granulosum

In contrast to the EphrinB2 staining the stratum pyramidale and stratum granulosum were striking in their absence of staining. Stratum oriens and stratum radiatum, particularly in the CA3 region, showed the strongest immunoreactivity. Again, in contrast with EphrinB2 staining, greater magnification revealed an absence of EphA4 from the cell body **(arrows in e and l)** and strong staining in axons, particularly in the CA3 region. When the stains were overlaid **(f and j)** and a spatial histogram charted **(c)** the reciprocal nature of the EphrinB2 EphA4 staining became clear. Neuronal cell bodies were identified with TOTO (**g and k).** A similar pattern of expression is seen in the amygdala. EphrinB2 staining is found in the lateral/basolateral and central amygdala (**l)**. Again, EphrinB2 is located at the membrane of cell bodies (**arrows in o**). EphA4 reactivity is stronger in the surrounding neuropil (**m and p).** Overlaying the images demonstrates the reciprocal relationship between ligand and receptor **(n and q).** The brain regions that the images **(a,b, d-q)** were taken from them are shown on a coronal section of mouse brain (http://mouse.brain-map.org/viewImage.do?imageId=79611194) **(r).**

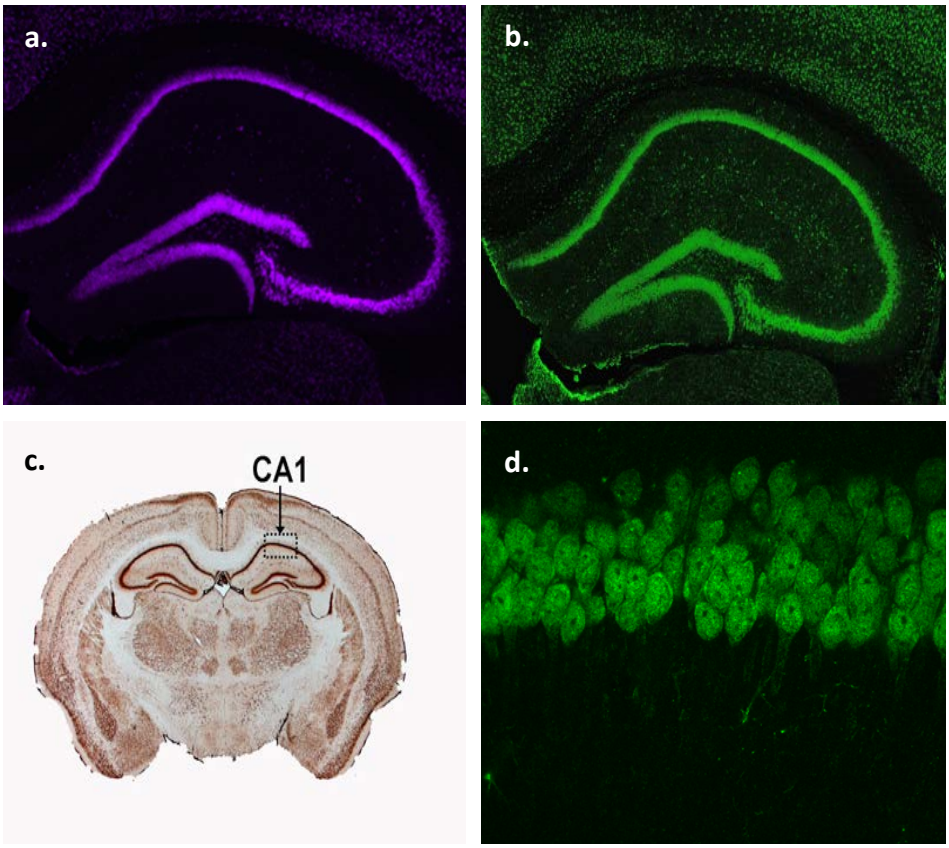


Figure 28. Expression of EphB2 coincides with NeuN in the mouse hippocampus. Whole mice brains were fixed in PFA and coronal slices of 70µm were cut. Slices were double stained for EphB2 and NeuN and binding of fluorescent secondary antibodies visualised using confocal microscopy. **(a)** and **(b).** Staining of EphB2 coincided with the staining of NeuN indicating a predominantly neuronal expression of EphB2. The staining of EphB2 revealed an expression within the stratum pyramidale and stratum granulosum, of the CA1/CA3 regions and dentate gyrus. Higher magnification at the CA1 region **(c)** revealed EphB2 expression at the neuronal cell body and extending into the dendrites **(d)**.

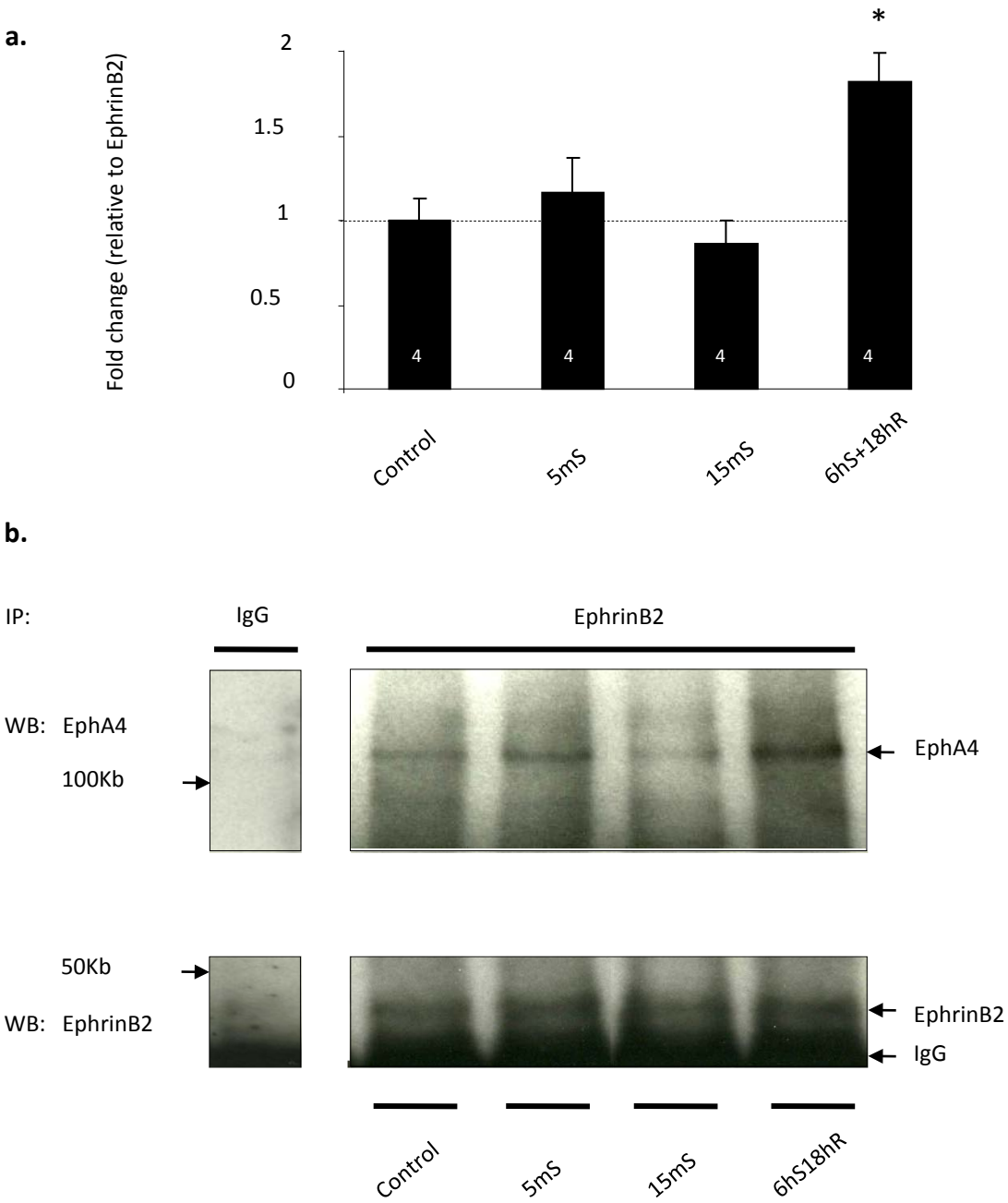


Figure 29. **EphrinB2-EphA4 interaction increases after a recovery period following stress. (b).** Control mice or mice were subjected to 5 minutes of stress, 15 minutes of stress or 6 hours of stress and an 18 hour recovery then their hippocampi were dissected and homogenised. EphrinB2 was immunoprecipitated and the resulting precipitates were analysed by Western blotting for EphA4 and EphrinB2. **(a)** Quantification revealed an increase in the interaction between EphrinB2 and EphA4 after 6 hours of stress and 18 hours recovery ($F_{(3, 12)} = 6.42$; $p<0.05$ control vs 6hS +18hR). * = $p<0.05$

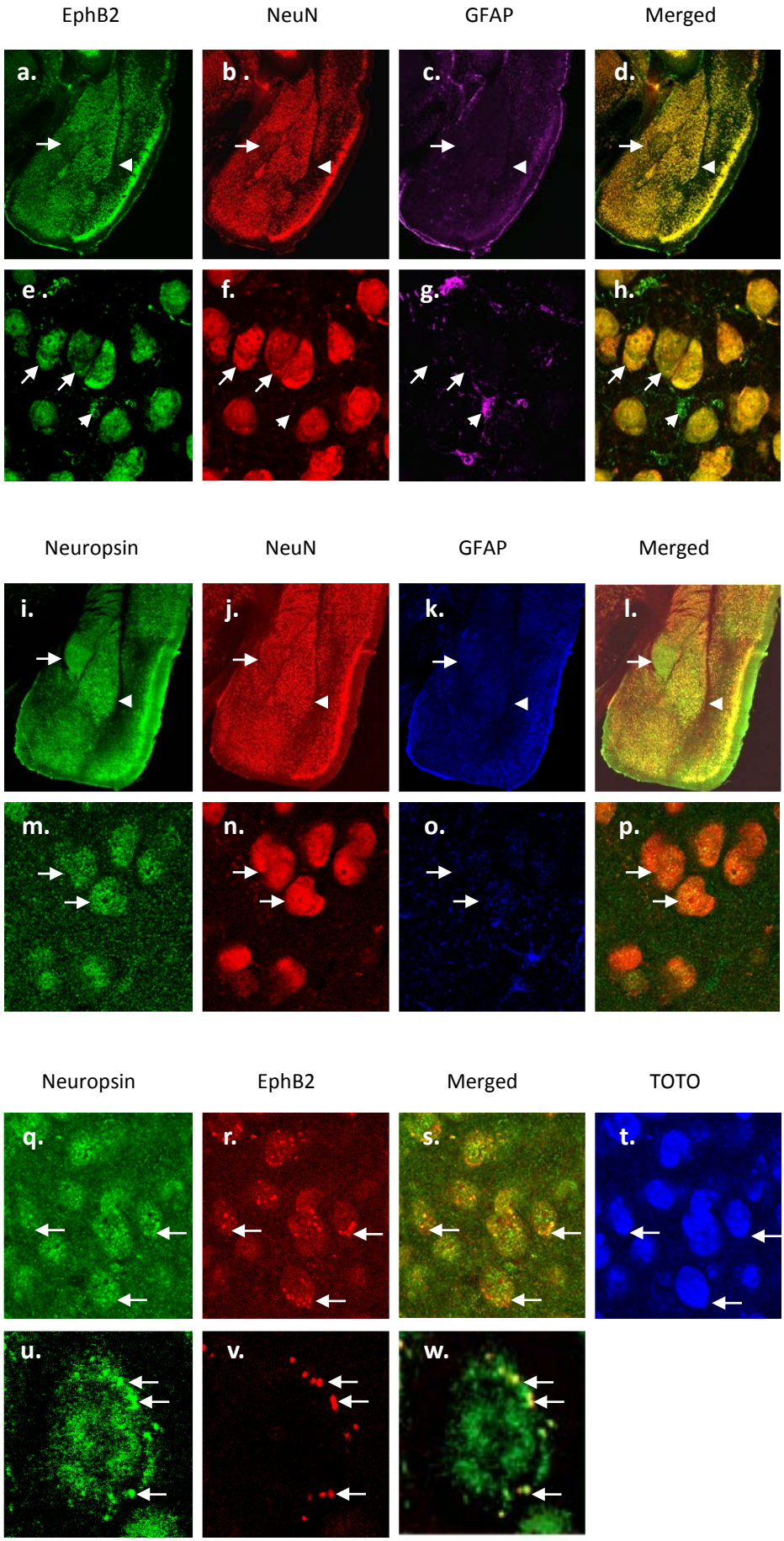

EphB2
NeuN
GFAP
Merged
a.
b .
c.
d.
e .
f.
g.
h.
Neuropsin
NeuN
GFAP
Merged
i.
j.
k.
l.
m.
n.
o.
p.
Neuropsin
EphB2
Merged
TOTO
q.
r.
s.
t.
u.
v.
w.

Figure 30. Neuropsin and EphB2 co-localise in neurons of the basolateral complex of the amygdala. With help from M. Mucha. Free-floating sections containing the amygdala were prepared from wild-type mouse brains and fixed with paraformaldehyde. Immunohistochemistry revealed high expression of EphB2 (green; **a** and **e**) in the lateral/basolateral and central (arrows in **a**-**d**) amygdala. To investigate which cell types express EphB2 in the amygdala we performed multiple co-labelling using antibodies against neuronal (NeuN; red) and astrocytic (GFAP; blue) markers in conjunction with the EphB2 staining. EphB2 highly co-localized with NeuN (**a**, **b** and merged in **d**; higher magnification in **e** and **f**; arrows) and to a lesser extent with with GFAP (**a**, **c** and merged in **h**; higher magnification in **e** and **g**; arrows) indicating that EphB2 is expressed by both neurons and astrocytes. (**b**) Neuropsin immunohistochemistry revealed high levels of this receptor in the lateral/basolateral and central amygdala (arrows in **i** and **m**) where EphB2 was highly expressed (**a** and **i**). Co-labelling with cell-specific markers demonstrated that neuropsin was expressed in neurons (**i** and **j**; higher magnification in **m-n**; arrows) but not in astrocytes (**i** and **k**; higher magnification in **m** and **o**; arrowheads). High levels of neuropsin were also observed in the neuropil which is consistent with its role as an extracellular protease (**l**, **m** and **q**). Double immunohistochemistry for neuropsin (**q** and **u**) and EphB2 (**r** and **v**) showed that they highly co-localize in the same cells in the lateral amygdala (**s** and **w**). Arrows point to EphB2-rich clusters where high levels of neuropsin were detected. Neuronal cell bodies were identified with TOTO (**t**)

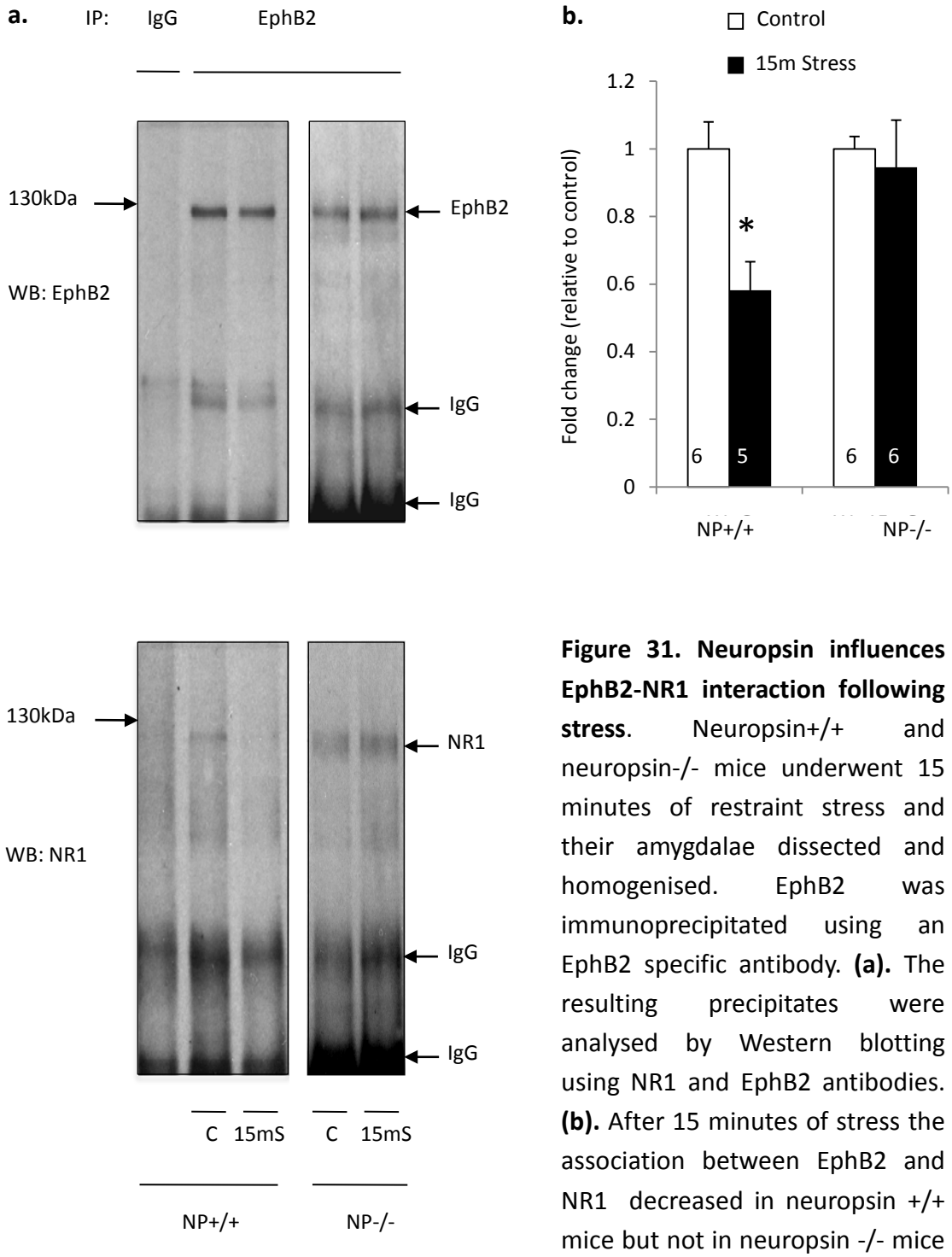


Figure 31. Neuropsin influences EphB2-NR1 interaction following stress. Neuropsin+/+ and neuropsin-/- mice underwent 15 minutes of restraint stress and their amygdalae dissected and homogenised. EphB2 was immunoprecipitated using an EphB2 specific antibody. **(a).** The resulting precipitates were analysed by Western blotting using NR1 and EphB2 antibodies. **(b).** After 15 minutes of stress the association between EphB2 and NR1 decreased in neuropsin +/+ mice but not in neuropsin -/- mice ($F_{(3,\ 19)} = 4.20$; $p<0.05$ NP+/+ control vs NP+/+ stressed). * = $p<0.05$.

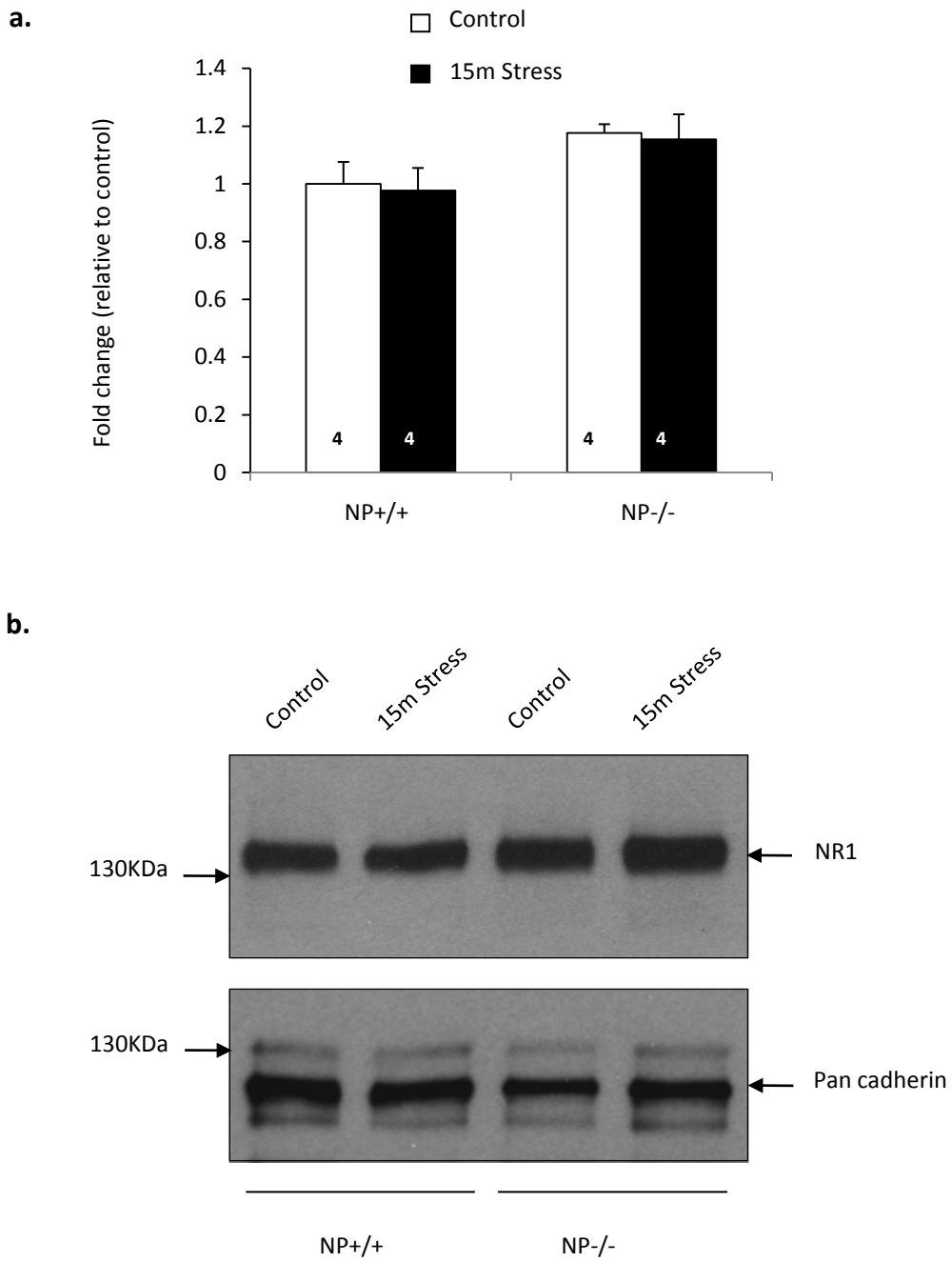


Figure 32. Neuropsin does not alter the membranous levels of the NMDA subunit NR1. Neuropsin+/+ and neuropsin-/- mice underwent 15minutes of restraint stress and their amygdalae were dissected and the cellular fractions were separated. The membrane fraction was blotted for NR1 and normalised using pan cadherin. **(a).** The levels of NR1 did not change following stress in both neuropsin+/+ or neuropsin-/- mice. **(b)** A representative blot against NR1 in amygdala samples taken from neuropsin+/+ and neuropsin -/- mice. The NR1 levels were normalised against levels of pan-cadherin.

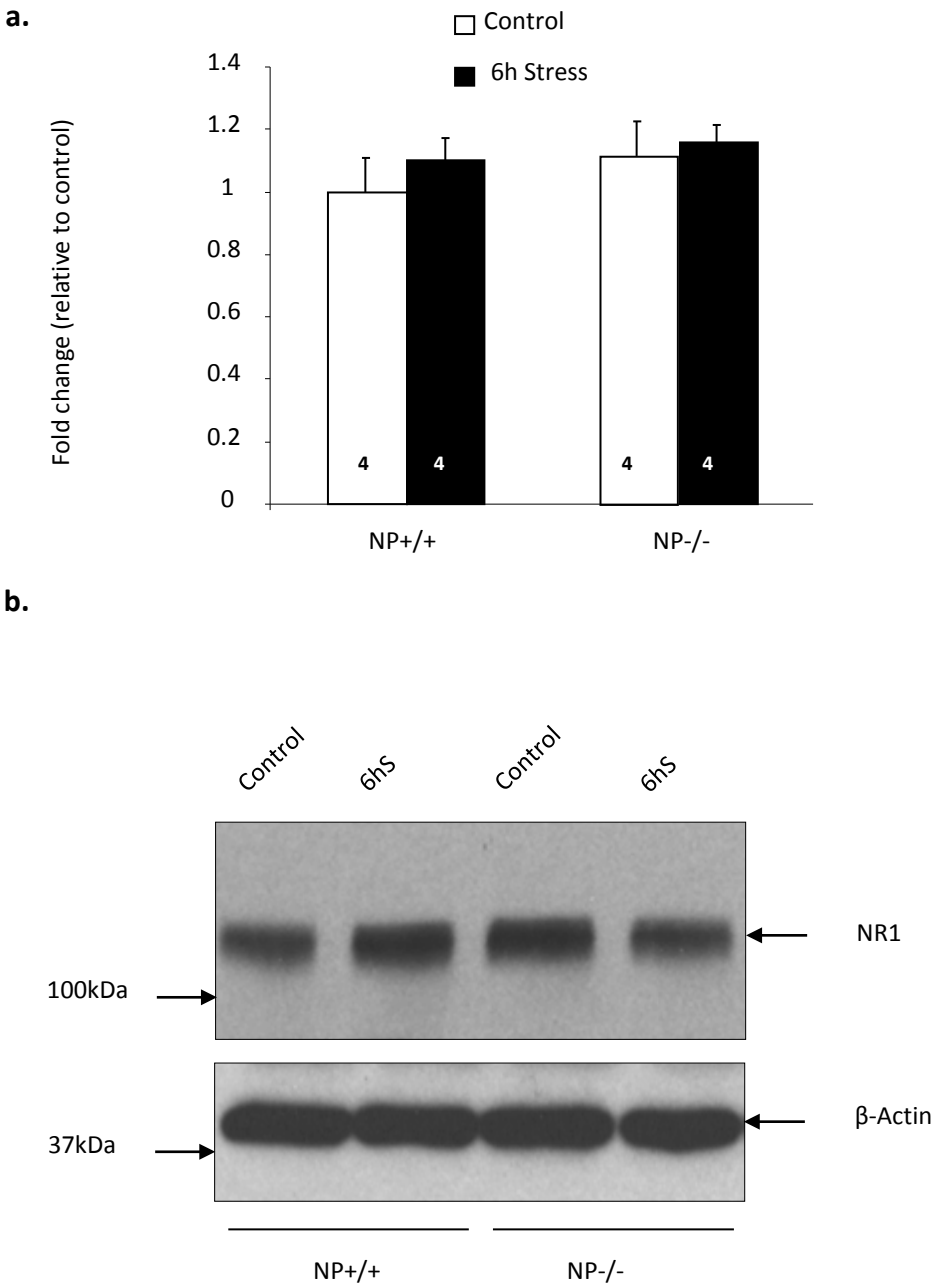


Figure 33. Total amygdala NR1 levels are not altered either by neuropsin or stress. Neuropsin+/+ and neuropsin-/- mice underwent 6 hours of restraint stress and their amygdalae were dissected and then homogenised. The homogenate was analysed by western blotting for NR1 and normalised using actin levels. (**a)** The levels of NR1 were the same in mice of both genotypes and did not change following stress. (**b)** A representative blot against NR1 in amygdala samples taken from neuropsin+/+ and neuropsin -/- mice. The NR1 levels were normalised against levels of actin.

Results

Eph gene expression following stress

Preliminary work (figures 22-24) indicated that Eph proteins may be critically involved in the stress response. This was revealed by investigations of the Eph gene expression in the hippocampus and amygdala. A group of wild-type mice were subjected to restraint stress, whilst a control group remained unstressed and the Eph gene expression was compared (Figure 21). qRT-PCR revealed an increase in EphB2 (Figure 21; 11-fold; T-test $p<0.001$) EphB6 (Figure 21; 5-fold; T-test $p<0.05$) and EphrinB2 (Figure 21; 2.1 fold; T-test $p<0.05$) gene expression in the hippocampus after restraint stress. Similar increases were observed in the amygdala. Compared to the hippocampus, EphB2 had a slightly lower upregulation (Figure 21; 2 fold; T-test $p<0.001$), EphB6 a slightly higher upregulation (Figure 21; 9-fold T-test $p<0.001$) and EphrinB2 a similar upregulation (Figure 21; 2.8-fold; T-test $p<0.05$). The other Eph gene expressions were unchanged, in both brain regions, following stress.

Eph protein expression following stress

To investigate whether the gene expression resulted in a protein increase, mice underwent the same stress protocol, and the protein levels of the EphA4, EphB2, EphB6 and EphrinB2 were measured by Western blotting. At the same time point as the gene expression upregulation (6hS), Western blotting revealed no differences in the level of Eph receptors or EphrinB2 in the

hippocampus or amygdala compared to unstressed control mice (Figure 22, Figure 23, Figure 24; T-test $p>0.05$ for all molecules).

To investigate whether a protein increase might be delayed relative to gene expression, wild-type mice were subjected to the same stress protocol but also allowed them to recover in their home cage for 6 hours or 18 hours before measuring the Eph protein level. When the protein expression was quantified in these samples, a 5.8- and 2.1-fold up-regulation of EphrinB2 in the hippocampus and amygdala, respectively, was observed (Figure 22; F = $p<0.01$ for the hippocampus and Figure 23; F = $p<0.05$ for the amygdala). This increase was more prominent after a longer duration of the recovery period (Figure 22; F = $p<0.05$ for 6hS + 6hR vs. $p<0.01$ for 6hS+18hR in the hippocampus and Figure 23; F = $p<0.05$ for 6hS + 18hR amygdala).

The protein levels of EphA4, and EphB6 remained unchanged at all time-points and regions examined (Figure 22 and Figure 23; F = ; $p>0.05$). Moreover, EphB2 showed a decrease in protein expression in the hippocampus (Figure 22; 6hS + 18hR; F = $p<0.05$).

EphA4 binds EphrinB2

To investigate EphrinB2 - Eph binding *in vitro,* EphA4-EGFP, EphB2-EGFP and EGFP alone were over-expressed in SHSY-5Y cells and incubated the cells with exogenous EphrinB2 Fc. Ephrin-Fc proteins contain the extracellular domain of the Ephrin protein, which is conjugated to an IgG fragment and is used to bind Eph receptors. Prior to the addition of EphrinB2 Fc, it was

clustered using Cy-5 conjugated anti-human IgG, which allowed for visualization of the exogenous Fc protein. Following fifteen minutes incubation, EphrinB2 clearly co-localised with clusters of both the EphB2-EGFP and EphA4-EGFP (Figure 25). In comparison, the EphrinB2 Fc associated uniformly with the cells that did not overexpress Eph receptors but were transfected with the empty EGFP construct (Figure 25). This confirms that EphrinB2 does act as a binding partner for both EphB2 and EphA4 *in vitro*.

To investigate EphrinB2 interaction with the Eph receptors *in vivo,* a co-immunoprecipitation experiment was performed. Antibodies specific to EphA4, EphB2 and EphB6 were used to immunoprecipitate the respective receptors from hippocampal and amygdala samples. The resulting material was then analysed by Western blotting, which revealed that EphrinB2 preferentially bound to EphA4 in both brain regions analysed (Figure 26). Successful immunoprecipitation of the receptors was demonstrated by a band of the correct molecular weight in the lanes where an Eph receptor antibody was used for the immunoprecipitation, whilst the absence of a band at the same molecular weight in the lane where unspecific IgG replaced the Eph-precipitating antibody demonstrated the opposite. When the same membranes were blotted for EphrinB2, a band was only found in the EphA4 precipitates, indicating a strong *in vivo* ligand and receptor interaction.

This relationship was further studied by immunohistochemistry to reveal the spatial distribution of EphA4 and EphrinB2 in the hippocampus and the amygdala. The hippocampus and the amygdala have elegant yet complex

morphology and cyto-architecture. The hippocampus is composed of four different regions, called CA1 to CA4, and is closely associated with the dentate gyrus. The amygdala is a group of several nuclei, which serve different functions. These regions may work in concert to coordinate stress-related responses, but are often autonomous when it comes to the regulation of gene and protein expression. Thus, immunohistochemistry may reveal spatial information regarding the expression and potential interaction of proteins restricted to particular areas within the hippocampus or amygdala. Such information may have gone undetected by Western blotting, which utilises homogenates of the whole structure. Our results show high levels of EphA4 and EphrinB2 protein in the hippocampus and amygdala, but the expression was not uniform within these structures. The analysis by confocal microscopy revealed a clear reciprocal spatial distribution of EphA4 and EphrinB2 **(**Figure 27).

EphA4, EphB2 and EphrinB2 staining in the hippocampus

Within the hippocampal formation, the expression of EphA4 was the strongest in CA3 region, intermediate in CA1-CA2 and the weakest in the dentate gyrus (Figure 27). EphA4 expression followed the pattern of the laminar organisation of the hippocampus, with its levels differing significantly among various anatomical layers. The strongest staining was observed in stratum oriens, which contains the basal dendrites of pyramidal neurons, as well as septal/commissural fibres from the contralateral hippocampus. The signal was almost equally strong in stratum radiatum composed of axonal projections from the contralateral hippocampus, Schaffer collateral fibres projecting from CA3,

apical dendrites of pyramidal cells of CA1, and interneurons. In particular, higher magnification revealed strong EphA4 staining on neuronal processes in CA3 region of the stratum radiatum. However, EphA4 levels were markedly lower in stratum lacunosum-moleculare which contains Schaffer collaterals and perforant path fibres projecting onto distal, apical dendrites of pyramidal cells. Relatively weak staining was observed in stratum moleculare of the dentate gyrus, the layer where commissural fibres from the contralateral dentate gyrus, perforant path processes and axonal inputs from the medial septum form synapses with the dendrites of the granule cells. In fact, two bands of staining were seen here: the outer third, receiving input from the lateral enterohinal area, expressing less EphA4, and the inner two-thirds, receiving input from the medial enterohinal area, expressing more EphA4. This result shows that the expression of EphA4 differs significantly among various hippocampal layers and is consistent with the expression of EphA4 in neuronal processes. Importantly, minimal staining of EphA4 was detected in stratum pyramidale or stratum granulosum, which harbour cell bodies of pyramidal cells/interneurons of the CA1-CA3 regions and granule cells of the dentate gyrus, respectively. This is in contrast and spatially complementary to EphrinB2 staining, which was strongest in the stratum pyramidale and granulosum. The staining was marginally weaker in the CA3 region reflecting the slightly less compact cell bodies in the CA3 region. Unlike EphA4 staining, EphrinB2 staining in the remaining strata - stratum oriens, radiatum, lacunosum-moleculare and moleculare – showed equal levels of staining, much weaker than staining from the stratum pyramidale and granulosum. Closer magnification revealed that the strong staining from the stratum pyramidale and granulosum was due to

strong EphrinB2 immunoreactivity from neuronal cell bodies. The cellular staining reflected that seen in transfection experiments of Eph receptor constructs (Figure 25). The fluorescence was localized towards the cell membrane, as expected from a transmembrane receptor. This was in contrast to EphA4 staining, which was absent from neuronal cell bodies. EphrinB2 staining was also highly visible in neuronal processes projecting from CA1 cell bodies into the stratum radiatum. EphB2 staining revealed a similar pattern of staining to EphrinB2 (Figure 28). The strongest fluorescence appeared in the stratum pyramidale of the CA1 and CA3 regions, and the stratum granulosum of the dentate gyrus. The staining was particularly strong from the cell body but did extend to the dendrite. The predominantly neuronal staining was confirmed by co-localisation with NeuN, a marker for neuronal cells.

EphA4 and EphrinB2 staining in the amygdala

The amygdala can be roughly divided into four groups of nuclei: basolateral, central, medial and cortical. While the lateral group has clear boundaries, the central, medial and cortical are less well-defined. Moreover, the amygdala neurons do not have clear polarity typical for the hippocampal ones, and therefore the amygdala is lacking a laminar structure. This makes it more difficult to interpret the expression pattern of a protein using histological methods. When immunohistochemistry was performed, the expression of EphA4 was evident in all groups of amygdala nuclei, but the levels differed significantly. The strongest signal was observed in the central amygdala, followed by the lateral/basolateral and medial/cortical amygdala (Figure 27). Unlike the hippocampus, the staining within the nuclei was uniform and diffuse,

making it difficult to associate the expression of EphA4 in the amygdala with any particular part of the cell. However, the absence of staining from the cell bodies, but presence of staining in the neuropil, reflecting staining within processes, was similar. Indeed, the reciprocal nature of the staining was the most striking similarity. EphrinB2 staining was also present in all the amygdala nuclei, although the medial nuclei showed the weakest staining. Although the cell bodies within the amygdala are not together, it was clear that they showed the highest EphrinB2 immunoreactivity. Higher magnification revealed the same neuronal body staining as the hippocampus. There was strong staining at the cell perimeter and intracellular puncta. Although the neuronal processes are not as easy to visualise in the longitudinal axis within the hippocampus, EphrinB2 staining was also found in neuronal processes.

The EphA4 – EphrinB2 interaction is regulated by stress

EphrinB2 was immunoprecipitated from hippocampal samples taken from mice at different time points after restraint stress. The precipitate was then probed for levels of EphA4 by Western blotting. At five minutes during the stress protocol, there were no differences in the interaction between EphA4 and EphrinB2; however, there appeared to be a trend towards a decrease in the interaction after fifteen minutes of stress (statistically non-significant). After eighteen hours following stress the interaction between EphrinB2 and EphA4 increased (Figure 29; $F_{(3, 12)} = 6.4$; $p<0.05$ control vs. 6hS +18hR).

EphB2 and neuropsin co-localise in vivo

There is no previous evidence for an interaction between EphB2 and neuropsin in the literature. To further investigate this interaction, immunohistochemistry was performed and the amygdala was examined. In order to learn which neuronal cell type expressed both neuropsin and EphB2, brain tissue was also stained for neuronal markers and astrocytic markers. EphB2 and neuropsin both displayed strong expression in the amygdala (Figure 30 – with help from M. Mucha). Throughout the amygdalar nuclei, the staining of both molecules was similar. The basolateral, central, medial and cortical nuclei all show strong reactivity for both neuropsin and EphB2. Closer magnification revealed that EphB2 staining was confined to cell bodies with highest fluorescence found in puncta towards the edges of the cell bodies. As in the hippocampus, the majority of the EphB2 staining co-localised with the NeuN, which highlighted that the EphB2 staining was both membranous, and within intracellular puncta. Staining with the astrocytic marker, GFAP revealed the EphB2 staining was also within astrocytes, although at a lower level than in neurons. Like EphB2, neuropsin staining also showed a high level of co-localisation with the NeuN. However, the staining is more evenly distributed throughout the cell body. Unlike EphB2, neuropsin staining does not co-localise with GFAP, indicating that astrocytes do not synthesise neuropsin. Neuropsin staining throughout the amygdala shows a more diffuse pattern than EphB2. Fluorescence is also observed in the neuropil beyond the boundary of the cell body, consistent with its role as an extracellular protease. Double immunohistochemistry of EphB2 and neuropsin reveals a high degree of co-localisation. Both receptor and protease are expressed by the same

amygdala neurons and their puncta co-localise to a high degree. Higher magnification shows that the interaction occurs predominantly at the border of the neuron. The staining indicates a spatial relationship that is likely to maximise the opportunity for their interaction (Figure 30).

The EphB2 and NMDA subunit interaction is regulated by stress

EphB2-NMDA interaction in the amygdala was investigated using immunoprecipitation. An EphB2 specific antibody was used to immunoprecipitate material from homogenised amygdalae of wild-type and NP-/- mice. The immunoprecipitate was probed for the NR1 subunit of the NMDA receptor by Western blotting. In non-stressed mice, NR1 immunoprecipitated with EphB2 in both wild-type and NP-/- mice (Figure 31). However following fifteen minutes of stress, this interaction was reduced in wild-type mice whilst remaining at the same level in NP-/- mice (Figure 31; $F_{(3, 19)} = 4.2$; $p<0.05$ NP+/+ control vs. NP+/+ stressed). The stress induced regulation of the EphB2 - NMDA interaction was not due to regulation of the total membranous NMDA receptor as this was unchanged following fifteen minutes of stress (Figure 32; $F_{(3, 12)} = 2.07$; $p>0.05$). It was also hypothesised that the stress-induced modulation of the EphB2 – NMDA interaction may result in a regulation of the NMDA amygdala expression level at a later time point following stress. However, following 6 hours of stress, the total amygdala NMDA level was not altered in either wild-type or NP-/- mice (Figure 33; $F_{(3, 12)} = 0.77$; $p>0.05$).

Discussion

Summary

Preliminary work had revealed that the gene and protein expression of Eph receptors and EphrinB2 was regulated by stress (Figure 21- 23). The discovery that plasmin cleaves EphA4 and that neuropsin cleaves EphB2 demanded that their role in stress was further investigated. This revealed different aspects of their function in different areas of the brain. The common theme in this chapter is the interactions between the receptors and their binding partners during stress. The Eph receptors display promiscuous ligand binding and physically interact with a number of partners at the cell membrane. Studies using an *in vitro* cell model revealed, in accordance with the literature, that EphrinB2 bound to both EphB2 and EphA4 (Figure 25). Immunoprecipitation studies revealed that EphA4 shows a strong *in vivo* interaction with EphrinB2 (Figure 26). When the *in vivo* positioning of the receptors and ligand were investigated immunohistochemically, it revealed that the majority of EphrinB2 was likely to interact with EphA4 (Figure 27). The EphA4-EphrinB2 interaction in the hippocampus was modulated by stress, increasing their interaction following stress (Figure 29).

The investigations of EphB2 focused on its regulation by neuropsin in the amygdala. Immunohistochemistry revealed co-localisation of EphB2 and neuropsin at the neuronal membrane – extracellular interface (Figure 30). An important receptor in experience-dependent plasticity is the NMDA receptor,

which interacts with EphB2. Investigations revealed that neuropsin regulates the interaction between EphB2 and NMDA in the amygdala during stress. Immunoprecipitation revealed that, fifteen minutes following stress, the interaction is decreased (Figure 31). This change in interaction occurs without a change in the membranous quantity of NMDA receptors and do not result in stress induced regulation of NMDA receptors in the amygdala (Figure 32 and Figure 33). The results described in this chapter reveal that Eph receptors and their modulation by proteases are central candidates in the regulation of stress-related neuronal physiology.

The Eph receptor and Ephrin gene expression changes

Preliminary studies revealed that the EphB2, EphB6 and EphrinB2 genes were up-regulated following stress (Figure 21). Is this pattern of upregulation consistent with models in the literature in which Eph receptors are upregulated? Eph receptors are upregulated in a wide range of scenarios in mature cells. This includes bone remodelling and bone disease, cancerous cells (colorectal and breast cancer), hypoxic cells (skin and bone marrow), neuronal cells following injury, and inflammatory cells (for review (Pasquale, 2008).

The neuronal upregulation of Eph receptors was observed in two studies using a model of neuronal injury in the hippocampus. In these studies, the gene upregulation of the Eph receptors was seen at a later time point than observed in our stress model. Whereas our gene upregulation was observed at six hours following stress, the injury models observed the peak gene upregulation three

and seven days following the injury (Wang *et al.*, 2005; Moreno-Flores & Wandosell, 1999). It is likely that the time frame in these cases reflect the different processes that are occurring in the different models. Wang *et al* and Moereno-Flores *et al* believe the Eph receptors are likely to be involved in axonal path finding, synaptogenesis or cell survival (Wang *et al.*, 2005; Moreno-Flores & Wandosell, 1999). Due to the traumatic nature of the injury model, the cellular functions occurring are involved in the regeneration of synapses and neurons (Miranda *et al.*, 1999). Our model may also contain elements of these functions, as stress results in neuronal and synaptic remodelling. For example, stress results in a modulation of synaptic plasticity, a process that may require morphological alteration of dendritic spines. There is overwhelming evidence for the Eph receptors to be involved in this process. However, the time frame for Eph function in the stress model is likely to be shorter, as the processes regulated by the Eph receptors in the injury models may only occur after inflammatory and reparative steps have been completed. It is also worth noting that upregulation of EphrinB1, but not EphrinB2, was seen in these models, perhaps indicating different signalling pathway. Other Eph molecules upregulated included EphB2, EphA5, EphA4 and EphrinA5, indicating coordinated molecular mechanisms, albeit different from those observed after stress.

A very similar time frame of Eph receptor upregulation to that seen in our paradigm occurs in a model of hypoxic mouse skin. Here, the authors found that EphB4 mRNA was upregulated six hours after hypoxia followed by an increase in the protein twenty-four hours after the hypoxic insult (Vihanto *et al.*,

2005). EphrinB2 mRNA was also upregulated and reached a peak at twenty-four hours, whilst the protein was upregulated from six hours persisting to forty-eight hours (Vihanto *et al.*, 2005). Within this model, the Ephrins are upregulated due to their role in angiogenesis, and so their function in this model is different to their function in our model. Nevertheless, the time scale of gene and protein upregulation of EphrinB2 described here is consistent with the time frame of upregulation of Eph receptors observed in our stress model.

Stress up-regulates the expression of EphrinB2 protein

To gain a more comprehensive picture of the hippocampus and amygdala's response to acute restraint stress, the regulation of the Eph proteins was measured (Figure 22-24). The protein expression of the genes that were significantly up-regulated (EphB2, EphB6 and EphrinB2) and also of EphA4 was measured. Although EphA4 did not show an increase in gene expression, it was decided to quantify its protein expression. This was based on the finding of its high gene expression in the hippocampus and the amygdala (Preliminary work and (Liebl *et al.*, 2003), its role in regulating spine morphology and plasticity, and the finding that protein abundance may increase without a preceding increase in mRNA (Ideker *et al.*, 2001).

Preliminary work revealed that significant increases in Ephrin mRNA levels after acute restraint stress did not necessarily lead to a corresponding increase in the Ephrin protein. Of the four proteins examined, only EphrinB2 showed a significant increase in response to stress. Furthermore this elevation did not immediately follow the mRNA increase indicating a delay in the translation of

the up-regulated mRNA. The response was greater in the hippocampus than the amygdala. EphB6 and EphA4 showed no significant change in protein concentration at any time period in both the hippocampus and the amygdala. Despite showing a robust increase in mRNA levels, the quantity of EphB2 protein decreased after the acute restraint stress in the hippocampus.

These results demonstrate the dissociation between gene and protein expression levels in biological systems, and highlight the importance of studying the central nervous system's response to environmental challenge at multiple levels. Most importantly, however, these results strengthen the possibility of EphrinB2 being an important mediator of the stress response.

Regulation of a gene expression is an important, but not the sole, factor in determining the level at which the protein is present and active in the cell. In fact, it has been shown that an up-regulation of a gene does not always lead to an increase in protein levels (Griffin *et al.*, 2002). The correlation between mRNA and the corresponding protein expression has been studied previously. The experiments clearly suggest that the quantity of the mRNA is an unreliable indicator of the quantity of the protein (Gygi *et al.*, 1999). Moreover, when a change in the gene expression occurs, it is not necessarily followed by a change in the abundance of the protein. In extreme circumstances, the increase in gene expression could be followed by a subsequent decrease in protein expression (Griffin *et al.*, 2002). Although these phenomena have mainly been studied in yeast, the mechanisms involved are also active in

eukaryotes (Gygi *et al.*, 1999) and therefore may explain the lack of correlation between EphB2 and EphB6 mRNA and protein.

One factor that could affect the translation of mRNA to protein is the availability of ribosomes. As the quantity of mRNA increases, the availability of ribosomes becomes the limiting factor in the translation. Furthermore, the cell is not able to increase the availability of ribosomal proteins rapidly as their mRNAs compete with the other mRNAs for binding to ribosomes (Lee *et al.*, 2003). The above situation is analogous to that seen in yeast galactosidase that converts galactose to glucose 6-phosphate. The presence of galactose causes a 500-fold increase in the GAL2 gene expression followed by only 10-fold rise in the protein levels (Griffin *et al.*, 2002). Other mechanisms of post-transcriptional regulation include the control of mRNA binding to ribosomes and the control of mRNA degradation (for review see (McCarthy, 1998).

The lack of an increase in EphB6 and a decrease in EphB2 protein levels could reflect an accelerated protein turnover and degradation. It has been demonstrated at the growth cone that the EphB-EphrinB complex is endocytosed to remove it from the cell membrane (Zimmer *et al.*, 2003). Furthermore, it has been shown that upon neuron-astrocyte contact full length EphB2 is trans-endocytosed from the neuron to the astrocyte (Lauterbach & Klein, 2006). It is possible that stress induces an increase in EphB2 protein synthesis, but also its subsequent degradation, resulting in a decrease in overall EphB2. This may be related to neuropsin cleavage of EphB2 (Figure 12-16). As discussed, the increase in EphB2 gene expression is neuropsin-

dependent (Figure 18. **EphB2 gene expression is increased 6 hours following stress.**). In the amygdala, neuropsin cleaves EphB2 in the first fifteen minutes following stress. However, it is possible that the neuropsin-dependent mechanisms of plasticity in the amygdala and hippocampus are different (Matsumoto-Miyai *et al.*, 2003; Attwood *et al.*, 2011). In the hippocampus, this may result in cleavage of EphB2 at a latter time point, correlating with the decrease in the EphB2 protein eighteen hours following stress, seen only in the hippocampus (Figure 22).

Composition of EphrinB2/Eph assemblies in vitro and in vivo

In order to understand the role of EphrinB2 during stress, it was attempted to establish its predominant Eph binding partner. Initially, an *in vitro* SHSY-5Y cell culture model over-expressing EphA4-GFP and EphB2-GFP was used. Following addition of soluble EphrinB2, it was clear that EphrinB2 showed high binding affinity to both EphA4 and EphB2 (Figure 25). This is in accordance with crystallography studies, which have characterized the binding interactions between EphrinB2 and the EphB2 and EphA4 receptors, as well as *in vitro* and *in vivo* studies that have indicated the function of these interactions (Himanen *et al.*, 2001; Qin *et al.*, 2010).

Although no quantification was performed, EphrinB2 bound to the overexpressed EphA4 and the overexpressed EphB2 in high abundance. This indicates that perhaps the weaker binding interaction of EphrinB2 and EphA4 shown by crystallography does not affect the interaction in this model. The crystallographic findings show the EphA4-EphrinB2 binding constant to be

approximately ten times weaker than the EphB2-EphrinB2 binding constant (Qin *et al.*, 2010). However, as the receptor was overexpressed and a liberal amount of EphrinB2 Fc was added to the medium, there is not likely to be significant competition between the native EphA4 and the overexpressed EphB2 (Figure 25).

Our functional *in vivo* interaction studies suggest that EphA4-EphrinB2 binding is more prominent compared to that of EphB2-EphrinB2 in the hippocampus and amygdala. The spatial expression, as determined by immunohistochemistry, and the functional binding, as determined by immunoprecipitation, indicate the predominant EphA4 interacting partner is EphrinB2 (Figure 26-28). The majority of Ephrin ligands that bind to Eph receptors in the brain are membrane-associated/tethered Ephrin molecules. This receptor-ligand interaction is therefore dependent on cellular Ephrin expression and must be taken into account when considering crystallography studies or our *in vitro* model, which use soluble Ephrin-Fc molecules. For example, in the hippocampus, the EphrinB2 staining can be seen in the dendrites of CA1 neurons that form synapses with axons projecting from CA3 neurons. The dendritic EphrinB2 staining appears to cut its way through the strong EphA4 stratum radiatum staining. This reciprocal distribution of EphA4 and EphrinB2 raises the possibility that the above molecules could, at least in part, interact trans-synaptically as the receptor and ligand. In comparison the EphB2 and EphrinB2 staining occur in the same neurons indicating a less prominent role in trans-synaptic signalling.

Further evidence for the interaction between EphA4 and EphrinB2 has been provided by our collaborators. Using *in situ* zymography together with immunohistochemistry Professor Wilczynski has shown that EphA4 and EphrinB2 co-localize in the hippocampus at sites of plasmin activity (Appendix 4). This techniques allows the activity of plasmin to be studied alongside the location of molecular targets in a coronal section of brain (Gawlak *et al., 2009)*

Overexpression of a particular Eph receptor *in vitro* is unlikely to reflect the precise and diverse interactions of the Eph receptors *in vivo.* For example, the *in vitro* neuronal cultures do not accurately model the complex environment in which hippocampal neurons exist. A stunning lay analogy that encapsulates this is the calculation that there is greater processing power within a single human brain than there is in all of the computers in the world combined (Micheva *et al.*, 2010). This is achieved by the number of synaptic connections, precisely organised through maturation from embryo to adult. The hippocampal structure is key in determining its function. Embryologically, it can be distinguished from thirteen to fourteen weeks, before folding into its characteristic laminar organisation over the following weeks (Kier *et al.*, 1997). The structural organisation allows the sequential connectivity of the hippocampal neuronal subtypes and thus the flow of information, creating the trisynaptic function. Although this classical model is simplified, it underlines the importance of structural positioning to the function of the neuronal cells. Each of the anatomically organised neuronal subtypes (CA1, CA3, dentate gyrus) possesses distinct molecular and functional characteristics. This is reflected in the Eph expression in the hippocampus, with pre- and post- synaptic Eph

protein expression regulating different neuronal functions (Chapter 1). The *in vitro* cultures can result in false representations of the *in vivo* function (Hensch *et al.*, 1998). The *in vitro* cellular models exist within an altered extracellular matrix without the complexity and brain circuitry observed *in vivo*. An important factor regulating neuronal function are neuroglia, which are critical for correct neuronal functioning. They regulate excitatory and inhibitory transmission as well as LTP (Theodosis *et al.*, 2008). *In vitro* cultures of neuronal cells do not maintain the same neuronal glia relationships that are critical *in vivo.* Indeed, the SHSY-5Y cell culture does not contain neuroglia at all and does not develop into a mature neuronal phenotype unless stimulated to do so (Agholme *et al.*, 2010). This cell line may also misrepresent the physiological state due to their inherent characteristics as neuroblastoma cells. They have pathological gains and losses in their chromosomes and fail to express a number of neuronal features (Agholme *et al.*, 2010).

Despite these differences, the *in vitro* cellular model does allow us to observe the interaction between the overexpressed Eph receptors and the EphrinB2 ligand. These findings, however, must be verified *in vivo*. In the context of my studies, it confirms that both EphA4 and EphB2 do bind to EphrinB2, and that at the concentrations used, their binding appears roughly similar. This is also useful in confirming the binding capabilities of the Eph receptors constructs, allowing confidence in further functional studies in which they may be used.

Regulation of EphA4 and EphrinB2 interaction by stress

The data presented in this thesis indicate that limbic Eph proteins are regulated in both the early stages and later stages of the stress response. Changes in the membranous EphB2 levels are seen following fifteen minutes of stress indicating a molecular dynamism at the neuronal membrane (Figure 17). Later in the stress response the Eph protein expression is also regulated with a rise in EphrinB2 protein in the hippocampus and amygdala eighteen hours following 6 hours of restraint stress (Figures 22 and 23). To investigate the molecular interactions over time, following the stress response, immunoprecipitation was used (Figure 29). This revealed that the interaction between EphrinB2 and EphA4 increased eighteen hours following six hours of restraint stress. The aim of the experiment was to normalise the quantity of EphA4 preciptated with EphrinB2 to EphrinB2 allowing analysis of the EphrinB2-EphA4 binding at different time points following stress. The time where the increase in interaction is observed is the same time point at which EphrinB2 expression is increased in the hippocampus (Figure 22). Although the experiment design does not allow for an inter-group comparison of EphrinB2 and therefore no definite conclusion can be made, it appears there is not the clear increase in the amount of EphrinB2 immunoprecipitated inkeeping with the Western blotting results at this time point (Figures 22 and 29). This may be due to a methodological cause or it may indicate that the rise in EphrinB2 protein described in Figures 22 has not been robustly replicated.

The immunoprecipitation experiment uses a different homogenisation buffer to that used for Western blotting, which may lead to a different quantity of

EphrinB2 extracted from the sample. A number of lysis buffers can be used for immunoprecipitation and the ‘HO’ buffer used was the third used by this investigator in order to gain robust immunoprecipitation. It may also be that the quantity of EphrinB2 pulled down by immunopreciptation is not representative of the total quantity of EphrinB2 in the sample. This could be caused by failure of the EphrinB2 antibody to efficiently immunoprecipitate EphrinB2 complexes formed during stress conditions. To investigate this alternative EphrinB2 antibodies could be used to immunopreciptaed the EphrinB2 complexes. The experiment could also be repeated immunopreciptationg EphA4 and probing for EphrinB2, such as demonstrated in an earlier experiment (Figure 26). EphA4 protein expression in the hippocampus was stable following stress and so the levels immunoprecipitated would not be expected to vary (Figure 22). However, given the immunoprecipitation result it may be prudent to re-test the hypothesis that EphrinB2 expression increases in the hippocampus at this time point. The Western blotting experiments could be repeated with more animals or an alternative method for measuring stress induced protein upregulation could be used. For example immunofluorescent staining for EphrinB2 in the hippocampus at similar timepoints following stress could be utilised.

The importance of subcellular location of Eph proteins

Without stress, EphA4 and EphrinB2 are prominent binding partners (Figure 29). As the animal undergoes stress the interaction between EphA4 and EphrinB2 increases (Figure 29). This indicates that both molecules must be located in the same cellular compartment for the interaction to occur. This may involve mobilization of EphA4 or/and EphrinB2 from their intracellular stores,

redistribution from other compartments, or novel molecular interactions that facilitate their binding. Due to the relatively weak binding between EphrinB2 and EphA4, it is less likely that EphA4 preferentially binds to EphrinB2 over EphrinA ligands (Qin *et al.*, 2010). Despite EphA4 being the most highly expressed Eph receptor in the hippocampus, most of it is not constitutively active (Tremblay *et al.*, 2007; Murai & Pasquale, 2003). In hippocampal neurons, much of EphA4 is stored in intracellular vesicles and the membranous expression of EphA4 is a dynamic process. Bouvier *et al* found that EphA4 is expressed in various vesicular organelles, including many synaptic vesicles at excitatory synapses. Furthermore the EphA4 cell membrane expression is increased following potassium depolarisation, indicating an experience-dependent membrane expression (Bouvier *et al.*, 2010). This is consistent with a sub-population of EphA4 that is transported to the membrane following stress, where it interacts with the increased EphrinB2 ligand. In the hippocampus, this is likely to be at the synapse, given its expression in synaptic vesicles and strong association with PSDs (Bouvier *et al.*, 2008).

The role of the EphA4-EphrinB2 interaction in synaptic plasticity

The alteration in EphA4-EphrinB2 interaction following stress may indicate the molecular mechanisms that regulate stress induced neuronal plasticity. Both EphA4 and EphrinB2 are required for hippocampal plasticity, but their specific interaction has never been shown to be critical in this respect. At the CA1-CA3 synapses, post-synaptic EphrinB2 is required for NMDA-mediated LTP (Grunwald *et al.*, 2004). At the same synapses, the extracellular, but not

intracellular, domains of EphA4 and EphB2 are also required for plasticity (Grunwald *et al.*, 2001). Therefore, it is possible that these receptors act as presynaptic partners activating EphrinB2. To investigate the role of EphA4 at the CA1 synapses, Filosa *et al* used conditional knockout animals in which EphA4 has been disrupted either in CA1 or CA3. This revealed that only the CA1, post-synaptic EphA4 was required for LTP (Filosa *et al.*, 2009). The authors attributed this effect to EphA4 interaction with astrocytic EphrinA3 to decrease glutamate transport resulting in LTP modulation, rather than to trans-synaptic interactions (Filosa *et al.*, 2009). This data poses an intriguing question: what is the function of presynaptic EphA4? Although it is not critical for LTP at these synapses, our data indicates it is still likely to interact with EphrinB2. The binding of EphrinB2 by EphA4 is likely to induce its phosphorylation.

At CA1-CA3 synapses, the LTP is dependent on the phosphorylation of EphrinB2 (Bouzioukh *et al.*, 2007). The mechanisms by which EphrinB2 regulates synaptic plasticity may occur through its intracellular domain: both its phosphorylated tyrosine residues, and its PDZ domain. When EphrinB2 is activated, five tyrosine residues are phosphorylated by Src kinases (Palmer *et al.*, 2002). This modification is critical for LTP but not for LTD. Rather, the PDZ domain is critical for both LTP and LTD (Bouzioukh *et al.*, 2007). The precise mechanisms by which the EphrinB2 reverse signalling differently affects LTP and LTD are not known. It may be due to differences in the activation of distinct NMDA subunits during LTP vs. LTD. The activation of Src family kinases by phosphorylated EphrinB2 is likely to lead to phosphorylated NR2A

NMDA sub-units (Lu *et al.*, 1998). The preferential activation of particular NMDA subunits has been shown to determine whether LTP or LTD is formed. Activation of NR2A subunits is implicated in the formation of LTP but not LTD, whilst the activation of NR2B subunits the formation of LTD but not LTP (Liu *et al.*, 2004). Less clear, but perhaps equally important, are the physical interactions between EphrinB2 and NMDA as the receptor has been shown to immunoprecipitate with the NR1 NMDA subunit (Calo *et al.*, 2005). A further mechanism by which EphrinB2's activation may regulate plasticity is through its interaction with AMPA receptors. The formation of LTD is also associated with the turnover of AMPA receptors at the synapse (Lee *et al.*, 2002). Upon activation, a critical serine residue on the intracellular tail of EphrinB2 is phosphorylated and leads to a glutamate interacting protein (GRIP) binding to the EphrinB2 PDZ domain (Essmann *et al.*, 2008). GRIP proteins also interact with AMPA receptors and the interaction between AMPA receptors acts to stabilize the glutamatergic receptor at the cell membrane. Therefore, by regulating the membranous AMPA receptors, EphrinB2 is likely to regulate plasticity (Essmann *et al.*, 2008).

The role of the EphA4-EphrinB2 interaction in morphological plasticity

The increased EphA4-EphrinB2 interaction following stress may also regulate neuronal plasticity through alteration in neuronal morphology. An integral part of neuronal plasticity is the ability of dendritic spines to alter their shape. Spines are formed from filopodia and progress through a number of stages before forming a mature mushroom shape (Lippman & Dunaevsky, 2005). This shape allows the neck to isolate calcium close to the synapse, separate from

the dendritic shaft. The morphogenesis of dendritic spines is highly dynamic, often occurring over a matter of minutes (Penzes *et al.*, 2003). Furthermore, the geometry of a spine correlates with its synaptic strength (Matsuzaki *et al.*, 2001). EphrinB reverse signalling promotes spine morphogenesis. Segura *et al* showed EphrinB ligands influence the Rac signalling pathway to increase mature mushroom shaped spines in relation to immature filopodia. They discovered that stimulation of EphrinB ligands led to the recruitment of the G-protein coupled receptor kinase interacting protein 1 (GIT1) to synapses through the adaptor protein Grb4 (Segura *et al.*, 2007). As GIT1 is a known regulator of Rac, they hypothesise that EphrinB signalling acts to localise Rac1 at the synaptic membrane to drive spine morphogenesis. Indeed, they showed that interference with either the binding of EphrinB to Grb4 or Grb4 to GIT1 impairs the EphrinB-mediated spine maturation (Segura *et al.*, 2007). A separate mechanism by which EphrinB2 signalling can alter morphology has been described. This is through the MAP kinase c-Jun NH2-terminal kinase (JNK) and is independent of the EphrinB2 tyrosine residues (Xu *et al.*, 2003). JNK is implicated in spine morphology through RAP kinases. These promote the growth of spines and mediate LTD and thus link EphrinB2 reverse signalling to rapid changes in dendritic spine functioning.

EphA4 is also known to regulate spine morphology in the hippocampus. Although the studies to date have investigated EphrinA stimulation of EphA4, it is possible that the stimulation by EphrinB2 leads to similar EphA4 mediated signalling and alterations in plasticity. As described above, EphA receptors are candidates for regulating astrocytic influence on neuronal plasticity. EphrinA3

is expressed in the hippocampus exclusively on astrocytic tips whilst its binding partners such as EphA4 or EphB2 are expressed in dendritic spines(Murai *et al.*, 2003). EphA4 knockout mice show abnormal spine morphology. Their dendritic spines are disorganised and irregular in shape and the irregularities couldn't be corrected by application of EphrinA3 (Murai *et al.*, 2003). Together with the finding that similar spine abnormalities are seen in EphrinA3 knockout mice, this indicated that EphA4 was the likely mediator of EphrinA3 induced spine changes (Carmona *et al.*, 2009). When the authors added EphrinA3 to wild-type brain slices, it caused a reduction in spine length and spine density (Murai *et al.*, 2003). The downstream molecular mechanism for these morphological changes implicates the RhoA GTPase. Fu *et al* found that EphrinA1 mediated spine retraction coincided with the activation of RhoA and could be abolished by inhibition of cyclin dependent kinase 5 (Cdk5). Indeed EphrinA1 had no influence on spine morphogenesis in Cdk5 knockout mice. EphrinA1 caused the recruitment and phosphorylation of Cdk5 which in turn activated a GEF, Ephexin1. The association between Ephexin1 and EphA4 is abolished in Cdk5 knockout animals and Cdk5 is necessary for EphA4 mediated RhoA activation (Fu *et al.*, 2007). The activation of RhoA explains how EphA signalling may control actin dynamics. However, the group led by K.Murai has discovered a parallel pathway that may also explain how EphA4 signalling causes spine retraction. They found that a phospholipase C (PLC) inhibitor prevented EphrinA mediated spine retraction. They demonstrated that EphA4 interacts with PLCγ1 and that it induces its phosphorylation (Zhou *et al.*, 2007). The effect of this signalling pathway was to alter the membrane associated fraction of cofilin. The authors suggest that the decrease in

membrane associated cofilin indicates its release in order to depolymerize actin filaments and therefore allow for the spine reduction seen following EphrinA stimulation (Zhou *et al.*, 2007). A further signalling pathway that the EphA-EphrinA interaction impacts is the β1-integrin signalling pathway. Murai's group have shown that EphrinA stimulation of EphA4 causes a decrease in phosphorylation of a number of proteins within the β1-integrin pathway. Furthermore, the inhibition of this signalling pathway leads to similar morphological effects on dendritic spines as the EphA4 mediated effects (Bourgin *et al.*, 2007). Taken together, it is clear that the EphA4 and EphrinB2 are centrally placed to mediate spine morphogenesis. There appears to be a number of pathways through which this effects, whether it is modulation of the actin cytoskeleton, or allowing morphological changes through altering the cells interaction with the extracellular matrix through the integrin system.

Co-localisation of EphB2 and Neuropsin in the amygdala

The staining of EphB2 and Neuropsin (Figure 30) is consistent with the literature, which has focused on their hippocampal location. The expression pattern of EphB2 was found to localise to dendritic regions of CA1 and dentate neurons, particularly those sub-compartments with a high NMDA receptor expression (Henderson *et al.*, 2001). Similarly, the hippocampal expression of neuropsin is likely to be in regions where NMDA receptors are active (Matsumoto-Miyai *et al.*, 2003). *In situ* hybridization shows that the highest expression of neuropsin in the hippocampus is in the CA1 and CA3 regions. The signal was weaker in the CA2 region and was not present in the stratum radiatum (Chen *et al.*, 1995). Studies using neuropsin-deficient mice found that

particular cells and synapses within the hippocampus were regulated by neuropsin. Consistent with the expression pattern of the neuropsin gene, CA1 and CA3 pyramidal cells were most affected, showing larger, elongated cell soma. The most marked changes were in the CA1 subfield of the stratum radiatum (Hirata *et al.*, 2001).

Stress disrupts the Ephb2-NMDA interaction through neuropsin activity

EphB2 is known to interact NMDA receptors (Dalva *et al.*, 2000). This interaction occurs in the amygdala and is altered following stress. Acute stress results in a decrease in the interaction between EphB2 and NMDA receptors, a process dependent on neuropsin (Figure 31). As the extracellular domain of EphB2 is necessary to mediate the EphB2 -NR1 interaction, it is logical that the EphB2- NMDA interaction will decrease following neuropsin cleavage of EphB2 (Dalva *et al.*, 2000) In the immunoprecipitation experiments described in this thesis the decrease in interaction was measured using an antibody that bound to the intracellular domain of EphB2. Therefore, our measurement will only show the interaction between full length EphB2 receptors and NR1 subunits. As the cleavage of EphB2 by neuropsin liberates an extracellular fragment most likely consisting of all but 23 amino acids of the EphB2 extracellular domain it is possible that EphB2 exodomain cleaved off by neuropsin maintains its interaction with NR1 subunits. However Dalva's work showed that the interaction between EphB2 and NR1 involves the EphrinB2 ligand in a ternary complex and may involve additional proteins. These factors

may stabilise the interaction, but once the extracellular fragment is liberated, it is unlikely that the complex will maintain its integrity.

A snapshot of the dynamic membrane following stress

It was reasoned that critical stress-related neuronal functions would occur within fifteen minutes of the stressful insult. The amygdala is activated following stress, as demonstrated by an increase in immediate- early gene, c-fos, following thirty minutes of restraint stress (Cullinan *et al.*, 1995). Furthermore, proteases are ideally poised to act rapidly in the neuronal environment. tPA is activated in the amygdala showing a four- fold increase in activity following thirty minutes of restraint stress (Pawlak 2003). Neuropsin also shows a rapid increase in activity following theta burst stimulation of hippocampal neurons. In this case, the peak activity of neuropsin occurs five to six minutes following stimulation (Matsumoto-Miyai *et al.*, 2003). Choosing a fifteen-minute time point has allowed us to observe rapid stress induced alterations in neuropsin, EphB2 and NMDA. However, given the dynamic nature of the membrane, it is important to consider that only a snapshot of the ongoing process has been taken. Although at fifteen minutes EphB2 receptors demonstrate a decreased interaction with NMDA receptors, it may be that at later time-points, the interaction continues to change. Given the number of ways in which EphB2 may modulate LTP, and the fluidity of the synaptic membrane, the observation that neuropsin disrupts the EphB2-NMDA interaction may not fully describe their relationship to neuronal physiology following stress.

The role of the EphB2-NMDA interaction in stress induced synaptic plasticity

The dynamic nature of the EphB2 - NMDA interaction is likely to reflect the synaptic activity in the amygdala following stress. During the first fifteen minutes of stress, neuropsin regulates membranous EphB2 (Figure 17). Furthermore, the interaction between EphB2 and NMDA receptors is decreased.

These changes may underlie the role of neuropsin in amygdala LTP. Mice deficient for neuropsin have a deficit in early LTP and decreased NMDA currents (Attwood *et al.*, 2011). What are the possible mechanisms that would underlie the regulation of LTP and NMDA function through EphB2 cleavage by neuropsin? Following NMDA activity, the extracellular protease, ADAM10, cleaves EphB2 (Litterst *et al.*, 2007). The remaining transmembrane and cytoplasmic domains are subjected to further proteolytic processing by γ-secretase, which leads to the release of an intracellular EphB2 fragment (Litterst *et al.*, 2007; Xu *et al.*, 2009). The liberated intracellular fragment contains the tyrosine kinase domain, which directly phosphorylates NMDA receptors (Xu *et al.*, 2009). The phosphorylation, which includes tyrosine residues on the NR1, NR2A and NR2B subunits, increases the surface expression of NMDA receptors (Xu *et al.*, 2009). However, it may also lead to an increase in the NMDA receptor activity (Sala & Sheng, 1999). As neuropsin cleaves EphB2 close to the cell membrane, it may activate this mechanism. The cleavage of EphB2 may also regulate its physical interactions with other

molecules. EphB2 binds to NMDA receptors and regulates their clustering (Takasu *et al.*, 2002). This interaction is studied in the context of our stress model and described in the next chapter of this thesis.

Previously, EphB2 receptors have been reported to cluster NMDA receptors, which leads to calcium influx in immature neurons (Takasu *et al.*, 2002). This is not likely to be occurring fifteen minutes following stress, as there is a decrease in the EphB2-NMDA interaction. However, EphB receptors are critical in regulating the localisation of NMDA receptors (Nolt *et al.*, 2011). Could the decrease in the interaction between EphB2 and NMDA reflect a reorganisation of NMDA receptors to enable their function at targeted synapses and neurons? Although the total quantity of membranous NR1 subunits does not change during fifteen minutes of stress (Figure 32), the decrease in the interaction may represent NMDA trafficking at the synapse. The dynamism of NMDA receptors is an integral part of the fluidity of the post-synaptic complex. NMDA receptors are trafficked laterally in the membrane between synaptic and extra-synaptic pools (Tovar & Westbrook, 2002). This movement varies depending on the subunit expression of NMDA receptors with NR2A-containing receptors showing more stability at the synapse, whilst NR2B containing- receptors are more mobile (Groc *et al.*, 2006). This movement allows for the distribution of NMDA receptors to their final synaptic site or for internalisation. Lateral to the PSD are clatharin coated pits that mediate the endocytosis of NMDA receptors (Blanpied *et al.*, 2002). The internalisation and membrane insertion of NMDA receptors is tightly regulated in order to influence synaptic transmission. In the adult hippocampus Shaffer

collateral- CA1 synapses, LTP results in the rapid insertion of NMDA to the postsynaptic membrane (Grosshans *et al.*, 2002). The insertion of NMDA receptors to the membrane is regulated by their phosphorylation (Scott *et al.*, 2001). Importantly, the phosphorylation of Ty-1472 on the NR2B NMDA subunit is critical for amygdala function. This tyrosine residue regulates the correct amygdala synaptic distribution of NMDA receptors, LTP formation and the expression of fear-mediated behaviour (Nakazawa *et al.*, 2006). As discussed, EphB2 is able to regulate the phosphorylation of NMDA receptor subunits, both indirectly through Src kinase (Grunwald *et al.*, 2001; Takasu *et al.*, 2002; Slack *et al.*, 2008) and directly following γ-secretase processing (Xu *et al.*, 2009). Thus, the stress-induced modulation of the EphB2- NMDA interaction may represent the functional necessity of the amygdala to regulate NMDA receptor subunit composition, synaptic mobility, and the balance between membrane insertion and internalisation.

The regulation of amygdala LTP and NMDA function may also occur through the dynamic trafficking of EphB2 receptors following stress. In neuropsin deficit mice, stress may result in the trafficking of EphB2 to the neuronal membrane (Figure 17). If this is occuring the EphB2 receptors that are trafficked to the membrane do not interact with NMDA receptors at this time point (Figure 31). It may be that an increase in the EphB2- EphrinB interaction could follow the increased trafficking of the EphB2 receptors. The literature indicates that NMDA function is facilitated by stimulation of EphB2 by EphrinB ligands (Dalva *et al.*, 2000). The stimulation of EphB2 receptors leads to the activation of Src kinase and phosphorylation of NMDA subunits (Grunwald *et al.*, 2001; Slack *et*

al., 2008). As discussed, the trafficking of NMDA receptors to the cell membrane following LTP is also regulated by Src phosphorylation (Grosshans *et al.*, 2002). Therefore the trafficking of EphB2 receptors to the membrane following stress may lead to NMDA-mediated LTP, through the activation of Src kinases and subsequent phosphorylation of NMDA receptors. The activation EphB receptors have also been shown to activate the ERK subfamily of MAP kinases which are critical in forming LTP (Kelleher *et al.*, 2004).

The regulation of NMDA receptors subunit composition

Neuropsin regulates the surface expression of EphB2 after fifteen minutes of stress and the EphB2 gene expression six hours following stress. However, the total membranous quantity of NR1 NMDA subunits remain stable at both fifteen minutes and six hours points following stress, with or without neuropsin activity (Figure 32 and Figure 33). As discussed, this may reflect the trafficking of the NMDA receptors during the initial stages of amygdala activity. Part of this may reflect regulation of the subunit composition of the membranous NMDA subunits. It is known that the localisation of NR2B containing NMDA receptors at the synapse is regulated by EphB2 (Nolt *et al.*, 2011). This data in this thesis indicates that, at the amygdala neuronal membrane, the proportion of NR1 subunits would remain similar. However, the proportion of NR2A and NR2B may change significantly. The regulation of the subunit composition allows the neuron to alter synaptic transmission. An increase in the proportion of NR2A subunits leads to a decrease in the NMDA- mediated current (Monyer *et al.*, 1994). Furthermore, at hippocampal CA1 synapses, NR2B subunits are

required for LTD whilst NR2A subunits are required for LTP (Liu *et al.*, 2004). The alteration of NMDA subunit composition has been observed in response to experience. Rats that were reared in the dark showed a lower level of NR2A protein levels compared to those reared with a normal light/dark cycle. This difference could be completely reversed within 24 hours of exposing the rats to a normal light/dark cycle, showing the ability of the cells to alter the subunit composition of the NMDA receptors (Quinlan *et al.*, 1999). When examined for shorter periods, the investigators found that the changes started occurring following just one hour of light exposure and continued to redress the subunit composition over the following few hours (Quinlan *et al.*, 1999). The rapid alterations in subunit expression and the distinct synaptic transmission properties that are regulated by subunit ratios indicate that the measurement of NR1 subunit membranous levels may not fully describe the activity and composition of NMDA receptors following stress.

Whole nuclei vs. circuit-specific effects

To be able to understand the synaptic dynamism that occurs following stress, biochemical analysis needs to focus on specific circuits rather than the amygdala as a whole. Experience-driven activity in the amygdala can result in the activation of specific neuronal subpopulations that form distinct neuronal circuits. Depending on which subset of neurons is activated, distinct behavioural states result (Herry *et al.*, 2008). A limitation of our experiments is that the amygdala has been analysed as a single entity. It is possible that the EphB2 receptors that are trafficked to the membrane following stress are targeted to a particular subset of neurons or synapses that require their

function. This may be an increase in their interaction with NMDA receptors, whilst, at other neurons or synapses, neuropsin facilitates a decrease in their interaction. Depending on the balance this could result in an observed decrease in the interaction between EphB2 and NMDA receptors. whilst failing to identify an increase in interaction in a particular subset of synapse/neurons.

Genetic regulation following neuropsin activity

The cleavage of EphB2 by neuropsin also leads to a regulation of EphB2 and NMDA-mediated gene expression (Attwood *et al.*, 2011). A genome wide micro- array found that the FKBP5 gene was differentially regulated in neuropsin knock out animals and that there was a neuropsin-dependent component to its upregulation following stress (Attwood *et al.*, 2011). The FKBP5 gene, which encodes FKBP51 protein, a molecular chaperone of the glucocorticoid receptor and its expression, has been linked with the development of anxiety, depression and post-traumatic stress disorder (Binder, 2009; Binder *et al.*, 2008; Binder *et al.*, 2004). Furthermore, the FKBP5 gene has previously been shown to be regulated by interference with EphB2 signalling (Genander *et al.*, 2009). *In vitro,* the addition of EphB2 antibodies inhibit the neuropsin-dependent component of stress-induced upregulation of the FKBP5 gene, implicating the neuropsin dependent cleavage of EphB2 in FKBP5 gene regulation. Furthermore, NMDA receptor stimulation mimicked the FKBP5 gene upregulation confirming the involvement of these receptors in the neuropsin pathway (Attwood *et al.*, 2011). This closely resembles the change in IEG gene expression observed by Takasu et al following EphB2-mediated NMDA clustering and increased calcium influx (Takasu *et al.*, 2002).

The EphB2 cleavage by neuropsin triggers a cascade of molecular events critical for the stress response.

Chapter 5. Eph receptors and anxiety-like behaviour

Introduction

Animal models of psychiatric pathology

The mouse restraint stress model used in this study not only leads to neurophysiological change, but also to behavioural change. In this chapter, the stress-dependent behaviour of mice, in which Eph proteins or neuronal proteases are altered, is investigated.

The psycho-social nature of psychiatric disorders has meant that the development of animal models has been difficult and their validity is limited. This has resulted in models that mimic specific aspects of that condition or its treatment rather than the disease itself (Van der Staay *et al.*, 2009; Belzung & Lemoine, 2011). In order to be considered relevant, a model of a psychiatric disorder must meet certain criteria. The basis of these criteria is built on those set out in 1984 by Willner in reference to depression (Belzung & Lemoine, 2011). Therefore, it is useful to discuss briefly predictive validity, construct validity and face validity.

Construct validity can be defined as the accuracy with which the model measures what it is intended to measure (Gould & Gottesman, 2006). In practice, this is rarely fulfilled and yet it is often argued to be the most important validation. A more helpful description of construct validity is the 'experimental substantiation of the theory underlying the disorder, its pathophysiology, neuronal and behavioural components' (van der Staay *et al.*,

2009). Construct validity will therefore evolve as the theory about the disorder evolves and the model is refined. The work described in this chapter is construct validation: the behavioural models are used to evaluate the molecular mechanisms described in the previous two chapters.

In the broader definition, predictive validity refers to the model's ability to make accurate predictions about the disorder in question. In psychiatric models, and as defined by Willner, it is the ability of the model to identify successful pharmacological agents. In other words, the pharmacological agent that relieves the symptoms of the patient also reverses the behaviour modelled by the animal (Belzung & Lemoine, 2011).

Face validity describes the phenomenological similarities between the model and the disorder. For example, a model of depression in which the animal appears to lose interest in an activity that would normally give it pleasure would have face validity, as a core symptom of clinical depression is anhedonia. The subjective component of psychiatric disorders precludes a model being developed on face validity alone. It is argued that, although behaviours between species may appear similar, their purpose may be different and therefore not represent the same biological or psychological mechanisms (Matthews *et al.*, 2005). Furthermore, a dependence on face validity excludes phylogenetically lower organisms and introduces the risk of anthropomorphism regarding the animal's behaviour (Sufka *et al.*, 2006; Matthews *et al.*, 2005).

Stress and animal behaviour

Mouse models have gained popularity in the age of molecular biology, largely due to the ability to manipulate the mouse genome in order to allow observation of the behavioural effect of a single gene (Holmes, 2001). To date, there are a wide range of behavioural models for examining the mouse response to stress. These can be divided into models that rely on either conditioned or unconditioned responses, and further subdivided into conflict or non-conflict, and actual versus potential exposure (Rodgers & Dalvi, 1997; Sousa *et al.*, 2006). Typically, the models involve observation of innate behavioural responses to stressful stimuli. For example, when a mouse is exposed to a predator odour, it is more likely to be immobile (freezing behaviour); immobility can therefore be used as a measure of anxiety (Hebb *et al.*, 2002).

The models used in this study focus on anxiety and fear-related behaviour. Fear and anxiety are thought to be distinct but overlapping emotional states. A key difference between these emotional states is that, whilst anxiety is aroused by nonspecific dangers or unconscious impulses, fear is the result of a specific danger (Davis, 1998). The open field (OF) and elevated plus maze (EPM) measure anxiety-like behaviour; unconditioned, non-conflict responses in which the animal experiences potential exposure to stress. Both the EPM and OF measure behaviour which is reproducibly altered by stress (such as the restraint stress model).

By contrast, fear conditioning measures conditioned, non-conflict responses where the animal is exposed to a threat. The responses measured in this model represent a different state to that potentiated by the elevated plus maze or OF. The experience of a noxious stimulus and re-exposure to that specific threat gives rise to both fear and anxiety. Fear conditioning has face validity as a model for stress-related disorders, as a neutral stimulus (the tone or chamber) is associated with a stimulus that induces a state of fear or anxiety (Davis, 1992; LeDoux, 2003).

The three tests described will allow investigation of overlapping molecular, genetic and behavioural pathways (Ramos, 2008; Turri *et al.*, 2001). It is important to employ more than one behavioural model to assess anxiety as it will result in a more comprehensive assessment of the animal's state and a subtler analysis of the differences between groups of animals (Ramos, 2008; Menard & Treit, 1999).

The Elevated Plus Maze

The origins of the elevated-plus maze lie in studies by Montgomery who investigated conflict theory. He found that the strength of fear/anxiety in rats negatively correlated with their exploratory behaviour. Montgomery used open and closed alleys to investigate rat exploratory behaviour and observed that rats preferred the closed alleys (Montgomery & Monkman, 1955). A standardized test utilizing this behaviour was then developed in the 1980s. The predictive validity of an x-maze with two open arms and two closed arms was

demonstrated as anxiolytic drugs increased the proportion of open arm entries made by rats (Handley & Mithani, 1984). The test was then adapted to its current ‘plus’ shape and validated by confirming its sensitivity to anxiolytic drugs as opposed to antidepressants (Pellow *et al.*, 1985). The test is also sensitive to stress. In rodents, prior exposure to stress results in more pronounced anxiety-like behaviour in the open arms, more defaecation, and reduced number of open arm entries. The elevated-plus maze has since been adapted for mice, guinea pigs, gerbils and voles (Lister, 1987; Rex *et al.*, 1994; Varty *et al.*, 2002; Hendrie *et al.*, 1997).

The EPM has a number of advantages compared to other anxiety models. Firstly, it is simple, cheap and avoids lengthy training. Furthermore, it does not rely on pain, thirst or appetite which the more traditional models, such as the Geller-Seifter or Vogel conflict tests utilize (Crawley, 2000). The elevated-plus maze is one of the models described as ethological as it relies on innate responses to ‘natural stimuli’ (Dawson & Tricklebank, 1995). Mice are naturally wary of well lit, open areas but are also exploratory, foraging animals. The test relies on this inner conflict between the urge of exploring novel environments and aversion towards risk exposure. This makes the elevated-plus maze an attractive test to model human anxiety for a number of reasons. Firstly, human anxiety is often based on a phobia of a novel situation or place. Secondly, inner conflict is a feature of psychodynamic theories of psychiatric pathology. Thirdly, the test measures a number of different behaviours, which measure different aspects of stress related behaviour.

The anxiety indices are calculated by the ratio of time spent in, or entries to, the open arm against the total time or total entries. An important confounding factor in the elevated-plus maze is locomotor activity. A lower number of entries to the open arm may be caused because of a lower activity level rather than higher anxiety (Pellow *et al.*, 1985). To control for this, the ratio of time spent in the closed arms (or number of entries to the closed arms) to the total time in the maze (or total number of entries made) is calculated. It is also standard practice to measure the motor activity of the rodents using the hole board or the OF in addition to the elevated-plus maze (Lister, 1987; Walf & Frye, 2007).

As the literature on the EPM has grown, a debate on the measurements of the animals' behaviour during the trials has emerged. The symptoms of anxiety are complex and overlap with other psychological illnesses. It has therefore been proposed that different types/aspects of anxiety can be detected within the EPM (File *et al.*, 1993). When rats are subjected to a second trial of the elevated-plus maze, they do not display the same anxiolytic benefits of benzodiazepines. This one trial tolerance is thought to represent a different anxiety state (File *et al.*, 1993).

To provide a more comprehensive analysis of anxiety behaviour in the EPM, defensive behaviours may also be measured (Rodgers & Johnson, 1995; Blanchard *et al.*, 2001). These behaviours include freezing, attack and flight typically seen in the presence of immediate danger (Blanchard *et al.*, 2001). There are also subtler defensive behaviours that are observed during the

elevated-plus maze testing which have been described as ethological behaviours. These include sniff duration, head dips and stretch attend postures. (Carola *et al.*, 2002; Rodgers & Johnson, 1995). It is argued that these parameters can provide a greater insight into the emotional state of an animal. There is evidence that suggests they correlate better than spatiotemporal behaviour with the animals' corticosterone response and show more sensitivity to some anxiolytic drugs (Rodgers *et al.*, 1999; Setem *et al.*, 1999). However, despite being described in the mid 1990s, they are not commonly utilized by most investigators using the EPM (Carobrez & Bertoglio, 2005). There is a lack of uniformity with regard to which ethological behaviours are used and how they are analysed (Wall & Messier, 2001). Furthermore, there is debate as to how to analyse a large number of behavioural indices and how they relate to anxiety (Wall & Messier, 2001). For these reasons, the author of this thesis has not used these measurements.

Stress and the Elevated Plus Maze

A wide range of stressors have been used to potentiate anxiety in the elevated-plus maze. These include predator odour, social defeat and forced swim but the key factor is that the stress must be inescapable (Korte & De Boer, 2003). The anxiety in the elevated-plus maze is robustly increased by restraint stress. This has been observed in rats, guinea-pigs and mice (Mamczarz *et al.*, 2009; Heinrichs *et al.*, 1994; Dunn & Swiergiel, 1999). Although an increase in anxiety behaviour in the elevated-plus maze in response to restraint stress depends on the species investigated and duration of the restraint, mice reproducibly demonstrate this feature (Table 2).

The inconsistencies seen in the results between laboratories are likely to be caused by methodological differences (Rodgers & Dalvi, 1997). A number of factors have been shown to alter behaviour in the elevated-plus maze. To improve consistency, all the animals should face the same arm at the start of the test (Pellow *et al.*, 1985). The illumination level, the time of day and circadian phase affect performance and so must be consistent between tests (Walf & Frye, 2007; Jones & King, 2001). It is established that the elevation of the maze is not the anxiogenic stimulus but results in the absence of spatial cues. It is helpful to have a small ledge at the edge of the open arms but the height of this correlates with the open arm exploration (Treit *et al.*, 1993). Our ledge is 5mm high, consistent with other set ups (Komada *et al.*, 2008). A range of other factors that must remain consistent include prior handling, single caging and prior maze experience (Walf & Frye, 2007; Brett & Pratt, 1990; Rodgers & Dalvi, 1997; File & Zangrossi, 1993; File *et al.*, 1993).

Mice strain	**Period of restraint**	**Type of restraint**	**Time until testing**	**Behavioural effects**	**Reference**
Swiss albino	6 hrs 8am - 2pm	Immobilisation, Four limbs taped to board	Not specified	Decrease in percentage of time spent in and	(Gilhotra & Dhingra, 2009)

				entries to the open arm	
Wild-type littermates	30 mins	50ml centrifuge tubes	Immediate first EPM then OF	Decrease in open arm entries and closed arm entries	(Dunn & Swiergiel, 1999)
Male DDY mice	1 hr, 15 hour or 5 days of 15 hr restraint	Wire cylinder	Immediate after 1 hour or 15 hours or 1 hour after 5x 15	No change in percentage of time spent in the open arm	(Hata *et al.*, 2001)
Wild-type littermates	2x12 mins separated by 6 days	Perspex tubes	6 days	Decreased time in closed arm, increased time and entries to open arm (prior exposure to stress)	(Harris *et al.*, 2001)

C57BL6	30 mins	Wire restrainers	Immediately	No differences in all classical measurements	(Yamada *et al.*, 2003)
C57BL6	10 mins	50ml polypropylene tube	Immediately	Decrease in percentage of time spent in open arms	(Venihaki *et al.*, 2004)
RC mice P50-63	14 days 6 hrs	Transparent plastic cylinder	24 hours	No differences in all classical measurements	(Chung *et al.*, 2005)
NMRI	30, 60, 120 mins	?	30mins	Decrease in % of time spent in open arms in 120 min restraint	(Hsu *et al.*, 2007)
129/SvJ×C57B	1 hrs	Transparent	30mins	Decrease in	

L/6J		plastic cylinder		% of time spent in open arms	(Bignante *et al.*, 2008)
NIH Swiss mice	2 hrs, 3 days	Perspex tubes	9 or 12 days 10 min acclimatization day before	Decrease in number of entries made to open arms. Increase in total time spent in closed arms	(Chotiwat & Harris, 2006)
C57/BL6x129/sv	25 mins	50ml centrifuge tubes	Immediately	Decreasing trend in open arm entries and time spent in open arm but not significant	(Swiergiel & Dunn, 2006)
C57/BL6	6 hrs or 21days x 6hrs	Wire mesh restrainers	Following morning	Decrease in open arm entries,	(Pawlak *et al.*, 2003)

				closed arm entries and head dips to open arms in both stress time points. Except for closed arm entries for 21x6	

Table 2. Literature review of stressed mice behaviour in the elevated plus maze.

The Open Field

The OF is the original behavioural test for rodent's emotionality, first described in 1934 (Hall, 1934). The OF is a simple test that consists of observing an animal in a relatively large (compared to the home cage), brightly lit, unfamiliar, enclosed environment. Originally, Hall correlated the lack of eating by starved animals in the OF with defecation, demonstrating the emotionality of the test (Hall, 1934). The OF relies on similar inner conflicts as the EPM. The animals are in a novel environment and the exploratory behaviour is countered by the preference for thigmotaxis. The main anxiety indices are based on ambulatory

behaviour. This includes the total activity of the animal, the time spent in the central part of the field and entries to the central part of the field(Ramos & Mormede, 1998; Prut & Belzung, 2003; Broadhurst, 1969). Traditionally total ambulation was the focus but it is now accepted that the time spent in the centre of the field, or conversely the time spent at the edge of the field, is a more accurate representation of anxiety. This is because they are less likely to be confounded by locomotion, exploration or escape behaviour. (Ramos & Mormede, 1998; Prut & Belzung, 2003; Steiner *et al.*, 1997).

By these measures, OF anxiety is reduced by benzodiazepines and 5-HT1a receptor agonists, which are used to treat generalized anxiety disorders (for review: Prut & Belzung, 2003). However, other classes of drugs such as selective serotonin reuptake inhibitors, which are used to treat other anxiety disorders such as panic attacks and obsessive compulsive disorders did not cause a reduction in the anxiety behaviour in the OF. It is suggested that the anxiety modelled is a particular type with specific pathophysiology separate from that which is manifested in other clinical disorders (Prut & Belzung, 2003).

Within the literature, it is stressed that the OF should not be used as a lone measure of anxiety. The obvious confounding factor is locomotion; indeed the OF is also used as a measure of locomotion (Steiner *et al.*, 1997). As with the EPM, the total locomotion must be controlled when measuring anxiety levels. Like the EPM, the OF allows for a more detailed analysis of behaviour and it has been suggested that the measurement of anxiety would be better served by analysing ethological behaviour (Lipkind *et al.*, 2004; Drai & Golani, 2001).

This has led to a detailed study of an animal's behaviour within the OF. Rather than simply measuring the time spent in the centre of the field, precise behavioural patterns are measured during the experiment. By doing this, the investigators found that the behaviour within the OF was not simply general activity but consisted of highly structured, typical behavioural patterns (Kafkafi *et al.*, 2003). By measuring these, it is hypothesised that behavioural endpoints that are more resistant to inter-laboratory or apparatus differences can be discovered (Kafkafi *et al.*, 2003; Crabbe *et al.*, 1999). The face validity of some of the behaviours observed, such as the shape of the path taken by the animal, is difficult to ascertain. However, this is not the case with all the measurements. For example, if the speed approaching the central area is slower than the speed it takes to leave the area, then the animal is considered more anxious (Lipkind *et al.*, 2004). Whilst the ethological behaviour of the EPM is relatively well validated, the behavioural endpoints suggested for the OF thus far lack the predictive validity that traditional measurements contain. One explanation is that ethological measures, such as defecation, also correlate with exploratory behaviour (Archer, 1973). Therefore, it is still too early for these measures to be used as reliable indices of anxiety.

Fear conditioning

Fear conditioning is a learning paradigm that takes advantage of principles discovered and pioneered by Ivan Pavlov. Classical fear conditioning consists of pairing a neutral, conditioned stimulus, such as a tone, with an aversive

unconditioned stimulus, a footshock. The unconditioned responses to footshock (defensive behaviour, autonomic activation, hypoalgesia, HPA axis activation, hyperarousal) are subsequently elicited by presentation of the tone only and can be used as a measurement of the animal's ability to form implicit, amygdala dependent, memory and learn about the anxiety/fear state of the animal (Rescorla, 1968; McAllister *et al.*, 1974; Crawley, 2000). In this paradigm, the conditioned responses are not only elicited by the conditioned stimulus but also by contextual stimuli. When the animals are returned to the chamber in which the footshock occurred, the animal also develop a fear state and display unconditioned responses. This process is dependent on the hippocampus. Therefore, compared to the EPM and OF, the use of memory formation can help investigate the role of different brain structures in the development of anxiety-like states.

There are further aspects of the two conditioned paradigms, referred to as cued (tone) and contextual (chamber), which reveal further information about the anxiety/fear state they invoke. They are characterised by a number of procedural differences that provide important information about the neural process involved in the conditioning. Firstly, the information processing is different. Cued conditioning is a response to a single distinct stimulus, whereas contextual conditioning involves the processing of a number of different cues. Secondly, the cued conditioning stimulus is related to the footshock temporally, whilst the context conditioning cues are constantly present. Thirdly, the cued conditioning tone is predictive of the footshock, whilst the context conditioning

cues are predictive only of a situation in which the footshock is expected to occur (Phillips & LeDoux, 1992; Phillips & LeDoux, 1994).

Cued conditioning

Evidence from a wide range of studies including lesion, stimulation, pharmacological, synaptic plasticity and human studies show that the association between the unconditioned and conditioned stimulus is dependent on the amygdala, and that this brain region is responsible for coordinating the response (Kim & Jung, 2006). The convergence of auditory and nociceptive information to the lateral amygdala has marked it as the locality for the association between the conditioned stimulus and the unconditioned footshock to occur (Rodrigues *et al.*, 2004; LeDoux, 2003; Maren, 2001). Lesion studies have shown that inactivation of the lateral amygdala inhibits tone-footshock association, as does a lesion to the mediate geniculate nucleus of the thalamus (Kim & Jung, 2006). Auditory information is also received by the lateral amygdala from the sensory cortex, which takes longer, but provides more detailed information on the sound. Electrophysiological studies have shown that firing properties within the lateral amygdala are altered during fear conditioning and, although this also occurs in the central amygdala and basolateral amygdala, the response latencies are longer than in the lateral amygdala (LeDoux, 2003).

Whilst the lateral amygdala is responsible for the association of the stimuli, the central amygdala controls the response of the animal to the footshock (Maren, 2001; Kim & Jung, 2006; LeDoux, 2003; Rodrigues *et al.*, 2004). There are

direct connections between the lateral and central amygdala, although the majority of communication is believed to be via other amygdaloid nuclei (LeDoux, 2007). The central amygdala has projections to a number of brain areas which initiate the behavioural and physiological responses typical in fear. By stimulating different areas of the central nucleus, investigators have initiated similar responses to those caused by fear conditioning, whilst lesion studies have identified specific central amygdala efferents that are responsible for behavioural and autonomic responses (Maren, 2001; LeDoux *et al.*, 1988).

Context conditioning

Unlike the cued conditioning, contextual conditioning relies on the hippocampus as well as the amygdala for expression (Maren, 2001; Kim & Jung, 2006; LeDoux, 2003). Lesions of the hippocampus made shortly before or after fear training cause large deficits in the contextual conditioning but not the cued conditioning (Phillips & LeDoux, 1992; Maren *et al.*, 1997). However, the lesion must occur within a time frame after the training, indicating that the hippocampus is temporarily involved and is not the site of memory storage (Maren *et al.*, 1997). Neuronal plasticity within the hippocampus is required for the contextual fear learning. Mice deficient in hippocampal LTP also display reduced contextual freezing but not cue-related freezing (Abeliovich *et al.*, 1993; Liu *et al.*, 2004). The current model for hippocampal function in contextual fear conditioning is that the hippocampus forms a configural representation of the contextual cues, that is to say, a single representation of a number of diffuse environmental cues, which is then presented to the amygdala and associated with the US (Maren, 2001; LeDoux, 2003; Rudy &

O'Reilly, 1999). Indeed, damage to hippocampal projections to the amygdala interferes with contextual conditioning (Maren & Fanselow, 1995). Some evidence implicates a hippocampal-independent mechanism for forming contextual fear memories. If the hippocampus is damaged, then it is possible that individual contextual cues could associate with the US independently rather than as a configural representation (Frankland *et al.*, 1998; Anagnostaras *et al.*, 2001). This model has been proposed because pre-training axon sparing lesions of the hippocampus do not cause contextual conditioning deficits (Frankland *et al.*, 1998; Cho *et al.*, 1999). However, the contextual conditioning is not as accurate, as mice also froze more in novel chambers as well as the training chamber (Frankland *et al.*, 1998). It has been speculated that the pre-training electrolytic lesions that did produce a contextual conditioning deficit also damaged the afferents to the nucleus accumbens, thereby impairing its role in contextual fear conditioning, suggesting its role is more significant than the hippocampus (Maren, 2001; Fanselow, 2000).

Stress and fear conditioning

From our own lives, we know that memory is affected by stress. Emotional events, good or bad, are likely to be remembered well and this phenomenon has been studied using animal models such as fear conditioning. Rats that receive various types of stress before fear conditioning consistently show increased freezing following both cued and contextual conditioning. The enhancement occurs immediately after and up to three months following the training (Cordero *et al.*, 2003; Rau & Fanselow, 2009; Kohda *et al.*, 2007). The

stressors include restraint stress, both acute (two hour restraint two days before the training) and chronic (twenty-one days of six hours restraint), which also potentiate fear learning (Cordero *et al.*, 2003; Sandi *et al.*, 2001). Cued fear conditioning has also been shown to be enhanced by stress post-training, which is in line with the influence of post-training stress in other learning paradigms (Hui *et al.*, 2006; Holahan & White, 2002).

There has been extensive investigation into the role of stress hormones in memory (for reviews see (Rodrigues *et al.*, 2009; Roozendaal, 2002). The fear conditioning model has shown hormones released during the stress response impact the functioning of the hippocampus and amygdala. Adrenaline and glucocorticoids, the major stress hormones, both act to increase fear learning. Adrenaline does not cross the blood brain barrier but activates noradrenaline signalling in the amygdala through activation of vagal afferents leading to the nucleus of the solitary tract (Roozendaal *et al.*, 2009)}. Infusion of epinephrine, which enhances noradrenaline signalling in the brain, enhances contextual fear learning, whilst depletion of noradrenaline in the brain impairs auditory fear learning (Frankland *et al.*, 2004; Selden *et al.*, 1990). Glucocorticoids act in concert with the noradrenergic system to enhance fear learning. The infusion of a glucocorticoid receptor antagonist into the basolateral amygdala or the ventral hippocampus before training led to decreased contextual freezing (Donley *et al.*, 2005). Corticosteroids given both systemically and directly to the basolateral amygdala and hippocampus enhance memory consolidation (reviewed in (Roozendaal, 2002). This includes enhancing

contextual and cued conditioning (Corodimas *et al.*, 1994; Hui *et al.*, 2004; Conrad *et al.*, 2004).

As we also know from our own lives, stress and its neurochemical mediators do not ubiquitously enhance memory. Chronic or severe stress is known to impair the ability of humans and animals in memory performance tasks. In animal models, a number of types of stress are also known to impair LTP in the hippocampus, which correlates with an impairment in performance of hippocampal dependent memory tasks (for reviews see (de Kloet *et al.*, 1999; Kim & Yoon, 1998). Critical to the effect of stress on memory is the timing of the stress in relation to the memory phase. Whilst glucocorticoids enhance memory consolidation, they impair memory retrieval (Roozendaal, 2002). For example, the performance of trained rats in a hippocampus-dependent memory task depends on the time footshock stress is given before a retention test. If it is given thirty minutes before the test, the rat's memory is impaired. However, if it is given four minutes or two hours before the test is taken, the memory formation is not impaired (de Quervain *et al.*, 1998). As the animal experiences different situations, the opposing effects of stress on memory consolidation and retrieval happen simultaneously. It is proposed that the stress-induced impairment of memory retrieval is adaptive, as it would inhibit previous memories from interfering with the memory formation of the new stressful situation (Roozendaal, 2002).

Fear conditioning using mice

The evidence that stress facilitates fear learning mostly comes from rat models. The impact of stress on fear conditioning in mice is less clear. Acute immobilisation and chronic variable stress prior to conditioning has been shown to increase context dependent freezing (Blank *et al.*, 2002; Sanders *et al.*, 2010). However, other studies show either no change in freezing or a decrease in freezing after training in both context- and tone-dependent conditioning following restraint stress or swim stress (Izquierdo *et al.*, 2006; Mongeau *et al.*, 2007; Ito *et al.*,). It is possible that the results of these mouse studies are biased by hyper-locomotion caused by the restraint (Ito *et al.,*). Indeed, when Sanders *et al* controlled for baseline freezing, mice did show stress induced learning (Sanders, 2009).

Eph receptors and behavioural tests

The effects of the Eph proteins on behaviour have not been investigated extensively. Gerlai *et al* studied EphA receptors in hippocampal memory tests. They developed immunoadhesion molecules by fusing the binding domain of EphA5 or EphrinA5 to the Fc portion of human IgG. When injected to the hippocampus, the hybrid molecules would either antagonize or stimulate endogenous EphA. The EpA5-Fc would act as an antagonist by binding to endogenous EphrinA ligands and therefore reduce the binding partners for endogenous EphA receptors. On the contrary, EphrinA5-Fc would act as an agonist of endogenous EphA5 receptors, as it contains two Ephrin binding domains and therefore mimicked the dimerisation of membrane bound EphrinA ligands necessary to cause stimulation. Following an eight day infusion, the

mice then underwent two behavioural tests designed to examine hippocampal-dependent memory function. Firstly, the animals performed the T-maze continuous spontaneous alternation task. This test examines the innate behaviour of mice to explore novel areas and relies on hippocampal working memory (Gerlai, 1998). Secondly, they used a context-dependent fear conditioning paradigm (Kim & Fanselow, 1992; Phillips & LeDoux, 1992). In both tests, using two strains of mice, an improvement in performance was observed in the mice that had been infused with the EphA stimulatory molecules, whilst a decrease in performance was observed in the mice that had been infused with the EphA antagonist (Gerlai *et al.*, 1999). The authors associated this alteration in behaviour with electrophysiological and genetic changes. In particular, they observed changes in the expression of the tubulin gene, indicating adaptations in the cytoskeleton of hippocampal neurons. Although the antagonist, EphA5-Fc, would reduce the binding partners for endogenous EphA5 receptors, it would also act to stimulate endogenous Ephrin ligands, which may compound the effects seen in the behavioural tests.

In a second study, the same group further demonstrated the role of EphA receptors in hippocampal memory formation, using the same immunoadhesion molecules but a different manipulation of memory. Following fear conditioning, the animals underwent a surgical anaesthesia ninety minutes after training, which resulted in a reduced level of context-dependent freezing. The investigators discovered that infusion with EphrinA5-Fc during the anaesthesia significantly ameliorated the anaesthesia-induced retrograde amnesia (Gerlai & McNamara, 2000/3). These findings also fit with the hypothesis that the

EphA receptors alter spine morphology, as common anaesthetic agents are known to inhibit dendritic spine motility (Kaech *et al.*, 1999).

The role of EphB receptors in behaviour has been investigated using genetically altered mice (Grunwald *et al.*, 2001). Mice that were deficient in the EphB2 gene were subjected to the Morris water maze to assess their learning and memory. Compared to the wild-type controls, the mutant mice required a longer time and had longer swim paths to find the hidden platform, indicating a deficit in hippocampal-based learning. During the reversal phase, where the mice had to find a new platform, the mutant mice demonstrated memory deficits as demonstrated by lack of preference towards the quadrant where the platform was hidden previously. This deficit in memory was rescued in a strain of mice that had a mutated EphB2 receptor lacking the intracellular domain, suggesting the kinase domain is not important in these functions. However, these results are difficult to interpret as the EphB2 -/- mice had a reduced swim speed, a tendency to float and showed impairment during the very first trial (Grunwald *et al.*, 2001). Nevertheless, due to the neurophysiological evidence discovered alongside the behavioural studies, it is likely that the some of the impairment seen is due to the absence of EphB2 from the hippocampus and other forebrain structures.

Figures

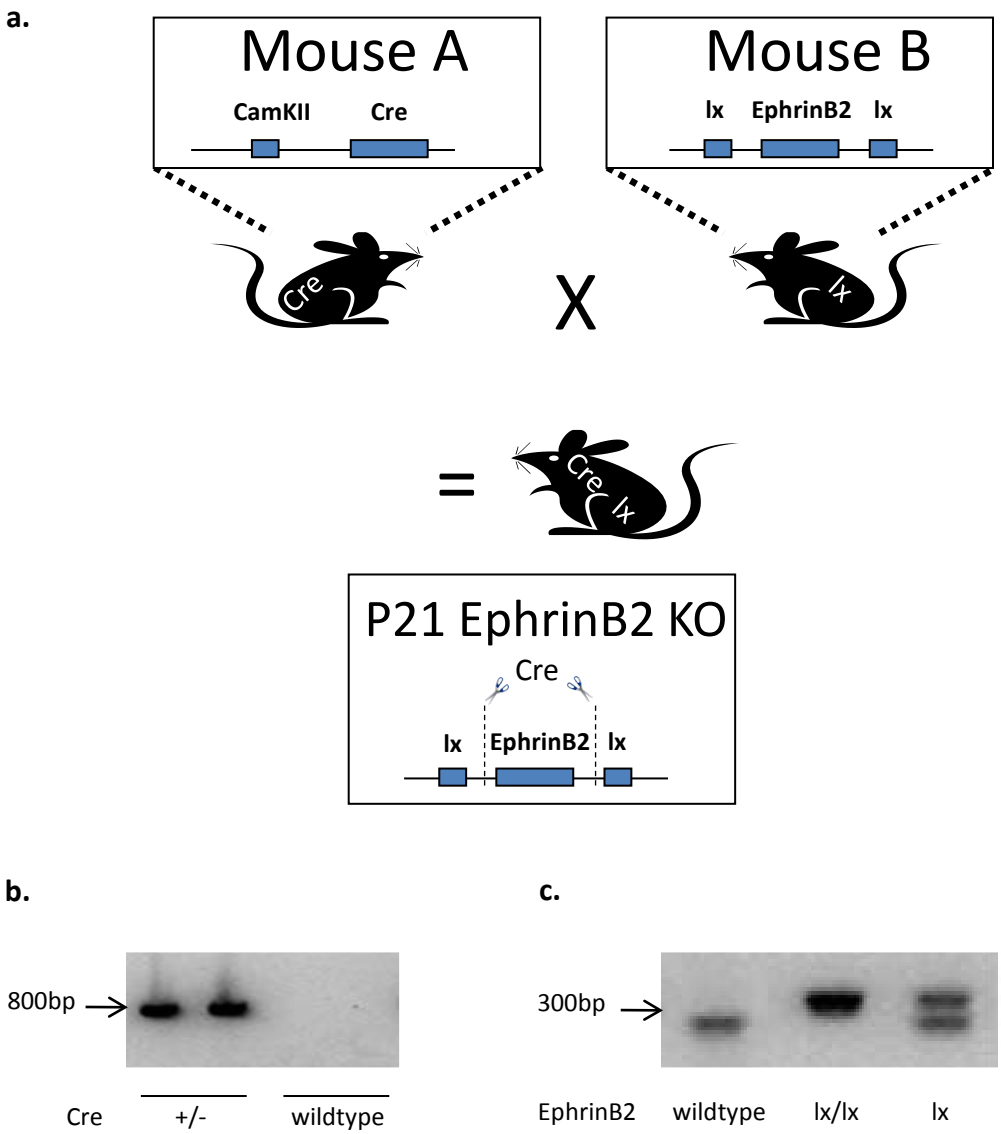


Figure 34. Utilising the Cre/loxP system to generate adult EphrinB2 deficient mice. (a). Adult EphrinB2 deficient mice are generated by crossing CAM kinase-Cre mice (Cre+/-) with EphrinB2 floxed mice (EphrinB2$^{lx/lx}$). At post natal day 21 Cre+/- mice produce forebrain specific Cre enzyme. This excises the floxed EphrinB2 gene from all cells producing Cre, Creating an adult, forebrain specific EphrinB2 deficient (EphrinB2-CaMKII-Cre) mouse. **(b)** Genotyping samples from Cre+/- mice. After PCR, Cre positive mice contain one PCR product at 800bp whilst wild type mice have no PCR product. **(c)** Genotyping samples from floxed EphrinB2 (EphrinB2l$^{x/lx}$) mice in our colony. After PCR, mice without the floxed inserts (wildtype) produce a single product at 240bp, mice with the floxed inserts (lx/lx) produce a single product at 350bp whilst mice with only one floxed insert (lx) contain both products.

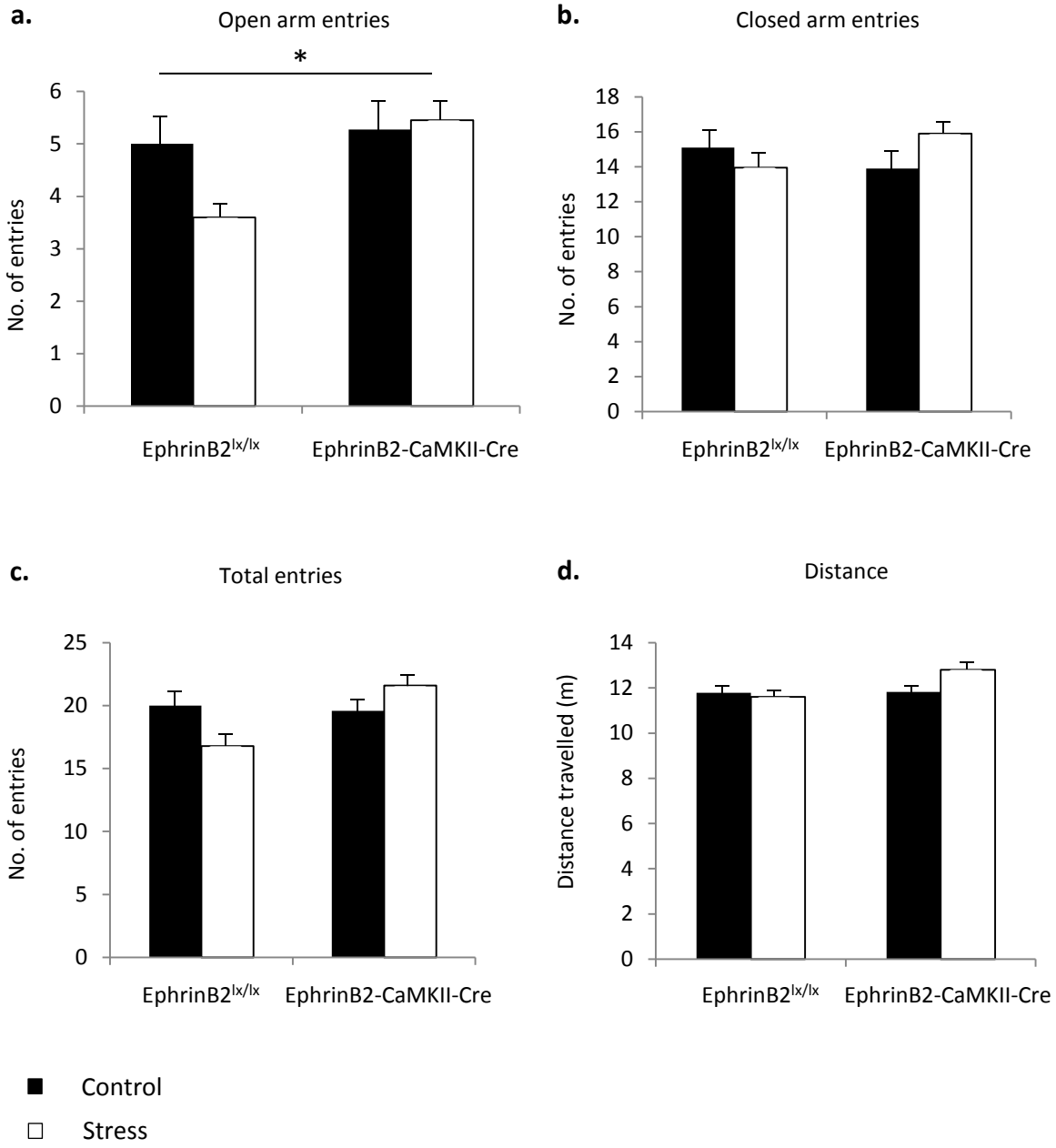


Figure 35. Conditional knockout EphrinB2 mice display less anxiety-like behaviour in the elevated plus maze. Anxiety-like behaviour of control and stressed EphrinB2-CaMKII-Cre and EphrinB2$^{lx/lx}$ mice was measured in the elevated plus maze. **(a).** EphrinB2-CaMKII-Cre mice entered the open arms more often than EphrinB2$^{lx/lx}$ mice ($F_{(1, 50)} = 4.27$; $p<0.05$ EphrinB2$^{lx/lx}$ vs EphrinB2-CaMKII-Cre). Although the biggest difference was observed between stressed EphrinB2-CaMKII-Cre and stressed EphrinB2$^{lx/lx}$ mice, the effect of stress did not reach statistical significance ($F_{(1, 50)}=1.22$; $p>0.05$ genotype vs. stress) **(b, c and d).** There was no effect of genotype or stress on the number of entries made to the closed arms, total entries or distance travelled.

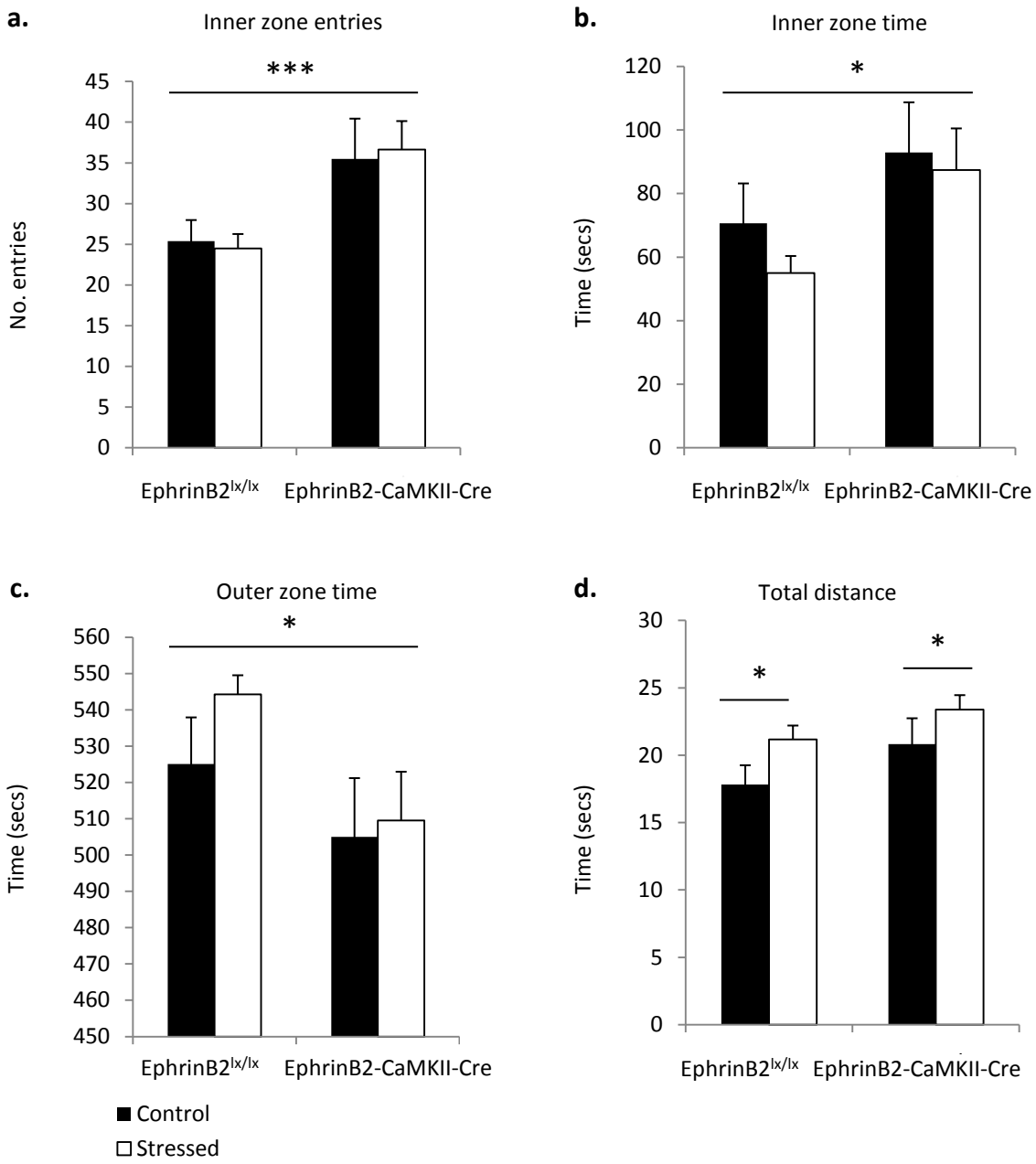


Figure 36. Conditional knockout EphrinB2 mice display less anxiety-like behaviour in the open field. Control and stressed EphrinB2-CaMKII-Cre and EphrinB2$^{lx/lx}$ littermates were observed in the OF **(a).** EphrinB2-CaMKII-Cre mice entered the centre zone more than the EphrinB2$^{lx/lx}$ mice, irrespective of stress ($F_{(1, 65)}$=12.52; P<0.001 EphrinB2$^{lx/lx}$ vs EphrinB2-CaMKII-Cre). **(b).** The EphrinB2-CaMKII-Cre mice also spent more time in the inner zone than the EphrinB2$^{lx/lx}$ mice, irrespective of stress ($F_{(1, 65)}$ = 5.35; p<0.05 EphrinB2$^{lx/lx}$ vs EphrinB2-CaMKII-Cre). **(c).** Conversely the EphrinB2-CaMKII-Cre mice spent less time in the outer zone than EphrinB2$^{lx/lx}$ mice, irrespective of stress ($F_{(1, 65)}$ = 5.15; p<0.05 EphrinB2$^{lx/lx}$ vs EphrinB2-CaMKII-Cre). **(d).** There were no differences between the total distances mice from both genotypes travelled. However mice from both genotypes travelled further following stress ($F_{(1, 65)}$ = 4.90; p<0.05 Control vs stress).

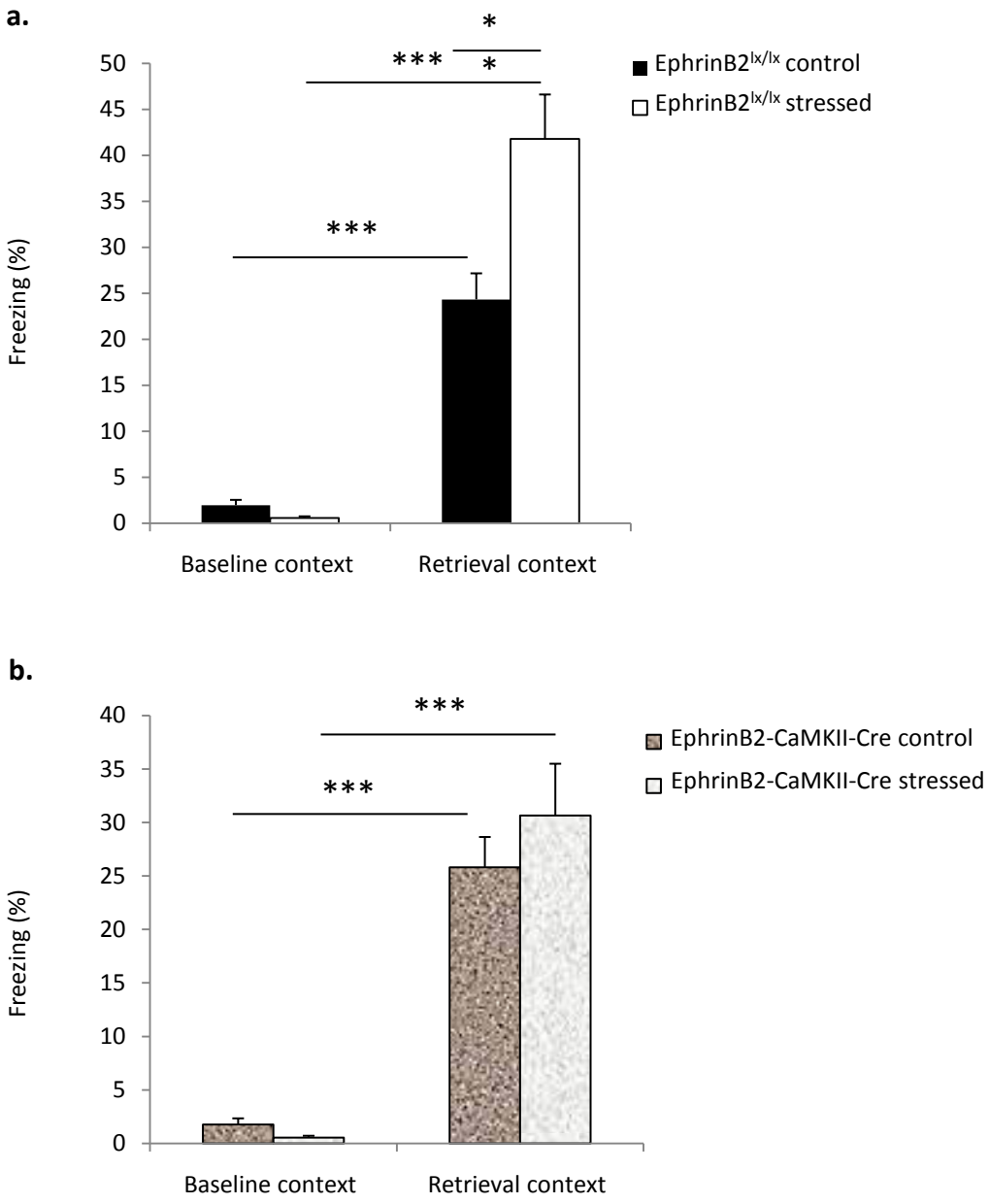


Figure 37. Conditional knockout EphrinB2 mice do not display a stress induced increase in freezing during fear conditioning context retrieval. Stressed and non-stressed EphrinB2-CaMKII-Cre mice and EphrinB2$^{lx/lx}$ littermates were subjected to fear conditioning and re-exposed to the same chamber 24 hours later. **(a)**. EphrinB2$^{lx/lx}$ mice demonstrated context fear conditioning showing increased freezing following re-exposure to the conditioning chamber ($F_{(1,\ 74)}$ =109.4; P<0.001 baseline vs retrieval context). During context retrieval stressed EphrinB2$^{lx/lx}$ mice showed more freezing compared to non-stressed littermates ($F_{(1,\ 74)}$ = 9.59; p<0.01 Stress vs contextual fear conditioning). **(b).** EphrinB2-CaMKII-Cre mice also demonstrated context fear conditioning, freezing more in retrieval than baseline ($F_{(1,\ 50)}$ = 90.48; p<0.001 baseline vs retrieval context). However EphrinB2-CaMKII-Cre mice did not demonstrate a stress induced increase in context retrieval freezing demonstrated by the EphrinB2$^{lx/lx}$ mice. **=p<0.01 ***=p<0.001

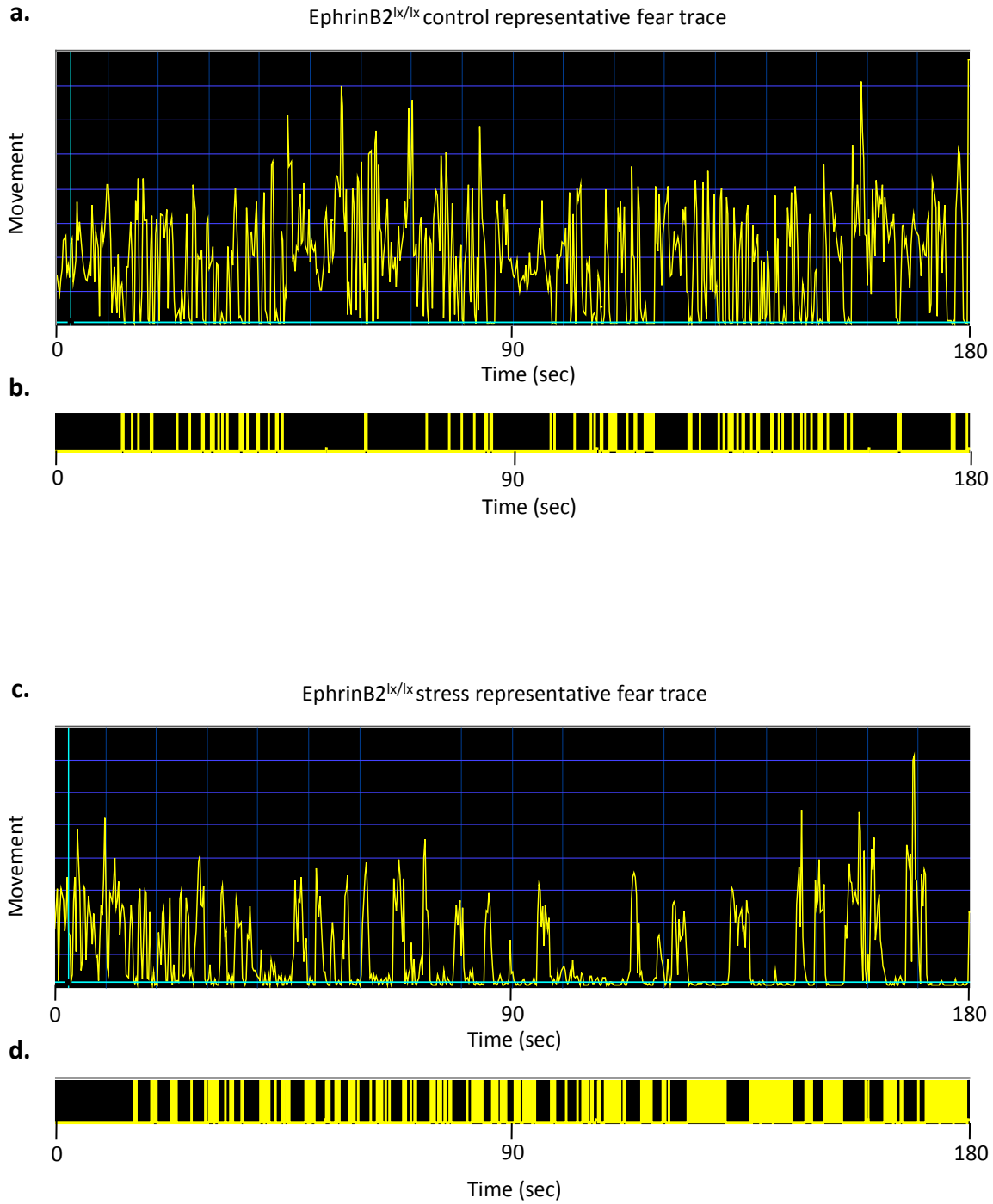


Figure 38. Representative traces of EphrinB2$^{lx/lx}$ mice during context retrieval. EphrinB2$^{lx/lx}$ mice were subjected to fear conditioning and re-exposed to the same chamber 24 hours later. **(a** and **c).** The trace represents the movement of a control EphrinB2$^{lx/lx}$ mouse and of a stressed EphrinB2$^{lx/lx}$ mouse, respectively, plotted against time during re-exposure to the fear conditioning chamber. **(b** and **d).** The vertical yellow bars represent periods of freezing during the 3 minutes. The stressed EphrinB2$^{lx/lx}$ mouse displayed more freezing following stress as seen by the higher proportion of vertical yellow bars in **(d)** compared to **(b)**

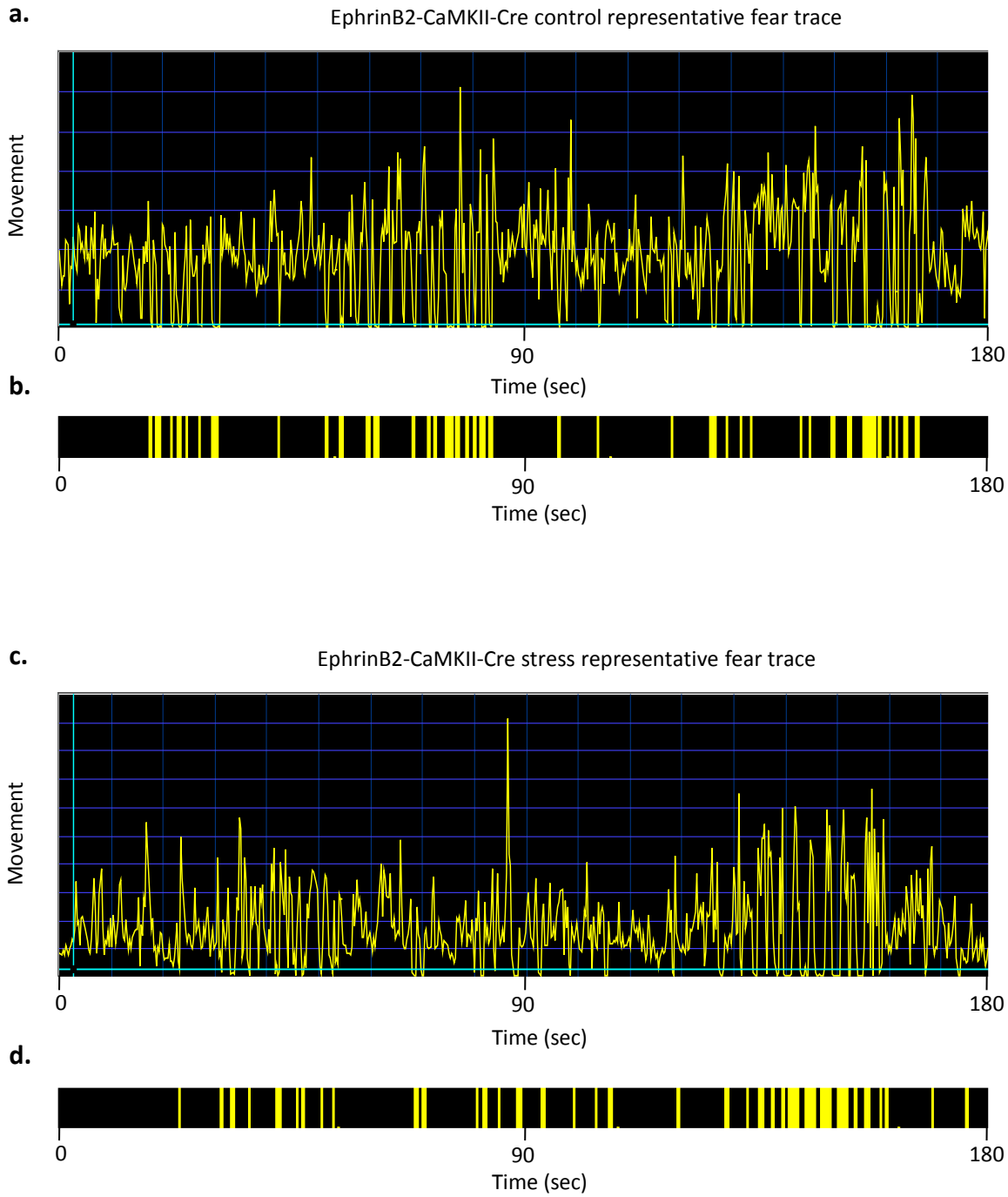


Figure 39. Representative traces of EphrinB2-CaMKII-Cre mice during context retrieval. EphrinB2-CaMKII-Cre mice were subjected to fear conditioning and re-exposed to the same chamber 24 hours later. **(a** and **c).** The trace represents the movement of a control EphrinB2-CaMKII-Cre mouse and of a stressed EphrinB2-CaMKII-Cre mouse, respectively, plotted against time during re-exposure to the fear conditioning chamber. **(b** and **d).** The vertical yellow bars represent periods of freezing during the 3 minutes. Unlike the stressed EphrinB2$^{lx/lx}$ mouse, the stressed EphrinB2-CaMKII-Cre mouse did not display more freezing than the control EphrinB2-CaMKII-Cre mouse as seen by a similar proportion of vertical yellow bars in both **(b)** and **(d)** respectively**.**

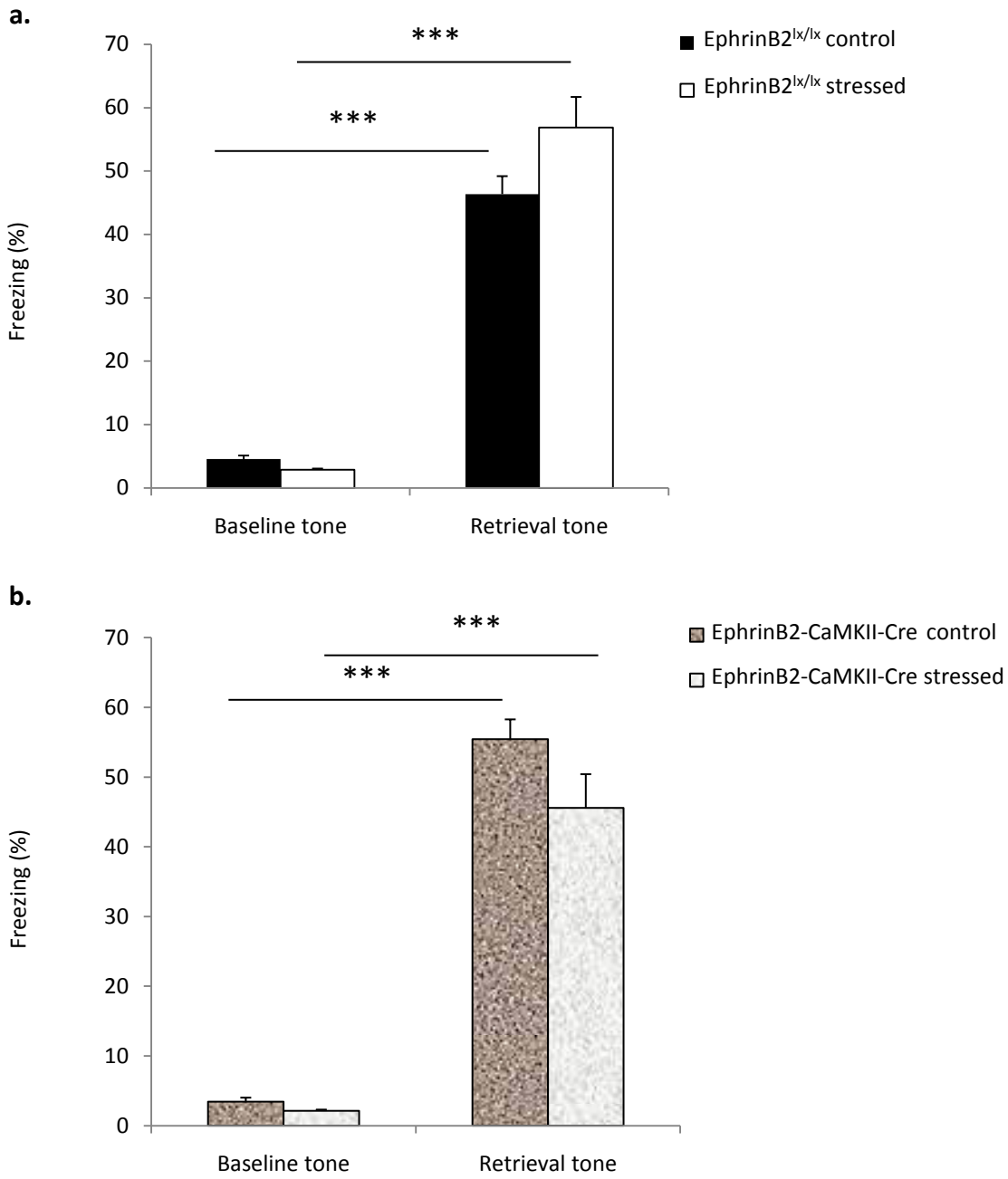


Figure 40. EphrinB2-CaMKII-Cre mice behave similarly to EphrinB2$^{lx/lx}$ mice during cued fear conditioning. Stressed and non-stressed EphrinB2-CaMKII-Cre mice and EphrinB2$^{lx/lx}$ littermates were subjected to fear conditioning. Forty-eight hours later they were placed in a new chamber and exposed to the same tone that was used during training. **(a).** EphrinB2$^{lx/lx}$ demonstrated tone fear conditioning, increasing their freezing upon re-exposure to the tone ($F_{(1, 74)}$ =142.4; P<0.001 baseline vs retrieval context). There were no significant differences between control or stressed groups. **(b).** EphrinB2-CaMKII-Cre mice demonstrated tone fear conditioning showing increased freezing upon re-exposure to the tone ($F_{(1, 74)}$ =142.4; P<0.001 baseline vs retrieval context). There were no significant differences between control or stressed groups. ***=p<0.001

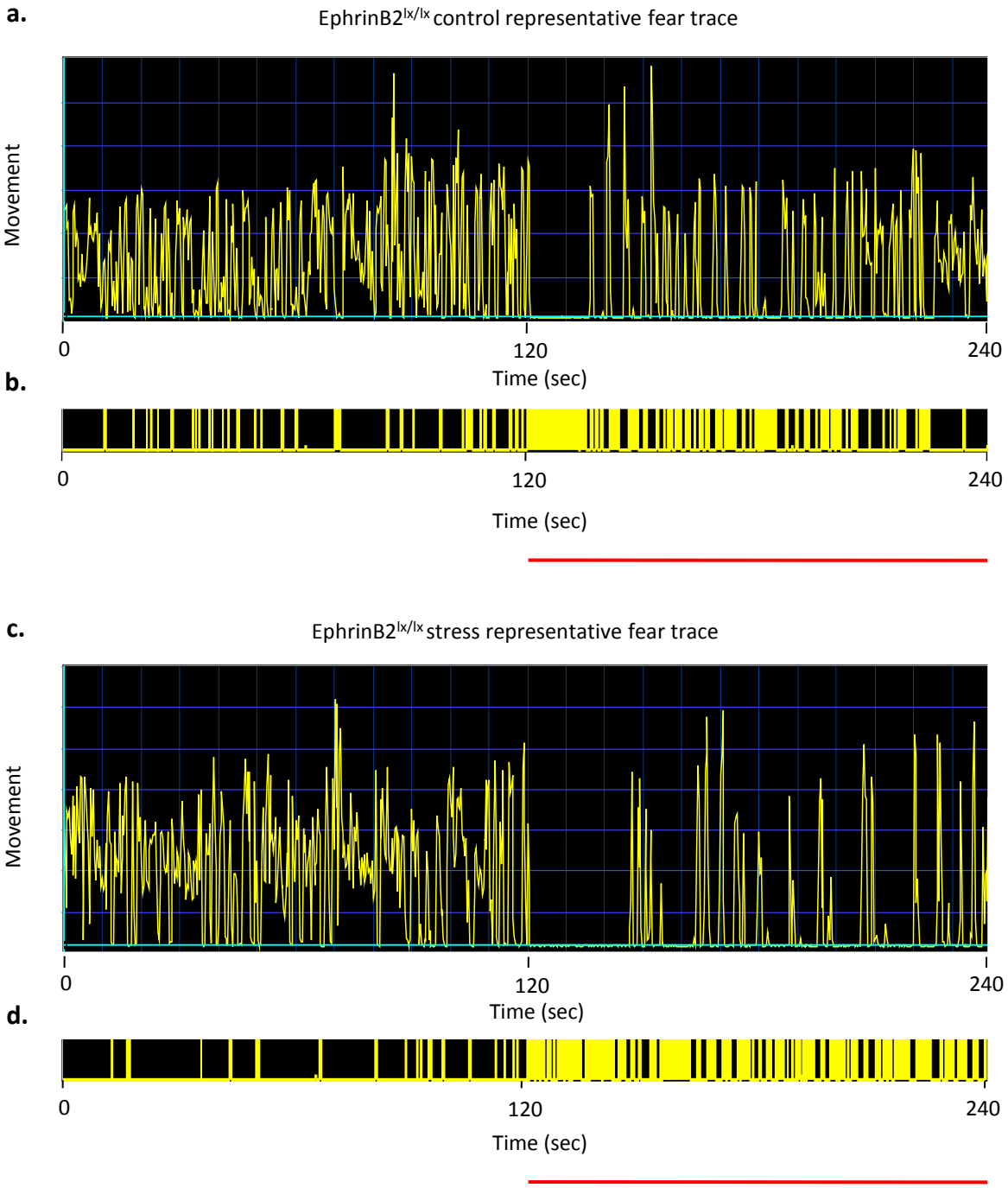


Figure 41. Representative traces of EphrinB2$^{lx/lx}$ mice during tone retrieval. EphrinB2$^{lx/lx}$ mice were subjected to fear conditioning. Forty-eight hours later they were placed in a new chamber and exposed to the same tone that was used during training (red line —). **(a** and **c).** The trace represents the movement of a control EphrinB2$^{lx/lx}$ mouse and of a stressed EphrinB2$^{lx/lx}$ mouse, respectively, plotted against time in the new chamber. **(b** and **d).** The vertical yellow bars represent periods of freezing during the 4 minutes. Both the control and stressed mice showed a high level of freezing following the onset of the tone, indicating successful cued conditioning. The stressed EphrinB2$^{lx/lx}$ mouse displayed a similar level of freezing to the control EphrinB2$^{lx/lx}$ mouse as seen by the vertical yellow bars in **(b)** and **(d).**

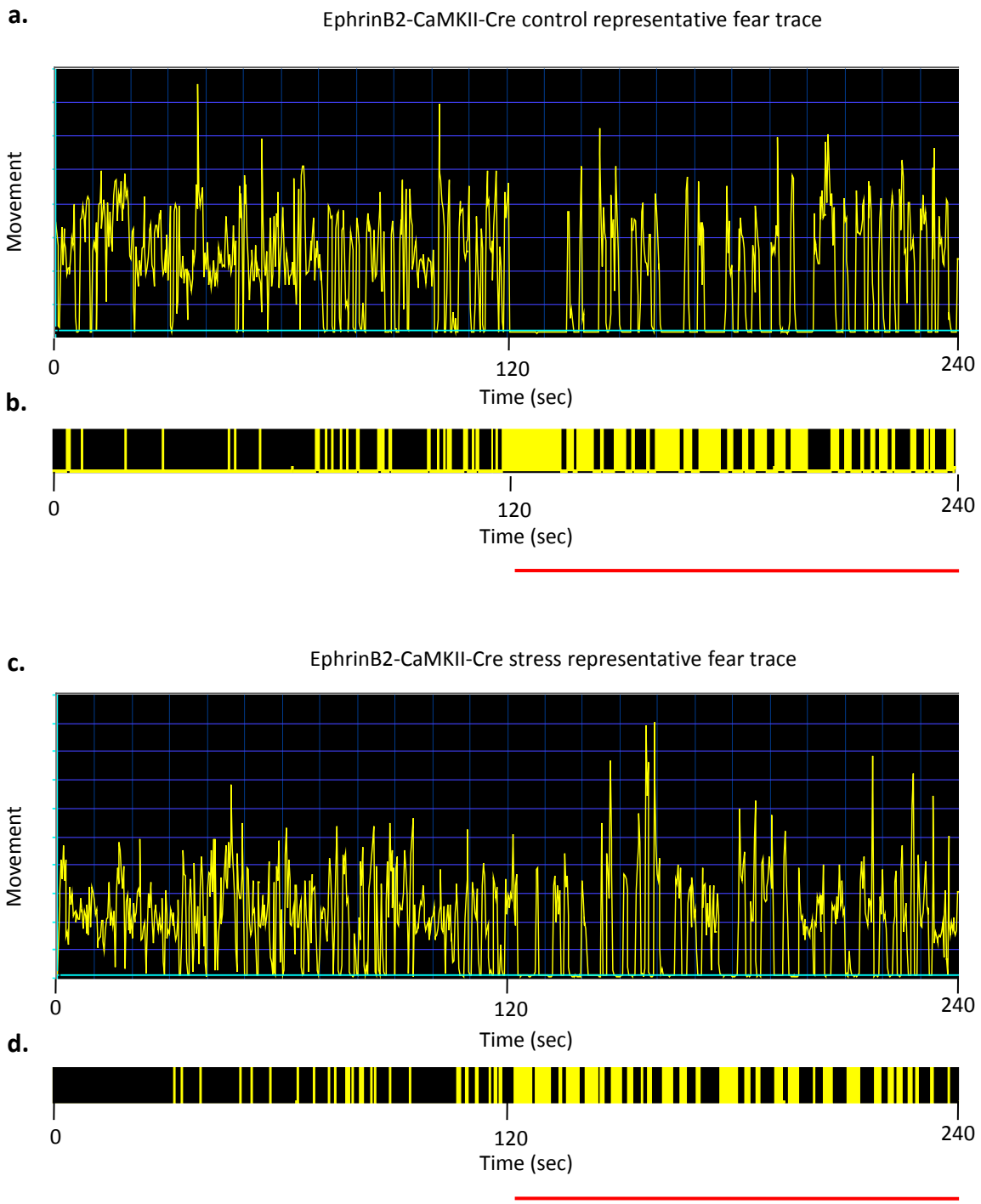
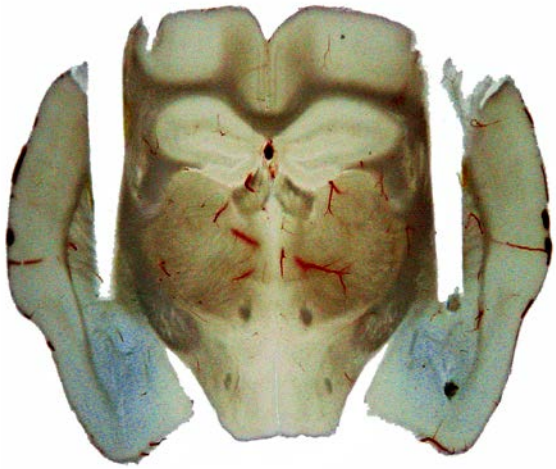


Figure 42. Representative traces of EphrinB2-CaMKII-Cre mice during tone retrieval. EphrinB2$^{lx/lx}$ mice were subjected to fear conditioning. Forty-eight hours later they were placed in a new chamber and exposed to the same tone that was used during training (red line ___). **(a.** and **c).** The trace represents the movement of a control EphrinB2-CaMKII-Cre mouse and of a stressed EphrinB2-CaMKII-Cre mouse, respectively, plotted against time in the new chamber. **(b** and **d).** The vertical yellow bars represent periods of freezing during the 4 minutes. The stressed EphrinB2-CaMKII-Cre mouse displayed a similar level of freezing to the control EphrinB2-CaMKII-Cre mouse as demonstrated by the vertical yellow bars in **(b)** and **(d).**

a.

b.



c.

- Anti-EphB2 - Stress
- IgG - Stress
- Anti-EphB2 - Control
- IgG - Control

d.

- Neuropsin - Stress
- ACSF - Stress
- Neuropsin - Control
- ACSF - Control

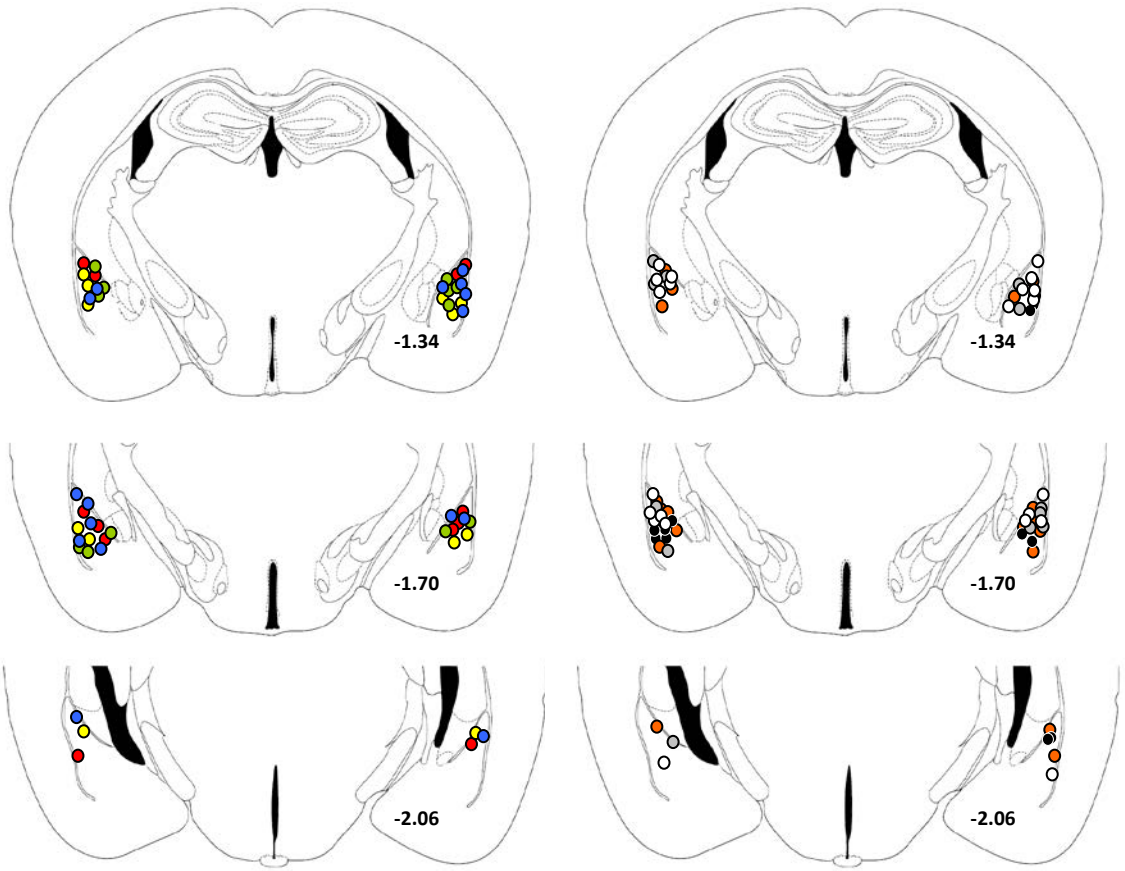


Figure 43. Cannulae placement in the amygdala of wild-type and neuropsin -/- mice. Mice underwent surgery to place bi-lateral cannulae in the amygdala. **(a).** Post-operative photograph showing a mouse with implanted cannulae. **(b).** Coronal section of brain from mouse with implanted cannulae**.** Following completion of the experiment and sacrifice of the mice, 1.5µl of a bromophenol blue solution was injected to show the target area of the injected solution. The dye confirmed the correct placement of the cannulae. **(c).** Diagram to show the placement of the cannulae in the mice used in the EphB2 antibody experiment (figure 44). Each coloured dot represents the placement of a cannula in an individual mouse, within an experimental group. The brains were sliced and the location of the cannula tip described in mm relative to bregma (see number on representative slice). **(d).** Diagram to show the placement of a cannula in the mice used in the neuropsin antibody experiment. Each coloured dot represents the placement of a cannula in an individual mouse, within an experimental group. The brains were sliced and the location of the cannula tip described in mm relative to bregma (see number on representative slice).

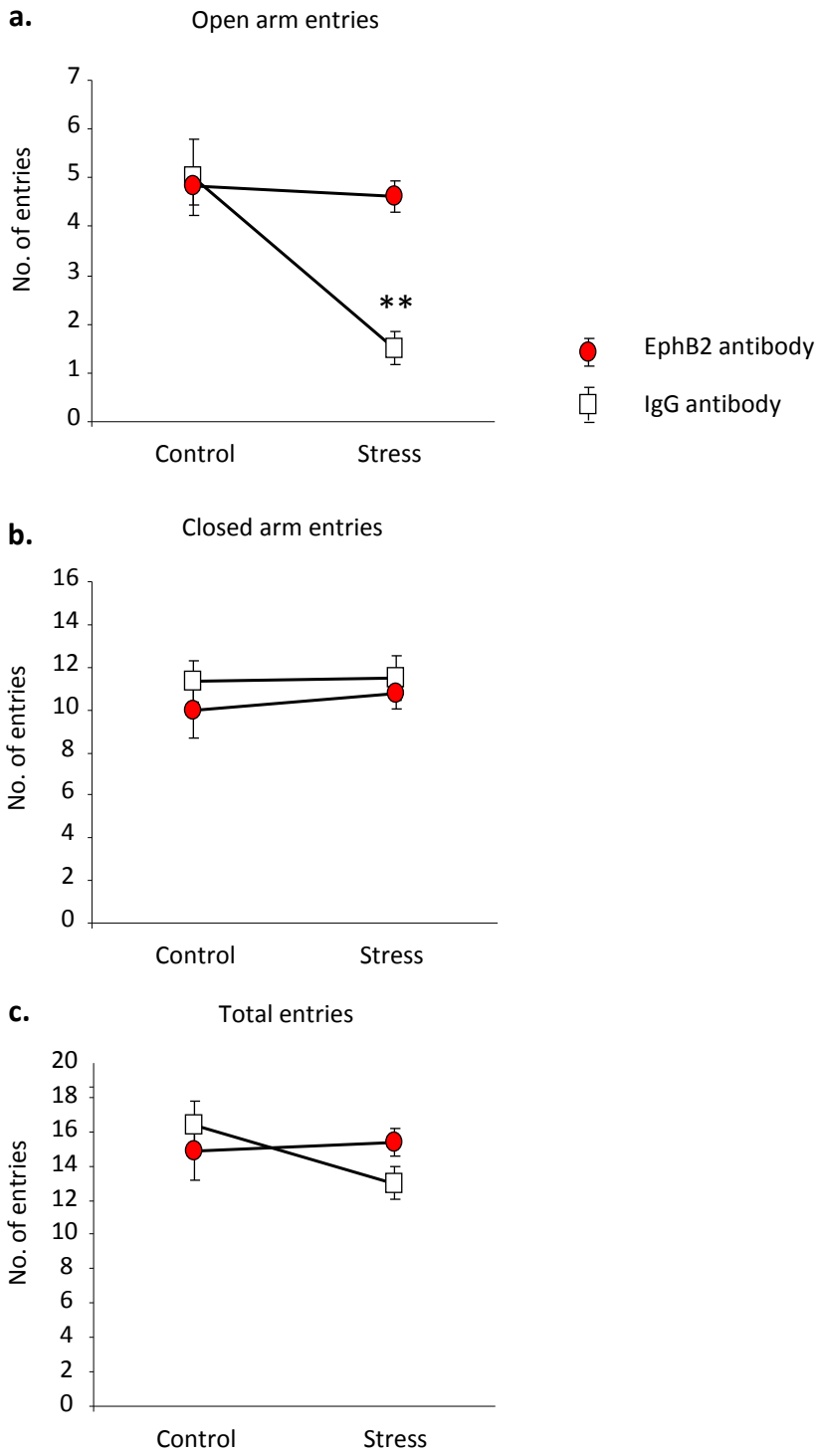


Figure 44. Injection of anti-EphB2 antibody to the amygdala of wild-type mice at the time of stress inhibits the development of anxiety like behaviour. Immediately before the onset of restraint stress either EphB2 or IgG antibody was injected to the amygdala via bilateral cannulae . **(a).** The injection of the EphB2 antibody rendered the animals resistant to the behavioural effects of the stress, as demonstrated by the lack of decrease in open arm entries by these mice compared to a stress induced decrease of open arm entries by those mice injected with IgG ($F_{(1, 22)} = 11.84$; $p<0.01$ Injected substrate vs tress) . This change occurred without affecting either group's general exploratory behaviour (**b and c**). Results are shown as mean±SEM. **= $p<0.01$

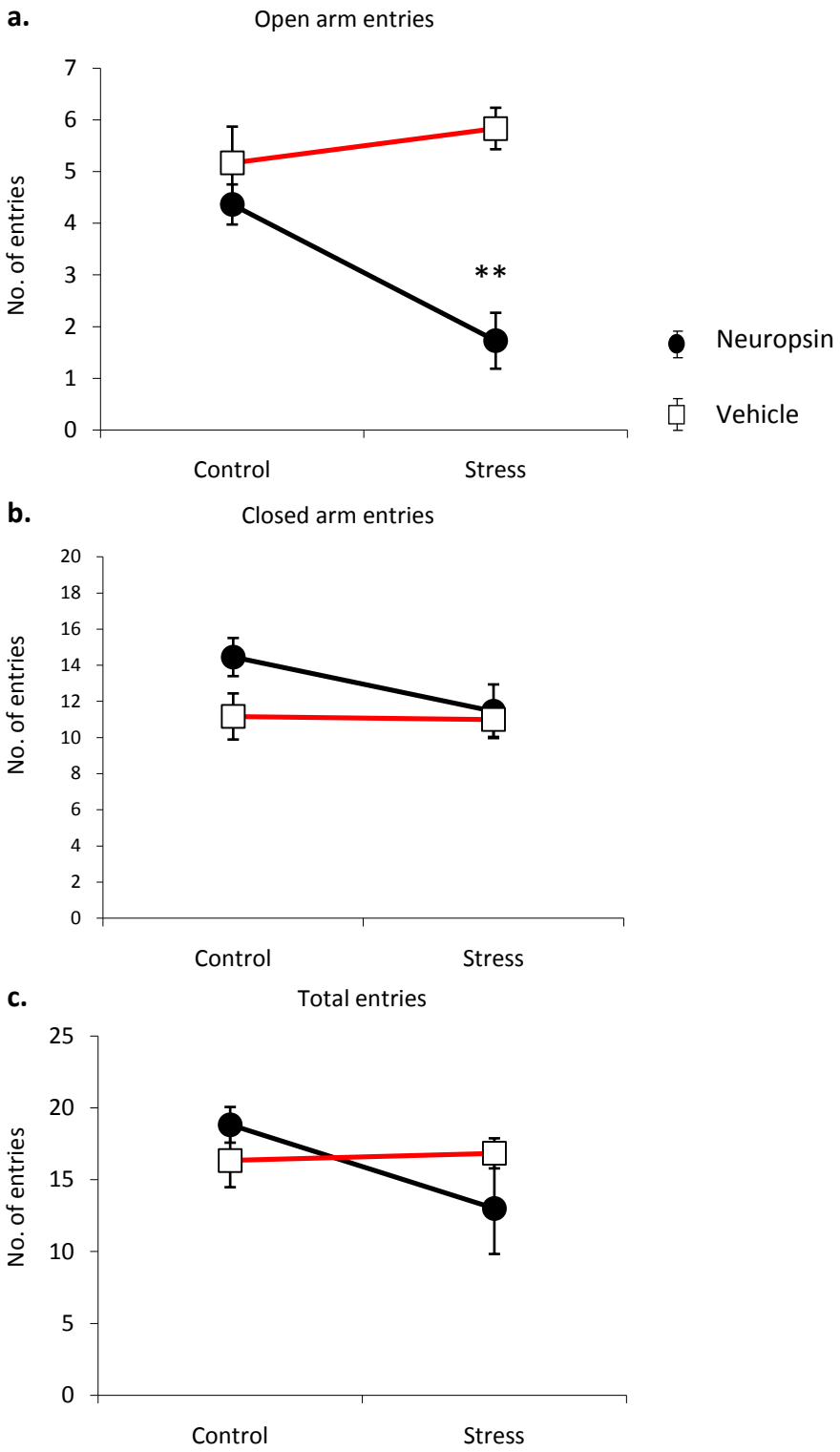


Figure 45. Injection of neuropsin to the amygdala of neuropsin -/- mice at the time of stress restores the development of anxiety like behaviour. Immediately before the onset of restraint stress neuropsin or vehicle was injected to the amygdala via bilateral cannulae. **(a).** The injection of neuropsin restored the stress induced anxiety like behaviour of NP-/- mice as indicated by the decrease in entries made by the mice to the open arm following restraint stress compared to mice injected with vehicle alone ($F_{(1,30)}$ =9.29; p<0.01 injected substrate vs stress). The injection did not affect the general exploratory behaviour of the mice (**b** and **c**). Results are shown as mean±SEM. **= p<0.01

Results

Generating adult EphrinB2 deficient mice

To create the conditional knockout animal, two strains were crossed (Figure 34). Firstly, mice in which the loxP sequence is inserted at both the 5' and the 3' ends of a critical exon of the target gene was used (in this case *efnb2* encoding EphrinB2). This strain was then crossed with animals that express Cre recombinase (in this case driven by the CamKII promoter). Once CamKII promoter is expressed, Cre recombinase is produced and targets the loxP sites, excising the target gene from the genome. As CamKII promoter is inactive during during embryonic development and the first three postnatal weeks this produces an adult mouse in which the target gene is disrupted.

The effect of EphrinB2 on anxiety-like behaviour

Conditional EphrinB2-CaMKII-Cre mice were tested for their anxiety-like behaviour both before and after six hours of restraint stress. The effect of stress on memory formation was also analysed by subjecting the mice to fear conditioning, again both before and after six hours of restraint stress. Throughout all the tests, the EphrinB2-CaMKII-Cre mice were compared to littermate EphrinB2 loxP mice (EphrinB2$^{lx/lx}$ mice), which contain the loxP sites that flank the EphrinB2 gene but do not contain the Cre knock-in.

The EPM revealed differences in the anxiety-like behaviour of the two genotypes. Taken as a whole group EphrinB2-CaMKII-Cre mice entered the

open arms of the EPM more than the EphrinB2$^{lx/lx}$ mice indicating a less anxious phenotype (Figure 35; $F_{(1, 50)} = 4.27$; $p<0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre). Control and stressed EphrinB2-CaMKII-Cre mice made 5.38 and 5.56 entries to the open arm, respectively, compared to 5.00 and 3.52 entries of control and stressed EphrinB2$^{lx/lx}$ mice, respectively. The group contributing most to the difference was the stressed EphrinB2$^{lx/lx}$ mice. However the effect of stress did not reach statistical signficance (Figure 35; $F_{(1, 50)} =1.22$; $p>0.05$ genotype vs stress). The number of closed arm entries (Figure 35; $F_{(1, 50)} =0.12$; $p>0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre), total number of entries (Figure 35; $F_{(1, 50)} =1.10$; $p>0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre) and distance travelled (Figure 35; $F_{(1, 50)} =2.18$; $p>0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre) by mice of both genotypes, in both control and stressed groups, did not differ, indicating no locomotor differences between the groups. EphrinB2lx/lx control n=7, stressed =21. EphrinB2-CaMKII-Cre control n=7, stressed =19.

The behaviour of the EphrinB2-CaMKII-Cre in the OF also revealed differences between the genotypes. The conventional measures of the OF indicated that the EphrinB2-CaMKII-Cre mice displayed a less anxious phenotype than the EphrinB2$^{lx/lx}$ mice. The EphrinB2-CaMKII-Cre mice made more inner zone entries than the EphrinB2$^{lx/lx}$ mice, making 35.5 and 36.6 entries with or without stress, respectively, compared to the 25.4 and 25.5 entries made by the control and stressed EphrinB2$^{lx/lx}$ mice, respectively (Figure 36; $F_{(1, 65)} =12.52$; $P<0.001$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre). The EphrinB2-CaMKII-Cre mice also spent greater time in the inner zone (Figure 36; $F_{(1, 65)} = 5.35$; $p<0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre) and less time spent in the outer

zone (Figure 36; $F_{(1, 65)} = 5.15$; $p<0.05$ EphrinB2$^{lx/lx}$ vs. EphrinB2-CaMKII-Cre), compared to EphrinB2$^{lx/lx}$ mice, irrespective of stress. Similarly to the EPM results, stress did not alter the anxiety-like behaviour of either genotype (Figure 36; Inner zone entries $F_{(1, 65)} = 0.001$; $p>0.05$ genotype vs stress, inner zone time $F_{(1, 65)} = 0.80$; $p>0.05$ genotype vs stress, outer zone time $F_{(1, 65)} = 0.96$; $p>0.05$ genotype vs stress). However, the stressed mice of both genotypes travelled a further distance following stress compared to the control animals of both genotypes (Figure 36; $F_{(1, 65)} = 4.90$; $p<0.05$ genotype vs stress). EphrinB2$^{lx/lx}$ control n=18, stressed =20. EphrinB2-CaMKII-Cre control n=12, stressed =19.

The effect of EphrinB2 on fear learning and its interaction with stress

EphrinB2-CaMKII-Cre mice and EphrinB2$^{lx/lx}$ mice were subjected to fear conditioning with or without stress. Without stress, the pre-training behaviour of both genotypes was the same. Both sets of mice displayed freezing at an expected rate, approximately two percent and four percent during context training and tone training, respectively (Figure 37 and Figure 40). This measurement indicates both genotypes responded to the novel environment in a similar manner.

Context conditioning

The day following training, the mice were returned to the conditioning chamber and allowed three minutes of exploration. During this period, all mice displayed conditioned learning demonstrated by an increase in freezing of at least twenty five percent (Figure 37**;** EphrinB2$^{lx/lx}$**,** $F_{(1, 74)} =109.4$; $P<0.001$ baseline vs.

retrieval context, EphrinB2-CaMKII-Cre $F_{(1, 50)}$ = 90.48; p<0.001 baseline vs. retrieval context). Whilst the non-stressed EphrinB2$^{lx/lx}$ mice show moderate freezing during testing, the stressed EphrinB2$^{lx/lx}$ mice show considerably more freezing (unstressed 25% vs. stressed 40%), with an increased number of freezing periods and longer freezing periods (Figure 37; $F_{(1, 74)}$ = 9.59; p<0.01 Stress vs. contextual fear conditioning). This is demonstrated by representative fear traces taken from non-stressed and stressed EphrinB2$^{lx/lx}$ mice during their return to the conditioning chamber (Figure 38) In contrast, the stressed EphrinB2-CaMKII-Cre mice do not show any increase in freezing compared to non-stressed EphrinB2-CaMKII-Cre mice following fear conditioning (Figure 37; $F_{(1, 50)}$ = 1.14; p>0.05 Stress vs. contextual fear conditioning). The representative fear traces show a similar pattern of freezing both with and without stress (Figure 39). EphrinB2lx/lx control n=17, stressed =22. EphrinB2-CaMKII-Cre control n=13, stressed =14.

Cued conditioning

To examine cued (tone-dependent) conditioning, on the day following context-dependent testing, the mice were exposed to a novel cage and, following two minutes of habituation, the conditioning tone was played for two minutes. During the tone, all of the mice of both genotypes, stressed and non-stressed, demonstrated learning as freezing increased from 4% to at least 40% (Figure 40; EphrinB2$^{lx/lx}$, $F_{(1, 74)}$ =142.4; P<0.001 baseline vs. retrieval tone, EphrinB2-CaMKII-Cre $F_{(1, 50)}$ = 144.3; p<0.001 baseline vs. retrieval tone). In contrast to the context-dependent conditioning, the facilitation of fear memory in EphrinB2$^{lx/lx}$ mice following stress was not seen in cued conditioning. Following stress, EphrinB2$^{lx/lx}$ mice did not display any increase in freezing compared to

non-stressed EphrinB2$^{lx/lx}$ mice (Figure 40; $F_{(1, 74)} = 2.29$; $p>0.05$ Stress vs. cued fear conditioning). The representative fear traces showed a clear increase in freezing during the tone, however, there was little difference between the freezing displayed by non-stressed EphrinB2$^{lx/lx}$ and stressed EphrinB2$^{lx/lx}$ mice (Figure 40). This was also the case with EphrinB2-CaMKII-Cre mice. They showed a similar level of freezing to EphrinB2$^{lx/lx}$ mice with or without stress (Figure 40**;** $F_{(1, 50)} = 1.16$; $p>0.05$ Stress vs. cued fear conditioning). The EphrinB2-CaMKII-Cre representative fear traces show a similar pattern to those of EphrinB2$^{lx/lx}$ mice (Figure 41 and Figure 42). EphrinB2lx/lx control n=17, stressed =22. EphrinB2-CaMKII-Cre control n=13, stressed =14.

Disruption of anxiety by blocking EphB2 in the amygdalae of wild-type mice

To investigate the effects of interfering with EphB2 in the amygdala on behaviour, anti-EphB2 antibodies were bilaterally injected to the amygdala of wild-type mice. Following investigations of anxiety-like behaviour, the mice brains were examined for location of the injection site and only mice with injections sites inside the amygdala were included in the data (Figure 43). Without stress, there were no behavioural differences between mice that received the EphB2 injection and those that received the injection of IgG antibody (Figure 44). Following stress, mice that received the IgG injection entered the open arms less frequently, indicating a development of anxiety-like behaviour. However, mice that received the injection of anti-EphB2 antibody prior to the restraint stress entered the open arms as often as those without stress, indicating a resistance to stress-induced anxiety (Figure 44; $F_{1, 22} =$

11.84; p<0.01 Injected substrate vs. stress). The number of closed arm entries (Figure 44; $F_{(1, 22)} = 0.085$; p>0.05 Injected substrate vs. stress) or total number of entries (Figure 44; $F_{(1, 22)} = 2.54$; p>0.05 Injected substrate vs. stress) did not differ between the genotypes with or without stress indicating no differences in locomotion between the groups. EphB2 antibody control n= 6, stressed = 8. IgG antibody control n= 6, stressed = 8

Restoration of anxiety by neuropsin delivery to the amygdala

Previous work in the lab demonstrated that NP-/- mice do not develop stress-induced anxiety-like behaviour in the EPM (Attwood et al., 2011). To investigate if the effect of neuropsin was acute or developmental, neuropsin was bilaterally injected into the amygdalae of NP-/- mice prior to subjecting them to restraint stress and analysing their anxiety-like behaviour. Following the behavioural investigations, the mice brains were examined for location of the injection site and those mice with injection sites outside the amygdala were excluded from the data (Figure 43). The results of the behavioural tests demonstrated that the anxiolytic phenotype of NP-/- mice was rescued by the injection of recombinant neuropsin to the amygdala, prior to restraint stress. Stressed NP-/- mice injected with the vehicle made the same number of open arm entries as the non-stressed NP-/- mice (Figure 45). However, the injection of recombinant neuropsin to the amygdalae of NP-/- mice before stress resulted in a reduction of open arm entries to the levels observed in wild-type animals following stress (Figure 45; $F_{(1,30)} =9.29$; p<0.01 injected substrate vs. stress). The number of closed arm entries (Figure 45; $F_{(1,30)} =1.06$; p>0.05 injected substrate vs. stress) or total number of entries (Figure 45; $F_{(1,30)}$

=3.42; p>0.05 injected substrate vs. stress) did not differ between the genotypes with or without stress, indicating no differences in locomotion between the groups (Figure 45). Neuropsin control n= 11, stressed = 11. Vehicle control n= 6, stressed n= 6.

Discussion

Summary

The molecular studies into the role of Eph proteins in the hippocampus and amygdala implicated particular members of the family in the regulation of stress-related neuronal physiology (Chapters 3 and 4). One member of the Eph family involved in stress-related responses is EphrinB2 . To investigate its role in anxiety-like behaviour, the CamKII Cre/loxP system has been utilised to knock out EphrinB2 expression in the forebrain of adult mice. Behavioural tests revealed that these mice displayed decreased anxiety-like behaviour. In the EPM, the EphrinB2-CaMKII-Cre mice entered the open arms more, despite a similar number of total entries, compared to the EphrinB2$^{lx/lx}$ mice. Furthermore, in the OF, the EphrinB2-CaMKII-Cre mice entered the inner zone more often and spent more time there compared to the EphrinB2$^{lx/lx}$ mice. During contextual fear conditioning, the EphrinB2-CaMKII-Cre also displayed less anxiety, albeit in a different manner. During this test, the behaviour only differed following stress; the EphrinB2-CaMKII-Cre did not develop the expected stress-induced increase in freezing during context-dependent fear conditioning.

Another Eph protein involved in stress-related responses is EphB2, which is cleaved by the protease neuropsin following stress. Mice that are deficient for neuropsin do not develop stress-related anxiety as measured by the EPM (Attwood *et al.*, 2011). To investigate the molecular pathway involving EphB2 and neuropsin in anxiety-like behaviour, bilateral cannulae were implanted to

the amygdala. First, neuropsin was delivered to the amygdalae of neuropsin knock-out mice immediately before stress. This showed that, unlike stressed NP-/- mice injected with the vehicle, these mice did develop anxiety-like behaviour. This result indicates that the effect of neuropsin in the amygdala to facilitate the development of behavioural signatures of stress is acute and not developmental. To investigate whether anxiety-like behaviour is EphB2 dependent, an anti-EphB2 antibody was delivered to the amygdalae of wild-type mice immediately before stress. This blocked the development of anxiety-like behaviour in the EPM. Thus, the experiments described in this chapter show that the roles of EphrinB2 and EphB2 are central to the development of anxiety-like behaviour in mice.

Rationale for using conventional and conditional knock-out animal strains

A major advance towards discovering the molecular mechanisms underlying behaviour has been the genesis of genetically modified animals. The first genetically modified mouse was developed in 1989, for which Martin Evans, Mario Capecchi and Oliver Smithies were awarded the Nobel Prize in Physiology and Medicine in 2007 (Thomas & Capecchi, 1987). Since then, the use of genetically modified animals has become the gold standard in demonstrating the behavioural significance of a molecular phenomenon. Due to the critical role EphrinB2 plays in the development of blood vessels, conventional knockout of this molecule leads to embryonic lethality (Wang *et al.*, 1998)**.** To overcome this problem, conditional knockout animals can be

used. These mice retain the complete genome during development before a targeted gene is disrupted when a certain age is reached.

Conditional knockouts also answer other criticisms of conventional knock-out technology. Retaining the gene of interest throughout development ensures that behavioural changes observed in in adulthood are not rooted in development. It also allows for the knockout to be anatomically restricted. To gain these advantages in the study of EphrinB2, the Cre/loxP method has been used in combination with the αCamKII promoter. This strain of mice shows a decreased hippocampal EphrinB2 mRNA expression, as well as a decreased hippocampus and cortex expression of EphrinB2 protein (Grunwald *et al.*, 2004). This can be explained by the genetic strains that Grunwald *et al* used. The αCamKII promoter is only active postnatally and it is only expressed in specific areas of the forebrain (Mayford *et al.*, 1996; Burgin *et al.*, 1990). Indeed, one of the first applications of this system, using αCamKII as the Cre-driving promoter, was to study the mechanisms of learning and memory (Tsien *et al.*, 1996). It therefore also well suited to this study.

The activity of αCamKII is tightly restricted to the forebrain and strongest in the hippocampus (Tsien *et al.*, 1996). Within the hippocampus, the expression is strong in the pyramidal cells of the CA1 region and in dentate gyrus, with the CA3 showing a weaker staining. There is also staining in the cortex and striatum but no staining at all outside these forebrain areas, such as the cerebellum (Tsien *et al.*, 1996). Within a tight forebrain expression, it is also reported to show expression in the amygdala (Mayford *et al.*, 1996). This is

important when considering the behavioural stress response, as the amygdala and hippocampus are critical for anxiogenesis and fear conditioning. Our immunohistochemistry has indicated that native EphrinB2 is expressed in the pyramidal cells of the CA1, CA3 and dentate gyrus along with amygdala neuronal expression (Attwood *et al.*, 2011). A small proportion of EphrinB2 co-localises with astrocytic staining in the amygdala (unpublished laboratory data). Thus, the EphrinB2-CaMKII-Cre mice will have disruption of EphrinB2 gene in the hippocampus and the amygdala, predominantly in neuronal cells.

The role of EphrinB2 in anxiety-like behaviour.

EphrinB2-CaMKII-Cre mice displayed decreased anxiety-like behaviour (Figure 35 and Figure 36). In the EPM it is expected that the control mice, in this case EphrinB2$^{lx/lx}$, will enter the open arms less following stress. The trend for this was observed but when all the groups were compared the effect of stress was not significant. However when the genotypes were compared irrespective of stress, the difference between the groups was statistically significant (Figure 35). This result by itself does not indicate whether the role of EphrinB2 is in regulating state anxiety or trait anxiety. Both trait and state anxiety are both believed to be important in the development of pathological anxiety. Trait anxiety refers to the underlying level of anxiety of a particular individual, whilst state anxiety describes the transient anxiety that an individual experiences following a stressful situation (Blanchard *et al.*, 2003; Wall & Messier, 2001). In the EPM results described above the trend indicates EphrinB2 regulates state anxiety; the stressed EphrinB2-CaMKII-Cre mice enter the open arms as many

times as the non-stressed EphrinB2-CaMKII-Cre mice, whereas the EphrinB2$^{lx/lx}$ mice have a trend towards a decrease in open arm entries following stress. In contrast, the statistical test indicates that EphrinB2 regulates trait anxiety; there is a difference between the genotypes irrespective of stress. This may be due to a lack of statistical power and an increase in the animals in the experiement may confirm that EphrinB2 regulates state anxiety.

As discussed above it is important to use more than on behavioural paradigm to gain insight into anxiety like behaviour. When the anxiety-like behaviour was measured in the OF this indicated a clearer role of EphrinB2 in trait anxiety rather than state anxiety. When measuring the anxiety indices (inner zone entries, inner zone time and outer zone time) it was clear that the EphrinB2-amKcre mice demonstrated less anxiety-like behaviour. Taken together the result from the EPM and the OF favour a role of EphrinB2 in trait anxiety rather than state anxiety. One factor that may have minimized the effect of stress on the open arm entries by both genotypes is locomotion. The OF revealed that stress increased the total distance travelled by both mice. Increased locomotion after stress may have skewed the results of the EPM in favour of a less anxious phenotype after stress, minimizing the effect of stress.

The role of locomotion in anxiety behaviour.

The behavioural markers of anxiety in the OF and the elevated plus maze rely on the internal conflict between the mouse needing to explore the new environment and staying safe. It is expected that stressed animals alter their

behaviour in the OF and demonstrate more anxiety (Roth & Katz, 1979). This was observed in NP+/+ mice, when they decreased their entries to the centre zone following stress (Attwood *et al.*, 2011) This is also seen in the literature with mice altering their centre field behaviour following stress or after the administration of anxiolytics (Meyer *et al.*, 2006; Choleris *et al.*, 2001). A compounding factor in this behaviour is the locomotor activity of the mouse, which can lead to a misinterpretation of the anxiety-like behaviour. The results from this chapter indicate that stress may affect the locomotion of genetically modified mice. Both EphrinB2-CaMKII-Cre and EphrinB2$^{lx/lx}$ mice travelled further distance in the OF when they had been subjected to stress compared to control mice (Figure 36). The increased distance travelled by the stressed mice may have affected the results of the test. It is common that mice increase their locomotion in the open field following stress (Mineur *et al.*, 2006; Pardon *et al.*, 2000). The locomotion of mice in the open field has been linked to a number of different factors as well as stress. The degree of lighting in the testing environment results in a differing locomotion of mice. In control conditions, mice have a higher locomotion in dim conditions, compared to illuminated conditions (Valentinuzzi *et al.*, 2000). However following stress, the opposite is true, when mice have a higher locomotion under bright lights (Strekalova *et al.*, 2005). This effect may explain why there was no stress induced increase in locomotion in the EPM (Figure 35). In the OF, the animal is continually exposed to brighter light, whilst in the EPM, the animal spends the majority of the time in the closed, poorly lit arms.

It has also been observed that mouse behaviour in the OF following stress varies throughout the year. In the spring-time, mice show a higher level of locomotion compared to the same strain of mice tested in the autumn (Meyer *et al.*, 2006). The behavioural experiments described in this thesis occurred in the spring time, perhaps contributing a further effect on the results of the OF. The behaviour in the open field also differs between mice strain. Carola *et al* analysed the behaviour of BALB/c and C57BL/6 mice and found that the second of these strains showed a higher locomotor activity than the first (Carola *et al.*, 2002). The EphrinB2-CaMKII-Cre mice are back-crossed to B6CG genetic background and are therefore closer to the C57BL/6 strain. A further factor than can influence stress induced locomotion is the age of the mouse with younger mice showing higher locomotion than adult mice, following stress (Ito *et al.*,). Furthermore, it may be dependent on the type of stress used. Studies using adult mice show that chronic stress does not increase locomotion and can actually decrease it (Conrad *et al.*, 1999; Strekalova *et al.*, 2004). Taken together, the literature shows there is a range of factors that could affect the results of the OF. In particular, the increased locomotor activity of both genotypes may have resulted in a masking of anxiety-like behaviour following stress. However, both genotypes were affected equally, and so differences in locomotion are unlikely to explain the less anxious behaviour of the EphrinB2-CaMKII-Cre mice.

The role EphrinB2 on hippocampus and amygdala dependent anxiety-like behaviour.

In EPM tests, the amygdala shows strong, early activation (as early as fifteen minutes after exposure to the EPM), whereas the hippocampus shows much less activation (Silveira *et al.*, 1993). This agrees with the dogma that the amygdala is important for anxiety-like behaviour. However, current evidence from lesion studies also suggests that the ventral hippocampus has a distinct role, separate from the amygdala, in regulation of anxiety-like behaviour (Bannerman *et al.*, 2003; McHugh *et al.*, 2004). This makes it difficult to determine whether hippocampal or amygdalae EphrinB2 deficiency is primarily responsible for the behavioural changes. In contrast, fear conditioning indicates that the role of EphrinB2 following stress is more important in the hippocampal function rather than the amygdala function. The fear conditioning results are similar to studies in which the hippocampus is lesioned prior to conditioning, which causes a disruption in contextual conditioning but not cued conditioning (Phillips & LeDoux, 1992; Maren *et al.*, 1997). Taken together, the pattern of results is more consistent with a critical role of EphrinB2 in hippocampal function during anxiogenesis.

This does not exclude a possible role of EphrinB2 in the amygdala in anxiogenesis. Cued conditioning produces a particular anxiety or fear state likely to be different to that produced by the EPM and OF. Cued conditioning represents a learned fear to a noxious stimulus, whilst the EPM and OF do not contain a specific noxious stimulus. Davies found that different parts of the amygdala complex are critical for a stimulus specific anxiety state and a less stimulus-specific anxiety state. He compared a shock potentiated startle (fear state) with a light potentiated startle and a CRH-induced startle (generalised

anxiety states), and hypothesised that different regions controlled the different anxiety/fear states. Indeed, the results showed that different regions of the amygdala complex were responsible for the behavioural response to the different stimuli (Davis, 1998). The lack of a stress-induced deficit seen in cued conditioning may be secondary to the specific anxiety state induced rather than a non-critical role of EphrinB2 in the amygdala. The paradigm-specificity of the result may also be secondary to the type of stress used. Chotiwat found that restraint stress resulted in anxiety behaviour in the EPM and light-dark, but not marble burying behaviour. These results are most likely due to different neural pathways regulating these different types of anxiety-like behaviour (Chotiwat & Harris, 2006). Therefore, EphrinB2 may play a role in the amygdala during the anxiety state during the EPM and OF, but not during a different anxiety/fear state induced by cued conditioning. It is difficult to tease apart the separate roles of the amygdala and hippocampus from behavioural studies. These two structures have extensive connections and work together to co-ordinate the stress response. Indeed, during stress, the hippocampus is regulated by the amygdala (Kim *et al.*, 2001). EphrinB2 is active in both regions and it may be a combination of amygdala and hippocampal deficiency that results in the behaviour observed.

The effect of stress on fear conditioning in mice

A discussed in the introduction to this chapter the experimental data in rats indicates that stress facilitates the learning response in fear conditioning. Rats freeze more to both the context conditioning and the tone conditioning

following a period of stress (Cordero *et al.*, 2003; Hui *et al.*, 2006). Like the OF, the effect of stress on fear conditioning in mice is not as clear. Whilst some studies show facilitation similar to that seen in rats (Sanders *et al.*, 2010; Blank *et al.*, 2002), others show no change (Mongeau *et al.*, 2007; Izquierdo *et al.*, 2006) or even a decrease in freezing (Ito *et al.*,). The data in this thesis agrees with a stress-induced facilitation of contextual conditioning (Figure 37). As discussed in regards to the OF, stress induced changes in locomotion can affect fear conditioning results. Hiroshi *et al* found that the fear conditioning was compromised by hyperlocomotion produced by chronic restraint stress. The studies that have shown a facilitation of contextual conditioning use either chronic variable stress of one hour immobilisation stress (Blank *et al.*, 2002; Sanders *et al.*, 2010). However, neither of these studies reported locomotion. Whilst the OF indicates that stress may increase the locomotion of the genetically modified mice, this did not hinder the stress induced facilitation in context conditioning in the studies described in this thesis. The results underline the importance of multiple behavioural tests to analyse stress-related behaviour.

The lack of facilitation of cued conditioning demonstrated in this thesis replicate a number of other studies on cued conditioning in stressed mice (Izquierdo *et al.*, 2006; Mongeau *et al.*, 2007). However, two studies by Sanders *et al* did have shown a stress-induced increase in cued conditioning. In the first study, chronic variable stress was applied in between conditioning and testing (Sanders, 2009) and in the second, only female mice were subjected to chronic variable stress (Sanders *et al.*, 2010). These protocols are

significantly different to the one used in this thesis and is likely to represent different neuronal mechanisms.

Timing of the EphrinB2 effects in the limbic system during the development of stress-induced anxiety

The critical role of EphrinB2 for the genesis of state and trait anxiety may indicate the importance of the biochemical changes following stress. The increase in the EphrinB2 gene was seen following six hours of stress, but there was a delayed increase in the EphrinB2 protein in the hippocampus and amygdala over the following eighteen hours after stress (Figures 22-24). This indicated that behavioural changes in EphrinB2-CaMKII-Cre mice would be most evident eighteen hours following the restraint stress. The effect of stress on mouse anxiety behaviour has been shown following various periods of restraint and various times of recovery following the stress (Table 2).

The hypothesis that pathological anxiety develops from state anxiety suggests that the changes occurring during state anxiety persist chronically. Although it has not been studied extensively, the literature suggests that single episodes of acute stress lead to persistent changes in anxiety-like behaviour. Mice that have been exposed to repeated restraint stress (two hours for three consecutive days) show exaggerated anxiety responses in the EPM twelve days after the original stress. The same mice also show an exaggerated stress response to the light-dark box twenty days following the original restraint (Chotiwat & Harris, 2006). This data indicates that the molecular mechanisms

that are engaged during an acute stress episode have chronic effects. Glucocorticoid responses are altered in rats twelve days following restraint stress (Harris *et al.*, 2004). In the hippocampus, acute stress results in the transformation of early LTP into late LTP (Ahmed *et al.*, 2006). Furthermore, altered synaptic plasticity is observed up to nine months following a social defeat stress model (Artola *et al.*, 2006). Morphological alterations in the hippocampus have also persisted for at least three weeks following social defeat stress. The particular Eph protein mechanisms that are likely to be engaged in the development of state anxiety following stress processes have been discussed in the preceding chapters of this thesis and include both synaptic and morphological plasticity.

Role of the neuropsin/EphB2 pathway in the stress response

Neuropsin brain expression pattern provides insight to the potential locus of its action. At the gene level, the highest expression of neuropsin is in the CA1 and CA3 regions of the hippocampus and the lateral amygdala (Chen *et al.*, 1995). It is expressed to a lesser extent in other areas of the limbic system, but is absent outside limbic structures. It is therefore likely that if the neuropsin gene is disrupted, then functions unique to the limbic system, such as anxiogenesis, will be affected. Indeed mice deficient for neuropsin do not develop stress-induced anxiety compared to wild-type mice. Following stress, wild-type mice reduced their number of entries to the open arms, whilst the stressed NP-/- animals entered the open arms with the same frequency as the non-stressed NP-/- group (Attwood *et al.*, 2011).

The brain locus of the neuropsin/EphB2 effect on stress-induced anxiety

In which limbic area does neuropsin exerts its effect on stress-induced anxiety? The data reported in the first two chapters of thesis indicate that the lack of anxiety in neuropsin knock-out animals may be attributed to its effect on EphB2 in the amygdala. If neuropsin is absent and EphB2 is not cleaved, the interaction of EphB2 with NMDA receptors is static, which results in attenuation of Fkbp5-dependent signalling. However, previous work by Shiosaka's group showed that neuropsin mRNA increases in the hippocampus following stress, and regulation of the hippocampal extracellular matrix by neuropsin may facilitate stress-induced neuronal plasticity (Matsumoto-Miyai *et al.*, 2003; Harada *et al.*, 2008). Anxiety-like behaviour may also be facilitated in the hippocampus by the synaptic tagging that requires neuropsin (Ishikawa *et al.*, 2008). To discover the locus of the neuropsin-dependent anxiety-like behaviour in the limbic system, neuropsin was injected directly to the amygdala of NP-/- animals immediately before stress. Our results indicate that neuropsin in the amygdala is critical to the genesis of anxiety-like behaviour following stress (Figure 45). Indeed, the injection of neuropsin not only rescued the anxiety-like behaviour but it also reproduced the dynamic interaction between EphB2 and NMDA receptors that was absent in neuropsin-deficient mice injected with vehicle (Attwood *et al.*, 2011).

What may be the role of neuropsin in the hippocampus in relation to anxiety-like behaviour? It is possible that neuropsin regulates separate aspects of

anxiety in different forebrain structures. Lesion studies show that the ventral hippocampus and the amygdala regulate different aspects of anxiety, for which the existence of separate neural circuits have been proposed. McHugh *et al* measured anxiety levels of rats using six different non-conditioned ethological tests, comparing rats with ventral hippocampal cytotoxic lesions against rats with amygdala cytotoxic lesions. They found that both groups of rats showed a reduction in anxiety-like behaviour, but that the anxiolysis resulting from amygdala lesions was displayed in different tests to the anxiolysis produced by hippocampal lesions. Both the amygdala and the ventral hippocampus lesions reduced the anxiety-like behaviour in the black/white two compartment test, but in the hyponeophagia test, the ventral hippocampal lesions reduced anxiety, whilst the amygdala lesions increased the anxiety-like behaviour. The hippocampal lesions also reduced anxiety in the social interaction test, whilst the amygdala-lesioned animals behaved similarly to the controls. In two other tests (spatial learning and spontaneous locomotor activity), both the ventral hippocampal and amygdala-lesioned animals behaved the same as the control animals. The authors also used the successive Alleys test, a modified form of the EPM, and found that lesions to the amygdala did not affect anxiety-like behaviour, whilst lesions to the ventral hippocampus did (McHugh *et al.*, 2004). It is important to note that the authors were measuring trait anxiety in rats, whilst the behavioural measurement utilised in this thesis is of state anxiety in mice. Taken together, our results and those described in the literature demonstrate that anxiety is not a singular, on-off behaviour. Depending on the type of paradigm used to measure anxiety and the situation preceding the test, the anxiety may be critically regulated by different brain structures or circuits.

In humans, different types of anxiety are associated with different brain regions being activated. During anxious apprehension (worry), patients show an increase in the right parietal lobe activity, whilst during anxious arousal (panic), the left hemisphere is activated (Heller *et al.*, 1997). However, recent evidence suggests that the amygdala circuitry is a common focus of abnormality in a number of anxiety-related clinical disorders in the human (Etkin & Wager, 2007). In our model, although the amygdala neuropsin rescue restored anxiety-like behaviour in the EPM, examination of these mice in different ethological behavioural tests may have revealed a deficit in anxiogenesis. Neuropsin in the amygdala is critical for the development of anxiety-like behaviour following restraint stress, but our work does not rule out the role of neuropsin in a different aspect of anxiety that are hippocampal dependent.

Neuropsin is secreted as an inactive zymogen and becomes rapidly activated in response to neuronal activity (Oka *et al.*, 2002). This allows for fast and spatially restricted regulation of experience-driven synaptic events. This was observed during neuropsin-dependent regulation of EphB2/NMDA association, which occurred as early as 15 minutes after stress (Figure 31). The neuropsin rescue experiment was designed to mimic the physiological sequence of events. Therefore, neuropsin was injected as an inactive zymogen, which meant that activity would be restricted to physiologically active synapses. However, the experiments reveal only a static snapshot of a highly dynamic and rapid process, and further studies are needed to further investigate the nuances of these rapid interactions.

The disruption of anxiety by interfering with the neuropsin/EphB2 pathway

The results show that neuropsin cleaves EphB2 to produce a number of downstream effects, which may alter anxiety-like behaviour. To investigate whether this cleavage event is critical in anxiety behaviour, EphB2 was blocked by injecting anti-EphB2 antibodies to the amygdala prior to stress and discovered that they inhibited the development of stress-induced, anxiety-like behaviour (Figure 44). The role of EphB2 in amygdala-dependent behaviour has not been studied previously. However, it has been demonstrated that EphB2 regulates memory and learning in the Morris water maze (Grunwald *et al.*, 2001; Cisse *et al.*, 2011). In the Morris water maze, mice that are deficient for EphB2 show deficits in memory, which are likely to be due to EphB2's role in hippocampal LTP formation (Grunwald *et al.*, 2001). Furthermore, in a mouse model of Alzheimer's disease, in which the performance in the Morris water maze deteriorates, the overexpression of EphB2 in the hippocampus rescues the performance deficit (Cisse *et al.*, 2011). Our amygdala data shows that the cleavage of EphB2 by neuropsin alters the EphB2-NMDA interaction (figure 31). It was also shown that neuropsin is critical to the early phase of amygdala LTP and that this is NMDA-mediated (Attwood *et al.*, 2011). Taken with the previously published behavioural data on hippocampus-dependent learning and LTP, this suggests that anti-EphB2 antibodies disrupt amygdala anxiogenesis by interfering with the neuropsin-EphB2-NMDA pathway, which likely alters the early LTP formation.

Chapter 6. Concluding remarks and future directions

Summary

This thesis has followed the development of two narratives regarding the interaction between Eph proteins and neuronal proteases in stress induced plasticity. The starting point was the discovery that two Eph receptors are susceptible to cleavage by particular neuronal proteases (Chapter 3). The role of these cleavage events was then explored on both a molecular level (Chapter 4) and a behavioural level (Chapter 5). The investigations into the separate cleavage events often started from the same question but required different methods and strategies in order to provide an answer.

One question presented to the investigator of stress-induced pathology is how transient psychological stimuli can lead to long-lasting behavioural changes. Proteases are attractive in the answer to this question, as they alter the molecular environment in a rapid and localised manner. In the case of tPA, plasmin and neuropsin, it is a simple molecular change that activates the zymogen, enabling it to alter the local molecular environment. Both strands of this thesis develop the hypothesis that the activity of a protease after stressful stimuli is critical to the development of long-lasting behavioural changes. By investigating how the protease may affect the Eph receptors' interaction with molecular binding partners in the period between the initial cleavage event and the development of behavioural changes, mechanisms by which the longer lasting effects occur have been described.

Conclusions

- EphA4 is identified as a novel substrate for plasmin.
- There is more than one plasmin cleavage site in EphA4 and a putative cleavage site of Arg^{520}-Ile^{521} close to the transmembrane domain is identified.
- EphrinB2 is identified as the principle binding partner of EphA4 in the hippocampus and amygdala.
- The stress response leads to an increase in ephrinB2 expression and an increased interaction between EphrinB2 and EphA4.
- EphrinB2 in the forebrain regulates anxiety and fear related behavioural signatures.
- EphB2 is identified as a novel substrate for neuropsin, likely cleaved at Gly^{517}-Arg^{518} within its extracellular domain.
- The stress response results in altered EphB2 dynamics in the amygdala in a number of ways:
    - EphB2 is cleaved by neuropsin within fifteen minutes following stress, altering the EphB2 membrane expression.
    - This cleavage event alters the EphB2 NMDA NR1 interaction.
    - This is followed by an increase in EphB2 gene expression.
- Anxiety-like behaviour is regulated by neuropsin and EphB2 dynamics in the amygdala.

EphA4, plasmin and EphrinB2

Chapter 3 describes the discovery that plasmin cleaves EphA4 close to the membrane. The evidence presented includes the cleavage demonstrated in SHSY-5Y cells, the cleavage of synthetic EphA4 Fc protein and the cleavage of native hippocampal EphA4 by exogenous plasmin. Mass spectrometry confirmed that the cleavage site is close to the membrane and that there is likely to be more than one extracellular cleavage site. To investigate this further amino-terminal oriented mass spectrometry of substrates (ATOMS) analysis could be used (Doucet & Overall, 2010). This labels the N-terminus of protein fragments after a cleavage event with heavy or light formaldehyde before isolation of these fragments and analysis by mass spectrometry. This eliminates the limitations of SDS-PAGE resolution by separating the cleaved protein fragments by precipitation rather than electrophoresis. In turn the protein sequences that have been cleaved before trypsin degradation are identified and accurate cleavage sites described.

The *in vitro* evidence described makes it likely that EphA4 is susceptible to cleavage by plasmin *in vivo*. Furthermore, the evidence from the literature and zymography studies (Appendix 4) indicate that plasmin is active in the same neural location that EphA4 is expressed. In the hippocampus, stress induces the activation of plasmin by tPA, altering neuronal morphology, hippocampal NMDA levels and mouse cognitive abilities (Pawlak *et al.*, 2005). Stress also increases the activity of tPA in the amygdala, which results in the development of anxiety-like behaviour (Pawlak *et al.*, 2003).

Unlike the cleavage of EphB2 by neuropsin, the cleavage of EphA4 by plasmin was not explicitly demonstrated following stress, which offers several opportunities and methods for further investigation. Firstly, using Western blotting, the quantity of membranous EphA4 could be quantified in the hippocampus before and after stress in both wild-type and mice deficient for plasmin. However, this is less likely to show a positive result than the investigation of EphB2 in neuropsin knock-out mice. EphA4 is much more abundantly expressed in the hippocampus than other Eph receptors (Tremblay *et al.*, 2007; Murai *et al.*, 2003) and in order to observe a significant proportion of EphA4 cleavage, high levels of cleavage would need to occur. To solve this problem of proportionality, high-resolution zymography could be used (Gawlak *et al.,* 2009). The combination of immunohistochemistry with high-resolution zymography could reveal a stress related plasmin-mediated cleavage of EphA4 at specific synaptic sites. For example pre or post-synaptic markers such as debrin, could be stained for along with Eph proteins to localise the plasmin activity precisely. This would give information regarding the hippocampal location of the cleavage activity and therefore insight into the downstream effects of this molecular event. As discussed our collaborators have developed this method preliminary images show promise as a proof of principle (Appendix 4).

The investigation into the role of EphA4 cleavage by plasmin led to investigating binding partners of the receptor. Although Eph binding of Ephrin is promiscuous, specific interactions regulate specific cellular responses (Pasquale, 2005). To learn about the function of the cleavage it was important

to examine which EphA4 interaction would be most likely to be altered by plasmin cleavage. In the hippocampus and the amygdala, immunoprecipitation and immunohistochemistry demonstrated the EphA4 – EphrinB2 interaction explained in Chapter 4. If plasmin were active in the hippocampus in the early stages following stressful stimuli, then the interaction between EphA4 and EphrinB2 would be decreased. Using immunoprecipitation, the quantity of the EphA4-EphrinB2 complex was compared after five minutes of stress, fifteen minutes of stress and at control levels. This did not show that stress had any significant effect on the interaction between the binding partners.

As discussed in Chapter 4, the neuronal molecular environment following stress is highly dynamic. Within fifteen minutes of a stressful event, EphB2 is cleaved by neuropsin. This was only revealed when the levels of membranous EphB2 were quantified in neuropsin knock-out mice. Therefore, despite the negative result regarding the EphA4-EphrinB2 interaction in the early stages following stress, the cleavage event may only be demonstrated if the interaction is investigated in plasmin-deficient mice.

It is also possible that the above results are hindered by methodological challenges. The abundance of EphA4 in the hippocampus may obscure a small physiologically important change in the interaction with EphrinB2. This could be overcome if the immunoprecipitation was performed in a specific area of the hippocampus in which the EphA4 cleavage by plasmin was high. This experiment could therefore be guided by the zymography described above. The zymography combined with immunohistochemistry could therefore also

provide further insight of how this interaction changes in the early period following stress.

The interaction between EphA4 and EphrinB2 is likely to cause phosphorylation of EphrinB2, initiating intracellular signalling (Qin *et al.*, 2010). Disruption of EphA4-EphrinB2 by plasmin is likely to cause a decrease in the phosphorylation of EphrinB2 and therefore regulate the function of the EphA4-EphrinB2 interaction. It could be speculated that - if plasmin is active in the hippocampus in the early period following stress and alters the EphA4-EphrinB2 complex - it will alter the signalling mechanisms of the complex. To investigate this, hippocampal homogenates from mice before and after 15 minutes of stress would be probed by Western blotting, using a phospho-EphrinB2 specific antibody. The experiment could be repeated in plasmin-deficient mice to discover if any stress induced changes in phosphorylation were plasmin-dependent.

Protein phosphorylation provides a rapid, reversible signalling mechanism that has been shown to initiate a number of neuronal functions. Whilst the experiment described above would indicate the importance of plasmin in phosphorylation of EphrinB2, it would not prove that this is dependent on plasmin cleavage of EphA4. Evidence that plasmin disrupts the EphA4-EphrinB2 interaction to alter the stress-induced phosphorylation of EphrinB2 would describe a novel and potentially critical early step in regulating the stress response in the hippocampus. This would be challenging to prove *in vivo* but the principles of the mechanism could be investigated *in vitro.* The

phosphorylation status of EphrinB2 could be measured using a phospho-EphrinB2 antibody following stimulation by the addition of EphA4 Fc to the cells. The effect of plasmin cleavage on the phosphorylation of EphrinB2 by EphA4 could then be measured by addition of plasmin to this *in vitro* system.

The EphrinB2 protein and gene expression levels robustly increase following restraint stress and during this, the interaction between EphrinB2 and EphA4 also increases (Chapter 4). The behavioural experiments using mice deficient for EphrinB2 indicate that the molecular events observed following stress are likely to be critical in regulating the anxiety-like behaviour caused by stress (Chapter 5). The Cre-loxP conditional knock-out mice have reductions in the floxed EphrinB2 gene in forebrain areas. To investigate this more thoroughly, EphrinB2 expression could be reduced more selectively in either the ventral hippocampus or the amygdala rather than both of these regions and other forebrain regions as seen in the CamKII Cre/loxP animals. This would be possible using an shRNA-based knockdown using a lentiviral delivery system. This technique has been previously used to investigate the role of amygdala FKBP5 expression on anxiety-like behaviour (Attwood *et al.*, 2011).

The Eph receptors and Ephrins display ligand-receptor promiscuity and yet comprise the largest family of receptor tyrosine kinases. Investigations have shown Eph-Ephrin interactions show functional redundancy as well as functional specificity (Pasquale, 2005). In reference to dendritic spine morphology, Henderson *et al* found that the knockout of one EphB receptor could be compensated by other Eph receptors. The largest abnormal

phenotype was demonstrated when three EphB receptors were knocked-out rather than just one. Why is it that other EphrinB receptors do not compensate for the removal of EphrinB2 in our model? It may be that by the time that the EphrinB2 expression is reduced (p21) the Ephrins' functions have already been set and the expression, function or localisation cannot be adapted to compensate. For example the compartmentalisation of Eph receptors changes through development and into adulthood (Henderson *et al.*, 2001). One way to investigate the role of other EphrinB's in our model would be to measure the stress response of EphrinB1 and EphrinB3 in the EphrinB2 lox mice to see if their expression alters following stress. If their expression increases this may indicate a degree of compensation at the genetic level. The lack of phenotypic compensation may also indicate that EphrinB2 specifically regulates the anxiety-like behaviour measured in chapter 5. This would explain the selective upregulation of EphrinB2 in response to stress, as opposed to EphrinB1 and EphrinB3, which are not changed in the wild-type mouse (Chapter 4). Conversely, it may be that EphrinB1 and EphrinB3 do play a role in anxiety-like behaviour and that if their expression was also reduced the anxiolytic phenotype would be further enhanced.

The discovery that EphrinB2 is also critical in the development of stress-induced changes in contextual fear conditioning confirms its critical role in stress-induced behaviour and indicates that the hippocampus is the likely locus of this regulation. It is possible that EphrinB2 is a critical factor in a common pathway regulating stress behaviour in all three of the behavioural paradigms used: they all measure fear behaviour (conditioned or

unconditioned). However, it cannot be excluded that EphrinB2 plays distinct roles in different pathways involved in the different behavioural tests utilized. Recently, it has been shown that EphrinB2 expression in the hippocampus is increased by contextual conditioning (Trabalza *et al.*, 2012). The stress-induced behavioural deficit during contextual conditioning described in this thesis likely stems from the effect of EphrinB2 during stress-induced memory formation, a separate molecular pathway to that of anxiogenesis. In clinical practice, stress is implicated in a number of psychiatric pathologies that can give rise to different pathological behaviours, resulting in different clinical diagnoses. It is hypothesised that, as the biochemical aetiology of psychiatric disorders is delineated, different clinical syndromes may be grouped together based on common biochemical pathways rather than common clinical symptoms. For example, different types of stress activate different neuronal pathways indicating that the stress response is not a unitary phenomenon. Therefore the large variety of clinical disorders that are either caused by or exacerbated by stress may benefit from treatments targeting specific stress pathways. These specific stress pathways would be identified by the type of causative stress, rather than the clinical pathology they produce (Pacak & Palkovits, 2001). The restraint stress model used throughout this thesis is a processive psychogenic stress. To investigate the role of EphrinB2 more broadly, measuring the behavioural and biochemical response of EphrinB2 to different types of stress would indicate if it was involved in a common stress pathway or a pathway specific to restraint stress.

EphB2 and neuropsin

The second theme of this thesis developed from the discovery that EphB2 is cleaved by neuropsin, another neuronal extracellular protease. Work with colleagues led to discoveries that this cleavage event results in a number of downstream events, from alterations in molecular interactions to changes in gene expression and changes in the electrical potentiation of the neurons (Attwood *et al.*, 2011). These changes lead to alterations in behaviour, furthering the hypothesis that psychological stress can be translated by molecular mechanisms into changed behaviour.

The evidence that EphB2 is cleaved by neuropsin is demonstrated in SHSY-5Y and HEK293 cells and *in vivo* following fifteen minutes of restraint stress (Chapter 3). Further *in vitro* validation of the cleavage, to gain information of the cleavage site by mass spectrometry for example, was hindered by technical difficulties. *In vitro,* recombinant neuropsin requires activation by lysyl endopeptidase (Kato *et al.,* 2001). Lysyl endopeptidase also cleaves EphB2, complicating analysis of cleavage of a synthetic EphB2-Fc (data not shown). However, this difficulty also served as an advantage when it came to injecting neuropsin to the amygdala of the mice deficient for neuropsin (Chapter 5): injecting an inactive zymogen ensured that its activity was restricted only to physiologically relevant synapses.

The cleavage of EphB2 by neuropsin within fifteen minutes of restraint indicates the rapid molecular changes occurring following a stressful event (Chapter 4). Due to the highly dynamic nature of a cellular environment, the

molecular biology employed here allowed only for a time-specific snapshot of stress-related events. To investigate the dynamic molecular interactions following stress, it would be informative to analyse samples collected at various time points in order to build up a more comprehensive data set. Alternatively, one could investigate morphological changes occurring using real-time *in vivo* imaging of the brain. Tobias Bonhoeffer has recently published data on the visual cortex using a two-photon microscope to image the mouse brain during the completion of a virtual reality task (Keller *et al.*, 2012). This system provides exciting possibilities of learning about the early stress response in a dynamic molecular and structural environment.

The cleavage of EphB2 by neuropsin occurs simultaneously with the insertion of new EphB2 receptors into the membrane (Chapter 4). To what extent is EphB2 trafficking important during the early stress response? It is known that EphB2 trafficking is important in dendrite morphology and that stress alters amygdala morphology (Hoogenraad *et al.*, 2005). At the same time as EphB2 trafficking occurs, the interaction between EphB2 and the NMDA subunit NR1 is decreased (Chapter 4). It is also known that NMDA trafficking is critical to its function (Tovar & Westbrook, 2002; Groc *et al.*, 2006; Blanpied *et al.*, 2002). Indeed, recent findings show that EphB receptors are key to precise synaptic localization of NMDA receptors and that they are able to influence the NMDA function at the synapse (Nolt *et al.*, 2011). It is likely that the lack of correct EphB2 trafficking underlies the decreased NMDA currents and lack of amygdala early LTP in neuropsin knock-out mice (Attwood *et al.*, 2011). Although EphB2 does not directly associate with NR2A and NR2B subunits, it

does stabilize NMDA receptors containing these subunits (Nolt *et al.*, 2011). The mechanisms underlying the amygdala stress response could therefore be further delineated by measuring the amygdala membranous levels of these subunits in the first fifteen minutes following stress. Other authors have used the flag-EphB2 construct expressed in cultured neurons to investigate EphB2 trafficking. The effects of neuropsin application to flag-EphB2 expressing neurons could illuminate the effect of EphB2 cleavage by neuropsin on EphB2 trafficking. NMDA constructs could also be used to investigate the effect of neuropsin on NMDA trafficking.

The role of neuropsin and EphB2 in stress-related anxiety-like behaviour was investigated using the elevated plus maze. Compared to the behavioural studies of EphrinB2 conditional knock-out mice, the EphB2/neuropsin behavioural studies were directed to investigate specific molecular interactions rather than to characterize the behavioural phenotype of an animal. This was achieved by utilizing direct access to the amygdala through intra-cranial cannulae. This technique allows for a direct application of molecular substrates to localized brain structures. The strength of this experiment design is that a molecular model can be validated using behavioural models of human pathology. This gives promise for further development in understanding molecular mechanism that regulates behaviour and their relation, the human pathology.

Appendices

a.

Mass observed	Mass Expected	Mass calculated	PPM	Miss	Peptide
791.4070	790.3997	790.3974	3.00	0	R.TDWITR.E
1244.5950	1243.5877	1243.5469	32.8	1	K.YYEKDQNER.S
1476.8250	1475.8177	1457.7620	37.8	0	R.VYPANEVTLLDSR.S
1502.8650	1501.8577	1501.8042	35.7	0	K.GLNPLTSYVFHVR.A
1953.9960	1952.9887	1952.8963	47.3	0	K.TDTIAADESFTQVDIGDR.I
2086.0090	2085.0017	2085.0717	-33.58	0	K.GFYLAFQDVGACIALVSVR.V
2270.0050	2268.9977	2268.8987	43.6	0	K.CPPHSYSVWEGATSCTCDR.G
2312.2090	2311.2017	2311.0968	45.4	0	R.TAAGYGDFSEPLEVTTNTVPSR.I

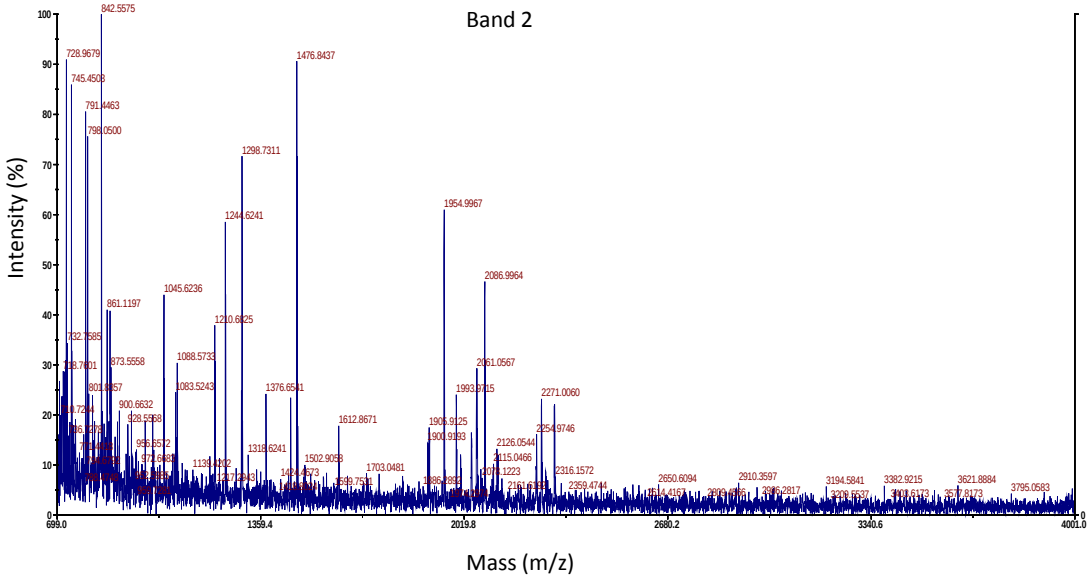
b.

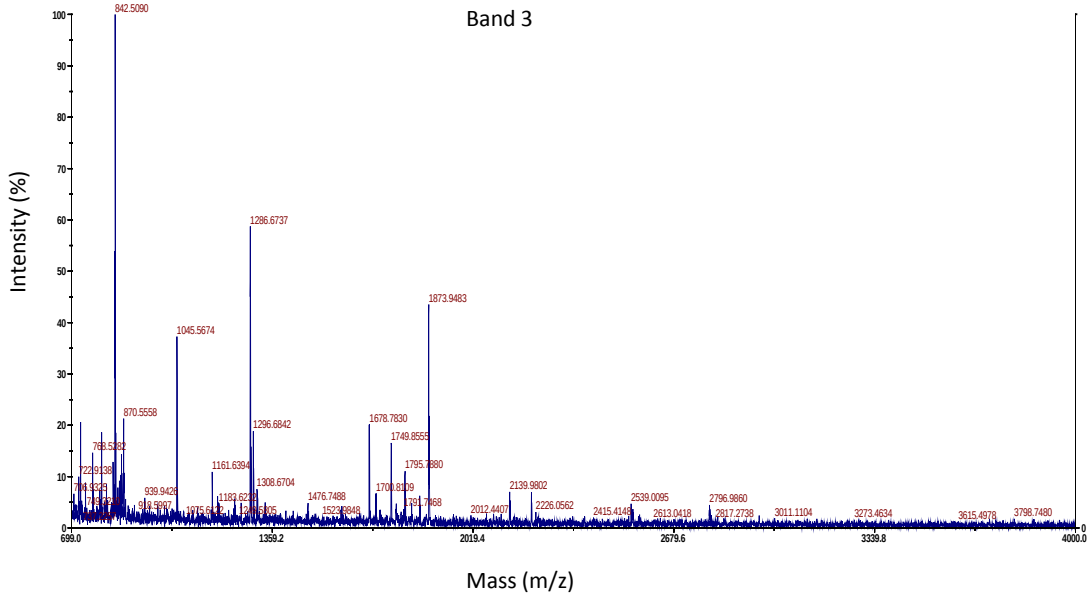
Mass observed	Mass Expected	Mass calculated	PPM	Miss	Peptide
745.4500	744.4427	744.4130	40.0	0	K.LNTEIR.D
791.4460	790.4387	790.3974	52.3	0	R.TDWITR.E
1244.6250	1243.6177	1243.5469	57.0	1	K.YYEKDQNER.S
1476.8480	1475.8407	1475.7620	53.3	0	R.VYPANEVTLLDSR.S
1953.9920	1952.9847	1952.8963	45.3	0	K.TDTIAADESFTQVDIGDR.I
2059.0510	2058.0437	2057.9524	44.4	0	K.CRPCGSGVHYTPQQNGLK.T
2086.0050	2084.9977	2085.0717	-35.50	0	K.GFYLAFQDVGACIALVSVR.V
2270.0000	2268.9927	2268.8987	41.4	0	K.CPPHSYSVWEGATSCTCDR.G
2312.1970	2311.1897	2311.0968	40.2	0	R.TAAGYGDFSEPLEVTTNTVPSR.I

c.

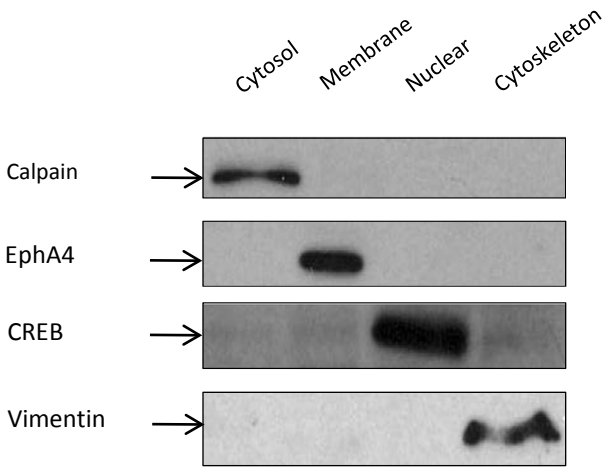
Mass observed	Mass Expected	Mass calculated	PPM	Miss	Peptide
841.3720	840.3647	840.3640	0.81	0	K.HHHHHH.-
1161.6300	1160.6227	1160.6223	0.33	0	K.NQVSLTCLVK.G
1286.6770	1285.6697	1285.6666	2.40	0	R.EPQVYTLPPSR.D
1677.7860	1676.7787	1676.7947	-9.53	0	K.FNWYVDGVEVHNAK.T
1872.9510	1871.9437	1871.9629	-10.23	1	R.EPQVYTLPPSRDELTK.N
2138.9830	2137.9757	2138.0202	-20.80	0	R.TPEVTCVVVDVSHEDPEVK

Appendix 1. Mascot data and peptides identified by mass fingerprinting of bands 1,2 and 3. Mascot data for band 1 (**a),** band 2 **(b)** and band 3 **(c)** of EphA4 FC cleavage by plasmin. Mass observed is the measured m/z ratio of the peptide. Mass expected is the measured mass of the peptide. Mass calculated is based on the identified sequence. PPM is the parts per million mass accuracy. Miss refers to the number of missed trypsin cleavage sites within the identified peptide. The identified peptide sequence is shown with amino-acids immediately prior to and following the identified sequence.

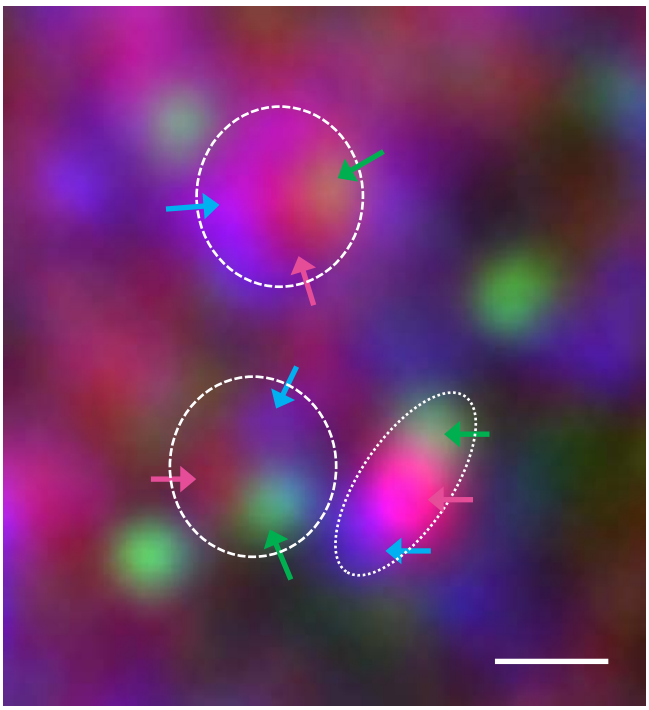




Appendix 2. **Peptide maps for band 2 and 3 of EphA4 cleavage by plamsin.** The mass spectometry peptide map for bands 2 and 3 of EphA4 cleavage by plasmin (Figure 10)**.** The peaks represent protein fragments produced by trypsin degradation. Each peak is analysed to identify its protein sequence.



Appendix 3. Verification of the purity of cellular fractions. Courtesy of S. Patel. Amygdalae were dissected and subcellular fractions purified using cellular protein fractionation kit as per the manufacturer's instructions (Perkin Elmer). Purity of individual fractions (membrane, cytosol, cytoskeletal and nuclear) were analysed by Western blotting using antibodies against markers of these cellular fractions (ephA4, calpain, vimentin and CREB, respectively). The results demonstrate high purity of the above fractions.



tPA (activity)
ephrinB2
EphA4

Appendix 4. Co-localisation of EphA4, EphrinB2 and plasmin activity in the hippocampus. Courtesy of Professor Wilczynski. Coronal mouse brain slices underwent *in situ* zymography and co-immunohistochemistry. Staining of EphrinB2 and EphA4 revealed co-localisation between EphA4, EphrinB2 and plasmin activity in the hippocampus.

Appendix 5. Neuropsin cleaves EphB2 in the amygdala to control anxiety (Publication from this thesis)

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