GPCR and RTK Regulation in Neurons: The Impact of Stress on GPCR and RTK signalling and Crosstalk

by

Nyasha Gondora

A thesis
presented to the University of Waterloo
in fulfilment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Pharmacy

Waterloo, Ontario, Canada, 2020

Examining Committee Membership

External Examiner	Dr. Cheryl McCormick
Supervisors	Dr. Michael Beazely and Dr. John Mielke
Internal Members	Dr. Praveen Nekkar Dr. Amy Newman
Internal-External Member	Dr. Robin Duncan

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Crosstalk between receptors allows for the integration of diverse and complex signalling pathways. Transactivation is a form of crosstalk between G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). RTKs are transactivated by GPCRs through two main mechanisms: ligand- independent intracellular pathways and triple membrane passing mechanisms involving GPCR mediated growth factor signaling.

Transactivation has neuroprotective potential; however, the physiological relevance of this pathway is not known. Interestingly, some stressors can up- or downregulate GPCR and RTK activity, as well as initiate certain transactivation pathways, leading to the question: could transactivation be a stress response? Given the impact of stress on GPCRs and RTKs, this thesis directly explored the impact of stress, more specifically acute chemical stress, in the form of the stress hormone corticosterone and chronic stress, in the form of Chronic Early Life Social Isolation (CELSI), on the 5HT7-TrkB transactivation pathway.

Another aim of this thesis was to analyze the effect of social isolation (CELSI) on the expression of proteins that are implicated in neuroplasticity and to explore if social isolation stress differentially primes the brain's response to stress or other stimuli. Coupling cell line based experiments with *ex vivo* tissue work, the overall aim of this thesis research was to gain a better understanding of some molecular mechanisms underlying the impact of stress on the brain.

Acknowledgements

I would like to thank my supervisors Dr. Beazely and Dr. Mielke for their constant support and guidance. Their depth of knowledge, strong dedication to mentorship and collaboration provided me with a wealth of opportunities for growth both academically and in extra-curricular pursuits. They are a dynamic duo- I am truly blessed to have had them as my mentors. I am also indebted to my thesis committee: Dr. Praveen Nekkar, Dr. Amy Newman and Dr. Jonathan Blay for their guidance during my graduate career.

I would like to acknowledge Christopher B. Pople, Gorvie Tandon and Brittani Brown who contributed directly to the work in this thesis. I owe a debt of gratitude to my colleagues Jeff Kruk and Nawaz Ahmed, who are geographically far but for years have remained a core part of my academic support system. I would like to acknowledge Morgan Robinson, Shannon Callender-Singh, Amna El ShatShat, Melinda Recchia and Krista Parsons for their exceptional support. I would also like to thank the entire graduate student body here at the School of Pharmacy for being an integral part of my journey.

I am grateful to the staff and faculty at the School of Pharmacy for granting me many opportunities to reach my academic potential and pursue my passion to teach. I would also like to extend my gratitude to the University of Waterloo and The Canadian Institute of Health Research Drug Safety and Effectiveness Cross Disciplinary Training program for funding various aspects of this research.

Last, but definitely not least I would like to thank all my friends and family for their unwavering encouragement and support.

Dedication

"If you want to go fast go alone, if you want to go far go together"- African Proverb.

I would like to dedicate this thesis to my family- to my parents, George and Evamary Gondora and to my sisters, Kudzaishe and Tinovongaishe Gondora. Thank you for not letting me walk this journey alone.

Table of Contents

Chapter 4:

List of Figures

Chapter 1

Chapter 2

Chapter 3:

Chapter 4:

List of Tables

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

List of Abbreviations

α – Alpha

ANOVA- Analysis of variance

AMP- Adenosine monophosphate

β- Beta

BAPTA- AM- 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)

BDNF- Brain- derived neurotrophic factor

Ca^{2+}- Calcium ions

CAMP- Cyclic adenosine monophosphate

CORT- Corticosterone or cortisol

DA- Dopamine

DAG- Diacyglycerol

DTT- Dithiothreitol

EDTA- Ethylenediaminetetraacetic acid

EGFR- Epidermal growth factor receptor

EGTA- Ethylene glycol tetra-acetic acid

γ- Gamma

GABA- γ-aminobutyric acid

GDP- Guanosine diphosphate

GEF- Guanine exchange factor

GPCR- G protein coupled receptor

Glu-Glutamate

GTP- Guanosine triphosphate

HPA axis- Hypothalamic-pituitary-adrenal axis

IP3- Inositol trisphosphate

K^+- Potassium

5-HT- 5-hydroxytryptamine

MAPK- Mitogen-activated protein kinase

MAPKK- Mitogen-activated protein kinase kinase

MAPKKK- Mitogen-activated protein kinase kinase kinase

NA- Norepinephrine; Noradrenaline

Na^+- Sodium

NADPH Oxidase-Nicotinamide adenine dinucleotide phosphate- oxidase

NMDA- N-methyl-D-aspartate

PDGFR- Platelet derived growth factor receptor

PDGF-BB- Platelet derived growth factor BB ligand

PIP2- Phosphatidylinositol 4, 5-bisphosphate

PKC- Protein kinase C

PLC- Phospholipase C

PMA- Phorbol 12-myristate 13-acetate

ROS- Reactive oxygen species

RTK- Receptor Tyrosine Kinase

TrkB-tropomyosin-receptor-kinase B

SDS-Sodium dodecyl sulfate

SSRI- Serotonin re-uptake inhibitor

VTA- Ventral tegmental area

CHAPTER 1

Introduction

Signal transduction describes the way in which cells communicate and respond to signals within the environment. It is a fundamental and highly dynamic process, which is dependent on the effective transfer and integration of intra- and extra-cellular signals across a dense network of receptors and signalling molecules [1]. This thesis explores how stress, in the form of a pharmacological stressor (corticosterone) and a rodent model of early life adversity (chronic early life social isolation) affects the expression and/or activation of key receptors, and ultimately how these changes affect receptor crosstalk.

1. Signal Transduction

Signal transduction between different classes of receptors can take many forms; this crosstalk is necessary for neuronal function and survival [2]. One of these forms of crosstalk is called transactivation, wherein one receptor is observed to activate another receptor. Specifically, transactivation is defined as the transient activation of receptor tyrosine kinases (RTKs) by G-protein coupled receptors (GPCRs) [3].

1.1 G-protein coupled receptors (GPCRs)

G-protein coupled receptors constitute the largest family of membrane receptor proteins, and approximately 70% of all clinically-used drugs target this family of receptors [4, 5]. GPCRs regulate cellular responses to neurotransmitters, hormones and environmental stimuli, and are implicated in basic physiological functions throughout all biological systems [4]. These membrane receptor proteins all contain seven transmembrane domains and are observed to interact with a heterotrimeric G protein complex at the C-terminus [6]. This heterotrimeric G protein complex is comprised of α, β and γ subunits, which are assembled in the inactive state [7]. Through the

exchange of GDP for GTP on the α subunit, the GPCR is activated, resulting in the cleavage of the α subunit from the βγ complex. The cleavage of this α subunit allows the receptor to bind various effector proteins [8, 9]. The α subunits are categorized into four main groups: $G\alpha_i$ is involved in the negative regulation of adenylate cyclase activity; $G\alpha_s$ stimulates adenylate cyclase activity; $G\alpha_q$ activates phospholipase C (PLC), and $G\alpha_{12}$ activates Rho GTPases [10, 11]. Additionally, the βγ subunit has been shown to play an integral role in the activation or modulation of a range of signalling pathways [11, 12].

This thesis will focus on two main GPCR families: serotonin (5HT) and dopamine receptors, with greater emphasis on 5HT receptors, and more specifically, the 5-HT7 receptor subtype.

1.2. Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter that is synthesized from L-tryptophan [13]. The central 5-HT system originates in the midbrain and brainstem raphe complex, and sends efferents throughout the brain [14]. Serotonin helps integrate complex brain functions such as cognition, sensory processing, and motor activity. In addition, serotonergic signaling has been implicated in the modulation of emotional behaviors, including fear, anxiety, and aggression [14]. Though serotonin is implicated in a variety of physiological functions, malfunction of its receptors (5-HT $_{1\text{-}7}$) or transporter (5HTT) is linked to a broad range of neuropathologies and mental illnesses [15]. The table below summarizes the roles, localization and clinical relevance of the seven 5HT receptor subtypes.

	Cellular Roles	Distribution	Physiological Roles	Relevant Drugs		Clinical Relevance
5HT 1A	$G\alpha_{i/o}$-coupled, ↓ cAMP, ↓ excitability, ↓ 5-HT release/ neuron firing (autoreceptors)	Cortex, limbic system, hypothalamus, raphe nuclei, and dorsal horn	Thermoregulation, Nociception, Emotion, Memory, Aggression (↓), Raphe nuclei neuron firing (↓), Oxytocin release (↑)	Agonists: Buspirone, Vortioxetine, Aripiprazole Antagonists: Pindolol	Agonists: 8-OH-DPAT Antagonists: WAY 100635-42	Anxiety, Depression
5HT 1B/ 1D	$G\alpha_{i/o}$-coupled, ↓ cAMP, ↓ excitability, ↓ 5-HT release (autoreceptors)	Basal ganglia, frontal cortex, hippocampus, striatum	Mesolimbic DA (↑), Reward sensitivity, Aggression (↓)	Agonists: Sumatriptan Antagonists: Yohimbine	Agonists: CP 93129 (1B selective) Antagonists: GR 127935-42	Migraine
5HT 1E	$G\alpha_{i/o}$-coupled, ↓ cAMP, ↓ excitability	Frontal/entorhinal cortex, caudate putamen, claustrum, hippocampus, amygdala	Migraine		Agonists: BRL 54443	
5HT 1F	$G\alpha_{i/o}$-coupled, ↓ cAMP, ↓ excitability	Dorsal raphe nucleus, hippocampus, cingulate/entorhinal cortex, claustrum	Migraine	Agonists: Sumatriptan	Agonists: BRL 54443, LY 334370, Lasmiditan	Migraine
5HT 2A	$G\alpha_{q/11}$-coupled, PLC → ↑ PKC/Ca^{2+}, ↑ excitability	Cortex, insula, limbic system, brain stem	Emotion, executive control, memory, ↑ Glu/DA/GABA release, ↑ hormone secretion	Agonists: Lisuride Antagonists: Most atypical antipsychotics	Agonists: LSD, DOI Antagonists: MDL 11939	Psychosis, Depression, Anxiety
5HT 2B	$G\alpha_{q/11}$-coupled, PLC → ↑ PKC/Ca^{2+}, ↑ excitability	Cerebellum, septum, hypothalamus, amygdala	Emotion, Nociception	Antagonists: Cyproheptadine	Agonists: α-Me-5-HT	Valvulopathy
5HT 2C	$G\alpha_{q/11}$-coupled, PLC → ↑ PKC/Ca^{2+}, ↑ excitability	Choroid plexus, cortex, hippocampus, amygdala, striatum, substantia nigra	Emotion, Anxiety (↑), Locomotion (↓), feeding (↓), ↓ DA/NA release, ↑ hormone release	Agonists: Lorcaserin Antagonists: Agomelatine, Trazodone	Agonists: LSD, DOI Antagonists: SB 242084	Depression, Anxiety, Appetite suppression
5HT 3A-E	Cys-loop superfamily, pentameric ligand-gated ion channel, $Na^{+}/K^{+}/Ca^{2+}$ entry, ↑ excitability	Brain stem, hippocampus, amygdala, and caudate nucleus	Nausea/emesis (↑), anxiety (↑), emotion, cognition, nociception (↑), reward	Agonists: Ethanol Antagonists: Ondansetron,	Agonists: SR 57227A Antagonists: Cocaine, GR 65630,	Emesis, Neuro-protection, depression, anxiety, Addiction

				Vortioxetine, Memantine	Zacopride	
5HT 4A-H	$G\alpha_s$-coupled, ↑ cAMP, ↑ excitability	DAergic systems, prefrontal cortex, septum, hippocampus	Memory, emotion, anxiety, DA release (↑)	Agonists: Metoclopramide Antagonists:	Agonists: Zacopride Antagonists: GR 113808	Alzheimer's, depression
5HT 5A-B	$G\alpha_{i/o}$-coupled, ↓ cAMP, ↓ excitability	Hippocampus, cortex, thalamus, hypothalamus, striatum, pons	Exploratory behavior	Agonists: Valerenic acid	Agonists: LSD Antagonists: SB-699551	
5HT 6	$G\alpha_s$-coupled, ↑ cAMP, ↑ excitability	Hippocampus, striatum, amygdala, cortex, nucleus accumbens, olfactory tubercle	Memory, cognition, depression	Antagonists: Clozapine, Quetiapine, Amitriptyline, Doxepin	Agonists: LSD Antagonists: SB 271046	Schizophrenia, depression, Alzheimer's
5HT 7A-D	$G\alpha_s$-coupled, ↑ cAMP, ↑ excitability	Thalamus, hypothalamus, amygdala, hippocampus, cortex, dorsal raphe nucleus	Circadian rhythm, sleep, emotion, cognition, memory, anxiety, migraine	Antagonists: Lurasidone, Amisulpride, Vortioxetine, Clozapine	Agonists: LP 44 Antagonists: SB 269770	Schizophrenia, depression

↑ And ↓ indicate effect of agonist/receptor activation. Note: Some partial agonists are listed as agonists, and some inverse agonists are listed as antagonists. Abbreviations: 5-HT – 5-hydroxytrypamine; serotonin, NE – norepinephrine; noradrenaline; DA – dopamine, GABA – γ-aminobutyric acid, Glu – Glutamate, cAMP – cyclic adenosine monophosphate, PLC – phospholipase C, PKC – protein kinase C, Ca^{2+} – calcium ions.

Table 1. *An outline of serotonin receptor function and pharmacology [16-44].*

1.3. Dopamine Receptor

Dopamine receptors are another class of GPCRs found in the vertebrate central nervous system (CNS). This class of GPCRs is implicated in the pathophysiology of neurological disorders like Parkinson's, schizophrenia, Tourette's, bipolar and Alzheimer's disease [45]. There are five dopamine receptor subtypes, which are classified according to their homology and pharmacology. These subtypes are divided into two categories: "D1-like" (D1) and "D2-like" (D2) dopamine receptors. D1-like receptors constitute D1 and D5 receptors, whereas, D2-like receptors include short and long isoforms of D2, D3 and D4 receptors [45]. In terms of relative abundance in the brain, D1 is observed to be the most abundant, followed by D2, D3, D4 and finally, D5 [46]. The

dopamine receptors within these two classes have significant amino acid sequence homology. Within the D1 class, D1 and D5 have 78% sequence homology; within the D-2 class, D2 and D3 have 46% sequence homology and D2 and D4 have 53% sequence homology [46]. D1 receptors are localized in the striatum, nucleus accumbens, olfactory bulb, amygdala, hippocampus and substantia nigra, whereas, D5 receptors are localized in the cortex, substantia nigra and hypothalamus. For D2-like receptors, D2 receptors are localized in the striatum, ventral tegmental area (VTA), olfactory bulb and cerebral cortex, whereas, D3 receptors are localized in the striatum, islands of calleja and cortex, and D4 receptors are localized in the frontal cortex, amygdala, hypothalamus, and nucleus accumbens [45].

D1 class of receptors are $G\alpha_s$-coupled, indicating that they stimulate adenylate cyclase activity, resulting in the subsequent increase of cellular cAMP. As such, these receptors are implicated in a variety of transduction pathways. For example, D1 receptors can modulate the electrochemical gradient in the striatum and kidney by inhibiting Na^+-K^+ ATPase through protein kinase A (PKA) and protein kinase C (PKC) signalling [47]. D1 receptors are also implicated in phospholipase C (PLC)-mediated increase of intracellular calcium, which can affect neurotransmitter release. In contrast, D2 receptors are $G\alpha_i$-coupled, indicating that these receptors are involved in the negative regulation of adenylate cyclase activity. In addition, D2 receptors activate pathways that are implicated in cell proliferation like the MAPK and Akt pathways, therefore they play a role in dopamine neuronal development [48].

1.4. Receptor Tyrosine Kinases (RTKs)

Receptor tyrosine kinases (RTKs) play a significant role in the regulation of many biological functions, including cell growth, differentiation, metabolism, and apoptosis [49]. RTKs are comprised of an extracellular ligand binding domain, a single transmembrane α-helix and a cytoplasmic tyrosine kinase domain. It is through ligand binding that RTKs are activated, which induces dimerization and the autophosphorylation of multiple intracellular tyrosine residues within the binding domain [50, 51]. The resulting phosphotyrosines serve as docking sites for other proteins involved in signaling cascades [50]. Further, the phosphorylation of specific tyrosine residues creates docking sites for Src homology 2 (SH2) domain and phosphotyrosine (PTB) domain containing proteins, leading to the activation of signal transduction pathways [50]. More than one SH2 domain-containing protein can bind to an activated RTK at once, allowing for the simultaneous activation of multiple signalling pathways [50]. One major pathway activated by RTKs is the MAPK pathway. In this signal cascade, RTK activation leads to the activation of the membrane bound RAS protein, the activated RAS protein phosphorylates and activates MAPKKK that phosphorylates MAPKK, and MAPKK, in turn, phosphorylates MAPK, ultimately leading to transcriptional regulation [52]. In addition, another major pathway involves the Phosphoinositide-3 Kinase, which phosphorylates inositol phospholipids in the cell membrane, subsequently leading to protein kinase activation [53].

This thesis will mainly focus on three RTKs, namely: the tropomyosin kinase B receptor (TrkB), the platelet derived growth factor receptor (PDGFR) and the epidermal growth factor receptor (EGFR).

1.5. Tropomyosin Kinase B receptor

Of the various RTKs is the tropomyosin kinase B receptor (TrkB), which belongs to the Nerve Growth Factor (NGF) family of receptors [54]. The TrkB receptor is largely expressed in the brain, with highest expression levels found in the cerebral cortex, hippocampus, thalamus, choroid plexus, granular layer of the cerebellum and the brain stem [55]. It is also expressed in the peripheral nervous system and in non-neuronal tissues such as the kidneys and the pancreas [55]. The TrkB receptor is activated by brain-derived neurotrophic factor (BDNF), but other neurotrophins, like neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), also bind to the TrkB receptor, albeit with lower affinity [56]. TrkB has 3 isoforms: TrkB-FL, T1 and T-Shc, and while all three isoforms have the same extracellular and transmembrane domains, isoforms T1 and T-Shc are truncated and therefore lack the tyrosine kinase activity of the TrkB-FL [57, 58]. The TrkB isoform T1 has been reported to negatively modulate the activity of TrkB-FL by forming heterodimers with TrkB-FL, and/or competing with it for neutrophin binding [57].

The TrkB receptor regulates neuronal growth, survival, differentiation, development and synaptic plasticity [59, 60]. TrkB mediates growth and survival through the PI3K/Akt signalling pathway, neuronal differentiation and neurite development is mediated through the MAPK pathway and synaptic plasticity is mediated through the PLCγ pathway [61, 62, 63]. The TrkB receptor is implicated in the pathophysiology of mental illnesses and neuropathologies like depression, schizophrenia, Alzheimer's disease and Huntington's disease and the ability to regulate TrkB in these disease states could improve clinical outcomes[64]. Further, activating TrkB in the context of some neuronal insults has been shown to decrease neuronal damage [65]. Therefore, the ability to activate TrkB and other RTKs in neuropathological states has great neuroprotective

potential. As such, understanding how transactivation pathways, initiated by GPCR signaling, might promote TrkB signaling will aid in our understanding of this neuroprotective pathway.

1.6. PDGF Receptor

The platelet derived growth factor receptors (PDGFRs) are classified into two major subtypes, PDGFRα and PDGFRβ. PDGFRs are activated by PDGF ligands, which can exist as one of four isoforms and these ligands are observed to interact with the receptor as dimers [66]. The possible dimer compositions are as follows: PDGF-AA, -BB, -AB,-CC and –DD. In addition, the PDGFRs subtypes, PDGFRα and PDGFRβ have differential affinity for the PDGF ligands, wherein, PDGFRα is observed to have a higher affinity for PDGF -A, -B and –C, whereas PDGFRβ tends to favor PDGF-B and -D [67]. PDGFRs are implicated in a variety of functions related to growth and development. In particular, PDGFRs play a significant role in the development of the brain, kidney, cardiovascular and respiratory systems. As well, they are specifically implicated in bone formation, erythropoiesis, and angiogenesis. Lastly, PDGFRs play a dual role in health and disease; for instance, they are implicated in tumor growth and in the mechanism of inflammatory diseases, however they also contribute to wound healing [68, 69]. PDGFRs are involved in many cross-talk pathways, and more specifically, undergo transactivation by 5-HT and dopamine receptors.

1.7. EGF Receptor

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the protein kinase superfamily comprised by three other members, erbB2/HER-2, erbB3/HER-3, and erbB4/HER-4. Six known agonists, including EGF and TGFα, activate EGFR; the activation of EGFR is observed to be the result of both ligand-dependent and ligand-independent mechanisms [70]. Interestingly, EGFRs show substantially increased basal signaling when over-expressed,

which underlies the role of this receptor in many cancers [71]. The EGFRs are implicated in a range of cellular functions, including: proliferation and survival, cell growth, apoptosis resistance, invasion, and migration. These outcomes are mediated through a variety of signalling pathways, namely, the Ras/Raf/mitogen-activated protein kinase pathway, the phosphatidylinositol 3-kinase/Akt pathway, phospholipase Cγ, Signal transducers and activators of transcription pathway and Src kinase pathways [72]. In addition, EGFRs are implicated in many pathologies including, cancers and vascular pathologies and diabetes [73, 74, 75]. The EGFR is a key target to explore when considering the intersection of stress and transactivation.

2. Transactivation: GPCR-RTK Crosstalk

Transactivation is a form of GPCR-RTK crosstalk that has been characterized extensively in many *in vitro* models. This section will describe the transactivation pathway, focusing on the two main mechanisms: ligand-independent (depicted in Figure 1) and ligand-dependent transactivation (depicted in Figure 3).

2.1. Ligand-independent transactivation

Ligand-independent transactivation involves the activation of a GPCR followed by the downstream activation of various secondary messengers that result in the phosphorylation and activation of the RTK [76]. These pathways are completely intracellular and occur in the absence of the RTK ligand [76]. Proteins that regulate the phosphorylation status of RTKs include phosphotyrosine kinases (PTKs), which increase RTK phosphorylation levels (through direct phosphorylation) and protein tyrosine phosphatases (PTPs), which reduce RTK phosphorylation (through dephosphorylation) [10]. These enzymes exist in a fine balance, deviation from which, can affect the phosphorylation equilibrium of RTKs [10]. As a result, the intracellular mechanisms through with GPCRs transactivate RTKs target the activity of PTKs and PTPs. Several

intracellular signaling effectors are involved in intercellular transactivation. Of these intracellular signalling effectors, this thesis will focus on: (i) membrane associated non-receptor tyrosine kinases and (ii) reactive oxygen species (ROS).

ROS induce transactivation through the inhibition of key phosphatases that dephosphorylate tyrosine residues, or by directly activating kinases through the modification of key protein-protein interactions, or triggering proteolysis of regulatory proteins that inhibit tyrosine kinase activity [77]. The combined effect of increased phosphorylation (due to increased PTK activity) and reduced dephosphorylation (due to inhibited PTPs) results in an overall increase in RTK phosphorylation and activation [10]. A study by Saito *et al.,* demonstrated that hydrogen peroxide (a ROS) can transactivate the PDGF-β receptor at the Ty1021 site [78]. A variety of signalling pathways in the brain produce, or are modulated by, ROS activity, for instance, mitochondrial dysfunction and inflammatory responses, respectively [79, 80]. Since cellular stress can produce ROS, it has the potential to trigger transactivation. Given the neuroprotective capacity of RTK activation and the capacity of various cellular stressors or pathological states to produce ROS, transactivation could be an evolutionarily conserved mechanism to promote pro-survival signal transduction.

Additionally, ligand-independent transactivation can also be achieved through the activity of membrane associated non-receptor tyrosine kinases, most notably the c-Src family, specifically Src, Pyk2, and Fyn. These non-receptor tyrosine kinases are activated by GPCRs and they in turn directly phosphorylate RTKs [10].

RTK transactivation, whether ROS mediated, or induced by non-receptor tyrosine kinases, is characterized by increased phosphorylation. This increased phosphorylation provides docking sites for the initiation of intracellular signalling cascades downstream of the RTKs, thus transactivation is able to "activate" a receptor in the absence of its ligand.

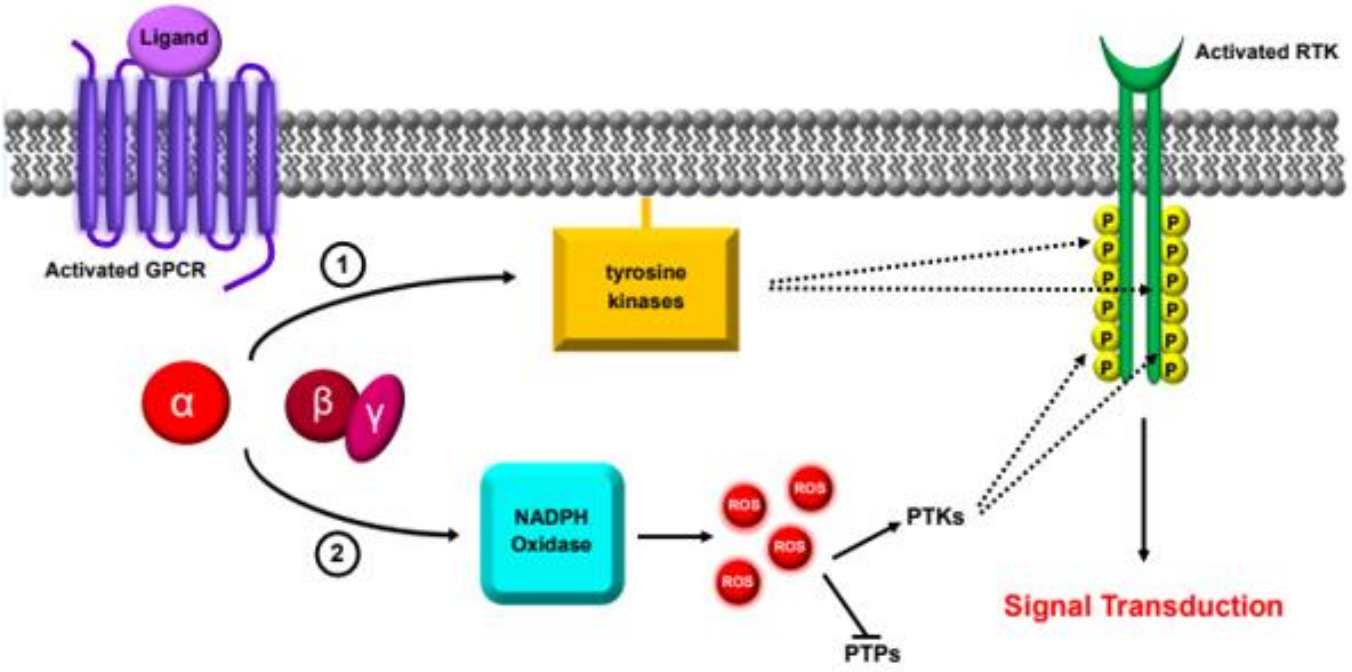


Figure 1. *Ligand-independent transactivation***.** The RTK is activated by the cognate GPCR through an intracellular, ligand independent mechanism. GPCRs can activate RTKs through: (1) the activation of membrane associated non-receptor tyrosine kinases e.g. c-Src that can directly phosphorylate the RTK OR (2) through the activation of NADPH oxidase, which releases reactive oxygen species (ROS). Increased ROS induces the inhibition of protein tyrosine phosphatases (PTPs) leading to enhanced phosphotyrosine kinase activity and subsequently increased RTK phosphorylation.

2.1.1 Characterization of ligand-independent transactivation

Our lab has previously characterized TrkB and PDGFβ receptor transactivation by several 5-HT and dopamine receptor ligands. We were able to demonstrate that in the human neuroblastoma-

derived SH-SY5Y cell line, 5-HT transactivation of the PDGFβ receptor results in phosphorylation at Y1021, Y751 and Y1009 sites [56]. These phosphorylation events are transient with a peak at 5 min and a return to baseline at approximately 15 min. As well, they are concentration-dependent, phosphorylation increases with increasing concentration of 5-HT, until 0.1 μM, after which it decreases [56].

We were also able to demonstrate 5HT transactivation of the TrkB receptor in SH-SY5Y cells [81]. However, unlike the acute timeframe observed in PDGFβ receptors, transactivation was observed at 2 hours in TrkB receptors [81]. This demonstrates that even though receptors may undergo transactivation by a similar mechanism, many key factors, for instance, the time profile of transactivation and the required drug concentrations, may differ. As a result, characterizing transactivation in a new model can be challenging, as multiple experimental factors need to be considered.

2.1.2 Characterization of the transactivation mechanism for TrkB and PDGFβ receptors

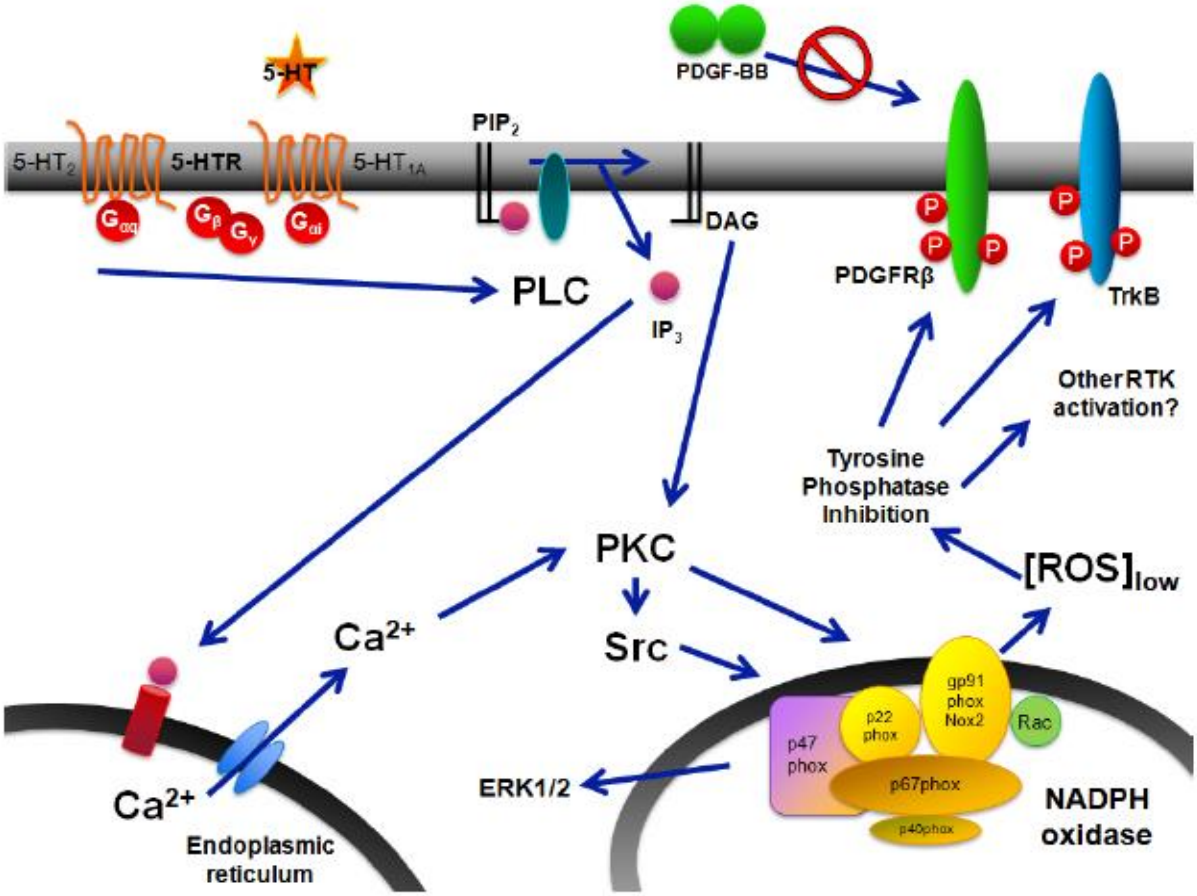


Figure 2. *Proposed mechanism for PDGFβ receptor transactivation* [82]. Proposed pathway for 5HT-induced transactivation of TrkB and PDGFβ receptors. This mechanism was mapped based on the response to treatment by various agonists and inhibitors of proteins implicated in the transactivation pathway.

As shown in Figure 2, 5-HT can transactivate PDGFβ receptors and TrkB receptors. Upon GPCR activation, PLC is activated; PLC in turn induces intracellular calcium release, and the subsequent activation of PKC. NADPH oxidase subunits assemble to produce ROS. ROS (generated through NADPH oxidase production or applied exogenously) temporarily oxidizes and inactivates PTPs, hindering them from dephosphorylating RTKs including PDGFβ and TrkB receptors, thus leading to an increase in RTK phosphorylation [10].

2.1.3 Transactivation is dependent on calcium, PLC, and PKC

Calcium signaling is a common requirement for numerous transactivation pathways. For example, Tanimoto *et al*., showed that sphingosine 1-phosphate transactivation of PDGFβ was calcium-dependent [83]. Previous work done in our lab to determine if 5-HT transactivation of the PDGFβ receptor is calcium-dependent demonstrated decreased receptor phosphorylation in cells that had been pre-treated with BAPTA-AM, an intracellular calcium chelator, whereas EGTA, an extracellular calcium chelator, did not have any effect on PDGFβ receptor phosphorylation [56]. This evidence suggests that transactivation is dependent on intracellular calcium. PLC and PKC were also investigated for their potential roles in this cascade. PLC cleaves the phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP2) to form secondary messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) [84]. IP3 induces opening of calcium channels on the endoplasmic reticulum [85]. Calcium activates calcium-sensitive PKC isoforms at the plasma membrane where they also bind phophatidylserine and DAG to become fully activated [86, 87]. Cells pretreated with U73122, a PLC inhibitor, showed a reduction in RTK transactivation. Gö 6983, a PKC inhibitor also blocked transactivation by 5-HT. These results showed that transactivation was dependent on PLC-mediated calcium release, and implicated PKC as part of this cascade [82].

2.1.4 Transactivation is dependent on reactive oxygen species (ROS), NADPH, and PKC

Earlier transactivation studies showed ROS dependency. For example, Chen *et al.,* (2004) showed that H_2O_2 was required for RTK transactivation and downstream signaling [88]. Previous work done in our lab to determine if this mechanism was ROS-dependent demonstrated an attenuation of receptor phosphorylation in cells pre-treated with ROS scavenger, *N*-acetyl-L-cysteine (1000 μM) [56]. To determine if NADPH oxidase function was necessary for transactivation, cells were treated with NADPH oxidase inhibitors, diphenyleneiodonium chloride (1 μM) and apocynin (100 μM). Transactivation was indeed blocked by both molecules, suggesting that NADPH oxidase is a part of the cascade, and most likely the source of ROS [56]. Importantly, PKC is known to activate NADPH oxidase [88]. Taken together, the generation of ROS by NADPH oxidase is likely downstream of PKC activation, which is in turn activated after PLC activation [56].

Thus far, we have discussed the general elements involved in ligand-independent transactivation, as well as the specific work previously conducted in our lab to characterize the mechanism of TrkB and PDGF transactivation by 5HT. The next section will focus on the other major mechanism of transactivation: ligand-dependent transactivation or triple membrane passing.

2.2. Triple membrane-passing signal (ligand-dependent transactivation)

The second major mechanism of transactivation is ligand-dependent transactivation, or triple membrane passing signalling. Canonical triple membrane passing transactivation mechanisms involve the activation of matrix metalloproteinase (MMPs), as well as, a disintegrin and metalloprotease (ADAMS) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTs). These proteins cleave the extracellular matrix (ECM), resulting in the release of the various growth factors, cytokines, and chemokines that are bound to the ECM [89, 90]. GPCRs can trigger the activation of MMPs through the activity of secondary messengers, including Src, Pyk, and calcium. MMP activation leads to the release of RTK ligands and subsequent RTK activation [91]. This mechanism is called triple membrane passing, because the signal passes through the membrane thrice: first, an extracellular signal transduces an internal response (GPCR activation); second, an intra-cellular message is externalized (activation of MMPs and ADAMS), and finally, the external signal "crosses" the membrane once again leading to a cellular response (RTK activation) by ECM cleaved ligands [92].

In summary, ligand-independent transactivation occurs in the absence of the RTK ligand and depends on an intracellular pathways to phosphorylate RTKs, either through the activity of non-receptor tyrosine kinases or through ROS inhibition of PTPs. On the contrary, in ligand-dependent transactivation, the RTK ligand activates the receptor directly; however, this activation requires on the cleavage of the membrane-tethered ligands, which upon release bind to and activate the RTK. Of these two mechanisms, the findings of this thesis align with the ligand-independent mechanism of transactivation.

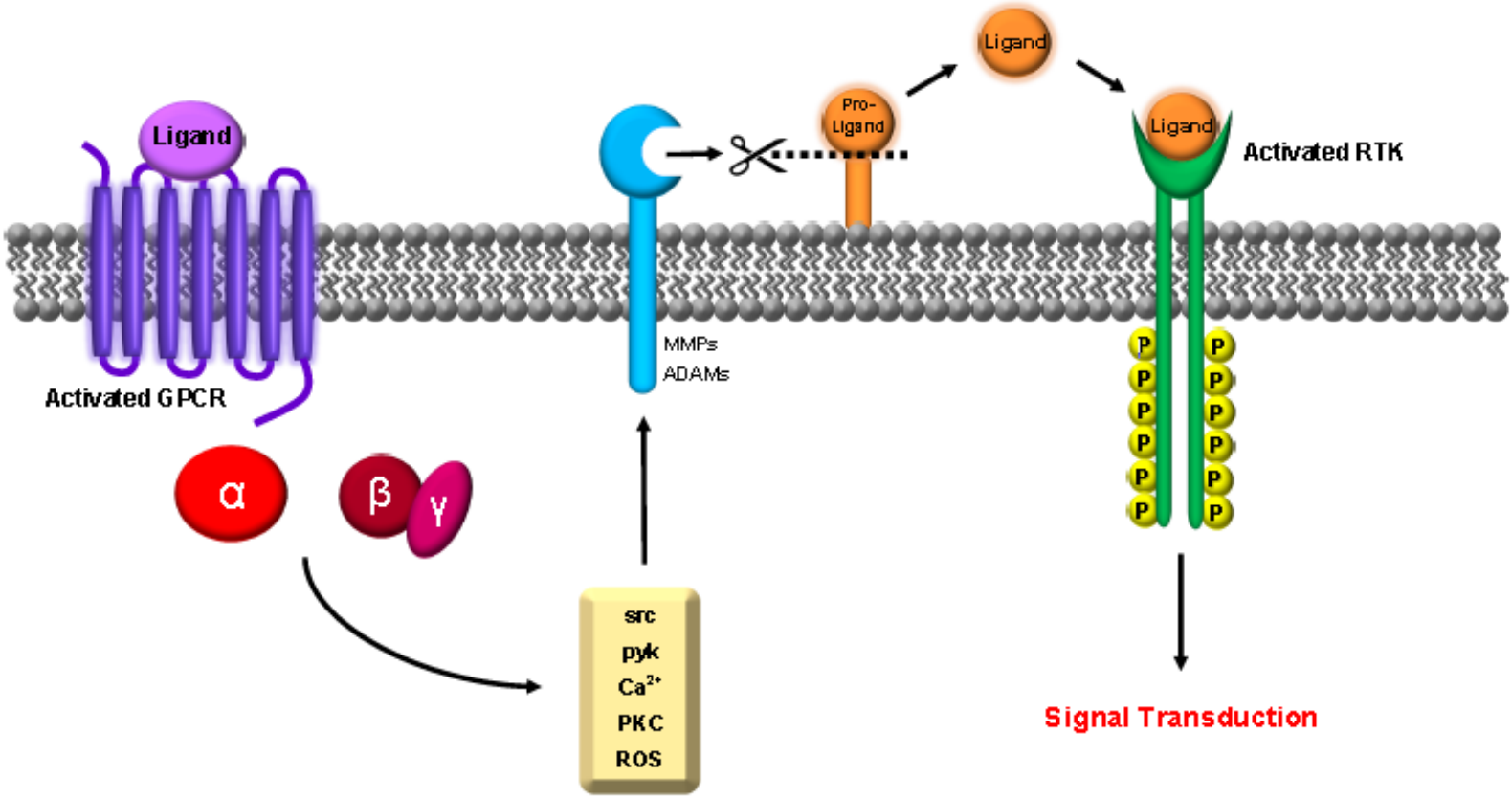


Figure 3. *Triple membrane-passing signal (ligand- dependent transactivation mechanism*). The activated GPCR recruits a secondary messenger (e.g. c-Src, Pyk2, calcium, PKC or ROS) which leads to the activation of the MMPs, ADAMS resulting in the cleavage of the membrane bound pro-ligand. The released ligand then binds the RTK resulting in its phosphorylation and activation.

3. Stress

3.1. Introduction to stress pathways

Stress is defined as any phenomenon that affects the homeostasis of the cellular environment. It can be induced endogenously through the dysfunction or dysregulation of cellular mechanisms required for homeostasis or by external causative factors [93]. In response to stress, cells activate adaptive mechanisms to counter the imbalance caused by the stressor [94]. Though stress differentially affects various tissues and organ systems, there are some key evolutionarily conserved multi-organ stress responses. In the brain, one such pathway is the hypothalamic-pituitary-adrenal (HPA) axis [94]. The HPA axis activation cascade involves the synthesis of corticotrophin-releasing factor (CRH) in the hypophysiotropic neurons of the paraventricular nucleus of the hypothalamus (PVN) [95]. CRH is released into the hypophyseal portal vessels that transport it to the anterior pituitary gland where it binds to its receptors inducing the release of adrenocorticotropic hormone (ACTH). ACTH in turn induces the release of cortisol/corticosterone (glucocorticoids) in the adrenal cortex [96]. The acute release of glucocorticoids is protective and activates the sympathetic nervous system, allowing the body to cope with the stressor. However, after a threshold concentration of glucocorticoids is reached, these glucocorticoids activate a negative feedback loop to inhibit the release of more CRH and ACTH [97].

In the brain, glucocorticoids bind the mineralocorticoid and glucocorticoid receptors. In the classic genomic pathway, once activated, cytoplasmic glucocorticoid receptors translocate to the nucleus where they regulate transcription. This genomic pathway can also be activated indirectly via membrane bound receptors and secondary messengers [97]. Recently, glucocorticoids have also been shown to have fast acting, non-genomic activity mediated through membrane bound receptors including GPCRs [98]. At the level of synaptic communication,

glucocorticoid action affects neurotransmission and ion channel activity through altering the release of excitatory amino acids and affecting the balance of key ions, like calcium [99].

Depending on its severity and frequency, stress can induce adaptive/ protective, or maladaptive responses. In the brain, acute glucocorticoid activation is adaptive and transiently promotes pro-survival signaling; however, chronic activation can induce negative results including, morphological and epigenetic changes, which can subsequently lead to pathological outcomes [100]. Acute stress typically enhances function through increasing synaptic transmission, long-term potentiation and learning for self-preservation. In contrast, mild chronic stress leads to the suppression of synaptic transmission, long-term potentiation, neurogenesis and dendritic remodelling. Further, severe chronic stress induces neurochemical distortions, impaired remodelling, mediates excitatory seizures, stroke, and head trauma [97].

Additionally, the impact of stress in the brain can be transient and reversible. For example, repeated restraint stress in rats reduces dendritic branching in medial prefrontal cortical pyramidal neurons that is reversible after a period (3 weeks) without the stressor [101]. Conversely, stress may also result in long-term changes in neuronal signaling and significantly impact development and behavior; the timing of the stressor can significantly affect its impact [97, 102]. Various models of early life adversity have demonstrated that stress during the developmental phase can affect the organism's developmental trajectory and lead to negative health outcomes in adulthood [103]. Section 5 provides more information on early life adversity.

3.2. RTK- Stress connection

There is abundant evidence in the literature to establish a correlation between stress and altered regulation or activity of RTKs. RTKs, specifically the neurotrophin family of receptors, are implicated in neuroprotective signalling. Various models of stress, both *in vivo* and *in vitro*, have been shown to activate RTKs. In particular, genetic and pharmacological manipulation of the BDNF-TrkB pathway has revealed a prominent role in the susceptibility to, and development of depression, as well as recovery from depressive behavior [104].

Various studies have demonstrated that elevated glucocorticoid secretion during chronic stress exposure is linked to the pathophysiology of depression, in part, by disruption of BDNF-TrkB signalling and subsequent effects on synaptic function. Restoration of this signalling can reverse stress-induced cellular and behavioral pathologies and may underlie the response to a diverse array of antidepressants [105]. Corticosterone regulates the expression of both BDNF and TrkB mRNA in the hippocampus, a potential locus of stress-induced neurological deficits and antidepressant action [106]. Studies conducted by Schaaf *et al.* demonstrated that corticosterone administration decreases BDNF mRNA in a dose dependent manner in the CA1, CA3 and dentate gyrus. Additionally, they showed that at low corticosterone concentrations, TrkB receptor expression increases in the dentate gyrus and CA3 [107], therefore, suggesting that the impact of corticosterone is dependent on the administered concentration.

Recently Barfield *et. al.*, reported that corticosterone exposure in the ventral hippocampus of group-housed male wildtype C57BL/6 mice during adolescence can lead to the modulation of goal-directed actions and behaviors; this mechanism was demonstrated to be TrkB-dependent. In this study, corticosterone induced key changes in the ventral hippocampus, including a shift from full-length TrkB to the truncated (inactive) form in many corticolimbic brain regions and

diminishing the phosphorylation of the TrkB substrate extracellular signal-regulated Kinase 42/44 (Erk 42/44). Administration of 7, 8-DHF (TrkB agonist) at doses that stimulated ERK42/22 corrected the corticosterone induced behavioral abnormalities [108]. Additionally, TrkB-dependent neurogenesis in the ventral hippocampus, as well as plasticity and activity of projections from this region to the prefrontal cortex, have been strongly implicated in the antidepressant and precognitive effects of ketamine [108].

BDNF increases neurotransmission of glutamate through the PLC gamma pathway [109]. A study demonstrated that 24-48-hour corticosterone treatment inhibits BDNF-stimulated PLC gamma activity and BDNF-triggered glutamate release in cultured cortical neurons, showing the potential connection to impaired glutamatergic transmission in stress-induced models of depression [109]. Additionally, other RTKs have been shown to mediate stress signalling under pathological conditions. For example, the EGF receptor can trigger endoplasmic reticulum stress in cardiac damage and micro vascular damage in diabetes type 1. Further, mechanical stressors have also been shown to activate many cellular responses in an EGFR dependent manner [110, 111]. These highlighted findings show the impact of stress on RTK signalling.

In summary, the observation that pharmacological stressors applied *in vitro* such as corticosterone, as well as more severe or pathological forms of *in vivo* stress, such as those described above, can modulate RTK activation, provides strong support for the RTK-stress connection.

3.3. GPCR (5HT)-stress connection

A correlation has been established between stress and serotonin function. For instance, upon acute stress exposure (such as a 5 minute forced swim stress test, or subcutaneous saline injection), 5-HT metabolism is increased in various regions of the rat brain, including the amygdala and medial prefrontal cortex [112]. In freely moving animals, extracellular 5-HT concentration in the brain is elevated after the application of aversive stimuli, such as psychological stress, immobilization or exposure to novel environments [112]. The relationship between 5-HT levels and stress is of critical importance, considering the implications of the serotonergic system in stress-related psychopathologies, such as anxiety disorder, or major depressive disorder [113].

As shown in Table 2, the seven 5-HT subtypes have differential responses to stress. Dysregulation of 5-HT_{1A} receptors have been linked to psychiatric disorders, such as depression and generalized anxiety [114]. In fact, there is also evidence of 5-HT_{1A} cross talk with RTKs *in vivo* in a genetic model of depression [115]. Stress hormones and glucocorticoid receptors may also modulate 5-HT_{1A} receptor signaling [116]. Similarly, the 5-HT subtype, 5-HT_{2A} receptor, has been implicated in specific features of stress-induced responses [117]. Previous studies have demonstrated that 5-HT_{2A} receptors can enhance GABAergic neurotransmission, which is altered by stress [118]. Additionally, 5-HT_3 receptor antagonists appear to be protective against long-term stress-induced behaviors [119] and 5-HT_4 receptor antagonists are also suggested to have anti-depressant properties [120]. Further, the 5-HT_{5A} receptor displays coupling properties similar to the 5-HT_{1A} receptor, and the 5-HT ligand [121]. However, details relating to the stress-induced behavioral consequences of serotonin signaling through 5-HT_{5A} receptors is relatively unknown [122]. The 5-HT_6 receptor agonists and antagonists have been proposed as possible means for treating psychiatric disorders such as depression [123].

As one of the more recently discovered serotonin receptors, the 5-HT_7 receptor is the least characterized [124]. 5-HT_7 mRNA levels are observed to increase following adrenalectomy in the CA3 sub-region of the hippocampus, indicating that glucocorticoids play a role in the regulation of the 5-HT_7 receptor [125, 126]. Low doses of corticosterone replacement prevents this increase, suggesting that tonic control of 5-HT_7 mRNA expression is affected by basal corticosterone levels in the hippocampus [125]. Alterations in serotonin release in the hippocampus and ventral striatum have been linked to 5-HT_7 receptor expression, but more specifically, to be caused as a result of glutamate receptors on the raphe nuclei in these regions [127]. Recent studies have demonstrated that chronic restraint stress-induced endocrine disruption may also be associated with the increase in function and expression of 5-HT_7 receptors [128]. It has been theorized that pharmacological blockades of adrenocortical 5-HT_7 receptors could provide some therapeutic benefit for overcoming endocrine disruption in stress-related syndromes [128]. Moreover, activation of 5-HT_7 receptors in cultured hippocampal neurons increases glucocorticoid receptor expression in a PKA-dependent manner [129]. The literature provides further evidence of the impact of stress on serotonin signalling.

Further examples of the individual contributions of each subtype are briefly reviewed in the following Table.

RECEPTOR	STRESSOR	OBSERVED OUTCOMES
5HT1A	Forced swim test	Decrease in 5-HT_{1A} autoreceptors density in the presynapse and increase in postsynaptic 5-HT_{1A} receptor labeling in the dorsal and medial raphe nucleus, and the hippocampus.
	Corticosterone	Decrease in potency of 5-HT_{1A} agonist 8-OH-DPAT, resulting in the inhibition of 5-HT cell firing rate, in brain stem.
5HT2A	Stress	Impairment of 5HT2A receptor mediated facilitation of GABAergic synaptic transmission in the BLA.
	Stress	Regulation of brain-derived neurotrophic factor (BDNF). Increased expression in BDNF mRNA in the frontal and parietal cortex; decreased mRNA expression in the hippocampal dentate gyrus.
5HT3	Chronic unpredictable stress	Ondansetron (5-HT_3 antagonist) demonstrated a reverse in: (i) chronic unpredictable stress-induced despair behavior in male rodents and a reduction in the time they spend immobile when subjected to forced swim test. (ii) Decrease in serotonin levels in the midbrain, prefrontal cortex and the cerebellum in chronically stressed mice
5HT4	Forced Swim test	$5HT_4$ agonists reduce immobility of rats subjected to the forced swim test.
5HT5	NA	Details relating to the stress-induced behavioral consequences of serotonin signaling through 5-HT_{5A} receptors is relatively unknown
5HT6	Unavoidable shock	Long-term treatment of rats with ST 1936, a 5-HT_6 agonist protects from development of escape deficit(ED), but does not revert rats already in a state of chronic ED.
5HT7	Chronic unpredictable stress	Upregulation of 5-HT_7 receptor mRNA expression in the hippocampus and hypothalamus of the rat brain
	Acute restraint stress	Increasing 5-HT_7 receptor mRNA
	halothane exposure forced swim tests, cold exposure, and restraint stress (for a period of one week)	Small change in 5HT7 receptor mRNA
	Repeated corticosterone administration	Increased hippocampal 5HT7 receptor expression

Table 2. An outline of the impact of stressors on 5HT receptor subtypes [112-129].

In summary, there is abundant evidence to support the modulation of RTK signalling and GPCR signalling by stressors. Specifically, the effect of stress on BDNF-TrkB and 5HT signalling is relevant because of the reported physiological and behavioral outcomes. One of the questions this thesis sought to explore is, given that stress affects 5HT signalling and TrkB signalling, does stress modulate 5HT and TrkB crosstalk (transactivation)? Our depth of experience in the study of receptors and in 5HT and TrkB receptor crosstalk, coupled with strong evidence in the literature of stress effects on these receptors, provided me sufficient basis to connect the pieces of a puzzle and address the question of stress effects on 5HT-TrkB transactivation. Given the neuroprotective potential of RTK activation, and thus transactivation, it is important that we explore this question, to gain insight into the molecular interplay between stress and transactivation pathways. Given the recent discovery, and hence relatively limited characterization of the 5HT7 receptor, I chose to specifically study 5HT7 transactivation of TrkB.

To my knowledge, I am the first to explore this specific question. Therefore, the following section provides both broad and specific evidence from various models and GPCR-RTK pairs to highlight of the impact of biological and environmental stressors on key transactivation pathways.

4. Transactivation and Stress Connection

Stress responses are diverse phenomena occurring at and coordinated between, many levels of organization within an organism, and are subserved at the cellular level by numerous interacting signaling pathways. Transactivation of RTKs represents a potential mechanism for the diversification and integration of signal transduction, as well as a method for the fine-tuning of effector recruitment [130-133].

4.1. Glucocorticoid-mediated effects on transactivation

The glucocorticoid receptor (GR) can transactivate Trk receptors *in vitro* via BDNF-independent, genomic mechanisms, as well as in the rat hippocampus via BDNF-dependent, nongenomic mechanisms, which may mediate glucocorticoid-induced enhancement of inhibitory avoidance memory [134, 135]. Altered BDNF-TrkB signaling appears critical for the expression of depressive phenotypes following chronic stress. Reduced BDNF-TrkB signaling is correlated with the development of depressive phenotypes; antidepressant response to pharmacologically diverse compounds involves enhanced activity in this pathway [136-141]. Importantly, diverse antidepressant drugs not only increase BDNF and TrkB phosphorylation, but may also transactivate TrkB in a BDNF- and 5-HT transporter-independent manner [138-140, 142]. The latter fact is particularly relevant, as several antidepressants have also been shown to increase GR activation as well [143-146]. As such, it is possible that GR-mediated transactivation of RTKs, particularly Trk neurotrophin receptors, may underlie both acute responses to psychological stressors as well as recovery from stress-induced behavioral disorders.

4.2. Acute stress mediated-effects on elements up- and down-stream of GPCRs and RTKs activation

Transactivation has been observed following stimulation of a diverse array of GPCRs with neuromodulators and peptides, including monoamines and adenosine [133, 147-151]. Acute exposure to diverse psychological stressors has been demonstrated to elevate extracellular concentrations of monoamines and adenosine, among other transmitters, and chronic exposure may enhance their release in response to future stressors [152-156]. Moreover, GRs in the prefrontal cortex play a critical role in the enhancement of local dopamine released by stress [157]. While transactivation has been demonstrated in acute tissues, the concentrations required to induce it frequently exceed extracellular concentrations of neurotransmitters such as dopamine as measured by microdialysis, as is the case for PDGFRβ transactivation-induced NMDA receptor inhibition in the prefrontal cortex [158, 159]. Nonetheless, microdialysis has poor temporal resolution and fails to capture endogenous transients produced by phasic firing of dopaminergic neurons, which can yield dramatically higher concentrations similar to those that produce transactivation, albeit briefly [159, 160]. Indeed, stressors and aversive stimuli not only increase tonic dopamine, but also enhance phasic dopamine release [161, 162]. Notably, stress exposure may increase cAMP/PKA signaling in regions including the prefrontal cortex, at least in part by monoaminergic transmission [152, 163-164]. This is particularly relevant as even short pulses of GR agonists can induce delayed ligand-independent transactivation of TrkB in a transcription-dependent manner *in vitro*, and signaling via $G\alpha_s$-coupled monoamine receptors, or cAMP/PKA has been shown to enhance glucocorticoid-mediated transcription [165-168]. Consistent with this idea, both PKA signaling and GR-mediated TrkB transactivation have independently been identified as critical for glucocorticoid-induced enhancement of inhibitory avoidance memory and

GR-mediated antidepressant effects [143, 169, 170]. Whether these events individually, or by interaction, bring about RTK transactivation *in vivo* as part of a normal physiological stress response, however, remains to be seen.

Hypoxia and ischemia may elicit even more dramatic increases in neurotransmitter concentration than psychological stress, particularly with respect to adenosine [171-174]. These insults also provide examples of stressors where transactivation is implicated in adaptive responses *in vivo*, most importantly within the brain [175]. Specifically, activation of the adenosine A1 and A2A receptors confer neuroprotective and neurotrophic effects via transactivation of EGF and Trk receptors and is similarly involved in cerebral ischemic preconditioning [175-177]. Similarly, transactivation of the EGF receptor by adenosine, acting via the A1 receptor, has been implicated in the cardioprotective effects of ischemic pre- and post-conditioning following later ischemia [178-180]. Additionally, cardioprotective roles for EGF receptor transactivation have also been described downstream of bradykinin B2 and δ opioid receptors as well [180-183].

Transactivation of the TrkB neurotrophin receptor by the A2A and 5-HT7 receptor has similarly been implicated in the LTP-like increase in synaptic strength seen in the spinal phrenic nerve following some forms of intermittent hypoxia [184-188]. Such results lend credence to the idea that transactivation may be a critical component of many stress response mechanisms acting across levels of organization, particularly with respect to early adaptive responses that promote resilience.

4.3. Chronic stress mediated-effects on elements up-and down-stream of GPCRs and RTKs activation

In addition to acute stressors, many aspects of the neurobiological response to chronic stress involve signaling pathways that may also induce transactivation of various RTKs and regulate

neuronal survival. Specifically, neuroinflammation is frequently observed in, and critical to the pathology of, chronic stress, psychiatric disorders including depression, ischemia, and neurodegenerative disorders [189-194]. Inflammation involves a cascade of signaling events and biological responses, which, notably, include generation of ROS by NADPH oxidase and mitochondria [195]. In fact, the generation of ROS and impairment of antioxidant enzyme systems is associated with the pathological effects of glucocorticoid exposure and chronic stress, as well as the expression of depressive behavior thereafter [136, 191, 196-200]. Generation of ROS by NADPH oxidase and/or mitochondria have also been implicated in many transactivation mechanisms through the activation (or inactivation) of various redox-sensitive proteins, such as Src-family kinases in ligand-independent transactivation or metalloproteinases in some triple-membrane passing transactivation [56, 201-208].

4.4. Factors affecting transactivation

Significant opportunity for transactivation to occur exists in both acute and chronic stress responses, but where these responses occur, when they are initiated, and whether they become disrupted or engaged at different stages of the stress response will determine the outcomes they confer. For example, although the GR can transactivate TrkB, chronic stress can decrease GR expression, thereby disrupting its interaction with TrkB and potentially producing stress-induced impairments [109, 208-210]. Both the glucocorticoid and 5-HT1a receptor, the latter a prominent target of antidepressants, have been reported to transactivate FGFR1 as part of signaling complexes, disruption of which were also identified in a genetic model of depression [115, 147, 211, 212]. Another notable mechanism by which stress could disrupt transactivation is the alteration of mitochondrial ROS signaling. Ischemic preconditioning, as well as both RTK activation and transactivation in the heart and brain, frequently require mitochondrial ROS

generation, likely via opening of redox-sensitive mitochondrial ATP-sensitive K+ channels (KATP) [202, 213-219]. Such ROS-induced mitochondrial ROS release can spread through cells in waves, propagating the signal [220-222]. Thus, whether transactivation is initiated may depend upon the interaction of numerous factors, but this signaling paradigm also has the potential to yield widespread, multifaceted effects on cellular signaling and stress responses.

To this point, we have discussed various stressors and models of stress that have been utilized to explore the impact of stress on GPCRs, RTKs and transactivation. The following section will focus on an *in vivo* model of stress that was explored in the context of our experimental studies.

5. Chronic Early Life Social Isolation (CELSI)

5.1. Early life adversity

The period from birth to early adolescence is a very crucial phase in an organism's development. In mammals, the early life phase is a time of rapid neural development [223]. A combination of genetic and environmental factors within this phase, govern an organism's ability to relate with the surrounding world. In fact, a substantial body of evidence shows that stress during the developmental phase can lead to negative health and behavioral outcomes in adulthood [223]. Felitti *et al*., reported a strong correlation between the extent of exposure to childhood abuse and many health-related risk factors in adulthood [224]. For example, this study showed that people who had experienced four or more categories of childhood abuse, compared to those who had experienced none, had 4- to 12-fold increased health risks for alcohol use disorder, drug abuse, depression, and suicide [224]. Studies have also shown that stress in the first few years of life can impact an individual's growth, intelligence, performance in skilled motor tasks and physical activity engagement [225]. Furthermore, a correlation has been established between childhood traumatic events and coronary artery disease, chronic pulmonary disease, cancer, and mental health problems [226]. Consistent evidence in the literature also suggests a link between early life adversity and later life onset of central sensitivity syndromes such as fibromyalgia or chronic widespread pain, headache or migraine, irritable bowel syndrome, temporomandibular join disorder, interstitial cystitis, endometriosis/vulvodynia and chronic pelvic pain [227]. This suggests that the impact of early life stress (ELS) is far reaching.

One of the most pronounced outcomes of ELS is depression, or the increased risk of depression. A study showed that women with exposure to one or more childhood adversities, such as family violence, parent psychopathology or alcoholism, had a higher likelihood of developing

depression. The women in this study were also shown to have an increased probability for the onset of depression following exposure to lower stress levels, compared to women who had not experienced such adversity [228]. A similar clinical study showed that depressed participants report a significant amount of ELS compared to controls; in fact, 62.5% of individuals with major depressive disorder (MDD) reported two, or more traumatic early life events compared to 28.4% of controls. This early life trauma was shown to be related to experiences of interpersonal violence such as emotional, sexual and physical abuse [229].

Overall, early life adversity has been shown to have detrimental effects in the human population, and many of these findings have been replicated in rodents using various models of early life adversity. Though the mechanisms underlying the impact of ELS have not been fully elucidated, various studies have traced these changes to molecular alterations at the genetic/epigenetic, protein or neurotransmitter level. These changes govern the observed behavioral differences and increased susceptibility to neuropsychopathologies [230]. For example, Wearick-Silva *et al.*, showed that ELS can induce changes in Dopamine receptor D1 (Drd1) and Dopamine Receptor D2 (Drd2), BDNF exon IV and TrkB receptor gene expression in key brain regions, namely, the mPFC, hippocampus, cerebellum and motor cortex [231]. ELS has also been shown to induce early onset cognitive decline or Alzheimer's disease, and it was shown that blocking glucocorticoid receptors rescued from this onset, which underlies the role of glucocorticoid receptors in stress related pathologies [232]. ELS has been shown to increase methylation in the NGF-1 gene, which regulates glucocorticoid receptor expression. Given that glucocorticoid activation regulates the negative feedback loop of HPA axis activation, attenuated GR expression results in less negative feedback, which in turn results in dysregulation of the HPA axis and increased levels of cortisol, CRH and ACTH [233]. Additionally, glucocorticoid receptors

have a low affinity for cortisol, so this binding is mainly facilitated under high levels of stress, which spike cortisol, therefore resulting in the compensatory downregulation of glucocorticoid receptors [97]. Therefore, as explained above reduced GR expression in the hippocampus would lead to a prolonged stress response as well as an increase in its magnitude. As a result, individuals with attenuated glucocorticoids have increased sensitivity to given stressors.

5.2. Chronic Early Life Social Isolation (CELSI)

Exposing mammals to social isolation and other adverse events in early life has been shown to have a significant impact on brain development and adult behavior [223]. Though the physiological and molecular mechanism(s) underlying these outcomes have not yet been fully elucidated, it is important to understand the capacity of chronic early life stress to alter the neurochemistry of the developing brain and explore the mechanism by which it does so.

In rodent models, pre- and post- weaning social isolation has been shown to induce behavioral changes, for example, increased locomotor activity in response to novel environments, increased responsiveness to psychostimulants and altered sensorimotor gating of the acoustic startle response [234, 235, 236]. Social isolation has also been reported to induce changes in the density and function of a variety of receptor-neurotransmitter systems in the brain [236, 237], subsequent sections will elaborate on some of these systems.

5.3. Behavioral changes in socially isolated rats

The behavioral changes observed in socially isolated rats, correlate to the behavioral phenotypes seen in human neuropsychological disorders, such as schizophrenia and depression. Previous studies reveal that socially isolated rats do not habituate normally when placed in a new environment, these rats exhibit motor hyperactivity and spend less time resting, compared with the group housed controls [223]. Furthermore, an abnormal expression of immediate early genes and

genes regulating cell differentiation and apoptosis have been observed in the pre-frontal cortex of these rats [238] which implies that abnormal PFC activity may be involved in this behavioural alteration. These behaviors are characteristic of the positive symptoms of schizophrenia and/or the 'anxiety' accompanying depression [239].

Pre-pulse inhibition (PPI) is a phenomenon in which a pre-stimulus, with a weaker signal, inhibits the response to a stronger signal. There is a strong co-relation in the neurobiology of PPI in humans and rodents [223]. PPI impairments likely reflect stimulus overload-induced cognitive fragmentation, and are characteristic in psychiatric disorders such as depression and schizophrenia [223]. Studies have shown that PPI impairments induced by social isolation can be reversed by, M100907 (selective 5-HT_{2A} receptor antagonist) in rats, and MKC-242/ozemozotan (5-HT_{1A} agonist) in mice [240].

5.4. ELS and TrkB/BDNF

As mentioned, brain-derived neurotrophic factor (BDNF) activates the TrkB receptor, and is involved in the regulation of proliferation, differentiation and survival of neuronal and non-neuronal cells in the CNS [223]. The neurotrophic factor theory of depression speculates that a decrease in BDNF is correlated with depression. In fact, BDNF has been shown to have antidepressant properties [241]. A study by Russo-Neustadt *et al*., showed that BDNF mRNA is up-regulated with chronic antidepressant treatment [242, 243]. These findings imply that chronic stress, or circumstances that have the capacity to induce relative levels of depression, will potentially affect the expression of BDNF and TRKB receptor in the brain.

In a similar trend, post-weaning social isolation has been reported to induce a decrease in BDNF levels in rats, suggesting that social isolation may induce depressive behaviors [260]. Further, in rats, stressful experiences such as immobilization have the capacity to reduce BDNF

mRNA expression up to 75% in the hippocampus [244]. However, re-exposure to cues previously associated with pain, for instance foot shock, reduces BDNF mRNA expression by about 21% [245]. A study conducted by Barrientos *et al*. observed that social isolation for 6 h was sufficient to cause a drastic decrease in BDNF mRNA in the dorsal and ventral dentate gyrus (60–80%), as well as the CA3 region (about 50%) of the hippocampus[244]. Additionally, we have shown that the activation of 5-HT7 receptors increases TrkB receptor expression. This observation may in part explain the ability of antidepressants (which prevent 5-HT reuptake) to promote TrkB/BDNF signalling [81].

5.5. ELS and 5-HT

5-hydroxytryptamine 5-HT is involved in excitatory and inhibitory neurotransmission [246]. Early life social isolation has been reported to alter the expression of 5-HT receptor in various regions of the brain. A study by Bibancos *et al*. showed that the gene expression of 5-HT receptor subtypes, 5-HT(1A), 5-HT(1B), 5-HT(2A), 5-HT(2C), 5-HT(3A), 5-HT(6) and 5-HT(7), were reduced in the pre-frontal cortex; 5-HT(1B), 5-HT(2A) and 5-HT(2C) were also shown to be reduced in both hypothalamus and midbrain. In contrast, the only alteration in the hippocampus was 5-HTR (6) overexpression [247]. A study by Preece *et al.*, reported an increase in 5-HT2A binding in prelimbic, motor and cingulate cortices. They also reported reduced 5-HT1A binding in the prelimbic cortex but increased binding in the hippocampus [248]. A study by Muchimapura *et al.,* showed that social isolation alters presynaptic 5-HT1B not the post-synaptic hippocampal 5-HT1A receptors [249]. These findings suggest that 5-HT receptor expression can be altered by social isolation.

5.6. ELS and NMDA

The N-methyl-D-aspartate (NMDA) receptor is a glutamate receptor that is implicated in both normal and pathological functions in the brain [250]. The NMDA receptor is a major regulator of neuroplasticity, playing a central role in functions like learning and memory, however, excessive activation of the NMDA receptor is associated with the pathophysiology of CNS injury syndromes like hypoxia-ischemia and as well as illnesses like schizophrenia [251, 252]. The NMDA receptor is a tetramer, comprising of the obligatory GluN1 subunits and the modulatory GluN2 (A-D) or GluN3 (A and B) subunits [253]. Various forms of stress, including ELS, can modulate glutamate levels, thus making the NMDA receptor a relevant target to explore [254]. Acute stressors have been shown to induce an increase in NMDA surface expression. Further, a study conducted by Turnock *et al*. reported a 1.26-fold increase in GluN2A transcripts in the prefrontal cortex of socially isolated rats [255]. Additionally, Zhao *et al*., showed up-regulation of GluN2A and GluN2B receptors in the hippocampus in socially isolated animals. These studies highlight the impact of stress on NMDA expression [256]

This evidence from NMDA, 5-HT, TrKB and BDNF studies show that the stress induced by social isolation can trigger changes in the neurochemistry of the brain, which could lead to adverse outcomes like depression and schizophrenia. Social isolation is a form of chronic stress; therefore, one can deduce from these findings that chronic stress has the capacity to induce changes in the brain during development. We seek to analyze the effect of social isolation on the expression of TrkB, BDNF, and the GluN2B subunit of the NMDA receptor. Observing and analyzing the changes that occur in these major signalling pathways will lead to a better understanding of the neurochemical changes that occur when a developing brain is exposed to chronic stress.

The next section, chapter 2, focuses on the characterization of transactivation in HT22 cells and *ex-vivo* brain slices, thus laying a foundation for further transactivation and stress studies. Chapter 3 explores the impact of CELSI on the expression of plasticity related proteins, namely, the TrkB receptor and the GluN2B subunit of the NMDA receptor, thus laying a foundation for further studies in which I explore the impact of various treatments on receptor expression in CELSI slices. Finally, chapter 4 explores the impact of pharmacologic manipulation of 5-HT7 and glucocorticoid receptors using LP12, CORT and CORT+LP12 treatment on TrkB-Y816 receptor activation, as well as TrkB receptor and GluN2B receptor expression. The work in chapter 4 allowed me to explore key questions, for instance, does CELSI affect transactivation? In addition, are there sex- or region-specific differences in transactivation?

Chapter 2

Overview

The activation of RTKs by GPCRs that occurs in the transactivation pathway facilitates signal integration through connecting two independent signalling cascades [10]. Though the transactivation pathway has been subject to extensive research since its discovery 25 years ago, some questions remain unanswered. For example, the exact *in vivo* role, or physiological relevance of this pathway remains to be elucidated [3, 10]. Despite these gaps in understanding, this pathway has garnered a great deal of interest because it has therapeutic potential, which is largely related to its capacity to induce RTK activation. The activation of RTKs is neuroprotective against a variety of neuronal insults [257]. However, the large size of RTK ligands limits their ability to cross the blood-brain-barrier and poses a challenge to harnessing this neuroprotective potential *in vivo*. As a result, transactivation offers a potential means to bypass this challenge and harness the neuroprotective potential of RTKs.

2.0 Introduction

The transactivation pathway has been most extensively studied in cell lines, and most predominantly in HEK293 cells. Recently, the Beazely lab demonstrated crosstalk between 5HT and PDGF receptors, as well as 5HT and TrkB receptors, in the SH-SY5Y cell line [56, 76, 81, 82]. Characterizing the above-mentioned pathway in the SH-SY5Y cell line provided a platform to transition these studies to more physiologically relevant models, such as, the HT22 cell line and *ex vivo* slices.

The HT22 cell-line is a murine-derived hippocampal cell line, which, due to its tissue origin, is a physiologically relevant model to explore neuronal processes such as transactivation [258]. Cell lines in general are robust models to explore signalling pathways, due to their relative

ease of maintenance, their rapid growth/turnover and the relative cost-effectiveness [259]. However, in these *in vitro* models, many environmental factors are highly controlled (e.g. temperature, humidity, PH, media composition). As such, there is some deviation from the dynamic nature of an *in vivo* environment, which can limit the degree of translation or replicability between *in vitro* and *ex vivo* studies. Additionally, receptor expression may differ between cell lines and animal, or human tissue [259]. Therefore, to gain a greater understanding of how the current *in vitro* findings relate to *in vivo* realities, the decision was made to study transactivation using *ex vivo* brain slices.

The overall aim of this thesis is to explore the relationship between transactivation and stress, and specifically, exploring the impact of psychosocial and pharmacological stressors on the transactivation pathway. In alignment with this overall aim, a sub-aim was to replicate transactivation both in cell lines and the *ex vivo* slice model reliably, in order to establish a viable model in which to explore the effect of stress. This chapter highlights foundational data exploring transactivation by comparing outcomes of different GPCR-RTK pairs. Additionally, the impact of a pharmacological stressor on RTK activation and/or expression was explored, and, lastly, the outcomes of combined transactivation and stress treatment on RTK activation was evaluated. Through the characterization of various GPCR- RTK transactivation partners, this study was an instrumental building block in addressing the overall aim of this thesis.

2.1. Study objectives

2.1.1 Replication of transactivation

The primary aim of this study was to replicate transactivation (increased RTK phosphorylation relative to control) reliably in the HT-22 cell line and in *ex vivo* brain slices. To achieve this aim, many GPCR agonist-RTK partners were used to screen for TrkB and PDGF receptor activation: 5-HT hydrochloride (general serotonin receptor agonist), 8-OH-DPAT (full 5-HT1A receptor agonist/partial 5-HT7 receptor agonist), LP12 hydrochloride (selective 5-HT7 receptor agonist), and quinpirole (Dopamine 2/3 receptor agonist). The main objective was to identify the most reliable GPCR-RTK candidate to carry forward into transactivation-stress studies, as well as to identity the best model (cell line vs. *ex vivo* slices) to proceed with.

2.1.2 Exploring pharmacological stress effects on RTKs

Given that the secondary goal of this study was to provide preliminary evidence for the impact of stress on transactivation, the second aim was to explore in naïve (non-stressed) brain tissue, how corticosterone (CORT), a pharmacological stressor, affected RTK activation. In simpler terms, does this pharmacological stressor affect baseline RTK activation?

2.1.3 Combined treatment (transactivating GPCR agonist and pharmacological stressor)

After achieving the above aims, the third aim was to explore the outcomes of a combined treatment of a transactivating agonist and corticosterone. The rationale being: (i) if the transactivating agonist induces transactivation and (ii) if CORT has an effect on RTK activation as well, what is the combined outcome?

2.1.4 Region differences

Brain slices from different regions: the hippocampus (HP) and prefrontal cortex (PFC) were used to explore if there were region specific differences.

2.1.5 Corroborating proteins (Src and Erk activation)

If RTK transactivation was observed, the activation of proteins and second messengers implicated in RTK transactivation as well as downstream signaling would also be expected. Using that line of reasoning, the final aim was to measure the activation of protein messengers upstream, or downstream of the RTK transactivation.

2.2. Hypotheses

In accordance with the aims, the following was hypothesized:

1. Based on previous findings with SH-SY5Y cells, I expected that the treatment of *ex vivo* brain slice tissue and/or HT-22 cells with GPCR agonists (5-HT hydrochloride (5-HT), LP12 hydrochloride (LP12), quinpirole and 8-OH-DPAT) would result in transactivation of TrkB and PDGF receptors, measured by an increase in tyrosine phosphorylation above baseline.
2. Based on various reports in the literature that showed that treatment of cells with CORT can induce RTK activation through unknown mechanisms [97], I anticipated that CORT treatment would increase RTK activation above baseline.
3. Based on the previously stated hypotheses that I would: (i) observe transactivation and (ii) that CORT would induce an increase in phosphorylation greater than baseline, I anticipated that the combined treatment with a transactivating GPCR agonist and CORT would result in either additive or synergistic effects.

4. The HP and PFC are differentially innervated and have different levels of receptor expression, among other differing factors, to support their respective functions [260]. Given that, I expected that the transactivation, or CORT-induced outcomes observed in these two regions would not present an identical profile, but would differ either in direction, or in magnitude.
5. In light of the classical mechanisms of receptor activation and signal transduction, I anticipated that if I observe transactivation, I would also observe the activation of proteins that activate, or are activated down-stream of, the RTK.

2.3 Materials and methods

2.3.1 Cell culture and western blot

HT22 cells were cultured in DMEM and HAM's F12 (1:1) (Fisher #SH20361), 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2, and media was changed every 3-5 days. Cells were trypsinized with 0.25% trypsin/0.1% EDTA, and passages 1-12 were used. For drug treatments and experimentation, the cells were seeded in full growth media for 24 h and once they reached 80% confluency, they were differentiated in neurobasal media (Gibco, Life Technologies) containing N2 supplement and 2 mM L-glutamine for an additional 24 h. Drug treatments were performed in neurobasal media.

After drug treatment, cells were washed with phosphate-buffered saline (PBS) and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1% Triton X-100; supplemented with Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario) prior to use. Cells were scraped, sheared using 26 gauge needles, and

centrifuged at 14,000 × g for 20 min at 4°C. Supernatants were collected, and then frozen at -80°C until the point of analysis by immunoblotting (Western blotting).

2.3.2 Slice preparation

Male Sprague-Dawley rats were anesthetized with CO_2 inhalant and euthanized by decapitation at post-natal day (PND) 70. The HP and PFC were immediately extracted from the brain, and slices were generated as per standard procedure, then allowed to recover for 90 minutes in artificial ACSF that contained (in mM): 127.0 NaCl (Sigma, Oakville, ON, Canada; all subsequent reagents from Sigma, unless otherwise noted), 26.0 $NaHCO_3$, 10.0 glucose, 2.0 $CaCl_2$, 2.0 KCl, 2.0 $MgSO_4$, and 1.2 KH_2PO_4 and was equilibrated with carbogen (95% O_2/5% CO_2), pH 7.37–7.43. After the 90-minute recovery period, slices were transferred to chambers containing the respective drugs dissolved to the desired concentration in ACSF. After the treatment period was over, slices were homogenized in non-ionizing lysis buffer (containing 10 mM Tris, 25 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl, 1% [v/v] Triton X-100, and 1% [v/v] NP-40, pH 7.4; protease inhibitor cocktail and sodium orthovanadate added on day of experiment) over ice with a Potter-Elvehjem homogenizer, and then centrifuged at 1000 x g for 10 min at 4ºC. Supernatants were collected then frozen at -80 ºC until the point of analysis by immunoblotting (Western blotting).

2.3.3 Western blot

Western blots were performed, and total protein was measured using a BCA protein assay (Thermo). Samples were heated in 3x loading buffer (240 mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v glycerol, 0.02% w/v bromophenol blue, 50 mM DTT, and 5% v/v β-mercaptoethanol) for 15 min at 75°C and 20-40 μg total protein was loaded into polyacrylamide gel wells. Proteins were separated by SDS-PAGE using electrophoresis buffer (25 mM Tris base, 190 mM glycine, 3.5 mM

sodium dodecyl sulfate), followed by transfer of proteins to a nitrocellulose membrane by electroblotting with transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris base, 150 mM NaCl, pH 7.6) plus 0.1% Tween (TBS-T) for 1 h at room temperature, or overnight at 4°C, followed by incubation with primary antibody added to blocking buffer for 1 h at room temperature, or overnight at 4°C. Membranes were washed three times with TBS-T, and then incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) in blocking buffer for 1 h at room temperature. Membranes were washed three additional times with TBS-T. Western chemiluminescent substrate (Luminata Crescendo-Millipore) was used to visualize proteins on a Kodak 4000MM Pro Imaging Station. Densitometric analyses of images were performed using Kodak Molecular Imaging software. After imaging, membranes were stripped and re-probed with other appropriate antibodies. Molecular weights of analyzed proteins are as follows: PDGFR and PDGF-Y1021 at 180 kDa, TrkB-FL and TrkB-Y816 at 145 kDa and bands were normalized to the loading control, β-actin at 42 kDA. Membranes were probed with the primary antibodies against, TrkB (Millipore; 1:1000 (rabbit)), pTrk816 (Millipore; 1:500 (rabbit)), PDGFRβ (Santa Cruz; 1:1000(rabbit)), PDGFRβ-Y1021 (Santa Cruz; 1:1000(rabbit)), β-actin (Santa Cruz; 1:1000 (mouse)). Anti-mouse (1:10000) and anti-rabbit (1:5000) horseradish peroxidase (HRP) enzyme-conjugated IgG secondary antibodies were used.

2.3.4 Statistics

Given our sample size, a conservative approach was taken regarding the assumptions that could be made around normality; as a result, our data were analyzed using non-parametric statistical analyses in GraphPad Prism™ 6. Specifically, the Wilcoxon Signed Rank Test (the distribution free equivalent of the 1-sample t-test) was used for analyses. TrkB and PDGFβ receptor

transactivation data were presented as fold change in receptor phosphorylation (TrkB at phosphorylation site Y816 and PDGF at phosphorylation site Y1021) relative to control. The respective cumulative fold changes were statistically analyzed using the Wilcoxon signed rank test. All error bars represent the standard error of the mean. Additionally, any inter-group comparisons were conducted using the Mann-Whitney U Test (the distribution-free equivalent of the 2 sample Student's t-test) and the Bonferroni calculation was applied to protect against the type I error. The Bonferroni calculation includes adjusting the alpha value by dividing the original α value by the number of groups being compared "n". The Cohen's d, effect size calculation that measures the standardized difference between two means was used to determine the biological relevance of the data. The general formula for Cohen's d calculation is d= (M1-M2)/S pooled; where M1 and M2 represent the means of the two groups being compared and S pooled, represents the pooled standard deviation for the two groups. Cohen's d calculations were completed using an online calculator [261]. Cohen's d conventional values for a small effect size (d = 0.2), a moderate effect size (d = 0.5), and a large effect size (d = 0.8) were used for interpreting the data. Outliers were identified and removed from the data set using Tukey's method. Tukey's method was chosen since it is a non-parametric method, thus aligning with the general statistical approach of this chapter. With Tukey's method, the outlier value is compared with computed upper and lower limits, which are referred to as "fences" for exclusion. Determination of the fences involves calculating the interquartile range (IQR), which is the difference between the upper quartile (75th percentile) and the lower quartile (25th percentile), for the dataset. The lower fence is obtained by subtracting 1.5 times the IQR from the 25th percentile and the upper fence is obtained by adding 1.5 times the IQR to the 75th percentile. Any values that fall outside of the calculated

range are outliers and can be removed from the dataset [262]. Additionally, any data that were subject to experimental error were also excluded.

Threshold p values are arbitrary, but are used as probabilities or estimates to determine whether the null hypothesis can be rejected. Typical p values range from 0.01 (more stringent), 0.05 (standard) and 0.10 (less stringent) [263]. Practical factors (e.g., cost, study time-line) limited the number animals that were used for this study, which, in conjunction with the sensitivity of the readouts, resulted in the selection of a less stringent p value threshold, $\alpha = 0.1$. In addition to p values, effect size calculations were also provided a means to determine the potential biological relevance of the given outcomes. Additionally, effect size calculations served as a viable secondary measure to determine the acceptance, or rejection of the null hypothesis.

2.3.5 Data overview

In order to determine transactivation (the fold change in phosphorylation relative to control), control and treatment samples were compared. Specifically, treatment data was divided by the control data to establish the fold change. The Western blot procedure is a semi-quantitative measure, which yields arbitrary densitometry values that correlate to the intensity of a given band, and therefore the relative abundance of a given protein. In this thesis, all control values appear as 1, as they are the baseline upon which a fold change is evaluated. Generally, the fold change in phosphorylation observed in transactivation is fairly modest on averaging ranging from 1.3 to about a 3 fold increase relative to control [81, 82].

2.4 Results

We have described transactivation as either acute (observed at a time-point shorter than 60 min), or chronic (observed at a time-point greater than 60 min). Importantly, transactivation is a transient phenomenon, meaning that, at a given time after drug application, signal (RTK phosphorylation)

increases from baseline, escalates to a peak, and then returns back to baseline, as the tyrosine is dephosphorylated. Therefore, the timing of the readout is important, as it is possible to miss the phenomenon entirely if an observation point is too soon, or too late. For example, in SH-SY5Y cells, peak phosphorylation for the PDGFβ receptor is observed at 5 min and returns to baseline by 15 min, whilst TrkB phosphorylation is observed at 2 h [56, 81]. Given these factors, a challenge associated with characterizing transactivation is determining the appropriate agonist concentration and treatment time that would enable one to capture the phenomenon near its peak. As a result, our characterization experiments were conducted at various agonist concentrations and treatment times. The two RTKs that we chose to evaluate transactivation were the PDGFβ and TrkB receptors, at the PDGFβ-Y1021 and TrkB-Y816 sites respectively, as these have been used previously as a proxy for RTK activation [56, 76, 82]. The Table below summarises a set of transactivation characterization experiments that were conducted over a period of 18 months.

2.4.1 *Ex vivo* slice studies

Drug	Agonist action	Region	Concentration	Time (min)	Phospho-site	Direction of effect	n	p value	d value
5-HT	5-HT receptors (5-HTRs)	HP	100 nM	5	PDGF-Y1021	=	7	0.57	0.44
		HP	100 nM	60	PDGF-Y1021	↑	3	1.00	0.61
		HP	100 µM	5	PDGF-Y1021	=	3	0.38	
LP12	5-HT7 receptor	HP	300 nM	5	PDGF-Y1021	↑	3	0.16	1.22
		HP	300 nM	45	PDGF-Y1021	↑	3	0.06 *	1.77
quinpirole (Q)	Dopamine 2/3 receptor	HP	10 µM	10	PDGF-Y1021	↑	5	0.04 **	1.07
		HP	10 µM	45	PDGF-Y1021	↑	3	0.38	0.96
8-0H-DPAT	5-HT 1-full & 5-HT7-partial agonist	HP	30 µM	5	TrkB-Y816	=	4	0.88	0.10
		HP	30 µM	15	TrkB-Y816	↑	4	0.38	0.95
		HP	30 µM	60	TrkB-Y816	↑	4	0.88	0.51
		PFC	30 µM	5	TrkB-Y816	↑	4	0.38	0.54
		PFC	30 µM	15	TrkB-Y816	↓	3	1.00	0.24
		PFC	30 µM	60	TrkB-Y816	↑	3	0.50	1.31
LP 12 (L)+ quinpirole (Q)	5-HT7 and dopamine 2/3 receptors	HP	L-300 nM Q- 10 µM	L- 45 Q-10	PDGF-Y1021	=	3	0.56	0.64
quinpirole (Q)+ 5-HT	Dopamine 2/3 and 5-HT receptors	HP	Q-10 µM 5-HT-100 µM	Q- 10 5-HT-5	PDGF-Y1021	↑	3	0.13	1.86

Table 3. A summary of outcomes of transactivation characterization experiments conducted in *ex vivo* slices. Please note ↑ = increase ↓ = decrease and "=" equals no notable change in phosphorylation relative to control; and * /** indicate statistical significance.

The preliminary data (Table 3) reflect that transactivation outcomes differ based on GPCR agonist and treatment time. We observed the following:

2.4.1.1 5-HT receptor activation (treatment: 5HT, LP12, 8-OH-DPAT)

Acute, 5 min treatment with 100 nM and 100 µM 5-HT in hippocampus *ex vivo* slices did not induce a notable change in PDGF-Y1021 receptor phosphorylation (transactivation). A longer treatment (1 h) showed a trend towards increased PDGF-Y1021 phosphorylation, but there was considerable variability in this outcome. We observed that the acute treatment of hippocampal slices with LP12 for 45 min increased PDGF-Y1021 phosphorylation, additionally, the low p value and high d value ($p = 0.06$, $d = 1.77$) suggest an effect of treatment. The treatment of hippocampal slices with 8-OH-DPAT for 5 min, 15 min and 60 min showed a trend towards an increase in TrkB-Y816 phosphorylation and this trend increased with treatment time. However, there was no statistical significance likely due to the variability in the data. The same treatment was conducted in PFC, and at 5 min and 60 min, we observed a trend towards an increase in TrkB-Y816 phosphorylation, whilst at 15 min the trend suggested a relative decrease in TrkB-Y816 phosphorylation. However, these outcomes were not statistically significant.

2.4.1.2 Dopamine receptor activation

Hippocampal treatment with quinpirole for 10 min resulted in PDGF transactivation and these findings were statistically significant. The findings with quinpirole for 45 min, showed a slight trend towards an increase in PDGF-Y1021 phosphorylation, but there was no significant difference. Given that a significant increase in PDGF-Y1021 phosphorylation was observed after a 45 min LP12 treatment and after a 10 min quinpirole treatment respectively, I sought to explore if the combined treatment would be additive and synergistic, and what I observed was neither; in fact, there was not much change. Additionally, I sought to explore the effect of a combined

treatment of quinpirole 10 min and 5-HT for 5 min and what I observed was a greater fold change than LP12+ quinpirole, but the difference was not statistically significant.

In summary, the findings show that LP12 (45 min) and quinpirole (10 min) transactivate *ex vivo* tissue in a statistically significant manner. The trends observed with the combined treatments suggest that the combined treatment have higher activation than individual treatments alone, but these outcomes were not additive or synergistic. The following sections highlight the data upon which we based future studies.

2.4.2. 5-HT7 activation of TrkB, Src, Erk

Based on preliminary studies, *ex vivo* HP and PFC slices were treated with 300 nM LP12 for 2 h. We blotted for TrkB and pTrkB, Src and pSrc, Erk and pErk to evaluate the impact of the treatment on the phosphorylation (activation) of these proteins.

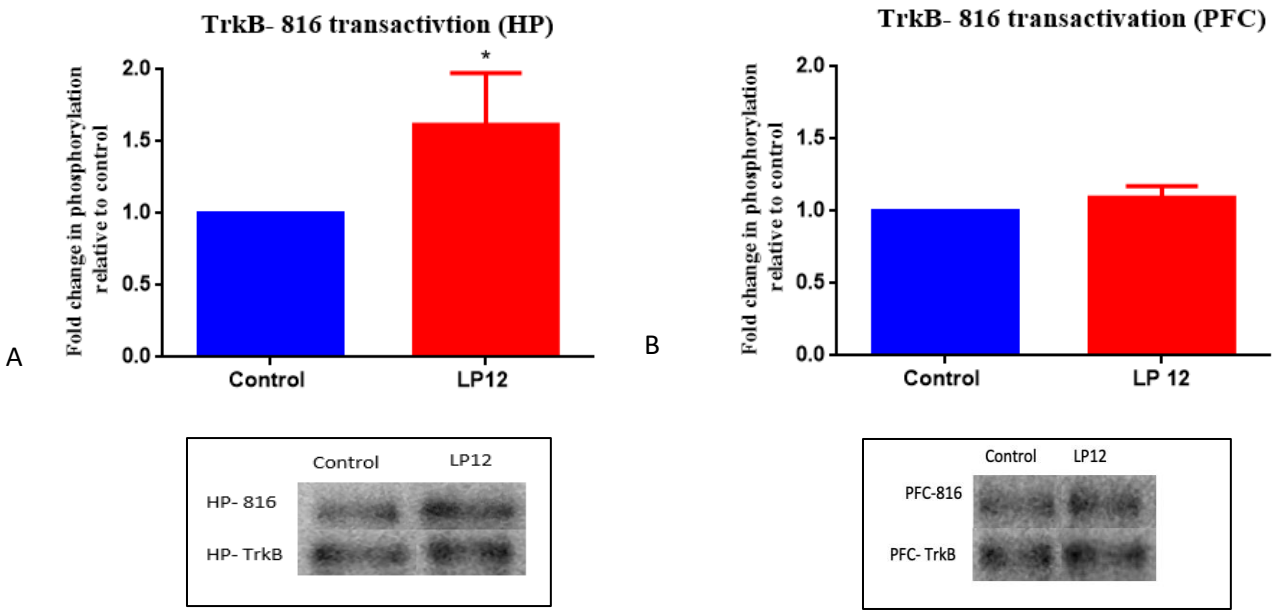


Figure 4. A) HP and B) PFC slices were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-816 immunoreactivity (* = $p < 0.1$; Wilcoxon Signed Rank Test compared to non-treated control. **LP12-HP**: n = 6, p = 0.063, d = 0.99; **LP12-PFC**: n = 6, p = 0.44, d = 0.66)

Our findings suggest that LP12 induces transactivation in the HP slices (p = 0.063, d = 0.99), but not in the PFC slices (p = 0.44, d = 0.66); these data highlight a regional difference in transactivation by LP12.

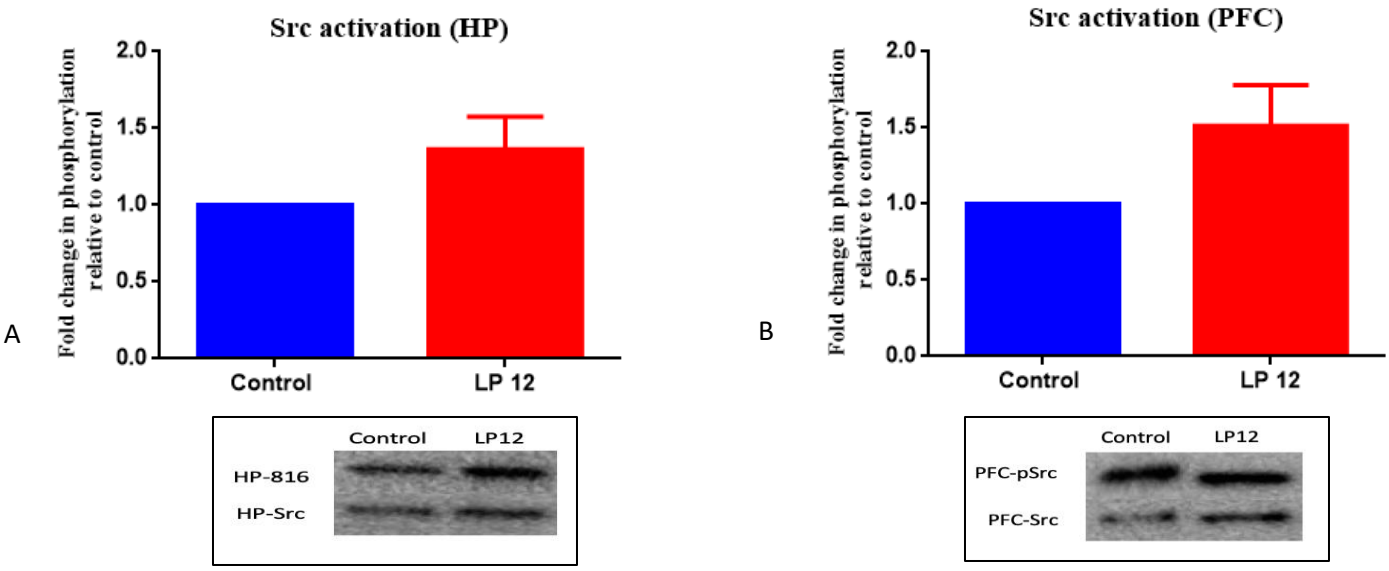


Figure 5. A) HP and B) PFC slices were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenized and samples evaluated by Western blots as described in the methods. The phospho-Src data were normalized to total Src expression and are expressed as the fold change in phospho-Src immunoreactivity (Wilcoxon Signed Rank Test compared to non-treated control. **Src-HP**: n = 5, p = 0.31, d = 1.08; **Src-PFC**: n = 5, p = 0.19, d = 1.20)

Our findings suggest that LP12 treatment leads to an increase in Src phosphorylation in both the HP (p = 0.31, d = 1.08) and the PFC (p = 0.19, d = 1.20) slices. Although these data did not reach the threshold for statistical significance, both the HP and PFC displayed large effect sizes, suggesting that both these outcomes may have biological relevance.

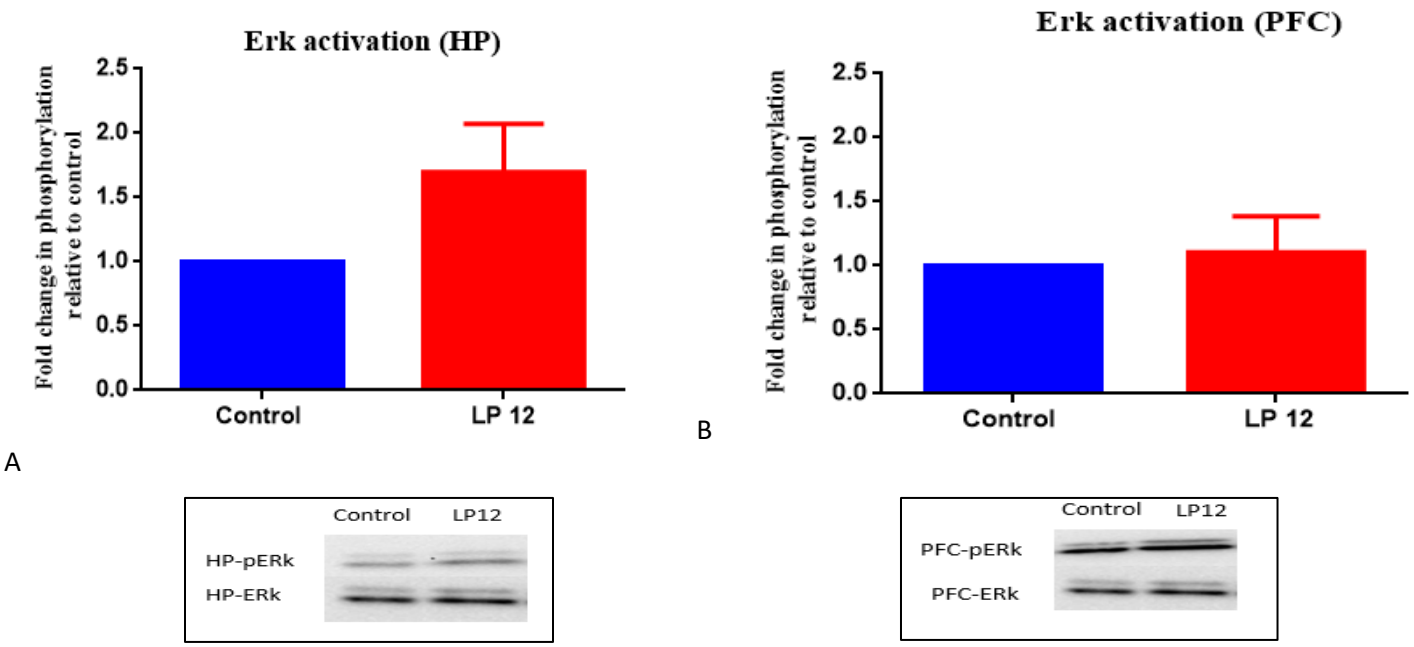


Figure 6. A) HP and B) PFC slices were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. The phospho-Erk data were normalized to total Erk 1/2 expression and are expressed as the fold change in phospho-Erk immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **Erk-HP**: n = 3, p = 0.25, d = 1.52; **Erk-PFC**: n = 4, p = 0.88, d = 0.27)

Our findings suggest that LP12 treatment leads to an increase in Erk phosphorylation in the HP (p = 0.25, d = 1.52) but not the PFC (p = 0.88, d = 0.27). Additionally, the effect size is very large in the HP, but small in the PFC, suggesting that these outcomes may have a biological relevance in the HP but not the PFC.

2.4.5. Corticosterone activation of TrkB

To evaluate the impact of CORT on TrkB, Src and Erk phosphorylation (activation), *ex vivo* brain slices were treated with 300 nM corticosterone for 1 h.

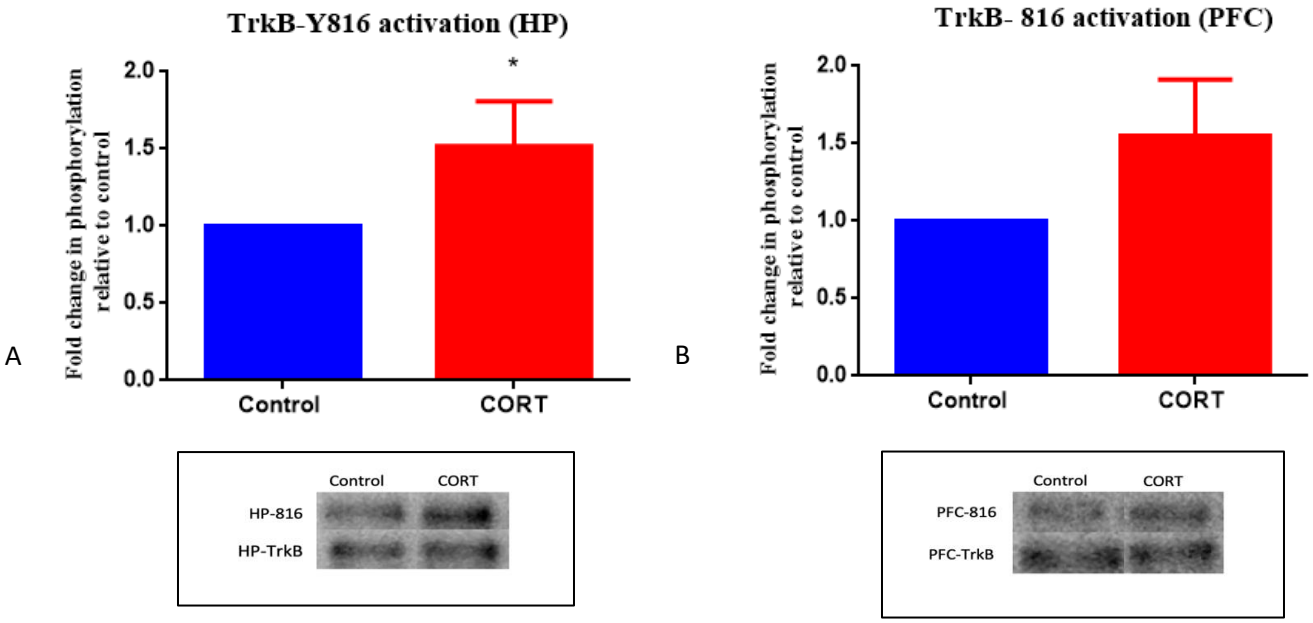


Figure 7. A) HP and B) PFC slices were treated with 300nM CORT for 1 hour. Following treatment, the slices were lysed and lysates were evaluated by western blots as described in methods. Data were normalized to total TrkB expression and are expressed as the fold change in phospho- 816 immunoreactivity (*= $p < 0.1$ (Wilcoxon Signed Rank Test) compared to non-treated control, **LP12- HP**: n = 6, p = 0.094, d = 1.03; **LP12-PFC**: n = 6, p = 0.16, d = 0.87)

Our findings suggest that CORT increases TrkB-Y816 phosphorylation in both the HP (p = 0.094, d = 1.03) and PFC (p = 0.16, d = 0.87). Even though the outcome is only statistically significant in the HP, both regions have large effect sizes, suggesting the biological relevance of these findings in both brain regions.

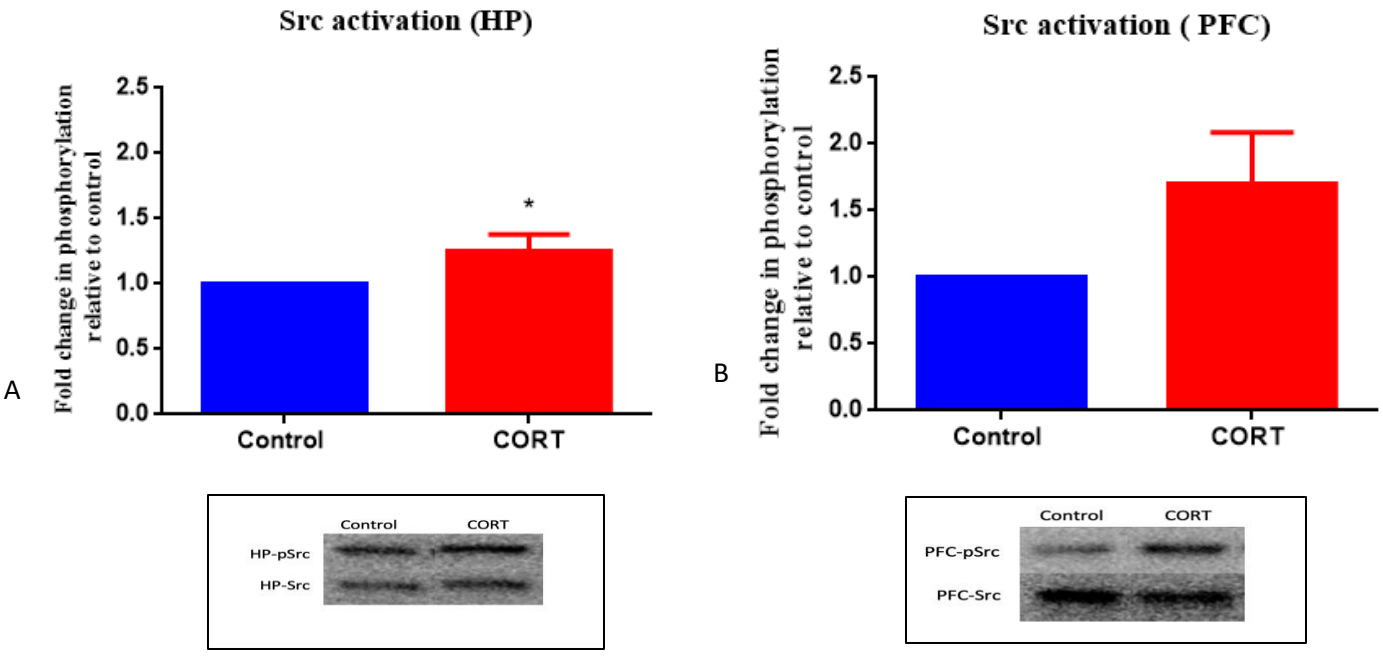


Figure 8. A) HP and B) PFC slices were treated with 300nM CORT for 1 h. Following treatment, the slices were lysed and lysates were evaluated by western blots as described in methods. Data were normalized to total Src expression and are expressed as the fold change in phospho- Src immunoreactivity. (*=p< 0.1 (Wilcoxon Signed Rank Test) compared to non-treated control, **Src-HP**: n = 5, p = 0.063, d = 1.21; **Src-PFC** n = 5, p = 0.13, d = 1.15)

Our findings suggest that corticosterone increases Src phosphorylation. Though the findings are only statistically significant in the HP (p = 0.063, d = 1.21) and not the PFC (p= 0.13, d= 1.15), the p value for the PFC, is close to the threshold for statistical significance and both the HP and PFC exhibit large effect sizes, suggesting that CORT causes a biologically relevant change in both regions.

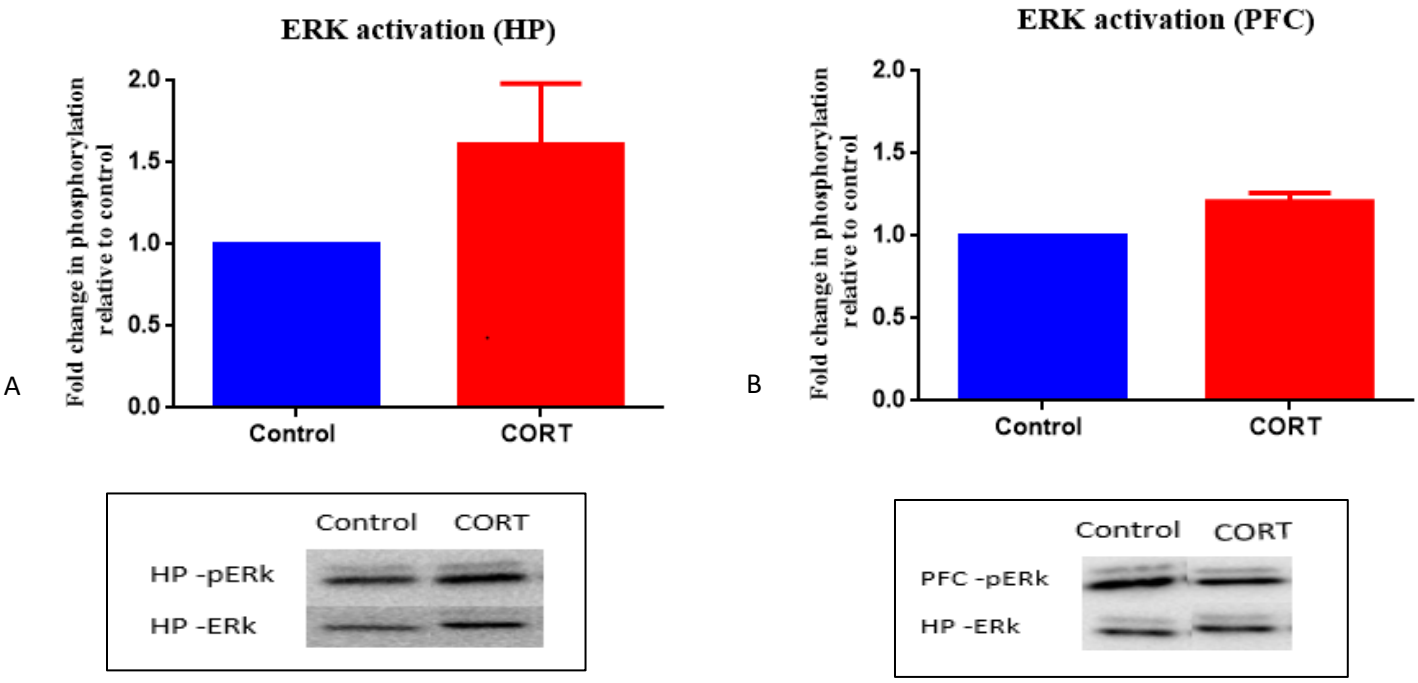


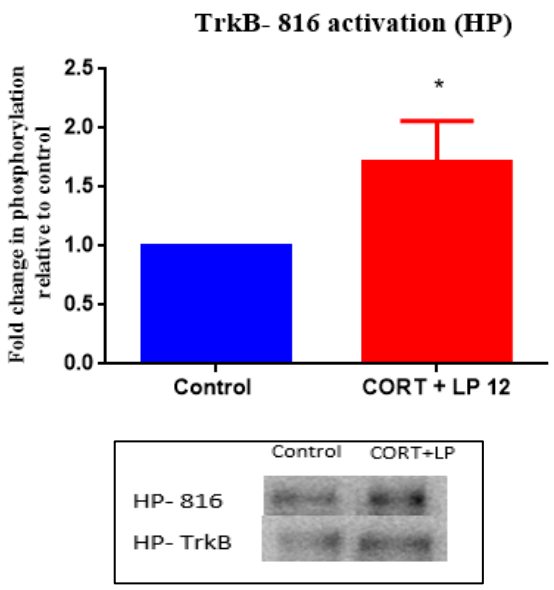
Figure 9. A) HP and B) PFC slices were treated with 300nM CORT for 1 h. Following treatment, the slices were lysed and lysates were evaluated by western blots as described in methods. Data were normalized to total TrkB expression and are expressed as the fold change in phospho-816 immunoreactivity. (*=p< 0.1 (Wilcoxon Signed Rank Test) compared to non-treated control, **Erk-HP:** n = 3, p = 0.25, d = 1.34; **ERK-PFC:** n = 4, p = 0.13, d = 2.78).

Our findings show that corticosterone may increase Erk phosphorylation, though the outcomes are not statistically significant, the combination of relatively low p values and very high d values in the HP (p = 0.25, d = 1.34) and PFC (p = 0.13, d = 2.78), suggest that these outcomes may be biologically relevant.

2.4.6. Combined- CORT+LP12 activation of TrkB

To evaluate the impact of a combined CORT and LP12 treatment on TrkB, Src and Erk phosphorylation (activation), *ex vivo* brain slices were treated with 300 nM LP12 for 2 h, then after LP12 treatment they were exposed to CORT for 1 h.

A



B

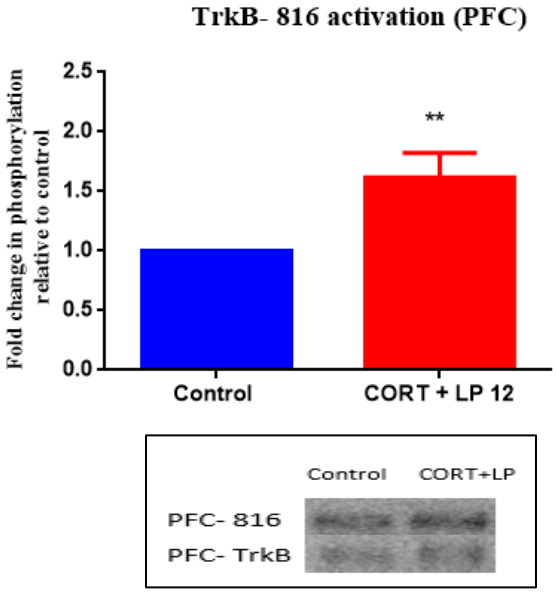


Figure 10. A) HP slices and B) PFC slices were treated with 300nM LP12 treatment for 2 hours followed by 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. The phospho-TrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-816 immunoreactivity. (* = $p < 0.1$, ** = $p < 0.05$; (Wilcoxon Signed Rank Test compared to non-treated control) **CORT+LP12 HP**: n = 3, p = 0.063, d = 1.19, **CORT+LP12 PFC**: n = 4, p = 0.03, d = 1.75.

These findings suggest that the combined CORT+LP12 treatment increases TrkB-Y816 phosphorylation. Additionally, these outcomes are statistically significant in both the HP (p =

0.063, d = 1.19) and the PFC (p = 0.03, d = 1.75), and the effect sizes, are large in both regions, suggesting the biological relevance of these results.

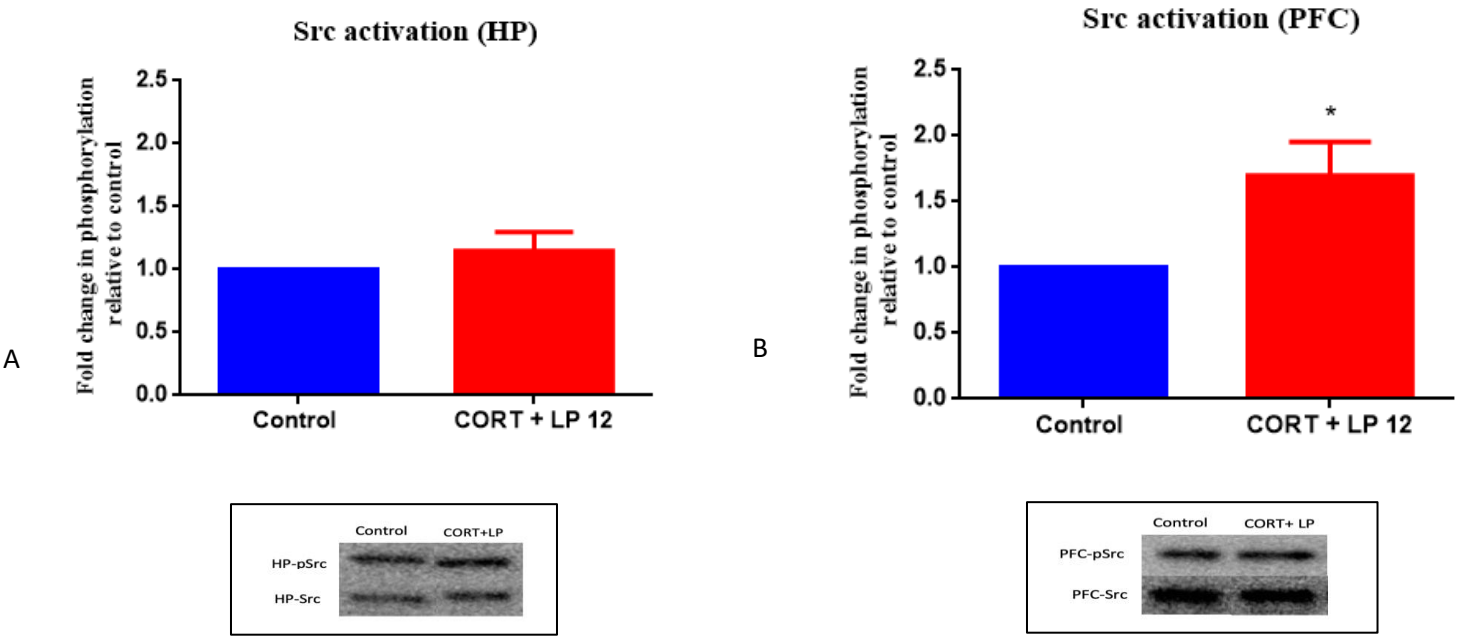
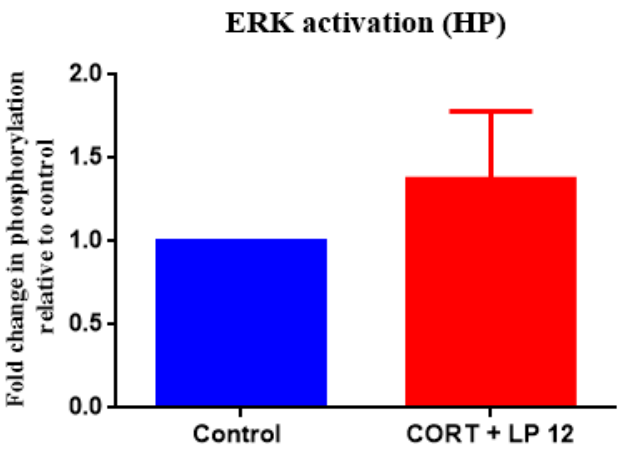


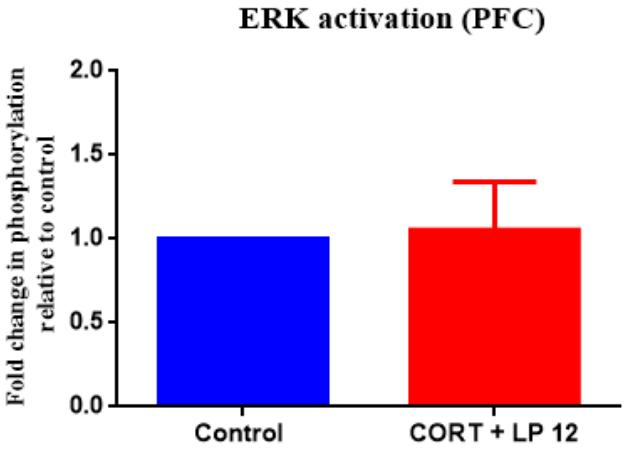
Figure 11. A) HP slices and B) PFC slices were treated with 300 nM LP12 for 2 h, then 300nM CORT for 1 h. Following treatment, the slices were homogenized and the samples were evaluated by Western blots as described in the methods. The phospo-Src data were normalized to total Src expression and are expressed as the fold change in phospho-Src immunoreactivity. (* = $p < 0.1$; Wilcoxon Signed Rank Test compared to non-treated control). **CORT+LP12 Src HP**: n = 5, p = 0.63, d = 0.61; **CORT+LP12 Src PFC:** n = 5, p = 0.063, d = 1.74).

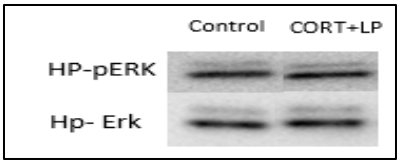
These findings suggest that the combined treatment of CORT+LP12 increases Src phosphorylation relative to baseline to a significant degree in the PFC (p= 0.063, d= 1.74), but not the HP (p= 0.63, d= 0.61).

A



B





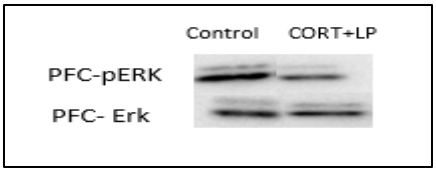


Figure 12. A) HP slices and B) PFC slices were treated with 300nM LP12 for 2 hours then 300nM CORT for 1 hour. Following treatment, the slices were lysed and lysates were evaluated by western blots as described in methods. Data were normalized to total TrkB expression and are expressed as the fold change in phospho- 816 immunoreactivity. (Wilcoxon Signed Rank Test) compared to non-treated control, **CORT+LP 12 HP:** n = 3, p = 0.5000, d = 0.74917; **CORT+LP12 PFC:** n = 4, p = 0.8750, d = 0.12340).

These findings suggest that the combined treatment of CORT+LP12 does not increase Erk phosphorylation in the HP (p = 0.50, d= 0.75), or the PFC (p= 0.88, d= 0.12).

2.4.7. Comparison of independent and combined treatments

We sought to evaluate if the combined CORT+LP12 treatment would exhibit additive or synergistic effects. Table 2 below shows a comparison (Mann-Whitney) of the LP12 OR CORT vs. CORT + LP12 treatments, to determine if there are significant differences between these conditions.

The statistical analyses below mainly show no difference between the independent and combined treatments, suggesting that there are neither additive nor synergistic effects at play.

Activation	Region	Comparisons	Mann-Whitney p values
TrkB-Y816	HP	LP12 vs. CORT+ LP12	0.79
	PFC	LP12 vs. CORT+ LP12	0.026 *
	HP	CORT vs. CORT+LP12	0.57
	PFC	CORT vs. CORT+LP12	0.57
Src	HP	LP12 vs. CORT+LP12	0.53
	PFC	LP12 vs. CORT+LP12	0.80
	HP	CORT vs. CORT+LP12	0.67
	PFC	CORT vs. CORT+LP12	0.67
Erk	HP	LP12 vs. CORT+LP12	0.90
	PFC	LP12 vs. CORT+LP12	0.66
	HP	CORT vs. CORT+LP12	0.90
	PFC	CORT vs. CORT+LP12	0.34

Table 4. A comparison of independent and combined treatments. * signifies a statistically significant difference between the combined treatments and independent treatments.

2.4.8 HT22 DATA

In addition to *ex vivo* slice work, studies were also conducted in HT22 cells; my goal was to characterize the transactivation profile in this new cell line. Given the complexity of characterizing transactivation in cell lines (establishing the effective time-frame and drug concentration), a conservative approach was chosen wherein I decided to study two receptors that were previously successful in activating transactivation in SH-SY5Y cells. Specifically, we studied 5HT1A receptors (activated by 8-OH-DPAT) and dopamine 2/3 receptors (activated by quinpirole); additionally, the concentrations chosen were based on previous SH-SY5Y work.

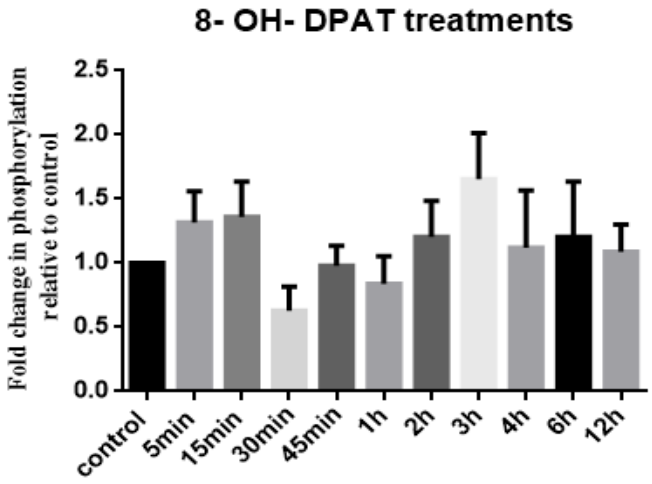


Figure 13. HT-22 cells were treated with 10 nM 8-OH-DPAT for the given times. Following treatment, the lysates were evaluated by Western blots as described in the methods. Data were normalized to total TrkB expression and are expressed as the fold change in phospho- 816 immunoreactivity. (8-OH-DPAT: **5 min**: n = 6, p = 0.22, d = 0.75 ; **15 min**: n = 6, p = 0.31, d = 0.75; **30 min**: n = 5, p = 0.13 d = 1.25 ; **45 min**: n = 5, p = 0.81 ,d = 0.085; **1 h**: n = 6, p = 0.56, d= 0.43 ; **2 h**: n = 9, p= 0.82, d = 0.35; **3 h:** n = 6,p = 0.16, d = 1.05 ; **4 h**: n = 3, p = 1.00, d = 0.22 ; **6 h**: n = 3,p = 0.75, d = 0.39 ; **12 h**: n = 3,p = 0.75, d= 0.34).

These data suggest that TrkB transactivation peaks at 3 h, but is relatively high at 5 min and 15 min. Though these data display great variability, they still point to the possibility that TrkB-Y816 can be transactivated by 5-HT1A/ 5-HT7 receptors in HT-22 cells. LP12 data (not shown) suggest that 5-HT7 can also activate TrkB receptors in HT-22 cells, however, the data display great variability making it difficult to define the exact outcome. Quinpirole data (below) reflect that 2 h, 3 h, 4 h and 6 h are the peak of TrkB-Y816 transactivation. These outcomes were statistically significant at the 2 h time point (p = 0.03, d = 1.22).

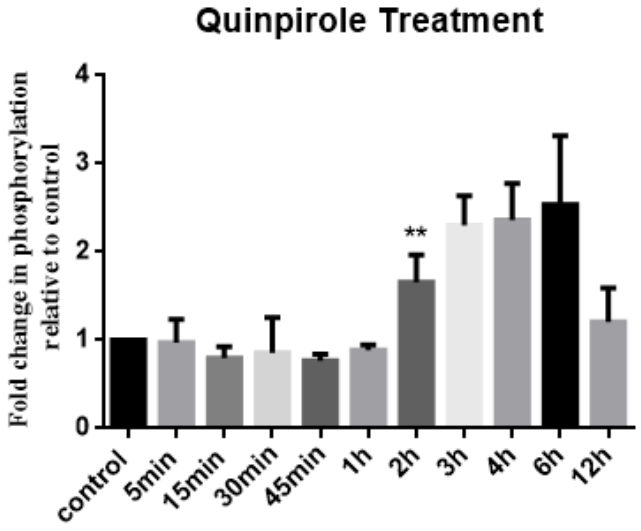


Figure 14. HT22 cells were treated with 10 μM quinpirole at given times. Following treatment, the lysates were evaluated by western blots as described in methods. Data were normalized to total TrkB expression and are expressed as the fold change in phospho-816 immunoreactivity. (* = p < 0.1, ** = p < 0.05; (Wilcoxon Signed Rank Test compared to non- treated control); quinpirole: **5 min**: n = 2 , d = 0.11; **15 min**: n = 2, d = 1.55; **30 min**: n = 2, d = 0.35; **45 min**: n = 2, d = 3.13; **1 h**: n = 3, p = 0.25 , d = 1.65; **2 h**: n = 6, p = 0.03, d= 1.22; **3 h**: n = 3, p = 0.25, d = 3.18; **4 h**: n = 4 , p = 0.13, d = 2.33; **6 h**: n = 4, p =0.25 , d = 1.39; **12 h**: n = 4, p = 0.63 , d = 0.37).

The peaks observed with 8-OH-DPAT (5 min, 15 min and 3 h) and quinpirole (3 h, 4 h and 6 h) reflect the time-points that require focus when further trying to characterize transactivation. The 2 h quinpirole treatment, shows that transactivation can be significantly observed in the HT22 cells, suggesting that further characterization (increase in sample size etc.) could lead to better outcomes at the observed peak times. Unfortunately, the timeline that would be required to fully characterize the HT22 cell line, as well as the clear data observed in slice, led to a switch in focus from HT-22 cells to *ex vivo* slice. However, future directions of this project will continue to explore transactivation in HT-22 cells.

2.5.0. Discussion

One of the aims of this chapter was to replicate transactivation in the *ex vivo* slice reliably, and this aim was achieved through testing various GPCR agonists at various concentrations and treatment times to determine the agonist providing the most reliable transactivation outcomes. Additionally, we sought to carry out a preliminary evaluation of transactivation in the HT-22 cell line. Lastly, we explored the impact of corticosterone (CORT) treatment, as well as a combined corticosterone and LP12 (CORT+LP12) treatment on slices as a means to evaluate the impact of the pharmacological stressor on TrkB-Y816 phosphorylation and to see how transactivation may be affected by stress.

Some of the preliminary data (not shown) exhibited greater variability than the later data. The preliminary data were generated using a relatively crude protocol in which brain slices were prepared manually. Given the variability in that data, steps were taken to refine our protocol to enable us to increase slice viability and increase the replicability of our data. Improving the protocol included the use of a McIlwain slicer to produce an exact and consistent thickness of slices instead of approximating brain slice thickness when manually cutting the slices.

Additionally, the increased precision enabled us to more clearly define an origin, or starting point, with every slice, which increased the consistency of tissue used across replicates. The refined protocol also included using interface perfusion chambers, rather than the submersion method, ultimately allowing for greater temperature and humidity control and arguably increased slice viability. However, when comparing the outcomes between the crude and refined protocols, we found that there was no difference in the magnitude of transactivation observed (the range remained between approximately1.3-3), however, we were able to decrease variability and replicate transactivation reliably. The increased reliability is likely due to the fact that with the refined protocol I was able to more accurately define the regions of study instead of crudely generating slices, and I was able to use the exact defined regions of the hippocampus and prefrontal cortex each time; additionally, we were able to control for slice thickness. Given the varying functions and receptor distribution along the axes of the HP and PFC, the ability to use specific regions enabled us to eliminate the inconsistent findings of the previous protocol.

We were successful in achieving our goal of replicating transactivation in *ex vivo* slices, as well as identifying transactivation in HT-22 cells. Interestingly, comparing the LP12 and CORT profiles of TrkB-Y816 activation, it is evident that they share a similar profile; both increase activation of TrkB-Y816 relative to baseline. Studies have shown that corticosterone activates an unknown GPCR whose activation leads to the activation of multiple secondary messengers like PLC, PKA and Phosphoinositide (PI3K) [98]. Further, the activation of these secondary messengers leads to the activation of the GluN2B subunit of the NMDA receptor, non-receptor tyrosine kinases, like Src, as well as the activation of the protective MAPK pathway [98]. Additionally, these secondary messengers have been shown to play a role in the activation of

RTKs, and some of these mechanisms include the activation of Src; therefore, these findings provide a potential mechanism through which CORT could activate TrkB.

In addition to addressing how CORT could activate TrkB, it is equally as important to consider why. For example, TrkB activation could be a protective mechanism. The line of reasoning that a stress hormone activates TrkB-Y816 in a similar fashion as LP12 transactivation, the latter being known to have neuroprotective potential, lends to the question: could transactivation be an adaptive stress response? Though this is an intriguing consideration, the data in this chapter cannot address this question. Given that various stressors have themselves been shown to trigger transactivation [110, 111], a future direction of this research would be to directly explore the stress-transactivation connection to determine if transactivation is indeed a stress response. The outcomes of such work would be significant, and would point towards the physiological relevance of transactivation, which is currently not known.

To explore any additive, or synergistic effects, CORT+LP12 treatments were conducted. Concerning TrkB-Y816 phosphorylation, we found that CORT+LP12 did not significantly increase TrkB-Y816 phosphorylation compared to CORT alone, or LP12 alone, meaning that additive, or synergistic effects were not likely. The lack of a combined effect may point to the possibility that CORT and LP12 could be activating TrkB through a similar pathway. Another alternative explanation is that TrkB-Y816 activation saturates at the level I observed and no further phosphorylation is possible. To determine if they are indeed activating in the same pathway, an experiment that could be conducted would be to measure activation of elements upstream, and/or downstream of transactivation in CORT slices, and indeed, I found that CORT activates Src and Erk1/2 in a similar fashion as LP12. To further verify this notion, the entire pathway could be tested by exploring CORT's effect on all the major proteins within the transactivation pathway.

Alternatively, one could argue that a greater concentration of LP12 and/or CORT could be required to elicit an additive change, or that different pairs of agonists vs. pharmacological stressors could elicit this effect. This could be tested simply by escalating the concentrations of one or both LP12/CORT in this experiment, or using high throughput screening of various transactivating agonist vs. stressors. Though there are many ways to determine the possibility of additivity or synergism, the data we have is sufficient to address the basic question we sought to explore.

Looking at the HT-22 data, it is evident that we can observe different patterns at the various times, but statistical significance was only observed with a 2 h quinpirole treatment. Our general null findings could mean that transactivation cannot be easily replicated in HT-22 cells, or at least replicated reliably, but given the relative ease with which transactivation has been observed in other cell lines, this explanation seems unlikely. Instead, different concentrations (higher and lower than the ones used) and times (more acute and chronic than the ones used) of the various agonists need to be explored; importantly, the peak times observed could be a guide of further studies in the characterization process. In addition, transfection of 5-HT7, dopamine and/or TrkB receptors could be conducted to increase the sensitivity of this cell line.

Overall, I was able to achieve the five aims, and replicate transactivation with 300 nM LP12 reliably, I was able to demonstrate activation of messenger proteins connected to TrkB (Src and Erk), and I was able to show that CORT does indeed activate TrkB-Y816. I was also able to show that though the HP and PFC often had similar trends, the outcomes differed in magnitude or direction. However, my hypothesis that CORT+LP12 combined treatment would yield additive, or synergistic effects was false, as it indeed turns out that they have similar activation levels. Given that I was able to reliably replicate transactivation in this study, this permitted me to lay a

foundation upon which I expanded in chapter 4 to explore the interconnection between transactivation and stress.

The following section, chapter 3, introduces chronic early life social isolation (CELSI), an in-vivo model of stress and primarily focuses on the impact of CELSI on the expression of plasticity related proteins.

CHAPTER 3

Overview

Social isolation is a method that is often used to understand the behavioral and physiological mechanisms that regulate interactions in social mammals. The impact of social isolation in early-life has significant adverse effects on the brain development and adult behavior of social animals. In addition, various studies have indicated a strong correlation between childhood trauma exposure and observed negative health outcomes in adulthood. While the exact physiological and molecular mechanisms underlying these outcomes have yet to be understood, evidence suggests that chronic early life stress alters neurochemistry, at the level of receptor function and neurotransmission. This chapter explores the impact of early life stress on the expression of two plasticity related proteins: the TrkB and the GluN2B subunit of the NMDA receptor.

3.1. Introduction

Chronic stress experienced during critical developmental stages in mammals has been linked to a myriad of negative outcomes in adulthood, including behavioral dysfunctions and an increased vulnerability to psychiatric illness [264]. A multitude of studies have repeatedly shown a large correlation between early life stressors and risk factors for diseases, including coronary artery disease, chronic pulmonary disease and cancer [265].

In rodent models, social isolation stress induces marked behavioral changes, which show signatures of neuropsychiatric disorders observed in humans, such as schizophrenia and depression [266]. For example, studies in which rodents were exposed to social isolation reported an increase in locomotor activity in response to novel environments. As well, other reports revealed a variety of changes, including an increase in responsiveness to psychostimulants, altered sensorimotor

gating of the acoustic startle response, and an impairment in pre-pulse inhibition [267, 268, 269, 270].

The molecular origins of the behavioral changes observed after isolation stress are beginning to be understood. For example, a study examining the pre-frontal cortex (PFC) of male Sprague-Dawley rats isolated at post-natal day 20, showed an abnormal expression- upregulation and downregulation of immediate early genes, as well as genes that regulate cell differentiation and apoptosis [271]. Given the role of the PFC in executive function and its role in social behaviour modulation, changes in its circuitry could explain some of the reported behavioural alterations, characteristic of the positive symptoms of schizophrenia and/or the anxiety accompanying depression [272]. Although the exact mechanisms underlying these social isolation-induced changes have not been fully elucidated, there is evidence to suggest that chronic early life stress alters brain neurochemistry at the level of receptor expression, and/or function and neurotransmission [273].

3.1.1 CELSI and TrKB/ BDNF

Of these alterations in brain neurochemistry, CELSI has been reported to decrease BDNF [273]. BDNF is a neurotrophic factor that activates the TrkB receptor, and, through TrkB signalling, BDNF regulates the proliferation, differentiation and survival of neuronal and non-neuronal cells in the CNS. Additionally, BDNF regulates neuronal plasticity, synaptogenesis, spine formation, LTP and neuronal excitability, as well as adult neurogenesis [274]. Neurotrophins, like BDNF, engage in cross-talk with, and have a significant role in the calibration of, many neurotransmitter systems. In fact, the fine-tuned interaction between neurotrophins and neurotransmitter systems regulates a homeostatic state, the disruption of which can trigger neuro-degeneration and inflammation [273]. Various mechanisms have been proposed to explain the interaction of early

life stress with BDNF. Of these proposed mechanisms is one suggested by Daskalakis *et al.*, wherein various models of early life stress epigenetically regulates BDNF expression through changes in the methylation patterns of BDNF at exon IV. This regulation was reported to be biphasic, with lower methylation of exon IV at post-natal day (PND) 21 and increased methylation in adolescence to adulthood [275].

The neurotrophic factor theory of depression posits that a reduction in neuronal levels of neurotrophic factors, like BDNF, contributes to the pathophysiology of depression [276]. In line with this theory, BDNF has been shown to have antidepressant properties [277, 278]. In addition, we have shown that the activation of 5-HT7 receptors can increase TrkB receptor expression, and this observation may partially explain the ability of antidepressants, which prevent 5-HT reuptake, to promote TrkB/BDNF signalling [279].

Given the fine-tuned connection between receptor and agonist expression, the evident impact of chronic stress on BDNF implies that there might also be stress induced changes in TrkB expression. To our knowledge, only one publication exists in the literature that explores the impact of CELSI on TrkB expression, therefore our findings will allow us to contribute significantly to this topic.

3.1.2. CELSI and NMDA/Glutamate

Alongside BDNF/TrkB pathways, the NMDA receptor is another major regulator of neuroplasticity [280]. The NMDA receptor is an ionotropic glutamate receptor, which is activated by the binding of glutamate and glycine. NMDA receptors have a dimorphic role in the CNS, as they play a major role in both neuronal function and neuronal death [273]. Normal, or healthy NMDA mediated-glutamergic signalling regulates synaptic-formation, function and learning and memory; however, excessive glutamergic signalling can contribute to excitotoxicity, which is

implicated in a broad range of neuropathologies, including depression, anxiety, epilepsy and schizophrenia [281]. Stress-induced changes on NMDA receptors have been studied extensively, and this work has highlighted the role of NMDA receptors in the pathophysiology of stress-induced depression. Studies conducted by Haj-Mirzaian *et al.*, have shown that adolescent rats under social isolation stress displayed a depressed-like phenotype in the forced swim test and splash test, which was reversed by sub-effective doses of MK-801, an NMDA antagonist [282].

In addition, social isolation stress in general increases glutamate levels in the brain, which may lead to excitotoxicity, thus resulting in various neuropathologies, including depression, anxiety, epilepsy and schizophrenia [254]. Given that social isolation stress has some impact on glutamate, the possibility exists that there may also be an effect on the expression of NMDA receptors. Further, studies have shown that isolation stress up-regulates GluN2B mRNA receptor expression in the hippocampus, and increases NMDA binding capacity in the frontal cortex, thus increasing NMDA signalling that could lead to excitotoxicity [283, 284]. Further, mice exposed to social isolation stress were demonstrated to have an altered response to ketamine, an NMDA antagonist, in the PFC, which highlights the ability of stress to affect circuitry in this region; this altered response includes a decrease in GABA expression [285].

Both TrkB and GluN2B receptors appear to be important regulators of neuroplasticity, and, as such, disruptions to their homeostatic states may trigger neuro-degeneration, and various other adverse effects. The effects of CELSI on these essential regulators, and the exact mechanisms of action these receptors undergo as a result of this CELSI, is an area that has not been extensively studied, and one that would help explain the observed results.

3.2 Study objectives

The aim of this study was to explore the impact of chronic early life social isolation (CELSI) on the expression of the TrkB and GluN2B receptors in a sex and region specific manner. To achieve this aim, the Western blot was used to measure differences in protein expression in the hippocampus and prefrontal cortex of male and female rats, respectively. Given the relative lack of female CELSI data (including TrkB data and GluN2B data, specifically) the findings of this study will be a significant contribution to the literature.

3.3. Hypotheses

3.3.1. TrkB/BDNF:

I hypothesized that CELSI would decrease BDNF protein expression, while increasing TrkB receptor expression. BDNF reduction plays a major role in the pathophysiology of depression; therefore, it was anticipated that since CELSI is a psychosocial stressor, it would increase the risk for depression in the rodents and lead to a decrease in BDNF protein expression. The rationale for an expected increase in TrkB expression is two-fold: i) the upregulation of TrkB would be a compensatory mechanism to counter the decrease in BDNF, thus providing a means to sustain TrkB-signalling, and ii) given the neuroprotective potential of TrkB signalling, increased TrkB expression could be a cellular mechanism to increase protective signaling to counter negative CELSI effects.

3.3.2. GluN2B:

As mentioned, numerous studies have demonstrated that early life stress (ELS), including CELSI, results in an increase in glutamate levels [254]. Given that glutamate activates NMDA receptors, I hypothesized that GluN2B expression would decrease, as a protective measure to reduce the potential for NMDA-mediated glutamate excitotoxicity.

3.3.3. Sex and region:

Various studies have observed that male and female rats differ in their threshold and response to stress [286]. While there are many molecular mechanisms that contribute to reported behavioral differences, it was hypothesized that stress would affect key neuronal proteins in a sex- specific manner; thus, stress would affect TrkB and GluN2B expression differently in male and female rats. Simply stated, if there are stress-induced expression differences, it was anticipated that males and females would not have an identical trend. Instead, I anticipated that trends would differ by sex, either in magnitude, or in direction.

Last, I hypothesized that we would observe region specific differences. The HP and PFC are uniquely innervated and have different levels of receptor expression in order to support their respective functions [260]. As a result, it is anticipated that if stress-induced expression changes are observed, these two regions will not present an identical profile, but will differ either in direction, or in magnitude.

3.4. Materials and Methods

3.4.1. Biological Assays

The findings in this chapter represent two cohorts. On post-natal day 21, male and female Sprague-Dawley rats were randomly separated into the treatment group, or control group and kept within that environment for 7 weeks. The rats were housed under a standard 12-hour light/dark cycle at a constant room temperature (23°C) and were given *ad libitum* access to standard rodent chow and water. Experiments with animals were carried out in accordance with the guidelines for animal use issued by the University of Waterloo Animal Care Committee and according to the protocols outlined by the Canadian Council of Animal Care.

The rats were anesthetized with CO_2 inhalant and euthanized by decapitation at post-natal day 70. The hippocampus and prefrontal cortex were immediately extracted from the brain, as per standard procedure, and homogenized. Samples were then frozen at -80°C until the point of analysis by immunoblotting (Western blotting). Western blots were performed, and total protein was measured using a BCA protein assay (Thermo). Samples were heated in 3x loading buffer (240 mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v glycerol, 0.02% w/v bromophenol blue, 50 mM DTT, and 5% v/v β-mercaptoethanol) for 15 min at 75°C and 20-40 μg total protein was loaded into polyacrylamide gel wells. Proteins were separated by SDS-PAGE using electrophoresis buffer (25 mM Tris base, 190 mM glycine, 3.5 mM sodium dodecyl sulfate), followed by transfer of proteins to a nitrocellulose membrane by electroblotting with transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris base, 150 mM NaCl, pH 7.6) plus 0.1% Tween (TBS-T) for 1 h at room temperature, or overnight at 4°C, followed by incubation with primary antibody added to blocking buffer for 1 h at room temperature, or overnight at 4°C. Membranes were washed three times with TBS-T, and then incubated with a secondary antibody conjugated to horse radish peroxidase (HRP) in blocking buffer for 1 h at room temperature. Membranes were washed three additional times with TBS-T. Western chemiluminescent substrate (Luminata Crescendo-Millipore) was used to visualize proteins on a Kodak 4000MM Pro Imaging Station. The images used for analyses were taken from the linear range of exposures. Densitometric analyses of images were performed using Kodak Molecular Imaging software. After imaging, membranes were stripped and re-probed with other appropriate antibodies. Molecular weights of analyzed proteins are as follows: TrkB, 145 kDa; GluN2B, 180 kDa; β-actin, 43 kDa. Membranes were probed with primary antibodies against GluN2B (Millipore; 1:1000), TrkB (Millipore;

1:1000), pTrkB-816 (Millipore; 1:500), β-actin (Santa Cruz; 1:1000). Anti-mouse (1:10000) and anti-rabbit (1:5000) horseradish peroxidase (HRP) enzyme-conjugated IgG secondary antibodies were used.

Capillary isoelectric focusing with UV whole column imaging detection (CIEF-UV-WCID) was used to quantitatively determine the relative amount of BDNF protein in GH and SI samples. With CIEF-UV-WCID, separation of proteins occurs inside a 5 cm capillary with a 200 μm ID. The entire length of the capillary is imaged consecutively, the narrow bone capillary provides high resolution and sensitivity. Proteins electro-migrate under the electric field and focus at their isoelectric point where the net charge is zero. The voltage is increased from 1-3 KV and the focusing is completed within 6 min.

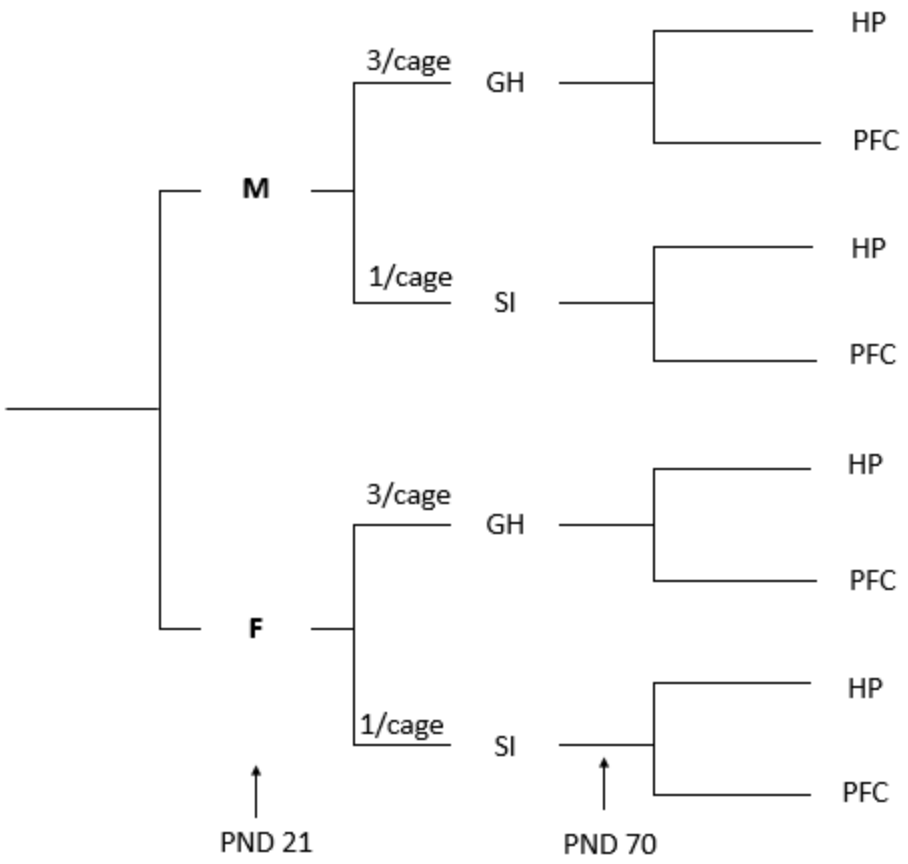


Figure 15. This schematic highlights the experimental protocol. At post-natal day 21 (PND =21) the rats were separated into their specific housing conditions. Group housed rats, were housed 3 rats per cage and socially isolated rats were housed 1 rat per cage. These housing conditions were maintained until PND 70, when these rats were sacrificed. Immediately after sacrifice, the HP and PFC were obtained and homogenized and later analyzed using the western blot technique.

3.4.2. Statistics

Given our sample size, a conservative approach was taken regarding the assumptions that could be made around normality; as a result, our data were analyzed using non-parametric statistical analyses in GraphPad Prism™ 6. Specifically, the Wilcoxon Signed Rank Test (the distribution free equivalent of the 1-sample t-test) and the Mann Whitney U Test (the distribution-free equivalent of the 2 sample Student's t-test) were used for analyses. Although our statistical approach is effective in determining the main effects, a major limitation is the inability of our chosen analytical strategy to elucidate interactions at play that might have contributed to the observed outcomes. Therefore, to address this limitation, in those cases where an interaction was suspected (given qualitative assessment of interaction graphs) a two-way ANOVA (which assumes that our data are taken from a population with a normal distribution) was also conducted to assess interactions between the given independent variables.

TrkB and GluN2B expression data were presented as the fold change relative to control, meaning that HP and PFC data from SI animals were normalized to data from their non-stressed (that is, GH), same sex siblings. Given that SI data was normalized to GH data and graphically presented as a fold change relative to the GH controls, the GH data became the baseline, in other words equals to 1. The respective cumulative fold changes were then statistically analyzed.

One of the aims of this study was to compare region-specific and sex-specific outcomes, so the following comparisons were conducted: (i) same receptor, same sex-region comparison (e.g. TrkB female-HP vs. TrKB female-PFC comparison) and (ii) same receptor, same region-sex comparison (e.g., TrkB HP-male vs. TrKB HP-female comparison). The Mann Whitney U Test was used to statistically analyze these comparisons; to protect against the type I error, the Bonferroni correction was applied (an adjusted alpha value was used, which was determined by

dividing the original α value by the number of groups being compared n). The Cohen's d, an effect size calculation that measures the standardized difference between two means, was used to assess the biological relevance of the data. The general formula for Cohen's d calculation is d = (M1-M2)/S pooled; where M1 and M2 represent the means of the two groups being compared and S pooled, represents the pooled standard deviation for the two groups. Cohen's d calculations were completed using an online calculator [261]. Cohen's d conventional values for a small effect size (d = 0.2), a moderate effect size (d = 0.5), and a large effect size (d = 0.8) were used for interpreting the data. Outliers were identified and removed from the data set using Tukey's method. Tukey's method was chosen since it is a non-parametric method, thus aligning with the general statistical approach of this thesis. With Tukey's method, the outlier value is compared with computed upper and lower limits, which are referred to as "fences" for exclusion. Determination of the fences involves calculating the interquartile range (IQR), which is the difference between the upper quartile (75th percentile) and the lower quartile (25th percentile), for the dataset. The lower fence is obtained by subtracting 1.5 times the IQR from the 25th percentile and the upper fence is obtained by adding 1.5 times the IQR to the 75th percentile. Any values that fell outside of the calculated range were regarded as outliers and removed from the dataset [262]. Additionally, any data that were subject to experimental error were also excluded.

The chosen threshold p value was 0.1. In addition to p values, effect size calculations were also provided a means to determine the biological relevance of the given outcomes, and served as a viable secondary measure to determine the acceptance, or rejection of the null hypothesis.

3.5. Results

3.5.1. GluN2B

This section describes the impact of CELSI on GluN2B expression in male and female Sprague-Dawley rats. In male rats we observed no statistically significant difference, but a trend towards an increase in GluN2B expression that is more notable in the HP ($p = 0.11$, $d = 0.84$) than the PFC ($p = 0.58$, $d = 0.58$). In females we observed a statistically significant decrease in GluN2B expression in the HP ($p = 0.03$, $d = 1.02$) and a trend towards a decrease in the PFC ($p = 0.15$, $d = 0.55$). Separate controls were used for the HP and PFC tissue (as shown in the representative images), however, given that the control is defined as the baseline (equal to 1, for both HP and PFC), one control bar was shown in the graphs in Figures 16- 19.

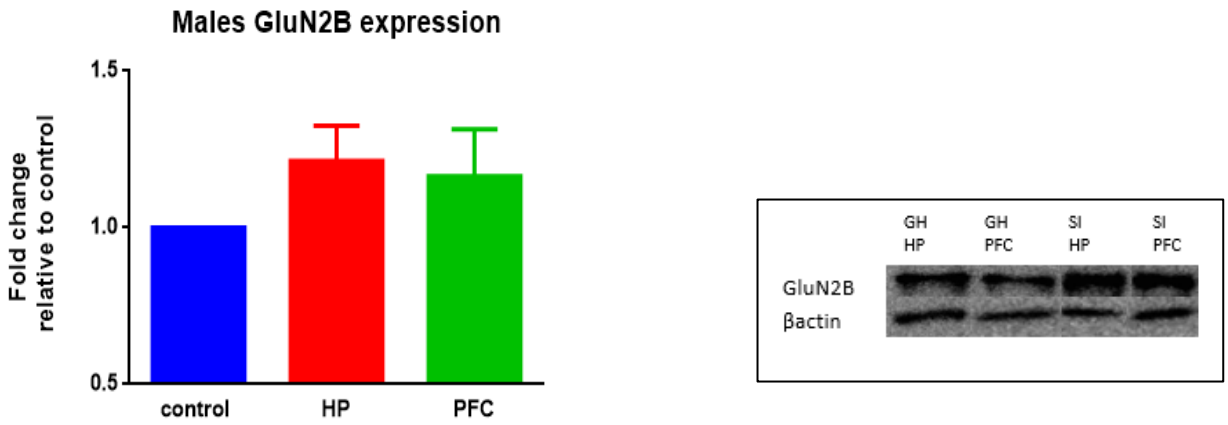


Figure 16. HP and PFC slices were obtained from GH (group housed- controls) and SI (socially isolated) male Sprague-Dawley rats. The slices were homogenised and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin protein expression and are expressed as the fold change in GluN2B immunoreactivity. GluN2B-HP n = 10, $p = 0.11$, $d = 0.84$ and GluN2B-PFC n = 7, $p = 0.58$, $d = 0.58$.

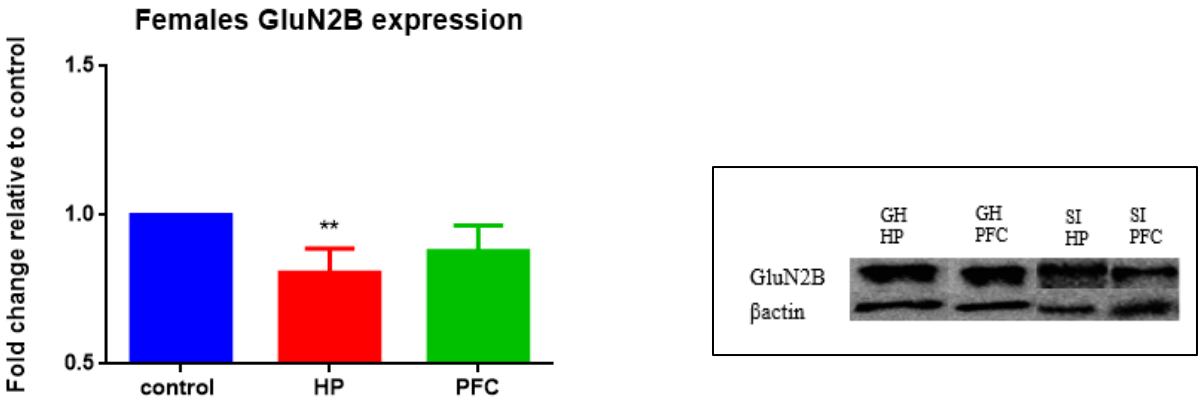


Figure 17. HP and PFC slices were obtained from GH (group housed) and SI (socially isolated) female Sprague-Dawley rats. The slices were homogenised and the samples were evaluated by Western blotting, as described in methods. Data were normalized to total β-actin protein expression and are expressed as the fold change in GluN2B immunoreactivity. (** = $p < 0.05$ (Wilcoxon Signed Rank Test)) compared to GH controls. GluN2B-HP n = 11, p = 0.03, d = 1.02 and GluN2B-PFC n = 13, p = 0.15, d = 0.55.

3.5.2. TrkB

This section describes the impact of CELSI on TrkB expression in male and female Sprague Dawley rats. In males, it was observed that TrkB expression was increased significantly (*$p < 0.1$) in the HP (p = 0.08, d = 0.68) and a trend towards an increase was observed in the PFC (p = 0.24, d = 0.68). In females, we observed a decrease in TrkB expression in the HP (p = 0.19, d = 0.64) and a statistically significant decrease in the PFC (p = 0.05, d = 0.80). The CIEF-UV-WCID trial data (n = 1) suggests a CELSI-induced decrease in BDNF.

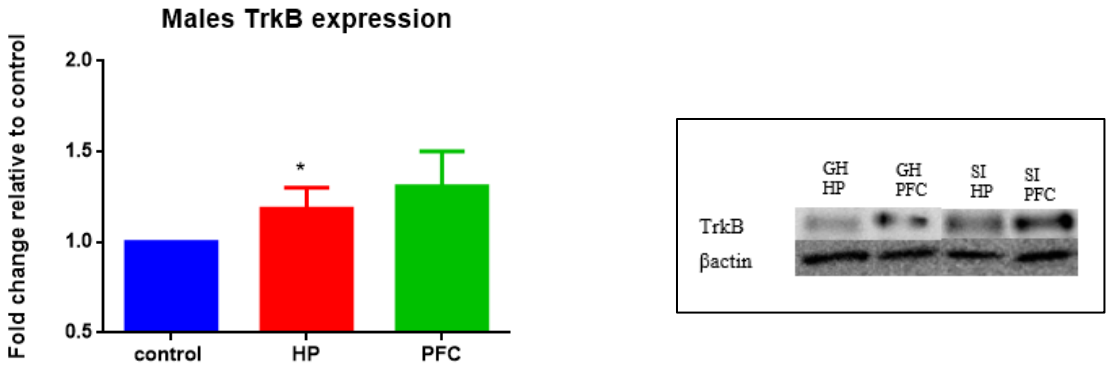


Figure 18. HP and PFC slices were obtained from GH (group housed) and SI (socially isolated) male Sprague-Dawley rats. The slices were homogenised and the samples were evaluated by Western blotting, as described in methods. Data were normalized to total β-actin protein expression and are expressed as the fold change in TrkB immunoreactivity respectively. (* = p <0.1 (Wilcoxon Signed Rank Test)) compared to GH controls. TrkB-HP n = 11, p = 0.08, d = 0.68 and TrkB-PFC n = 11, p = 0.24, d = 0.68.

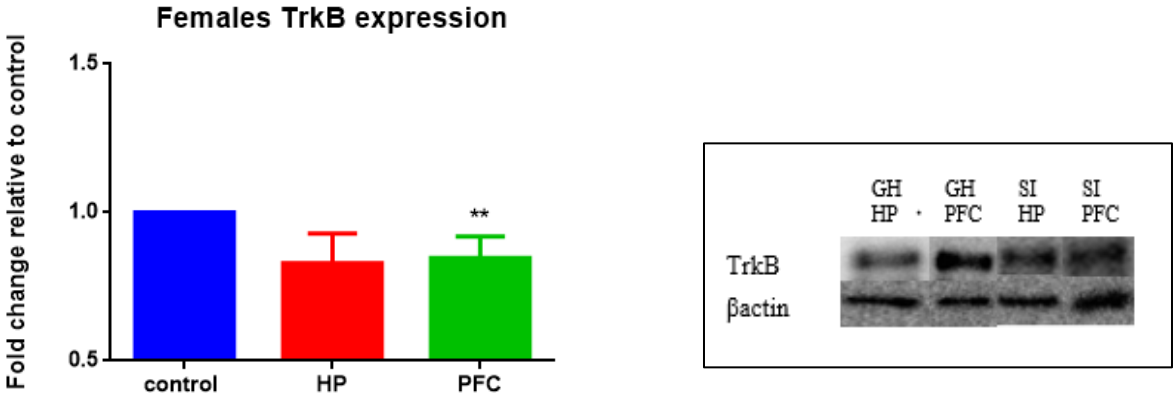
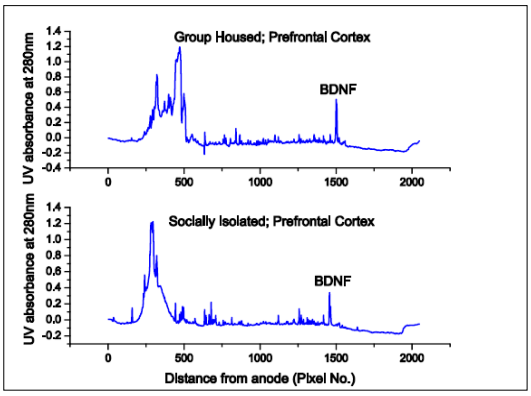


Figure 19. HP and PFC slices were obtained from GH (group housed-control) and SI (socially isolated) female Sprague Dawley rats. The slices were lysed and lysates were evaluated by western blots, as described in methods. Data was normalized to total β-actin protein expression and are expressed as the fold change in TrkB immunoreactivity. (*=p <0.1, **=p < 0.05 (Wilcoxon Signed

Rank Test)) compared to controls. TrKB-HP n=14, p=0.19, d=0.64 and TrkB-PFC n=14, p=0.05, d= 0.80.

A 

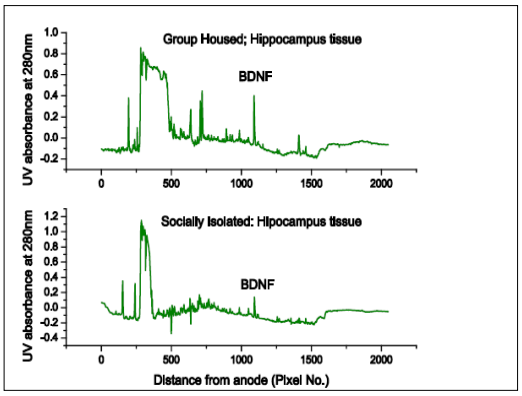
B 

Figure 20. CIEF-UV-WCID was used as a trial (n=1), to qualitatively determine relative amounts of BDNF in female samples. A comparison of egrams GH (group housed-control) and SI (socially isolated) homogenates from the (A) prefrontal cortex and (B) hippocampus respectively.

3.5.3. Sex and region comparisons

Our findings show that there are no statistically significant differences in region (HP vs. PFC), however, there are significant sex differences reflected by the large effect size and small p- values.

Receptor	Sex	Comparison	p Values	Effect Size (d Values)
GluN2B	Male	HP vs. PFC	> 0.9999	0.13
	Female	HP vs. PFC	0.64	0.25
TrkB	Male	HP vs. PFC	0.93	0.23
	Female	HP vs. PFC	0.72	0.05

Table 5. CELSI region comparisons. This table shows the region comparison (HP vs. PFC) within the same sex and same receptor. Significance was determined by the Mann-Whitney U Test (Bonferroni correction was applied) and Cohen's d values.

Receptor	Region	Comparison	p Values	Effect Size (d Values)
GluN2B	HP	Male vs. Females	0.01	1.29
	PFC	Male vs. Females	0.16	0.80
TrkB	HP	Male vs. Females	0.03	0.93
	PFC	Male vs. Females	0.03	0.93

Table 6. CELSI sex comparisons. This table shows the sex comparison- (males vs. females) within the same receptor and region. Significance was determined by the Mann Whitney U Test (Bonferroni correction was applied) and Cohen's d values.

3.5.4. TrkB sex and region comparisons.

Our data (Table 5 and 6) reflect that there are no statistically significant differences between HP and PFC. However, in alignment with our hypothesis, the outcomes observed for the HP and PFC are not identical. For instance, with the female data, significant outcomes are observed in the PFC but not the HP and the opposite is observed in the male data.

When exploring sex differences, it is apparent looking at the large effect size values (d = 0.93 and d = 0.93), that there is a biologically relevant sex difference, and this is corroborated by the relatively small p values. Males had an increase in TrkB expression whilst females had a decrease. Overall, my hypothesis for TrkB stands true as, for both region and sex comparisons, we observed a difference in magnitude in HP vs. PFC and with regards to sex, a difference in direction. Additionally, a two-way ANOVA was conducted to explore if there was an interaction of sex (male vs. female) and brain region (HP vs. PFC) in TrkB expression, and the outcomes reflect that there was no interaction.

3.5.5. GluN2B sex and region comparisons.

Our data (Table 5 and 6) reflect that there was no observed statistically significant difference in GluN2B expression between the HP and PFC. However, in alignment with our hypothesis, there is a difference, though not statistically significant, between HP and PFC. Concerning direction of

effect, there was no pattern, as the HP is higher than PFC in males, but the fold change is very similar in females, although the PFC appears slightly greater than the HP.

When exploring sex differences, there was a statistically significant difference between males and females in the HP ($p = 0.01$ and $d = 1.29$), and a strong trend towards a difference in the PFC ($p = 0.16$ and $d = 0.80$). The low p values and the high d values, suggest that these outcomes are biologically relevant. Males had an increase in GluN2B expression whilst females had a decrease. Overall with GluN2B our hypothesis stands true and for both region and sex comparisons, as we observed a difference in magnitude in HP vs. PFC and a difference in direction for males vs. females. Additionally, a two-way ANOVA was conducted to explore if there is an interaction of sex (male vs. females) and brain region (HP vs. PFC) in GluN2B expression, the outcomes reflect that there is no interaction.

In summary, the data in Table 5 reflects that brain region (HP vs. PFC) is not a critical factor when it comes to TrkB or GluN2B expression differences, whereas Table 6 reflects that sex plays a significant and biologically relevant role in expression differences, and overall no interactions of sex or brain region are observed.

3.6. Discussion

This study sought to evaluate the impact of early life adversity on the expression of two plasticity related proteins, the GluN2B subunit of the NMDA receptor and the TrkB receptor. Our findings suggest that CELSI affects GluN2B and TrkB expression in a -sex specific manner. Additionally, a small sample trial of CIEF-UV-WCID demonstrated the possibility of BDNF protein changes in CELSI.

3.6.1 TrkB/BDNF

A recent review by Murinova *et al*. confirms our own observation, that there is only one publication (Djouma *et al.*, 2006) that explores the impact of CELSI on TrkB protein expression [287]. In this publication, Djouma *et al*. reported an increase in TrkB expression in the HP; however, different outcomes were reported across cortical regions, with a decrease in expression observed in the cingulate cortex and the piriform cortex, but an increase in expression found in the retrosplenial cortex [288]. These findings suggest that in the cortical regions, changes in TrkB expression are region specific. To our knowledge, no study has been published that explores TrkB changes in female HP and PFC.

As indicated previously, we hypothesized that we would observe a decrease in BDNF expression and a corresponding increase in TrkB expression, after CELSI. We observed a statistically significant increase in TrkB expression in the HP of males, and a trend towards an increase in the PFC. In contrast, a decrease in TrkB expression in the HP was observed in females, and a statistically significant decrease in the PFC was observed. Thus, the outcomes of the male data support our hypothesis, whilst the female data reflect a trend that is opposite to what was expected.

Given the limited CELSI-TrkB data in the literature, we explored CELSI-BDNF studies to provide an avenue to glean potential TrkB outcomes. We found that many studies report a CELSI-induced decrease in BDNF expression in the HP [289-298]. Of the few studies conducted in females, no change was reported in both the HP and PFC [290, 299] suggesting that, either, no change is observed in BDNF after stress in females, or alternatively, that the sample size is not large enough to determine an effect. Nevertheless, the findings from this study contribute to the much-needed literature on TrkB female studies.

The aforementioned interpretation of our findings begs the question: if an increase in TrkB expression in a given region implies an adaptive response to stress, what does a decrease in expression mean with regards to resilience to stress? In this regard, a study exploring BDNF and TRK gene expression changes, in a cohort of animals exposed to ELS, demonstrated that, within the same cohort, some animals exhibited superior performance in behavioral tests (stress resilient), whereas other animals had inferior performance (stress susceptible). Furthermore, this study demonstrated that animals with superior vs. inferior performance had differing patterns of BDNF and TrkB mRNA in key brain regions, including the hippocampus and PFC [231]. This underlies the fact that even within a cohort of sibling rodents, genetic variability might be limited, but significant enough to cause an array of outcomes.

To validate the interpretation that an increase in TrkB expression is indeed a neuroprotective response to stress, we would need to compare the baseline phosphorylation (activation) of the TrkB receptor between control and stressed tissues. Additionally, the expression and activation of secondary messengers downstream of TrkB activation could be measured because receptor changes do not always fully translate into functional changes. Therefore, to get a clearer understanding of this observation, we need to also explore various markers of TrkB signalling, for example, AKT, Erk 1/2 or CREB to name a few.

Given the complex interplay between TrkB/BDNF signalling pathways with glucocorticoid, dopaminergic and serotonergic signalling during the developmental phase, ELS effects would have to be explored in the context of some of these other systems in order to glean a full understanding of the impact of ELS, as the TrkB-BDNF signalling does not function in isolation. Being one of few known studies that explicitly explore the impact of CELSI on TrkB expression, our work is valuable and contributes further evidence to the literature to help

understand the big picture of ELS effects in this system. Further, to address the above-mentioned limitations from this study, a follow-up study was conducted (explained in chapter 4) that explores: i) baseline TrkB phosphorylation (activation) in CELSI vs. control tissue and ii) the impact of CELSI on a 5HT-TrkB crosstalk specific pathway.

In addition to studying TrkB expression levels, a small sample CIEF-UV-WCID trial was conducted in female samples (n = 1) after repeated failure to obtain clear BDNF data using the Western blot technique. (The Western blot difficulties were largely related to antibody detection). Due to the limited volume of male samples, CIEF-UV-WCID was only conducted in female samples. The outcomes suggest a decrease in BDNF protein levels in both the HP and PFC of socially isolated rats relative to their group housed controls. Given the sample size, it is not possible to determine the true direction of effect concerning BDNF expression, however, given that this measure was sensitive enough to detect some change even in such a sample size, suggests to us that some change could be occurring at the level of BDNF protein. This outcome aligns with our hypothesis that states that CELSI is a psychosocial stressor that will induce a measure of depression in the rats, and reduce BDNF expression. This finding is also supported by the BDNF trends that have been heavily studied and reported in male animals. Next steps include determining BDNF protein levels in CELSI vs. control animals in a sex and region manner.

In summary, we observed an opposite trend in males and females, with males having an increase in TrkB expression and females having a decrease. We also observed a decrease in BDNF in females, but given the limited sample size, more replicates need to be run to determine the true direction of effect. Overall, these findings are promising and contribute to the literature on CELSI effects on TrkB.

3.6.3. GluN2B

As mentioned previously, stress modulates NMDA receptor expression and signalling [254]. In fact, various markers of stress have been shown to regulate NMDA receptor expression. For instance, hormonal changes (androgens and estrogens) and changes in neurotransmitters, like dopamine, that are implicated in mood and emotion have been shown to affect NMDA receptor expression [300]. As a result, a stressor like CELSI will likely affect NMDA receptor expression, since it can induce a depressed phenotype, and activate various stress markers. When exploring the timing of the stressor, both acute and chronic stressors have been shown to affect NMDA receptor expression [254]. More precisely, ELS has been shown to increase glutamergic signalling, specifically the extra-synaptic signalling, which, in the context of GluN2B receptors, is implicated in negative, excitotoxic signalling [254]. The negative impact of extra-synaptic signalling is largely because it antagonizes the pro-survival signalling of the synaptic receptors, in fact, a major shift in the balance of synaptic vs. extrasynaptic NMDA receptors can drive cell death and has been implicated in neurodegenerative diseases like Alzheimer's diseases [254].

My hypothesis suggested that a decrease in GluN2B expression would be observed, as a protective measure against excessive NMDA mediated glutamergic signalling. Interestingly, my findings suggest that there is a trend towards an increase in GluN2B expression in the HP and PFC in males, however it is not statistically significant. In contrast, I observed a statistically significant decrease in GluN2B expression in the HP and a trend towards a decrease in the PFC in females. Therefore, the female data support our hypothesis, whilst the male data do not match the expected outcomes. Specifically, the male HP data includes a p value of the HP ($p = 0.11$) that is very close to the parameters for statistical significance, and the large effect size ($d = 0.84$) also suggests that the effect of this change is significant. However, for the PFC, the effect size is moderate ($d = 0.58$).

To put these findings into context and to explore what could underlie the observed increase in males and the decrease in females, a viable interpretation could be that this decrease in GluN2B expression in females is a neuroprotective mechanism; in contrast, males could be viewed as susceptible to stress induced glutamergic signalling. If males do indeed exhibit susceptibility to stress-induced excitotoxicity, that could explain their upregulation of TrkB, which may provide protection against glutamate excitotoxicity.

The NMDA receptor is implicated in dynamic signalling and crosstalk with many receptors, therefore one way to improve this study would be to explore the activation of proteins directly downstream of NMDA/GluN2B activation or exploring GluN2B in the context of other signalling pathways. To address these points, our following study (chapter 4) explores how TrkB activation affects GluN2B expression.

In summary, in males we observed an increase in GluN2B, which is contrary to our hypothesis, whilst in the females we observed a decrease in GluN2B expression, which aligns with our expectations. To put these findings in context of the bigger picture, further work needs to be done to elucidate GluN2B changes in relation to related pathways.

CHAPTER 4

Overview

In chapters 2 and 3, transactivation was successfully replicated in tissue slices, and it was demonstrated that CELSI regulates TrkB and GluN2B expression in a sex- and region- specific manner. Having identified these expression changes, the next goal was to study the impact of CELSI on TrkB signalling. Given that TrkB plays a role in functions like neuronal development and survival, the receptor communicates extensively with other receptors through various crosstalk mechanisms. As a result, I chose to study if CELSI affects a specific transactivation pathway: 5-HT7-TrkB transactivation (this pathway was characterized in previous work).

4.1. Introduction

TrkB transactivation, like direct activation of TrkB, has neuroprotective potential, but, despite this potential, the *in vivo* role of transactivation has not yet been elucidated [10]. Evidence points to the fact that many different stressors, including oxidative stress, UV radiation, and shear stress can induce transactivation through unknown mechanisms [302, 303, 304]. Additionally, there is evidence of transactivation in various human tissues, like the heart where it is thought to play a protective role [305]. Therefore, exploring the connection between stress and transactivation will provide more understanding as to the physiological relevance of this pathway.

The possible transactivation-stress connection inspired the question- is transactivation part of the stress response? Although seemingly simple, this question is highly complex and has to be addressed using a dynamic approach that considers the myriad of molecular players that would need to be identified to fully characterize such a pathway. The aim of this chapter is to provide important pieces of the puzzle, which will lay a sturdy foundation upon which future studies addressing this complex question can be built.

The first step was the establishment of a transactivation model (HT-22 cell line, or *ex vivo* brain slices) that was more physiologically relevant than previous models. Once established, the model was used to study the effect of stress upon transactivation. Studying "stress effects" included evaluating the impact of a pharmacological stressor (i.e., CORT) on RTK activation, and/or expression to see how an acute form of stress affects transactivation, before testing a more chronic model in the form of CELSI. The most reliable GPCR-RTK transactivation candidates (5-HT7 and TrkB) were identified, and transactivation was replicated reliably with 300 nM LP12. In addition, CORT was found to activate TrkB in a manner similar to LP12. The study further demonstrated that, while the HP and PFC often had similar trends, the magnitude, or direction of the outcome differed. As such, this study provided the foundation needed to expand and explore the interconnection between transactivation and stress.

Following chapter 2, we explored the impact of stress on the expression of two plasticity related proteins: the TrkB receptor and the GluN2B subunit of the NMDA receptor. The findings suggested that CELSI affects GluN2B and TrkB expression in a sex specific manner. In an attempt to build upon these two studies, this chapter will take a direct approach to exploring the impact of CELSI on the response to LP12 treatment, CORT treatment and CORT+LP12 treatment. Considering the complex interplay between TrkB and GluN2B signalling pathways during the developmental phase (e.g., with dopaminergic and serotonergic signalling), we explored the effect that early life stress had on these proteins in the context of the transactivation pathway. As such, in order to clarify the transactivation-stress connection, this chapter provides an exploration of the impact of CELSI on 5-HT-TrkB crosstalk.

4.2. Study objectives

The aim of this study was to explore the impact of CELSI on transactivation, and to explore whether this chronic, developmental stressor primes the brain for differential response to agonist treatment, by comparing the outcomes of TrkB activation and expression, as well as GluN2B expression, on tissue slices treated with LP12, CORT, or CORT+LP12.

4.2.1. Intra-sex comparisons

The aim of intra-sex comparisons was to evaluate the impact of the treatment(s) on TrkB activation and expression, as well as GluN2B expression within a given sex. Intra-male analyses explored if there were differences between group housed (GH) and socially isolated (SI) male outcomes of LP12, CORT, and CORT+LP12 treatment. Additionally, HP and PFC differences were explored. Prior to this point, all reported *ex vivo* slice work was conducted in male brain slices. The work presented below was our first attempt to replicate transactivation in female brain slices, making this study relevant in helping us understand if there are sex-related differences in transactivation outcomes. As such, observing the trends in CELSI control (non-stressed) female slices was imperative to laying a foundation for our understanding of baseline transactivation in female tissue. Additionally, it was also important to compare the female control outcomes to those of male control slices (from chapter 2) as this comparison helped us understand baseline transactivation in male vs. female tissue. Female intra-sex comparisons, explored if there were differences between group housed (GH) and socially isolated (SI) outcomes of LP12, CORT and CORT+LP12 treatment. Additionally, with the treatment subset, HP and PFC differences will be examined.

4.2.2. Inter-sex comparisons

The aim of inter-sex comparisons was to compare male and female outcomes in TrkB activation. Male vs. female differences in the LP12, CORT and CORT+LP12 activation of TrkB were evaluated. Additionally, region specific differences in these outcomes were also considered.

4.3. Hypotheses

In line with the study objectives, the following hypotheses were made:

1. Various studies have observed that male and female animals display differences in their response to stress. Based on our preliminary findings, it was expected that stress would affect TrkB and GluN2B expression in a sex-specific manner.
2. Additionally, it was hypothesized that region specific differences in males would be observed, (as observed in preliminary findings wherein there was an observable difference in the HP and PFC regions). Additionally it was anticipated that these regions would differ either in the direction, or in magnitude of transactivation.
3. Based on the findings of chapter 2, it was anticipated that LP12 transactivation of the TrkB receptor would be replicated in male derived non-stressed (control) slices.
4. Based on literature demonstrating that early-life stress can induce TrkB-Y816 phosphorylation, it was anticipated that there would be a difference in transactivation between socially isolated (stressed) vs. group-housed (control) slices, which would differ either in magnitude, or in direction.
5. It was expected that CORT treatment in socially isolated vs. group-housed animals would induce different outcomes, in line with literature suggesting that ELS can modify the stress response later on in the lifespan.

6. Similarly, in slices from group-housed female animals, it was hypothesised transactivation and CORT activation would be observed, as was observed in slices from non-stressed male animals. Given that both male and female neural tissue express the same receptors (though the relative abundance may differ), hypotheses 3-5 were also applied to female-derived tissue.

4.4. Materials, Methods and Statistics.

A cohort of 40 rats at age PND 21 was used (20 males and 20 females); within each sex, 10 rats were group housed and 10 were socially isolated. At the end of the isolation period (approximately PND 70-71), each rat was sacrificed and the HP and PFC were extracted (as described in previous chapters). The extracted tissue was subjected to one of three treatments: LP12, CORT, or CORT+LP12. Tissue from all three treatments was subjected to Western blotting for TrkB-Y816, TrkB and GluN2B proteins (as described in previous chapters). Figure 21 provides an outline of the experimental procedure. Please note that the general materials, methods and statistics used for this chapter do not differ from those used in previous chapters.

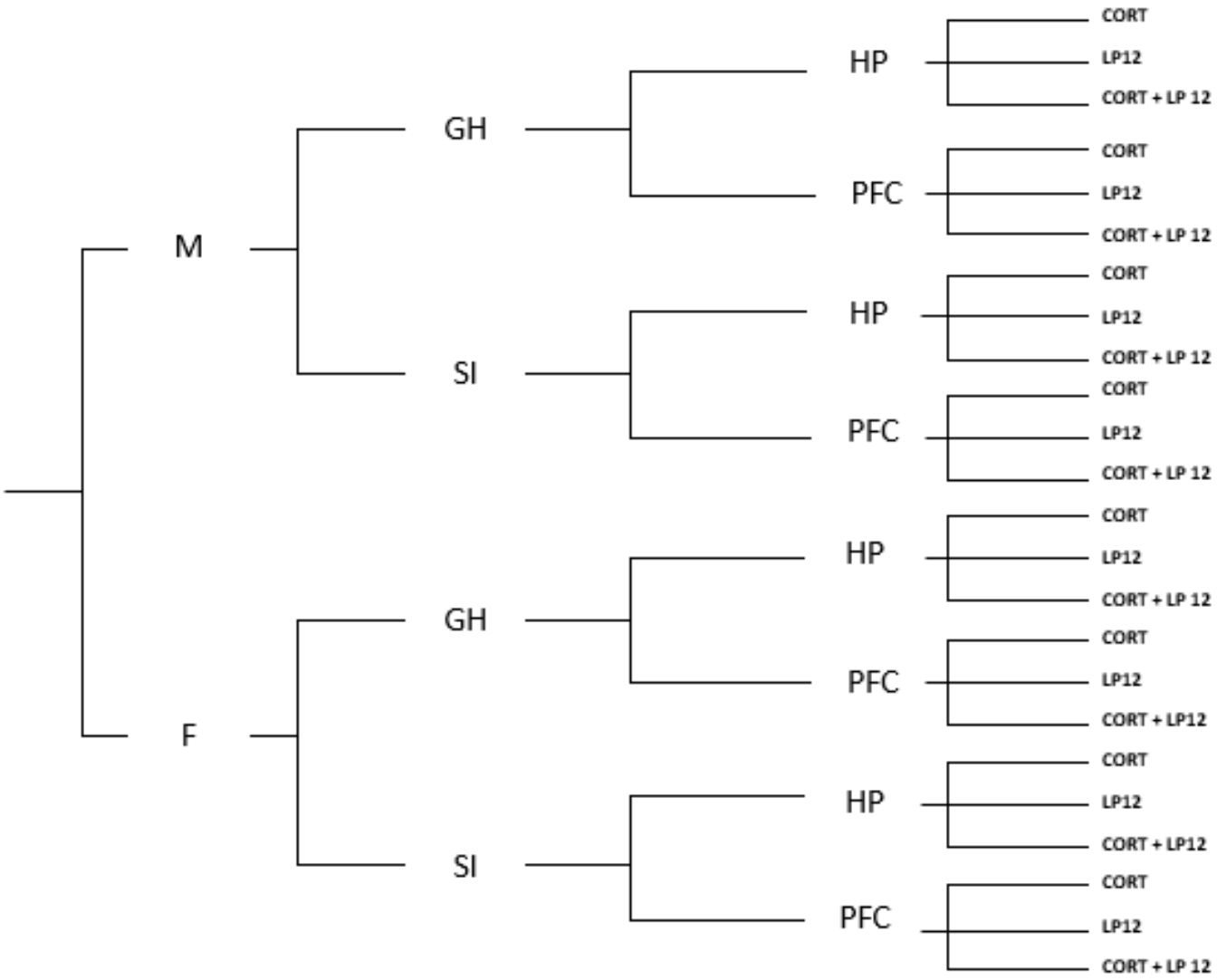


Figure 21. This schematic outlines the experimental procedure. HP and PFC tissue was obtained from male and female rats that had been group housed, or socially isolated. The tissue was subsequently treated with LP12, CORT, or CORT+LP12 and all treatments were blotted with TrkB-Y816, TrkB, and GluN2B antibodies. **M** = male, **F** = female, **GH** = group housed, **SI** = socially isolated, **HP** = hippocampus, **PFC** = prefrontal cortex, **LP12** = LP12 hydrochloride, **CORT** = corticosterone, **816** = blotted with TrkB-Y816 antibody, **TrkB** = blotted with TrkB antibody, **GluN2B** = blotted with GluN2B antibody.

4.5. Results

4.5.1. LP12, CORT and CORT+LP12 activation of the TrkB-Y816 receptor in male rats

Based on preliminary studies (described in chapter 2), *ex vivo* HP and PFC slices were treated with 300 nM LP12 for 2 h, 300 nM CORT for 1 h, or a combined treatment of CORT+ LP12.

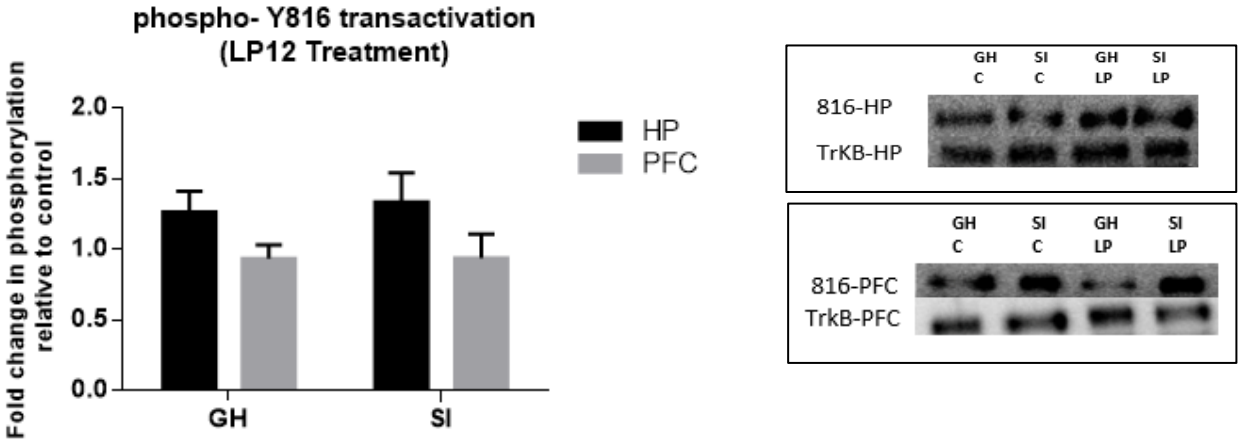


Figure 22. HP and PFC slices were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 12, p = 0.15, d = 0.74; **GH-PFC**: n = 14, p = 0.71, d = 0.26; **SI-HP**: n = 8, p = 0.15, d = 0.81; **SI-PFC**: n = 8, p = 0.64, d = 0.18.

The data in Figure 22 suggests that LP12 may induce transactivation in the group housed (GH) and socially isolated (SI) – HP tissue; though these findings are not statistically significant, the relatively low p values and the high d values (0.74 and 0.81, respectively) indicate that a biologically significant effect may be present. Notably, there was no statistically significant change in TrkB-Y816 phosphorylation in PFC tissue from either GH, or SI animals, which, together with

the low effect sizes, strengthens our ability to conclude that transactivation does not occur in the PFC. These outcomes show great similarity to those observed in chapter 2, wherein LP12 was found to induce transactivation in the HP, while a significant change was not observed in the PFC. Additionally, when considering comparisons within the housing groups, the low p values and high d values, shown in Table 7 (TrkB-Y816, LP12 activation), suggest the biological relevance of the differences between GH-HP vs. GH-PFC and SI-HP vs. SI-PFC. Conversely, the high p values and low d values for the intra-region comparisons suggest that there is no notable difference between GH-HP and SI-HP vs GH-PFC and SI-PFC. In summary, the outcomes observed are similar in magnitude and direction across the housing conditions (SI and GH), which might suggest that brain region, not isolation stress, is a more important factor influencing transactivation in male animals. Additionally, a two-way ANOVA was conducted to explore if there is an interaction of housing (GH vs. SI) and brain region (HP vs. PFC) in TrkB transactivation, the outcomes reflect that there is no interaction. Furthermore, the findings of the ANOVA show that there is a statistically significant effect of brain region ($p = 0.0209$), corroborating the earlier statement that brain region rather than housing is driving the observed outcomes.

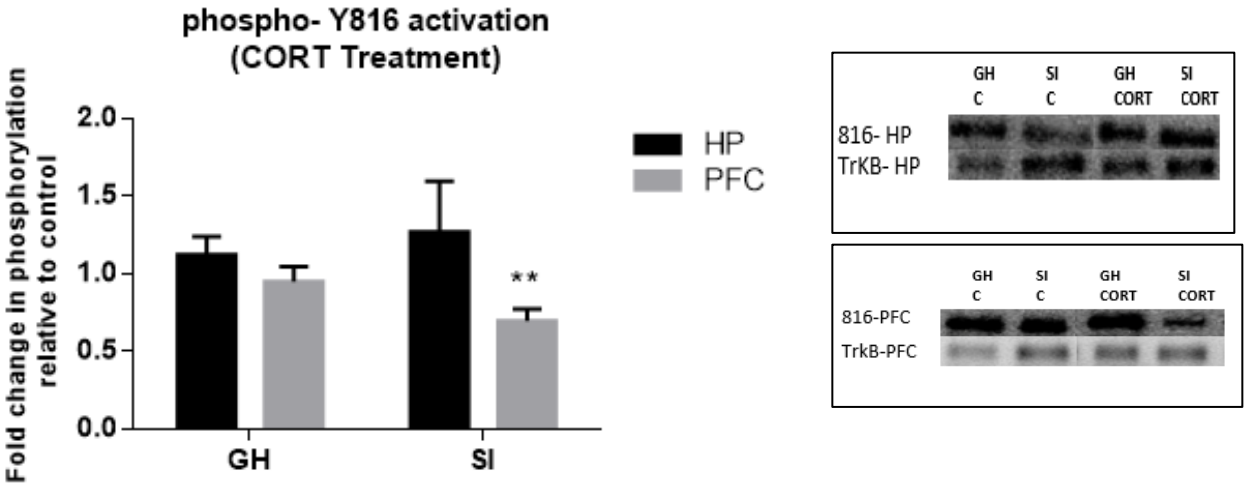


Figure 23. HP and PFC slices were treated with 300 nM CORT for 1 h. Following treatment, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (* = $p < 0.1$; ** = $p < 0.05$; Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 13, p = 0.50, d = 0.41; **GH-PFC**: n = 13, p = 0.68, d = 0.20; **SI-HP**: n = 8, p = 0.84, d = 0.42; **SI-PFC**: n = 8, p = 0.016, d = 1.97.

The outcomes displayed in Figure 23 suggest that CORT has no effect on TrkB-Y816 activation in HP tissue from the GH and SI animals. Additionally, a trend towards a decrease in TrkB-Y816 phosphorylation was observed in PFC tissue; in particular, this outcome was statistically significant (p = 0.016), and had a very large effect size (d = 1.97) in the SI-PFC tissue. When taking a closer look at intra-housing comparisons and intra-region comparisons (Table 7), the difference in TrkB-Y816 between SI-HP vs. SI-PFC seems to be biologically relevant, whilst the GH-HP vs GH-PFC does not; finally, the difference between GH-PFC vs. SI-PFC appears relevant, whilst the difference between GH-HP vs. SI-HP does not. Overall, these findings display clear region dependent patterns that are observed in both housing conditions, once again pointing

to the impact of brain region on these outcomes. Given these outcomes, a two-way ANOVA was conducted to explore if there is an interaction of housing (GH vs. SI) and brain region (HP vs. PFC); however, the outcomes reflect that there is no interaction. Furthermore, the findings of the ANOVA show that there is a statistically significant effect of brain region ($p = 0.027$), affirming the aforementioned suggestion that brain region rather than housing is driving the observed outcomes.

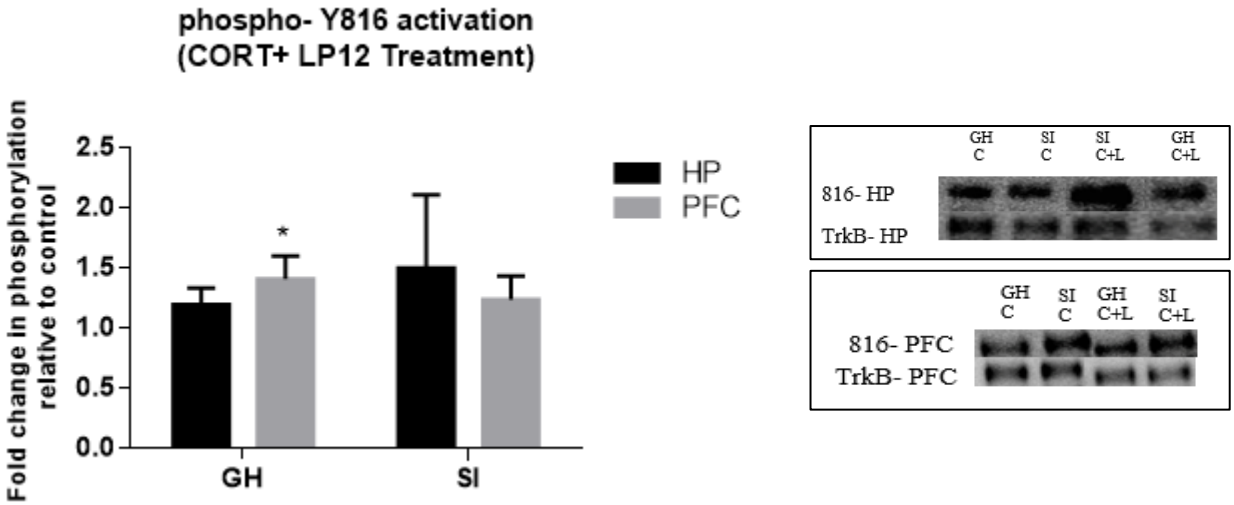


Figure 24. HP and PFC slices were treated with 300 nM LP12 for 2 h, then 300 nM CORT for 1 h. Following treatment, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (* = $p < 0.1$; Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: $n = 11$, $p = 0.21$, $d = 0.61$; **GH-PFC**: $n = 13$, $p = 0.068$, $d = 0.84$; **SI-HP**: $n = 6$, $p = 0.44$, $d = 0.12$; **SI-PFC**: $n = 7$, $p = 0.81$, $d = 0.14$.

The outcomes displayed in Figure 24 show a statistically significant difference in the GH-PFC group that is not observed in other groups. Further, the GH-PFC group has both statistical significance ($p = 0.068$) and a large effect size ($d = 0.84$), while the other conditions reflect high p

values and low d values, suggesting that the changes observed in these groups is likely due to some combination of chance and error, specifically the SI-HP group which had the greatest variability about the mean. When considering intra-housing comparisons and intra-region comparisons, the lack of statistical significance, coupled with low-medium effect sizes (Table 7) suggests that these trends are likely not biologically relevant.

In summary, no interactions of brain region and housing were observed. Data show that LP12 likely transactivates TrkB-Y816 in HP slices (GH and SI), and that CORT has no effect on HP slices (GH and SI) but induces a significant decrease in SI-PFC tissue. These findings suggest that: i) LP12 has greater capacity to activate TrkB-Y816 than CORT and ii) TrkB-Y816 activation is more likely to be influenced by brain region (HP vs. PFC) than housing condition (GH vs. SI). The combined CORT+LP12 treatment shows a significant difference in the GH-PFC tissue; this is a key finding, given that separate LP12 and CORT treatments showed a reduction in TrkB-Y816 activation in the GH-PFC. The unexpected finding implies that CORT+LP12, through some mechanism, induces activation that either CORT, or LP12 alone could not achieve.

	Comparison	p-value	d-value
TrkB-Y816 activation (LP12 treatment)	GH- HP vs. GH-PFC	0.12	0.75
	SI-HP vs. SI-PFC	0.19	0.74
	GH-HP vs. SI-HP	0.78	0.13
	GH-PFC vs. SI-PFC	0.91	0.0042
TrkB-Y816 activation (CORT treatment)	GH-HP vs. GH-PFC	0.47	0.44
	SI-HP vs. SI-PFC	0.19	0.86
	GH-HP vs. SI-HP	0.78	0.21
	GH-PFC vs. SI-PFC	0.10	0.86
TrkB-Y816 activation (CORT+LP12 Treatment)	GH-HP vs. GH-PFC	0.68	0.36
	SI-HP vs. SI-PFC	0.52	0.26
	GH-HP vs. SI-HP	0.51	0.44
	GH-PFC vs. SI-PFC	0.73	0.52

Table 7. Region and housing comparisons in male TrkB-Y816 activation. To explore region and housing specific differences in TrkB-Y816 activation, the Mann-Whitney U test (α=0.05 (Bonferroni correction) and corresponding effect size calculations were performed.

4.5.2. LP12, CORT and CORT+LP12 effect on expression of the TrkB receptor and GluN2B subunit in male rats

Expression data show some changes in male TrkB and GluN2B expression based on LP12, CORT, or CORT+LP12 treatment.

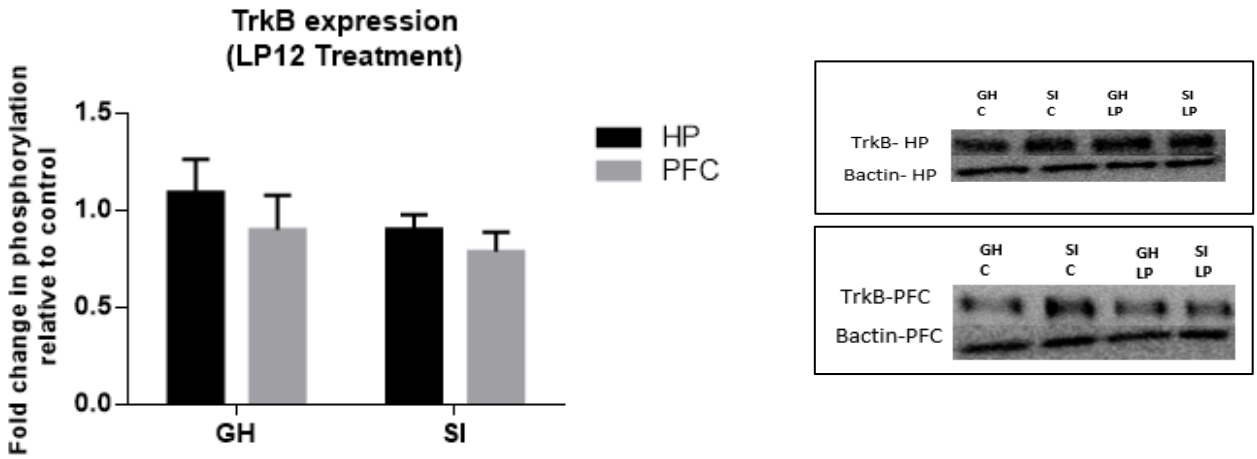


Figure 25. HP and PFC slices from male Sprague-Dawley rats were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenized and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control.**GH-HP**: n = 7, p = 0.94, d = 0.30; **GH-PFC**: n = 8, p = 0.64, d = 0.28, **SI-HP**: n = 9, p = 0.50, d = 0.60; **SI-PFC**: n = 8, p = 0.15, d = 1.045.

LP12 treatment has no effect on TrkB expression in the GH-HP and GH-PFC tissue; however, there is a slight decrease in the SI-HP and SI-PFC, which may be biologically relevant in the SI-PFC, where a relatively low p value (0.15) and high d (1.045).

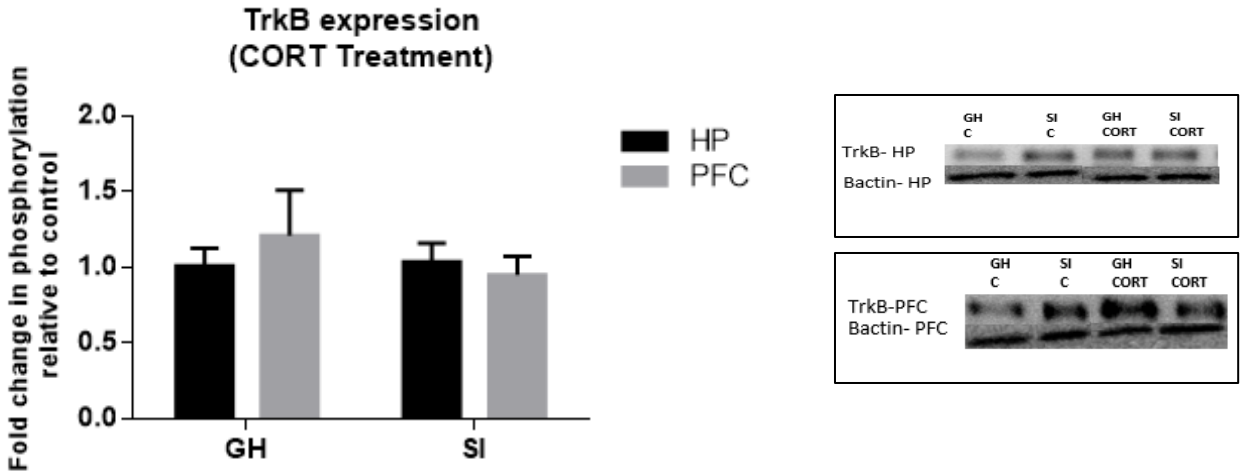


Figure 26. HP and PFC slices from male Sprague-Dawley rats were treated with 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 8, p = 0.95, d = 0.047; **GH-PFC**: n = 8, p = 0.84, d = 0.35, **SI-HP**: n = 8, p = 0.38, d = 0.52; **SI-PFC**: n = 8, p = 0.74, d = 0.20.

CORT treatment does not seem to have an effect on TrkB expression, suggesting that CORT treatment for 1 h may not be generally sufficient to induce changes in receptor expression in male tissue.

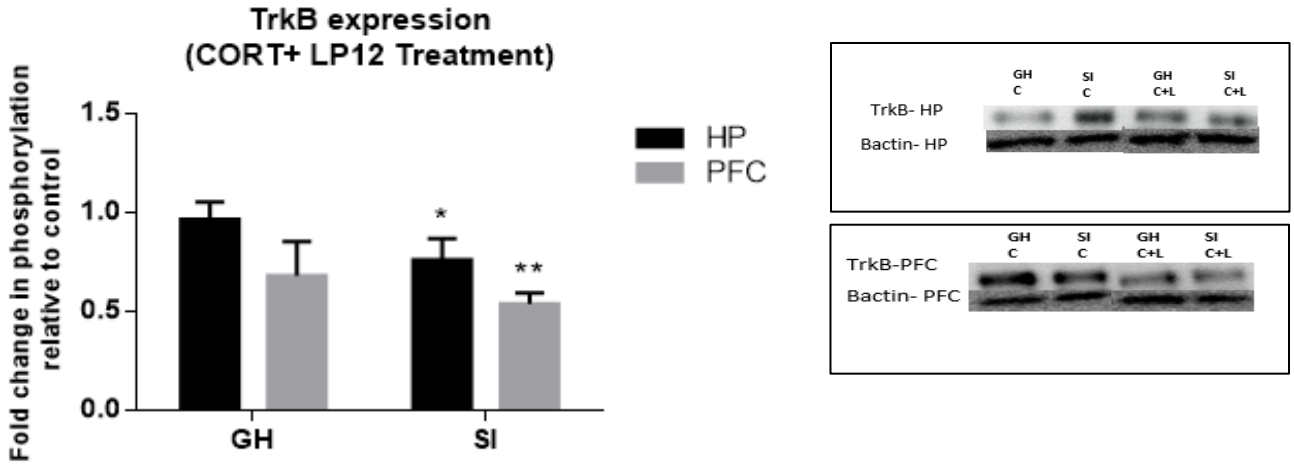


Figure 27. HP and PFC slices from male Sprague-Dawley rats were treated with 300 nM LP12 for 2 h and then 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 7, p = 0.58, d = 0.20; **GH-PFC**: n = 7, p = 0.11, d = 0.98, **SI-HP**: n = 8, p = 0.055, d = 1.13; **SI-PFC**: n = 6, p = 0.031, d = 4.94.

Most CORT+LP12 data show a general decrease in TrkB expression in both GH and SI groups; notably, the decrease is statistically significant in SI regions: SI-HP, (p = 0.055) and SI-PFC, (p = 0.031). The CORT+LP12 data reflect that, overall, TrkB expression is higher in the HP than the PFC in both GH and SI groups, which is corroborated by the low p values and high d values in GH and SI: HP vs. PFC comparisons (Table 8) suggesting that these outcomes are biologically relevant. A two-way ANOVA was conducted to explore if there was any interaction between housing and brain region, and no interaction was observed; however, a statistically significant effect of brain region (p = 0.0410) was observed.

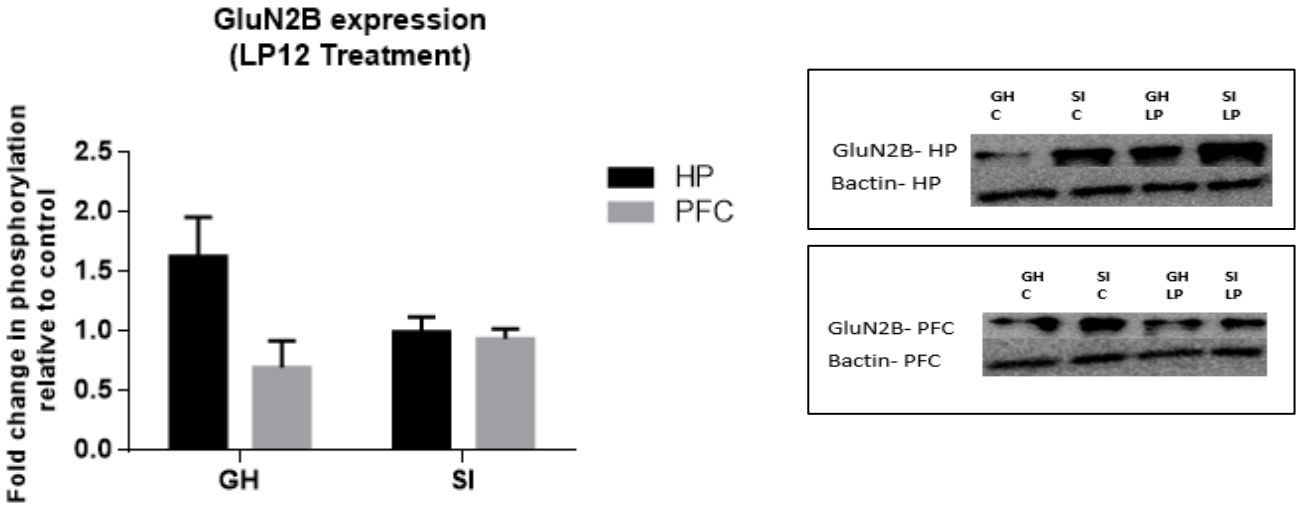


Figure 28. HP and PFC slices from male Sprague-Dawley rats were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin expression and are expressed as the fold change in GluN2B immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 0.16, d = 1.11; **GH-PFC**: n = 4, p = 0.36, d = 0.96; **SI-HP**: n = 6, p = 1.00, d = 0.024; **SI-PFC**: n = 5, p = 0.63, d = 0.46.

For GluN2B expression, the data suggest that LP12 treatment may have increased expression in the GH-HP group (p = 0.16, d = 1.11) and may have caused a decrease in the GH-PFC (p = 0.36, d = 0.96) group, but no change in either of the SI groups. Though the findings in the GH tissue are not statistically significant, the high effect sizes suggest that these data may be biologically relevant. Further, region and housing comparisons show a biologically relevant difference between GH-HP vs. GH-PFC (d = 1.45) and GH-HP vs. SI-HP (d = 1.05) groups (Table 8). A two-way ANOVA was conducted- to explore if there is any interaction between housing and

brain region, and no interaction was observed; however, a statistically significant effect of brain region ($p = 0.042$) was observed.

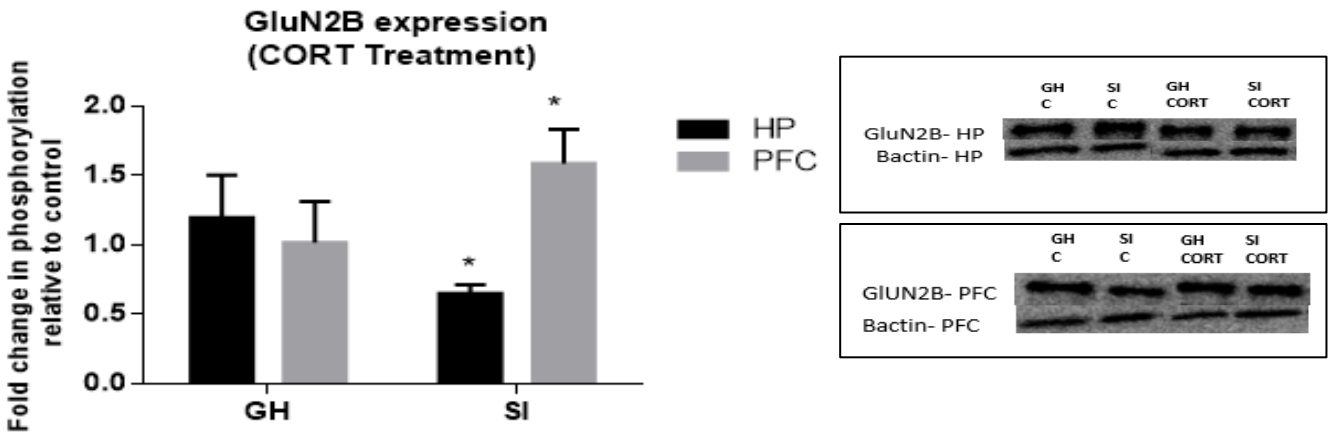


Figure 29. HP and PFC slices from male- Sprague-Dawley rats were treated with 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blots as described in the methods. Data were normalized to total β-actin expression and are expressed as the fold change in GluN2B immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 1.00, d = 0.38; **GH-PFC**: n = 4, p = 0.88, d = 0.040; **SI-HP**: n = 5, p = 0.063, d = 3.54; **SI-PFC**: n = 5, p = 0.063, d = 1.51.

The high p values and low d values suggest that CORT treatment had no effect on GluN2B expression in the GH groups; however, in the SI groups there was a significant decrease in HP expression and a significant increase in PFC expression; SI-HP: p = 0.063 and SI-PFC: p = 0.063. Further, region and housing comparisons (Table 8) show a biologically relevant difference between SI-HP vs. SI-PFC (d = 2.3) and GH-HP vs. SI-HP (d = 1.02), suggesting region differences in the SI group and housing specific differences in the HP, respectively. Further, a two-way ANOVA was conducted to explore if there was any interaction between housing and brain

region, and a statistically significant interaction of housing and brain region (p = 0.044) was observed.

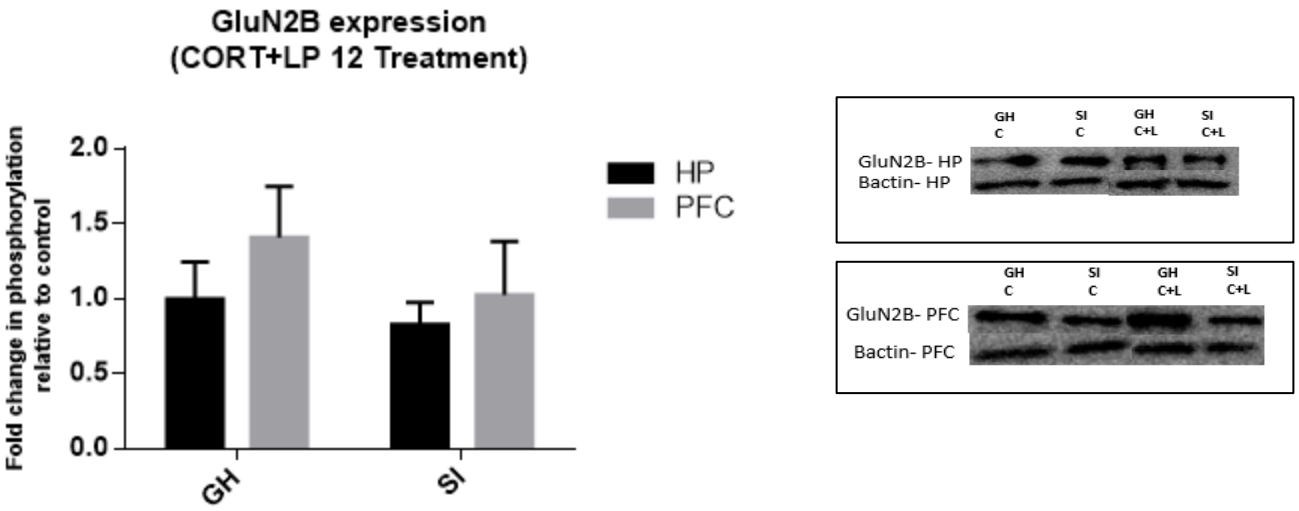


Figure 30. HP and PFC slices from male Sprague-Dawley rats were treated with 300 nM LP12 for 2 h and then 300 nM CORT for 1 h. Following treatment, the slices were homogenised and samples were evaluated by Western blots as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (*=p< 0.1 Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 5, p = 0.63, d = 0.01; **GH-PFC**: n = 3, p = 0.50, d = 0.96, **SI-HP**: n = 5, p = 0.31, d = 0.74; **SI-PFC**: n = 3, p = 1.00, d = 0.055.

The CORT+LP12 data suggest that there may be no change in GluN2B expression, except for the GH-PFC, in which a possible increase was observed (d = 0.96); however, the GH-PFC group also had the smallest sample size of the groups examined.

In summary, the findings show that LP, CORT and CORT+LP12 can affect TrkB and GluN2B expression in male brain slices. Though many of the observed changes appear to be driven by brain region, some effects are as a result of the housing condition. For TrkB, CORT+ LP12

treatment induces a decrease in TrkB expression in the SI-HP and SI-PFC groups; for GluN2B, CORT treatment significantly reduces SI-HP GluN2B expression, but significantly increases SI-PFC expression. In addition, in the GluN2B data a trend is observed in which individual treatment with either LP12, or CORT leads to changes in expression such that GH-HP > SI-HP and GH-PFC < SI-PFC, suggesting a housing specific difference.

	Comparisons	p-value	d-value
TrkB-LP12	GH- HP vs. GH-PFC	0.2785	0.41
	SI-HP vs. SI- PFC	0.3148	0.45
	GH- HP vs. SI- HP	0.4643	0.54
	GH- PFC vs. SI-PFC	0.8541	0.27
TrkB-CORT	GH- HP vs. GH-PFC	0.8541	0.31
	SI-HP vs. SI- PFC	0.8541	0.07
	GH- HP vs. SI- HP	0.5604	0.29
	GH- PFC vs. SI-PFC	0.9319	0.40
TrkB-CORT+LP12	GH- HP vs. GH-PFC	0.0728	0.78
	SI-HP vs. SI- PFC	0.0806	0.97
	GH- HP vs. SI- HP	0.2303	0.76
	GH- PFC vs. SI-PFC	0.7005	0.42
GluN2B-LP12	GH- HP vs. GH-PFC	0.1143	1.45
	SI-HP vs. SI- PFC	0.6277	0.22
	GH- HP vs. SI- HP	0.3052	1.05
	GH- PFC vs. SI-PFC	0.6825	0.69
GluN2B-CORT	GH- HP vs. GH-PFC	0.8286	0.27
	SI-HP vs. SI- PFC	0.0519	2.33
	GH- HP vs. SI- HP	0.1255	1.02
	GH- PFC vs. SI-PFC	0.1905	1.00
GluN2B-CORT+LP12	GH- HP vs. GH-PFC	0.3593	0.72
	SI-HP vs. SI- PFC	0.7857	0.40
	GH- HP vs. SI- HP	0.6667	0.37
	GH- PFC vs. SI-PFC	0.7000	0.63

Table 8. Region and housing comparisons in male TrkB and GluN2B expression. To explore region and housing specific differences in TrkB and GluN2B expression, Mann-Whitney ($\alpha = 0.05$ (Bonferroni correction)) and corresponding effect size calculations were performed.

4.5.3. LP12, CORT and CORT+LP12 activation of the TrkB-Y816 receptor in female rats

Based on preliminary studies, *ex vivo* HP and PFC slices were treated with 300 nM LP12 for 2 h, 300 nM CORT for 1 h, or a combined treatment of CORT+LP12.

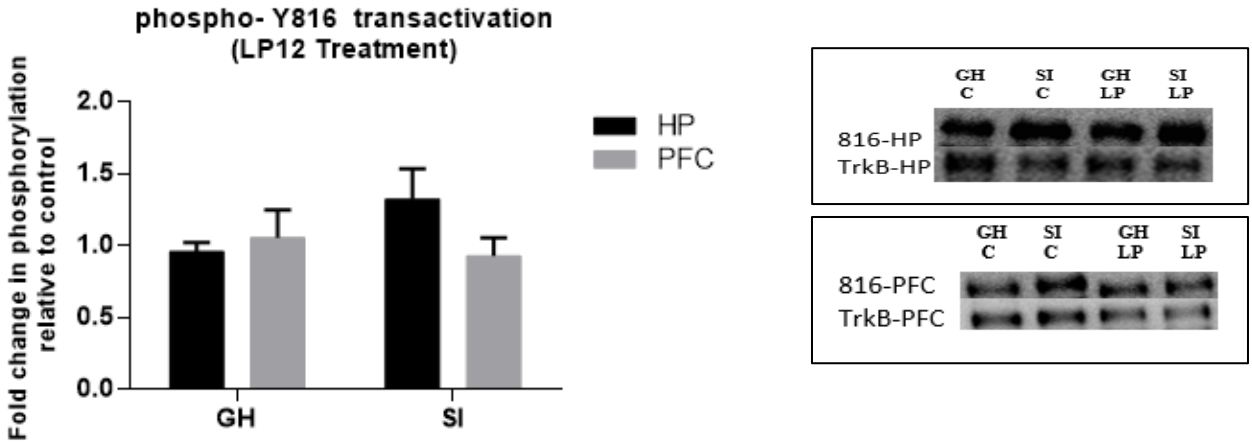


Figure 31. HP and PFC slices were treated with 300 nM LP12 for 2 h, the slices were then homogenized, and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 9, p = 0.65, d = 0.29; **GH-PFC**: n = 8, p = 1.00, d = 0.14; **SI-HP**: n = 8, p = 0.25, d = 0.76; **SI-PFC**: n = 8, p = 0.64, d = 0.27.

These data show that LP12 may induce transactivation in the SI-HP tissue, the p value (0.25) is not statistically significant, but a large effect size (d = 0.79) is observed. The d value suggests that treatment leads to an effect that is large enough to have biological relevance, but the p value does not allow us to strongly state that the effect is not chance-driven. Additionally, the small effect sizes and high p values for the remaining groups (shown in the Figure 31 legend) suggest that transactivation is not likely in the other three conditions. Since transactivation appeared to be present in the SI-HP group, but not the GH-HP group, this suggests that the HP

(but not the PFC) is sensitive to an isolation- (housing) difference. When considering intra-housing comparisons for SI-HP vs. SI-PFC, Table 9 shows a large effect size (d = 0.8). Intra-region differences (GH-HP vs. SI-HP) also display a large effect size (d = 0.82), suggesting the biological relevance of these outcomes. A two-way ANOVA was conducted to explore if there is any interaction between housing and brain region, and no interaction was observed.

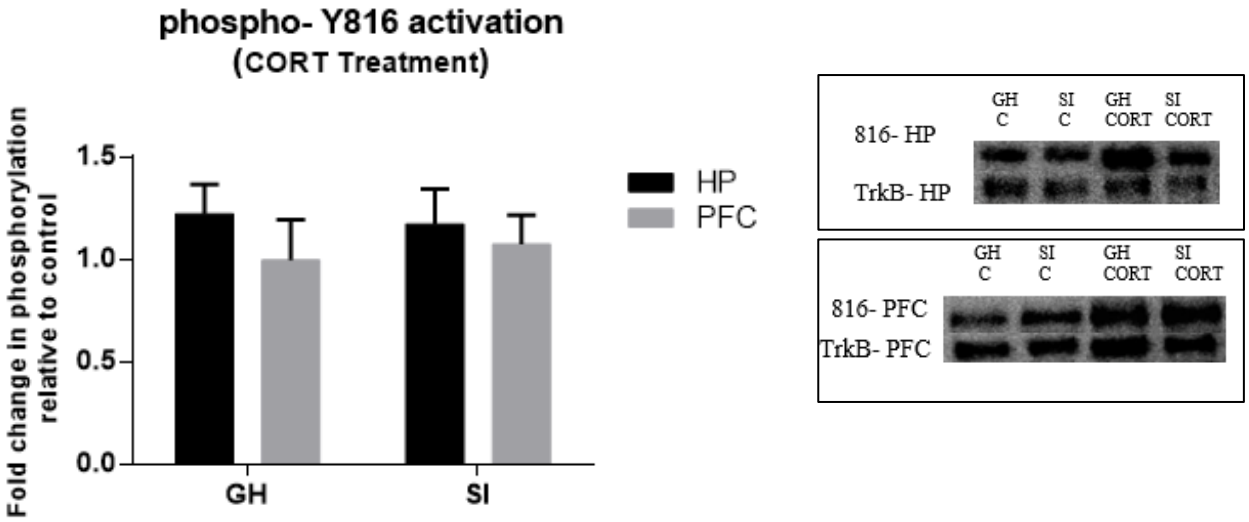


Figure 32. HP and PFC slices were treated with 300 nM CORT for 1 h, the slices were homogenized, and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 8, p = 0.15, d = 0.77; **GH-PFC**: n = 8, p = 0.95, d = 0.0088; **SI-HP**: n = 7, p = 0.38, d = 0.54; **SI-PFC**: n = 6, p = 1.00, d = 0.30.

These findings suggest that corticosterone may activate TrkB-Y816 in GH-HP tissue, however, activation is not observed in the PFC slices. Generally, these data show that there is no isolation specific change as both GH and SI slices from within the brain regions have similar

outcomes. The data in Table 9 suggest that there are no significant intra-housing or intra-region differences in TrkB-Y816 activation as a result of CORT treatment.

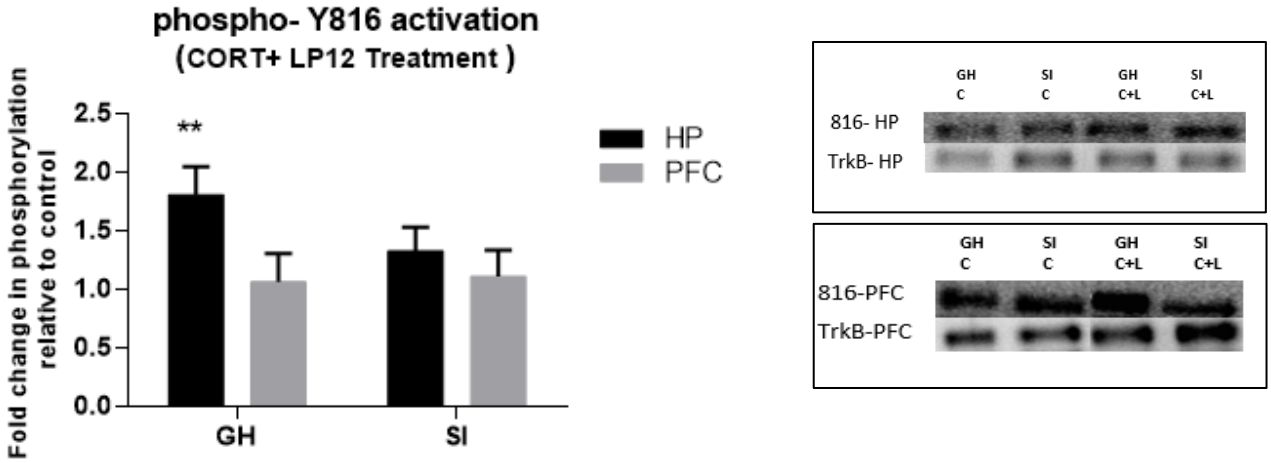


Figure 33. HP and PFC slices were treated with 300 nM LP12 for 2 h and then CORT for 1 h, the slices were homogenized, and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (* = $p < 0.1$, ** = $p < 0.05$; Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 9, p = 0.020, d = 1.58; **GH-PFC**: n = 8, p = 0.95, d = 0.15; **SI-HP**: n = 7, p = 0.22, d = 0.88; **SI-PFC**: n = 8, p = 0.74, d = 0.26.

The findings suggest that the combined CORT+LP12 treatment, increases TrkB-Y816 phosphorylation in GH- HP tissue and this outcome is statistically significant. This treatment may also increase SI- HP phosphorylation, however no changes were observed with GH-PFC or SI-PFC tissue. It is interesting to note that the profile of these data show similarity with those observed in figure 32 (female-CORT); the difference in the HP between the GH and SI groups suggests an interaction between housing condition and brain region. Whilst all other intra-housing and intra-

region comparisons show no notable difference in CORT+LP12 induced changes in TrkB-Y816 activation, the data in Table 9 suggest that the difference between GH-HP and GH-PFC ($p = 0.074$, $d = 1.051$) is biologically relevant. A two-way ANOVA was conducted to explore if there is any interaction between housing and brain region, and no interaction was observed; however, a statistically significant effect of brain region was observed ($p = 0.0495$)

In summary, in female animals LP12 transactivation may only occur in the SI-HP group, and CORT and CORT+LP12 outcomes show a trend in which TrkB-Y816 activation is more apparent in the HP, but not the PFC, groups. Interestingly, these outcomes match the finding in the male animals that TrkB-Y816 activation is primarily region specific.

	Comparison	p value	d value
TrkB-Y816 activation (LP12 treatment)	GH- HP vs. GH-PFC	0.93	0.23
	SI-HP vs. SI-PFC	0.27	0.80
	GH-HP vs. SI-HP	0.14	0.82
	GH-PFC vs. SI-PFC	0.85	0.27
TrkB-Y816 activation (CORT treatment)	GH-HP vs. GH-PFC	0.43	0.46
	SI-HP vs. SI-PFC	0.60	0.24
	GH-HP vs. SI-HP	0.75	0.11
	GH-PFC vs. SI-PFC	0.34	0.17
TrkB-Y816 activation (CORT+LP12 Treatment)	GH-HP vs. GH-PFC	0.074	1.051
	SI-HP vs. SI-PFC	0.46	0.37
	GH-HP vs. SI-HP	0.25	0.75
	GH-PFC vs. SI-PFC	0.70	0.069

Table 9. Region and housing comparisons in female TrkB-Y816 activation. To explore region and housing specific differences in TrkB-Y816 activation, the Mann-Whitney U test ($\alpha = 0.05$; Bonferroni correction) and corresponding effect size calculations were performed.

4.5.4. LP12, CORT and CORT+LP12 effect on TrkB and Glu2NB in female rats

Expression data show that there are changes in female TrkB and GluN2B expression based on LP12, CORT, or CORT+LP12 treatment.

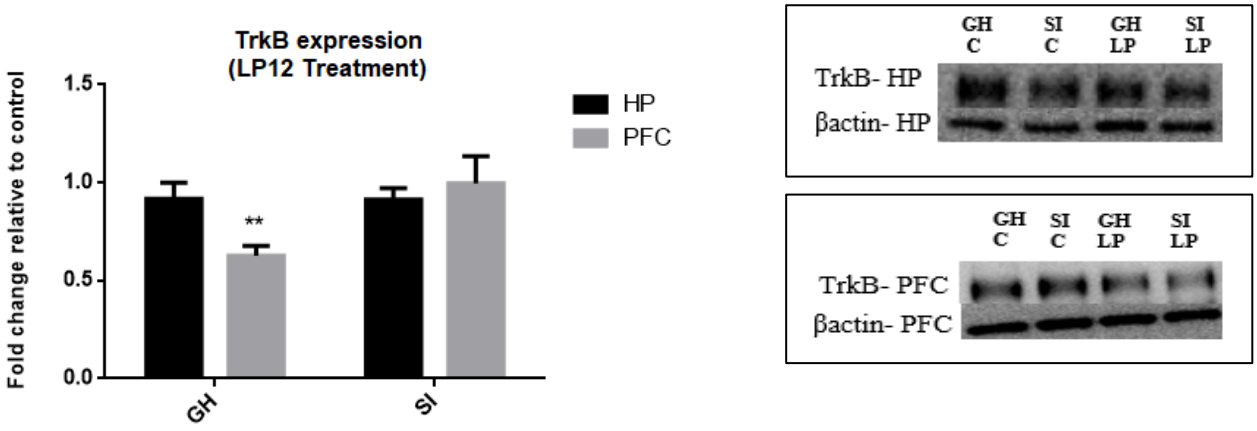


Figure 34. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (* = $p < 0.1$, ** = $p < 0.05$ Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 7, p = 0.38, d = 0.53; **GH-PFC**: n = 8, p = 0.0078, d = 3.75; **SI-HP**: n = 5, p = 0.19, d = 0.93; **SI-PFC**: n = 8, p = 0.95, d = 0.014.

LP12 treatment did not affect TrKB expression in the GH-HP, or SI-HP slices, however a 40% decrease, was observed in GH-PFC slices (p = 0.0078, d = 3.75) (but was not observed in SI-PFC slices). Additionally, a significant difference (p = 0.0059 d = 1.58) was observed between GH-HP and GH-PFC groups (Table 10), reflecting a region specific difference within the GH

slices. Further, the difference observed between GH-PFC and SI-PFC was also significant (p = 0.049, d = 1.25), suggesting a housing specific effect (Table 10).

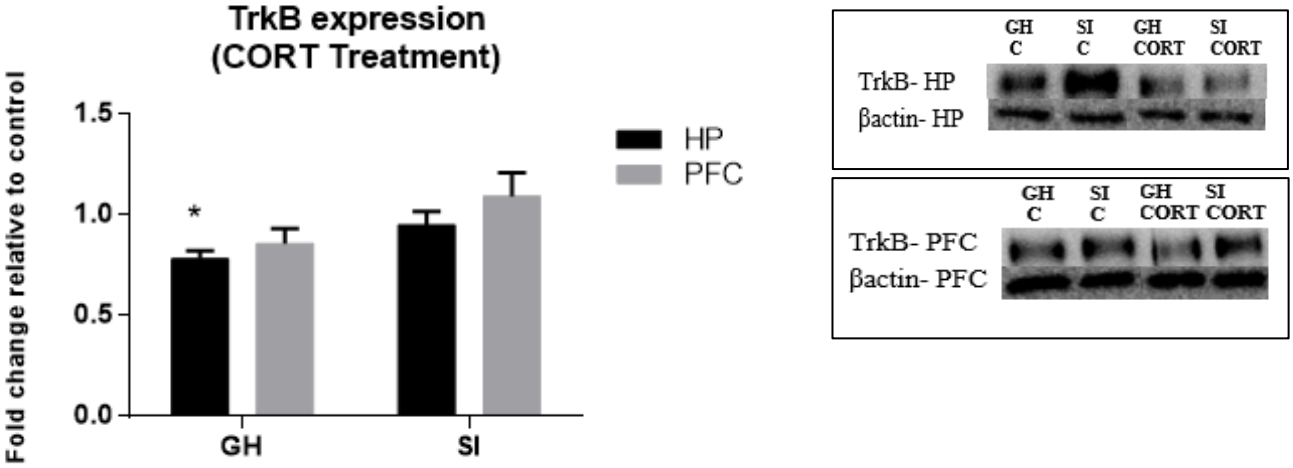


Figure 35. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (* = $p < 0.1$ Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 0.031, d = 3.10; **GH-PFC**: n = 9, p = 0.13, d = 0.90; **SI-HP**: n = 5, p = 0.63, d = 0.47; **SI-PFC**: n =8, p = 0.46, d = 0.41.

CORT treatment does not have an effect on TrkB expression in the SI-HP and SI-PFC groups, but may cause a modest decrease in expression (of 10-15%) in the GH-HP group (p = 0.031, d = 3.10) and a similar decrease is observed in the GH-PFC group. A two-way ANOVA was conducted to explore if there was any interaction between housing and brain region, and no interaction was observed; however, a statistically significant effect of housing was observed (p = 0.034).

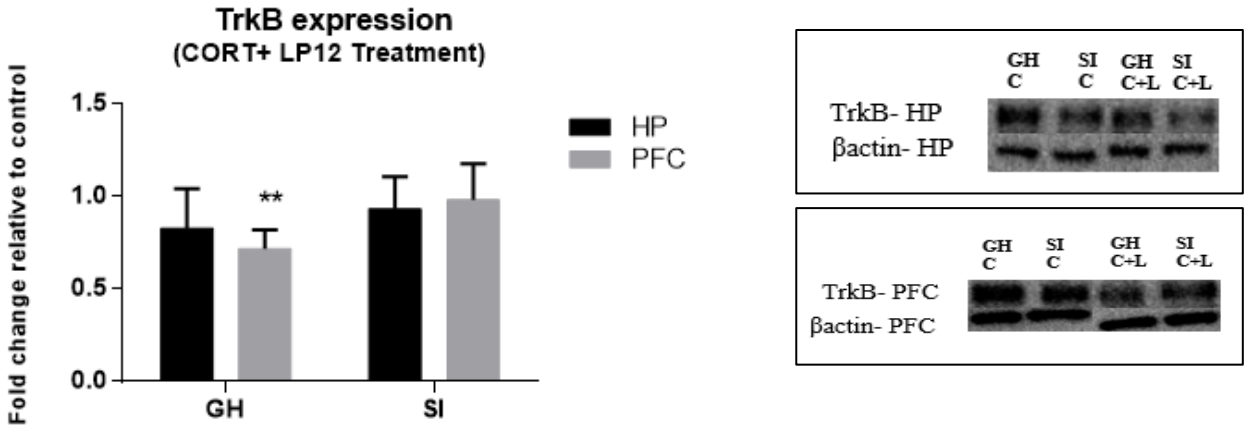


Figure 36. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM LP12 for 2 h followed by 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (* = $p < 0.1$, ** = $p < 0.05$ Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 7, p = 0.38, d = 0.42; **GH-PFC**: n = 9, p = 0.020, d = 1.29; **SI-HP**: n = 5, p = 1.00, d = 0.24; **SI-PFC**: n = 7, p = 0.94, d = 0.047.

CORT+LP12 data show a statistically significant decrease in TrkB expression in the GH-PFC group, while the high p values and low d values suggest no change in the GH-HP, SI-HP and SI-PFC groups.

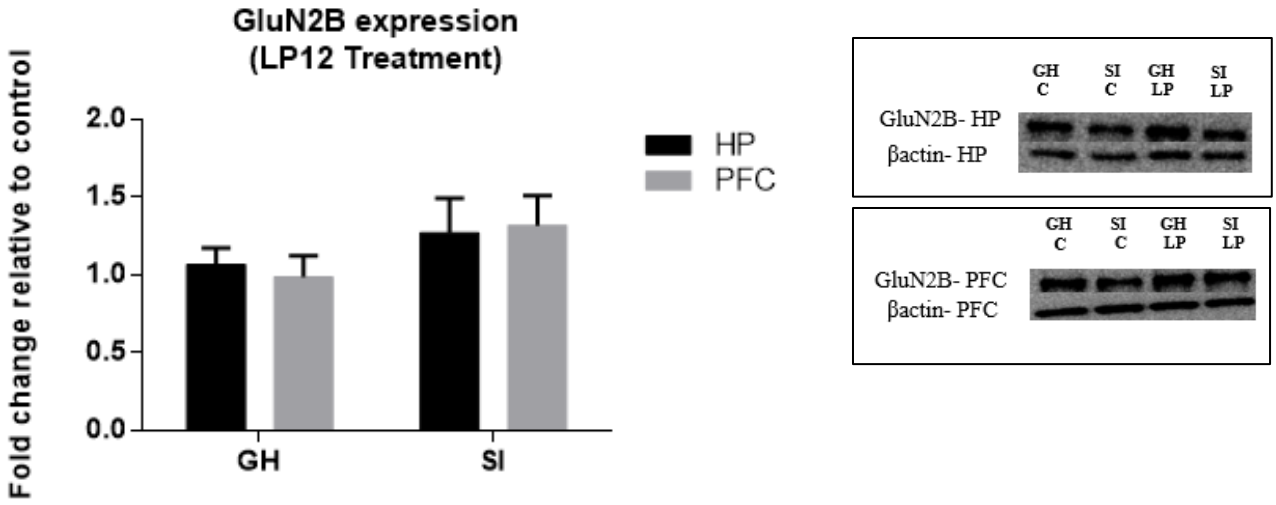


Figure 37. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM LP12 for 2 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in GluN2B immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 0.69, d = 0.35; **GH-PFC**: n = 8, p = 0.55, d = 0.053; **SI-HP**: n = 5, p = 0.31, d = 0.77; **SI-PFC**: n = 7, p = 0.30, d = 0.87.

LP12 treatment causes a modest increase in GluN2B expression, which given the relatively low p values and the high d values in the SI-HP and SI-PFC groups, may be biologically relevant. However, no effects are observed in the GH-HP and GH-PFC groups.

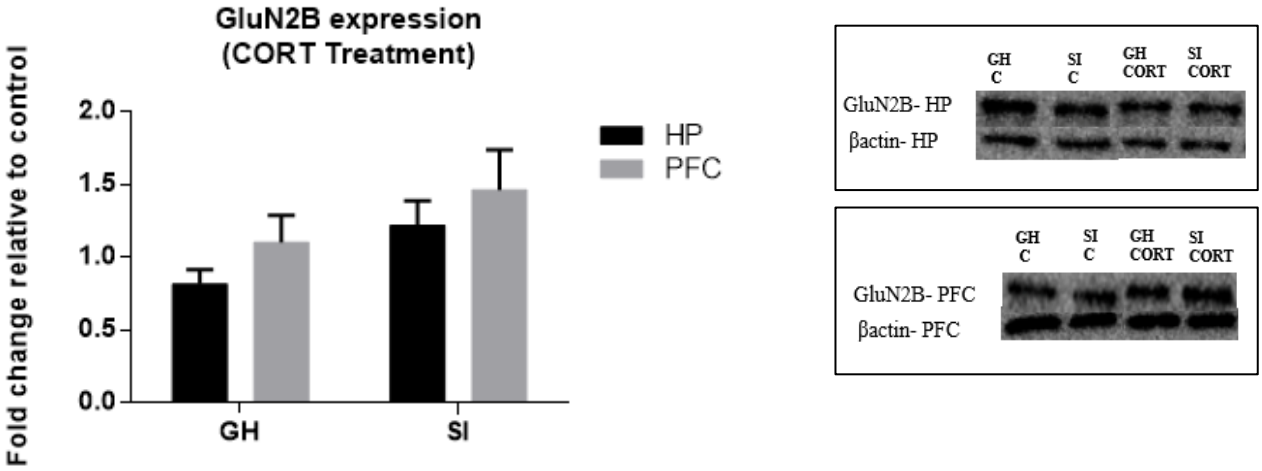


Figure 38. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in GluN2B immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 0.16, d = 1.10; **GH-PFC**: n = 8 , p = 0.74, d = 0.27; **SI-HP**: n = 4, p = 0.38, d = 0.91; **SI-PFC**: n = 7, p = 0.22, d = 0.89.

Several trends are observed with GluN2B after CORT treatment, with a modest increase in expression observed in the SI-HP and SI-PFC groups (d = 0.90 and 0.89, respectively), while there is a decrease in GH-HP, and GH-PFC remains close to baseline.

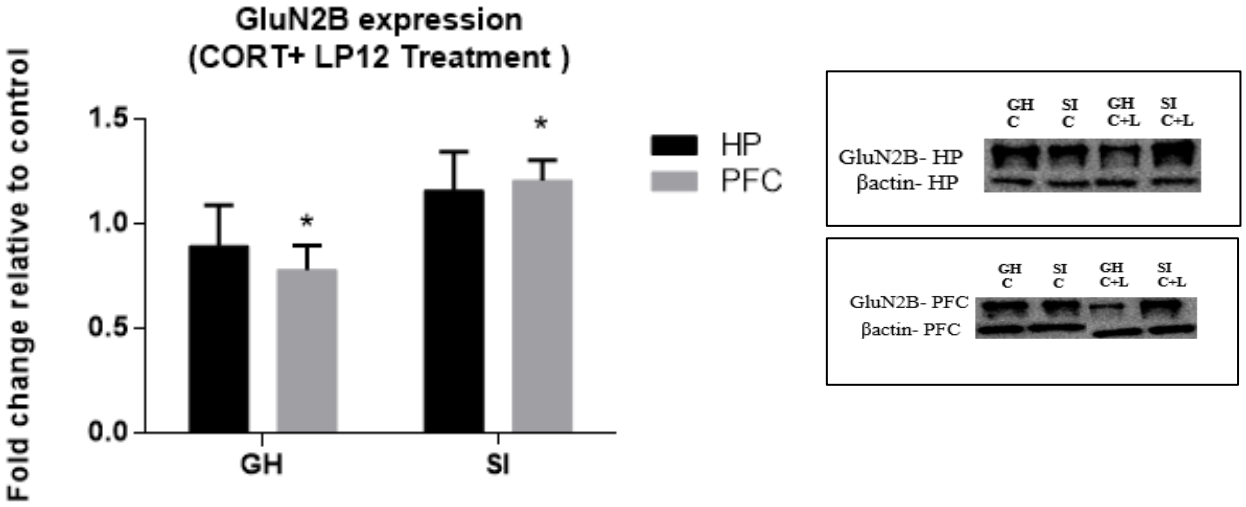


Figure 39. HP and PFC slices from female Sprague-Dawley rats were treated with 300 nM LP12 for 2 h and then 300 nM CORT for 1 h. Following treatment, the slices were homogenised and the samples were evaluated by Western blotting as described in methods. Data were normalized to total β-actin expression and are expressed as the fold change in TrkB immunoreactivity (* = $p < 0.1$, Wilcoxon Signed Rank Test) compared to non-treated control. **GH-HP**: n = 6, p = 0.56, d = 0.31; **GH-PFC**: n = 8, p = 0.078, d = 0.94; **SI-HP**: n = 3, p = 0.50, d = 0.69; **SI-PFC**: n = 7, p = 0.078, d = 1.13.

For CORT+LP12, a statistically significant increase was observed in GluN2B expression in the SI-PFC group, while a statistically significant decrease was observed in the GH-PFC group. Additionally, the difference observed between GH-PFC vs. SI-PFC was clearly significant (p = 0.029 and d = 1.44), suggesting a housing specific effect. A two-way ANOVA was conducted to explore if there was any interaction between housing and brain region, and no interaction was observed; however, a statistically significant effect of housing was observed (p = 0.038).

In summary, to varying degrees, LP12, CORT and CORT+LP12 treatments all cause a decrease in TrKB expression in the GH-PFC group. Additionally, the changes observed in GluN2B expression, suggest a housing specific effect, as there are differences in magnitude and/or direction between GH vs. SI outcomes.

	Comparisons	p-value	d-value
TrkB-LP12	GH- HP vs. GH-PFC	0.0059	1.58
	SI-HP vs. SI- PFC	0.8827	0.28
	GH- HP vs. SI- HP	0.6313	0.02
	GH- PFC vs. SI-PFC	0.0499	1.26
TrkB-CORT	GH- HP vs. GH-PFC	0.6549	0.44
	SI-HP vs. SI- PFC	0.2720	0.57
	GH- HP vs. SI- HP	0.0823	1.33
	GH- PFC vs. SI-PFC	0.1135	0.84
TrkB-CORT+LP12	GH- HP vs. GH-PFC	0.7292	0.24
	SI-HP vs. SI- PFC	0.9369	0.11
	GH- HP vs. SI- HP	0.4293	0.21
	GH- PFC vs. SI-PFC	0.2509	0.62
GluN2B-LP12	GH- HP vs. GH-PFC	0.3430	0.24
	SI-HP vs. SI- PFC	0.7096	0.09
	GH- HP vs. SI- HP	0.7706	0.52
	GH- PFC vs. SI-PFC	0.2303	0.73
GluN2B-CORT	GH- HP vs. GH-PFC	0.7193	0.70
	SI-HP vs. SI- PFC	0.5879	0.43
	GH- HP vs. SI- HP	0.1143	1.37
	GH- PFC vs. SI-PFC	0.3916	0.56
GluN2B-CORT+LP12	GH- HP vs. GH-PFC	0.8352	0.28
	SI-HP vs. SI- PFC	0.8667	0.17
	GH- HP vs. SI- HP	0.3810	0.65
	GH- PFC vs. SI-PFC	0.0289	1.44

Table 10. Region and housing comparisons in female TrkB and GluN2B expression. To explore region and housing specific differences in TrkB and GluN2B expression, the Mann-Whitney U test ($\alpha = 0.05$ with Bonferroni correction) and corresponding effect size calculations were performed.

4.5.5. Inter-sex comparisons

This section will focus solely on male vs. female comparisons in TrkB-Y816 transactivation, as well as CORT and CORT+LP12 TrkB-Y816 activation.

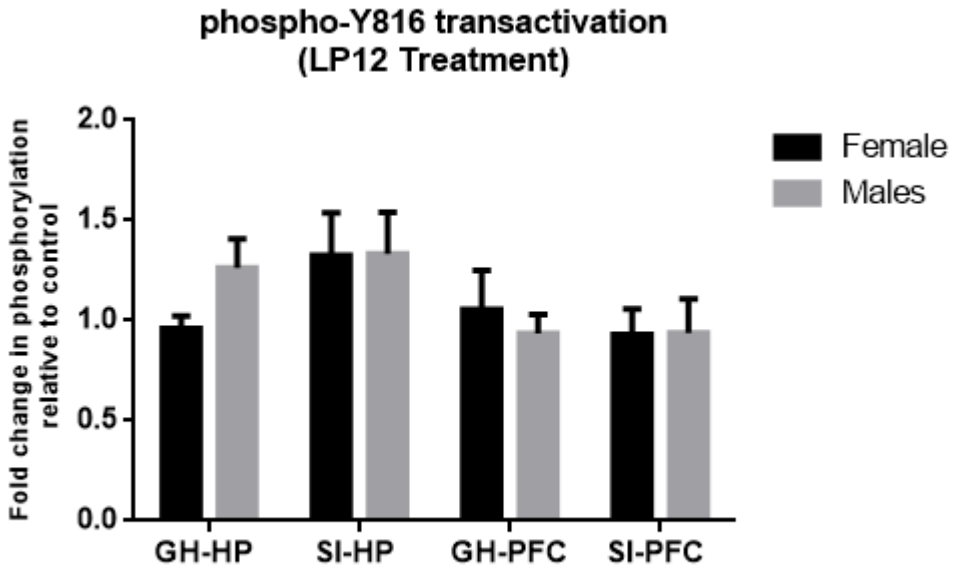


Figure 40. male and female comparisons for TrkB-Y816 transactivation. HP and PFC slices were treated with 300 nM LP12 for 2 h, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity. The Mann-Whitney U test was used to compare sex differences in each region (males vs. females): **GH-HP**, p = 0.13; **GH-PFC**, p = 0.86; **SI-HP**, p = 0.85; **SI-PFC**, p = 0.93.

The greatest difference was observed in the GH-HP, wherein TrkB-Y816 phosphorylation was greater in the male samples than the female samples. In the GH-PFC group, there was no transactivation observed in either male, or female tissue. Comparing male and female data for SI-HP and SI-PFC, it is evident that they have similar values, suggesting that the effect of isolation stress on TrkB-Y816 transactivation does not vary by sex. Furthermore, some degree of

transactivation seems to be present in SI-HP groups, but not the SI-PFC ones. Overall, transactivation is observed in male HP tissue from both housing conditions, but is observed only in female tissue from the SI-HP group. A two-way ANOVA was conducted to explore if there was any interaction between sex and housing within a given brain region (i.e., comparing males/females vs. GH/SI in either HP, or PFC); overall, no interactions were observed.

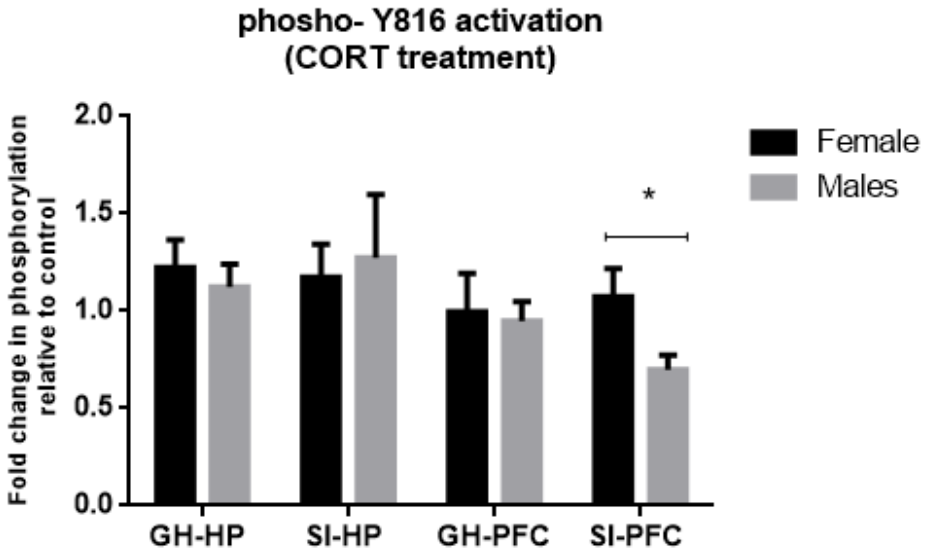


Figure 41. male and female comparisons for CORT activation of the TrkB-Y816 receptor. HP and PFC slices were treated with 300 nM CORT for 1 h, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. The Mann-Whitney U test (* = $p < 0.05$) was used to compare sex differences in each region (males vs. females): **GH-HP**, $p = 0.54$; **GH-PFC**: $p = 0.89$; **SI-HP**: $p = 0.93$; **SI-PFC**: $p = 0.013$.

The data suggest that the difference in TrkB-Y816 activation caused by CORT treatment is statistically significant in only the SI-PFC group ($p = 0.013$). Overall, the male and female comparisons for the GH-HP, SI-HP and GH-PFC conditions suggest that CORT activation of phospho-Y816 does not vary by sex. A two-way ANOVA was conducted to explore if there was any interaction between sex and housing within a given brain region (e.g., comparing males/females vs. GH/SI in HP); overall, no interaction was observed.

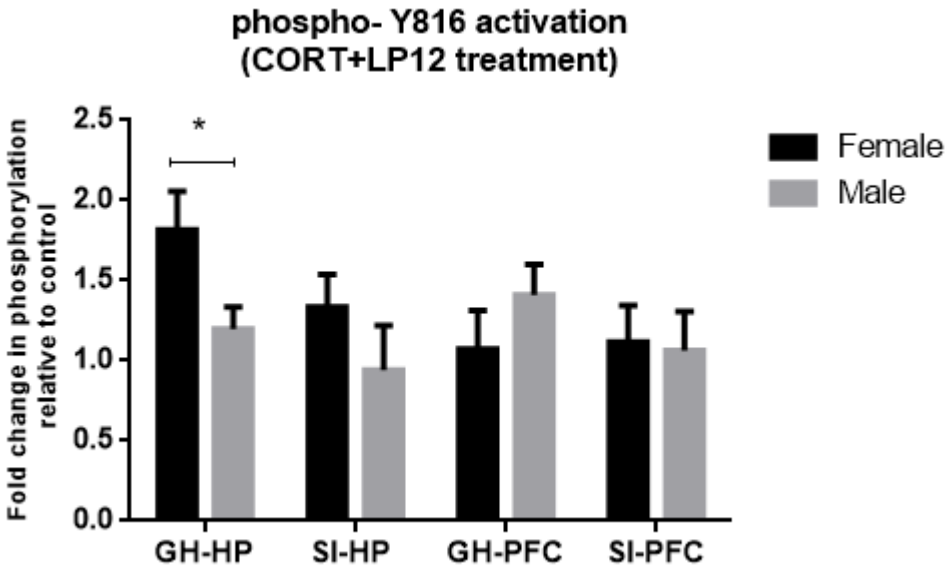


Figure 42. male and female comparisons for CORT+LP12 activation of the TrkB-Y816 residue. HP and PFC slices were treated with 300 nM CORT for 1 h, the slices were homogenized and samples evaluated by Western blots as described in the methods. The pTrkB data were normalized to total TrkB expression and are expressed as the fold change in phospho-Y816 immunoreactivity (Wilcoxon Signed Rank Test) compared to non-treated control. The Mann-Whitney U test (* = $p < 0.05$) was used to compare sex differences in each region (males vs. females): **GH-HP**, $p = 0.016$; **GH-PFC**: $p = 0.26$; **SI-HP**: $p = 0.23$; **SI-PFC**: $p = 0.99$.

These data suggest that female HP tissue has a higher CORT+LP12-induced TrkB-Y816 activation than male HP tissue, particularly in the GH-HP group ($p = 0.016$). The high p value ($p = 0.99$) in the SI-PFC suggests that there is no difference between male and female groups in that condition. Overall, these findings suggest that CORT+LP12 activation appears to differ by sex in all conditions except the SI-PFC. A two-way ANOVA was conducted to explore if there was any interaction between sex and housing within a given brain region (e.g., comparing males/females vs. GH/SI in HP), and, overall, no interaction was observed; however, a statistically significant effect of sex ($p = 0.025$) was observed in the HP.

In summary, LP12 and CORT data seem comparable across male and female tissue for most groups, suggesting that TrkB-Y816 activation is not sex specific. CORT+LP12 data were more variable, and overall outcomes differed by sex and region. Overall, the male and female comparison show that transactivation is observed in male HP (for both the GH and SI groups), but is only observed in female SI-HP tissue, suggesting that transactivation may be independent of sex, but influenced by brain region.

4.6. Discussion

The aim of this study was to evaluate within a given sex the impact of housing condition (group housed, GH vs. socially isolated, SI) on whether the treatments (LP12, CORT and CORT + LP12) affected transactivation, TrkB expression, or GluN2B expression. Additionally, I also sought to explore if there would be any region-specific differences in the outcomes. Based on previous findings, I hypothesized that I would observe: (i) sex-specific differences, (ii) region specific differences, (iii) that I would be able to replicate transactivation in male slices (in line with chapter 2 findings), and (iv) that I would observe transactivation in female slices.

4.6.1. TrkB-Y816 Activation in male animals

The male data suggest that transactivation may occur in the GH-HP and SI-HP regions but there is no evidence of such an effect in the PFC. The observed changes seem to be more dependent on brain region than housing condition, which suggests that social isolation may not affect LP12-induced 5-HT7 transactivation of the TrkB receptor observed at 2 h. It is imperative that this finding is not generalized to all forms of transactivation, as shifting parameters (for instance, treatment time, agonist concentration, initiating GPCR and target RTK) could change this outcome. To further clarify our outcomes, the sample size should be increased to see if the threshold for statistical significance will be crossed for some of our groups that displayed a null finding. Additionally, the magnitude of transactivation is generally modest which can make observing an effect challenging.

If the current findings do indeed turn out to be the trend, the benefit would be that transactivation, which is a neuroprotective pathway, could be stable, and that various molecular changes that come with stress would not hinder, or affect the pathway. Another question that needs to be explored concerns the factors contributing to the observed region specificity. For example,

could receptor abundance, co-expression of various receptors, or other mediators be important? These answers would be imperative to explore in future studies to gain a clearer understanding of the therapeutic relevance of this pathway. The combined CORT+LP12 treatment shows an increase in the GH-PFC condition, showing that co-treatment with two agonists that activate TrkB-Y816 has the capacity to induce PFC activation, even when the compounds had no effect on their own. These findings support our hypothesis, which stipulates that we would observe region specific differences in TrkB-Y816 activation in males.

4.6.2. TrkB-Y816 Activation in female rats

The hypothesis was that transactivation would be observed in female animals. The findings reflect that there appears to be LP12 transactivation in only one group (the SI-HP), which suggests a housing specific difference in the HP. In line with this argument, there is a possibility that this outcome could be a priming effect, in which transactivation is not observed in non-stressed rats, but activated in stress-exposed slices. Further, I observed that CORT modestly increases TrkB-Y816 activation in hippocampal slices from the GH. Given that TrkB-Y816 activation was not observed in the GH-HP following LP12 treatment (but it was observed in SI-HP), but that CORT treatment induced TrkB-Y816 activation in the GH-HP, acute stress could influence TrkB-Y816 activation in female HP tissue. CORT+LP12 treatment causes a significant increase in TrkB-Y816 in the GH-HP, and a modest increase in SI-HP, whilst no change was observed in the PFC; these outcomes appear to be independent of housing conditions. In line with what was observed in male tissue, it appears that there is a region-specific change in females as TrkB-Y816 activation is primarily observed in the HP and not the PFC.

Reflecting on my original hypothesis, it is important to note that this is the first time I attempted to characterize transactivation in female rat slices, and thus the parameters used were

those optimized in male animals. The rationale behind this decision was the desire to explore sex-specific differences, based on findings in Chapter 3 that demonstrated sex-specific differences in response to TrkB and GluN2B expression changes. Additionally, I hypothesized that since female brains possess the required receptors (5-HT7 and TrkB), I should be able to observe transactivation in female slices. Eighteen months of preliminary data were collected before transactivation could be optimized in male slices, meaning that even though I observed transactivation in one region, it is a notable achievement that it was achieved on the first attempt. By that same token, similar characterization efforts may be required with female tissue. Therefore, the limited transactivation might be a function of the experimental conditions rather than the biological relevance. Increasing the sample size and exploring various concentrations of transactivating partners would be beneficial in understanding this outcome, and help to adequately interpret the female findings.

4.6.3. Sex-specific differences

Male and female rats have been shown to have different thresholds in their capacity to cope with stress, as well as differences in the behavioral manifestation of their stress response [286]. Differences in stress adaptability reported between male and female animals could underlie different changes in neurotransmission and neuronal structures. Based on this rationale, I hypothesized that I would observe sex specific differences; specifically, I anticipated that there would be a difference in the magnitude, or direction of effects. Surprisingly, the male and female data were comparable across many of the conditions in LP and CORT treatments, suggesting the effect to be more dependent on brain region and/or housing condition than sex. Given our p value thresholds, for TrkB-Y816 transactivation we are unable to reject the null hypothesis that there is no difference between female and male tissue. For CORT treatment a significant difference was observed in SI-PFC; likewise, for CORT+LP12 a significant difference was observed in the GH-

HP, but overall no difference was observed for all other CORT, or CORT+LP12 treatment conditions.

Since CELSI is an established rodent stressor, which has been shown to induce behavioral, structural and neurochemical changes in rodent brains [223], this finding does not bring into question the ability of CELSI to induce stress, rather it might be a reflection on the stability of the TrkB transactivation pathway against stress.

4.6.4. Does stress have a priming effect on the brain, as is observed by CORT treatment?

One of my aims was to explore if CELSI, an *in vivo* model of stress, would have a "priming" effect on HP and PFC slices that would pre-dispose them to respond differently to an acute stressor compared to non-stressed slices. To address this question, I chose to maintain TrkB-Y816 as our read-out, due to evidence in the literature [254, 302, 303, 305] that TrkB can be activated by stress. Additionally, my own CELSI treatments in slice showed that CORT can activate TrkB. Looking at the CORT data for male and female slices respectively, there is some possibility of mild priming effects. For the female rats, this was discussed in section 4.6.2. For the male rats, social isolation appears to decrease TrkB-Y816 activation in both the HP and PFC, although this outcome is statistically significant only in the latter group. Further, when looking at the intra-region and intra-housing comparisons, the SI-HP vs. SI-PFC, and GH-PFC vs. SI-PFC have large effect sizes, suggesting that the magnitude of the observed differences may have biological significance.

4.6.5. GluN2B and TrkB expression data

TrkB expression

In males, LP12 treatment and CORT treatment generally had limited to no effect on TrkB expression. On the contrary, CORT+LP12 data shows a general decrease in TrkB expression

compared to untreated controls in both GH and SI groups. In females, LP12 treatment and CORT treatment either had no effect or induced a decrease in TrkB expression.

The outcomes in which treatment has no effect on expression may be an indicator that the given treatments (drug concentration and duration of treatment) are not sufficient to induce changes in receptor expression in slice. To better understand these results it would be essential to evaluate the corresponding changes in the BDNF protein, as for instance, this downregulation of TrkB could be in response to a significant increase in BDNF.

GluN2B expression

In males, LP12 treatment induces changes in GluN2B expression in the GH but no change was observed in the SI. Further, the data suggests that CORT has no effect on GluN2B expression in the GH group; but significant changes were observed in SI groups. The CORT+LP12 data suggest that there may be no change in GluN2B expression. In females, LP12 treatment generally had no effect on GluN2B expression. For CORT treatment as well as CORT+LP12 treatment opposite trends are observed in SI vs. GH groups for GluN2B expression. A predominant trend reflected in both the male and female data is the contrasting outcomes observed between GH and SI, suggesting that social isolation may have a priming effect that results in different outcomes to drug treatment.

4.6.6. Summary of findings

In summary, our findings of this study suggest that CELSI does not significantly affect transactivation, that there are minimal sex-specific effects, and limited priming effects, and that the major determinant of outcome is region. Overall, these outcomes make a powerful case for the therapeutic potential of a transactivation, since the pathway demonstrates such stability in the face of many factors.

5.0 Conclusions

This thesis had three major aims; the foundational goal was to replicate transactivation in a more physiologically relevant model. We achieved this by demonstrating reliable replication of transactivation with 300 nM LP12 for 2 h. This finding laid the foundation for subsequent studies that were focused on exploring the impact of stress on transactivation. The second aim of this thesis was to explore the impact of stress on plasticity related proteins, specifically, the TrkB and GluN2B proteins. Our findings show that CELSI affected TrkB and GluN2B receptor expression in a region and sex specific manner.

Having captured the changes in expression, the next step was to understand how CELSI affects receptor cross-talk. Given that TrkB and GluN2B play a significant role in neuronal development and are involved in many forms of receptor crosstalk, we chose to study their activation, or expression in the context of a cross-talk pathway. The pathway that we chose to study was the 5-HT7-TrkB transactivation pathway. Transactivation has been shown to be neuroprotective, so we sought to understand how CELSI affects transactivation, and if there are CELSI induced priming effects that would affect response to a later-stage stressor. Our findings suggest that CELSI does not significantly affect transactivation, that there are minimal sex-specific effects, and limited priming effects, and that the major determinant of outcome is brain region. These findings make a powerful case for the therapeutic potential of a transactivation, since the pathway demonstrates such stability in the face of many factors.

Overall, the four chapters of this thesis have addressed the questions that it sought to explore. Future directions include further studies to explore the mechanism by which stressors; specifically corticosterone induces transactivation-like effects.

References

1. Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Chapter 15, Signal-Transduction Pathways: An Introduction to Information Metabolism. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21205/
2. V. Di Liberto, G. Mudò, N. Belluardo, Crosstalk between receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCR) in the brain: Focus on heteroreceptor complexes and related functional neurotrophic effects. *Neuropharm.* 152, 67-77 (2019).
3. Daub H., Weiss F.U., Wallasch C., Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature. 1996;6565:557–560
4. Rosenbaum DM, Rasmussen SGF, Kobilka BK (2014) The structure and function of G-protein-coupled receptors Daniel. Nature 459:356–363 . doi: 10.1038/nature08144.The
5. Yamaguchi E (2005) GPCR-Based Drug Discovery. In: Shahi GS (ed) Biobusiness: A Strategic Perspective, Volume 1. Lulu Press, Morrisville, NC, pp 122–124
6. Ferguson S (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 53:1–24
7. González-Maeso J (2011) GPCR oligomers in pharmacology and signaling. Mol Brain 4:1–7 . doi: 10.1186/1756-6606-4-20
8. Van Eps N, Preininger AM, Alexander N, et al (2011) Interaction of a G protein with an activated receptor opens the interdomain interface in the alpha subunit. Proc Natl Acad Sci 108:9420–9424 . doi: 10.1073/pnas.1105810108
9. Neer EJ (1995) Heterotrimeric C proteins: Organizers of transmembrane signals. Cell 80:249–257 . doi: 10.1016/0092-8674(95)90407-7
10. Cattaneo F, Guerra G, Parisi M, et al (2014) Cell-surface receptors transactivation mediated by G protein-coupled receptors. Int J Mol Sci 15:19700–19728 . doi: 10.3390/ijms151119700
11. Schappi JM, Krbanjevic A, Rasenick MM (2014) Tubulin, actin and heterotrimeric G proteins: Coordination of signaling and structure. Biochim Biophys Acta - Biomembr 1838:674–681 . doi: 10.1016/j.bbamem.2013.08.026
12. Ahmed SM, Daulat AM, Meunier A, Angers S (2010) G protein βγ subunits regulate cell adhesion through Rap1a and its effector Radil. J Biol Chem 285:6538–6551 . doi: 10.1074/jbc.M109.069948

13. Filip M, Bader M (2009) Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacol Rep 61:761–77 . doi: 10.1016/S1734-1140(09)70132-X
14. Lesch KP, Zeng Y, Reif A, Gutknecht L (2003) Anxiety-related traits in mice with modified genes of the serotonergic pathway. Eur J Pharmacol 480:185–204 . doi: 10.1016/j.ejphar.2003.08.10
15. L (2006) Serotonin as a modulator of glutamate- and GABA-mediated neurotransmission: implications in physiological functions and in pathology. Curr Neuropharmacol 4:101–114 . doi: 10.2174/157015906776359540
16. Aznar, S., & Hervig, M. E.-S. (2016). The 5-HT2A serotonin receptor in executive function: Implications for neuropsychiatric and neurodegenerative diseases. Neuroscience & Biobehavioral Reviews, 64, 63–82. doi: 10.1016/j.neubiorev.2016.02.008
17. Bortolozzi, A., Díaz-Mataix, L., Scorza, M. C., Celada, P., & Artigas, F. (2005). The activation of 5-HT receptors in prefrontal cortex enhances dopaminergic activity. Journal of Neurochemistry, 95, 1597–607. doi: 10.1111/j.1471-4159.2005.03485.x
18. de Boer, S. F., & Koolhaas, J. M. (2005). 5-HT1A and 5-HT1B receptor agonists and aggression: a pharmacological challenge of the serotonin deficiency hypothesis. European Journal of Pharmacology, 526, 125–39. doi: 10.1016/j.ejphar.2005.09.065
19. Dietz, B. M., Mahady, G. B., Pauli, G. F., & Farnsworth, N. R. (2005). Valerian extract and valerenic acid are partial agonists of the 5-HT5a receptor in vitro. Molecular Brain Research, 138, 191–197. doi: 10.1016/j.molbrainres.2005.04.009
20. Fakhfouri, G., Mousavizadeh, K., Mehr, S. E., Dehpour, A. R., Zirak, M. R., Ghia, J.-E., & Rahimian, R. (2015). From chemotherapy-induced emesis to neuroprotection: Therapeutic opportunities for 5-HT3 receptor antagonists. Molecular Neurobiology, 52(3), 1670–1679. doi: 10.1007/s12035-014-8957-5
21. Filip M, Bader M (2009) Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacol Rep 61:761–77 . doi: 10.1016/S1734-1140(09)70132-X
22. Granados-Soto, V., Argüelles, C. F., Rocha-González, H. I., Godínez-Chaparro, B., Flores-Murrieta, F. J., & Villalón, C. M. (2010). The role of peripheral 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1Eand 5-HT1F serotonergic receptors in the reduction of nociception in rats.

Neuroscience, 165, 561–568. doi: 10.1016/j.neuroscience.2009.10.020

23. Hedlund PB (2010) The 5-HT7 receptor and disorders of the nervous system: an overview. Psychopharmacol (Berl) 206:345–354 . doi: 10.1007/s00213-009-1626-0
24. Howell LL, Cunningham KA (2015) Serotonin 5-HT2 receptor interactions with dopamine function: implications for therapeutics in cocaine use disorder. Pharmacol Rev 67:176–97 . doi: 10.1124/pr.114.009514
25. Jordan, S., Koprivica, V., Chen, R., Tottori, K., Kikuchi, T., & Altar, C. A. (2002). The antipsychotic aripiprazole is a potent, partial agonist at the human 5-HT1A receptor. European Journal of Pharmacology, 441(3), 137–40. doi: 10.1016/S0014-2999(02)01532-7
26. King, M. V., Marsden, C. A., & Fone, K. C. F. (2008). A role for the 5-HT(1A), 5-HT4 and 5-HT6 receptors in learning and memory. Trends in Pharmacological Sciences, 29(9), 482–92. doi: 10.1016/j.tips.2008.07.001
27. Lucas, G., Rymar, V. V., Du, J., Mnie-Filali, O., Bisgaard, C., Manta, S., … Debonnel, G. (2007). Serotonin(4) (5-HT(4)) receptor agonists are putative antidepressants with a rapid onset of action. Neuron, 55(5), 712–25. doi: 10.1016/j.neuron.2007.07.041
28. Millan, M. J., Marin, P., Kamal, M., Jockers, R., Chanrion, B., Labasque, M., … Mannoury la Cour, C. (2011). The melatonergic agonist and clinically active antidepressant, agomelatine, is a neutral antagonist at 5-HT(2C) receptors. The International Journal of Neuropsychopharmacology, 14(6), 768–83. doi: 10.1017/S1461145710001045
29. Millan, M. J., Newman-Tancredi, A., Audinot, V., Cussac, D., Lejeune, F., Nicolas, J. P., … Gobert, A. (2000). Agonist and antagonist actions of yohimbine as compared to fluparoxan at α2-adrenergic receptors (AR)s, serotonin (5-HT)(1A), 5-HT(1B), 5-HT(1D) and dopamine D2and D3receptors. Significance for the modulation of frontocortical monoaminergic transmission and depressive states. Synapse, 35(2), 79–95. doi: 10.1002/(SICI)1098-2396(200002)35:2<79::AID-SYN1>3.0.CO;2-X
30. Muchimapura, S., Mason, R., & Marsden, C. A. (2003). Effect of isolation rearing on pre- and post-synaptic serotonergic function in the rat dorsal hippocampus. Synapse (New York, N.Y.), 47(3), 209–17. doi: 10.1002/syn.10167
31. Newman-Tancredi A (2011) Biased agonism at serotonin 5-HT 1A receptors: preferential postsynaptic activity for improved therapy of CNS disorders. Neuropsychiatry (London) 1:149–164 . doi: 10.2217/npy.11.12

32. Nichols, D. E., Frescas, S., Marona-Lewicka, D., & Kurrasch-Orbaugh, D. M. (2002). Lysergamides of isomeric 2,4-dimethylazetidines map the binding orientation of the diethylamide moiety in the potent hallucinogenic agent N,N-diethyllysergamide (LSD). Journal of Medicinal Chemistry, 45(19), 4344–9. doi: 10.1021/jm020153s
33. Nikiforuk, A. (2012). Selective blockade of 5-HT7 receptors facilitates attentional set-shifting in stressed and control rats. Behavioural Brain Research, 226(1), 118–123. doi: 10.1016/j.bbr.2011.09.006
34. Puig, M. V., Watakabe, A., Ushimaru, M., Yamamori, T., & Kawaguchi, Y. (2010). Serotonin modulates fast-spiking interneuron and synchronous activity in the rat prefrontal cortex through 5-HT1A and 5-HT2A receptors. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 30(6), 2211–22. doi: 10.1523/JNEUROSCI.3335-09.2010
35. Reuter, U., Israel, H., & Neeb, L. (2015). The pharmacological profile and clinical prospects of the oral 5-HT1F receptor agonist lasmiditan in the acute treatment of migraine. Therapeutic Advances in Neurological Disorders, 8(1), 46–54. doi: 10.1177/1756285614562419
36. Riad, M., Garcia, S., Watkins, K. C., Jodoin, N., Doucet, E., Langlois, X., Descarries, L. (2000). Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. The Journal of Comparative Neurology, 417(2), 181–94. https://doi.org/10.1002/(SICI)1096-9861(20000207)417:2<181::AID-CNE4>3.0.CO;2-A
37. Riga, M. S., Sánchez, C., Celada, P., & Artigas, F. (2016). Involvement of 5-HT3 receptors in the action of vortioxetine in rat brain: Focus on glutamatergic and GABAergic neurotransmission. Neuropharmacology, 108, 73–81. doi: 10.1016/j.neuropharm.2016.04.023
38. Rizzi, C. A., Mierau, J., & Ladinsky, H. (1997). Regulation of plasma aldosterone levels by metoclopramide: a reappraisal of its mechanism from dopaminergic antagonism to serotonergic agonism. Neuropharmacology, 36(6), 763–8. doi: 10.1016/S0028-3908(97)00025-7
39. Shen, F., Smith, J. A. M., Chang, R., Bourdet, D. L., Tsuruda, P. R., Obedencio, G. P., & Beattie, D. T. (2011). 5-HT(4) receptor agonist mediated enhancement of cognitive function in vivo and amyloid precursor protein processing in vitro: A pharmacodynamic and pharmacokinetic assessment. Neuropharmacology, 61(1–2), 69–79. doi: 10.1016/j.neuropharm.2011.02.026

40. Steward, L. J., Ge, J., Stowe, R. L., Brown, D. C., Bruton, R. K., Stokes, P. R. A., & Barnes, N. M. (1996). Ability of 5-HT4 receptor ligands to modulate rat striatal dopamine release in vitro and in vivo. British Journal of Pharmacology, 117(1), 55–62. doi: 10.1111/j.1476-5381.1996.tb15154.x
41. Walstab, J., Rappold, G., & Niesler, B. (2010). 5-HT(3) receptors: role in disease and target of drugs. Pharmacology & Therapeutics, 128(1), 146–69. doi: 10.1016/j.pharmthera.2010.07.001
42. Wesołowska, A. (2010). Potential role of the 5-HT6 receptor in depression and anxiety: an overview of preclinical data. Pharmacological Reports, 62(4), 564–77. doi: 10.1016/S1734-1140(10)70315-7
43. Yan, Q. S., & Yan, S. E. (2001). Activation of 5-HT(1B/1D) receptors in the mesolimbic dopamine system increases dopamine release from the nucleus accumbens: a microdialysis study. European Journal of Pharmacology, 418(1–2), 55–64. doi: 10.1016/S0014-2999(01)00913-X
44. Zhang, G., & Stackman, R. W. (2015). The role of serotonin 5-HT2A receptors in memory and cognition. Frontiers in Pharmacology, 6(October), 225. doi: 10.3389/fphar.2015.00225
45. Mishra A, Singh S, Shukla S. Physiological and Functional Basis of Dopamine Receptors and Their Role in Neurogenesis: Possible Implication for Parkinson's disease. *J Exp Neurosci*. 2018;12:1179069518779829. Published 2018 May 31. doi:10.1177/1179069518779829
46. M. Jaber, S. W. Robinson, C. Missale, M. G. Caron, Dopamine receptors and brain function. *Neuropharmacology.* **35**, 1503-1519 (1996).
47. Nishi A, Fisone G, Snyder GL, et al. Regulation of Na+, K+-ATPase isoforms in rat neostriatum by dopamine and protein kinase C. J Neurochem. 1999;73:1492–1501
48. Yan Z, Feng J, Fienberg AA, Greengard P. D(2) dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. *Proc Natl Acad Sci U S A.* 1999;96(20):11607–11612. doi:10.1073/pnas.96.20.11607
49. Paul MK, Mukhopadhyay AK (2004) Tyrosine kinase – Role and significance in Cancer. Int J Med Sci 1:101–115 . doi: 10.7150/ijms.1.101
50. Lemmon MA, Schlessinger J (2011) Cell signaling by receptor-tyrosine kinases. Biochemistry 141:1117–1134 . doi: 10.1016/j.cell.2010.06.011.Cell

51. Schlessinger J (2014) Receptor Tyrosine Kinases : Legacy of the First Two Decades Receptor Tyrosine Kinases : Legacy of the First Two Decades. Cold Spring Harb Perspect Biol 6:1–13 . doi: 10.1101/cshperspect.a008912
52. Cargnello M, Roux PP (2011) Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiol Mol Biol Rev 75:50–83 . doi: 10.1128/MMBR.00031-10
53. Cantrell DA (2001) Phosphoinositide 3-kinase signalling pathways. J Cell Sci 114:1439–1445
54. Barbacid M (1994) The Trk family of neurotrophin receptors. J Neurobiol 25:1386–1403 . doi: 10.1002/neu.480251107
55. Gupta VK, You Y, Gupta VB, et al (2013) TrkB receptor signalling: Implications in neurodegenerative, psychiatric and proliferative disorders. Int J Mol Sci 14:10122–10142 . doi: 10.3390/ijms140510122
56. Kruk JS, Vasefi MS, Heikkila JJ, Beazely MA (2013) Reactive Oxygen Species Are Required for 5-HT-Induced Transactivation of Neuronal Platelet-Derived Growth Factor and TrkB Receptors, but Not for ERK1/2 Activation. PLoS One 8:1–11 . doi: 10.1371/journal.pone.0077027
57. Vidaurre OG, Gascón S, Deogracias R, et al (2012) Imbalance of neurotrophin receptor isoforms TrkB-FL/TrkB-T1 induces neuronal death in excitotoxicity. Cell Death Dis 3:1–12 . doi: 10.1038/cddis.2011.143
58. Luberg K, Wong J, Weickert CS, Timmusk T (2010) Human TrkB gene: Novel alternative transcripts, protein isoforms and expression pattern in the prefrontal cerebral cortex during postnatal development. J Neurochem 113:952–964 . doi: 10.1111/j.1471-4159.2010.06662.x
59. Huang EJ, Reichardt LF (2001) Neurotrophins: Roles in Neuronal Development and Function. Annu Rev Neurosci 24:677–736 . doi: 10.1146/annurev.neuro.24.1.677
60. Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc B Biol Sci 361:1545–1564 . doi: 10.1098/rstb.2006.1894
61. Kim MS, Lee WS, Jeong J, et al (2015) Induction of metastatic potential by TrkB via activation of IL6/ JAK2/STAT3 and PI3K/AKT signaling in breast cancer. Oncotarget 6:40158–40171
62. Hashmi F, Liu M, Shen S, Qiao L-Y (2016) Phospholipase C gamma mediates endogenous brain-derived neurotrophic factor-regulated calcitonin gene-related peptide expression in

colitis-induced visceral pain. Mol Pain 12:174480691665708 . doi: 10.1177/1744806916657088

63. Morooka T, Nishida E (1998) Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J Biol Chem 273:24285–24288 . doi: 10.1074/jbc.273.38.24285
64. Angoa-Pérez M, Anneken JH, Kuhn DM (2017) Journal of Psychiatry and Psychiatric Pathophysiology of Psychiatric and Neurological Disorders. 1:
65. Harada C, Azuchi Y, Noro T, et al (2015) TrkB signaling in retinal glia stimulates neuroprotection after optic nerve injury. Am J Pathol 185:3238–3247 . doi: 10.1016/j.ajpath.2015.08.005
66. Magnusson P.U., Looman C., Ahgren A., Wu Y., Claesson-Welsh L., Heuchel R.L. Platelet-derived growth factor receptor-beta constitutive activity promotes angiogenesis *in vivo* and *in vitro*. Arterioscler. Thromb. Vasc. Biol. 2007;27:2142–2149
67. Chen PH, Chen X, He X. Platelet-derived growth factors and their receptors: structural and functional perspectives. *Biochim Biophys Acta*. 2013;1834(10):2176–2186. doi:10.1016/j.bbapap.2012.10.015
68. Rajkumar VS, Shiwen X, Bostrom M, et al. Platelet-derived growth factor-beta receptor activation is essential for fibroblast and pericyte recruitment during cutaneous wound healing. *Am J Pathol*. 2006;169(6):2254–2265. doi:10.2353/ajpath.2006.060196
69. Alvarez R.H., Kantarjian H.M., Cortes J.E. Biology of platelet-derived growth factor and its involvement in disease. Mayo Clin Proc. 2006 Sep;81(9):1241-57.doi: 10.4065/81.9.1241
70. Scaltriti M and Baselga J. The Epidermal Growth Factor Receptor Pathway: A Model for Targeted Therapy. Clin Cancer Res September 15 2006 (12) (18) 5268-5272; DOI: 10.1158/1078-0432.CCR-05-1554
71. A. Wells, EGF receptor. *The International Journal of Biochemistry & Cell Biology.* **31**, 637-643 (1999).
72. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cell Mol Life Sci*. 2008;65(10):1566–1584. doi:10.1007/s00018-008-7440-8
73. Bethune G, Bethune D, Ridgway N, Xu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. *J Thorac Dis*. 2010;2(1):48–51.

74. Makki N, Thiel KW, Miller FJ Jr. The epidermal growth factor receptor and its ligands in cardiovascular disease. *Int J Mol Sci*. 2013;14(10):20597–20613. Published 2013 Oct 15. doi:10.3390/ijms141020597
75. Li Z, Li Y, Overstreet JM, et al. Inhibition of epidermal growth factor receptor activation is associated with improved diabetic nephropathy and insulin resistance in Type 2 diabetes. Diabetes. 2018;67(9):1847–1857. doi:10.2337/db17-1513
76. Kruk JS, Vasefi MS, Gondora N, et al (2015) Fluoxetine-induced transactivation of the platelet-derived growth factor type β receptor reveals a novel heterologous desensitization process. Mol Cell Neurosci 65:45–51 . doi: 10.1016/j.mcn.2015.02.013
77. Adrain C, Freeman M (2014) Regulation of receptor tyrosine kinase ligand processing. Cold Spring Harb Perspect Biol 6:1–17 . doi: 10.1101/cshperspect.a008995
78. Saito S, Frank GD, Mifune M, et al (2002) Ligand-independent trans -activation of the platelet-derived growth factor receptor by reactive oxygen species requires protein kinase C-δ and c-Src. J Biol Chem 277:44695–44700 . doi: 10.1074/jbc.M208332200
79. Kausar S, Wang F, Cui H. The role of mitochondria in reactive oxygen species generation and its implications for neurodegenerative diseases. *Cells*. 2018;7(12):274. Published 2018 Dec 17. doi:10.3390/cells7120274
80. Popa-Wagner A , Mitran S, Sivanesan S, Chang E et al (2013) ROS and Brain Diseases: The Good, the Bad, and the Ugly. Oxidative Medicine and Cellular Longevity.https://doi.org/10.1155/2013/963520
81. Samarajeewa A, Goldemann L, Vasefi MS, et al. 5-HT7 receptor activation promotes an increase in TrkB receptor expression and phosphorylation. *Front Behav Neurosci*. 2014;8:391. Published 2014 Nov 7. doi:10.3389/fnbeh.2014.00391
82. Kruk JS. Transactivation of platelet-derived growth factor receptor type β: Mechanisms and potential relevance in neurobiology, University of Waterloo; 2013.
83. Tanimoto T, Lungu AO, Berk BC. Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells. *Circulation research.* Apr 30 2004;94(8):1050-1058.
84. Mellor H, Parker PJ. The extended protein kinase C superfamily. *The Biochemical journal.* Jun 1 1998;332 (Pt 2):281-292.
85. Liu B, Wu D. Analysis of G protein-mediated activation of phospholipase C in cultured cells.

Methods in molecular biology (Clifton, N.J.). 2004;237:99-102.

86. Zeng L, Webster SV, Newton PM. The biology of protein kinase C. *Advances in experimental medicine and biology.* 2012;740:639-661.
87. Newton AC. Protein kinase C: structure, function, and regulation. *The Journal of biological chemistry.* Dec 1 1995;270(48):28495-28498.
88. Chen K, Thomas SR, Albano A, Murphy MP, Keaney JF, Jr. Mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling. *The Journal of biological chemistry.* Aug 13 2004;279(33):35079-35086.
89. Wang Z (2016) Transactivation of epidermal growth factor receptor by g protein-coupled receptors: Recent progress, challenges and future research. Int J Mol Sci 17: . doi: 10.3390/ijms17010095
90. Klein T, Bischoff R (2011) Physiology and pathophysiology of matrix metalloproteases. Amino Acids 41:271–290 . doi: 10.1007/s00726-010-0689-x
91. Leserer M, Gschwind A, Ullrich A (2000) Transactivation. IUMBF Life 49:405–409
92. George AJ, Hannan RD, Thomas WG (2013) Unravelling the molecular complexity of GPCR-mediated EGFR transactivation using functional genomics approaches. FEBS J. 280:5258–5268
93. Kagias K, Nehammer C, Pocock R (2012) Neuronal responses to physiological stress. Front Genet 3:1–17 . doi: 10.3389/fgene.2012.00222
94. Herman JP (2013) Neural control of chronic stress adaptation. Front Behav Neurosci 7:1–12 . doi: 10.3389/fnbeh.2013.00061
95. Myers B, McKlveen JM, Herman JP (2012) Neural regulation of the stress response: The many faces of feedback. Cell Mol Neurobiol 32:683–694 . doi: 10.1007/s10571-012-9801-y
96. Keller-Wood ME, Dallman MF (1984) Corticosteriod inhibition of ACTH secretion. Endocr Rev 5:1–24 . doi: 10.1210/edrv-5-1-1
97. McEwen, B., Bowles, N., Gray, J. *et al.* Mechanisms of stress in the brain. *Nat Neurosci* **18,** 1353–1363 (2015) doi:10.1038/nn.4086
98. Yang S, Roselli F, Patchev A, Yu S et al (2013) Non-receptor-tyrosine Kinases Integrate Fast Glucocorticoid Signaling in Hippocampal Neurons. The journal of biological chemistry vol. 288, no. 33, pp. 23725–23739.

99. Popoli M, Yan Z, McEwen BS, Sanacora G (2012) The stressed synapse: The impact of stress and glucocorticoids on glutamate transmission. Nat Rev Neurosci 13:22–37 . doi: 10.1038/nrn3138

100. Vyas S, Rodrigues AJ, Silva JM, et al (2016) Chronic stress and glucocorticoids: From neuronal plasticity to neurodegeneration. Neural Plast 2016: . doi: 10.1155/2016/6391686

101. J. J. Radley et al., Reversibility of apical dendritic retraction in the rat medial prefrontal cortex following repeated stress. Experimental Neurology. 196, 199-203 (2005).

102. Harris A, Seckl J (2011) Glucocorticoids, prenatal stress and the programming of disease. Hormones and Behavior 59 (2011) 279–289

103. Pechtel P, Pizzagalli DA. Effects of early life stress on cognitive and affective function: an integrated review of human literature. *Psychopharmacology (Berl)*. 2011;214(1):55–70. doi:10.1007/s00213-010-2009-2

104. Serra MP, Poddighe L, Boi M, et al. Effect of Acute Stress on the Expression of BDNF, trkB, and PSA-NCAM in the Hippocampus of the Roman Rats: A Genetic Model of Vulnerability/Resistance to Stress-Induced Depression. *Int J Mol Sci*. 2018;19(12):3745. Published 2018 Nov 24. doi:10.3390/ijms19123745

105. Budziszewska B, Jaworska-Feil L, Kajta M, Lasoń W. Antidepressant drugs inhibit glucocorticoid receptor-mediated gene transcription - a possible mechanism. Br J Pharmacol. 2000;130(6):1385–1393. doi:10.1038/sj.bjp.0703445

106. Schaaf, M.J., Hoetelmans, R.W., de Kloet, E.R. and Vreugdenhil, E. (1997), Corticosterone regulates expression of BDNF and trkB but not NT-3 and trkC mRNA in the rat hippocampus. J. Neurosci. Res., 48: 334-341. doi:10.1002/(SICI)1097-4547(19970515)48:4<334::AID-JNR5>3.0.CO;2-C

107. M. J. M. Schaaf, J. de Jong, E. R. de Kloet, E. Vreugdenhil, Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Research.* **813**, 112-120 (1998).

108. Barfield ET, Gerber KJ, Zimmermann KS, Ressler KJ, Parsons RG, Gourley SL. Regulation of actions and habits by ventral hippocampal trkB and adolescent corticosteroid exposure. *PLoS Biol*. 2017;15(11):e2003000. Published 2017 Nov 29. doi:10.1371/journal.pbio.2003000

109. Numakawa T, Kumamaru E, Adachi N, Yagasaki Y, Izumi A, Kunugi H. Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for

glutamate release via a glutamate transporter. *Proc Natl Acad Sci U S A*. 2009;106(2):647–652. doi:10.1073/pnas.0800888106

110. Galán M, Kassan M, Choi SK, et al. A novel role for epidermal growth factor receptor tyrosine kinase and its downstream endoplasmic reticulum stress in cardiac damage and microvascular dysfunction in type 1 diabetes mellitus. *Hypertension*. 2012;60(1):71–80. doi:10.1161/HYPERTENSIONAHA.112.192500

111. He P., Shen N., Gao G et al (2016) Periodic Mechanical Stress Activates PKCδ-Dependent EGFR Mitogenic Signals in Rat Chondrocytes via PI3K-Akt and ERK1/2. Cell Physiol Biochem 2016;39:1281-1294. https://doi.org/10.1159/000447833

112. Adell A, Casanovas JM, Artigas F (1997) Comparative Study in the Rat of the Actions of Different Types of Stress on the Release of 5-HT in Raphe Nuclei and Forebrain Areas. Neuropharmacology 36:735–741

113. Linthorst ACE, Peñalva RG, Flachskamm C, et al (2002) Forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotropin-releasing hormone receptor-dependent mechanism. Eur J Neurosci 16:2441–2452 . doi: 10.1046/j.1460-9568.2002.02400.x

114. Briones-Aranda A, López-Rubalcava C, Picazo O (2002) Influence of forced swimming-induced stress on the anxiolytic-like effect of 5HT1A agents in mice. Psychopharmacology (Berl) 162:147–155 . doi: 10.1007/s00213-002-1046-x

115. Borroto-Escuela *et al.*, Disturbances in the FGFR1-5-HT1A heteroreceptor complexes in the Raphe-hippocampal 5-HT system develop in a genetic rat model of depression. *Frontiers in Cellular Neuroscience.* **11**, 309 (2017).

116. Briones-Aranda A, Rocha L, Picazo O (2005) Influence of forced swimming stress on 5-HT1Areceptors and serotonin levels in mouse brain. Prog Neuro-Psychopharmacology Biol Psychiatry 29:275–281 . doi: 10.1016/j.pnpbp.2004.11.011

117. Jaggar M, Weisstaub N, Gingrich JA, Vaidya VA (2017) 5-HT2A receptor deficiency alters the metabolic and transcriptional, but not the behavioral, consequences of chronic unpredictable stress. Neurobiol Stress 7:89–102 . doi: 10.1016/j.ynstr.2017.06.001

118. Jiang X, Xing G, Yang C, et al (2009) Stress Impairs 5-HT2A Receptor-Mediated Serotonergic Facilitation of GABA Release in Juvenile Rat Basolateral Amygdala. Neuropsychopharmacology 34:410–423 . doi: 10.1038/npp.2008.71

119. Gupta D, Radhakrishnan M, Kurhe Y (2014) 5HT3receptor antagonist (ondansetron) reverses depressive behavior evoked by chronic unpredictable stress in mice: Modulation of hypothalamic-pituitary-adrenocortical and brain serotonergic system. Pharmacol Biochem Behav 124:129–136 . doi: 10.1016/j.pbb.2014.05.024

120. Lucas G, Rymar V V., Du J, et al (2007) Serotonin4(5-HT4) Receptor Agonists Are Putative Antidepressants with a Rapid Onset of Action. Neuron 55:712–725 . doi: 10.1016/j.neuron.2007.07.041

121. Goodfellow NM, Bailey CDC, Lambe EK (2012) The Native Serotonin 5-HT5A Receptor: Electrophysiological Characterization in Rodent Cortex and 5-HT1A-Mediated Compensatory Plasticity in the Knock-Out Mouse. J Neurosci 32:5804–5809 . doi: 10.1523/JNEUROSCI.4849-11.2012

122. Beckman D, Santos LE, Americo TA, et al (2015) Prion protein modulates monoaminergic systems and depressive-like behavior in mice. J Biol Chem 290:20488–20498 . doi: 10.1074/jbc.M115.666156

123. Scheggi S, Marchese G, Borsini F, et al (2011) Effects of the 5-HT6 receptor agonist ST 1936 on depression- and anhedonia-like experimental models. Behav Brain Res 224:35–43 . doi: 10.1016/j.bbr.2011.05.019

124. Hedlund PB (2009) The 5-HT7receptor and disorders of the nervous system: An overview. Psychopharmacology (Berl) 206:345–354 . doi: 10.1007/s00213-009-1626-0

125. Yau JLW, Noble J, Seckl JR (2001) Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus. Neurosci Lett 309:141–144 . doi: 10.1016/S0304-3940(01)02054-7

126. Le Corre S, Sharp T, Young AH, Harrison PJ (1997) Increase of 5-HT7 (serotonin-7) and 5-HT1A (serotonin-1A) receptor mRNA expression in rat hippocampus after adrenalectomy. Psychopharmacol 130:368–374 . doi: 10.1007/s002130050252

127. Healy DJ, Meador-Woodruff JH (1999) Ionotropic glutamate receptor modulation of 5-HT6 and 5-HT7 mRNA expression in rat brain. Neuropsychopharmacology 21:341–351 . doi: 10.1016/S0893-133X(99)00043-3

128. Nikiforuk A (2015) Targeting the Serotonin 5-HT7 Receptor in the Search for Treatments for CNS Disorders: Rationale and progress to date. CNS Drugs 29:265–275 . doi: 10.1007/s40263-015-0236-0

129. P. Laplante, J. Diorio, M. J. Meaney, Serotonin regulates hippocampal glucocorticoid receptor expression via a 5-HT7 receptor. *Developmental Brain Research.* **139**, 199-203 (2002).

130. Dalton, G. D., & Howlett, A. C. (2012). Cannabinoid CB 1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/ threonine kinases to activate ERK in neuronal cells. *British Journal of Pharmacology*, *165*(8), 2497–2511. https://doi.org/10.1111/j.1476-5381.2011.01455.x

131. Swift, J. L., Godin, A. G., Doré, K., Freland, L., Bouchard, N., Nimmo, C., Sergeev, M., De Koninck, Y., Wiseman, P. W., Beaulieu, J.-M. (2011). Quantification of receptor tyrosine kinase transactivation through direct dimerization and surface density measurements in single cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(17), 7016–21. https://doi.org/10.1073/pnas.1018280108

132. Lee, F. S., Rajagopal, R., & Chao, M. V. (2002). Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors. Cytokine & Growth Factor Reviews, 13(1), 11–7.

133. Meitzen, J., Luoma, J. I., Stern, C. M., & Mermelstein, P. G. (2011). β1-Adrenergic receptors activate two distinct signaling pathways in striatal neurons. *Journal of Neurochemistry*, *116*(6), 984–95. https://doi.org/10.1111/j.1471-4159.2010.07137.x

134. Chen, D. Y., Bambah-Mukku, D., Pollonini, G., & Alberini, C. M. (2012). Glucocorticoid receptors recruit the CaMKIIα-BDNF-CREB pathways to mediate memory consolidation. Nature Neuroscience, 15(12), 1707–1714. https://doi.org/10.1038/nn.3266

135. Jeanneteau, F., Garabedian, M. J., & Chao, M. V. (2008). Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(12), 4862–7. https://doi.org/10.1073/pnas.0709102105

136. Kubera, M., Obuchowicz, E., Goehler, L., Brzeszcz, J., & Maes, M. (2011). In animal models, psychosocial stress-induced (neuro) inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *35*(3), 744–759. https://doi.org/10.1016/j.pnpbp.2010.08.026

137. Neto, F. L., Borges, G., Torres-Sanchez, S., Mico, J. A., & Berrocoso, E. (2011). Neurotrophins role in depression neurobiology: a review of basic and clinical evidence. *Current Neuropharmacology*, *9*(4), 530–52. https://doi.org/10.2174/157015911798376262

138. Wohleb et al., 2016. Integrating neuroimmune systems in the neurobiology of depression. doi: 10.1038/nrn.2016.69

139. Rantamäki, T., Hendolin, P., Kankaanpää, A., Mijatovic, J., Piepponen, P., Domenici, E., Chao, M. V., Männistö, P. T., Castrén, E. (2007). Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cγ signaling pathways in mouse brain. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *32*(10), 2152–62. https://doi.org/10.1038/sj.npp.1301345

140. Rantamäki, T., Knuuttila, J. E. A., Hokkanen, M. E., & Castrén, E. (2006). The effects of acute and long-term lithium treatments on trkB neurotrophin receptor activation in the mouse hippocampus and anterior cingulate cortex. *Neuropharmacology*, *50*(4), 421–427. https://doi.org/10.1016/j.neuropharm.2005.10.001

141. Zhang, Y., Gu, F., Chen, J., & Dong, W. (2010). Chronic antidepressant administration alleviates frontal and hippocampal BDNF deficits in CUMS rat. *Brain Research*, *1366*, 141 https://doi.org/10.1016/j.brainres.2010.09.095

142. Rantamäki, T., Vesa, L., Antila, H., Di Lieto, A., Tammela, P., Schmitt, A., Lesch, K.-P., Rios, M., Castrén, E. (2011). Antidepressant drugs transactivate TrkB neurotrophin receptors in the adult rodent brain independently of BDNF and monoamine transporter blockade. *PloS One*, *6*(6), e20567. https://doi.org/10.1371/journal.pone.0020567

143. Anacker, C., Zunszain, P. A., Cattaneo, A., Carvalho, L. A., Garabedian, M. J., Thuret, S., Price, J., Pariante, C. M. (2011). Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. *Molecular Psychiatry*, *16*(7), 738–750. https://doi.org/10.1038/mp.2011.26

144. Pariante, C. M., Kim, R. B., Makoff, A., & Kerwin, R. W. (2003). Antidepressant fluoxetine enhances glucocorticoid receptor function in vitro by modulating membrane steroid transporters. *British Journal of Pharmacology*, *139*(6), 1111–1118. https://doi.org/10.1038/sj.bjp.0705357

145. Pariante, C. M., Makoff, A., Lovestone, S., Feroli, S., Heyden, A., Miller, A. H., & Kerwin, R. W. (2001). Antidepressants enhance glucocorticoid receptor function in vitro by modulating the membrane steroid transporters. *British Journal of Pharmacology*, *134*(6), 1335–43. https://doi.org/10.1038/sj.bjp.0704

146. Pariante, C. M., Pearce, B. D., Pisell, T. L., Owens, M. J., & Miller, A. H. (1997). Steroid-Independent Translocation of the Glucocorticoid Receptor by the Antidepressant Desipramine. *Molecular Pharmacology*, *52*(4), 571–581. https://doi.org/10.1124/mol.52.4.571

147. Borroto-Escuela, D. O., Romero-Fernandez, W., Mudó, G., Pérez-Alea, M., Ciruela, F., Tarakanov, A. O., Narvaez, M., Di Liberto, V., Agnati, L. F., Belluardo, N., Fuxe, K. (2012). Fibroblast growth factor receptor 1- 5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity. *Biological Psychiatry*, *71*(1), 84–91. https://doi.org/10.1016/j.biopsych.2011.09.012

148. Du, T., Li, B., Liu, S., Zang, P., Prevot, V., Hertz, L., & Peng, L. (2009). ERK phosphorylation in intact, adult brain by α2-adrenergic transactivation of EGF receptors. *Neurochemistry International*, *55*(7), 593–600.

149. Hoffman & Mitchell, 2011. Spinal 5-HT7 receptor activation induces long-lasting phrenic motor facilitation. doi: 10.1113/jphysiol.2010.201657

150. Iwakura, Y., Nawa, H., Sora, I., & Chao, M. V. (2008). Dopamine D1 receptor-induced signaling through TrkB receptors in striatal neurons. *Journal of Biological Chemistry*, *283*(23), 15799–15806. https://doi.org/10.1074/jbc.M801553200

151. Wiese et al., 2007. Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. doi: 10.1073/pnas.0705267104

152. Fujino, K., Yoshitake, T., Inoue, O., Ibii, N., Kehr, J., Ishida, J., Nohta, H., Yamaguchi, M. (2002). Increased serotonin release in mice frontal cortex and hippocampus induced by acute physiological stressors. *Neuroscience Letters*, *320*(1–2), 91–95. https://doi.org/10.1016/S0304-3940(02)00029-0

153. Holly, E. N., & Miczek, K. A. (2016). Ventral tegmental area dopamine revisited: effects of acute and repeated stress. *Psychopharmacology*, *233*(2), 163–186. https://doi.org/10.1007/s00213-015-4151-3

154. Scaccianoce, S., Navarra, D., Di Sciullo, A., Angelucci, L., & Endröczi, E. (1989). Adenosine and pituitary-adrenocortical axis activity in the rat. *Neuroendocrinology*, *50*(4), 464–8. https://doi.org/10.1159/000125264

155. Yoshioka, M., Matsumoto, M., Togashi, H., & Saito, H. (1995). Effects of conditioned fear stress on 5-HT release in the rat prefrontal cortex. *Pharmacology Biochemistry and Behavior*,

51(2–3), 515–519. https://doi.org/10.1016/0091-3057(95)00045-X

156. Yoshioka, M., Matsumoto, M., Togashi, H., & Saito, H. (1996). Effect of conditioned fear stress on dopamine release in the rat prefrontal cortex. *Neuroscience Letters*, *209*(3), 201–203. https://doi.org/10.1016/0304-3940(96)12631-8

157. Butts, K. A., Weinberg, J., Young, A. H., & Phillips, A. G. (2011). Glucocorticoid receptors in the prefrontal cortex regulate stress-evoked dopamine efflux and aspects of executive function. *Proceedings of the National Academy of Sciences*, *108*(45), 18459–18464. https://doi.org/10.1073/pnas.1111746108

158. Trantham-Davis et al., 2004. Mechanisms Underlying Differential D1 versus D2 Dopamine Receptor Regulation of Inhibition in Prefrontal Cortex. doi: 10.1523/JNEUROSCI.3179-04.2004

159. Robinson, D. L., Venton, B. J., Heien, M. L. A. V, & Wightman, R. M. (2003). Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. *Clinical Chemistry*, *49*(10), 1763–1773. https://doi.org/10.1373/49.10.1763

160. Robinson, D. L., Phillips, P. E. M., Budygin, E. A., Trafton, B. J., Garris, P. A., & Wightman, R. M. (2001). Sub-second changes in accumbal dopamine during sexual behavior in male rats. NeuroReport, 12(11), 2549–2552. https://doi.org/10.1097/00001756-200108080-00051

161. Anstrom, K. K., Miczek, K. A., & Budygin, E. A. (2009). Increased phasic dopamine signaling in the mesolimbic pathway during social defeat in rats. *Neuroscience*, *161*(1), 3–12. https://doi.org/10.1016/j.neuroscience.2009.03.023

162. Budygin, E. A., Park, J., Bass, C. E., Grinevich, V. P., Bonin, K. D., & Wightman, R. M. (2012). Aversive stimulus differentially triggers subsecond dopamine release in reward regions. *Neuroscience*, *201*, 331–337. https://doi.org/10.1016/j.neuroscience.2011.10.056

163. Arnsten, A. F. T., Wang, M. J., & Paspalas, C. D. (2012). Neuromodulation of Thought: Flexibilities and Vulnerabilities in Prefrontal Cortical Network Synapses. *Neuron*, *76*(1), 223–239. https://doi.org/10.1016/j.neuron.2012.08.038

164. Barsegyan, A., Mackenzie, S. M., Kurose, B. D., McGaugh, J. L., & Roozendaal, B. (2010). Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. *Proceedings of the National Academy of Sciences*, *107*(38), 16655–16660. https://doi.org/10.1073/pnas.1011975107

165. Colangelo, A. M., Mallei, A., Johnson, P. F., & Mocchetti, I. (2004). Synergistic effect of

dexamethasone and β-adrenergic receptor agonists on the nerve growth factor gene transcription. *Molecular Brain Research*, *124*(2), 97–104. https://doi.org/10.1016/j.molbrainres.2004.01.011

166. Rangarajan, P. N., Umesono, K., & Evans, R. M. (1992). Modulation of glucocorticoid receptor function by protein kinase A. *Molecular Endocrinology*, *6*(9), 1451–7. https://doi.org/10.1210/mend.6.9.1435789

167. Schmidt, P., Holsboer, F., & Spengler, D. (2001). B2-Adrenergic Receptors Potentiate Glucocorticoid Receptor Transactivation via G Protein By-Subunits and the Phosphoinositide 3-Kinase Pathway. *Molecular Endocrinology*, *15*(4), 553–564. https://doi.org/10.1210/me.15.4.553

168. Schmidt, M., & Michel, M. C. (2011). How Can 1 + 1 = 3? β2-Adrenergic and Glucocorticoid Receptor Agonist Synergism in Obstructive Airway Diseases. *Molecular Pharmacology*, *80*(6), 955–958. https://doi.org/10.1124/mol.111.075481

169. Roozendaal, B., Hahn, E. L., Nathan, S. V, de Quervain, D. J.-F., & McGaugh, J. L. (2004). Glucocorticoid effects on memory retrieval require concurrent noradrenergic activity in the hippocampus and basolateral amygdala. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(37), 8161–9. https://doi.org/10.1523/JNEUROSCI.2574-04.2004

170. Roozendaal, B., Okuda, S., De Quervain, D. J. F., & McGaugh, J. L. (2006). Glucocorticoids interact with emotion-induced noradrenergic activation in influencing different memory functions. *Neuroscience*, *138*(3), 901–910. https://doi.org/10.1016/j.neuroscience.2005.07.049

171. Baker, A. J., Zornow, M. H., Scheller, M. S., Yaksh, T. L., Skilling, S. R., Smullin, D. H., Larson, A. A., Kuczenski, R. (1991). Changes in Extracellular Concentrations of Glutamate, Aspartate, Glycine, Dopamine, Serotonin, and Dopamine Metabolites After Transient Global Ischemia in the Rabbit Brain. *Journal of Neurochemistry*, *57*(4), 1370–1379. https://doi.org/10.1111/j.1471-4159.1991.tb08303.x

172. Pedata, F., Corsi, C., Melani, A., Bordoni, F., & Latini, S. (2001). Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. *Annals of the New York Academy of Sciences*, *939*, 74–84. https://doi.org/10.1111/j.1749-6632.2001.tb03614.x

173. Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S., & Sandberg, M. (1987). Extracellular Adenosine, Inosine, Hypoxanthine, and Xanthine in Relation to Tissue

Nucleotides and Purines in Rat Striatum During Transient Ischemia. *Journal of Neurochemistry*, *49*(1), 227–231. https://doi.org/10.1111/j.1471-4159.1987.tb03419.x

174. Van Wylen, D. G. L., Park, T. S., Rubio, R., & Berne, R. M. (1986). Increases in Cerebral Interstitial Fluid Adenosine Concentration during Hypoxia, Local Potassium Infusion, and Ischemia. *Journal of Cerebral Blood Flow & Metabolism*, *6*(5), 522–528. https://doi.org/10.1038/jcbfm.1986.97
175. Heurteaux, C., Lauritzen, I., Widmann, C., & Lazdunski, M. (1995). Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(10), 4666–70. https://doi.org/10.1073/PNAS.92.10.4666
176. Xie, K.-Q., Zhang, L.-M., Cao, Y., Zhu, J., & Feng, L.-Y. (2009). Adenosine A 1 receptor-mediated transactivation of the EGF receptor produces a neuroprotective effect on cortical neurons in vitro. *Acta Pharmacologica Sinica*, *30*(30), 889–898. https://doi.org/10.1038/aps.2009.80
177. Zhong, M., Song, W., Xu, Y., Ye, Y., & Feng, L. (2015). Paeoniflorin ameliorates ischemic neuronal damage in vitro via adenosine A1 receptor-mediated transactivation of epidermal growth factor receptor. *Acta Pharmacologica Sinica*, *36*(3), 298–310. https://doi.org/10.1038/aps.2014.154
178. Krieg, T., Qin, Q., McIntosh, E. C., Cohen, M. V, & Downey, J. M. (2002). ACh and adenosine activate PI3-kinase in rabbit hearts through transactivation of receptor tyrosine kinases. *American Journal of Physiology. Heart and Circulatory Physiology*, *283*(6), H2322-30. https://doi.org/10.1152/ajpheart.00474.2002
179. Williams-Pritchard, G., Knight, M., Hoe, L. S., Headrick, J. P., & Peart, J. N. (2011). Essential role of EGFR in cardioprotection and signaling responses to A 1 adenosine receptors and ischemic preconditioning. *American Journal of Physiology-Heart and Circulatory Physiology*, *300*(6), H2161–H2168.
180. Xi, L., Das, A., Zhao, Z.-Q., Merino, V. F., Bader, M., & Kukreja, R. C. (2008). Loss of Myocardial Ischemic Postconditioning in Adenosine A1 and Bradykinin B2 Receptors Gene Knockout Mice. *Circulation*, *118*(14_suppl_1), S32–S37. https://doi.org/10.1161/CIRCULATIONAHA.107.752865
181. Förster, K., Kuno, A., Solenkova, N., Felix, S. B., & Krieg, T. (2007). The delta-opioid

receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation. *American Journal of Physiology. Heart and Circulatory Physiology*, *293*(3), H1604-8. https://doi.org/10.1152/ajpheart.00418.2007

182. Methner, C., Donat, U., Felix, S. B., & Krieg, T. (2009). Cardioprotection of bradykinin at reperfusion involves transactivation of the epidermal growth factor receptor via matrix metalloproteinase-8. *Acta Physiologica*, *197*(4), 265–271. https://doi.org/10.1111/j.1748-1716.2009.02018.x
183. Schultz, J. J., Hsu, A. K., & Gross, G. J. (1997). Ischemic preconditioning and morphine-induced cardioprotection involve the delta (delta)-opioid receptor in the intact rat heart. *Journal of Molecular and Cellular Cardiology*, *29*(8), 2187–95. https://doi.org/10.1006/jmcc.1997.0454
184. Hoffman, M. S., & Mitchell, G. S. (2009). Spinal 5-HT7 Receptor Agonist Induced Phrenic Motor Facilitation Requires TrkB Signaling. *The FASEB Journal* , *23*(1 Supplement), 607.4-607.4.
185. Hoffman, M. S., & Mitchell, G. S. (2011). Spinal 5-HT7 receptor activation induces long-lasting phrenic motor facilitation. *The Journal of Physiology*, *589*(6), 1397–1407. https://doi.org/10.1113/jphysiol.2010.201657
186. Golder, F. J., Ranganathan, L., Satriotomo, I., Hoffman, M., Lovett-Barr, M. R., Watters, J. J., Baker-Herman, T. L., Mitchell, G. S. (2008). Spinal Adenosine A2a Receptor Activation Elicits Long-Lasting Phrenic Motor Facilitation. *Journal of Neuroscience*, *28*(9), 2033–2042. https://doi.org/10.1523/JNEUROSCI.3570-07.2008
187. McGuire, M., Zhang, Y., White, D. P., & Ling, L. (2004). Serotonin receptor subtypes required for ventilatory long-term facilitation and its enhancement after chronic intermittent hypoxia in awake rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *286*(2), R334–R341. https://doi.org/10.1152/ajpregu.00463.2003
188. Nichols, N. L., Dale, E. A., & Mitchell, G. S. (2012). Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. *Journal of Applied Physiology*, *112*(10), 1678–1688. https://doi.org/10.1152/japplphysiol.00060.2012
189. Attwells, S., Setiawan, E., Wilson, A. A., Rusjan, P. M., Mizrahi, R., Miler, L., Xu, C., Richter, M. A., Kahn, A., Kish, S. J., Houle, S., Ravindran, L., Meyer, J. H. (2017). Inflammation in the Neurocircuitry of Obsessive-Compulsive Disorder. *JAMA Psychiatry*,

74(8), 833. https://doi.org/10.1001/jamapsychiatry.2017.1567

190. Cheng, Y., Pardo, M., Armini, R. de S., Martinez, A., Mouhsine, H., Zagury, J.-F., Jope, R. S., Beurel, E. (2016). Stress-induced neuroinflammation is mediated by GSK3-dependent TLR4 signaling that promotes susceptibility to depression-like behavior. *Brain, Behavior, and Immunity*, *53*, 207–222. https://doi.org/10.1016/j.bbi.2015.12.012

191. Gárate, I., Garcia-Bueno, B., Luis, J., Madrigal, M., Caso, J. R., Alou, L., Gomez-Lus, M. L., Micó, J. A., Leza, J. C. (2013). Stress-Induced Neuroinflammation: Role of the Toll-Like Receptor-4 Pathway. *BPS*, *73*, 32–43. https://doi.org/10.1016/j.biopsych.2012.07.005

192. Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell*, *140*(6), 918–34. https://doi.org/10.1016/j.cell.2010.02.016

193. Patel, J. P., & Frey, B. N. (2015). Disruption in the Blood-Brain Barrier: The Missing Link between Brain and Body Inflammation in Bipolar Disorder? *Neural Plasticity*, *2015*, 1–12. https://doi.org/10.1155/2015/708306

194. Zhang, Y., Liu, L., Liu, Y.-Z., Shen, X.-L., Wu, T.-Y., Zhang, T., Wang, W., Wang, Y.-X., Jiang, C.-L. (2015). NLRP3 Inflammasome Mediates Chronic Mild Stress-Induced Depression in Mice via Neuroinflammation. *International Journal of Neuropsychopharmacology*, *18*(8), pyv006-pyv006. https://doi.org/10.1093/ijnp/pyv006

195. Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling*, *20*(7), 1126–1167. https://doi.org/10.1089/ars.2012.5149

196. Che, Y., Zhou, Z., Shu, Y., Zhai, C., Zhu, Y., Gong, S., Cui, Y., Wang, J.-F. (2015). Chronic unpredictable stress impairs endogenous antioxidant defense in rat brain. *Neuroscience Letters*, *584*, 208–213. https://doi.org/10.1016/j.neulet.2014.10.031

197. Lee, Y. J., Choi, B., Lee, E. H., Choi, K. S., & Sohn, S. (2006). Immobilization stress induces cell death through production of reactive oxygen species in the mouse cerebral cortex. *Neuroscience Letters*, *392*(1–2), 27–31. https://doi.org/10.1016/j.neulet.2005.08.065

198. Schiavone, S., Jaquet, V., Sorce, S., Dubois-Dauphin, M., Hultqvist, M., Bäckdahl, L., Holmdahl, R., Colaianna, M., Cuomo, V., Trabace, L., Krause, K. H. (2012). NADPH oxidase elevations in pyramidal neurons drive psychosocial stress-induced neuropathology. *Translational Psychiatry*, *2*. https://doi.org/10.1038/tp.2012.36

199. Seo, J.-S., Park, J.-Y., Choi, J., Kim, T.-K., Shin, J.-H., Lee, J.-K., & Han, P.-L. (2012). NADPH oxidase mediates depressive behavior induced by chronic stress in mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *32*(28), 9690–9. https://doi.org/10.1523/JNEUROSCI.0794-12.2012

200. Tang, V., Young, A., Tan, H., Beasley, C., & Wang, J.-F. (2013). Glucocorticoids Increase Protein Carbonylation and Mitochondrial Dysfunction. *Hormone and Metabolic Research*, *45*(10), 709–715. https://doi.org/10.1055/s-0033-1345119

201. Cao, H., Sanguinetti, A. R., & Mastick, C. C. (2004). Oxidative stress activates both Src-kinases and their negative regulator Csk and induces phosphorylation of two targeting proteins for Csk: caveolin-1 and paxillin. *Experimental Cell Research*, *294*(1), 159–71. https://doi.org/10.1016/j.yexcr.2003.11.010

202. Chen, K., Thomas, S. R., Albano, A., Murphy, M. P., & Keaney, J. F. (2004). Mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling. *Journal of Biological Chemistry*, *279*(33), 35079–35086. https://doi.org/10.1074/jbc.M404859200

203. Frank, G. D., Mifune, M., Inagami, T., Ohba, M., Sasaki, T., Higashiyama, S., Dempsey, P. J., Eguchi, S. (2003). Distinct mechanisms of receptor and nonreceptor tyrosine kinase activation by reactive oxygen species in vascular smooth muscle cells: role of metalloprotease and protein kinase C-delta. *Molecular and Cellular Biology*, *23*(5), 1581–9. https://doi.org/10.1128/MCB.23.5.1581–1589.2003

204. Ohtsu, H., Dempsey, P. J., & Eguchi, S. (2006). ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *American Journal of Physiology - Cell Physiology*, *291*(1), C1-10. https://doi.org/10.1152/ajpcell.00620.2005.

205. Meng, T.-C., Fukada, T., & Tonks, N. K. (2002). Reversible Oxidation and Inactivation of Protein Tyrosine Phosphatases In Vivo. *Molecular Cell*, *9*(2), 387–399. https://doi.org/10.1016/S1097-2765(02)00445-8

206. Shah, B. H., Shah, F. B., & Catt, K. J. (2006). Role of metalloproteinase-dependent EGF receptor activation in α 1 -adrenoceptor-stimulated MAP kinase phosphorylation in GT1-7 neurons. *Journal of Neurochemistry*, *96*(2), 520–532. https://doi.org/10.1111/j.1471-4159.2005.03585.x

207. Zhang, G.-X., Lu, X.-M., Kimura, S., & Nishiyama, A. (2007). Role of mitochondria in

angiotensin II-induced reactive oxygen species and mitogen-activated protein kinase activation. *Cardiovascular Research*, *76*(2), 204–12. https://doi.org/10.1016/j.cardiores.2007.07.014

208. Arango-Lievano, M., Lambert, W. M., Bath, K. G., Garabedian, M. J., Chao, M. V, & Jeanneteau, F. (2015). Neurotrophic-priming of glucocorticoid receptor signaling is essential for neuronal plasticity to stress and antidepressant treatment. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(51), 15737–42. https://doi.org/10.1073/pnas.1509045112

209. Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *39*(1), 112–119. https://doi.org/10.1016/j.pnpbp.2012.05.018

210. Lambert, W. M., Xu, C.-F., Neubert, T. A., Chao, M. V., Garabedian, M. J., & Jeanneteau, F. D. (2013). Brain-Derived Neurotrophic Factor Signaling Rewrites the Glucocorticoid Transcriptome via Glucocorticoid Receptor Phosphorylation. *Molecular and Cellular Biology*, *33*(18), 3700–3714. https://doi.org/10.1128/MCB.00150-13

211. Cheung, L. W. T., Leung, K. W., Wong, C. K. C., Wong, R. N. S., & Wong, A. S. T. (2011). Ginsenoside-Rg1 induces angiogenesis via non-genomic crosstalk of glucocorticoid receptor and fibroblast growth factor receptor-1. *Cardiovascular Research*, *89*(2), 419–425. https://doi.org/10.1093/cvr/cvq300

212. Borroto-Escuela, D. O., Pérez-Alea, M., Narvaez, M., Tarakanov, A. O., Mudó, G., Jiménez-Beristain, A., Agnati, L. F., Ciruela, F., Belluardo, N., Fuxe, K. (2015). Enhancement of the FGFR1 signaling in the FGFR1-5-HT1A heteroreceptor complex in midbrain raphe 5-HT neuron systems. Relevance for neuroplasticity and depression. *Biochemical and Biophysical Research Communications*, *463*(3), 180–6. https://doi.org/10.1016/j.bbrc.2015.04.133

213. Arabian, M., Aboutaleb, N., Soleimani, M., Ajami, M., Habibey, R., & Pazoki-Toroudi, H. (2018). Activation of mitochondrial KATP channels mediates neuroprotection induced by chronic morphine preconditioning in hippocampal CA-1 neurons following cerebral ischemia. *Advances in Medical Sciences*, *63*(2), 213–219. https://doi.org/10.1016/j.advms.2017.11.003

214. Myers, T. J., Brennaman, L. H., Stevenson, M., Higashiyama, S., Russell, W. E., Lee, D. C.,

& Sunnarborg, S. W. (2009). Mitochondrial Reactive Oxygen Species Mediate GPCR–induced TACE/ADAM17-dependent Transforming Growth Factor-α Shedding. *Molecular Biology of the Cell*, *20*(24), 5236–5249. https://doi.org/10.1091/mbc.e08-12-1256

215. Yuan, F., Fu, H., Sun, K., Wu, S., & Dong, T. (2017). Effect of dexmedetomidine on cerebral ischemia-reperfusion rats by activating mitochondrial ATP-sensitive potassium channel. *Metabolic Brain Disease*, *32*(2), 539–546. https://doi.org/10.1007/s11011-016-9945-4

216. Zhang, G.-X., Lu, X.-M., Kimura, S., & Nishiyama, A. (2007). Role of mitochondria in angiotensin II-induced reactive oxygen species and mitogen-activated protein kinase activation. *Cardiovascular Research*, *76*(2), 204–12. https://doi.org/10.1016/j.cardiores.2007.07.014

217. Frijhoff, J., Dagnell, M., Augsten, M., Beltrami, E., Giorgio, M., & Östman, A. (2014). The mitochondrial reactive oxygen species regulator p66Shc controls PDGF-induced signaling and migration through protein tyrosine phosphatase oxidation. *Free Radical Biology and Medicine*, *68*, 268–277. https://doi.org/10.1016/j.freeradbiomed.2013.12.022

218. Kalogeris, T., Bao, Y., & Korthuis, R. J. (2014). Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology*, *2*, 702–714. https://doi.org/10.1016/J.REDOX.2014.05.006

219. Kim, Y.-M., Kim, S.-J., Tatsunami, R., Yamamura, H., Fukai, T., & Ushio-Fukai, M. (2017). ROS-induced ROS release orchestrated by Nox4, Nox2, and mitochondria in VEGF signaling and angiogenesis. *American Journal of Physiology - Cell Physiology*, *312*(6), C749–C764. https://doi.org/10.1152/ajpcell.00346.2016

220. Brady, N. R., Elmore, S. P., van Beek, J. J. H. G. M., Krab, K., Courtoy, P. J., Hue, L., & Westerhoff, H. V. (2004). Coordinated behavior of mitochondria in both space and time: a reactive oxygen species-activated wave of mitochondrial depolarization. *Biophysical Journal*, *87*(3), 2022–34. https://doi.org/10.1529/biophysj.103.035097

221. Brady, N. R., Hamacher-Brady, A., Westerhoff, H. V., & Gottlieb, R. A. (2006). A Wave of Reactive Oxygen Species (ROS)-Induced ROS Release in a Sea of Excitable Mitochondria. *Antioxidants & Redox Signaling*, *8*(9–10), 1651–1665. https://doi.org/10.1089/ars.2006.8.1651

222. Jou, M.-J. (2008). Pathophysiological and pharmacological implications of mitochondria-targeted reactive oxygen species generation in astrocytes☆. *Advanced Drug Delivery*

Reviews, *60*(13–14), 1512–1526. https://doi.org/10.1016/j.addr.2008.06.004

223. Fone KC, Porkess MV. Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders. *Neuroscience and biobehavioral reviews.* Aug 2008;32(6):1087-1102.

224. Felitti VJ, Anda RF, Nordenberg D, et al. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. American journal of preventive medicine. May 1998;14(4):245-258.

225. Bolton JL, Molet J, Ivy A, Baram TZ. New insights into early-life stress and behavioral outcomes. *Curr Opin Behav Sci*. 2017;14:133–139. doi:10.1016/j.cobeha.2016.12.012

226. Shonkoff JP, Boyce WT, McEwen BS. Neuroscience, molecular biology, and the childhood roots of health disparities: building a new framework for health promotion and disease prevention. JAMA : the journal of the American Medical Association. Jun 3 2009;301(21):2252-2259.

227. Jones, GT., Psychosocial Vulnerability and Early Life Adversity as Risk Factors for Central Sensitivity Syndromes. Current Rheumatology Reviews. Volume 12 , Issue 2 , 2016. 10.2174/1573397112666151231113438

228. Hammen, C., Henry, R., & Daley, S. E. (2000). Depression and sensitization to stressors among young women as a function of childhood adversity. *Journal of Consulting and Clinical Psychology*, *68*(5), 782–787. https://doi.org/10.1037/0022-006X.68.5.782

229. Williams, L., Debattista, C., Duchemin, A. *et al.* Childhood trauma predicts antidepressant response in adults with major depression: data from the randomized international study to predict optimized treatment for depression. *Transl Psychiatry* **6,** e799 (2016) doi:10.1038/tp.2016.61

230. Hambrick, E.P Brawner, T., and Perry, B. Timing of Early-Life Stress and the Development of Brain-Related Capacities. Front. Behav. Neurosci., 06 August 2019 | https://doi.org/10.3389/fnbeh.2019.00183

231. L. E. Wearick-Silva *et al.*, Dual influences of early life stress induced by limited bedding on walking adaptability and Bdnf/TrkB and Drd1/Drd2 gene expression in different mouse brain regions. *Behavioural Brain Research.* **359**, 66-72 (2019)

232. Lesuis, S.L., Weggen, S., Baches, S. *et al.* Targeting glucocorticoid receptors prevents the

effects of early life stress on amyloid pathology and cognitive performance in APP/PS1 mice. *Transl Psychiatry* **8,** 53 (2018) doi:10.1038/s41398-018-0101-2

233. Liu PZ, Nusslock R. How Stress Gets Under the Skin: Early Life Adversity and Glucocorticoid Receptor Epigenetic Regulation. *Curr Genomics*. 2018;19(8):653–664. doi:10.2174/1389202919666171228164350

234. Del Arco A, Zhu S, Terasmaa A, Mohammed AH, Fuxe K. Hyperactivity to novelty induced by social isolation is not correlated with changes in D2 receptor function and binding in striatum. Psychopharmacology. Jan 2004;171(2):148-155.

235. Jones GH, Marsden CA, Robbins TW. Increased sensitivity to amphetamine and reward-related stimuli following social isolation in rats: possible disruption of dopamine-dependent mechanisms of the nucleus accumbens. Psychopharmacology. 1990;102(3):364-372.

236. Heidbreder CA, Weiss IC, Domeney AM, et al. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience. 2000;100(4):749-768

237. Cilia J, Hatcher PD, Reavill C, Jones DN. Long-term evaluation of isolation-rearing induced prepulse inhibition deficits in rats: an update. *Psychopharmacology.* Jun 2005;180(1):57-62.

238. Levine JB, Youngs RM, MacDonald ML, et al. Isolation rearing and hyperlocomotion are associated with reduced immediate early gene expression levels in the medial prefrontal cortex. *Neuroscience.* Mar 2 2007;145(1):42-55.

239. Cilia J, Hatcher PD, Reavill C, Jones DN. Long-term evaluation of isolation-rearing induced prepulse inhibition deficits in rats: an update. *Psychopharmacology.* Jun 2005;180(1):57-62.

240. Sakaue H, Nishizawa A, Ogawa W, et al. Requirement for 3-phosphoinositide-kependent dinase-1 (PDK-1) in insulin-induced glucose uptake in immortalized brown adipocytes. The Journal of biological chemistry. Oct 3 2003;278(40):38870-38874.

241. Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* Apr 15 2002;22(8):3251-3261.

242. Russo-Neustadt AA, Beard RC, Huang YM, Cotman CW. Physical activity and antidepressant treatment potentiate the expression of specific brain-derived neurotrophic factor transcripts in the rat hippocampus. *Neuroscience.* 2000;101(2):305-312.

243. Ivy AS, Rodriguez FG, Garcia C, Chen MJ, Russo-Neustadt AA. Noradrenergic and serotonergic blockade inhibits BDNF mRNA activation following exercise and antidepressant. *Pharmacology, biochemistry, and behavior.* Apr 2003;75(1):81-88.
244. Barrientos RM, Sprunger DB, Campeau S, et al. Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. *Neuroscience.* 2003;121(4):847-853.
245. Rasmusson AM, Shi L, Duman R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology.* Aug 2002;27(2):133-142.
246. Frazer A, Morilak DA. What should animal models of depression model? *Neuroscience and biobehavioral reviews.* 2005;29(4-5):515-523.
247. Bibancos T, Jardim DL, Aneas I, Chiavegatto S. Social isolation and expression of serotonergic neurotransmission-related genes in several brain areas of male mice. *Genes, brain, and behavior.* Aug 2007;6(6):529-539.
248. Preece MA, Dalley JW, Theobald DE, Robbins TW, Reynolds GP. Region specific changes in forebrain 5-hydroxytryptamine1A and 5-hydroxytryptamine2A receptors in isolation-reared rats: an in vitro autoradiography study. *Neuroscience.* 2004;123(3):725-732.
249. Muchimapura S, Mason R, Marsden CA. Effect of isolation rearing on pre- and post-synaptic serotonergic function in the rat dorsal hippocampus. *Synapse (New York, N.Y.).* Mar 2003;47(3):209-217.
250. Newcomer JW, Farber NB, Olney JW. NMDA receptor function, memory, and brain aging. *Dialogues Clin Neurosci*. 2000;2(3):219–232.
251. Collingridge, G. The role of NMDA receptors in learning and memory. *Nature* **330,** 604–605 (1987) doi:10.1038/330604a0
252. Balu DT. The NMDA Receptor and Schizophrenia: From Pathophysiology to Treatment. *Adv Pharmacol*. 2016;76:351–382. doi:10.1016/bs.apha.2016.01.006
253. Lee CH, Lü W, Michel JC, et al. NMDA receptor structures reveal subunit arrangement and pore architecture. *Nature*. 2014;511(7508):191–197. doi:10.1038/nature13548
254. Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nat Rev Neurosci. 2011;13(1):22–37. Published

2011 Nov 30. doi:10.1038/nrn3138

255. Turnock-Jones JJ, Jennings CA, Robbins MJ, et al. Increased expression of the NR2A NMDA receptor subunit in the prefrontal cortex of rats reared in isolation. *Synapse (New York, N.Y.).* Oct 2009;63(10):836-846.

256. Zhao X, Sun L, Jia H, et al. Isolation rearing induces social and emotional function abnormalities and alters glutamate and neurodevelopment-related gene expression in rats. *Progress in neuro-psychopharmacology & biological psychiatry.* Oct 1 2009;33(7):1173-1177.

257. A. Hennigan, R. M. O'Callaghan, Á. M. Kelly, Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. *Biochem. Soc. Trans.* **35**, 424-427 (2007).

258. He M, Liu J, Cheng S, Xing Y, Suo WZ. Differentiation renders susceptibility to excitotoxicity in HT22 neurons. *Neural Regen Res*. 2013;8(14):1297–1306. doi:10.3969/j.issn.1673-5374.2013.14.006

259. Kaur G, Dufour JM. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis*. 2012;2(1):1–5. doi:10.4161/spmg.19885

260. McEwen, B., Nasca, C. & Gray, J. Stress Effects on Neuronal Structure: Hippocampus, Amygdala, and Prefrontal Cortex. *Neuropsychopharmacol* **41,** 3–23 (2016) doi:10.1038/npp.2015.171

261. Becker 1999. Effect size calculator. https://www.uccs.edu/lbecker/

262. Lee S, Lee DK. What is the proper way to apply the multiple comparison test?. *Korean J Anesthesiol*. 2018;71(5):353–360. doi:10.4097/kja.d.18.00242

263. Dahiru T. P - value, a true test of statistical significance? A cautionary note. *Ann Ib Postgrad Med*. 2008;6(1):21–26. doi:10.4314/aipm.v6i1.64038Mce

264. Fone KC, Porkess MV. Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders. *Neuroscience and biobehavioral reviews.* Aug 2008;32(6):1087-1102.

265. Felitti VJ, Anda RF, Nordenberg D, et al. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. *American journal of preventive medicine.* May 1998;14(4):245-258.

266.Shonkoff JP, Boyce WT, McEwen BS. Neuroscience, molecular biology, and the childhood roots of health disparities: building a new framework for health promotion and disease prevention. *JAMA : the journal of the American Medical Association.* Jun 3 2009;301(21):2252-2259.

267. Del Arco A, Zhu S, Terasmaa A, Mohammed AH, Fuxe K. Hyperactivity to novelty induced by social isolation is not correlated with changes in D2 receptor function and binding in striatum. *Psychopharmacology.* Jan 2004;171(2):148-155.

268. Jones GH, Marsden CA, Robbins TW. Increased sensitivity to amphetamine and reward-related stimuli following social isolation in rats: possible disruption of dopamine-dependent mechanisms of the nucleus accumbens. *Psychopharmacology.* 1990;102(3):364-372.

269. Heidbreder CA, Weiss IC, Domeney AM, et al. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. *Neuroscience.* 2000;100(4):749-768.

270. Sakaue H, Nishizawa A, Ogawa W, et al. Requirement for 3-phosphoinositide-kependent dinase-1 (PDK-1) in insulin-induced glucose uptake in immortalized brown adipocytes. *The Journal of biological chemistry.* Oct 3 2003;278(40):38870-38874.

271. Levine JB, Youngs RM, MacDonald ML, et al. Isolation rearing and hyperlocomotion are associated with reduced immediate early gene expression levels in the medial prefrontal cortex. *Neuroscience.* Mar 2 2007;145(1):42-55.

272.Cilia J, Hatcher PD, Reavill C, Jones DN. Long-term evaluation of isolation-rearing induced prepulse inhibition deficits in rats: an update. *Psychopharmacology.* Jun 2005;180(1):57-62.

273. Mumtaz F, , Khan MI, Zubair M, Dehpour AR. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomedicine & Pharmacotherapy September 2018; 105: 1205–1222

274. R.H. Lipsky, A.M. Marini, Brain-derived neurotrophic factor in neuronal survival and behavior related plasticity, Ann. N.Y. Acad. Sci. 1122 (1) (2007) 130–143.

275. Daskalakis NP, De Kloet ER, Yehuda R,Malaspina D, Kranz TM. Early Life Stress Effects on Glucocorticoid—BDNF Interplay in the Hippocampus. Front Mol Neurosci. November 16 2015; 8: 68.

276. Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *The Journal of*

neuroscience : the official journal of the Society for Neuroscience. Apr 15 2002;22(8):3251-3261.

277.Russo-Neustadt AA, Beard RC, Huang YM, Cotman CW. Physical activity and antidepressant treatment potentiate the expression of specific brain-derived neurotrophic factor transcripts in the rat hippocampus. *Neuroscience.* 2000;101(2):305-312.

278. Ivy AS, Rodriguez FG, Garcia C, Chen MJ, Russo-Neustadt AA. Noradrenergic and serotonergic blockade inhibits BDNF mRNA activation following exercise and antidepressant. *Pharmacology, biochemistry, and behavior.* Apr 2003;75(1):81-88.

279. Samarajeewa A, Goldemann L, Vasefi MS, Ahmed N, Gondora N, Khanderia C, Mielke JG, and Beazely MA. 5-HT7 receptor activation promotes an increase in TrkB receptor expression and phosphorylation. Front Behav Neurosci. November 7 2014; 8: 391.

280.Coyle JT. NMDA receptor and schizophrenia: a brief history. *Schizophrenia bulletin.* Sep 2012;38(5):920-926.

281.E.A. Waxman, D.R. Lynch, N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease, Neuroscientist 11 (1) (2005) 37–49.

282.Haj-Mirzaian, et al., Attenuation of oxidative and nitrosative stress in cortical area associates with antidepressant-like effects of tropisetron in male mice followingsocial isolation stress, Brain Res. Bull. 124 (2016) 150–163.

283.Zhao X, Sun L, Jia H, et al. Isolation rearing induces social and emotional function abnormalities and alters glutamate and neurodevelopment-related gene expression in rats. *Progress in neuro-psychopharmacology & biological psychiatry.* Oct 1 2009;33(7):1173-1177.

284. C. Toua, et al., The effects of sub-chronic clozapine and haloperidol administration on isolation rearing induced changes in frontal cortical N-methyl-D-aspartate and D1 receptor binding in rats, Neuroscience 165 (2) (2010) 492–499.

285. A. Napolitano, et al., In vivo neurometabolic profiling to characterize the effects of social isolation and ketamine-induced NMDA antagonism: a rodent study at 7.0 T, Schizophr. Bull. 40 (3) (2013) 566–574.

286. Verma R, Balhara YP, Gupta CS. Gender differences in stress response: Role of developmental and biological determinants. *Ind Psychiatry J*. 2011;20(1):4–10. doi:10.4103/0972-6748.9840

287. Murínová J, Hlaváčová N, Chmelová M, Riečanský I. The Evidence for Altered BDNF Expression in the Brain of Rats Reared or Housed in Social Isolation: A Systematic Review. *Front Behav Neurosci*. 2017;11:101. Published 2017 May 31. doi:10.3389/fnbeh.2017.00101

288.Djouma E., Card K., Lodge D. J., Lawrence A. J. (2006). The CRF1 receptor antagonist, antalarmin, reverses isolation-induced up-regulation of dopamine D2 receptors in the amygdala and nucleus accumbens of fawn-hooded rats. Eur. J. Neurosci. 23, 3319–3327. 10.1111/j.1460-9568.2006.04864.x

289. Pisu M. G., Dore R., Mostallino M. C., Loi M., Pibiri F., Mameli R., et al. . (2011a). Down-regulation of hippocampal BDNF and Arc associated with improvement in aversive spatial memory performance in socially isolated rats. Behav. Brain Res. 222, 73–80. 10.1016/j.bbr.2011.03.021

290.Pisu M. G., Garau A., Boero G., Biggio F., Pibiri V., Dore R., et al. . (2016). Sex differences in the outcome of juvenile social isolation on HPA axis function in rats. Neuroscience 320, 172–182. 10.1016/j.neuroscience.2016.02.009

291.Pisu M. G., Mostallino M. C., Dore R., Maciocco E., Secci P. P., Serra M. (2011b). Effects of voluntary ethanol consumption on emotional state and stress responsiveness in socially isolated rats. Eur. Neuropsychopharmacol. 21, 414–425. 10.1016/j.euroneuro.2010.07.006

292.Ravenelle R., Santolucito H. B., Byrnes E. M., Byrnes J. J., Donaldson S. T. (2014). Housing environment modulates physiological and behavioral responses to anxiogenic stimuli in trait anxiety male rats. Neuroscience 270, 76–87. 10.1016/j.neuroscience.2014.03.060

293.Uys M., Shahid M., Sallinen J., Dreyer W., Cockeran M., Harvey B. H. (2016). The α2C-adrenoceptor antagonist, ORM-10921, has antipsychotic-like effects in social isolation reared rats and bolsters the response to haloperidol. Prog. Neuro Psychopharmacol. Biol. Psychiatry 71, 108–116. 10.1016/j.pnpbp.2016.07.002

294.Han X., Wang W., Xue X., Shao F., Li N. (2011). Brief social isolation in early adolescence affects reversal learning and forebrain BDNF expression in adult rats. Brain Res. Bull. 86, 173–178. 10.1016/j.brainresbull.2011.07.008

295.Li M., Du W., Shao F., Wang W. (2016). Cognitive dysfunction and epigenetic alterations of the BDNF gene are induced by social isolation during early adolescence. Behav. Brain Res. 313, 177–183. 10.1016/j.bbr.2016.07.025

296.Evans J., Sun Y., McGregor A., Connor B. (2012). Allopregnanolone regulates neurogenesis and depressive/anxiety-like behaviour in a social isolation rodent model of chronic stress. Neuropharmacology 63, 1315–1326. 10.1016/j.neuropharm.2012.08.012

297.Sun Y., Evans J., Russell B., Kydd R., Connor B. (2013). A benzodiazepine impairs the neurogenic and behavioural effects of fluoxetine in a rodent model of chronic stress. Neuropharmacology 72, 20–28. 10.1016/j.neuropharm.2013.04.021

298. Ma J., Wu C. F., Wang F., Yang J. Y., Dong Y. X., Su G. Y., et al. . (2016). Neurological mechanism of Xiaochaihutang's antidepressant-like effects to socially isolated adult rats. J. Pharm. Pharmacol. 68, 1340–1349. 10.1111/jphp.12616

299.Wall V. L., Fischer E. K., Bland S. T. (2012). Isolation rearing attenuates social interaction-induced expression of immediate early gene protein products in the medial prefrontal cortex of male and female rats. Physiol. Behav. 107, 440–450. 10.1016/j.physbeh.2012.09.002

300.Cepeda C, André VM, Jocoy EL, et al. NMDA and Dopamine: Diverse Mechanisms Applied to Interacting Receptor Systems. In: Van Dongen AM, editor. Biology of the NMDA Receptor. Boca Raton (FL): CRC Press/Taylor & Francis; 2009. Chapter 3. Available from: https://www.ncbi.nlm.nih.gov/books/NBK5280/

301.C. Nasca *et al.*, Stress dynamically regulates behavior and glutamatergic gene expression in hippocampus by opening a window of epigenetic plasticity. *Proc. Natl. Acad. Sci. USA.* **112**, 14960-14965 (2015).

302. Fernandez-Patron, C (2007) Therapeutic potential of the epidermal growth factor receptor transactivation in hypertension: a convergent signaling pathway of vascular tone, oxidative stress, and hypertrophic growth downstream of vasoactive G-protein-coupled receptors? Canadian Journal of Physiology and Pharmacology, 2007, 85(1): 97-104, https://doi.org/10.1139/y06-097

303.Weiss. F.U., Daub H., Ullrich A. (1997) Novel mechanisms of RTK signal generation. Current Opinion in Genetics & Development. Volume 7, Issue 1, February 1997, Pages 80-86. https://doi.org/10.1016/S0959-437X(97)80113-X

304. Shear stress induces $G\alpha_{q/11}$ activation independently of G protein-coupled receptor activation in endothelial cells. Nathaniel G. dela Paz, Benoît Melchior, and John A. Frangos. American Journal of Physiology-Cell Physiology 2017 312:4, C428-C437
305. Forrester SJ, Kawai T, O'Brien S, Thomas W, Harris RC, Eguchi S. Epidermal Growth Factor Receptor Transactivation: Mechanisms, Pathophysiology, and Potential Therapies in the Cardiovascular System. *Annu Rev Pharmacol Toxicol*. 2016; 56:627–653. doi:10.1146/annurev-pharmtox-070115-095427