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Title	Investigating the regulatory role of the nuclear receptor TLX in IL-1ß-induced changes in hippocampal neurogenesis
Authors	Ó Léime, Ciarán S.
Publication date	2017
Original Citation	Ó Léime, C. S. 2017. Investigating the regulatory role of the nuclear receptor TLX in IL-1ß-induced changes in hippocampal neurogenesis. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-11-02 03:18:16
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UNIVERSITY COLLEGE CORK

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Investigating the regulatory role of the nuclear receptor TLX in IL-1β-induced changes in hippocampal neurogenesis

Thesis presented by

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under the supervision of

Dr. Yvonne Nolan

Prof. John F. Cryan

for the degree of

Doctor of Philosophy

October, 2017

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Preface

All work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Dr. Yvonne Nolan and Prof. John Cryan between September 2013 and August 2017 in the Department of Anatomy and Neuroscience, University College Cork, Ireland. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at any other University.



Ciarán Ó Léime
October 2017

Abstracts and publications arising from this work

Journal articles:

O'Leime, C. S., Cryan, J. F. & Nolan, Y. M. 2017. Nuclear Deterrents: Intrinsic Regulators of IL-1beta-induced Effects on Hippocampal Neurogenesis. *Brain Behav. Immun.* PMID: 28751020 **(Published)**

O'Leime, C. S., Kozareva, D. A., Hoban A. E., Long-Smith, C. M., Cryan, J. F. & Nolan, Y. M. 2017. TLX is an intrinsic regulator of the negative effects of IL-1β on proliferating hippocampal neural progenitor cells. *FASEB J.* **(Published)**

Ó'Léime, C. S., Hoban, A E., Hueston, C. M., Stilling, R., Moloney, G., Cryan, J. F., & Nolan, Y. M. 2017. The orphan nuclear receptor TLX regulates hippocampal transcriptome changes induced by IL-1β. *Brain Behav. Immun* **(Under revision)**

Ó'Léime, C. S., Hueston, C. M., Cryan, J. F., & Nolan, Y. M. 2017. Enhanced hippocampal cell proliferation disrupts object recognition but not location recognition in rats. *Neurobiol. Learn Mem.* **(Submitted)**

Peer reviewed abstracts:

Ó'Léime, C. S., Hoban A. E., Kozareva, D. A., Hueston, C. M., Stilling R. M., Cryan, J. F. & Nolan, Y. M. 2017. The orphan nuclear receptor TLX regulates hippocampal transcriptome changes induced by IL-1β. Society for Neuroscience Annual Conference, 11^{th}-15^{th} November 2017, Washington DC, USA.

Ó'Léime, C. S., Kozareva, D. A., Long-Smith, C. M., Cryan, J. F. & Nolan, Y. M. 2016. TLX as a protective modulator against IL-1β-induced impairment in hippocampal neurosphere growth. Society for Neuroscience Annual Conference, 12^{th}-16^{th} November 2016, San Diego, USA.

Ó'Léime, C. S., Long-Smith, C. M., Cryan, J. F. & Nolan, Y. M. 2016. IL-1β negatively impacts upon expression of both inflammatory and neurogenesis-associated signalling molecules in hippocampal neural stem cells *in vitro.* Eurogenesis, 11^{th}-13^{th} July, Bordeaux, France.

Ó'Léime, C. S., Cryan, J. F. & Nolan, Y. M. 2016. IL-1β negatively impacts upon expression of both inflammatory and neurogenesis-associated signalling molecules in hippocampal neural stem cells *in vitro.* Psychoneuroimmunology Research Society, 8th-11th June 2016, Brighton, UK.

Other publications not featured in this work:

Kozareva, D. A., Hueston, C. M., Ó'Léime, C. S., S., Crotty, S., Dockery, P., Cryan, J. F. & Nolan, Y. M. 2017. Absence of the neurogenesis-dependent nuclear receptor TLX induces inflammation in the hippocampus. *J Neuroimmunol*. PMID: 28844503 **(Published article)**

Kozareva DA., Hueston CM., Ó'Léime C.S., Cryan JF., Nolan YM. "TLX, a regulator of neural stem cell self-renewal, is required for microglial integrity in the adult mouse dentate gyrus" (2015) SFN Conference, Chicago. **(Peer reviewed abstract)**

Hueston CM., Ó'Léime C.S., Kozareva DA., Cryan JF., Nolan YM. "Lentiviral overexpression of interleukin-1β in the hippocampus induces neurogenesis-associated cognitive deficits in adult male Sprague-Dawley rats" (2015) SFN Conference, Chicago. **(Peer reviewed abstract)**

Acknowledgements

First of all I would like to sincerely thank my supervisors Dr. Yvonne Nolan and Prof. John Cryan for their guidance, wisdom and support over the past four years. I am incredibly grateful that you gave this opportunity and provided me with this great experience as I begin my career.

To all the staff and students in the Department of Anatomy & Neuroscience who have helped me along the way I thank you all. Particularly I would like to thank Tara Foley and Gerard Moloney. Thank you guys for all your help with getting everything set up in the BSI and WGB and all your technical help. Gerry, I look forward to meeting you in Croke Park when Donegal and Dublin meet next September and Tara thank you very much for introducing me to the climbing wall. Nothing like hanging off a wall by your fingertips to de-stress after long day in the lab! I would also like to offer my sincere thanks to all the staff at the Biological Services Unit. Particularly, Jay, Aine, Brian, Noelle, Kieran, Trish, Fachna, Vince, and James I thank you all for your help during the last few months of animal work and being the best hosts!

To all the students and postdocs who have come and gone over the last four years I thank you for all the fun and laughs both science and non-science related. Particularly I would like to thank Seamus, Erin, Cara, Danka, Ken, Shauna, Alan, and Katie. You guys have been the greatest for all the craic and laughs throughout this journey and I count myself extremely lucky to have met such brilliant people. To the gym nuts Shauna and Katie, thank you for making me feel beautiful on the inside at least ☺ To Cara and Alan, thank you guys for being the greatest postdocs ever! To

Seamus, Erin, and Ken, coffee (strong tea in Donegal terms) is just not the same without you guys even without Seamus's endless rants on the merits of Bitcoin.

To my family I cannot thank you enough. I would not be where I am today if it wasn't for all your support from day one. To my parents, Sharon and Padraig, I could not have wished for better parents. I owe everything to you and am grateful everyday for the love, encouragement and support you give me every day of my life. To my brothers, Eoin and Cormac, I know this will earn me some slagging but you guys have been my best friends along the way. You have been an endless source of laughs and the *very* occasional source of wisdom (I'll let you two argue over who provided the most wisdom). I can always count on you guys to knock me down a few pegs when I get too ahead of myself and so I thank you for that.

Finally, I want to thank my partner in crime. Emily, you have been a constant support for the last 7 years and I thank you from the bottom of my heart for sticking with me through everything. Without you there this just would not have been possible. I am the luckiest guy to have met someone who will travel the world with me, put up with my whims and soundly beat me in any debate! I love you.

Author contributions

The author conducted all the work in this thesis independently with the exception of the following contributions:

Chapter 2

Danka Kozareva packaged all lentiviral constructs. Alan Hoban assisted in PCR analysis on lentiviral treated neurospheres. All authors listed helped prepared the manuscript for publication.

Chapter 3

Prof. Elizabeth Simpson provided the initial breeding mice used to generate the animals for this study. Cara Hueston set up the breeding colony in house and assisted with the stereotaxic surgeries. Alan Hoban conducted network and enrichment analysis on differentially regulated genes. All authors listed helped prepare the manuscript for publication.

Chapter 4

Cara Hueston assisted in the stereotaxic surgeries. All authors listed helped prepare the manuscript for publication

Chapter 5

Cara Hueston established the cafeteria diet feeding schedule. Alan Hoban assisted in conducting the PCRs and ELISA.

Abstract

Hippocampal neurogenesis is the process by which new neurons are born within the dentate gyrus (DG). This process begins during embryonic development and persists throughout life. Neurogenesis encompasses proliferation, differentiation and integration of neural progenitor cells (NPCs) into the surrounding neural network. Each stage is regulated by a host of intrinsic and extrinsic factor such as intracellular signalling molecules, exercise, environmental enrichment, diet and learning. TLX is an orphan nuclear receptor and transcription factor, which promotes the proliferation of NPCs, maintains the neurogenic pool of cells within the DG, and has been shown to promote hippocampal neurogenesis-associated cognition. Conversely, the pro-inflammatory cytokine IL-1β is a major mediator of the anti-neurogenic effects of hippocampal neuroinflammation, and previous work from the group has shown that IL-1β can suppress the expression of TLX within proliferating NPCs.

The aims of this thesis were to investigate the interactions between TLX and IL-1β both *in vitro* and *in vivo,* and to determine the behavioural outcome of enhancing TLX and IL-1β, as well as well as in response to dietary intervention *in vivo*. We demonstrate that IL-1β suppresses TLX expression and neurogenesis (neurosphere expansion) *in vitro*, and that these effects are mediated by the NF-κB pathway. Restoration of TLX expression is sufficient to attenuate the negative effects of IL-1β on neurogenesis. We have shown using an RNA sequencing approach that TLX expression maintains a reduced inflammatory transcriptional profile in the hippocampus at baseline, and regulates the transcriptional response to IL-1β *in vivo*. We demonstrate that lentiviral-mediated overexpression of TLX does not enhance hippocampal neurogenesis-associated cognitive processes *in vivo* but that it impairs

object recognition memory in rats. This suggests that enhancing cell proliferation is not sufficient to promote certain hippocampal-associated cognitive processes, and may even have a detrimental effect on cognitive behaviour. Finally, we show that an adolescent cafeteria diet which induces negative effects on hippocampal-associated memory, does not induce lasting cognitive defects when rats are switched to standard chow diet in adulthood. Lentiviral-mediated overexpression of IL-1β does not impact upon cognitive behaviours in rats fed a cafeteria diet throughout adolescence. However, we show that this chronic low-grade hippocampal IL-1β-mediated inflammation promotes fear memory in adulthood.

In summary, TLX and IL-1β can enhance and repress hippocampal neurogenesis respectively. Determining the role that TLX has on neurogenesis-associated cognition and how it can interact with IL-1β may position TLX as a novel therapeutic target for the treatment of neuroinflammatory-associated disorders where hippocampal neurogenesis is impaired.

Abbreviations

AAV	adeno-associated virus
Aβ	amyloid beta
AD	Alzheimer's disease
Adol.	Adolescent
ANOVA	analysis of variance
APP	amyloid precursor protein
APS	ammonium persulfate
BBB	blood brain barrier
bFGF	basic fibroblast growth factor
BDNF	brain derived neurotrophic factor
BrdU	5'-bromo-deoxyuridine
BSA	bovine serum albumin
CA	cornu ammonis
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
CMS	chronic mild stress
CMV	cytomegalovirus
CNS	central nervous system
CREB	cAMP response element binding protein
CUS	chronic unpredictable stress
DAPI	4',6-diamidino-2-phenylindole
DG	dentate gyrus
DIV	days *in vitro*
DCX	doublecortin
dH_2O	distilled water
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	embryonic day
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay

ER	estrogen receptor
FBS	fetal bovine serum
FDR	false discovery rate
g	grams
GABA	γ-aminobutyric acid
GCL	granule cell layer
GFP	green fluorescent protein
GFAP	glial fibrillary acidic protein
GO	gene ontology
GR	glucocorticoid receptor
GSK-3β	glycogen synthase kinase-3 beta
h	hour
HBSS	hanks balanced salt solution
HD	Huntingtin's disease
HDAC	histone deacetylase
HPA	hypothalamic-pituitary-adrenal
i.c.v.	introcerebroventricular
IκB	inhibitory kappa B
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IKK	IκB kinase
IL-1	interleukin-1
IL-1R1	interleukin-1 receptor type 1
IL-1R2	interleukin-1 receptor type 2
IL-1RA	IL-1 receptor antagonist
JNK	c-Jun N-terminal Kinase
kDA	kilo Dalton
KO	knockout
LPS	lipopolysaccharide
LTP	long term potentiation
LSD1	lysine specific demethylase
LV	lentivirus
MAPK	mitogen activated protein kinase
mg	milligrams

MHC	major histocompatibility complex
Min	minutes
miR	micro RNA
ml	millilitre
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
MYD88	myeloid differentiation factor 88
NDS	normal doneky serum
NFκB	nuclear factor kappa B
ng	nanogram
NMDA	N-methyl-D aspartate
NPC	neural precursor cell
NR2E1	nuclear receptor subfamily 2 group E member 1
NSAID	non steroid anti-inflammatory drug
NSC	neural stem cell
OEX	overexpression
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
PD	Parkinson’s disease
PFA	paraformaldehyde
PPAR	peroxisome proliferator-activated receptor
qRT-PCR	quantitative reverse transcriptase PCR
rcf	relative centrifugal force
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNAse	ribonuclease
RT	room temperature
rtPCR	reverse transcriptase PCR
RPM	revolutions per minute
SDHA	succinate dehydrogenase A
SDS	sodium dodecyl sulfate
SGZ	subgranular zone

SSRI	selective serotonin reuptake inhibitor
SVZ	subventricular zone
PAGE	polyacrylamide gel electrophoresis
TACE	TNF-α converting enzyme
TGF	transforming growth factor
TLL	tailless
TLR	toll like receptor
$TLX^{-/-}$	TLX knockout
TNFα	tumour necrosis factor-α
TNF-R1	tumour necrosis factor-receptor 1
TU	transfection units
µg	microgram
µM	micromolar
µl	microlitre
UV	ultraviolet
WT	wildtype

Chapter 1: General introduction

Chapter sections published in Brain, Behavior and Immunity.

1.1: The hippocampus

The limbic system is a group of forebrain structures that are involved in motivation, emotion, learning and memory. The hippocampus, a component of the limbic system, is a bilateral curved structure situated within the medial temporal lobe. The hippocampus consists of three main regions which as a whole constitute the hippocampal formation. These three regions are the hippocampus proper or Cornu ammonis (CA), the subiculum and the dentate gyrus (DG). The hippocampus proper can be subdivided transversely into 4 main layers, the *Stratum Oriens* which consists of basal dendrites and axon collaterals of pyramidal neurons (the main excitatory neurons of the hippocampus), the *Stratum Pyramidae* which consists of the cell bodies of the pyramidal neurons, the *Stratum Radiatum* which consists of dendrites and recurrent axon collaterals of the pyramidal cells and finally the *Stratum Moleculare* containing distal dendrites of the pyramidal cells and some incoming axons from the perforent pathway (see figure 1.1 for diagram of hippocampal substructure locations) (Nolte & Sundsten 2002). The *Stratum Moleculare* faces the DG and it is within the DG that neurogenesis occurs. The DG consists of three main layers, the *Stratum Granulare*, the *Stratum Moleculare* and the *Stratum Multiforme.* It is within the *Stratum Granular*, specifically the Subgranular Zone (SGZ) of the DG, that neural precursor cells reside and proliferate throughout adulthood (Altman & Das 1965).

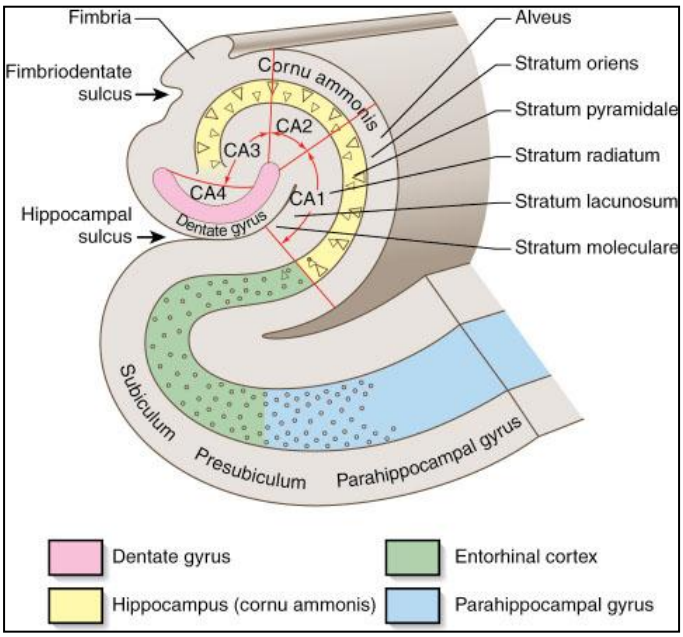


Figure 1.1: Hippocampal Structure (Kandel et al. 2000)**.** A diagram of a human hippocampus cut in the coronal plane with major substructure labelled.

The main external neuronal inputs into the hippocampus come from the entorhinal cortex (EC) which communicates signals from cortical regions, the amygdala and the olfactory cortex to the distal dendrites of the granule cells within the *Stratum Moleculare* (Kandel et al. 2000). After the DG, the signal is relayed on to the CA3 region of the hippocampus proper. From here the signals are sent to the CA1 region. Neurons present within the subiculum, CA1 and CA3 regions project axons into the fornix which is the main output pathway. This is the main information processing pathway within the hippocampus and is known as the trisynaptic circuit (Kandel et al. 2000; Nolte & Sundsten 2002).

1.1.1: Function of the hippocampus

Functionally, the hippocampus has a major role in memory formation and recall. Primarily, the hippocampus is known to be involved in declarative memory (Squire 1992) and spatial awareness (Olton 1977; Ekstrom et al. 2003). Two types of memory that have been widely studied in relation to the role of the hippocampus and memory formation are declarative (explicit) and non-declarative (implicit) memory. Declarative memory involves the conscious recall of previous experiences or factual knowledge about people, places and things (Kandel et al. 2000). Declarative memory can also be subdivided into episodic memories concerning specific events and semantic memories concerning knowledge of words etc. (McClelland et al. 1995). Non-declarative memory is an unconscious form of memory and is involved in performing specific tasks such a riding a bike. Much of the early work in understanding the differences between declarative and non-declarative memory came from studies involving a human patient referred to as patient H.M (Milner et al. 1968). In 1952 at the age of 27, patient H.M. underwent an experimental procedure to remove parts of the medial temporal lobe including the hippocampus, amygdala and parahippocampal gyrus to alleviate severe seizures he suffered as a result of a childhood bicycle accident. As a result, patient H.M. suffered from severe but very specific memory loss. His non-declarative memory remained largely intact in that he could retain his command of language and IQ post surgery. However, he was not able to process new memories post surgery. This was one of the first cases linking memory and the medial temporal lobe, a large part of which is the hippocampus.

More recent studies have expanded on this showing that declarative memory can be impaired by hippocampal damage resulting in retrograde or anterograde amnesia

(Squire et al. 2004). However, non-declarative memory can remain intact after hippocampal impairment (McClelland et al. 1995). Despite the hippocampus showing function in memory development and recall, it has also been shown to have a role in emotion and anxiety and that functions appear to be specific to sub-regions within the hippocampus. This functional segregation of the hippocampus has been shown via several lesion studies where the ventral area has been shown to be more involved with anxiety and emotional behaviours, with the dorsal hippocampus being more involved with spatial learning and memory (Bannerman et al. 2004; McHugh et al. 2011; Kjelstrup et al. 2002; Bast et al. 2009). Another indication of the role of the hippocampus in the regulation of mood and emotion comes from the fact that it is part of the limbic system (Sahay and Hen, 2007). The limbic system of brain structures also includes the amygdala and prefrontal cortex (PFC). This system relays synaptic inputs which inhibits the paraventricular nucleus (PVN) of the hypothalamus. The PVN is part of the hypothalamic-pituitary-adrenal (HPA) axis. This axis involves the transduction of neuronal stress responses into endocrine responses by releasing corticotrophin-releasing factor (CRH) from the PVN which stimulates adrenocorticotropic hormone (ACTH) from the pituitary, ultimately leading to glucorticoid release from the adrenal glands (Lopez et al., 1999). Heightened activation of this axis is evident after stress and chronic activation has been implicated in the etiology of depression and other mood disorders (Kessler, 1997, Sapolsky, 2000, Sapolsky, 1996, Lupien et al., 2009).

1.1.2: Experimental assessment of hippocampal-associated cognition

Various behavioural techniques have been developed to assess hippocampal-associated spatial cognition including contextual fear conditioning and spatial

navigation in the Morris water maze, radial arm maze, Barnes maze, Y-maze, or T-maze and pattern separation (Wills et al., 2014, Sahay et al., 2011a, Clelland et al., 2009). Most of these behavioural paradigms rely on relational learning, e.g. the location of the platform in the Morris water maze in relation to visual cues around the arena and/or the innate exploratory behaviour of rodents (Morris et al., 1982, Crusio et al., 1989).

The Y-maze, T-maze and radial arm maze can be used to assess spontaneous alternation behaviour in rodents (Hughes, 1997, Hughes, 2004, Gerlai, 2001). This type of behaviour involves rodents' tendency to explore a novel environment. In the context of a maze, e.g. a Y-maze, continued spontaneous alternation behaviour rodents is commonly used as a quick test of spatial working memory and was first used for testing drug effects on spatial memory (Kokkinidis and Anisman, 1976b, Kokkinidis and Anisman, 1976a, Drew et al., 1973, Swonger and Rech, 1972). In this test, rodents are placed in one arm of the maze and allowed to explore freely during which time the sequence of arm entries is recorded. These sequences are used then to calculate the proportion of arms entered that were different to previous entries which is usually reported as percentage alternation in the Y-maze (Hughes, 2004). Alternation is determined from successive entries of three arms on an overlapping triplet set where three different arms are entered (Sarter et al., 1988). Therefore, this task requires the rodents to spatially differentiate what arm they last entered and subsequently enter a previously unexplored arm. The T-maze can also be used to assess continued spontaneous alternation behaviour and it has been demonstrated that in normal mice approximately 70% of these triplets will be three consecutively different arms. Impairments in hippocampal function by excitotoxic

lesions have been shown to impair the percentage alternation in rodents (Gerlai, 1998) thus indicating that this task is hippocampal-associated.

Contextual fear conditioning is another behavioural paradigm that has been shown to be sensitive to hippocampal dysfunction (Kim and Fanselow, 1992, Phillips and LeDoux, 1992). This test involves an acquisition phase of placing an animal within a specific context and inducing a fear response within said context via a small footshock. After a defined delay period, the animal is then returned to the context but this time is not exposed to a footshock. The animals freezing behaviour is recorded and animals that have normal hippocampal function are able to recall the context and associated footshock and thus freeze more. Addition of a tone or cue before the shock during the acquisition phase can also be used to differentiate hippocampal-associated behaviour and non-hippocampal-associated behaviour. Freezing behaviour in this cued fear conditioning paradigm has been shown to be maintained even after a hippocampal lesion and is suggested to involve the amygdala (Phillips and LeDoux, 1992). Hippocampal lesion studies using contextual fear conditioning as a behaviour read-out have highlighted interesting discrepancies in performance depending on when the lesions are carried out. For example, rodents who received a hippocampal lesion 1 day after the acquisition phase showed a significant impairment in context recall (Kim and Fanselow, 1992). However, if lesions to the hippocampus are carried out prior to the acquisition phase, up to 28 days prior, rodents are still able to acquire a fear response to the context (Kim and Fanselow, 1992). This indicates that rodents are able to recruit non hippocampal based neuronal circuits to aquire a contextual fear response if there is hippocampal dysfunction during the memory acquisition phase. However, once the contextual fear memory is

acquired, subsequent hippocampal disruption results in impaired retrieval of that memory.

The Morris water maze involves assessing the ability of rodents to locate and escape from a platform within a large arena filled with opaque water. In this test the platform is hidden and the latency for the rodent to locate the platform by relying on external visual cues around the arena is recorded (Morris, 1984). This paradigm requires that the animal be trained to locate the hidden platform placed just beneath the surface of the water. To achieve this, the animal must be given a sufficient amount of external visual cues outside the arena, however, the number and type of visual cues vary widely across studies. It has been shown that lack of any visual cues significantly slows the animal from successfully locating the platform whereas by simplifying the external cues (cues closer to arena) rodents showed faster acquisition of the platform location during the acquisition/training phase (Morris, 1984, D'Hooge and De Deyn, 2001, Lamberty and Gower, 1991). After a defined delay, rodents are returned to the maze with the platform removed. In this test phase the amount of time the animal spends in the quadrant where the platform was is recorded and is used to indicate the spatial memory performance of the animal. This task has shown robust reproducibility in mice across laboratories where latency for rodents to find the platform are similar across testing sites and when factors such as apparatus used, training protocol, animal age and animal housing are accounted for (Crabbe et al., 1999).

Similar to contextual fear conditioning, performance in the Morris water maze is strongly associated with hippocampal function as several lesion studies have shown

that when the hippocampus is damaged rodents are significantly impaired in learning the location of a hidden platform (Morris, 1984, Morris et al., 1982, Pearce et al., 1998). Additionally, it has been suggested that the dorsal hippocampus is more critical in performance in the Morris water maze than the ventral hippocampus (Moser et al., 1993, Moser et al., 1995).

The ability to distinguish between similar experiences is important in episodic memory and is a function of the hippocampus (Yassa and Stark, 2011). This process is widely known as pattern separation. Pattern separation involves taking similar sensory inputs and transforming them into dissimilar outputs to ensure the sensory inputs can be recalled as different experiences (Sahay et al., 2011a). In its simplest form, assessing pattern separation in rodents involves assessing behaviour of rodents in two similar contexts. If the rodents successfully distinguish the two contexts their behaviour will be different in each context. For example, they may show increased exploratory behaviour in the novel context (Clelland et al., 2009, Bekinschtein et al., 2013a). Pattern separation load can then be adjusted by increasing or decreasing the similarity between the two contexts. Hippocampal DG lesion studies have demonstrated that when the hippocampus is damaged, rodents fail to distinguish between two similar contexts compared to controls (Hunsaker et al., 2008). Additionally, it has been demonstrated that enhancing hippocampal neurogenesis promotes pattern separation (Sahay et al., 2011a).

1.2: Adult neurogenesis

Within the adult brain of many mammalian species including rodents and non-human primates, neurogenesis occurs in two specific regions known as neurogenic

niches, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the DG in the hippocampus (Suh et al., 2009, Eriksson et al., 1998, Alvarez-Buylla and Lim, 2004). In these niches it has been shown that neural progenitor cells (NPCs) have the ability to replenish themselves via symmetrical division, or differentiate into specialized cells of the CNS, such as mature neurons or astrocytes via asymmetrical division (Gage et al., 1995, Fisher, 1997, Zhao et al., 2008). In humans, while there is clear evidence to show ongoing neurogenesis in the DG of the hippocampus, there appears to be negligible levels of SVZ neurogenesis (Bergmann et al., 2012, Spalding et al., 2013). More recently it has also been proposed that neurogenesis occurs within the striatum of humans (Ernst et al., 2014).

Evidence of adult neurogenesis first began to appear as early as the 1960s where radiolabelling studies using adult rats showed that there was an increase in newborn cells within the DG of the hippocampus (Altman and Das, 1965). This study demonstrated proliferating cells within the hippocampus of rats by injecting the rats with thymidine-H^3. Thymidine-H^3 is utilised in the production of chromosomal DNA in dividing cells (Hughes et al., 1958, Leblond et al., 1959). Thus the uptake of this compound within cells of the hippocampus was proof that dividing cells exist within the adult brain. Then in the 1980s, it was first demonstrated that these newborn cells can functionally integrate into the surrounding CNS neuronal circuitry in songbirds (Paton and Nottebohm, 1984). NPCs were then isolated from the adult mammalian brain in the 1990s (Reynolds and Weiss, 1992, Richards et al., 1992)**.** With the introduction of BrdU, a thymidine analogue capable of labelling proliferating cells and allowing lineage tracing of these newborn cells, neurogenesis was finally

demonstrated within humans (Eriksson et al., 1998, Kuhn et al., 1996). It has been shown that these proliferating cells are NPCs that are capable of differentiating into mature neurons, astrocytes, or oligodendrocytes via asymmetrical division (Gage, 2000, Zhao et al., 2008).

Hippocampal neurogenesis begins during embryonic development and persists throughout life. Multipotent neural progenitor cells (NPCs) undergo a stepwise process of proliferation, differentiation into newborn neurons and functional integration of those new neurons into the surrounding circuitry (See figure 1.2) (Alvarez-Buylla and Lim, 2004). Each of these cellular processes is under strict regulation by a wide range of intracellular and extracellular factors. Reductions in hippocampal neurogenesis have been associated with neurological and psychiatric disorders, such as Alzheimer's disease and major depression, as well as with aging (Verret et al., 2007, Sahay and Hen, 2007, Kuhn et al., 1996, Maruszak and Thuret, 2014). This then raises the question; can therapies aimed at promoting or protecting hippocampal neurogenesis be used to subsequently improve cognitive decline in these conditions? With our greater understanding of the promoters and suppressors of the various cellular processes of neurogenesis, we will stand well-equipped to address this question in future research.

It has been proposed that neurogenesis also occurs within the striatum of humans, however this has yet to be widely validated (Ernst et al. 2014). The term niche refers to a micro environment within certain tissue types that possess the ability to maintain a stable population of proliferating stem or progenitor cells (Morrison and Spradling, 2008). Thus a niche should be an environment that if its resident stem cells are

depleted it should be able to take up and maintain a newly introduced population of stem cells.

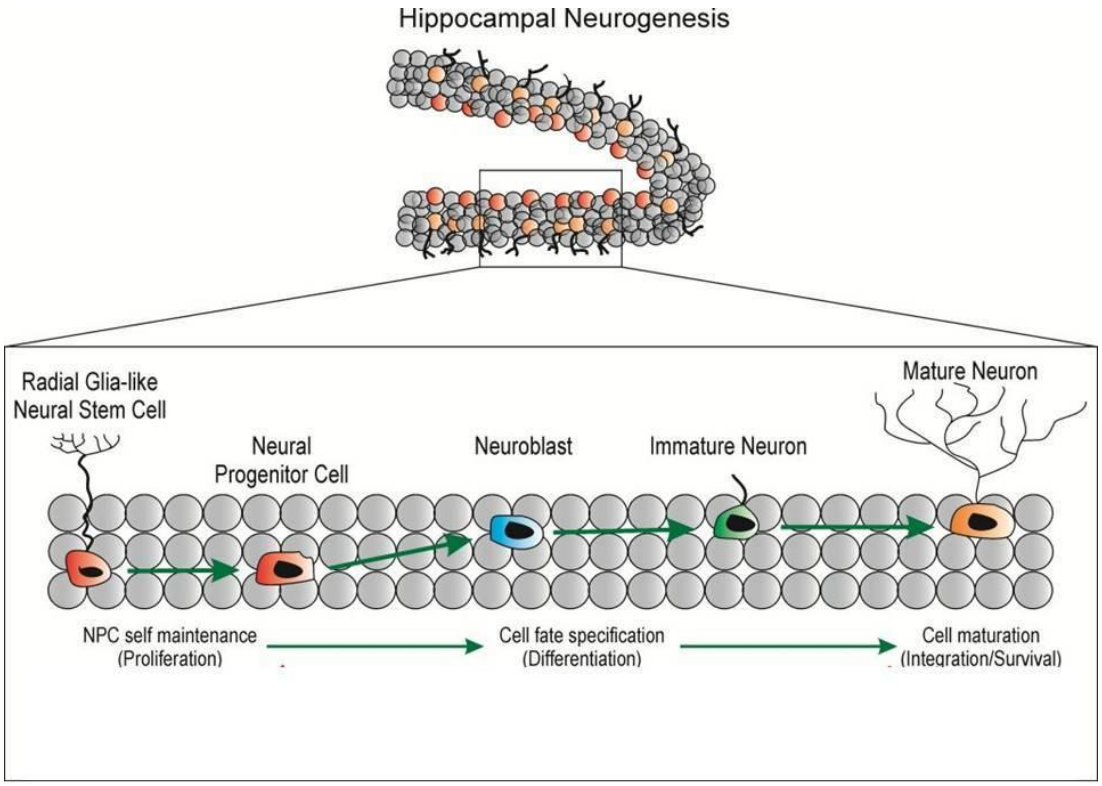


Figure 1.2. Cells and processes involved in hippocampal neurogenesis. Adapted from (O'Leime et al., 2017a).

1.2.1: The neural stem cell (NSC) and neural progenitor cell (NPC)

Multipotent NSCs reside within the embryonic and adult hippocampus and can give rise to NPCs (Gage, 1998, Kriegstein and Alvarez-Buylla, 2009, Eriksson et al., 1998). Potentiality of cells is a term used to describe the ability of certain cells to form other cell types. Totipotent refers to stem cells that can form any cell type, pluripotent refers to stem cells that are more lineage restricted and thus cannot form placental cells while mulitpotent cell types can only form cells resident in the organ that the multipotent cells are situated. In the case of multipotent NPCs, they can form

specialised cells of the central nervous system (CNS) (Fisher, 1997, Kennea and Mehmet, 2002). The distinctions between NPCs and NSCs are not yet clearly defined yet it is thought that NPCs are an intermediary between NSCs and differentiated cells and have a more limited capacity for self-renewal compared to NSCs (Gage, 1998, Gage, 2000, Sohur et al., 2006, Olynik and Rastegar, 2012). However, broadly speaking, NSCs and NPCs can be called NPCs (Sohur et al., 2006). Therefore, for simplicity in this thesis NPCs will refer to all undifferentiated cells that have the ability to self-replicate and differentiate into various cellular lineages of the central nervous system (CNS), such as neurons, astrocytes or oligodendrocytes.

To date NPCs have been isolated from multiple adult rodent brain regions and can be isolated from all embryonic brain regions (Brewer and Torricelli, 2007, Shanley and Sullivan, 2006, Reynolds and Weiss, 1992). NPCs were first isolated from adult mice in 1992 (Reynolds and Weiss, 1992). Reynolds and Weiss demonstrated the isolated NPCs could be induced to differentiate into neurons and astrocytes *in vitro* thus demonstrating the multipotentiality of these cells. The NPCs within the neurogenic niches of the mammalian brain is maintain by a careful balance of symmetrical and asymmetrical division (Zhao et al., 2008). NPCs divide symmetrically to produce two identical daughter cells from a single parent cell and maintain the NPC pool. NPCs can also asymmetrically divide as they begin to differentiate with a signal parent cell producing one NPC and another lineage fate determined cell. This lineage fate determined cell can either become a neuron, astrocyte or oligodendrocyte as it differentiates further (Breunig et al., 2007).

1.2.2: Cells and processes of adult hippocampal neurogenesis

The progression of NPCs in the adult hippocampus towards functionally integrated mature neurons is a multi step process. As such, a model has been developed to outline the main features of the NPCs and the development processes they undergo in all mammalian species (See figure 1.3) (Kempermann et al., 2004a). This model proposes 6 developmental milestones. The first is the proliferation of 'type 1' NPCs, or early progenitor cells. These cells are radial glial-like cells with a distinct morphology of a triangular soma with apical processes along the vertical axis (Seri et al., 2001, Filippov et al., 2003). These cells display the astrocytic marker glial fibrillary acidic protein (GFAP) and the marker for NPCs nestin. They make up approximately two thirds of the nestin expressing cells within the murine SGZ (Filippov et al., 2003, Kronenberg et al., 2003). However, these cells divide very slowly as they only account for approximate 5% of dividing cells in the SGZ at any given time (Kronenberg et al., 2003). Since this discovery, another type 1 cell has been identified with a horizontal morphology and more rapid rate of proliferation (Lugert et al., 2010).

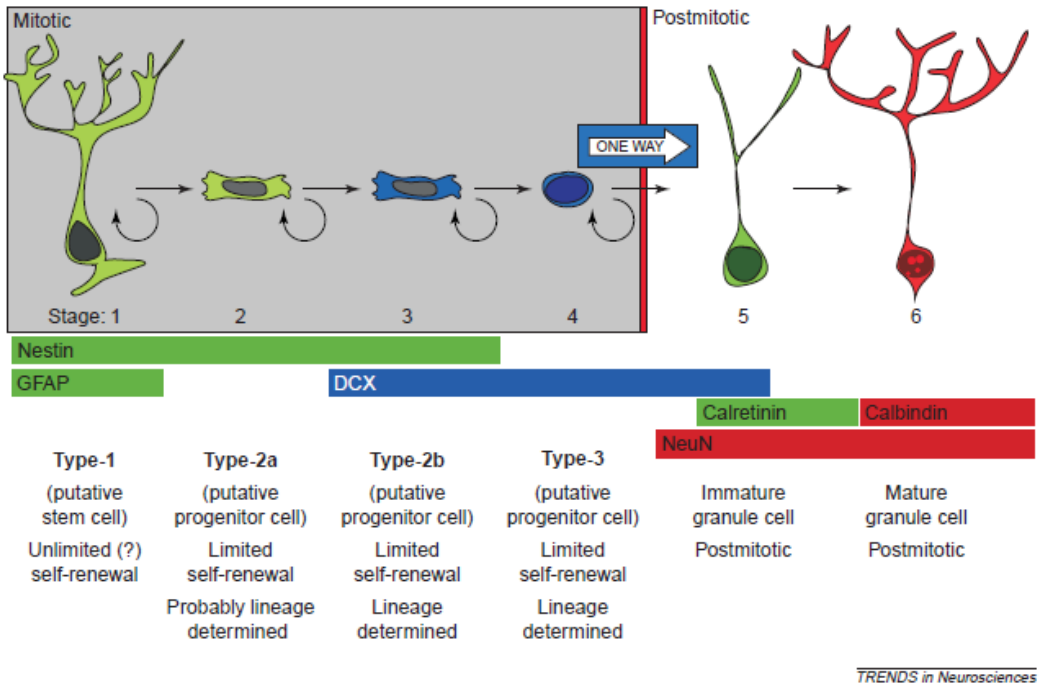


Figure 1.3: Propose model outlining the various steps NPCs take as they undergo the neurogenic process (Kempermann et al., 2004a).

In stages 2 to 4, these type 1 cells divide asymmetrically to produce type 2 cells. Type 2 cells are morphologically different to type 1 cells in that they have a more flattened longitudinal shape. However they are still classed as NPCs (Kronenberg et al., 2003, Filippov et al., 2003). They are GFAP negative and are highly proliferative (Kronenberg et al., 2003). Type 2 cells are subdivided into two categories both of which express nestin, name type 2a and type 2b (Kempermann et al., 2004a, Kronenberg et al., 2003). The difference between these two types is that type 2a cells exlusively express nestin whereas type 2b express both nestin and the marker for immature neurons, doublecortin (DCX) (Filippov et al., 2003, Kronenberg et al., 2003, Brown et al., 2003b, Brandt et al., 2003). The expression of DCX indicated that type 2b cells are lineage determined. Type 3 cells proliferating neuroblasts and are a continuation of the type 2b cells. They exclusively express DCX and the

appearance of these cells represents a major morphological change from what was NPC into what will become a neuron (Brandt et al., 2003). These cells are restricted to a neuronal lineage and express the polysialated form of neural cell adhesion molecule (PSA-NCAM) (Kempermann et al., 2004a).

During stage 5, type 3 cells then leave the cell cycle and become postmitotic. Approximately 3 days after the first cell division, they transiently express the calcium binding protein calretinin and the neuronal marker NeuN (Kempermann et al., 2004a). During this stage, newborn neurons begin their migration from the SGZ towards the granule cell layer (GCL). These new neurons then begin to extend their axons to the CA3 region of the DG and become tonically activated by GABA approximately 1 week after their birth (Brandt et al., 2003, Espósito et al., 2005)

Finally, stage 6 involves the integration and final maturation of these post-mitotic type 3 cells into the granular cell layer (GCL) of the DG. After 2-3 weeks they switch from expressing calretinin to calbindin while maintaining NeuN expression. At the same time they begin to develop dendritic GABAergic synaptic inputs (Espósito et al., 2005). However, functionally it takes approximately 5 weeks for these new neurons to become indistinguishable from the surrounding neurons. This was demonstrated by quantifying the expression of neuronal activation markers such as c-fos in newborn neurons versus mature neurons after simulation by a learning based cognitive task, in this case the Morris water maze (Jessberger and Kempermann, 2003). This study demonstrated that as newborn neurons mature, they gradually increased their expression of markers of neuronal activation until by 5 weeks they had similar levels of expression to older neurons. Despite a clear model

available for the processes of adult neurogenesis, no clear model is available to date for embryonic neurogenesis (Kempermann et al., 2004a).

1.2.3: Embryonic vs. adult hippocampal neurogenesis

Within the hippocampus, pyramidal neurons are formed within the CA1 and CA3 regions during embryonic development and reside there throughout adulthood (Altman and Bayer, 1990). Granule cells in the DG begin their development during the embryonic period and continue to be produced post-natally (Altman and Bayer, 1990). Development of the DG begins at embryonic day (E) 14 in the rat. At this timepoint, precursor cells from the dentate neuro epithelium on the medial wall of the lateral ventricle become postmitotic (Schlessinger et al., 1978, Laplagne et al., 2007). Once these cells become post mitotic they migrate to the external shell of the outer blade of the DG. By E18 a secondary dentate shell is evident as a result of proliferating neuroblasts migrating to form the granular cell layer (GCL). Mature granule neurons are evident 2-3 days before birth with a tertiary dentate shell around the hilus region evident at birth. These granule neurons continue to be produced post-natally as the DG continues to form until it is fully developed by postnatal day (P) 30. By this time NPCs are established in the SGZ where neurogenesis occurs throughout adulthood (Gage, 2000, Altman and Bayer, 1990). When comparing rodent hippocampus and DG development to human development, it is noteworthy to consider that humans have a much longer gestational period as well as having a larger hippocampal formation. The DG granule neurons in humans can be identifiable at the 11.5^{th} gestational week (Humphrey, 1967). Using the proliferation marker Ki67, it was found that proliferating cells are widespread in various hippocampal regions in developing humans at the 14^{th} week and as gestation

progresses these proliferating cells become more restricted to the hilus region of the DG (Seress, 2007). Thus, taking rodent gestation to be approximately 3 weeks and human gestation to be on average 40 weeks, it can be seen that appearance of granule neurons appear much earlier in humans than rodents. However, this may be due to the evolutionary differences between rodents and humans as well as the larger hippocampal formation in humans.

The continuation of neurogenesis throughout adulthood was largely discovered by employing thymidine-H^3 or bromodeoxyuridine (BrdU) labelling of mitotic cells within the hippocampus. To date, it has been demonstrated in most mammalian species (Gould et al., 1997, Kukekov et al., 1999, Ming and Song, 2005, Kornack and Rakic, 1999, Eriksson et al., 1998). Within the adult brain, neurogenesis appears to be largely confined to two main areas known as neurogenic niches. These niches are known as the SGZ of the DG in the hippocampus and the SVZ of the lateral ventricle. Within these niches, adult neurogenesis involves slow proliferation of a pool of early progenitors cells, more rapid proliferation of progenitors that are more restricted in their lineage fate and finally integration of newborn neurons into the surrounding circuitry (Ehninger and Kempermann, 2008). It is estimated that in the adult human hippocampus there are around 700 new neurons produced each day (Spalding et al., 2013). Interestingly, it is suggested that humans replace approximately 35% of their DG through neurogenesis whereas rodents are estimated to replace only around 10% (Ninkovic and Gotz, 2007, Imayoshi et al., 2008). Furthermore, it is well established that there is an age-related decline in neurogenesis in rodent models and now this phenomenon has been demonstrated in humans. (Spalding et al., 2013, Knoth et al., 2010). Pathology-associated deficits in

neurogenesis observed in various experimental models of Alzheimer's disease (AD) and Parkinson's disease (PD) have also been mirrored in adult human neurogenesis (Crews et al., 2010, Höglinger et al., 2004, Zeng et al., 2016, Klein et al., 2016). Thus, traits in rodent models of adult neurogenesis appear to be shared in human hippocampal neurogenesis and so this provides validation for these models.

The cellular processes involved in embryonic and adult neurogenesis are very similar with the two main differences between each primarily arising from differences in the tissues that the NPCs are situated in (Kempermann et al., 2004a). Firstly, adult NPCs require a microenvironment conducive to neurogenesis (the neurogenic niche) within a larger non-neurogenic environment. In effect, adult NPCs need to be protected from the wide range of potential anti-neurogenic influences, which may reside within the surrounding brain tissue. In this regard, it has been shown that adult rat NPCs derived from the non-neurogenic spinal cord region show self-renewal and multipotentiality properties once transplanted into the DG (Shihabuddin et al., 2000). This indicates that the microenvironment in the DG is crucial for providing support for neurogenesis to occur. Secondly, adult neurogenesis is an individualistic process with cells at all stages of development present in the same microenvironment at the same time whereas embryonic neurogenesis is a highly coordinated process with large groups of cells undergoing the same developmental process at the same time (Kempermann et al., 2004a). Despite these differences, individual adult NPCs have been shown to reiterate all the cellular processes of embryonic NPCs during developmental neurogenesis i.e. proliferation, differentiation and functional integration of newborn neurons (Kempermann et al., 2004a, Zhao et al., 2008). Additionally, the intracellular mechanistic features that regulate each stage of

neurogenesis are suggested to be the same in the DG throughout all stages of development (Pleasure et al., 2000). Therefore, promoters and inhibitors of neurogenesis are likely to have mechanistically similar effects on embryonic NPCs and adult derived NPCs.

1.3: Functional role of adult hippocampal neurogenesis

Functionally, neurogenesis has been linked to certain hippocampal cognitive tasks such as spatial learning, memory and regulation of emotion (Ming and Song, 2011, Cameron and Glover, 2015, van Praag et al., 2005, Shors et al., 2002). However, its exact role remains unclear with a large body of conflicting reports within the literature assessing its involvement in various hippocampal-associated cognitive tasks (Deng et al., 2010). Hippocampal neurogenesis has been suggested to play a role in the stress response and adaptation of the hippocampus in terms of learning and memory to certain stressors (Cameron and Glover, 2015, Levone et al., 2015, Snyder et al., 2011). Additionally, hippocampal neurogenesis has been proposed to be involved in the regulation of anxiety as well as facilitating antidepressant action (Revest et al., 2009, Santarelli et al., 2003).

1.3.1: Neurogenesis in learning and memory

A growing body of evidence over the past two decades suggests that newly born neurons in the adult hippocampus contribute to hippocampal dependent learning and memory (Deng et al., 2010). Techniques to manipulate neurogenesis in rodents have provided direct evidence of the role of hippocampal neurogenesis in behaviour. These include irradiation of the hippocampus (Huo et al., 2012, Madsen et al., 2003, Saxe et al., 2006), as well as transgenic animal models (Snyder et al., 2016,

Cummings et al., 2014, Saxe et al., 2006) and viral mediated techniques targeting neurogenic promoters specifically within NPCs (Murai et al., 2014, Jessberger et al., 2009b, Taliaz et al., 2010) to ablate or enhance neurogenesis. The majority of these techniques used to ablate hippocampal neurogenesis have demonstrated impairments in hippocampal associated cognition, such as novel object and novel location recognition, fear conditioning, long-term spatial memory in a Morris water maze test and pattern separation, an ability to distinguish two similar contexts (Jessberger et al., 2009b, Saxe et al., 2006, Snyder et al., 2005, Dupret et al., 2008, Deng et al., 2009, Sahay et al., 2011a, Winocur et al., 2006, Tronel et al., 2012, Clelland et al., 2009). It has been shown that neurogenesis and its functional consequences on cognition can be selectively enhanced by enhancing NPC survival. Specifically, a subsequent improvement in pattern separation has been reported in transgenic mice where NPC survival was enhanced by selectively ablating the pro-apoptotic gene *Bax* in NPCs (Sahay et al., 2011a). It has also been established that increasing hippocampal neurogenesis via physical exercise induces an improvement in cognitive performance (Voss et al., 2013, van Praag et al., 2005, Ryan and Kelly, 2016). However, enhancing neurogenesis is complex due to its multifactorial process of proliferation, differentiation and survival of the newborn neurons, and thus it may be difficult to definitively uncover the role of enhanced neurogenesis by manipulating one aspect of the process.

It should be noted that it has also been reported that ablation of hippocampal neurogenesis had no effect on some of these hippocampal-dependent tasks (Hernandez-Rabaza et al., 2009, Wojtowicz et al., 2008, Groves et al., 2013). Furthermore, a meta-analysis of the relevant literature revealed no significant effects

of ablation of adult neurogenesis on spatial memory or cued fear conditioning (Groves et al., 2013), although the meta-analysis showed high levels of heterogeneity among the studies. Thus, while there is general consensus that adult hippocampal neurogenesis is required for pattern separation, the functional role of hippocampal neurogenesis has not yet been clearly demonstrated. It has been suggested that the heterogeneity in the findings to date may be due to differences in species, method of ablation, behavioural test used or in the variation in stress levels (Groves et al., 2013, Dranovsky and Leonardo, 2012, Glasper et al., 2012).

One proposed cellular mechanism for learning and memory is via changes in hippocampal synaptic plasticity. Long term potentiation (LTP) is an electrophysiological correlate of changes in synaptic plasticity and it has been shown that adult born neurons contribute to LTP formation within the DG of rats (Snyder et al., 2001). Ablating neurogenesis by using either pharmacological suppression of proliferation, or hippocampal irradiation inhibited LTP formation in the DG (Wang et al., 2008, Garthe et al., 2009). Conversely, LTP can itself increase the proliferation of NPCs in the DG if it is induced in the medial perforant pathway, which relays synaptic input to the DG (Bruel-Jungerman et al., 2006). This reciprocal relationship between neurogenesis and LTP is suggested to underlie the beneficial effects of learning a new task on neurogenesis and vice versa (Snyder et al., 2001, Deng et al., 2009, Deng et al., 2010).

It is generally believed that neurogenesis can promote the formation of new memories however it has also been suggested that hippocampal neurogenesis can promote forgetting of old memories (Frankland et al., 2013, Welberg, 2014, Akers et

al., 2014, Epp et al., 2016). As hippocampal neurogenesis ultimately involves the generation of new synaptic inputs from new neurons into the existing hippocampal circuitry, it is believed that these new inputs can interfere with existing neuronal circuitry (Akers et al., 2014). It has been demonstrated that increasing neurogenesis in adult rats after contextual fear conditioning by either voluntary running, administration of memantine or fluoxetine or conditional deletion of p53 impairs contextual recall (Akers et al., 2014). Thus, it appears that increased levels of hippocampal neurogenesis can impair the stability of existing memories and indeed it has been shown that reducing hippocampal neurogenesis can promote the stability of these memories (Epp et al., 2016). Additionally, this neurogenesis-associated forgetting has been suggested to be involved in the absence of memories from the first few years of life however conclusive evidence of this yet still needed (Welberg, 2014)

1.3.2: Neurogenesis in mood and antidepressant function

It is well established that prolonged periods of heightened stress exposure can be detrimental to brain function and emotion. These effects have been clearly demonstrated within the hippocampus where chronic stress has been demonstrated to induce hippocampal atrophy, neuronal death, impairments in neurogenesis and in hippocampal-associated behaviours (Hueston et al., 2017, Sapolsky, 1996, Malberg and Duman, 2003, Hanson et al., 2011). The fact that the hippocampus appears to be involved in some aspects of regulating the stress response and the clear deleterious effects of stress on the hippocampus has naturally led researchers to question the role hippocampal neurogenesis has on the development of depression (Mirescu and

Gould, 2006, Dranovsky and Leonardo, 2012, Dranovsky and Hen, 2006, Sahay and Hen, 2007, Snyder et al., 2011).

The functional link between hippocampal neurogenesis and depression began to emerge when it was noticed that most clinically used antidepressants, electroconvulsive therapy and environmental interventions such as exercise all enhanced hippocampal neurogenesis (Malberg et al., 2000, Madsen et al., 2000, Perera et al., 2007, Duman, 2004, van Praag et al., 1999c). It is evident from the literature that rodent and non-human primate models of depression have impairments in hippocampal neurogenesis (Czeh et al., 2001, Kronenberg et al., 2009, Tanti and Belzung, 2013) and patients with major depression show reduced hippocampal volume which could be linked to reduced neurogenesis (Eisch and Petrik, 2012, Small et al., 2011, Fotuhi et al., 2012). It has also been demonstrated that ablation of neurogenesis by irradiation blocked the behavioural effects of antidepressants in mice exposed to chronic unpredictable stress (Santarelli et al., 2003). This study used focal irradiation of the hippocampus to ablate neurogenesis and showed that treatment with antidepressants did not reverse the increase in latency for these mice to feed in a novel environment, a task usually sensitive to antidepressant treatment. This has led to the theory that some antidepressants work by altering neurogenesis and that neurogenesis itself is an important process in mood regulation (O'Leary and Cryan, 2014, Tanti and Belzung, 2013, Eisch and Petrik, 2012). Structurally, the link between neurogenesis and mood regulation has been associated with the ventral hippocampus. Evidence for this comes from the fact that connections from the ventral hippocampus are made with structures associated with the HPA axis. It has also been demonstrated that the mood stabilising drug lithium preferentially

promotes neurogenesis in the ventral hippocampus (O'Leary et al., 2012). However, it must be noted that in this study this effect was only apparent in stressed animals and not in non-stressed animals thus highlighting the potential role of neutral hippocampal neurogenesis in stress responses.

However, it is unlikely that impaired neurogenesis is an etiological factor in depression or other mood disorders. Support from this comes from the fact that ablation of neurogenesis does not induce depression-like or anxiety-like behaviours in rodents at baseline (Santarelli et al., 2003). Additionally, blocking neurogenesis does not induce anxiety-like behaviour in tests such as the open field, light-dark box, or elevated plus maze nor do mice lacking neurogenesis (via irradiation of the hippocampus or genetic ablation of glial fibrillary acidic protein-positive neural progenitor cells) display depression-like behaviour in the forced-swim test (Holick et al., 2008, Airan et al., 2007, Saxe et al., 2006). Thus, this theory has seen some modifications in recent years and it is now believed that neurogenesis can be regulated by stress and antidepressants 'under certain as-yet-undefined conditions' rather than neurogenesis being the underlying mediator of stress, depression and antidepressant treatment (Hanson et al., 2011). Thus, the exact role of hippocampal neurogenesis in mood regulation remains a complex field of research.

It has been suggested that the environment that the new neurons are born into will determine their functional significance with regard to both learning and memory and mood regulation (Dranovsky and Leonardo, 2012, Glasper et al., 2012). For example, it has been proposed that neurons born in conditions of low stress contribute to learning and memory while neurons born in conditions of high stress

contribute to anxiety-like and escape behaviours (Glasper et al., 2012). Indeed, evidence now suggests that there is a critical role for hippocampal neurogenesis in the stress response and in the regulation of emotion (Cameron and Glover, 2015, Levone et al., 2015, Snyder et al., 2011). Moreover, recent evidence indicates that there is overlap between the role of neurogenesis in learning and emotion by demonstrating that inhibition of adult neurogenesis in the mouse DG reduces defensive behaviour to ambiguous threat cues but does not if the same negative experience is reliably predicted (Glover et al., 2017). This indicates that neurogenesis is required for adapting to a high stress situation of uncertainty whereas adaptation to a predictable stressor may be neurogenesis-independent. This then suggests that neurogenesis is a means of fine-tuning behavioural responses to environmental cues.

1.3.3: Hippocampal neurogenesis in disease

As there is strong evidence currently available to support the role of hippocampal neurogenesis in promoting hippocampal function (learning and memory, and emotional regulation), there is unsurprisingly a wealth of evidence linking neurogenesis to a myriad of neurological diseases affecting the hippocampus. Altered hippocampal neurogenesis has been associated with various preclinical models of neurological and psychiatric disorders such as epilepsy, schizophrenia and neurodegenerative diseases. Many of these diseases are associated with impaired hippocampal-associated cognition. However, whether abnormal neurogenesis contributes to the development of these diseases or results as a consequence remains to be determined (Danzer, 2008, Hsieh and Eisch, 2010).

Whether neurogenesis is increased or decreased depends on the disease state, and increases in neurogenesis do not always represent a positive response. In various rodent models of epileptogenesis including pilocarpine models of temporal lobe epilepsy (TLE) and direct limbic system electrode stimulation where prolonged seizure activity is produced, there is also a robust increase in NPC proliferation (Bengzon et al., 1997, Parent et al., 1997, Scott et al., 1998). This enhancement of proliferation also translates to increased neuronal differentiation thus indicating successful neurogenesis (Parent et al., 2006). However, the timing of seizure induction is an important factor in determining the effects on neurogenesis. Shortly after kainic acid induced TLE (5 days), there is an increase in NPC proliferation however after 5 months there is a decrease in neurogenesis compared to control animals (Hattiangady et al., 2004). Indeed, in human hippocampal tissue there is evidence of decreased neurogenesis in epilepsy patients (Mathern et al., 2002, Fahrner et al., 2007). Epilepsy is also associated with aberrant displacement of NPCs and axonal reorganisation such that these newborn neurons integrate abnormally into the surrounding circuitry and promote network hyperexcitabiliy that is associated with epilepsy (Parent, 2007). This aberrant displacement of NPCs and axonal reorganisation is thus suggested to contribute to epileptogenesis and impairments in cognition (Balu and Lucki, 2009).

Schizophrenia, like depression, has been strongly associated with reduced hippocampal volume (MacDonald and Schulz, 2009). This may be as a result of impaired neurogenesis, however, the most robust evidence linking schizophrenia to impaired neurogenesis comes from assessing the role of Disrupted-in-Schizophrenia-1 (DISC1) gene in schizophrenia. The mutated form of DISC1 is an indicator of

schizophrenia susceptibility while its normal form appears to be critical for neurogenesis (Enomoto et al., 2009, Ming and Song, 2009, Mao et al., 2009, Kim et al., 2009). In preclinical studies using various models of schizophrenia including mice expressing mutated DISC1 and repeated phencyclidine (PCP) administration, there is a decrease in NPC proliferation and impaired survival and integration of newborn neurons (Duan et al., 2007, Liu et al., 2006, Maeda et al., 2007, Reif et al., 2007). Additionally, mutated DISC1 has been shown to be highly expressed specifically in the hippocampi of patients diagnosed with schizophrenia (Nakata et al., 2009). It has also been demonstrated that NPC proliferation is reduced in the hippocampi of schizophrenia patients and it is suggested that this might contribute to some of the cognitive deficits observed in these patients (Reif et al., 2006). Interestingly, drugs used to counteract symptoms of schizophrenia such as antipsychotics (clozapine), NMDA antagonists (memantine), and acetylcholinesterase inhibitors (donepezil) generally enhance various specific stages of neurogenesis and so these may produce their therapeutic effects in part by reversing the impairments in neurogenesis (Maeda et al., 2007, Kotani et al., 2008, Namba et al., 2009, Balu and Lucki, 2009).

Alzheimer's disease (AD) is characterised by neuronal loss and the build-up of hyperphosphorylated tau and amyloid beta (Aβ) protein into plaques within the basal forebrain, cortex, hippocampus and amygdala (Hardy and Selkoe, 2002). The hippocampus is one of the first structures to be affected in this disease (Fox et al., 1996). There are conflicting reports demonstrating both increased and decreased hippocampal neurogenesis in various AD animal models as well as in clinical samples of hippocampal tissue (Haughey et al., 2002, Jin et al., 2004a, Jin et al.,

2004b). Additionally, Aβ has been demonstrated to stimulate hippocampal NPC proliferation *in vitro* (Lopez-Toledano and Shelanski, 2004). In an AD mouse model expressing a double or triple mutation in the amyloid precursor protein (APP) there is enhanced hippocampal NPC proliferation coupled with increased spine density and longer dendrites in newborn neurons (Jin et al., 2004a, Sun et al., 2009). Indeed, in humans it has been demonstrated that there is an increase in newborn neurons in the SGZ and CA1 region of the hippocampus in post-mortem tissue from AD patients (Jin et al., 2004b). However, it has been suggested that this may be a compensatory mechanism to replace neuronal loss (Winner et al., 2011). There is also evidence demonstrating that neurogenesis is decreased in human post-mortem tissue and that this may be mediated by increased bone morphogenic protein 6 (BMP6) expression which is known to suppress embryonic neurogenesis (Crews et al., 2010, Nakashima and Taga, 2002). Thus, as AD is a complex and multifaceted disease, adult hippocampal neurogenesis may be differentially affected depending on the stage of the disease, the degree of plaque build-up, or even the age at which the disease begins (Winner et al., 2011).

Hunttington's disease (HD) and Parkinson's disease (PD) are neurodegenerative disorders with similar disease etiology to that of AD. Both HD and PD are associated with protein aggregates, neuronal loss and lead to devastating impairments in cognition, particularly in HD, with PD characterised by initial motor dysfunction and late stage cognitive impairments (Winner et al., 2011, Collins et al., 2012). HD and PD have been shown to be associated with impaired hippocampal neurogenesis *in vivo* in mice models of these diseases (Lazic et al., 2004, Kohl et al., 2007, Crews et al., 2008). However, as with AD, HD and PD are multifaceted neurodegenerative

diseases and so the exact impact on hippocampal neurogenesis may differ depending on the disease state (Winner et al., 2011).

1.4: Regulators of hippocampal neurogenesis

Hippocampal neurogenesis is regulated by a wide range of intrinsic signalling molecules as well as by external factors influenced by the surrounding environment (Aimone et al., 2014). Intrinsic regulators of hippocampal neurogenesis include intracellular signalling pathways within NPCs, such as Wnt, Notch, NF-κB and mitogen-activated protein kinase (MAPK) pathway (Johnson et al., 2009, Aimone et al., 2014, Mu et al., 2010, Lie et al., 2005, Breunig et al., 2007, Faigle and Song, 2013). These intrinsic factors regulate gene expression within NPCs in order to control their proliferation, differentiation and maturation into neurons or glia (Mu et al., 2010). Expression and function of these intrinsic regulators can be altered by external environmental factors, which subsequently lead to altered hippocampal neurogenesis. The main external environmental factors that influence hippocampal neurogenesis include stress, exercise, diet, learning, environmental enrichment and environmental toxins (Besnard and Sahay, 2016, Stangl and Thuret, 2009, Deng et al., 2010, Brown et al., 2003a, van Praag et al., 1999c). Voluntary exercise, learning, environmental enrichment and a healthy diet have all been shown to promote neurogenesis (Gould et al., 1999, An et al., 2008, van Praag et al., 1999c, van Praag et al., 1999a). However, the mechanism underlying their positive effects on neurogenesis is the subject of much research. For example, enhanced serum concentration of brain derived neurotrophic factor (BDNF) and increased levels of dietary nutrients, such as flavonoids (enriched in foods such as cocoa and blueberries) have been proposed as mediators of the pro-neurogenic effects of diet

and exercise, respectively (Erickson et al., 2011, An et al., 2008). Environmental factors that negatively regulate neurogenesis include stress, an unhealthy diet and environmental toxin exposure (Gould et al., 1998, Lindqvist et al., 2006, Sava et al., 2007, Desplats et al., 2012). Interestingly, these negative regulators are all associated with neuroinflammation as evidenced by increased microglial activation and increased pro-inflammatory cytokine release (Tynan et al., 2010, Goshen et al., 2008, Goshen and Yirmiya, 2009, Boitard et al., 2014, Purisai et al., 2007). Pro-inflammatory cytokines released from activated microglia can affect many aspects of hippocampal neurogenesis including the proliferation, differentiation and maturation of NPCs. Therefore, they are robust manipulators of hippocampal neurogenesis (Green et al., 2012, Green and Nolan, 2012b, Ryan et al., 2013, Keohane et al., 2010, Yirmiya and Goshen, 2011, Borsini et al., 2015). While microglia are the predominant source of pro-inflammatory cytokines within the brain (Hanisch and Kettenmann, 2007), it should be noted that astrocytes are also susceptible to stimulation to release cytokines. However, they produce relatively low levels of cytokines but have been shown to potentiate the release of cytokines from microglia in response to an inflammatory insult (Barbierato et al., 2013, Chen et al., 2015).

Successful hippocampal neurogenesis requires and is regulated by a wide range of intrinsic and extrinsic signals provided by the microenvironment in which the neural progenitor cells reside known as the neurogenic niche. Environmental factors influencing NPCs within these neurogenic niches include other NPCs, surrounding mature cells, cilia, secreted factors and neurotransmitters (Alvarez-Buylla & Lim 2004; Ma et al. 2005; Faigle & Song 2013). The neurogenic niche is the main source of factors that determine whether NPCs undergo symmetrical or asymmetrical

division. Indeed, it has been demonstrated that cells derived from the spinal cord, traditionally not a neurogenic niche, display multipotentiality traits when transplanted into the DG (Shihabuddin et al., 2000). Additionally, local interneurons within the SGZ of the DG can release GABA which is known to enhance dentritic development and synaptic integration of newborn neurons (Ge et al., 2006, Ge et al., 2007). Thus, the neurogenic niche within which the NPCs reside is the main determinant of fate of the cells rather than intrinsic factors within the cells themselves (Olsson et al., 1998, Brustle et al., 1997).

It is thought that, within the SVZ and SGZ in particular, there are specific factors present that promote the differentiation and integration of new neurons. Within the SGZ, NPCs reside within close proximity to a dense layer of granular neurons including both mature neuronal and immature neuronal phenotypes. There are also glial cells present including astrocytes and oligodentrocytes which are believed to play a role in maintaining NPCs within the niche. For example, it has been demonstrated *in vitro* that hippocampal astrocytes and not astrocytes from non-neurogenic spinal cord tissue, promote the neuronal differentiation and survival of newborn neurons derived from adult hippocampal NPCs (Lim and Alvarez-Buylla, 1999, Song et al., 2002). Astrocytes are known to express a number of secreted and membrane attached factors such as cytokine (IL-1β and IL-6) and also growth factor related proteins (insulin-like growth factor binding protein 6) that are known to regulate proliferation and differentiation of NPCs (Barkho et al., 2006). It has also been demonstrated that the Wnt signalling pathway is involved in astrocyte-mediated promotion of neurogenesis, as inhibition of this pathway impairs the neurogenesis supportive role of astrocytes (Lie et al., 2005). Within the SVZ, NPCs are situated in

close proximity to ependymal cells of the lateral ventricles and these ependymal cells express various factors such as noggin (bone morphogenic protein antagoinist) and pigment epithelium-derived factor which can act to regulate NPC self-renewal (Ramirez-Castillejo et al., 2006, Lim et al., 2000).

Microglia can also regulate NPCs activity. Not only do microglia remove the remains of apoptotic neurons, it has also been suggested that activation of microglia is required for the beneficial effects of environmental enrichment on neurogenesis (Ziv et al., 2006). Additionally, under inflammatory conditions or after tissue damage, the release of either pro- or anti-inflammatory cytokines by activated microglia can also regulate NPCs activity either positively or negatively depending on the balance of these cytokines (Ekdahl et al., 2009).

NPCs in the SVZ and SGZ are also situated near the vasculature. Indeed within the SGZ, dense clusters of proliferating NPCs are found anatomically close to capillaries (Palmer et al., 2000). This then makes NPCs susceptible, not just to factors produced locally within the neurogenic niche, but also peripheral factors that can circulate in the blood stream and have direct effects on NPCs (Alvarez-Buylla and Lim, 2004). It has been shown that infusion of vascular endothelial growth factor (VEGF) can promote NPC proliferation in the SGZ and SVZ (Cao et al., 2004). Additionally, this effect was shown to be blocked by expression of a dominant negative VEGF receptor type 2, and that VEGF is required for the neurogenic effects of exercise and environmental enrichment. The close proximity of NPCs to the vasculature can also partly explain the beneficial effects of certain dietary factors on neurogenesis (Hueston et al., 2017). For example, flavonoids (a plant metabolite found in foods

such as blueberries, tea, and cocoa) and blueberries have been shown to enhance proliferation in chronically stressed and aged rats, respectively (An et al., 2008, Casadesus et al., 2004). It is suggested that these beneficial effects may be mediated in part by increasing circulating levels of brain derived neurotrophic factor (BDNF) (An et al., 2008). As well as receiving beneficial effects of peripheral stimuli, NPCs can also receive negative effects via the vasculature. For example stress and hippocampal pituitary adrenal (HPA) axis activation can increase circulating levels corticosterone (CORT) (cortisol in humans), which in turn can suppress NPCs activity (Wurmser et al., 2004).

The importance of the vasculature in delivering factors to the neurogenic niche that can regulate hippocampal neurogenesis has been clearly demonstrated using heterochronic parabiosis (Villeda and Wyss-Coray, 2013). In this study, young mice were exposed to the systemic environment of older mice by suturing young and old mice together such that a shared circulatory system was generated (Conboy et al., 2005). In these paired mice, there is a significant impairment in neuronal differentiation and survival as well as synaptic plasticity in the young mice however the opposite occurs in the older mice (Villeda and Wyss-Coray, 2013). This study shows that the neurogenic niche and residing NPCs are susceptible to circulating factors that can enter the niche via the vasculature. Thus, the neurogenic niche under baseline conditions maintains expression of factors conducive to neurogenesis. However, it is susceptible to detrimental changes in these factors which in turn can inhibit NPC activity and neurogenesis.

1.4.1: Extrinsic regulators of hippocampal neurogenesis

Hippocampal neurogenesis is a highly dynamic physiological process and as such is sensitive to a wide range of extrinsic factors. Extrinsic factors are known to both enhance and suppress neurogenesis depending on the specific factor (Aimone et al., 2014). Commonly studied extrinsic regulators of neurogenesis include environmental enrichment, exercise, learning, diet and stress (van Praag et al., 1999c, van Praag et al., 1999a, Kempermann et al., 1997a, Gould et al., 1999, Stangl and Thuret, 2009, Dranovsky and Hen, 2006).

1.4.1.1: Environmental enrichment

An enriched environment is generally defined as an environment that provides sensory, social and motor stimulation (Aimone et al., 2014). This paradigm involves placing animals in enlarged enclosures with added tunnels, toys, running wheels and other animals for social interaction and was originally demonstrated to induce enhanced neuroplasticity in rodents (Rosenzweig, 1966, Rosenzweig et al., 1978, Walsh et al., 1969). Environmental enrichment (EE) was first demonstrated to increase the number of newborn cells as labelled by BrdU and measured 40 days after mice were exposed to EE (Kempermann et al., 1997a). This study did not demonstrate an increase in proliferation of NPCs and it largely believed that EE promotes the survival of newborn neurons rather than enhancing their proliferation (Kempermann et al., 2010). However, more recently an increase in proliferation by Ki67 labelling of proliferating cells after 40 days of EE was demonstrated (Karelina et al., 2012).

EE-induced newborn neurons are suggested to correlate with improved performance in hippocampal neurogenesis-associated behaviours such as spatial navigation in the Morris water maze (Kempermann et al., 1998, Kempermann et al., 1997a). The mechanism by which EE enhances neurogenesis is complex but it is suggested to be mediated in part through the increased production of neurotrophins and growth factors such as VEGF and BDNF within the hippocampus. In rats, shRNA-mediated knockdown of VEGF prevents the pro-neurogenic effects of EE (Cao et al., 2004). In mice lacking mitogen/stress-activated kinase 1 (MSK1), a downstream target of BDNF, there is no effect of EE on NPC proliferation suggesting that BDNF/MSK1 signalling is a critical component of the effects of EE (Karelina et al., 2012). It should be noted though that increases in VEGF after EE have been reported in rats but not in mice and so there may be species specific differences in the mechanisms of EE-induced neurogenesis (Kuzumaki et al., 2011).

1.4.1.2: Exercise

In many EE paradigms, running wheels were added to the environment and it was since discovered that voluntary exercise alone is one of the strongest environmental enhancers of neurogenesis (van Praag et al., 1999c, van Praag et al., 1999a). Rodents who are allowed access to a running wheel in their homecage show increases in synaptic strength and plasticity, hippocampal-associated cognition, expression of growth factors and proliferation of NPCs (Wang and van Praag, 2012, van Praag et al., 1999a, van Praag et al., 1999c). Indeed, it has been suggested that the positive effects of EE are predominantly a result of increased exercise activity (Kobilo et al., 2011). One study where mice were divided into four groups (control, running, EE and EE plus running), only the groups with access to running wheels demonstrated

increases in NPC proliferation, newborn neurons and neuronal survival (Kobilo et al., 2011). Notwithstanding this, it is believed that EE and exercise have additive effects on neurogenesis, with EE enhancing the survival of newborn neurons and exercise promoting NPC proliferation (Kempermann et al., 2010, Fabel et al., 2009).

Aerobic exercise has been demonstrated to promote hippocampal neurogenesis-associated spatial navigation behaviour in the MWM. Mice who were allowed access to a running wheel displayed reduced latencies to find the hidden platform (van Praag et al., 1999a). Additionally, exercise has been shown to reverse the detrimental effects of aging on neurogenesis and related cognition (van Praag et al., 2005). Like with EE, BDNF has been suggested to be a major component in mediating the effects of exercise on neurogenesis (Bekinschtein et al., 2011). BDNF mRNA expression was found to be increased in rats after exercise and was strongly correlated with the distance the rats ran on the running wheels (Neeper et al., 1995). Moreover, blockade of BDNF signalling in rats was shown to prevent exercise-induced improvements in MWM performance, and intracerebroventricular injection of BDNF can mimic exercise induced improvements in object recognition (Gomez-Pinilla et al., 2008, Griffin et al., 2009).

1.4.1.3: Learning

It is widely accepted that hippocampal neurogenesis has been linked with improved learning and memory in certain hippocampal-associated tasks (Leuner et al., 2006). Interestingly, the process of learning has been linked in turn with enhancing neurogenesis (Gould et al., 1999, van Praag et al., 1999a). Evidence for a positive relationship between learning and neurogenesis first appeared in studies assessing

neurogenesis in songbirds during seasonal fluctuations in song learning behaviour (Alvarez-Buylla et al., 1990, Goldman and Nottebohm, 1983). Evidence then came from rodent studies where rats trained in trace eyeblink conditioning and spatial learning in the MWM displayed an increase in the number of newborn neurons after training (i.e learning) (Gould et al., 1999). Moreover, the effects of learning appeared to require learning hippocampal-dependent tasks, as hippocampal independent tasks (i.e. delay eyeblink conditioning and cued navigation in the MWM) did not increase the number of newborn neurons (Gould et al., 1999). Since then, studies have been carried out to try and elucidate the complex and seemingly reciprocal relationship between learning and neurogenesis.

The process of learning is suggested to comprise of two main phases (Dobrossy et al., 2003). The first phase which involves rapid improvement in performance was found to have no effect on the number of newborn neurons in rats, whereas the second phase which involves slow increases in performance increased the number of newborn neurons and their survival after MWM training (Dobrossy et al., 2003). This effect was also demonstrated in trace eyeblink conditioning in rats (Leuner et al., 2004). The number of learning or training sessions has been shown to determine the impact on neurogenesis with few trials increasing cell survival and more trials having no effect or even decreasing cell survival (Gould et al., 1999, Dobrossy et al., 2003, Olariu et al., 2005, Ambrogini et al., 2004, Snyder et al., 2005). However, this may be task dependent as 200 trace eyeblink training trials did not enhance cell survival whereas 800 trials did (Leuner et al., 2004). Moreover, it has more recently been suggested that for neurogenesis to be enhanced by learning, the learning paradigm must be sufficiently difficult, yet must be successfully learned (Curlik and

Shors, 2011). There is a clear linkage between successful neurogenesis and learning and vice versa. However, the ability of one to regulate the other is complex and may be task difficulty and duration dependent.

1.4.1.4: Diet

Diet is a readily modifiable factor that can influence cognition, mood, and hippocampal neurogenesis (Stangl and Thuret, 2009, Zainuddin and Thuret, 2012, Murphy et al., 2014). In humans it is suggested that the increase in 'Western' diets high in processed foods, fats and sugars is contributing to increased cognitive and emotional disorders (Jacka et al., 2010, Kanoski and Davidson, 2011). Conversely, healthy diets such as the Mediterranean diet that are high in vegetables, fruits, fish, and nuts and are rich in macronutrients such as omega-4, flavonoids promote healthy cognition (Willett et al., 1995, Feart et al., 2010, Panza et al., 2004).

There are several ways in which diet can be modulated to potentially alter hippocampal neurogenesis, including altering calorie intake, meal texture and/or meal composition (Stangl and Thuret, 2009). Low calorie diets have been shown to increase memory performance in aged humans (Witte et al., 2009). Likewise in rodents, calorie restriction has been shown to enhance survival of new neurons and improve spatial learning in the radial arm maze and MWM (Lee et al., 2002a, Lee et al., 2002b, Lee et al., 2000, Stewart et al., 1989). Interestingly, intake of soft textured diet in rats has been associated with decreased proliferation in the hippocampus, however the mechanism for this is not fully understood (Aoki et al., 2005). It has since been demonstrated that liquid diet intake does impair proliferation in the hippocampus but does not alter the rate of new neuron production thus ultimately not

affecting neurogenesis (Patten et al., 2013). Diets rich in flavonoids, a compound found in berries, green tea, parsley and cocao, can increase NPC proliferation and performance in hippocampal-related memory tasks (Wang et al., 2011, Yoo et al., 2010, Lee et al., 2010). Additionally, in rats exposed to chronic unpredictable stress, a flavonoid rich diet reversed the stress-induced decrease in proliferating NPCs within the DG (An et al., 2008). Similar pro-neurogenic and improved neurogenesis-associated cognitive effects have been demonstrated with omega-3 fatty acids, polyphenols, magnesium, and zinc (Kawakita et al., 2006, Valente et al., 2009, Abumaria et al., 2013, Levenson and Morris, 2011).

Conversely, diets high in fat and/or sugar have been demonstrated to impair hippocampal neurogenesis and associated cognition. For example, access to sucrose in drinking water for just two hours daily impaired hippocampal-associated spatial pattern separation and this coincided with a reduction in newborn neurons in the hippocampus of rats (Reichelt et al., 2016). Likewise, high fat diet intake has been shown to impair NPC proliferation and is also linked with impairments in spatial memory in the MWM in rats (Lindqvist et al., 2006, Molteni et al., 2002). The combination of both high fat and high sugar diet into a single 'cafeteria diet' has also been shown to impair hippocampal-associate spatial memory after just 5 days of consuming the diet in rats (Beilharz et al., 2014). Interestingly, it has been shown that young mice (P21) fed a high fat diet for 11 weeks exhibit impaired cognitive performance in the radial arm maze and a reduced number of newborn neurons whereas adult mice fed the same diet do not (Boitard et al., 2012). Similarly, rats displaying cognitive deficits in MWM performance after juvenile high fat diet intake displayed no deficits after adult high fat diet intake (Boitard et al., 2014). However,

in rats fed a high fat diet for 3 months and then switched to standard chow for 3 months, impairments in MWM performance are reversed (Boitard et al., 2016). Taken together, these studies indicate that the adolescent period is a particularly plastic period yet also vulnerable to diet induced impairments in hippocampal neurogenesis-associated cognition (Hueston et al., 2017).

The mechanisms by which diet influences neurogenesis are complex. For many dietary modulations, whether there is a direct interaction between nutrients in the various diets and NPCs or if the diets regulate neurogenesis indirectly, remain to be elucidated. In many cases it may be a combination of both. For example, omega-3 fatty acids have been shown to directly stimulate the birth of new neurons *in vitro* while also enhancing pro-neurogenic BDNF expression which could indirectly stimulate neurogenesis (Kawakita et al., 2006, Wu et al., 2004). Interestingly, the pro-neurogenic effect of calorie restriction was not evident in $BDNF^{-/+}$ mice where there is reduced BDNF signalling (Lee et al., 2002a). This suggests that BDNF may mediate some of the effects of certain diets on neurogenesis. Alternatively, the effects of diet on neurogenesis may, in part, be mediated by its effects on the gut microbiota composition. As diet is the leading factor in microbiota alterations, and microbiota composition has been demonstrated to affect hippocampal neurogenesis, this represents another possible indirect mechanism through which diet can manipulate neurogenesis (Ogbonnaya et al., 2015, Heberden, 2016, De Filippo et al., 2010, McAllan et al., 2014).

1.4.1.5: Stress

Stress has widely been demonstrated as a potent manipulator of neurogenesis (Aimone et al., 2014, Levone et al., 2015, Schoenfeld and Gould, 2012). Stressors known to affect neurogenesis can either be physical (e.g. restraint stress, footshock), social (e.g. social defeat or subordination stress), or odour (e.g. predator odour) based. Additionally, these stressors can either be chronic or acute (Schoenfeld and Gould, 2012, Gould et al., 1997, Tanapat et al., 2001, Malberg and Duman, 2003). Chronic social defeat stress, where an aggressor rodent is placed in the cage with the test subject, has been demonstrated to decrease NPC proliferation in several animal models (Czeh et al., 2001, Czeh et al., 2007, Ferragud et al., 2010). Similar anti-neurogenic effects have been demonstrated after chronic stress paradigms using restraint stress and footshock, which impair NPC proliferation and neuronal differentiation in rodents however, the effects on differentiation may result from impaired proliferation (Dagyte et al., 2009, Bain et al., 2004).

Acute stressors are generally a single stressful event and results are more variable than chronic stress. Acute exposures to predator odour or acute social defeat have been shown to robustly reduce NPC proliferation and neuronal differentiation in rodents (Tanapat et al., 2001, Lagace et al., 2010). Acute restraint stress have shown conflicting results with some studies showing no effect on NPC proliferation in adult rats (Pham et al., 2003), and others showing decreased proliferation in rats and an increase in mice (Bain et al., 2004). Chronic restraint stress has also yielded species dependent results, with rats exhibiting reduced newborn neuron survival but enhanced survival in mice (Snyder et al., 2009). The conflicting results have been suggested to be a result of variations in species and strain differences in stress

susceptibility (Buynitsky and Mostofsky, 2009). Indeed, it has also been suggested that variation in neurogenesis across species and strains may determine their responses to stressors, and increased neurogenesis may even promote stress resilience (Levone et al., 2015).

Stress is associated with HPA activation and release of glucocorticoids from the adrenal glands into the bloodstream (Schoenfeld and Gould, 2012). Corticosterone itself can cross the blood brain barrier and has been demonstrated to suppress NPC proliferation (Cameron and Gould, 1994b, Wong and Herbert, 2006). Conversely, adrenalectomy has been shown to result in enhanced NPC proliferation (Gould et al., 1992, Cameron and Gould, 1994a). This suggests that stress effects on circulating glucocorticoids mediate its effects on neurogenesis. However, it has been demonstrated that the pro-inflammatory cytokine interleukin-1 beta (IL-1β) mediates the effects of stress, specifically on neurogenesis (Koo and Duman, 2008).Thus, the mechanism of stress-induced impairments in neurogenesis may rely on multiple factors including glucocorticoid and cytokine expression.

1.4.2: Intrinsic regulators of hippocampal neurogenesis

As well as extrinsic regulators of neurogenesis, there are a host of intracellular intrinsic regulators of neurogenesis. These include members of several signalling pathways and associated transcription factors that act to regulate gene expression within NPCs, which in turn maintains the various stages of neurogenesis under strict control (Horgusluoglu et al., 2017, Aimone et al., 2014, Zhao et al., 2008). As neurogenesis is a complex and dynamic process, so too are the intrinsic factors that regulate it, with many overlapping signalling pathways which are differentially

activated depending on the stage of neurogenesis they regulate (Aimone et al., 2014, Urbán and Guillemot, 2014).

1.4.2.1: Signalling pathways in neurogenesis

Several critical signalling pathways are involved in regulating various stages of hippocampal neurogenesis including NPC proliferation. Arguably some of the most important of these pathways include Wnt, Notch, and Sonic hedgehog (Shh) signalling pathways (Aimone et al., 2014, Balu and Lucki, 2009). Wnt proteins and their cognate receptors are expressed in hippocampal NPCs (Lie et al., 2005). Wnt proteins are cystein rich secreted glycoproteins and can signal via two distinct pathways, the canonical β-catenin pathway and non-canonical β-catenin-independent pathway (Nusse and Varmus, 1982, Willert et al., 2003). Typically, it is signalling via the canonical β-catenin pathway that regulates Wnt effects on hippocampal neurogenesis (Willert et al., 2003). Wnt proteins can be released by astrocytes near the NPCs within the DG which in turn promotes NPC proliferation and neuronal differentiation via activation of Wnt signalling-dependent activation of NeuroD1 (Kuwabara et al., 2009, Song et al., 2002). Additionally, it has been demonstrated that overexpression of Wnt3 increased NPC proliferation and neuronal differentiation within the hippocampus while blockade of Wnt signalling reduced proliferation and differentiation (Lie et al., 2005). Glycogen synthase kinase 3 beta (GSK3β) is a negative regulator of Wnt signalling via inhibiting the Wnt co-signalling molecule β-catenin, and plays an important role in maintaining control of Wnt signalling to regulate normal neurogenesis, particularly during embryonic development (Ikeya et al., 1997, Reya et al., 2003, Zeng et al., 1997, Salinas and

Zou, 2008). Conversely, GSK-3β may mediate some of the anti-proliferative effects of IL-1β associated inflammation on NPCs (Green and Nolan, 2012b).

Notch signalling regulates a wide range of aspects involved in neurogenesis including NPC proliferation, differentiation, and survival (Louvi and Artavanis-Tsakonas, 2006, Faigle and Song, 2013). Notch receptors are single-pass transmembrane receptors that, when activated by surface ligands on neighbouring cells, are cleaved to release an intracellular fraction that translocates to the nucleus (Schroeter et al., 1998). Overexpression of activated Notch in adult mice has been demonstrated to induce an increase in NPC proliferation thus, maintaining the NPC pool (Breunig et al., 2007). Likewise, inhibition of Notch signalling led to a decrease in NPC proliferation and premature neuronal differentiation (Breunig et al., 2007). In addition to this, Notch deletion led to dendritic arborisation whereas Notch1 overexpression led to increased dendritic complexity (Breunig et al., 2007). Thus, Notch signalling acts to maintain NPC in a proliferative state and also regulates the dendritic complexity of newborn neurons.

Sonic hedgehog (Shh) is an extracellular soluble signalling protein that has been demonstrated to be vitally important in regulating nervous system development (Ruiz i Altaba et al., 2002). Post-natally, Shh has been shown to be involved in the formation and maintenance of a hippocampal NPC pool (Han et al., 2008). Shh signals via two different receptors, patched (Ptc) and smoothened (Smo), both of which are expressed on hippocampal NPCs (Lai et al., 2003, Traiffort et al., 1998). In the absence of a Shh ligand, Ptc represses signal transduction from the Smo receptor thus repressing downstream signalling. After binding of Shh to Ptc,

disinhibition of Smo leads to activation of an intracellular signalling cascade resulting in the differential regulation of Shh target genes (Philipp and Caron, 2009). Overexpression of active Shh with the DG of rats has been demonstrated to increase cell proliferation and survival without affecting neuronal differentiation (Lai et al., 2003). Interestingly, recruitment of Shh signalling is suggested to be a mechanism through which electroconvulsive shock therapy promotes hippocampal NPC proliferation (Banerjee et al., 2005). Conversely, administration of cyclopamine, a Shh inhibitor, reduced cell proliferation (Lai et al., 2003, Banerjee et al., 2005). Additionally, Smo knockout mice show significant impairments in NPC proliferation within the SGZ (Machold et al., 2003, Han et al., 2008).

1.4.2.2: Nuclear receptors in neurogenesis

Nuclear receptors (NRs) are a superfamily of intracellular ligand regulated transcription factors and are common drug targets: approximately 13% of all Food and Drug Administration (FDA) approved drugs target NRs (Rask-Andersen et al., 2011). The NR superfamily shares a similar three domain structure. These domains include a highly conserved DNA binding domain, a ligand binding domain, and a variable N-terminal transactivation domain, which binds various co-activators and co-repressors. As NRs are transcriptional regulators, they govern many aspects of development, reproduction and metabolism via suppression or promotion of gene expression (Sun and Shi, 2010). Many nuclear receptors have been demonstrated to regulate neurogenesis including glucocorticoid receptors (GRs), estrogen receptors (ERs), peroxisome proliferator activated receptors (PPARs) and the orphan nuclear receptor tailless homolog (TLX or NR2E1) (See figure 1.4 and 1.5) (O'Leime et al.,

2017a). As TLX is a major topic in this thesis, it will be discussed in greater detail in section 1.5.3.

Glucocorticoid receptors (GRs) have a pivotal role to play in the stress response and immune function (Garcia et al., 2004, Busillo and Cidlowski, 2013). The main activators of GRs are adrenal glucocorticoid hormones or stress hormones. Corticosterone (CORT), or cortisol in humans, is a lipophilic glucocorticoid hormone produced from cholesterol that readily passes the blood brain barrier and can enter neuronal cells through the cell membrane. CORT is produced mainly by the adrenal glands, however there is evidence to suggest that it is also produced within the hippocampus where there is an abundance of enzymes required for its production (Beyenburg et al., 2001, Gomez-Sanchez et al., 1996, Stromstedt and Waterman, 1995). Two types of GR exist, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GRs are expressed in about 50% of all proliferating NPCs with MRs below detectable levels, and as NPCs begin to express the immature neuronal marker doublecortin (DCX), the expression of GR within these cells is significantly reduced but is restored again once the NPCs mature into neurons (Garcia et al., 2004, Cameron et al., 1993, Boku et al., 2009). This suggests that GRs initially mediate the effects of CORT on NPCs proliferation and are then subsequently involved in the regulation of neuronal activity. Mice lacking GR display impairments in hippocampal neurogenesis-associated behaviours. For example, deficits in contextual fear conditioning in GR knockdown mice is coupled with reduced NPC proliferation and accelerated neuronal differentiation (Fitzsimons et al., 2013).

Peroxisome Proliferator Activated Receptors (PPARs) consist of three main subtypes; PPARα, PPARβ/δ, and PPARγ. They can be activated by endogenous lipid compounds, such as prostaglandins and other fatty acids, as well as synthetic compounds from the diabetic drug class thiazoladinediones (Ahmadian et al., 2013). Much of the focus on the role of PPARs in hippocampal neurogenesis to date has focused on PPARγ, although all PPAR subtypes have been shown to be expressed in the DG of the hippocampus in adult rats and in mouse NPCs from the SVZ (Bordet et al., 2006, Sun and Shi, 2010, Moreno et al., 2004, Cimini et al., 2007). PPAR deficient NPCs failed to proliferate whereas treatment with the PPARγ agonist rosiglitazone induced cell growth and inhibited differentiation of NPCs into neuronal cells (Wada et al., 2006). Both PPARγ and PPARβ/δ activation in NPCs in the SVZ of adult mice leads to increased proliferation in these cells (Bernal et al., 2015). These authors also demonstrated that PPARγ activation increased the expression of PPARβ/δ, suggesting that these receptor isotypes work in tandem to promote proliferation of NPCs. It should be noted however, that the variation of PPAR effects on neurogenesis may be due to the wide variety of compounds used to modulate their activity with each one having differing dosage and time dependent effects (Ghoochani et al., 2012, Wada et al., 2006). Indeed, it has been shown that with doses of the PPARγ agonists rosiglitazone or pioglitazone in excess of 30μM, cell proliferation of embryonic NPCs is significantly reduced (Wada et al., 2006).

Two types of estrogen receptors (ERs) exist; ERα and ERβ. Both are expressed within the hippocampus of rodents and humans, with ERα displaying a slightly higher expression profile in rodents and ERβ slightly higher in humans (Mitterling et al., 2010, Pérez et al., 2003, Towart et al., 2003, Osterlund et al., 2000, González et

al., 2007). The sex or estrogen hormones estradiol, estrone, and estriol bind to ERs. Estradiol is the most potent and has been the focus of most research to date on the role of the ERs in hippocampal function (McEwen et al., 2012, McEwen and Milner, 2007, Vasudevan and Pfaff, 2008). Estradiol is synthesised from cholesterol, mainly in the ovaries in females and estrogen has also been shown to be locally produced by hippocampal neurons in male rats (Hojo et al., 2004). It has been shown in rats, that ERs are expressed within adult and postnatal hippocampal NPCs (Mazzucco et al., 2006, Pérez et al., 2003). Administration of estradiol to female rats or meadow voles has been shown to increase NPC proliferation, however this effect was only observed after 4 hours of treatment whereas with 48 hours of treatment there was a suppression of cell proliferation within the DG (Ormerod and Galea, 2001, Ormerod et al., 2003). Estradiol induces a rapid increase in serotonin levels that has been suggested to mediate estradiol effects on NPC proliferation in the DG of rats (Banasr et al., 2001). With prolonged estradiol exposure, adrenal activity is increased and so suppression of the proliferation of NPCs within the DG ensues (Ormerod et al., 2003). Thus, normal nuclear receptor mediated signalling play a critical role in regulating neurogenesis, however excessive or impaired signalling through these receptors can result in dysregulation.

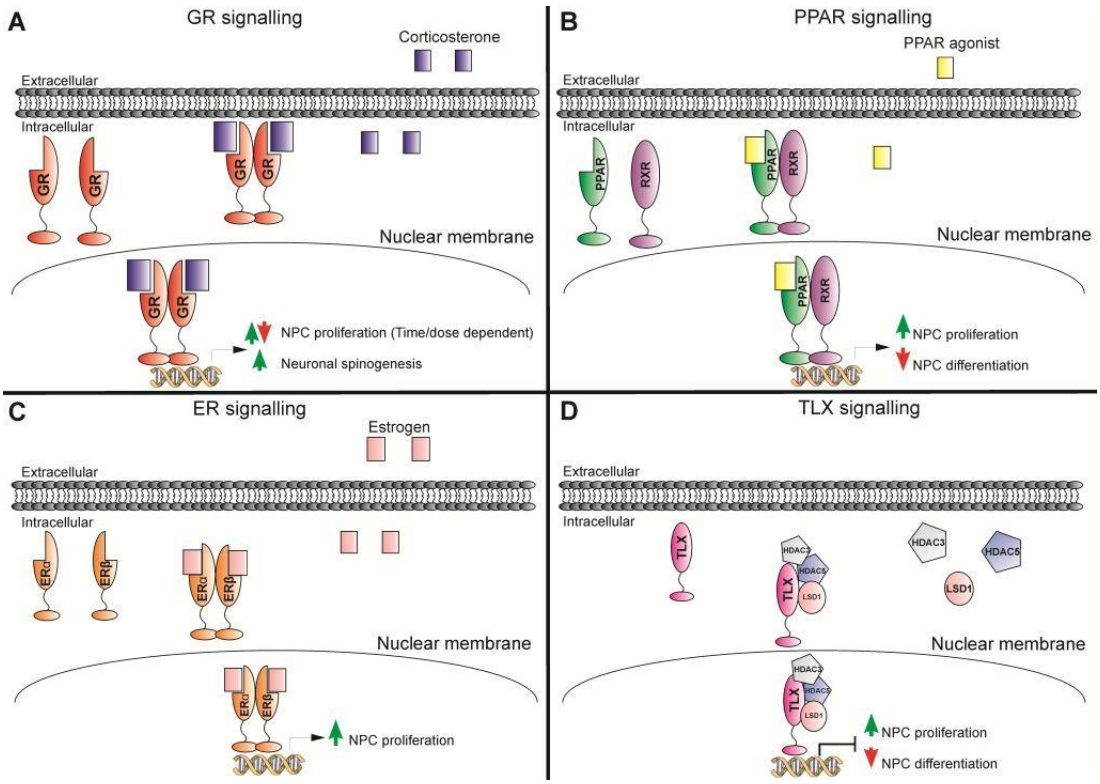


Figure 1.4: Nuclear Receptor signalling. A) Glucocorticoid receptor (GR) signalling. B) PPAR receptor signalling. C) Estrogen receptor (ER) signalling. D) NR2E1 (TLX) receptor signalling. GRs can be activated by ligands, such as corticosterone in rodents, once activated they form homodimers and regulate gene transcription. PPARs are activated by a wide variety of synthetic and endogenous ligands and can form heterodimers with the retinoid x receptor (RXR) to regulate gene transcription. ERs form either homo or heterodimers with other ER subtypes once activated by ligands, such as estradiol. NR2E1 is an orphan nuclear receptor and as such has no known ligand yet it recruits various co-signalling molecules, such as HDAC3/5 and LSD1 to repress gene expression. (O'Leime et al., 2017a).

1.4.3: TLX (NR2E1)

Homolog of the drosophila tailless gene (TLX) is an orphan nuclear receptor, which is also known as nuclear receptor subfamily 2 group E member 1 (NR2E1), is

predominantly expressed in the eye and forebrain (Monaghan et al., 1995). Indeed, the expression of TLX is mainly restricted to the mammalian CNS (Li et al., 2012). In the adult, TLX is mainly expressed in the DG in the hippocampus and the SVZ zone of the lateral ventricles. Like most nuclear receptors, TLX has at least three structural domains, a DNA binding domain, an N-terminal transactivation domain and a moderately conserved ligand-binding domain (LBD) (Yu et al., 1994, Bain et al., 2007, Benod et al., 2014a). TLX functions primarily as a transcriptional repressor of downstream target genes. To do so, TLX recruits transcriptional co-repressors, such as the epigenetic modulators lysine-specific histone demethylase 1 (LSD1), and HDACs 3 and 5, which repress gene expression and in turn suppress the expression of anti-proliferative genes to regulate NPC proliferation (Sun et al., 2007, Sun et al., 2010, Sun et al., 2011). Genes regulated by TLX include *Ascl1, Pou5f1, Pax2, Mir9, Mir137, Pten, p21, Cdkn1a, Sirt1, and Wnt7a* (Islam and Zhang, 2014). Importantly, repression of cyclin dependent kinase inhibitor *p21* and the tumour suppressor gene *Pten* by TLX activation, promote the proliferation of NPCs (Sun et al., 2007). At a cellular level, TLX has been demonstrated to be critical for maintaining neural stem cell proliferation in both the developing embryonic forebrain and dorsal midbrain, and within the neurogenic niches of the adult brain (Monaghan et al., 1995, Shi et al., 2004, Sun et al., 2007).

TLX expressing NPCs from the SGZ, SVZ, and olfactory bulb proliferate and are capable of differentiating into all neural cell types whereas NPCs lacking TLX fail to proliferate (Shi et al., 2004). The importance of TLX was further demonstrated by the fact that reintroduction of TLX into NPCs restored all of the deficits in proliferation observed in the cells lacking TLX (Shi et al., 2004). Moreover,

overexpression of TLX within hippocampal NPCs has been shown to enhance the proliferation phase of neurogenesis and subsequently improve learning and memory processes in mice (Murai et al., 2014). TLX knockout mice show deficits in hippocampal neurogenesis and impaired learning and memory as well as a heightened stress response (Monaghan et al., 1997, Young et al., 2002, O'Leary et al., 2016a). More recently, it has been demonstrated that mice with a spontaneous deletion of TLX (not genetically engineered to delete TLX) display impairments in hippocampal neurogenesis-associated cognitive tasks (O'Leary et al., 2016). Specifically, this study demonstrated that $TLX^{-/-}$ mice displayed impaired spatial memory as tested by spontaneous alternations in the Y-maze. Additionally, it was reported that male adolescent mice displayed impaired contextual fear conditioning performance indicating a role for TLX in sex and age dependent effects on certain hippocampal neurogenesis-associated tasks (O'Leary et al., 2016a).

Most work to date assessing the relevance of TLX in hippocampal neurogenesis-associated cognition has focused on genetic reductions (germline knockouts or inducible knockouts) in TLX expression (O'Leary et al., 2016b). For example, TLX knockout in mice (cre-lox deletion) resulted in impaired performance in the Morris water maze spatial memory task (Zhang et al., 2008). This impairment in cognition was also associated with impaired hippocampal neurogenesis. However, it has been demonstrated that enhancing TLX expression specifically in NPCs can promote hippocampal neurogenesis and enhance performance in the Morris water maze task (Murai et al., 2014). This study demonstrated the potential for TLX as a therapeutic target to selectively enhance hippocampal neurogenesis and associated cognition. Thus, it would appear the TLX is intrinsically linked with both the cellular

mechanisms and functional outcomes of hippocampal neurogenesis and as such its role in mitigating neuropathology-induced deficits in neurogenesis will be of interest for future research.

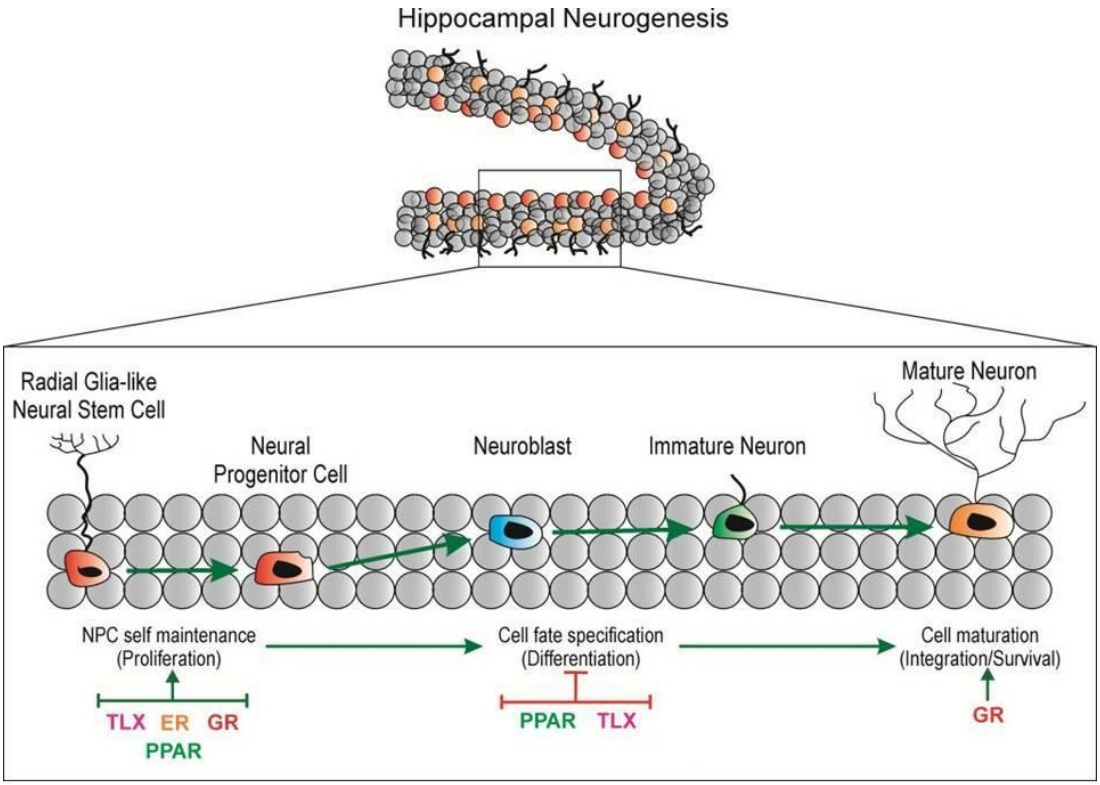


Figure 1.5. Nuclear receptors regulate various aspects of hippocampal neurogenesis (O'Leime et al., 2017a).

1.5: Experimental manipulation of neurogenesis

To determine the role of hippocampal neurogenesis in various behaviours, researchers use different methods to either ablate or enhance hippocampal neurogenesis and thus determine the consequent effects on behaviour. As neurogenesis involves multiple processes (i.e. proliferation of NPCs, differentiation, integration, and survival of newborn neurons), various methods have been developed to target specific or several of these processes, and these methods have greatly

enhanced our understanding of the functional role of hippocampal neurogenesis (Imayoshi et al., 2011).

1.5.1: Irradiation

Exposure of the brain to radiation occurs during many clinical situations particularly as a form of cancer therapy (Burger et al., 1979, Sheline et al., 1980). It has been demonstrated with aggressive radiation therapy treatments, that there is significant tissue destruction and subsequent morphological and cognitive impairments in both humans and animal models (Caveness, 1977, Schultheiss et al., 1995, Hopewell, 1979, Fike and Gobbel, 1991, Van der Kogel, 1991). However, even after lower doses of ionizing radiation, cognitive impairments are evident despite little effect on morphology (Abayomi, 1996, Butler et al., 1994, Kramer et al., 1992). Cognitive impairments that have been typically reported include impaired attention and memory performances which are indicative of impairments in hippocampal function (Crossen et al., 1994, Imperato et al., 1990, Roman and Sperduto, 1995). It has since been demonstrated that radiation directed at the hippocampus of adult rats induces a long-lasting suppression of proliferating cells (Tada et al., 2000).

Radiation-induced suppression of neurogenesis has been used to assess the role of hippocampal neurogenesis in rodents subjected to various behavioural paradigms such as MWM, Barnes maze, contextual fear conditioning and novel object recognition (Wong-Goodrich et al., 2010, Winocur et al., 2006, Rola et al., 2004, Ji et al., 2014, Rao et al., 2011, Conner et al., 2010). Additionally, radiation-mediated ablation of neurogenesis was used to demonstrate that some antidepressants function by enhancing neurogenesis (Santarelli et al., 2003). All of these behaviours have

been demonstrated to be impaired weeks to months after hippocampal irradiation. However, these results may not be a consequence of impaired neurogenesis alone. Radiation treatment has been demonstrated to induce a significant inflammatory response with increased oxidative stress and cytokine expression (Hong et al., 1995, Kataoka, 2013, Nakatsukasa et al., 2008). Interestingly, treatment of rats with the anti-inflammatory drug indomethacin is sufficient to prevent the detrimental effects of radiation on neurogenesis (Monje et al., 2003). This then suggests, that radiation is not an entirely effective method of assessing the functions of neurogenesis as multiple non-neurogenesis effects may result due to increased inflammation.

1.5.2: Transgenic animals

Neurogenesis can be selectively ablated or enhanced using transgenic animals. Site specific recombinases (SSRs) are enzymes used as part of genome editing technology to delete or insert specific DNA sequences into specific regions of the genome (Nagy, 2000, Wu et al., 2007). A commonly used recombinase is Cre receombinase (Nagy, 2000). Cre recognises loxP recognition sites within DNA and effectively excises any DNA sequence found between two loxP recognition sites (Imayoshi et al., 2011). Control of Cre-mediated recombination of specific genes is commonly achieved by fusing the Cre protein to the mutated ligand-binding domain (LBD) of the estrogen receptor to produce a ligand-dependent chimeric CreER recombinase (Metzger et al., 1995, Feil et al., 1997). In these transgenic mice, CreER is activated by administration of the synthetic ER antagonist tamoxifen which results in genetic recombination by Cre recombinase (Indra et al., 2000, Li et al., 2000). Moreover, this genetic recombination cannot be induced by natural ER ligands such as estradiol, thus providing robust control over gene expression.

Using this CreER recombinase genome editing technology, researchers can generate transgenic mice in which the CreER recombinase gene is driven by NPC specific promoters such as the *Nestin* promoter or glial fibrillary acidic protein (GFAP) promoter (Imayoshi et al., 2006, Lagace et al., 2007, Favaro et al., 2009). This results in only NPCs expressing CreER. These transgenic mice can then be used to cross breed with reporter mice which have ubiquitous expression of a reporter gene such as (green fluorescent protein) GFP. The expression of this reporter gene is prevented by transcriptional STOP cassette which is flanked by two loxP sites. Thus, when CreER is activated by tamoxifen, the STOP sequence is removed allowing for transcription of the reporter specifically in CreER expressing cells, i.e. NPCs. These CreER-reporter transgenic mice are used widely for visualising and tracing the lineage of NPCs in response to various stimuli.

Neurogenesis can be successfully manipulated using transgenic animals also. In nestin-CreER transgenic mice where loxP flanked *Bax* (a pro-apoptotic gene) is expressed specifically in nestin expressing NPCs, administration of tamoxifen to these mice removed *Bax* expression within NPCs of these mice (Sahay et al., 2011a, Hill et al., 2015). This enhances survival of NPCs which in turn results in increased new neuron survival as they differentiate, as well as improved pattern separation performance and reduced anxiety and depression-like behaviours in CORT treated mice (Sahay et al., 2011a, Hill et al., 2015). There are other forms of transgenic mice commonly used to study neurogenesis apart from CreER mice. These include GFAP-TK or nestin-TK mice in which the expression of herpes simplex virus thymidine kinase (HSV-TK) is driven by either GFAP or nestin promoters thus localising HSV-

TK expression largely to NPCs. Expression of thymidine kinase renders the cells susceptible to the anti-viral drug valganciclovir and when these animals are administered this drug, GFAP or nestin expressing cells are ablated. This method has been used to ablate neurogenesis and demonstrate impairments in specific fear learning paradigms with ambiguous rather than reliably predicted cues (Glover et al., 2017, Snyder et al., 2011). Additionally, transgenic models in which the nuclear receptor TLX is overexpressed specifically in NPCs have been used to demonstrate that increase TLX-induced NPC proliferation promotes hippocampal neurogenesis-associated spatial navigation in the MWM (Zhang et al., 2008, Murai et al., 2014). These extremely targeted manipulations of neurogenesis are valuable in unravelling the exact role neurogenesis plays in behaviour.

1.5.3: Spontaneous deletion animal models

Spontaneous deletion animal models are models in which there is a deletion of s specific gene which has not been experimentally induced (i.e. targeted knockouts). Of particular interest to the study of neurogenesis is the TLX spontaneous deletion mouse model. In this model, a spontaneous germline genetic disruption occurs, which results in the complete deletion of the TLX gene without affecting the transcription of neighbouring genes (Kumar et al., 2008, Young et al., 2002). The occurrence of this specific spontaneous deletion has been investigated in a variety of mouse strains including C57BL/6J, 129P3/JEms and Bl6129F1 (Young et al., 2002). The majority of work has been carried out on the 129P3/JEms and crossbred Bl6129F1 strains as these strains do not display the hydrocephalus evident in the C57BL/6J strain (Young et al., 2002, Kozareva et al., 2017a, O'Leary et al., 2016). As TLX is a critical regulator of hippocampal neurogenesis, spontaneous deletion

TLX mice have severely impaired hippocampal neurogenesis as well as significant impairments in associated cognitive processes such as contextual fear conditioning and spontaneous alternation in the Y-maze (O'Leary et al., 2016, Shi et al., 2004). Additionally, these mice display a broad range of behavioural abnormalities including increased aggressiveness and anxiety-like behaviour as well as hyperactivity (Roy et al., 2002, O'Leary et al., 2016a). As these mice lack TLX from development, it could be that the behavioural effects observed are due to developmental deficits in TLX rather than deficits in adulthood. Nonetheless, the fact these mice relatively easy to produce means they represent a valuable alternative to developing transgenic models.

1.5.4: Viral vectors

Viral vectors are a common method of gene delivery which hijacks the ability of viruses to transfer their genes into a host cell (Deglon and Hantraye, 2005). Various types of viral vectors exist, each with their own advantages and disadvantages. Important aspects to consider when utilising viruses to manipulate hippocampal neurogenesis include whether the virus can transfect mitotic cells, post-mitotic cells or both, the size of the gene that is being transferred, and whether the transferred gene is to be inserted into the host genome for prolonged expression or remain extranuclear for short term expression (Walther and Stein, 2000). Additionally, viral vectors can be used to transfer short hairpin RNA (shRNA) molecules which can be used to block endogenous gene expression (Bahi and Dreyer, 2012). The most commonly used viral vectors for CNS gene transfer include viruses derived from adeno-associated virus (AAV), herpes simplex virus and lentiviruses (LV) (Senut et al., 2000, Natsume et al., 2001, Osinde et al., 2008). Many of these have been used

to manipulate aspects of neurogenesis or assess the effects of prolonged expression of various protein factors on neurogenesis (Jessberger et al., 2009b, Ryan et al., 2013, Lagace et al., 2008, Xu et al., 2015, Aelvoet et al., 2015, Pechnick et al., 2011, Wu et al., 2013, Yoshimura et al., 2003).

AAV was originally discovered as a contaminant in laboratory stocks of adeno virus (Monahan and Samulski, 2000). AAV are small, non-pathogenic viruses that are capable of transferring small amounts of DNA into the host cells. AAVs transfer DNA sequences which can site specifically intergrate into the host genome. In human cells, it was initially suggested that approximated 70% of transductions result in successful integration of viral transferred genes into the host genome (Kotin et al., 1990, Weitzman et al., 1994). However, AAV transferred genetic material does not generally integrate, and indeed integration after AAV transfection has been associated with tumorigenesis in mice (Deyle and Russell, 2009). However, AAVs do provide long-term stable gene expression and can transfect dividing and non-dividing cells (Bouard et al., 2009). The AAV genome itself is small (4.7kb) but very little of the viral genome is required for successful transduction of host cells and transfer of desired genetic material (Lim et al., 2010).

Due to their ability to induce stable expression of the transferred genetic material and low immune response to the virus itself, AAV-derived viral vectors have been used in clinical trials for various CNS related pathologies including transfer of neurotrophic factors in neurodegenerative disease such as AD (Lim et al., 2010). AAV has also been used to induce sustained IL-1β expression within the hippocampus, which in turn was shown to suppress neurogenesis (Wu et al., 2013).

However, one of the main drawbacks with AAVs is their inability to transfer larger genetic sequences and so it is difficult to transfer a gene encoding a protein of interest and a tag to identify successfully transduced cells. The ability to mark and track transduced cells is important in tracking NPCs as they progress through the neurogenesis process.

HSV vectors are similar to AAV in that they transfer DNA, however they are much larger vectors and can transfer much larger amounts of genomic material (Hibbitt and Wade-Martins, 2006). However, they do not possess the ability to target dividing cells therefore they cannot target NPCs (Bouard et al., 2009). They can modulate neurogenesis indirectly by transducng surrounding neurons to induce the expression of factors like fibroblast growth factor 2 (FGF2) which is known to stimulate NPC prolfieration (Yoshimura et al., 2003, Reynolds and Weiss, 1992).

LVs are members of the retrovirus family of viruses which includes human immunodeficiency virus (HIV), as well as simian (SIV), equine (EIAV) and feline (FIV) immunodeficiency viruses (Escors and Breckpot, 2010). As such, the LV genome is a single stranded RNA (ssRNA) sequence of about 9.7kb in length (Lim et al., 2010). This RNA based genomic material includes transcripts for virus packaging, reverse transcription, nuclear localisation and nuclear integration of reverse transcribed genes into the host genome (Lim et al., 2010). LVs have been the focus of some research to ascertain their application as clinical gene delivery vectors (Sakuma et al., 2012). This is due to their ability to transfect a broad range of cell types including post-mitotic cells or slowly dividing cells which in many cases NPCs are (Kempermann et al., 2004a). Classical retrovirus cannot transduce post-

mitotic cells as they require shedding of the nuclear membrane of the host cell (which occurs during mitosis) to successfully transduce cells (Naldini, 1998). However, following reverse transcription of ssRNA from modern LV vectors into doublestranded DNA (dsDNA) within the host cell cytoplasm, dsDNA can gain entry to the nucleus of the host cell via active transport mechanisms (Escors and Breckpot, 2010). The dsDNA is then integrated into the host DNA which results in long-term expression of the viral genomic material.

As the LV vector genome is based on HIV, it is very important that the aspects of the LV genome that are HIV based (i.e. not the gene being transduced) are not capable of replication. In this regard, steps have been taken to maximise the biosafety of LV vectors such as removal of the majority of viral genes that could contribute to viral self replication (Manfredsson and Mandel, 2011). Additionally, preclinical studies using LV vectors use LVs with a FIV, SIV or EIAV genomic background to minimise the ability of the virus to replicate in humans (Manfredsson and Mandel, 2011). LVs have a wide range of beneficial aspects in their use in preclinical and clinical research into gene delivery. This includes their ability to transduce post-mitotic or slowing proliferating cells, their low potential of producing self replicating viruses, their lack of viral gene expression and their relatively high capacity for transferring genetic material (Naldini et al., 1996a, Naldini et al., 1996b, Zufferey et al., 1997, Dull et al., 1998, Blomer et al., 1997, Zufferey et al., 1998).

LVs have been used to transfer interfering RNA (iRNA) into the hippocampus of rats to suppress hippocampal neurogenesis (Jessberger et al., 2009b). This study used LVs to transfer iRNA designed to inhibit Wnt signalling which resulted in impaired

proliferation of NPCs and subsequent impairments in spatial and object recognition. Additionally, it has been demonstrated that NPCs can be induced to overexpress IL-1β which in turn reduces their proliferation *in vitro* (Ryan et al., 2013). Conversely, overexpression of PPARδ in the hippocampus of rats enhances NPC proliferation and rescues depressive-like behaviours after chronic mild stress (Ji et al., 2015). However, LV vectors themselves can cause a local immune response when injected intrahippocampally (Kunitsyna et al., 2016). This study did not observe any impairment in hippocampal-associated learning and memory as assessed by contextual fear conditioning. Therefore, although extremely useful in transferring large amounts of genetic material and being able to target hippocampal NPCs, LV vectors, future generation of LVs should aim to minimize viral effects on immune activation.

1.6: Neuroinflammation in the CNS

Neuroinflammation was initially characterised by the infiltration of peripheral immune cells in response to central viral or bacterial infection, ischemic stroke, or multiple sclerosis (Crotti and Glass, 2015, Hensley, 2010, Aguzzi et al., 2013). However, the local production of inflammatory mediators such as cytokines within the CNS is another major component of neuroinflammation. Under pathological conditions as well as neurodegenerative disorders where neuroinflammation is evident, the mechanisms of hippocampal neurogenesis deregulation at an intracellular level are the focus of much ongoing research. It has been shown that inflammation negatively affects hippocampal neurogenesis (Ekdahl et al., 2009, Monje et al., 2003, Heine et al., 2004).

Neuroinflammation has been associated with many psychiatric and neurodegenerative disorders where cognitive dysfunction is evident (Raison et al. 2006; Leonard 2007). Moreover, cognitive dysfunction in pathological conditions and through aging has been strongly linked with decreased hippocampal neurogenesis (Kuzumaki et al. 2010; Kohman & Rhodes 2013). From the evidence to date, it appears that interplay exists between hippocampal neurogenesis, inflammation and cognitive function in aging and pathological conditions.

1.6.1: Neuroinflammation in neurodegenerative and psychiatric disorders

Neurodegenerative diseases such as AD, PD and HD have been linked with central neuroinflammatory processes such as sustained microglial activation and cytokine release (Schellenberg and Montine, 2012, Samii et al., 2004, Silvestroni et al., 2009). However, these neurodegenerative pathologies do not display the characteristics initially associated with neuroinflammation such as peripheral immune cell infiltration from the blood stream (Crotti and Glass, 2015). Psychiatric disorders associated with neuroinflammation include depression, bipolar disorder and schizophrenia (Rao et al., 2010, Raison et al., 2006, Hope et al., 2009, Schiepers et al., 2005, O'Brien et al., 2006, Dowlati et al., 2010). The close association between evidence of neuroinflammation and neurodegenerative and psychiatric disorders has led to the suggestion that neuroinflammation itself may be an important component to the aetiology and/or progression of these pathologies (Ransohoff, 2016, Raison et al., 2006).

The first indication for the involvement of neuroinflammation in neurodegenerative disorders came from the observation of active microglia in brain autopsy tissue of

patients with AD and PD (McGeer et al., 1988b, McGeer et al., 1988a, Rogers et al., 1996). Since then, it has been demonstrated that there is a significant increase in the expression pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 within brain tissue and plasma of AD patients (Fillit et al., 1991, Swardfager et al., 2010, Forlenza et al., 2009). Similar increases in cytokine expression within the striatum, substantia nigra and plasma of PD patients have also been observed (Mogi et al., 1994a, Mogi et al., 1994b, Hunot et al., 1999, Dobbs et al., 1999). Interestingly, increased occurrences of single nucleotide polymorphisms (SNPs) in the *Il1b* gene are associated with the development of AD (Griffin et al., 2000, Nicoll et al., 2000, Di Bona et al., 2008). A SNP in the *Il1b* gene, that introduces a *Taq1* restriction site into the gene, results in a fourfold increase in IL-1β production and also a 1.6 fold increased chance of developing AD (Di Bona et al., 2008). Additionally, reactive microglia have been shown to localise around amyloid-β (Aβ) plaques in the hippocampus of AD patients, and Aβ plaques themselves can induce the release of cytokines from microglia which potentially exacerbates cell death and neurodegeneration (McGeer et al., 1993, Sheng et al., 1995a, Block and Hong, 2005).

Increased expression of pro-inflammatory cytokine related enzymes have been observed in PD patients (Collins et al., 2012). Several pre-clinical models of PD are also associated with an increased inflammatory profile within the brain. For example, in rodent models using 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce parkinsonian symptoms, there is an increase in the expression of TNFα in the mid-brain and substantia nigra (Ferger et al., 2004, Nagatsu and Sawada, 2006). Additionally, inhibition of TNF synthesis or signalling

in the MPTP mouse model of PD prevents striatal neuronal toxicity, and blockade of TNF signalling in the 6-hydroxydopamine model attenuates motor impairments in rats (Ferger et al., 2004, McCoy et al., 2006). It has been demonstrated that α-synuclein, the protein that aggregates to form Lewy bodies in PD, can induce the activation and release of cytokines from microglia (Hoenen et al., 2016). Indeed, it has previously been demonstrated that microlia aggregate around Lewy bodies and this may promote disease progression (Zhang et al., 2005).

Unlike AD and PD which are multifactorial pathologies associated with several genetic mutations and environmental factors, HD is caused by a single mutation in the Huntington gene (*Htt*) which results in progressive neuronal loss and severe cognitive impairments (MacDonald et al., 1993). Although HTT protein is expressed ubiquitously throughout the body, *Htt* mRNA is expressed threefold higher within immune cells suggesting the dysregulation of this gene can also have a direct impact on inflammatory responses (Soulet and Cicchetti, 2011). Activated microglia are evident in pre-symptomatic mutated *Htt* gene carriers, even up to 15 years before the predicted disease onset (Tai et al., 2007). Increases in plasma IL-6 are also evident during the same timeframe microglia activation occurs (Bjorkqvist et al., 2008). Additionally, in cerebrospinal fluid of HD patients there are increased concentrations of TNFα, IL-6 and IL-8 (Bjorkqvist et al., 2008). Thus, from the evidence within the literature there is a clear link between neuroinflammation and neurodegeneration. However, whether neuroinflammation plays a causal role or is a consequence of neuronal loss in specific neurodegenerative disorders remains the focus of much research.

There is mounting evidence linking psychiatric disorders, particularly depression, and neuroinflammation (Raison et al., 2006, Miller et al., 2009, Pariante, 2017, Khansari and Sperlagh, 2012, Na et al., 2014). For example, there is an increase in pro-inflammatory cytokine expression in depressed patients (Maes et al., 1991, Raison et al., 2006), pro-inflammatory cytokines themselves induce depressive behaviours in rodents (Smith, 1991, Goshen et al., 2008), pro-inflammatory cytokine production is suppressed by antidepressant drugs (Xia et al., 1996, Shen et al., 1999, Reynolds et al., 2005) and pro-inflammatory cytokines can disrupt HPA axis regulation (Maes et al., 1993a, Maes et al., 1993b). These studies and others have given support to the 'cytokine hypothesis' of depression which was first put forward in 1991 (Smith, 1991). This hypothesis proposes that excessive release of cytokines is the cause of depression. However, not all depressed patients exhibit neuroinflammation. It has been suggested that pre-exposure during early life to a stressful event or trauma is a leading cause of inflammation related depression (Danese et al., 2011, Danese et al., 2007, Danese et al., 2008). However, it has been demonstrated that treatment of rodents with antidepressants reduces cytokine-induced depressive behaviours as well as cytokine production (Castanon et al., 2002, Obuchowicz et al., 2006a). This suggests that cytokines, when increased in depressed patients, can potentially contribute to depressive symptoms.

Increased expression of pro-inflammatory cytokines have also been detected in *post-mortem* brain tissue of bi-polar and schizophrenia patients (O'Brien et al., 2006, Hope et al., 2009, Rao et al., 2010). The mood stabiliser lithium, which is commonly used to treat bi-polar disorder, has also been demonstrated to reduce pro-inflammatory TNFα expression and increase anti-inflammatory IL-10 expression in

rat microglia *in vitro* (Wang et al., 2010, Martin et al., 2005). Similar to lithium, antipsychotic drugs such as perazine used to treat schizophrenia can also reduce cytokine expression (Obuchowicz et al., 2006b). Thus, neuroinflammation appears to be involved in both neurodegenerative diseases and psychiatric disorders, however the molecular mechanisms of these interactions remain to be fully elucidated.

1.6.2: Activation and regulation of microglia

Within the brain, microglia are the main immune cell type and provide support for the surrounding neuronal circuits. Microglia have a distinct lineage compared to other glial cells in the CNS such as astrocytes and oligodendrocytes and they make up approximately 12% of all neuroglial cells within the CNS (Lawson et al., 1990, Aguzzi et al., 2013). They colonise the brain during embryonic development before the formation of the blood brain barrier (BBB), after which they remain confined to the brain throughout life (Ajami et al. 2007). Microglia are derived from mesodermal precursor cells that infiltrate the CNS during development and they express cell surface markers such as Fc, CD11b and Iba1 which are also expressed on macrophages (de Haas et al., 2008, Ohsawa et al., 2004). The overlap in marker expression between microglia and macrophages alludes to the phagocytic functions of microglia.

Microglia can either be inactive or active depending on their response to their environment. In normal conditions, microglia adopt a 'resting' phenotype. While in this phenotype, microglia have a small soma and many branching, long, thin processes. These processes act as detectors for any detrimental insult to the brain. It has been shown that microglia are highly motile in this phenotype as they survey

their environment (Nimmerjahn et al. 2005). When microglia become activated by an insult such as an infection, they retract their processes, increase the expression of activation markers and release certain cytokines to combat the insult (Kettenmann et al. 2013). Active microglia can be identified by their morphological features as well by the fact that they have increased expression of cell surface antigen expression such as major histocompatibility complex class II and membrane receptors such as CD45 (de Haas et al., 2008, Ford et al., 1995). Activation of microglia is a normal and necessary process to protect the brain from prolonged injury. Indeed, as it has been suggested that half of all newborn neurons die via apoptosis within a few days, microglia play an important role in clearing up cell remnants (Sierra et al., 2010, Kempermann et al., 2003, Dayer et al., 2003, Biebl et al., 2000). However, if microglia remain in this activated state chronically they become maladaptive and can damage the brain. In response to stress, infections or age, sustained microglia activation is a common occurrance (Ekdahl et al. 2009).

Activation of microglia is a complex process as microglia can be classically activated which leads to increased inflammatory responses or they can be alternatively activated which can lead to a neuroprotective phenotype (Kohman and Rhodes, 2013). Classical activation of microglia results in the release of a host of cytokines and this activation state is the main source of inflammatory cytokines within the brain (Hanisch 2002; Rothwell et al. 1996). Activation of microglia occurs rapidly within minutes after detection of a host of factors such as viral infections, lipopolysaccharide (LPS) exposure, neuronal death and cytokines (Davalos et al., 2005, Hanisch and Kettenmann, 2007, Colton and Wilcock, 2010). LPS is a bacterial endotoxin commonly used to induce microglial activation *in vitro*

and *in vivo.* To this end, LPS-induced activation of microglia is mediated via toll-like receptor-4 (TLR4) binding on microglia and has been shown to significantly increase their production of inflammatory cytokines such as TNFα, IL-1β and IL-6 (Kohman and Rhodes, 2013).

Microglia can also shift to an alternatively activated state, in which release of pro-inflammatory cytokines is reduced and cell death is attenuated. The main initiators of this shift come from cytoactive agents released by cells around the site of injury or infection such as regulator T_H2 immune cells (Colton, 2009). These cytoacive agents are predominantly anti-inflammatory cytokines such as IL-4 and IL-13 (Butovsky et al., 2006, Colton, 2009). Microglia themselves once in this alternative activated state can release other anti-inflammatory cytokines such as IL-10 and TGFβ, as well as growth factors such as insulin-like growth factor (IGF), neural growth factor (NGF) and brain derived neural growth factor (BDNF) (Kohman and Rhodes, 2013). The expression of alternatively activated microglia typically occurs after their pro-inflammatory classically activated phase (Cacci et al., 2008, Thored et al., 2009). It is thought that this transition occurs to limit neuronal damage during the pro-inflammatory phenotype while also promoting neuronal repair. Indeed, this transition is also vitally important in maintaining hippocampal neurogenesis during periods of neuroinflammation (Kohman and Rhodes, 2013).

1.6.3: Neuroinflammation and hippocampal neurogenesis

It was initially demonstrated that hippocampal neurogenesis is impaired by LPS induced classical activation of microglia (Monje et al., 2003, Ekdahl et al., 2003). Monje et al. demonstrated that after intraperitoneal (i.p) injection of LPS to rats,

there was a significant reduction in proliferation and neuronal differentiation as well as reduced newborn cell survival (Monje et al., 2003). These impairments were correlated with a 240% increase in the density of activated microglia within the DG and were prevented by co-administration of the non-steroidal anti-inflammatory indomethacin. Additionally, they demonstrated using co-cultures of microglia and NPCs *in vitro* that the impairments of neuronal differentiation were mediated by cytokine release rather than microglia-NPC direct interactions (Monje et al., 2003). These data were also supported by another study that demonstrated that 4 weeks of cortical LPS infusion in rats induced significant microglia activation as well as impairments in neuronal differentiation (Ekdahl et al., 2003). This study also demonstrated that inhibition of microglial activation using minocycline attenuated the effects of LPS on neurogenesis indicating that microglial activation is critical for the effects of inflammation on neurogenesis.

Inflammation has generally been shown to be a robust suppressor of neurogenesis as it is capable of impacting on all aspects of neurogenesis. For example, in addition to the data described above, it has been shown that 5 hours after i.p injections of LPS there was a significant reduction in proliferation of NPCs in the DG of rats (Fujioka and Akema, 2010). As well as impacting the proliferation, differentiation and survival of newborn neurons (Monje et al., 2003, Ekdahl et al., 2003, Fujioka and Akema, 2010), inflammation can also impact upon the integration into the surrounding circuitry of those new neurons that do manage to survive chronic inflammatory conditions. For example, it has been demonstrated that chronic LPS-induced inflammation in rats resulted in increased inhibitory neuronal signalling specifically to new neurons within the hippocampus (Jakubs et al., 2008). This study

demonstrated that although all neurons assessed within the hippocampus displayed enhanced frequency of spontaneous excitatory postsynaptic currents, only the newborn neurons (relative to pre-existing neurons) displayed enhanced inhibitory characteristics. Specifically, these new neurons born during chronic inflammation displayed increased frequency of spontaneous inhibitory postsynaptic currents as well as increased density of inhibitory synapses in their distal dendrites (Jakubs et al., 2008). It has also been demonstrated that new neurons born during periods of chronic inflammation have reduced participation in hippocampal contextual processing (Belarbi et al., 2012). These studies demonstrate that not only can inflammation impact all processes of neurogenesis, but that these effects may be long-lasting and even result in impairments in the functional role of neurogenesis.

Another aspect in which inflammation can regulate neurogenesis is by microglial-mediate synaptic pruning of newborn neurons (Hong and Stevens, 2016, Schafer et al., 2012, Paolicelli and Gross, 2011, Tremblay et al., 2010). Using their highly motile processes, microglia can engulf synapses and regulate their connectivity (Paolicelli and Gross, 2011, Hong and Stevens, 2016). Under physiological conditions, the extent interaction between microglia and specific neuronal synapses is highly regulated by the activity of these synapses with microglia pruning less active neuronal inputs (Tremblay et al., 2010, Schafer et al., 2012). Thus microglial synaptic pruning can be seen as a way of optimising neuronal connectivity. Furthermore, the mechanism behind this microglial-mediated synaptic pruning appears to involved the complement system of proteins as mice lacking C1q, C3, or CR3 display impaired synaptic connectivity (Schafer et al., 2012). Conversely, in pathological conditions were increased inflammation and microglial activation such

as in AD, there is also an increase in compliment driven microglial pruning which results in excessive synapse loss (Hong and Stevens, 2016, Hong et al., 2016).

Despite the wealth of evidence demonstrating that inflammation is detrimental to neurogenesis, it must be stated that general immune function is of critical importance in maintaining neurogenesis. For example, it is has been shown that immune deficient severe combined immunodeficiency (SCID) mice have impaired proliferation of NPCs both at baseline and after pro-neurogenic environmental enrichment as well as impaired spatial cognition (Ziv et al., 2006). Additionally, it was found that these effects could be attenuated by reintroduction of T-cells with CNS-specific antigens. Another study demonstrated that expression of the antigen recognition receptor, Toll-like receptor-2 (TLR2), on NPCs is important in maintaining neuronal differentiation (Rolls et al., 2007). Thus, under baseline conditions, normal immune function is required to maintain hippocampal neurogenesis.

1.6.4: Cytokines that impact upon hippocampal neurogenesis

As well as studies demonstrating the effects of LPS-induced inflammation on neurogenesis, there are several studies that have assessed the impact of specific cytokines such as TNFα, IL-6, IL-10 and IL-1β on neurogenesis. Cytokines are generally small proteins of about 8-30kDA in size. They are usually released in precursor form from immune cells such as monocytes, macrophages and lymphocytes in the periphery as well as from microglia and astrocytes within the CNS (Kim et al., 2016). Within the CNS they are released mainly by microglia but also by astrocytes in response to infection, injury or even chronic stress (Barbierato

et al., 2013, Sorrells et al., 2009, Nathan, 2002, Woodroofe, 1995). However, active cytokines released in the periphery can cross the BBB to exert their effects in the CNS. This generally occurs during periods of inflammation where the inflammatory response can result in increased BBB permeability (Stolp et al., 2005). Indeed the pro-inflammatory cytokines TNFα and IL-1β have themselves been shown to break down the BBB (Quagliarello et al., 1991, Blamire et al., 2000, de Vries et al., 1996).

Cytokines may act as autocrine or paracrine mediators of cell activity within the CNS (Rothwell et al., 1996). Generally, cytokines act as signal communicators between immune cells and neurons or NPCs and these signals are roughly divided into either pro- or anti-inflammatory, either promoting or inhibiting inflammation, respectively (Kim et al., 2016). IL-1β, TNFα and IL-6 are three of the most widely studied pro-inflammatory cytokines with regard to hippocampal neurogenesis. Alternatively, IL-10 and IL-4 are widely known anti-inflammatory cytokines. However, it is not entirely accurate to divide cytokines into either pro- or anti-inflammatory, as it has been suggested that the inflammatory outcome of certain cytokines may depend on their concentration, the cells they target, the timing of activation or the type of activation signal (Cavaillon, 2001). Under normal conditions, cytokines maintain a relatively low concentration and it has even been shown that certain cytokines such as IL-1β are required to maintain normal hippocampal neurogenesis-associated cognition (Goshen et al., 2007, Pitossi et al., 1997). However, during pathological conditions the expression of cytokines can increase up to 100-fold more than under normal conditions (Lee et al., 2002c).

1.6.4.1: TNFα

TNFα is a pro-inflammatory cytokine released in its inactive form as a 25kDa protein from microglia. This pro-TNFα is then proteolytically cleaved by TNFα converting enzyme (TACE) into its active, soluble, 17kDa form (Kriegler et al., 1988, Gearing et al., 1994, McGeehan et al., 1994). However, both pro-TNFα and cleaved TNFα have been shown to have varying degrees of biological activity (Decker et al., 1987). TNFα can bind to two receptors, namely tumour necrosis factor-receptor 1 (TNF-R1) and tumour necrosis factor-receptor 2 (TNF-R2) both of which are expressed on neurons, NPCs and glia (Kinouchi et al., 1991, Dopp et al., 1997, Widera et al., 2006). TNF-R1 is thought to be the main mediator of the biological activity of TNFα with cell death being the main outcome (Goetz et al., 2004, Tartaglia et al., 1993).

The effects of TNFα on neurogenesis are found to be variable depending on what stage of the neurogenic process NPCs are in when they encounter TNFα (Yirmiya and Goshen, 2011). Initial *in vitro* studies demonstrated that when TNFα expression was increased, NPC proliferation was reduced (Ben-Hur et al., 2003, Monje et al., 2003). However, another study demonstrated that, when NPCs under proliferative conditions were exposed to TNFα, there was no effect on proliferation, yet when NPCs undergoing differentiation were exposed to TNFα, there was a reduction in neuronal differentiation and an increase in astrocyte differentiation (Keohane et al., 2010). The effects of TNFα on NPC proliferation had previously been found to be dose dependent, where high concentrations induced cell death but lower concentrations may even act to promote NPC proliferation (Bernardino et al., 2008).

Thus, the varying effects of TNFα on neurogenesis may be a result of varying dosages.

It has been demonstrated that inhibition of TNF signalling using the TNF inhibitor pentoxifylline, prevented impairments in neuronal differentiation induced by LPS activated microglia *in vitro* (Liu et al., 2005). This suggests that TNFα may be the predominant mediator of activated microglia-induced suppression of neuronal differentiation. Additionally, it has been demonstrated that in TNF-R1 knockout mice as well as mice with both TNF-R1 and TNF-R2 knocked out there is a reduction in the number of new born neurons (Iosif et al., 2006). This reduction was not evident in TNF-R2 knockout mice which suggests that TNF-R1 is also the primary receptor mediating the effects of TNF on neurogenesis.

Assessment of TNFα-induced changes in cognitive function associated with neurogenesis has yielded conflicting reports, with one study demonstrating an enhancement in spatial memory in the MWM in TNF knockout (KO) mice compared to WT mice (Golan et al., 2004), while other studies demonstrated no differences (Gerber et al., 2004, Scherbel et al., 1999). However, TNFα KO mice were more susceptible to memory impairments after brain injury compared to WT (Gerber et al., 2004). Furthermore, aged mice with TNFα overexpression (OEX) displayed spatial memory impairments whereas young mice with TNFα OEX did not (Golan et al., 2004). These studies indicate that TNFα may mediate impairments in neurogenesis-associated behaviour during injury or with aging.

1.6.4.2: IL-6

IL-6 is a single chain 21kDA glycoprotein and is another commonly studied pro-inflammatory cytokine (Simpson et al., 1997, Yirmiya and Goshen, 2011). IL-6 signals via its cognate receptor, the IL-6 receptor (IL-6R), which is a type 1 cytokine receptor and tranduces IL-6 signals via its association with the transmembrane protein gp130 (Simpson et al., 1997). Gp130 is expressed in nearly all tissue types and within the CNS, IL-6R is expressed highest within the hippocampus of rats (Gadient and Otten, 1994). Initially, IL-6 was demonstrated to impair hippocampal plasticity as it was shown to impair LTP formation in the DG of rodents (Li et al., 1997, Bellinger et al., 1995, Tancredi et al., 2000). These effects on hippocampal plasticity were mediated via the IL-6 receptor because when this receptor was blocked by antibodies, IL-6 administration to hippocampal slices did not impair LTP formation (Li et al., 1997).

As well as its detrimental effects on hippocampal LTP, IL-6 has been shown to negatively impact upon hippocampal neurogenesis. For example, astrocyte mediated overexpression of IL-6 has been shown to reduce hippocampal neurogenesis by up to 63% in mice (Vallieres et al., 2002). This astrocyte-mediated IL-6 overexpression robustly impaired neurogenesis by suppressing proliferation, differentiation and survival of NPCs. However, these effects were not mediated by an increase in cell death or any increase in glial cell activation or expression (Vallieres et al., 2002). Using conditioned media from activated microglia, NPCs where shown to have reduced neuronal differentiation *in vitro* (Monje et al., 2003). This study also demonstrated similar effects with the addition of just IL-6 to NPCs and both the conditioned media and IL-6 effects were reversed by addition of an IL-6 neutralizing

antibody. This approach of treating NPCs with conditioned media from activated microglia was also used to demonstrated that IL-6 released from microglia promote astrocyte differentiation, an effect which was blocked by administration of a neutralizing IL-6 antibody (Nakanishi et al., 2007). Thus, the evidence suggests that IL-6 acts to suppress neurogenesis and promote NPC differentiation into astrocytes.

IL-6 has not been demonstrated to robustly affect hippocampal neurogenesis-associated spatial memory (Yirmiya and Goshen, 2011). One study demonstrated that intracerebroventricular (i.c.v.) administration of IL-6 in rats prior to training in the MWM did not affect spatial memory in this task, whereas i.c.v. administration of IL-1β over the same time period did impair spatial memory performance (Oitzl et al., 1993). Alternatively, IL-6 KO mice have been shown to have improved spatial memory performance in the radial arm maze compared to WT mice (Braida et al., 2004), and acute blockade of IL-6 improved acquisition in a forced alternation task (another method of assessing spatial memory) (Balschun et al., 2004). This has led to the suggestion that IL-6 may act to negatively regulate memory formation. However, IL-6 has been shown to have both pro- and anti-inflammatory properties depending on the magnitude and duration of its expression, and elevations in its expression in humans has been shown to correlate with improved memory retention scores in certain conditions (Jones et al., 2005, Kozora et al., 2001, Shapira-Lichter et al., 2008). This dual inflammatory role of IL-6 may then account for some of the discrepancies between the effects of IL-6 on neurogenesis and neurogenesis-associated behaviours.

1.6.4.3: IL-10

IL-10 was first discovered as a factor secreted by T-helper 2 immune cells that was able to suppress further cytokine production (Fiorentino et al., 1989). As its function as an anti-inflammatory cytokine, IL-10 acts to prevent the development of inflammatory or autoimmune reactions by limiting the immune response to pathogens (O'Garra and Vieira, 2007). IL-10 has been shown to be produced and secreted by microglia and astrocytes within various brain regions including the hippocampus and it is thought that the secretion of IL-10 after an inflammatory stimulus such as LPS is a form of negative feedback mechanism (Lobo-Silva et al., 2016, Martin et al., 2005, Lieberman et al., 1989, Huang et al., 2009). Specifically, IL-10 has been demonstrated to inhibit LPS-induced increases in TNFα and IL-6 in the CNS (Sheng et al., 1995b, Agnello et al., 2000), and IL-1β within the hippocampus of rats (Lynch et al., 2004).

IL-10 signals via the IL-10 receptor family which, when activated, initiates the Jak-stat signalling cascade (Moore et al., 2001). Activation of IL-10 receptors have also been shown to inhibit the NF-κB pathway, a commonly recruited pathway by pro-inflammatory cytokines, by inhibiting IκB kinase and preventing NF-κB DNA binding in monocytic cell lines (Schottelius et al., 1999). Within neurons, IL-10 signalling has been shown to enhance neuronal survival, potentially in part by its ability to enhance the expression of anti-apoptotic genes such as Bcl-2 and Bcl-xl and suppressing pro-apoptotic caspase-3 expression (Moore et al., 2001, Zhou et al., 2009a, Zhou et al., 2009b).

IL-10 largely impacts upon hippocampal neurogenesis by indirectly suppressing pro-inflammatory cytokine expression and blocking their effects on NPCs. For example, IL-10 overexpression in the APPPS1 mouse model of AD, enhanced hippocampal neuronal differentiation and survival (Kiyota et al., 2012). Additionally, this study suggested that the effects of IL-10 were largely mediated by their anti-inflammatory effects on microglia rather than their direct action on NPCs. Although there is no definitive evidence to suggest that the IL-10 receptor is expressed on hippocampal NPCs, there are reports that this receptor is expressed on NPCs from the SVZ (Perez-Asensio et al., 2013). Additionally, it was demonstrated in SVZ NPCs, that IL-10 maintains NPCs in an undifferentiated proliferative state and that this effect may be mediated by IL-10 receptor-mediated activation of ERK/Stat3 pathway (Perez-Asensio et al., 2013, Pereira et al., 2015). Therefore, it is possible that hippocampal NPCs may also express IL-10 receptors allowing IL-10 itself to directly regulate NPC function. In combination with its anti-inflammatory mechanisms, IL-10 positions itself as a strong pro-neurogenic cytokine.

1.6.4.4: IL-1β

IL-1β is a pro-inflammatory cytokine that plays a role in hippocampal neuroinflammation due to its heightened receptor expression profile in the hippocampus (Farrar et al., 1987). It is part of the IL-1 family of proteins which also includes IL-1α and the interleukin-1 receptor anatgonist (IL-1RA) (Rothwell and Luheshi, 2000). IL-1α and IL-1β were first sequenced and cloned in 1984 followed by IL-1RA in 1985 (Arend et al., 1985, Lomedico et al., 1984, Auron et al., 1984). IL-1β is produced predominantly by microglia but also by pyramidal neurons and astrocytes in the hippocampus (Bandtlow et al., 1990, Lechan et al., 1990, Eriksson

et al., 1999, Barbierato et al., 2013). IL-1β is initially synthesised as a 31kDA inactive precursor protein which is then cleaved into its 17kDa active form by caspase-1 (Dinarello, 1997). This active form is then released into the extracellular matrix, where it binds to its cognate transmembrane receptor interleukin receptor type 1 (IL-1R1) (Hanisch and Kettenmann, 2007, Rothwell et al., 1996, Green and Nolan, 2012b). Alternatively, IL-1β can bind to IL-1R2, which is a decoy receptor and does not produce a physiological response (McMahan et al., 1991). IL-1RA is the endogenous antagonist of IL-1β which binds with almost equal affinity as IL-1β to IL-1R1 (Seckinger et al., 1987, Hannum et al., 1990). IL-1RA is expressed and produced by both pyramidal neurons and microglia within the hippocampus (Eriksson et al., 1999).

As well as influencing the function of mature neurons, IL-1β can directly influence NPCs via interaction with IL-1R1 on both embryonic and adult NPCs (Green and Nolan, 2012b, Ryan et al., 2013). The expression of IL-1R2 has not been demonstrated on NPCs. However, it has been shown to be mainly expressed on microglia, which suggests it plays a role in dampening cytokine-induced activation of microglia (Pinteaux et al., 2002). Upon binding to IL-1R1, IL-1β induces an intracellular signalling cascade, which ultimately leads to altered gene expression of IL-1β target genes and a heightened pro-inflammatory response (Sims et al., 1993). Classical IL-1β signalling in NPCs involves activation of the NF-κB pathway. NF-κB is composed of homo- or heterodimeric combinations of the Rel family of proteins consisting of p52, p65 (Rel A), c-Rel, RelB, and p50. These proteins work in combination or with other co-factors, such as creb binding protein (CBP) to regulate the transcription of NF-κB target genes (Sheppard et al., 1999). However, in

mature neurons, IL-1β can also activate the MAPK pathway which suggests that IL-1β may recruit different pathways within NPCs as they proceed through the stages of neurogenesis (Koo and Duman, 2008, Srinivasan et al., 2004, O'Leime et al., 2017a). The MAPK pathway is a signal transduction pathway that can be activated by various cytokines and growth factors that act on cell surface kinase-linked receptors (Johnson and Lapadat, 2002). Once this pathway is activated there is a cascade of protein phosphorylation, which ultimately leads to altered gene expression and regulation of cell division, apoptosis, and tissue regeneration (Rang et al., 2012). See figure 1.6 for schematic of both pathways.

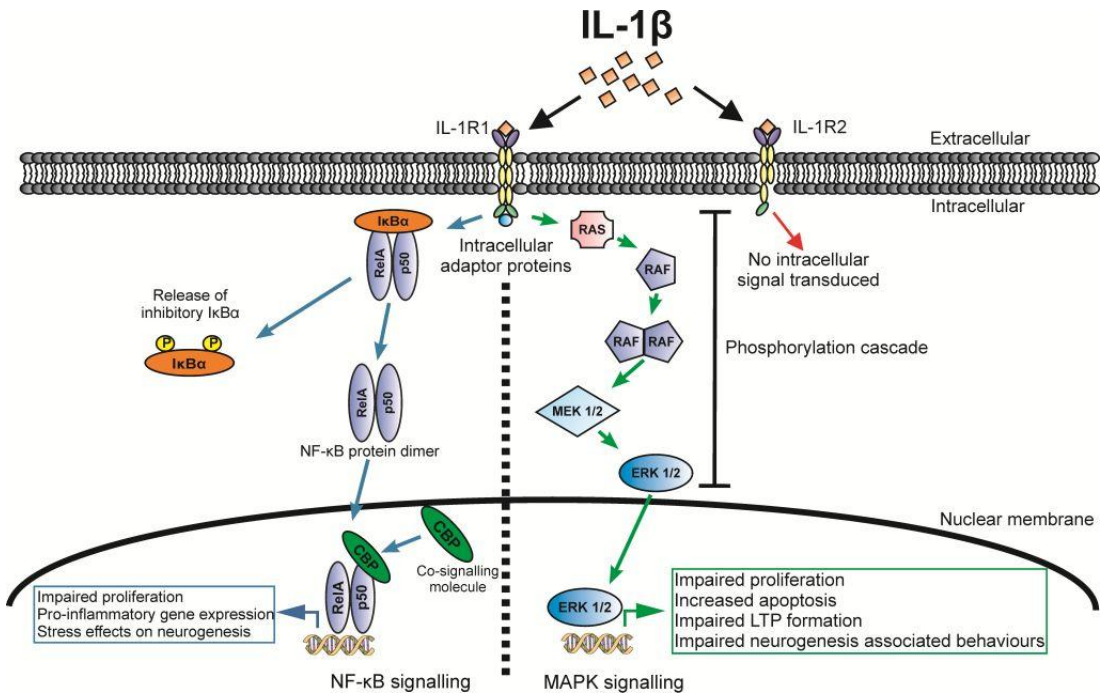


Figure 1.6 Schematic of IL-1β signalling cascades. Within neurogenic cells, IL-1β signals via either NF-κB or MAPK pathways. Interleukin-1 beta (IL-1β), interleukin receptor type 1 (IL-1R1), interleukin receptor type 2 (IL-1R2), creb binding protein (CBP), long term potentiation (LTP). (O'Leime et al., 2017a).

IL-1β has been shown to negatively impact upon the proliferation, differentiation and survival of hippocampal NPCs in cultures of rat hippocampal NPCs (Green et al., 2012, Ryan et al., 2013). There is further *in vitro* evidence to suggest that IL-1RA can reverse IL-1β-induced suppression of NPC proliferation as well as its promotion of astrogenesis in cultures of rat hippocampal neurospheres (Green and Nolan, 2012b, Ryan et al., 2013, Koo and Duman, 2008). It should also be noted that IL-1β may differentially influence NPCs or respond to changes in neurogenesis depending on the time during the lifespan at which changes in either inflammatory insult or neurogenesis occur. For example, an increase in hippocampal IL-1β was demonstrated in juvenile rats but not in adult rats subjected to whole brain irradiation (which ablates neurogenesis) (Blomstrand et al., 2014) and thus suggests that different inflammatory mechanisms may be involved in regulating hippocampal neurogenesis at different ages. Likewise, inflammatory-induced changes in neurogenesis during embryonic or postnatal development may result in overall changes in the number of proliferating cells in the adult DG, which may consequently cause structural changes in the hippocampus and ultimately lead to changes in behaviour (Green and Nolan, 2014). Development of anti-inflammatory interventions to circumvent inflammatory-induced impairments in neurogenesis and associated hippocampal functions should also take into consideration the timing during the lifespan of the intervention. Taken together, the evidence to date suggests that the signalling mechanism of IL-1β in NPCs during both development and in the adult hippocampus represents an important avenue for future research.

It has also been shown that IL-1β can suppress neuronal differentiation in a human hippocampal progenitor cell line (Zunszain et al., 2012b). While this study also

reported an increase in cell proliferation in response to IL-1β, which is contrary to what has been shown in rodent cell models, the increase in cell proliferation does not necessarily indicate enhanced neurogenesis. It is possible that an increase in cell proliferation in response to IL-1β leads to a phenotypic shift towards astrocyte rather than neuronal production, as has been previously demonstrated in response to IL-1β in primary cultures of rat NPCs (Green et al., 2012). It is also possible that the signalling mechanisms underlying IL-1β-induced changes in cell proliferation in human and rodent NPCs are not comparable, but this has yet to be explored in more detail. In addition, it is likely that immortalised cells, as is the case for the human hippocampal progenitor cell line, may respond differently to cytokine stimulation compared to cultured primary cells or indeed NCPs *in vivo.* This may be due to genotypic and phenotypic variation which can come about due to serial passaging of cell lines over time (Pan et al., 2009). Notwithstanding this, these discrepancies highlight the importance of assessing not only the proliferation status of NPCs but also their differentiation phenotype, as only the complete progression from proliferating NPCs to integrated mature neurons represents successful neurogenesis.

While there is evidence to suggest that low expression levels of IL-1β are required for hippocampal-related learning and memory it is well established that chronically elevated IL-1β has detrimental effects on memory and cognition (Yirmiya and Goshen, 2011, Lynch, 2015). For example, it has been demonstrated that transgenic overexpression or pharmacological administration of IL-1RA impairs contextual fear conditioning and spatial navigation in the MWM in rodents, indicating that baseline IL-1β is indeed required to facilitate certain forms of memory (Goshen et al., 2007). Indeed, this study demonstrated a slight increase in IL-1β expression 24 hours after

fear conditioning, indicating its role in fear learning (Goshen et al., 2007). Other *in vivo* studies have provided evidence that neurogenesis is a mediator of IL-1β-induced changes in cognitive behaviours. For example, impairments in performance in the MWM and reduced neurogenesis due to elevated levels of hippocampal IL-1β in rodents can be rescued by administration of IL-1RA (Ben Menachem-Zidon et al., 2014). These studies demonstrate that IL-1RA is an important tool to examine the effects of IL-1β signalling on hippocampal neurogenesis and associated behavioural tasks. They also highlight the complexity of assessing the contribution of IL-1β to hippocampal function. Generally it is thought that slight elevations in IL-1β could potentially facilitate hippocampal-associated cognition whereas a substantially or chronically elevated IL-1β is detrimental (Goshen et al., 2007). See table 1 for a summary of evidence showing the effects of IL-1β on hippocampal neurogenesis and associated cognition.

The link between IL-1β signalling and TLX is only beginning to be studied. So far, it has been shown that IL-1β decreases TLX expression in both adult and embryonic hippocampal NPCs (Green and Nolan, 2012b, Ryan et al., 2013). Furthermore, co-treatment of hippocampal NPCs with IL-1β and IL-1RA or siRNA to inhibit IL-1R1 expression restored TLX expression in hippocampal NPCs (Ryan et al., 2013). Little is currently known about the molecular interactions between IL-1β and TLX signalling, however, based on evidence from the literature, the NF-κB pathway may be a potential mediator between IL-1β and TLX. The expression of p52, p65, p50, and CBP has been reported in NPCs from embryonic and rat forebrain where they have been shown to be involved in a range of processes, such as proliferation of NPCs and regulation of the effects of environmental enrichment on neurogenesis

(Shingo et al., 2001, Lopez-Atalaya et al., 2011, Kaltschmidt and Kaltschmidt, 2009). RelB and c-Rel are expressed on migratory NPCs involved in SVZ neurogenesis and thus may play a role in the regulation of NPC migration (Denis-Donini et al., 2005). Many of the target genes of TLX, such as *Cdkn1a* and *Pten* are also regulated by NF-κB signalling in embryonic mouse fibroblasts. NF-κB targets these genes to enhance their expression while TLX represses their expression (Xia et al., 2007). None of these signalling interactions have been demonstrated in hippocampal NPCs however and this represents an important area for future research as NF-κB has been shown to be a involved in IL-1β-mediated suppression of neurogenesis (Koo and Duman, 2008, Koo et al., 2010).

1.6.4.5: Cytokine network

Despite the insights gained into how IL-1β signals intracellularly, it is important to acknowledge that the presence of other cytokines in the microenvironment can alter the effects of IL-1β and vice versa. This interaction may also explain some of the contradictory effects of IL-1β. There is clear evidence to show that IL-1β can negatively modulate cognitive processes associated with hippocampal neurogenesis however, there is also evidence to show positive effects. Indeed, it has been suggested that IL-1β is a requirement for certain forms of cognition and memory formation. An example of this is the fact that IL-1β has been shown to both improve and impair contextual fear conditioning (Goshen et al.; Gonzalez et al. 2009; Gonzalez et al. 2013). Instead of these effects being a direct result of IL-1β signalling, it is possible that the inflammatory network that IL-1β induces may differ depending on the intensity of IL-1β and/or the presence of other cytokines. For example, it has been reported that IL-1β can lead to increases in TNFα and IL-6,

other members of the IL-1 family, as well as cytokine receptors (Anisman et al. 2008; Moore et al. 2009; Shaftel et al. 2007; Skelly et al. 2013). In a similar fashion, TNFα and IL-6 have been shown to alter the expression of other cytokines (Balschun et al. 2004; del Rey et al. 2013; Skelly et al. 2013). Thus the expression status of cytokines other than IL-1β could be determined by the expression level of IL-1β itself. Decreased IL-4 expression has been shown to correlate with upregulation of IL-1β and subsequent impairments in memory while increases in IL-4 can prevent the inflammatory induced deficits in memory in models of AD (Maher et al. 2004; Maher et al. 2005; Kiyota et al. 2010; Kiyota et al. 2012). In addition, IL-10 has been shown to reduce the effects of IL-1β or LPS on hippocampal plasticity (Kelly et al. 2001; Lynch et al. 2004). This network of cytokine activity adds a level of complexity to the signalling of any one cytokine and may account for the complexity and variability reported regarding the effects of cytokines on cognitive processes.

Species	IL-1B Manipulation	IL-1B Signalling Mechanism	*In vitro* hippocampal effects	*In vivo* hippocampal effects	Overall effect on neurogenesis	Reference
E18 rat	10ng/ml in culture media	Upregulation of GSK-3β	↓ NPC proliferation ↑ Astrocytic differentiation ↓ Neuronal differentiation	-	↓	Green & Nolan 2012 Green et al. 2012
Adult rat	10ng/ml in culture media	IL-1R1 binding	↓ NPC proliferation ↓ Neuronal differentiation	-	↓	Ryan et al. 2013
Adult rat	Interferon alpha mediated increase in IL-1β	IL-1R1 binding	↓ NPC proliferation	-	↓	Kaneko et al. 2006
Postnatal day 1 rats	5, 10, and 10ng/ml in culture media	IL-1R1 binding	↓ Neuronal differentiation (Serotonergic neurons)	-	↓	Zhang et al. 2013
Human hippocampal progenitor cells	10mg/ml in culture media	-	↑ NPC proliferation ↓ Neuronal differentiation	-	↓	Zunszain et a. 2012
E16 rat	0.8 – 500ng/ml in culture media	↑ SAPK/JNK	↓ NPC proliferation ↓ Neuronal differentiation	-	↓	Wang et al. 2007
Aged adult mice	Inhibition of IL-1β activation by caspase-1 cleavage	-	-	↑ proliferation	↑	Gemma et al. 2007
Adult mice	MyD88 knockout (Disruption of IL-1R1 dependent signalling)	IL-1R1 independent	-	↓ NPC proliferation	↓	Wu et al. 2013
Adult rat	20ng/ml or 200ng/ml ICV	↑NF-κB	-	↓ Cell proliferation	↓	Koo & Duman 2008
Mice	OEX of IL-1RA	-	-	Impaired MWM performance	↓	Goshen et al. 2007
Mice	Transplantation of NPC OEX IL-1RA	-	-	Prevention of stress induced impairments in fear conditioning	↑	Ben Menachem-Zidon et al. 2008
Mice	Transplantation of NPC OEX IL-1RA	-	-	Improved MWM performance in model of AD		Ben Menachem-Zidon et al. 2014
Rats	Intrahippocampal IL-1β infusion (5ng/0.25μl)	↑ MAPK (p-ERK)	-	Impaired fear conditioning Prevented by MAPK inhibition		Gonzalez et al. 2013

Table 1: Summary of literature assessing the effects of IL-1β on hippocampal neurogenesis.

1.7: Aims

The overall aim of this thesis is to investigate the function of TLX in hippocampal neurogenesis and hippocampal-associated cognition in the presence and absence of the pro-inflammatory cytokine IL-1β. Inflammation is known to be a potent suppressor of hippocampal neurogenesis and IL-1β is a key player in hippocampal inflammation. IL-1β can suppress the expression of TLX in proliferating NPCs and so we aim to further elucidate the functional interactions between IL-1β and TLX in the hippocampus and hippocampal NPCs. We also focus on how these two regulators of neurogenesis can impact individually upon hippocampal-associated cognition. We hypothesize that TLX can be manipulated to firstly mitigate the effects of IL-1β on NPC proliferation in vitro, secondly regulate transcriptome responses to IL-1β in vivo and thirdly alter cognitive performance in hippocampal associated tasks. Additionally, we hypothesize that sustained IL-1β expression in conjunction with adolescent consumption of a diet high in fat and sugar can impact upon hippocampal cognitive processes. To test these hypotheses, our aims were:

Aim 1: To determine the effect on NF-κB inhibition on IL-1β-induced suppression of TLX expression in primary cultures of rat hippocampal NPCs (Chapter 2)

Aim 2: To examine whether TLX overexpression can prevent the effects of IL-1β on neurogenesis *in vitro* (Chapter 2).

Aim 3: To determine the transcriptome changes in the hippocampus of TLX knockout mice (Chapter 3)

Aim 4: To determine transcriptome changes in the hippocampus of TLX knockout mice induced by hippocampal IL-1β administration. (Chapter 3).

Aim 5: To determine if TLX overexpression *in vivo* can promote hippocampal neurogenesis-associated behaviours in rats (Chapter 4).

Aim 6: To determine whether cafeteria diet during adolescence can impair hippocampal neurogenesis-associated cognition in rats (Chapter 5).

Aim 7: To assess whether hippocampal IL-1β overexpression during adulthood can exacerbate adolescent cafeteria diet-induced effects on hippocampal neurogenesis-associated cognition in rats (Chapter 5).

Chapter 2

TLX is an intrinsic regulator of the negative effects of IL-1β on proliferating hippocampal neural progenitor cells

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Published in FASEB J

Abstract

Hippocampal neurogenesis is a lifelong process whereby new neurons are produced and integrate into the host circuitry within the hippocampus. It is regulated by a multitude of extrinsic and intrinsic regulators and is believed to contribute to certain hippocampal-dependent cognitive tasks. Hippocampal neurogenesis and associated cognition have been demonstrated to be impaired following increases in levels of the pro-inflammatory cytokine IL-1β in the hippocampus; such as occurs in various neurodegenerative and psychiatric disorders. IL-1β also suppresses the expression of TLX. TLX is an orphan nuclear receptor which functions to promote NPC proliferation while suppressing neuronal differentiation. Therefore, manipulation of TLX represents a potential strategy to prevent the anti-proliferative effects of IL-1β. In this study, we assessed the mechanism underlying IL-1β-induced changes in TLX expression and determined the protective capacity of TLX in mitigating the effects of IL-1β on embryonic rat hippocampal neurosphere expansion. We demonstrated that IL-1β activated the NF-κB pathway in proliferating NPCs and that this pathway activation was responsible for IL-1β-induced changes in TLX expression. Additionally, we report that enhancing TLX expression prevents IL-1β-induced suppression of neurosphere expansion. Thus, we highlight TLX as a potential protective regulator of the anti-proliferative effects of IL-1β on hippocampal neurogenesis.

1: Introduction

Hippocampal neurogenesis is a process by which new neurons are born from neural progenitor cells (NPCs) in the hippocampus. The process begins during embryonic development and persists into adulthood, where it is thought to contribute to hippocampal-dependent cognitive function (Kohman and Rhodes, 2013, Zhao et al., 2008). Evidence of this functional role of hippocampal neurogenesis in the adult has been shown *in vivo* due to its involvement in various cognitive tasks such as Morris water maze (Drapeau et al., 2007, van Praag et al., 1999c, van Praag et al., 1999a, van Praag et al., 2005), novel object and novel location recognition (Jessberger et al., 2009a, Rola et al., 2004, Madsen et al., 2003), and pattern separation in rodents (Sahay et al., 2011b, Clelland et al., 2009, Creer et al., 2010, Bekinschtein et al., 2011). Additionally, adult hippocampal neurogenesis has been demonstrated to be impaired in conditions where there is a subsequent impairment in hippocampal cognition, such as stress, depression, aging, and neurodegeneration (Lazarov et al., 2010, Na et al., 2014, Santarelli et al., 2003, Tanti and Belzung, 2013, Yirmiya and Goshen, 2011). Many of these conditions have been associated with increased inflammation and expression of the pro-inflammatory cytokine interleukin-1β (IL-1β) in the brain (Maes et al., 2012, Godbout and Johnson, 2009, Leonard, 2007, Nolan et al., 2013).

Inflammation is well established as having negative consequences on central nervous system function and on hippocampal neurogenesis. Release of IL-1β occurs from activated microglia as a result of stress, infection, neurodegeneration, or indeed with aging (Raison et al., 2006, Leonard, 2007, Kettenmann et al., 2013, Yirmiya and Goshen, 2011). The cognate receptor for IL-1β is the IL-1 receptor type 1 (IL-1R1)

and this receptor is highly expressed on glia, neurons, and NPCs in the hippocampus of the rodent brain (Farrar et al., 1987, Parnet et al., 1994, Green et al., 2012, Ryan et al., 2013). After binding to IL-R1, IL-1β induces an intracellular signalling cascade leading to altered gene transcription and a heightened pro-inflammatory response (Rothwell et al., 1996, Green and Nolan, 2012b, Sims et al., 1993). In most cell types, ligation of IL-1β to IL-1R1 activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway (DiDonato et al., 1997). NF-κB is composed of homo- or heterodimeric combinations of the Rel family of proteins, consisting of p52, p65 (Rel A), c-Rel, RelB, and p50. These proteins work in combination or with other co-factors, such as creb binding protein (CBP), to regulate the transcription of NF-κB target genes (Sheppard et al., 1999). Consequences of sustained elevation of IL-1β in the hippocampus include impaired synaptic plasticity and hippocampal dependent learning and memory (Vereker et al., 2000, Goshen et al., 2008, Pugh et al., 1999, Green and Nolan, 2012b, Ryan et al., 2013). Indeed the NF-κB pathway has been suggested to mediate the effects of IL-1β on long term potentiation (LTP) (Tong et al., 2012, Vereker et al., 2000, Lynch, 2004). It has also been demonstrated that IL-1β can recruit the NF-κB pathway to suppress hippocampal neurogenesis. For example, it has been demonstrated that inhibition of the NF-κB pathway prior to extrinsic administration of IL-1β to hippocampal NPCs *in vitro* prevented an IL-1β-induced suppression of proliferation in these cells (Koo and Duman, 2008). However it is uncertain how the activation of this pathway can affect intrinsic regulators of hippocampal neurogenesis.

The study of signalling mechanism within embryonic NPCs provides valuable insights into signalling mechanisms which may occur within adult NPCs (Hodge and

Hevner, 2011, Kriegstein and Alvarez-Buylla, 2009, Liu and Zhao, 2009, Qu and Shi, 2009). IL-1R1 is expressed on both embryonic and adult NPCs (Green et al., 2012, Ryan et al., 2013) and IL-1β has been shown to negatively impact upon various aspects of hippocampal neurogenesis such as proliferation, differentiation, and survival (Green et al., 2012, Ryan et al., 2013, Koo and Duman, 2008). TLX (Orphan nuclear receptor tailless homolog), or Nr2e1 (nuclear receptor subfamily 2 group E member 1), is an orphan nuclear receptor and an intrinsic regulator of neurogenesis that is required to maintain the pool of NPCs within the hippocampus by promoting NPC proliferation and suppressing differentiation (Shi et al., 2004, Zhao et al., 2010). Studies using transgenic models show that TLX can contribute to hippocampal dependent learning and memory (Shi et al., 2004, Murai et al., 2014, O'Leary et al., 2016b). For example in $TLX^{-/-}$ mice, impairments in hippocampal-associated cognitive function and hippocampal neurogenesis have been reported (Monaghan et al., 1995, Roy et al., 2002). It has also been demonstrated that IL-1β negatively regulates the expression of TLX within both adult and embryonic NPCs, with subsequent impairments in NPC proliferation (Green and Nolan, 2012b, Ryan et al., 2013, O'Leary et al., 2016a). However, the associated signalling remains largely unknown. Likewise, whether TLX manipulation can prevent the effects of IL-1β on NPC expansion has not been investigated. Here we identify the NF-κB signalling pathway as a key mediator of the effects of IL-1β on TLX expression and show that restoration of TLX expression is sufficient to prevent the negative effects of IL-1β on NPC expansion *in vitro*. These data along with previous data from ours and others groups highlight TLX as a potential regulator of IL-1β-induced impairments in hippocampal neurogenesis.

2: Experimental Methods

2.1: Preparation and treatments of rat hippocampal NPCs

Embryonic day (E) 18 rat hippocampi (Biological Services Unit, UCC, Cork, Ireland) were cultured for 6 days *in vitro* (DIV) as previously described (Green et al. 2012; Keohane et al. 2010). Neurospheres were kept under proliferative conditions in T25 flasks at a density of $2x10^6$ cells/10ml of proliferation media (DMEM:F12 (Sigma-Aldrich), 2% B27 supplement (Invitrogen), 1% penicillin/streptomycin (Sigma-Aldrich), 10ng/ml EGF (Sigma-Aldrich), 10ng/ml FGF (Millipore), 200mM L-glutamine (Sigma-Aldrich), 33mM D-glucose (Sigma-Aldrich)). At 6DIV the neurospheres were dissociated using 0.1% trypsin-EDTA enzyme (Sigma-Aldrich) and treated as a single cell suspension. $5x10^4$ cells were plated in proliferation media onto poly-D-lysine (Sigma-Aldrich) coated coverslips for immunocytochemical analysis and $5x10^5$ cells were seeded per well in proliferation media in six well plates for PCR analysis. In the first set of experiments, recombinant rat IL-1β (10ng/ml) (R&D Systems) was added to the media for 10min, 30min, 1h, 2h, 4h or 6DIV to measure mRNA expression levels of TLX, p21 and IκBα, and p65 and TLX protein levels. A second series of NPC cultures were treated with IL-1β (10ng/ml) for 8hr, 16hr, and 24hr to assess the effects of longer term treatment of IL-1β on TLX and p21 mRNA expression. In a subsequent series of experiments, IL-1β (10ng/ml) was added to the proliferation media with or without the NF-κB inhibitor, JSH-23 (25μM) (Sigma-Aldrich) for 3h *in vitro* and mRNA expression levels of TLX, p21 and IκBα, as well as protein levels of TLX were assessed. We chose to use JSH-23 at a concentration of 25μM throughout this study as this concentration had previously been shown to suppress NF-κB signalling in cultured adult rat derived hippocampal NPCs without affecting viability (Koo et al., 2010).

2.2: Lentiviral production and transduction of NPCs with TLX lentivirus

Mouse TLX (Nr2e1) was cloned into a pCDF-CMV-IRES-GFP lentiviral FIV backbone using standard cloning methods. Transgene expression of TLX was driven by a cytomegalovirus (CMV) promoter which was connected via an internal ribosomal entry sequence (IRES) to a GFP reporter. Lentiviral particles were produced by transfecting a HEK 293T producer cell line with the lentiviral Nr2e1 plasmid or lentiviral GFP control plasmid and pPACKF1^{TM} Lentivector Packaging Kit (System Biosciences, 2438 Embarcadero Way, Palo Alto, CA, 94303, USA). Harvested viral supernatant was aliquoted and stored at -80°C until use. Packaged viruses had titres of approximately $1x10^6$ TU/ml. On the day of seeding, $5x10^5$ dissociated NPCs were plated in each well of a 6 well plate along with $7x10^4$ TU/ml of lentivirus overexpressing either TLX or GFP. After 24 hours, recombinant rat IL-1β (10ng/ml) or phosphate buffered saline (PBS) was added. Cells were allowed to proliferate as neurospheres and were kept under proliferative conditions for a further 6DIV.

2.3: PCR

Total cellular RNA was extracted from the dissociated NPCs derived from the neurospheres using a Total Mammalian RNA extraction kit (Sigma-Aldrich) following the manufacturer's instructions. Extracted RNA was treated with DNase (Sigma-Aldrich) to remove any DNA contamination. Complimentary DNA (cDNA) was prepared using the High capacity cDNA reverse transcription kit (Applied Biosystems). PCR was carried out on a 96 well plate using a StepOnePlus Real-Time PCR machine (Applied Biosystems) using the following primers: TLX F:

GCTTTCTTCACAGCGGTCAC, R: GCAGACACAGCGGTCAACT; IκBα F: TGAAGTGTGGGGCTGATGTC, R: AGGGCAACTCATCTTCCGTG; p21 F: GTATGCCGTCGTCTGTTCGG, R: GCAGAAGACCAATCGGCGCT; and SHDA F: CCCACTAACTACAAGGGACAGG, R: TTGGCACCATGCACTGAG. The PCR consisted of the following steps: 95°C for 3 minutes to denature the cDNA followed by 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Each reaction consisted of 1µl of sample (5ng/ul), 10µl of Sybr MasterMix (KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX™ for ABI instruments, Sigma-Aldrich), 0.1µl of both forward and reverse primers, and 8.8µl of RNase free H_2O. RNA elution solution was used instead of samples to ensure no amplification occurred as a result of self complimentary primers. Results were normalised to succinate dehydrogenase (SDHA) and analysed using the ΔΔCT method (Livak and Schmittgen, 2001).

2.4: Immunocytochemistry

Cells were incubated in 5% donkey serum overnight at 4°C to block non-specific antibody binding. Cells were then incubated with antibodies against TLX (Santa Cruz, sc292096, 1:100, donkey anti-rabbit) or p65 (Abcam, ab7970, 1:100, donkey anti-rabbit) at 4°C overnight and subsequently incubated with the appropriate fluorescently tagged secondary antibody (Alexafluor, Invitrogen) for 2h at room temperature in the dark. The cells were counterstained with 4'6-diamidino-2-phenylindole (DAPI) (1:2500) to identify the nuclei. For each protein a sample of cells was incubated with blocking solution and secondary antibody, but without prior incubation with primary antibody, as a negative control.

2.5: MTT viability analysis of treated NPCs

After 6DIV of IL-1β treatment or 3 hours of JSH-23 with and without IL-1β treatment, NPCs were seeded at a density of 2 x 10^4 cells in a 96-multiwell plate, in proliferation media. Cells were incubated with 0.5mg/mL Thiazolyl Blue Tetrazolium Bromide (Sigma) in proliferation media (without IL-1β or JSH-23) under conditions of 5% CO2 at 37 °C for 3 hours. The solution was removed and cells were lysed using DMSO. Lysates were transferred to a fresh 96-multiwell plate and the absorbance 129 was read on a Multiskan spectrophotometer (Thermo Fisher) at an absorbance wavelength of 540nm and a reference wavelength of 690nm. Each experiment was carried out three times and in technical triplicate.

2.6: Neurosphere diameter analysis, cell counts, and densitometry

Neurospheres were imaged using an inverted microscope (IX70, Olympus) at 10x magnification each day for 5DIV. At 6DIV neurospheres were dissociated to assess changes in TLX and p21 expression as previously described. For diameter analysis, images of five individual neurospheres were captured per field of view from four fields of view per flask per time point. Neurosphere diameter was measured using ImageJ (version 1.47, NIH, Bethesda, MD, USA). At least 20 spheres were analysed per condition. Each experiment was repeated at least 3 times. Immuno-labelled cells were imaged using an upright microscope (BX53, Olympus). Positively-stained cells were counted from five random fields of view per coverslip. For each condition, cells from four coverslips were counted and each experiment was repeated three times. Merged images of DAPI with TLX or p65 immunolabelled cells were used to analyse densitometry of nuclear staining from ten randomly selected TLX or p65-positive cells per field of view using ImageJ software. Twenty micrographs per

condition were analysed from three independent experiments. Background fluorescence intensity was controlled for by thresholding. Data were expressed as arbitrary units of the intensity ratio of nuclear: nuclear + cytoplasmic TLX and each dataset resulting from IL-1β treatment was normalised to its respective control.

2.7: Statistical Analysis

Unpaired Student's t-test and one-way or two-way ANOVA followed by a Bonferroni *post hoc* test were performed where appropriate to determine which conditions were significantly different from each other. Results are displayed as mean with SEM and deemed significant when $p<0.05$.

3: Results

3.1: IL-1β suppresses neurosphere expansion

Two-way ANOVA revealed a main effect of IL-1β (10ng/ml) treatment ($p<0.001$) and treatment duration ($p<0.001$). *Post hoc* analysis showed that IL-1β suppressed neurosphere expansion under proliferative conditions after 4 and 5DIV (*$p<0.05$ vs. Ctrl) (Fig. 2.1A, B). Under the same conditions at 6DIV, IL-1β induced a significant increase in the mRNA expression of the tumour suppressor gene p21 in dissociated NPCs (**$p<0.01$ vs. Ctrl) (Fig. 2.1C). IL-1β also induced a significant decrease in the mRNA expression of TLX, a known promoter of cell proliferation in NPCs, at this timepoint (**$p<0.01$) (Fig. 2.1D). This is consistent with a previous report demonstrating that IL-1β reduced the protein expression levels of TLX in hippocampal NPCs after 7 DIV (Green and Nolan, 2012b). IL-1β did not induce a reduction in cell viability at this dose and over this timeframe (Fig. 2.1E) which supports previous data (Green et al., 2012).

3.2: IL-1β induces rapid activation of NF-κB

To assess whether IL-1β administration induced translocation of the NF-κB subunit p65 to the nucleus in hippocampal NPCs, an indicator of NF-κB activation, we assessed the ratio of nuclear to cytoplasmic expression of p65 (RelA), a key signalling component of the NF-κB pathway. As NF-κB signalling has been shown to be rapidly activated in human glial cells after IL-1β exposure (Griffin and Moynagh, 2006), we assessed p65 translocation to the nucleus from 10 minutes to 4 hours after IL-1β administration. IL-1β administration induced a significant increase in nuclear translocation of p65 protein at 10 minutes after IL-1β indicating increased NF-κB activation (F (5, 114) = 19.21, *** = $p<0.001$ vs. Ctrl) (Fig. 2.2A, C). A

negative feedback mechanism was evident due to the fact that mRNA expression of IκBα, a negative regulator and target gene of p65, was significantly increased 10 minutes after IL-1β (F (5, 18) = 6.56, *** = p<0.001 and * = p<0.05 vs. Ctrl) (Fig. 2.2B). The increase in IκBα expression was maintained for up to 4 hours which coincided with a decrease in nuclear p65 protein expression at 4 hours after IL-1β (** = p<0.01 vs. Ctrl) (Fig. 2.2A).

3.3: IL-1β induces suppression of TLX expression

As we observed NF-κB activation induced by IL-1β in hippocampal NPCs, we next assessed the effects of IL-1β on TLX expression in hippocampal NPCs over the same time frame. After 30 minutes of IL-1β exposure there was a decreased mRNA expression of TLX in proliferating NPCs (F (5, 12) = 3.17, *p<0.05) (Fig. 2.3A). This resulted in a significant decrease in TLX protein after 1 hour of IL-1β exposure (F (5, 114) = 20.75, ***p<0.001) (Fig. 2.3B, C). We assessed TLX mRNA expression after an extended treatment with IL-1β and show that it remained reduced after 8 hours of IL-1β exposure, normalised at 16 hours, and was significantly lower that control again at 24 hours after IL-1β treatment. F (3, 3) = 18.88, ***p<0.001, **p<0.01) (Fig. 2.3D) This corroborates previous data showing a reduction in TLX protein expression at 24h in response to IL-1β (Ryan et al., 2013). As TLX functions as a transcriptional suppressor, we assessed the effect of IL-1β on the expression of the TLX target gene and suppressor of proliferation, p21. IL-1β induced a significant increase in p21 mRNA expression after 2 hours (F (5, 18) = 3.25, ** = p<0.01 vs. Ctrl), however this effect was not significant after 4 hours (Fig. 2.3E). Therefore, we extended our treatment times and observed that after 8 hours of IL-1β exposure there was no change in p21 expression compared to control. However, after 16 and 24

hours p21 mRNA expression was significantly higher than controls (F (3, 8) = 12.16, ** = p<0.01) (Fig. 2.3F).

3.4: IL-1β mediates its effects on TLX expression via NF-κB pathway activation

To assess whether the IL-1β-induced activation of NF-κB pathway is responsible for the IL-1β-induced decrease in TLX expression, we pre-treated hippocampal NPCs with JSH-23, an inhibitor of NF-κB activation (Koo et al., 2010). We demonstrate that when this pathway is inhibited, the IL-1β-induced reduction in TLX mRNA expression is attenuated (F (3, 8) = 12.14,*** = p<0.001 vs. Ctrl; + = p<0.05 vs. IL-1β) (Fig. 2.4A). The changes in TLX mRNA expression were mirrored by the changes in TLX protein expression (F (3, 796) = 75.57, *** = p<0.001 vs. Ctrl; +++ = p<0.001 vs. IL-1β) (Fig. 2.4B, C). Treatment of NPCs with JSH-23 had no effect on the IL-1β -induced increase in in the mRNA expression of the TLX target gene p21 (Fig. 2.4D) but attenuated the IL-1β -induced activation of NF-κB target gene IκBα (Fig. 2.4E). The attenuation of IL-1β-induced decrease in TLX by JSH-23 was not due to a reduction in total cell viability, as neither IL-1β nor JSH-23 reduced cell viability compared to control during this acute incubation period (Fig. 2.4F).

3.5: Increased TLX expression prevents IL-1β-induced suppression of neurosphere expansion

To assess whether TLX could prevent the IL-1β-induced decrease in neurosphere expansion we transduced neurospheres with lentiviruses expressing either GFP or TLX and showed that they did not significantly impair neurosphere expansion compared to control non lentiviral transduced neurospheres across all timepoints (Fig. 2.5A, B). Two-way ANOVA revealed a main effect of treatment with lentivirus

with or without IL-1β (F (5, 216) = 29.575, $p<0.001$) and treatment duration (F (5, 216) = 120.422, $p<0.001$) and an interaction effect of treatment x treatment duration (F (25, 216) = 1.780, $p<0.05$). *Post hoc* analysis showed that IL-1β treatment of GFP-transduced neurospheres impaired neurosphere expansion to a similar extent as non-lentiviral transduced neurospheres treated with IL-1β at 4DIV, 5DIV, and 6DIV ($p<0.05$) (Fig. 2.5A) while IL-1β treatment of TLX-transduced neurospheres did not (Fig. 5.5B). Moreover, neurospheres transduced with lentivirus overexpressing TLX significantly attenuated IL-1β-induced impairment in neurosphere expansion at 4DIV, 5DIV, and 6DIV ($p<0.01$) (Fig. 2.5B, C). IL-1β-treated GFP-transduced neurospheres showed impaired expansion compared to GFP-transduced controls at 6DIV ($p<0.001$) (Fig. 2.5D) and this effect was not apparent in IL-1β-treated TLX-transduced neurospheres compared to TLX-transduced controls (Fig. 2.5D, E). To confirm the efficacy of the lentiviral transduction, we assessed TLX mRNA expression in GFP and TLX transduced neurospheres at 6DIV. In the absence of IL-1β, there was no significant difference in TLX mRNA expression between TLX and GFP transduced neurospheres (Fig. 2.5F). However, after treatment with IL-1β for 6DIV, TLX transduced neurospheres maintained a significantly higher expression of TLX mRNA compared to GFP transduced neurospheres (F (3, 25) = 7.73, # = $p<0.05$ between GFP + IL-1β and TLX + IL-1β, ** = $p<0.01$ between GFP and GFP + IL-1β, $$$ = $p<0.001$ between TLX and GFP + IL-1β) (Fig. 2.5F). Both GFP and TLX overexpressing cells display an increase in the mRNA expression of IκBα in response to IL-1β treatment however the increase in expression only reaches significance in the TLX-transduced neurospheres (F (3, 19) = 5.71, +++ = $p<0.001$ vs. TLX) (Fig 2.5G).

4: Discussion

The present study demonstrates that TLX may protect against IL-1β-induced suppression of hippocampal NPC proliferation, and thus TLX is an important regulator of neuroinflammatory-induced changes in hippocampal neurogenesis. Specifically, we have shown that IL-1β induces a decrease in hippocampal NPC neurosphere expansion, which is consistent with previous reports (Ryan et al., 2013, Green and Nolan, 2012b). This decrease is associated with a significant reduction in TLX expression, a regulator of NPC proliferation, and an increase in p21 expression, a mediator of cell cycle arrest and reduced proliferation (Sun et al., 2007, Sharpless and DePinho, 2004, Waga et al., 1994). Additionally, we demonstrate that IL-1β recruits the NF-κB pathway in embryonic hippocampal NPC neurospheres and that pharmacological inhibition of this pathway is sufficient to reverse the negative effects of IL-1β on TLX expression. Finally, we show that restoration of TLX expression in the presence of IL-1β protects against IL-1β-induced suppression of neurosphere expansion. This suggests that TLX can mitigate the anti-proliferative effects of IL-1β on hippocampal NPCs.

We, and others, have previously established that IL-1β induces a decrease in hippocampal NPC proliferation in cultures of embryonic and adult neurospheres (Green and Nolan, 2012b, Ryan et al., 2013, Koo and Duman, 2008), and neurosphere expansion has been correlated with increased proliferation of NPCs from both the hippocampus and subventricular zone of the lateral ventricles (Widera et al., 2006, Murai et al., 2014). We also observed that IL-1β at the dosage and duration of treatment used in the current study does not affect cell viability. We did however, demonstrate that IL-1β decreased the mRNA expression of TLX after short

treatment duration for up to 24 hours as well as after a longer treatment duration of 6 days. This supports previously published data reporting an IL-1β- induced decrease in the protein expression of TLX at both 24 hours (Ryan et al., 2013) and 7 days (Green and Nolan, 2012b). As TLX promotes cell proliferation, the suppression of neurosphere expansion in response to IL-1β is potentially mediated via reduced expression levels of TLX. Additionally, we observed an increase in the mRNA expression of the TLX target gene p21 after IL-1β treatment for up to 5 days. p21 expression in suppressed by TLX and when its expression is increased it induces cell cycle arrest and reduces proliferation (Waga et al., 1994, Pechnick et al., 2011, Sun et al., 2007). Therefore, based on the current study and previous reports (Green and Nolan, 2012b, Ryan et al., 2013), the ability of IL-1β to reduce neurosphere expansion is likely due to a reduction in NPC proliferation rather than an induction of cell death.

The NF-κB pathway has been shown to be responsible for inhibiting cell proliferation *in vitro* and *in vivo* in several cell types including rat hippocampal NPCs, mouse embryo fibroblasts, and human epithelial cells (Seitz et al., 2000, Chen et al., 2006, Koo and Duman, 2008). IL-1β has previously been shown to recruit the NF-κB pathway to suppress hippocampal NPC proliferation *in vivo* (Koo and Duman, 2008). NF-κB is rapidly activated in response to IL-1β in human glial cells (Griffin and Moynagh, 2006) and thus we hypothesised that a rapid activation may also occur in hippocampal NPCs. Indeed, we observed translocation of the p65 subunit to the nucleus (an indicator of NF-κB activation (Xie et al., 2016, Zhu et al., 2015)) of hippocampal NPCs within 10 minutes of IL-1β administration. We also demonstrated activation of the NF-κB target gene IκBα within this time frame.

Inhibition of NF-κB activation using JSH-23 (Koo and Duman, 2008, Arias-Salvatierra et al., 2011, de Vries et al., 2014, Kesanakurti et al., 2013) abrogated the IL-1β-induced decrease in TLX mRNA and protein expression in hippocampal NPCs after 3 hours of exposure to IL-1β and also attenuated the IL-1β -induced increase in IκBα mRNA expression. Thus, the NF-κB pathway appears to be a potential mediator of IL-1β-induced changes, not only on NPC proliferation as previously shown (Koo et al., 2010, Koo and Duman, 2008) but also on TLX expression. It is tempting to speculate that suppression of TLX by IL-1β via enhanced NF-κB activation is the predominant mechanism which determines the outcome of IL-1β-induced changes in proliferation of NPCs. The data herein would suggest that IL-1β-induced activation of NF-κB results in the suppression of TLX gene expression resulting in a subsequent decline in TLX protein expression and NPC proliferation. This mechanistic pathway would be more likely than a direct protein interaction between TLX and NF-κB signalling due to the fact that changes in TLX and p65 expression occur at largely different timepoints and locations within the NPCs.

We did not observe an effect of NF-κB inhibition on expression of the TLX target gene p21 in response to IL-1β. JSH-23 has been established as a selective NF-κB inhibitor but any non-specific signalling effects that JSH-23 treatment may have in hippocampal NPCs that may impact upon target genes cannot be ruled out (Shin et al., 2004, Kumar et al., 2011, Arias-Salvatierra et al., 2011). However, we observed that JSH-23 treatment attenuated the IL-1β-induced increase in the NF-κB target gene IκBα therefore our data are a proof of concept that NF-κB signalling is at least partially responsible for the effects of IL-1β on TLX expression. It should be noted

though that IL-1β does not exclusively recruit NF-κB signalling pathways components to mediate its effects on hippocampal NPC proliferation (Wang et al., 2007, Zunszain et al., 2012a, O'Leime et al., 2017a). Specifically it has previously been demonstrated that IL-1β treatment for 48 hours induced the activation of SAPK/JNK signalling in embryonic rat forebrain NPCs *in vitro* and that SAPK/JNK signalling, and not the NF-κB pathway, mediated IL-1β-induced decrease in proliferation of these NPCs (Wang et al., 2007). The differences reported in that study may be related to brain region, embryonic age of the NPCs, or treatment duration of IL-1β. The results also suggest that, at specific stages in development, IL-1β may recruit different pathways to exert its effects on NPCs, or indeed, longer incubations with IL-1β may result in different signalling pathway activation. We have previously shown that IL-1β can increase the activity of GSK-3β, a negative regulator of Wnt signalling pathway (Wu and Pan, 2010), in proliferating hippocampal embryonic NPCs with subsequent reductions in proliferation (Green and Nolan, 2012b). Additionally, that study demonstrated that when GSK-3β was inhibited, the IL-1β-induced suppression of NPC proliferation and TLX expression were ameliorated. However, that study assessed the effects of chronic IL-1β administration and so GSK-3β, and potentially Wnt signalling, may regulate the chronic effects of IL-1β on TLX and NPC proliferation. Interestingly, GSK-3β can interact with NF-κB signalling to regulate cytokine production, and so the protective effects of inhibiting GSK-3β after IL-1β exposure may be indirectly mediated by the subsequent effects on NF-κB signalling (Green and Nolan, 2012a, Martin et al., 2005). Assessment of the differential activation of specific pathways in response to individual activators as NPCs proliferate, differentiate, and integrate into the surrounding neuronal circuitry provides an interesting avenue for future research.

An interesting insight into the transcriptional effects of IL-1β in NPCs is the increase in p21 expression after IL-1β treatment of proliferating NPCs demonstrated in this study. p21 is a potent suppressor of the cell cycle, and subsequently is an important regulator of cell proliferation (Sharpless and DePinho, 2004). TLX has previously been shown to suppress the expression of p21 in neural stem cell (NSC) cultures (unknown source) which is correlated with an increase in NPC proliferation (Sun et al., 2007). However, the study did not attempt to manipulate the increase in p21 expression (either knockdown or other method of inhibition) to assess the direct role of p21 on NSC proliferation (Sun et al., 2007). The importance of p21 in regulating the proliferation of hippocampal NPCs has since been established in $p21^{-/-}$ mice where there is a significant increase in NPC proliferation (Pechnick et al., 2011) and so it is reasonable to suggest that the mechanism by which TLX regulates proliferation involves the regulation of p21 expression. We demonstrate here that when TLX expression is suppressed by IL-1β, there is a concurrent increase in p21 expression which suggests that IL-1β may suppress NPC proliferation by enhancing p21 expression. This theory is supported by findings showing an increase in hippocampal IL-1β expression, a subsequent increase in hippocampal p21 expression, and reduced hippocampal NPC proliferation and neuronal differentiation in a mouse model of inflammatory bowel disorder (Zonis et al., 2015).

Deficits in neurogenesis in TLX null mice have been reported by several research groups (Murai et al., 2014, Sun et al., 2007, Monaghan et al., 1997, Li et al., 2008). For example, Shi and colleagues have demonstrated that NPCs of $TLX^{-/-}$ mice have prolonged cell cycles and impaired proliferation during embryonic development (Li

et al., 2008). This was also demonstrated in adult hippocampal NPCs, where there was an impairment in NPC proliferation within the hippocampus of adult mice with an induced recombination of the TLX gene (Zhang et al., 2008). Functionally, deficits in TLX expression and subsequent suppression of hippocampal neurogenesis have been demonstrated to impair spatial memory in mice, which is a hippocampal-associated cognitive process (Zhang et al., 2008). More recently, it has been shown that overexpressing TLX can rescue the proliferative defects in mice lacking TLX, and improve hippocampal neurogenesis-associated cognition as evidenced by improved performance in the Morris water maze (Murai et al., 2014). Taken together, these data show that TLX can be manipulated to positively regulate hippocampal neurogenesis and associated cognition. However, little has been done in assessing whether TLX can mitigate the negative effects of an external insult such as inflammation on hippocampal neurogenesis. In the current study, we show that overexpression of TLX rescued the negative effects of IL-1β on hippocampal neurosphere expansion. Thus, TLX may act as a regulator of IL-1β-induced deficits in NPC expansion but whether this effect translates *in vivo* remains to be determined. As we have demonstrated that NF-κB signalling may mediate the suppressive effects of IL-1β on TLX expression, the ability of TLX to counter the effects of IL-1β on neurosphere expansion may not be mediated by suppressing NF-κB signalling. This is due to the fact that both GFP and TLX overexpressing neurospheres both show a similar increase in IκBα expression following IL-1β treatment. As previously discussed, IL-1β can recruit multiple pathways to regulate NPC proliferation and so TLX may potentially interact with pathways other than NF-κB to mitigate the effects of IL-1β on neurosphere expression (Song et al., 2013, O'Leime et al., 2017a, Wang et al., 2007). Notwithstanding, our study demonstrates that TLX can prevent the

functional effects of IL-1β on neurosphere expansion and so future studies will involve identifying the specific signalling interactions between these two regulators of NPC proliferation. It should be noted, however, that overexpression of TLX can lead to hyperproliferation of NPCs and increased glioma formation *in vivo* (Liu et al., 2010, Park et al., 2010). However this may be overcome with the advent of pharmacological ligands for TLX. Indeed efforts have been made to identify ligands for this orphan nuclear receptor in order to target and manipulate TLX function (Benod et al., 2014b). Moreover, ligands may provide a controllable regulation of TLX function and thus avoid the potential side-effects of enhanced TLX expression.

In conclusion, the current study provides evidence that IL-1β-induced downregulation of TLX is mediated by the NF-κB pathway, and that enhanced TLX expression prevents IL-1β-induced reduction in NPC neurosphere expansion potentially via an NF-κB independent mechanism. We propose that the IL-1β-mediated suppression of TLX results in increased p21 expression which leads to reduced cell proliferation. Further research on the role of TLX in modulating neuroinflammatory-induced deficits in hippocampal neurogenesis will pave the way to the development of new therapeutic strategies for hippocampal-dependent cognitive disorders.

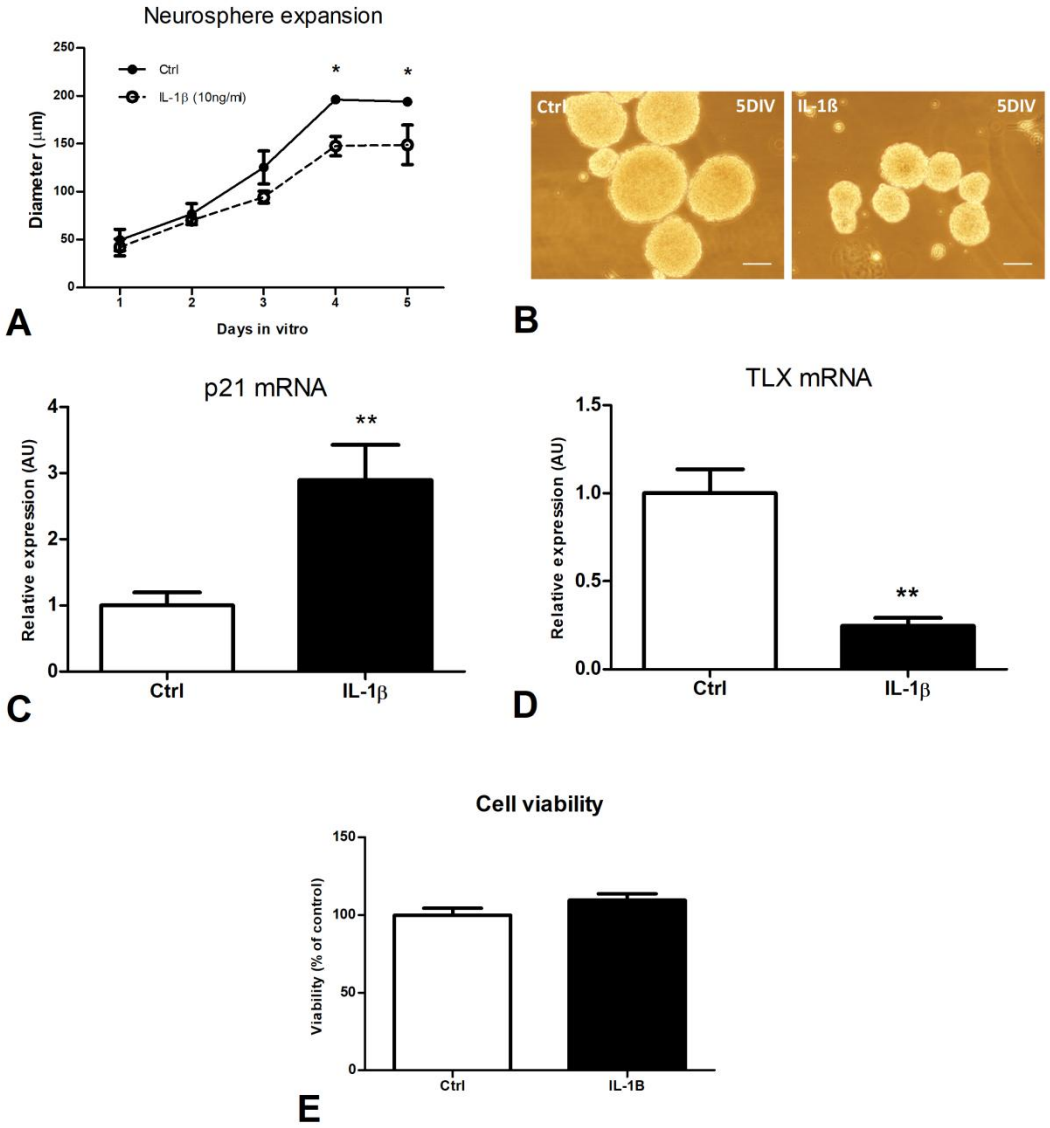


Figure 2.1: IL-1β-induced suppression of hippocampal neurosphere expansion is associated with increased p21 expression and reduced TLX expression. **A)** Neurosphere diameter from IL-1β-treated and untreated cultures (n=3) (Two-way ANOVA with Bonferroni post hoc). **B)** Representative images of control and IL-1β-treated neurospheres at 5DIV. Scale bar = 100μm. **C)** Relative mRNA expression of p21 in untreated and IL-1β-treated cultures after 5DIV under proliferative conditions (n=8) (Student's t-test). **D)** Relative mRNA expression of TLX in untreated and IL-1β-treated cultures after 5DIV under proliferative conditions (n=8). **E)** Cell viability of NPCs treated with IL-1β. Data are expressed as mean ± SEM *p<0.05, **p<0.01 (Student's t-test).

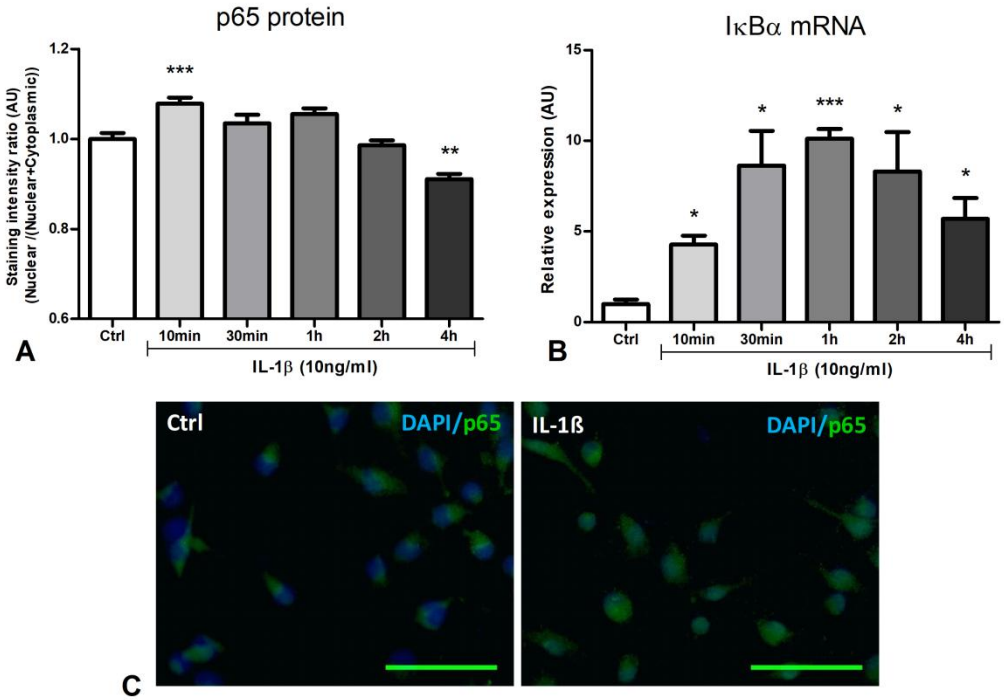


Figure 2.2: IL-1β induces rapid activation of NF-κB signalling in proliferating hippocampal NPCs. Ratio of nuclear:cytoplasmic p65 staining intensity (AU = Arbitrary Units) (n=3) (A) and relative mRNA expression of IκBα (B) in untreated and IL-1β-treated NPCs (n=3). **C)** Representative images of p65 staining. Scale bar = 5μm. Data are expressed as mean ± SEM *=$p<0.05$, **$p<0.01$, and ***$p<0.0001$ (ANOVA with Bonferroni post-hoc).

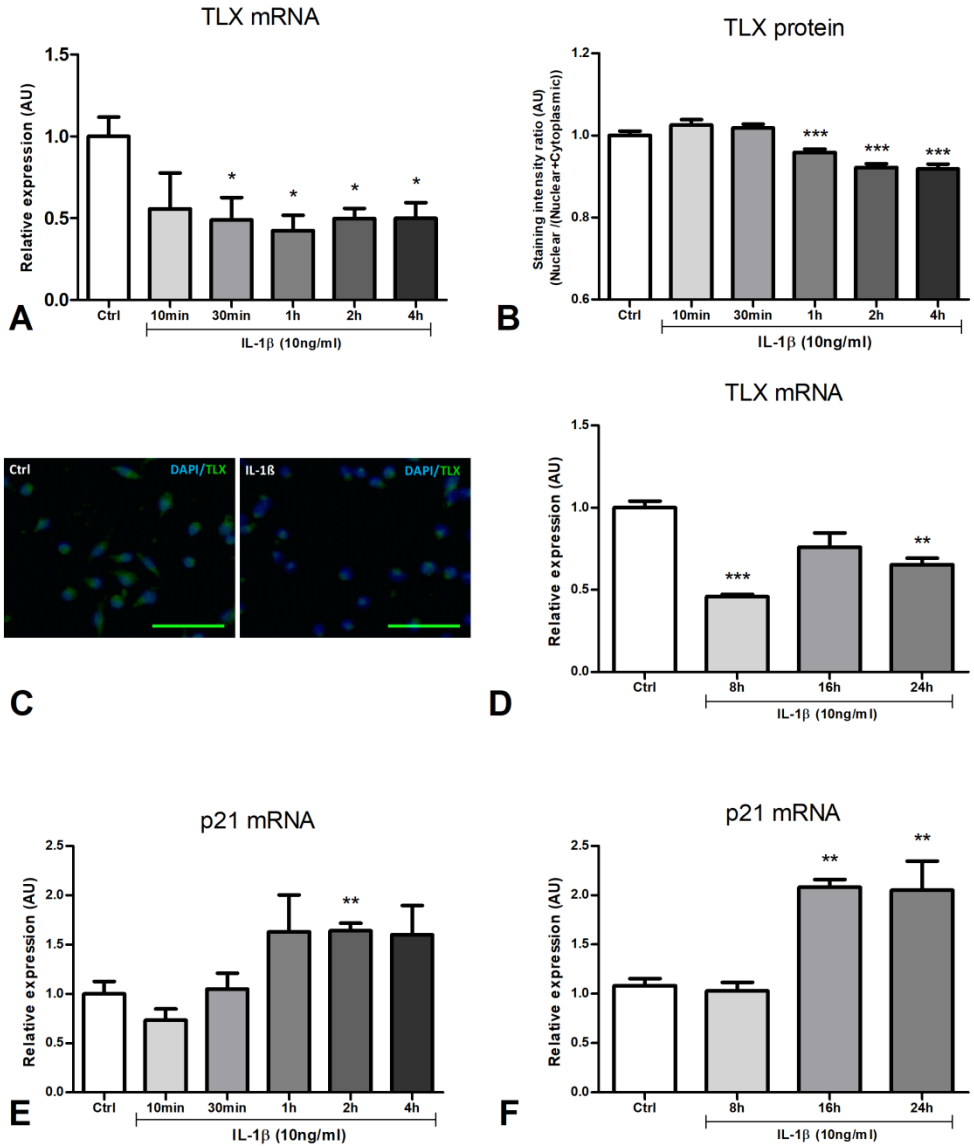


Figure 2.3: IL-1β suppresses TLX expression. Relative mRNA expression of TLX (n=3) **(A)** and ratio of nuclear:cytoplasmic TLX staining intensity (AU = Arbitrary Units) (n=3) **(B)** in untreated and IL-1β-treated NPCs at 10min, 30min, 1h, 2h and 4h post IL-1β treatment **C)** Representative images of TLX staining. Scale bar = 5µm. **D)** Relative mRNA expression of TLX in untreated and IL-1β-treated NPCs at 8h, 16h, and 24h post IL-1β treatment (n=3). **E)** Relative mRNA expression of p21 in untreated and IL-1β-treated NPCs at 10min, 30min, 1h, 2h and 4h post IL-1β treatment(n=3). **F)** Relative mRNA expression of p21 in untreated and IL-1β-treated NPCs at 8h, 16h and 24h post IL-1β treatment (n=3). Data are expressed as mean ± SEM where **p<0.01, and ***p<0.0001 compared to ctrl (ANOVA with Bonferroni post-hoc).

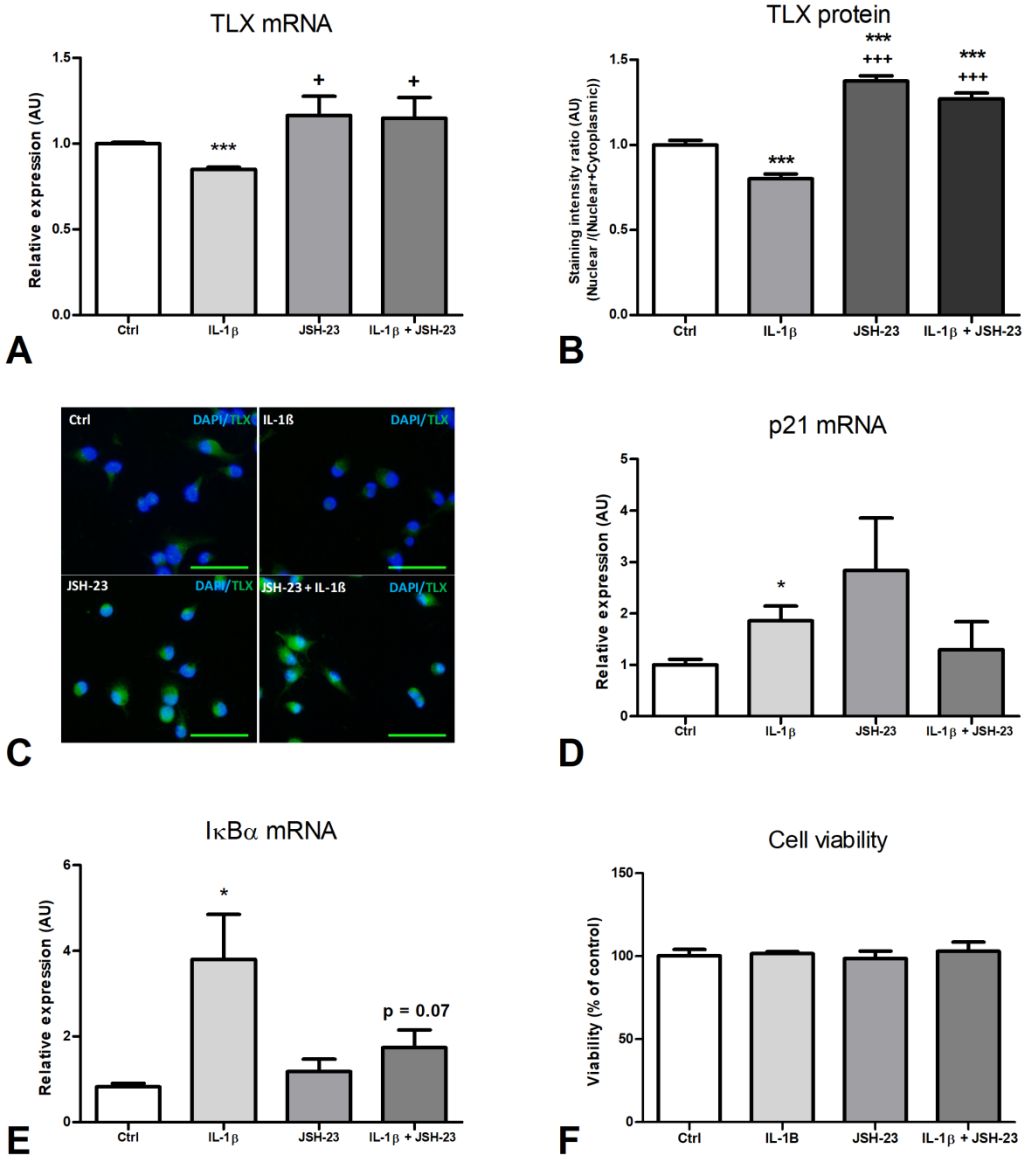


Figure 2.4: Inhibition of NF-κB signalling prevents IL-1β-induced decrease in TLX expression. Relative mRNA expression of TLX **(A)** and nuclear TLX protein expression **(B)** in untreated, IL-1β-treated, JSH-23 treated, and IL-1β+JSH-23 treated hippocampal NPCs. **C)** Representative images of TLX staining. Scale bar = 5μm. Relative mRNA expression of p21 **(D)** and IκBα **(E)** in untreated, IL-1β-treated, JSH-23 treated, and IL-1β+JSH-23 treated hippocampal NPCs. **F)** Cell viability after untreated, IL-1β-treated, JSH-23 treated, and IL-1β + JSH-23 treated hippocampal NPCs. Data are expressed as mean ± SEM where ***p<0.001 compared to vehicle and +++p<0.001 compared to IL-1β (ANOVA with Bonferroni post-hoc).

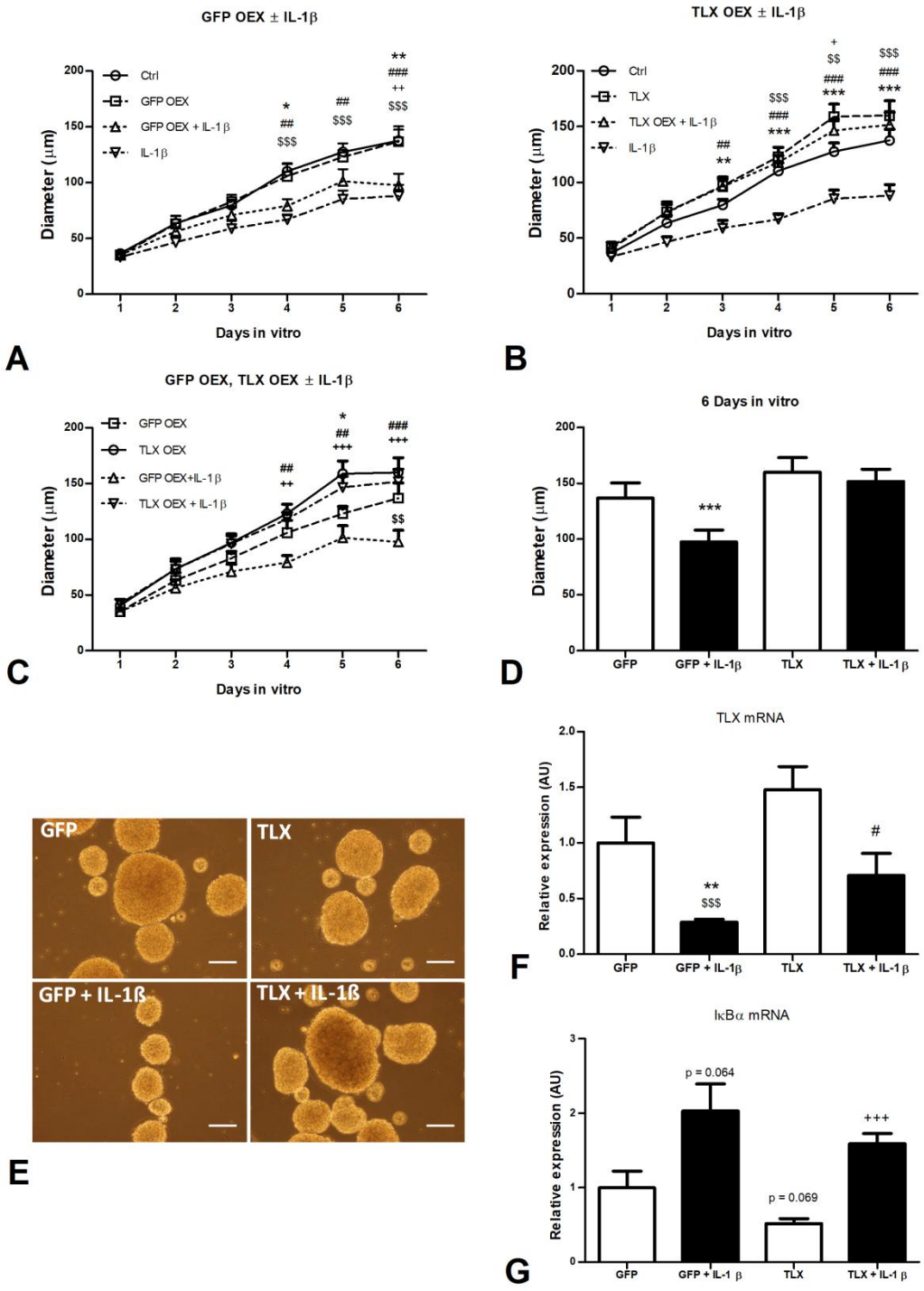


Figure 2.5: TLX overexpression (OEX) protects IL-1β-induced suppression of neurosphere growth. **A)** Effect of GFP OEX on IL-1β-induced changes in neurosphere growth. *p<0.05, **p<0.01, Ctrl compared to GFP+IL-1β. $$$p<0.001, Ctrl compared to IL-1β. ++p<0.01 GFP compared to GFP+IL-1β. ##p<0.01, ###p<0.001 GFP compared to IL-1β (Two way ANOVA with Bonferroni posthoc, n=7). **B)** Effect of TLX OEX on IL-1β-induced changes in neurosphere growth. $$p<0.01, $$$p<0.001, Ctrl compared to IL-1β. +p<0.05 Ctrl compared to TLX.

##p<0.01, ###p<0.001 TLX compared to IL-1β. **p<0.01, ***p<0.001 TLX+IL-1β compared to IL-1β (Two way ANOVA with Bonferroni posthoc, n=7). **C)** Comparison of GFP OEX and TLX OEX on IL-1β induced changes in neurosphere growth. *p<0.05 GFP compared to TLX. $$p<0.01 GFP compared to GFP+IL-1β. ++p<0.01, +++p<0.001 TLX compared to GFP+IL-1β. ##p<0.01, ###p<0.001 TLX+IL-1β compared to GFP+IL-1β (Two way ANOVA with Bonferroni posthoc, n=7). **D)** Comparison of GFP OEX and TLX OEX on IL-1β-induced changes in neurosphere growth at 6DIV, ***p<0.001 GFP compared to GFP+IL-1β (One way ANOVA with Bonferroni post hoc test, n=7). **E)** Representative images of GFP OEX and TLX OEX neurospheres, untreated or IL-1β-treated. Scale bar = 100μm. Relative mRNA expression of **(F)** TLX, **p<0.01 GFP compared to GFP+IL-1β. $$$p<0.001 TLX compared to GFP+IL-1β. # p<0.05 GFP+IL-1β compared to TLX+IL-1β (Two way ANOVA with Bonferroni posthoc, n=7), and **(G)** IκBα, +++P<0.001 TLX compared to TLX+IL-1β (Two way ANOVA with Bonferroni posthoc, n=7), after lentiviral treatment of neurospheres with and without IL-1β treatment.. Data are expressed as mean ± SEM.

Chapter 3

The orphan nuclear receptor TLX regulates hippocampal transcriptome changes induced by IL-1β

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Submitted to Brain, Behavior, and Immunity (Under revision)

Abstract

TLX is an orphan nuclear receptor highly expressed within neural progenitor cells (NPCs) in the hippocampus where is regulates proliferation. Inflammation has been shown to have negative effects on hippocampal function as well as on NPC proliferation. Specifically, the pro-inflammatory cytokine IL-1β has been shown to suppress NPC proliferation as well as TLX expression in the hippocampus. However, it is unknown whether TLX itself is involved in regulating the inflammatory response in the hippocampus. To explore the role of TLX in inflammation, we assessed changes in the transcriptional landscape of the hippocampus of TLX knockout mice ($TLX^{-/-}$) compared to wildtype (WT) littermate controls with and without intrahippocampal injection of IL-1β using a whole transcriptome RNA sequencing approach. We demonstrated that there is an increase in the transcription of genes involved in the promotion of inflammation and regulation of cell chemotaxis (*Tnf, Il1b, Cxcr1, Cxcr2, Tlr4*) and a decrease in the expression of genes relating to synaptic signalling (*Lypd1, Syt4, Cplx2*) in cannulated $TLX^{-/-}$ mice compared to WT controls. We demonstrate that mice lacking in TLX share a similar increase in 176 genes involved in regulating inflammation (e.g. *Cxcl1, Tnf, Il1b*) as WT mice injected with IL-1β into the hippocampus. Moreover, $TLX^{-/-}$ mice injected with IL-1β display a blunted transcriptional profile compared to WT mice injected with IL-1β. Thus, $TLX^{-/-}$ mice, which already have an exaggerated inflammatory profile after cannulation surgery, are primed to respond differently to an inflammatory stimulus such as IL-1β. Together, these results demonstrate that TLX regulates hippocampal inflammatory transcriptome response to brain injury (in this case cannulation surgery) and cytokine stimulation.

1: Introduction

The hippocampus is one of two regions of the adult brain that neural progenitor cells (NPCs) reside throughout the lifespan (Gage, 2000, Zhao et al., 2008). Specifically within the dentate gyrus (DG) of the hippocampus, these NPCs are core components of neurogenesis (i.e. the birth of new neurons) and this process is thought to contribute to hippocampal cognitive functions such as spatial memory (Kempermann et al., 2004b, Shors et al., 2002) as well as playing a role in the regulation of emotion (O'Leary and Cryan, 2014). For hippocampal neurogenesis to occur, NPCs must successfully progress from a proliferative state to fully mature integrated neurons (Deng et al., 2010). This progression is under strict regulation by a host of intrinsic and extrinsic factors (Suh et al., 2009). One of these regulators is the orphan nuclear receptor subfamily 2 group E member 1 (Nr2e1 or TLX) (Niu et al., 2011, Li et al., 2012, Shi et al., 2004, Zhang et al., 2008). TLX is required to maintain NPCs in a proliferative state and to prevent ectopic neural differentiation (Shi et al., 2004, Zhao et al., 2010). Its expression in the adult mouse brain is localised to the neurogenic niches and specifically within the NPCs of these niches (Monaghan et al., 1995). Mice lacking TLX display reduced hippocampal volume as well as impaired neurogenesis due to the fact that NPCs fail to proliferate (Shi et al., 2004). TLX deficient mice also have impaired long-term potentiation (LTP) in the DG and display deficits in hippocampal neurogenesis-associated spatial memory cognition (O'Leary et al., 2016b, Christie et al., 2006, Roy et al., 2002, O'Leary et al., 2016a). Although there are profound behavioural and cellular defects observed in mice lacking the TLX, to date it has not been determined how the whole hippocampal transciptome may be affected by the loss of this NPC-specific transcription factor. Moreover, whether intrinsic regulators of neurogenesis, such as TLX, can regulate

wider hippocampal transcriptional responses to negative stimuli such as inflammation is not fully known.

It has been established that hippocampal inflammation can induce deficits in hippocampal neurogenesis and associated cognitive function (O'Leime et al., 2017a, Kohman and Rhodes, 2013, Yirmiya and Goshen, 2011). Interestingly, it has been demonstrated that NPCs themselves can regulate the inflammatory environment when transplanted into a region of tissue damage (Pluchino et al., 2005, Martino and Pluchino, 2006). Interleukin-1β (IL-1β) is a pro-inflammatory cytokine whose receptor, IL-1 receptor type 1 (IL-1R1) is expressed in proportionately higher levels in the hippocampus compared to other brain regions (Parnet et al., 1994, Farrar et al., 1987). IL-1R1 is also expressed on NPCs in the hippocampus (Green et al., 2012; Ryan et al., 2013). IL-1β is predominantly responsible for the negative effects of neuroinflammation on hippocampal neurogenesis and hippocampal-related impairments due to its receptor expression profile (Ryan et al., 2013, Green and Nolan, 2012b, Kelly et al., 2003, Koo and Duman, 2008, Hein et al., 2010). It is noteworthy that IL-1β has been demonstrated to inhibit the expression of TLX in both embryonic and adult hippocampal NPCs (Green and Nolan, 2012b, Ryan et al., 2013). However, it is unknown whether TLX itself can regulate the IL-1β-induced inflammatory response within the hippocampus. To address this, we compared the transcriptome changes in the hippocampus of TLX knockout ($TLX^{-/-}$) mice to wild type (WT) mice following an inflammatory stimulus with IL-1β.

2: Methods

2.1: Animals

Postnatal day (P) 52 male $TLX^{-/-}$ and wildtype (WT) littermate control mice (on a cross BL6/129S1 background) were used in this study (n=4-8). Breeding pairs were kindly provided by Prof. Elizabeth Simpson, University of British Colombia and were generated as previously described (Wong et al., 2010). Briefly, male TLX heterozygous mice ($TLX^{-/+}$) on a 129S1 background were crossbred with female BL6 $TLX^{-/+}$ mice to generate TLX wildtype controls ($TLX^{+/+}$), $TLX^{-/+}$, and TLX knockout ($TLX^{-/-}$) pups with offspring genotypes followed Mendelian inheritance (See figure 3.1 for breeding summary and numbers of mice used per group). All pups were weaned at P21 and tailsnips were taken for genotype analysis which was carried out using an Extract N' Amp kit (Sigma-Aldrich) as per the manufacturer's instructions. After genotyping, the animals were single housed under standard housing conditions (temperature 21^{o}C and relative humidity 55%), with food and water available *ad libitum*. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

2.2: Experimental design

For RNA sequencing analysis, $TLX^{-/-}$ and WT mice were injected with either IL-1β or vehicle (phosphate buffered saline (PBS)) via cannulation, thus there were four experimental groups for the RNA sequencing section of this study (Figure 3.1, Table 3.1). Hippocampal tissue from an additional group of non-cannulated $TLX^{-/-}$ and WT

mice was taken for PCR analysis to assess the effect of cannulation on cytokine (IL-1β and TNFα) expression.

2.3: Stereotaxic surgery for hippocampal cannulation

At P52 (4 days prior to IL-1β or PBS microinjection), $TLX^{-/-}$ and WT mice were anaesthetised using a mixture of ketamine (0.25ml), xylazine (0.2ml) and sterile 0.9% NaCl (2.05ml) at a dose of 0.1mls/10g (i.p.) and placed in a Kopf stereotaxic frame. A guide cannula (*Plastics1*, Gauge: 22, Pedestal Length: 4mm, Projection: 1.5mm) was implanted unilaterally into the dorsal hippocampus at the following co-ordinates: AP -1.7, ML -1.2 and DV -1.5 relative to bregma. Animals were randomly implanted on the left or right hemisphere. The cannula was secured in place using superglue as a base layer followed by dental cement. After the dental cement had hardened sufficiently, animals were administered Carprofen (0.1ml/30g, i.p.) and 0.5ml of 5% Glucose (5g/100ml; i.p.), and returned to their home cage.

2.4: Intrahippocampal microinjections

At P56 mice were unilaterally injected into the hippocampus with 1µl of either recombinant mouse IL-1β (10ng/µl; R&D systems) or PBS (filtered using a 0.2µm sterile filter) and infused at a rate of 0.5ul/min using an automated 'Pico Plus' microinjector (Harvard Apparatus, Kent, UK). The injection cannula projected a further 0.5mm past the guide cannula to give a total depth of 2.0mm and was left in place for an additional 2 min for diffusion before the needle was withdrawn. Mice were sacrificed by decapitation one hour after injection. This timepoint was chosen as we have previously demonstrated that IL-1β can induce a significant reduction in TLX gene expression in hippocampal NPCs at this timepoint (O'Leime et al.,

2017b). Moreover, we aimed to assess the immediate gene expression changes induced by IL-1β rather than secondary gene expression changes induced by the release of other regulators of gene expression as a result of IL-1β injection. The injection needle was attached to the guide cannula and mice were allowed to move freely during the injection. After one hour, left or right hippocampi were removed, flash frozen on dry ice and stored at -80°C.

2.5: Hippocampal RNA Extraction

Total RNA was extracted and DNase treated from hippocampal tissue from both WT and $TLX^{-/-}$ animals using the mirVana^{TM} total RNA extraction kit (Ambion/Life Technologies, Dublin, Ireland) and Turbo DNA-free kit (Ambion/life technologies) as per the manufacturer's instructions. The total concentration of extracted RNA was quantified using a Nanodrop 2000 (Thermo Scientific, UK) and was stored at -80°C until sent for sequencing.

2.6: mRNA sequencing

Equal volumes of total hippocampal RNA from each animal (no pooling of samples was conducted) was sent for sequencing by Exiqon (Vedbaek, Denmark) and conducted on an Illumina NextSeq500 sequencer with an average of 30 million reads with a 50 basepair paired-end read length. Annotation of the obtained sequences was performed using the reference genome annotation: Mus musculus (organism), GRCm38 (reference genome), Ensembl_70 (annotation reference).

2.7: Differential gene expression and functional enrichment analysis

Data analysis was conducted by Exiqon using XploreRNA automated analysis software. The data analysis pipeline used in this software is based on the Tuxedo software package which is a combination of open-source software and makes use of peer-reviewed statistical methods. Additionally, Exiqon employs specialised software developed at Exiqon to interpret and enhance the readability of the finalised results. The components of the data analysis pipeline used at Exiqon for RNA sequencing include Bowtie2 (v. 2.2.2.), Tophat (v. 2.0.11.), and Cufflinks (v. 2.2.1.). Briefly, Bowtie2 is a sequence aligner used by Tophat to align the sequencing reads to the reference genome (GRCm38, UCSC Genome brower and Ensembl_70 (annotation reference)). Cufflinks uses the alignment results from Tophat and assembles the aligned sequences into transcripts to construct a map of the transcriptome. Cufflinks assembles the aligned reads into transcript isoforms based on exon usage and also determines transcriptional start sites. Additionally, Exiqon performs fragment bias correction which corrects for sequence bias during library preparation. Cuffdiff is used when comparing groups to calculate the number of fragments per kilobase of transcript per million mapped fragments (FPKM) and to determine differential gene expression and regulation based on the assembled transcripts from the submitted samples using the Cufflinks output. An adjusted p-value of ≤ 0.05 was considered significantly differentially regulated. Differentially regulated genes were analysed for enrichment of Gene Ontology (GO) terms and KEGG pathways using the DAVID Bioinformatics Resources (v6.8) (Huang da et al., 2009).

2.8 Quantitative real-time PCR (qRT-PCR)

Validation of RNA sequencing was performed using specific PrimerTime® qPCT assays sourced from IDT (Integrated DNA Technologies) (Table 2). cDNA was reversed transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the SureCycler® 8800 (Agilent Technologies) and diluted to a final concentration of 10ng/μl. All qRT-PCR was performed in 3 technical replicates for each biological sample on a LightCycler® 480 Instrument II (Roche). Quantification of gene expression was analysed using the $\Delta\Delta$ C_t method (Livak and Schmittgen, 2001). RNA sequencing data was validated for four genes that contributed to the enrichment for TNF signalling in our KEGG pathway analysis using the following primers from Integrated DNA Technologies (IDT): SDHA (Mm.Pt.56a.12170577), IL-1β (Mm.PT.58.41616450), IL-6 (Mm.Pt.58.10005566), TNF (Mm.PT.58.12575861).

2.9 STRING analysis

The STRING database, which stems from computational predictions was used to investigate direct (physical) and indirect (functional) associations between differentially regulated genes. An interaction network was created using a list of differentially regulated genes between WT and $TLX^{-/-}$ and genes that were commonly up-regulated in WT vs. $TLX^{-/-}$ and WT vs. WT + IL1B (172 genes). The string network was visualized using the following parameters: excluded any genes that there was no evidence of association of connectivity, minimum required interaction score was set at high confidence (0.007), and meaning of network edges was set to line thickness indicating strength of data support. KEGG pathway analysis was also conducted in STRING which indicated among the 172 up-regulated genes

that TNF signalling pathway was the most significantly enriched pathway $p<0.001$ (red nodes).

2.10 Statistics

An adjusted p value (q value, Benjamini-Hochberg method) of ≤ 0.05 for RNA sequencing data was considered to indicate significantly differentially regulated gene expression. For Gene Ontology (GO) and KEGG analysis a cut off of 0.05 FDR p value was used to determine significance. For RT-PCR validations, one-way ANOVA followed by Tukey's *post-hoc* analysis or student's *t*-test was used where appropriate to determine statistical significance which was established at $p \leq 0.05$.

3: Results

3.1: TLX regulates inflammatory gene expression in the hippocampus of mice.

By performing pairwise analysis of whole transcriptome RNA sequencing data, we compared differentially expressed genes in the hippocampus between vehicle injected cannulated WT and $TLX^{-/-}$ mice. We identified a large number of differentially expressed genes in the hippocampus of $TLX^{-/-}$ mice compared to WT controls (1542 genes) (Figure 3.2A, B). Of these 1542 genes, the majority were up-regulated (1272 genes) with a smaller subset down-regulated (274 genes) in $TLX^{-/-}$ mice (Figure 3.2A, B, C). Next we assessed the biological functions of these up- and down-regulated genes in $TLX^{-/-}$ mice. We observed a significant enrichment in GO terms such as regulation of inflammatory response, cytokine production, and cellular responses to cytokine stimulus (e.g. *Tnf, Il1b, IL6, Tlr2, Nfkbia* and *Tlr4)* (Figure 3.2D). GO analysis on the list of down-regulated genes observed in $TLX^{-/-}$ mice revealed an enrichment in genes involved in synpatic signalling such as *Syt4*, *Syt17*, and *Lypd1* compared to WT control (Figure 2.2E).

3.2: $TLX^{-/-}$ mice display similar transcriptional profile to that of WT mice injected intrahippocampally with IL-1β.

We found a large number of genes to be similarly up-regulated in $TLX^{-/-}$ and WT + IL-1β mice compared to WT mice (Figure 3.3A, B). Of the 1272 genes whose expression increased in $TLX^{-/-}$ mice compared to WT mice, 176 or 13.8% these genes are also increased in WT + IL-1β. We assessed the biological function of these genes and and observed an orcastrated enrichment in GO terms for cellular response to cytokines (e.g. *Cxcl1*, *Il1b*, *Tnf, Ccl2*), regulation of inflammatory response (e.g. *Tlr2, Ccl4, Ccl7, Ier3*), and apoptotic processes (e.g. *Ier3, Cyr61, LgalsS3, Perp*)

(Figure 3.3C, E). KEGG pathway analysis revealed a significant enrichment for genes involved in inflammatory pathway signalling such as TNF signalling (*Tnf, Fos, Jun, Il1b*), cytokine-receptor interaction (*Csf3, Il6, Ccl2, Il1b, Il1a*), NF-κB signalling (*Nfkbia, Lbp, Ptgs2*), and TLR signalling (*Tlr2, Il1b, Nfkbia, Lbp*) (Figure 3.3D, E). This indicated that in $TLX^{-/-}$ mice, there is a similar dyresgulation of inflammatory gene expression to that induced by IL-1β within the hippocampus of WT mice. Additionally, KEGG pathway analysis using two web based algorythims (DAVID and STRING) indicated that TNF signalling was the most significant and prominent pathway to be enriched in this subset of differentially up-regulated genes (172) (Figure 3.4A, B).

3.3: Cannulated $TLX^{-/-}$ mice have a significantly different cytokine response to IL-1β

WT + IL-1β mice show a down-regulation in 7 genes and an up-regulation in 221 genes within the hippocampus compared to cannulated WT mice (Figure 3.5A, B). $TLX^{-/-}$ + IL-1β mice display a much reduced transcriptional response as they display a differential transcriptional response in 38 genes and only 5 of those were up-regulated compared to $TLX^{-/-}$ mice (Figure 3.5A, B). GO enrichment analysis only showed significant enrichment among down-regulated genes (33 genes) between $TLX^{-/-}$ and $TLX^{-/-}$ + IL-1β groups of mice (Figure 3.5A). Specifically, we observed enrichment in GO terms such as cell (including leukocytes and neutrophils) chemotaxis, (*Cxcl13, Ccr1, Trem1*), defence to bacterium (*Lyz2, Lyz1*), response to LPS (*Lcn2, Cxcl13*), and acute inflammatory response (*S100a8, Saa3*) (Figure 3.5C).

3.4: TLX potentially regulates TNF signalling indirectly via two separate gene interactions and cannulation induces a significant increase in IL-1β and TNFα expression in TLX$^{-/-}$ mice

As we identified TNF signalling as a possible mechanism through which TLX regulates inflammation, we again used STRING analysis to identify the link between TLX (*Nr2e1*) and TNF signalling. We identified *Top2a* and *Bcl6b* as two genes from all differentially regulated genes (1542 genes) in TLX$^{-/-}$ mice compared to WT mice that may interact with TLX and potentially mediate the regulatory effects of TLX on TNF signalling (Figure 3.6A). We confirmed our sequencing results using qRT-PCR that TNFα and IL-1β, which have been shown from our KEGG analysis to be highly involved in regulating TNF signalling, have elevated expression levels in cannulated TLX$^{-/-}$ mice (Figure 3.6B, C). We also assessed the expression of these genes in non-cannnulated mice. Non-cannulated TLX$^{-/-}$ mice have similar hippocampal expression of IL-1β and TNFα to that of non-cannulated WT mice (Figure 3.6B, C). However, after hippocampal cannulation surgery, there is a significant increase in IL-1β ($F(3,24)=12.34$, ***=$P<0.001$) and TNFα ($F(3,24)=10.12$, ***=$P<0.001$) expression in TLX$^{-/-}$ mice only (Figure 3.6B, C). There is a non-significant trend towards an increase in these cytokines in WT mice after hippocampal cannulation surgery.

Discussion

In the present study, we demonstrate that TLX acts to regulate the transcription of genes involved in maintaining a normal inflammatory profile after cannulation surgery and in response to an IL-1β stimulus in the hippocampus of mice. Firstly, we demonstrated an orchestrated up-regulation in genes involved in inflammatory processes in the hippocampus of $TLX^{-/-}$ mice when compared to WT mice. WT mice injected with IL-1β into the hippocampus display a significant up-regulation in genes involved in cellular responses to cytokines and inflammatory signalling pathways, and these genes overlap with the genes increased in $TLX^{-/-}$ mice. We showed that $TLX^{-/-}$ mice have a blunted transcriptional response to IL-1β after cannulation surgery compared to cannulated WT mice and we highlight that TLX may act to regulate inflammatory responses via TNF signalling. Finally, we outline that there was no difference in the expression levels of IL-1β and TNFα between $TLX^{-/-}$ and WT naïve mice but that these cytokines were significantly increased in $TLX^{-/-}$ mice after surgery. Taken together, these data indicate that the absence of TLX results in disruption of the hippocampal inflammatory transcriptome response.

There was a large dysregulation in the transcriptional landscape of the hippocampus of $TLX^{-/-}$ mice evident with 1542 genes differentially expressed compared to WT mice. The majority of these genes (1272 in total) were up-regulated, which is in keeping with the current literature describing TLX as a transcriptional repressor (Sun et al., 2007, Islam and Zhang, 2014). Similarly, whole transcriptome analysis of another strain of TLX knockout mice revealed a large number of differentially regulated genes (1721) in NPCs from the subventricular zone of the lateral ventricles (Niu et al., 2011). Most likely these genes are not all targets of TLX but rather are

up-regulated as a knock-on effect due to a lack of regulation of TLX target genes. These genes showed enrichment for GO terms such as the regulation of inflammatory processes and included genes such as *Tnf*, *Tlr2*, *Tlr4*, and *Il1b*. We have recently demonstrated that there is a significant increase in microglial cell density and activation (increased somal area) in the DG of $TLX^{-/-}$ mice compared to WT counterparts under baseline conditions, while there is no change in the number of apoptotic cells in the DG of these mice (Kozareva et al., 2017a, Kozareva et al., 2017b). Thus, there is evidence to suggest that TLX is intrinsically linked with inflammatory processes in the hippocampus as the absence of TLX leads to increased microglial activation at baseline and a dysregulated inflammatory gene expression profile.

As proliferating NPCs are the predominant source of TLX within the hippocampus (Shi et al., 2004), it could be suggested that NPCs may function to normalise inflammatory gene expression. Moreover, it has been demonstrated that TLX is not expressed, or expressed at a very low level within cerebral cortex microglia (EMBL gene expression atlas (http://www.ebi.ac.uk/gxa/genes)), and thus it is likely that the regulatory role of TLX on inflammation within the hippocampus is mediated by its functions within NPCs (Zhang et al., 2014, Li et al., 2012). To this end, data from transplantation studies have demonstrated that NPCs themselves have indirect beneficial effects on the surrounding tissue by modulating the inflammatory environment (Pluchino et al., 2005, Martino and Pluchino, 2006).

Among the genes that were down-regulated in $TLX^{-/-}$ mice compared to WT mice, a significant enrichment for genes involved in synaptic signalling such as *Syt4* and

Syt17, was observed. This supports a previous report, also using mice with a spontaneous deletion of TLX, which demonstrates an impairment in LTP formation in the DG in $TLX^{-/-}$ mice compared to WT mice (Christie et al., 2006). Interestingly, that study demonstrated that LTP was specifically impaired in $TLX^{-/-}$ mice in the DG and not the CA1 region of the hippocampus. As the DG is the primary area where NPCs are found, and TLX is primarily expressed in proliferating NPCs, it can be suggested that impairments in synaptic plasticity resulting from TLX deletion occurs as a results of its disfunction or absence in NPCs. The dysregulation in synaptic gene expression and impairment in LTP may also underlie the hippocampal-dependent behavioural deficits that we and others have previously observed in these $TLX^{-/-}$ mice (O'Leary et al., 2016a, Christie et al., 2006, Young et al., 2002, O'Leary et al., 2016b).

Apart from genes involved in the regulation of inflammation, there are a large number of genes involved in other processes such as intracellular signal transduction, cell chemotaxis, and organ development that are differentially expressed in $TLX^{-/-}$ mice. These mice have a spontaneous deletion and are thus devoid of TLX from embryonic development through to postnatal development and adulthood (Young et al., 2002). Therefore, the wide ranging biological processes dysregulated in these mice are possibly due to the absence of TLX during critical moments of various organ development rather than a specific lack of TLX in adulthood (Monaghan et al., 1997, Roy et al., 2002). Further studies should assess the inflammatory profile of mice with a conditional knockout of TLX in adulthood to address the role that loss of TLX during embryonic development has on the development of the immune system and its function in adulthood.

As we observed a significant dysregulation in inflammatory gene expression in $TLX^{-/-}$ mice and because we have previously reported enhanced microglial activation in the DG of $TLX^{-/-}$ mice, we aimed to assess whether an inflammatory cytokine stimulus could induce similar transcriptome changes in WT mice. The pro-inflammatory cytokine IL-1β, whose receptor is expressed on NPCs in the hippocampus (Green et al., 2012), has previously been shown to downregulate TLX expression in NPCs *in vitro* (Green and Nolan, 2012b, Ryan et al., 2013, Farrar et al., 1987). In response to hippocampal IL-1β injection in WT mice, we observed a significant increase in inflammation and chemotaxis related genes such as *Cxcl1, Il1b, Tnf,* and *Tlr2*. This is supported by reports that increased cytokine expression or administration of exogenous cytokines to the hippocampus can induce further endogenous cytokine expression (Anisman et al., 2008, Moore et al., 2009, Shaftel et al., 2007, Skelly et al., 2013, Balschun et al., 2004, del Rey et al., 2013). We did not observe a significant decrease in TLX expression, However, this may be due to the fact that TLX is only expressed in a subset of cells within the hippocampus and as we extracted RNA from the whole hippocampus, any changes in specific TLX-expressing cells may be not be detected.

With regard to the similarity in transcriptome changes in $TLX^{-/-}$ mice and IL-1β-injected WT mice compared to WT mice, there were 176 genes (of the 1272 genes up-regulated in $TLX^{-/-}$ mice) also up-regulated in WT mice in response to IL-1β. It should be noted that RNA sequencing analysis only highlighted similarly upregulated genes however, and did not compare the magnitude of gene expression change between the two groups. Notwithstanding, these data suggest that the absence

of TLX expression leads to an inflammatory state in the hippocampus similar to that induced by IL-1β in WT mice. KEGG pathway analysis of the overlaping genes shared between $TLX^{-/-}$ and WT mice injected with IL-1β compared to WT mice reveal that TNF signalling is a common pathway. This then suggests that TLX may act to repress TNF signalling, which in turn is increased by knocking down TLX expression or by increasing IL-1β expression. It has been established that IL-1β can induce the expression of other cytokines such as TNFα, and that TNFα can itself suppress proliferation of TLX expressing NPCs and subsequent neuronal differentiation (Monje et al., 2003, Ben-Hur et al., 2003, Keohane et al., 2010). Likewise, TNFα can induce the expression of other cytokines such as IL-6 and IL-10 (del Rey et al., 2013, Skelly et al., 2013). Therefore, for future studies on the mechanism of the regulatory role of TLX in inflammation, it will be important to assess the effects of TLX on various cytokines and their respective signalling pathways. From the current study it appears that TLX maintains normal inflammatory status in the hippocampus and that the TNF signalling pathway may be a major pathway through which TLX carries out this function.

We conducted STRING analysis on the up-regulated genes in $TLX^{-/-}$ compared to WT mice enriched for TNF signalling. This analysis predicts potential protein-protein interactions based on evidence from the literature and from online protein databases. The thickness of the interconnecting lines depicted in Figure 3.6 is indicative of the amount of evidence supporting the interaction between any two proteins. We observed that TLX does not appear to directly interact with TNF protein. Rather, TLX may interact with TNF signalling proteins via its potential interactions with Top2a and Bcl6b proteins. Both of these genes are up-regulated in

our sequencing data suggesting that they may be repressed by TLX signalling. However, the evidence for an interaction between TLX and Top2a and Bcl6b proteins is not strong, as indicated by the thickness of the connecting lines in Figure 3.6A. Specifically, STRING analysis highlighted these two proteins due to the fact that structural homologs of the proteins (TLX, Top2a, and Bcl6b) have been shown to interact in human tissue and there is greater evidence for the interactions between protein homologs of TLX and Bcl6b (Ku et al., 2009, Diner et al., 2015). Nuclear transrepression is also another potential mechanism by which TLX regulates inflammation (Glass and Saijo, 2010). Nuclear transrepression involves the 'tethering' of nuclear receptor to other signalling factors in order to prevent their signalling. This is a common mechanism through which glucocorticoid receptors and PPARs act to suppress inflammatory-related NF-κB signalling (Glass and Ogawa, 2006, Glass and Saijo, 2010, De Bosscher et al., 2003, Delerive et al., 1999). It may be possible that TLX regulates inflammation via transrepression of TNF signalling or other inflammation-related pathways. However, to date no such transrepression mechanism has been described for TLX and any component of TNF signalling.

Somewhat surprisingly, we observed a drastically different alteration in the transcriptional landscape of $TLX^{-/-}$ mice in response to IL-1β compared to WT mice injected with IL-1β. $TLX^{-/-}$ mice have a blunted response with only 38 differentially expressed genes compared to 228 genes differentially regulated in the WT mice after IL-1β injection. Moreover, enrichment could only be achieved for the down-regulated genes in the $TLX^{-/-}$ mice in response to IL-1β, and this revealed an enrichment in GO terms such as cell chemotaxis (*Cxcl13* and *Ccr1*). These genes are important for NPC-neuron-microglia communication (Cartier et al., 2005, Li,

2013). A possible explaination for this blunted response is that the $TLX^{-/-}$ mice already have an elevated inflammatory response to the cannulation surgery. Specifically, we observed a significant increase in the expression of IL-1β and TNFα in $TLX^{-/-}$ mice only after surgery. Thus, it could be suggested that these mice have reached an inflammatory plateau after cannulation surgery and are resistant to further cytokine stimulus. Additionally, these data indicate that $TLX^{-/-}$ mice may be more sensitive to brain injury compared to WT mice. Despite the fact that we did not see any differences in IL-1β or TNFα expression levels between $TLX^{-/-}$ and WT mice at baseline, we have previously reported increased microglial activation in $TLX^{-/-}$ mice which indicates a heightened inflammatory state in these mice at baseline (Kozareva et al., 2017a). It should also be noted that IL-1R1 is expressed on endothelial cells throughout the brain (Matsuwaki et al., 2014). These cells also express genes involved in the TNF signalling pathway and are thus critical in the regulation of neuroinflammation (Liu et al., 2015). Thus mechanistically, it is possible that the absence of TLX can disrupt both NPC-endothelial and NPC-microglia communication which subsequently results in blunted responses to stimulation by inflammatory cytokines as well as an enhanced inflammatory response to cannulation surgery.

Disruption of TLX as a result of spontaneous deletion results in abnormalities in motor, cognitive and anxiety-related behaviours. The most striking behavioural phenotype is increased aggression (Young et al., 2002; O'Leary et al., 2016 review). The $serotonin_{2A/C}$ receptor has been shown to mediate the aggressive phenotype of $TLX^{-/-}$ mice (Juarez et al., 2013) and interestingly, from our GO enrichment of genes for biological processes we observed an increase in expression in genes associated

with serotonin transport and release (*Cd300a, Syk, Fcerig, Lgals3* and *Fcgr3*) in $TLX^{-/-}$ compared to WT mice. Hyperactivity has also been consistently observed in $TLX^{-/-}$ mice, and we have recently reported a progressive decline in motor performance on the accelerating rotarod (O'Leary et al., 2016) which implicate corticostriatal pathways. With regard to hippocampal-associated behaviours, $TLX^{-/-}$ mice display poor performance in working memory as assessed by spontaneous alternations in the Y-maze, as well as and contextual fear conditioning (O'Leary et al., 2016a). Extensive evidence in the literature shows that hippocampal adminstration of IL-1β results in impairments in hippocampal-associated spatial learning and memory tasks and in LTP (Moore et al., 2009, Kohman and Rhodes, 2013, Vereker et al., 2000). Although we did not assess behavioural outcomes of IL-1β administration to $TLX^{-/-}$ mice in the current study, it is possible that IL-1β may not exacerbate the already robust deficits in cognitive behaviours due to the significant dysregulated and indeed pro-inflammatory transcriptional profile of the TLX-/- mice in this study.

In conclusion, this study provides valuable insights into the role of TLX as a regulator of inflammation and lays the ground work for future studies assessing the effects of TLX on inflammatory and cognitive processes. Moreover, as TLX is a nuclear receptor and thus has the potential to be targeted therapeutically (Benod et al., 2014b), future studies should aim to identify selective ligands for TLX and determine the ability of modulating TLX activity to mitigate the effects of extensive inflammation on hippocampal-associated cognition.

Experimental group	Abbreviation	n	Pairwise comparison
Wild type + PBS	WT	6	WT vs. WT + IL-1β
TLX knockout + PBS	$TLX^{-/-}$	4	WT vs. $TLX^{-/-}$
Wild type + IL-1β	WT + IL-1β	7	WT + IL-1β vs. $TLX^{-/-}$ + IL-1β
TLX knockout + IL-1β	$TLX^{-/-}$ + IL-1β	6	$TLX^{-/-}$ + IL-1β vs. $TLX^{-/-}$

Table 3.1: Meaningful pairwise comparisons for gene expression changes conducted using RNA sequencing (4 unique comparisons).

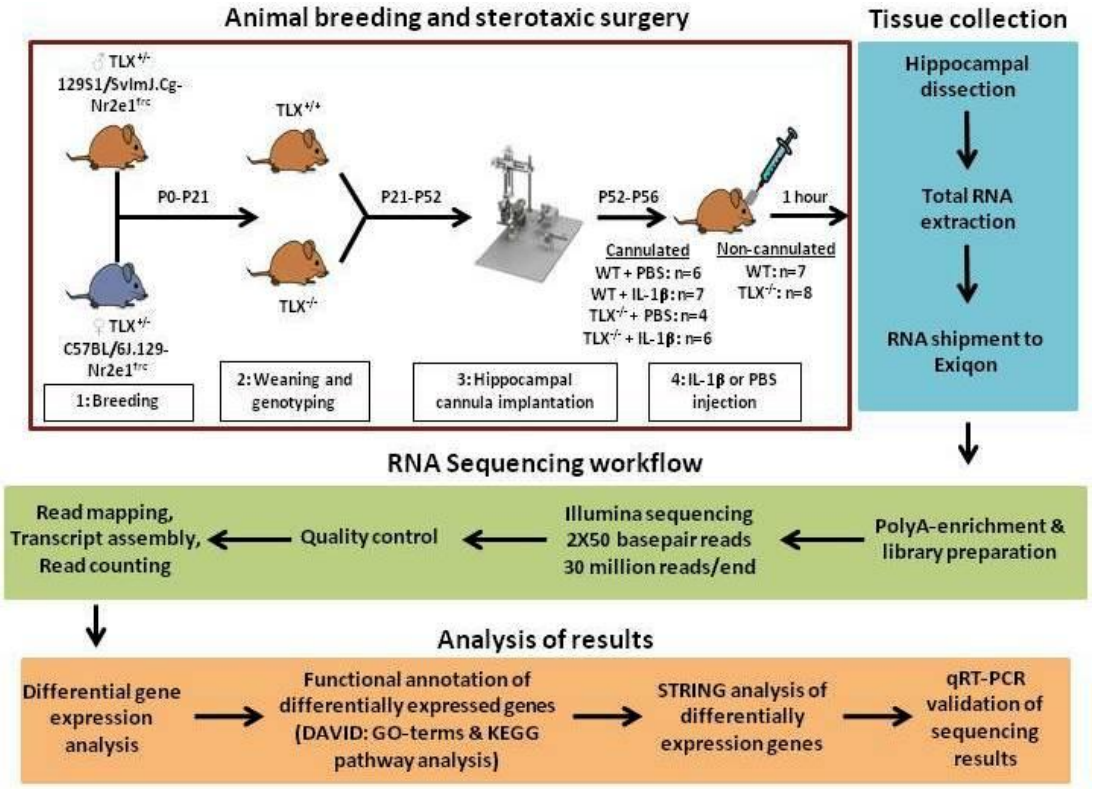


Figure 3.1: Schematic of experimental design and timeline. WT, wildtype; $TLX^{-/-}$, TLX knockout; PBS, Phosphate buffered saline; IL-1β, Interleukin-1 beta; WT + PBS, Wildtype PBS injected mice; WT + IL-1β, Wildtype IL-1β injected mice; $TLX^{-/-}$ + PBS, TLX knockout PBS injected mice; $TLX^{-/-}$ + IL-1β, TLX knockout IL-1β injected mice.

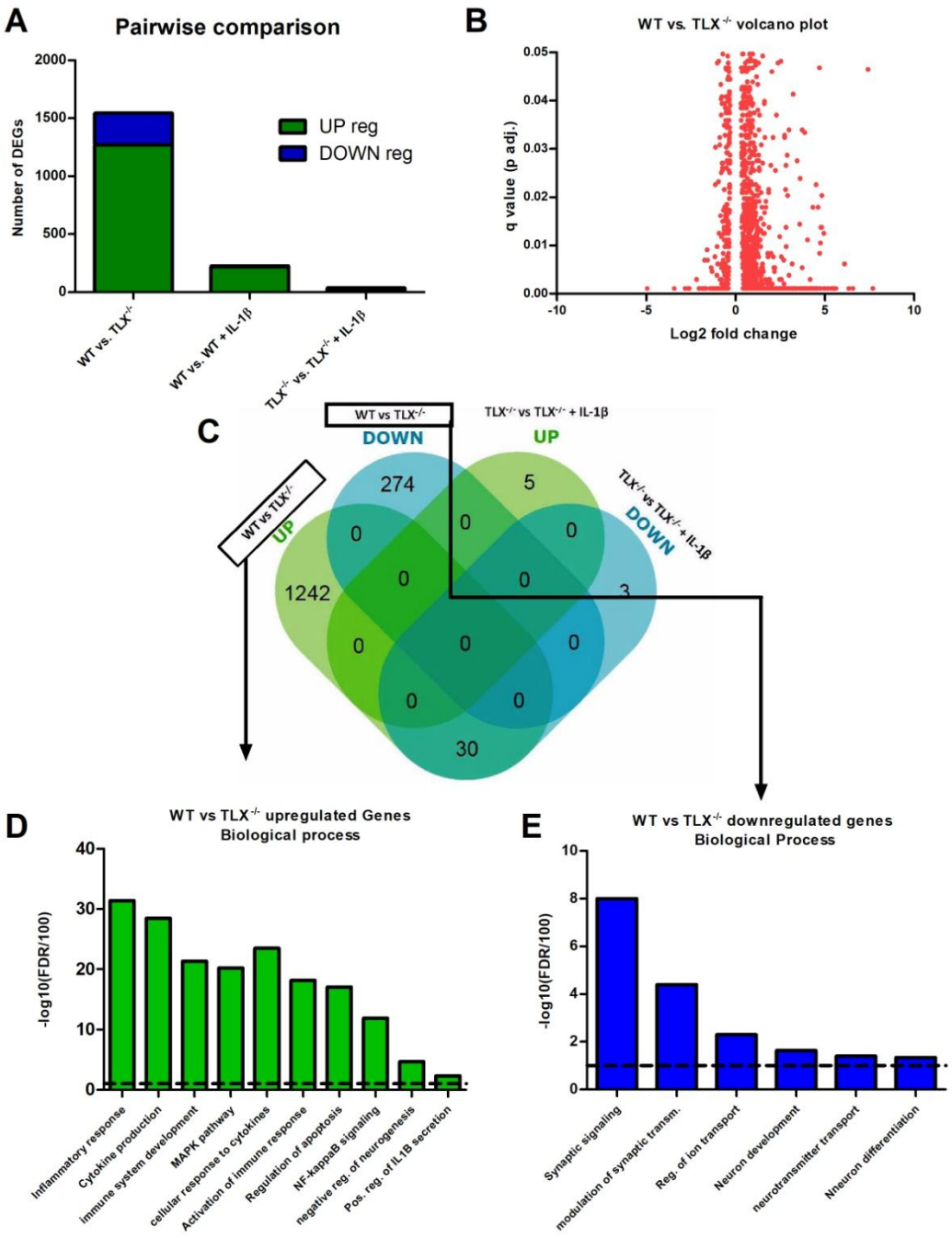


Figure 3.2: TLX$^{-/-}$ mice display elevated transcription of inflammatory genes in the hippocampus compared to WT controls. **A)** The number of differentially expressed genes (DEGs) between all pairwise comparisons across the groups. **B)** A volcano plot depicting the number of differentially regulated genes between WT and TLX$^{-/-}$ mice. Each gene is graphed as the fold change (log 2) versus q value (p adjusted value) for multiple comparisons. **C)** Venn diagram outlining the number of DEGs either up-regulated (green circles) or down-regulated (blue circles) across all groups. **D)** GO enrichment in biological processes for up-regulated genes comparing TLX$^{-/-}$

to WT mice. Dotted line represents significance level, with values above this line deemed significant. **E)** GO enrichment in biological processes for down-regulated genes comparing $TLX^{-/-}$ to WT mice. Dotted line represents significance level, with values above this line deemed significant.

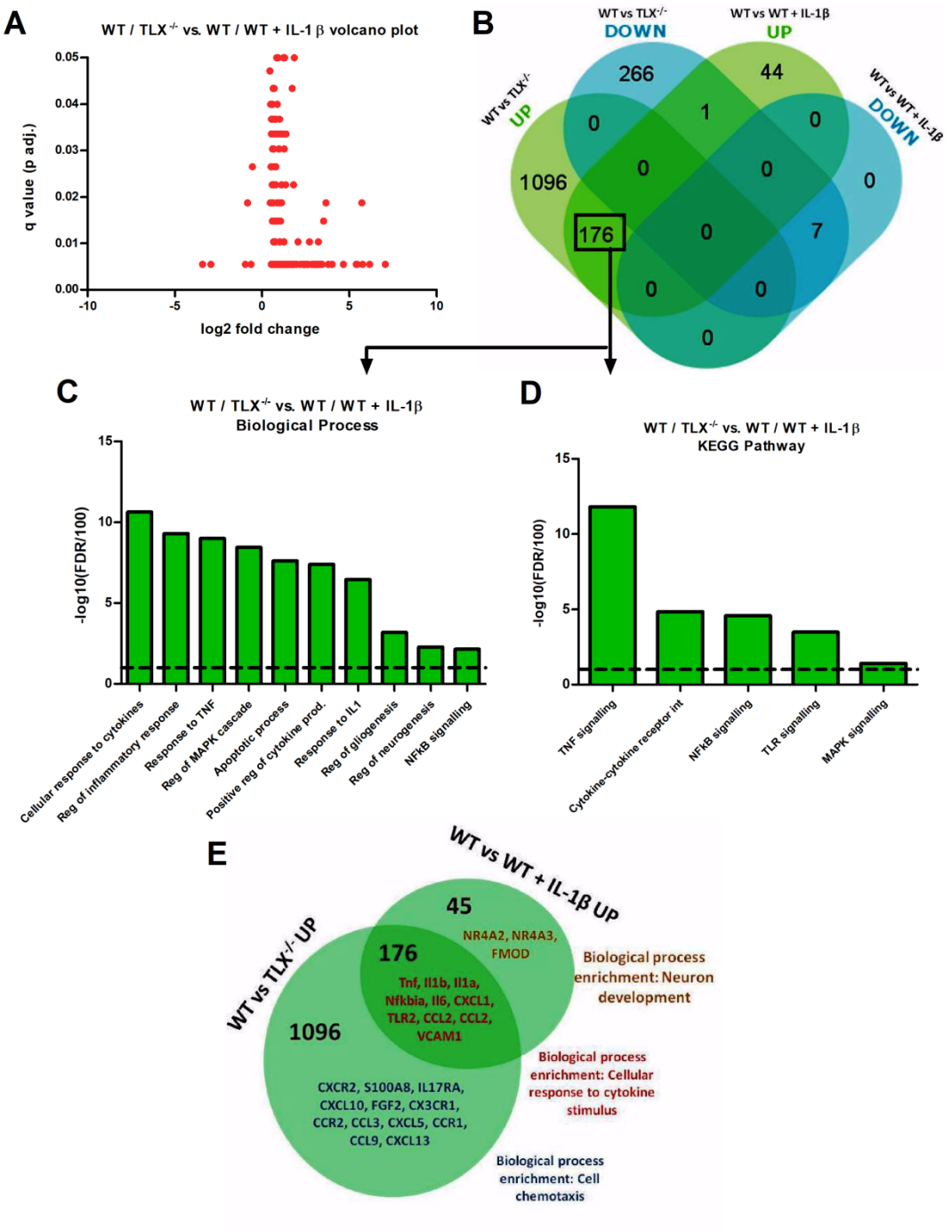


Figure 3.3: IL-1β induces the transcription of inflammatory-related genes in WT mice similar to that in TLX$^{-/-}$ mice. **A)** Volcano plot of all DEGs when comparing WT + IL-1β and WT mice vs. WT and TLX$^{-/-}$ mice. **B)** Venn diagram of all DEGs either up-regulated (green circles) or downregulated (blue circles) and the overlap of these genes when comparing TLX$^{-/-}$ and WT mice. **C)** GO enrichment in KEGG pathway for up-regulated genes comparing the overlap of WT vs. WT + IL-1β and WT vs. TLX$^{-/-}$ mice. The dotted line represents significance level, with values above this line deemed significant. **D)** GO enrichment in biological processes for up-

regulated genes comparing the overlap of WT vs. WT + IL-1β and WT vs. $TLX^{-/-}$ mice. The dotted line represents significance level, with values above this line deemed significant. **E)** Venn diagram comparing the overlap in gene transcriptional changes between WT vs. WT + IL-1β and WT vs. $TLX^{-/-}$ mice.

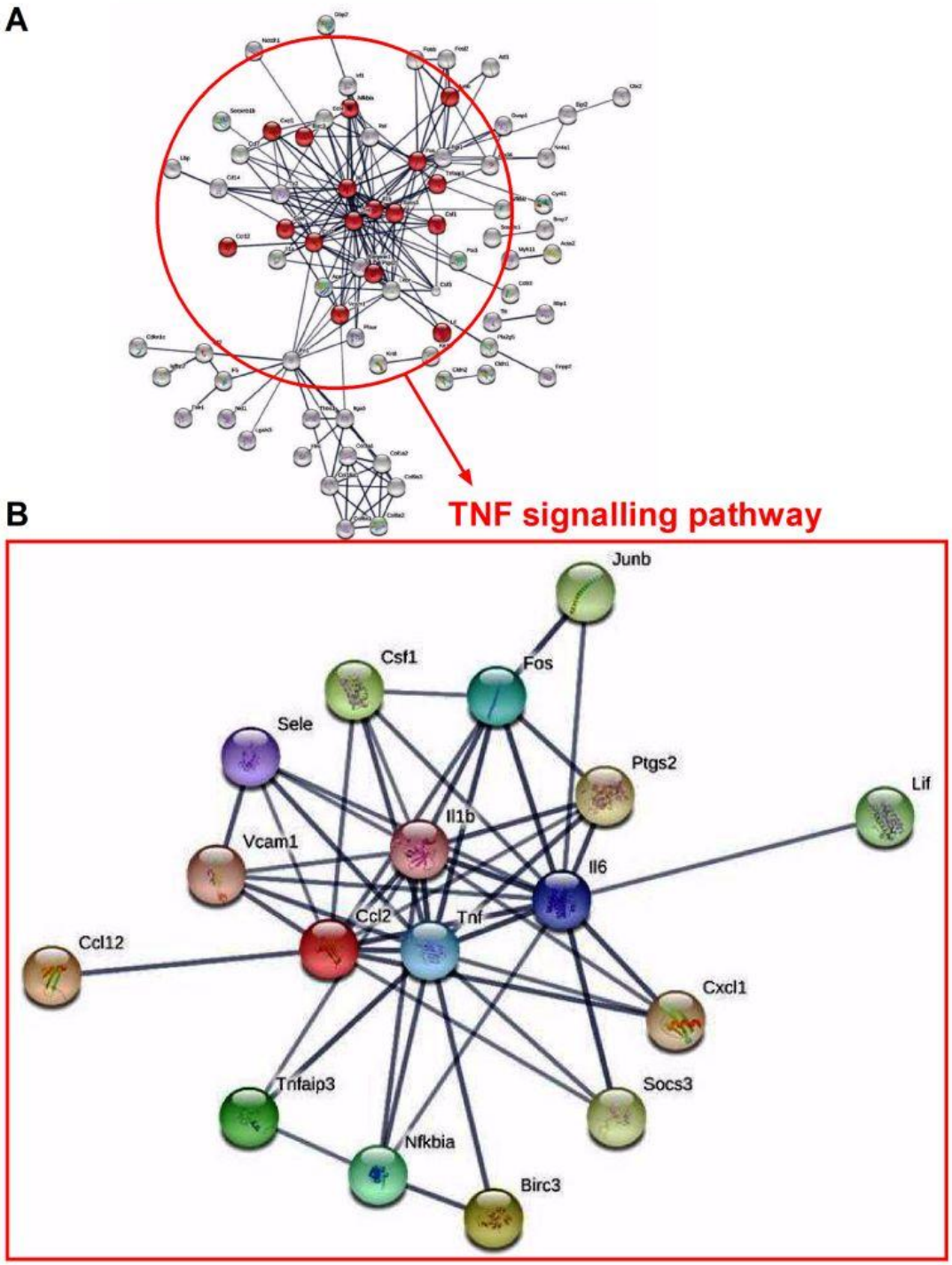


Figure 3.4: String analysis showing interactions between genes similarly increased in TLX$^{-/-}$ mice and WT mice injected with IL-1β compared to WT mice that are involved in TNF signalling. Line thickness between nodes indicates the strength of the evidence to support the interaction.

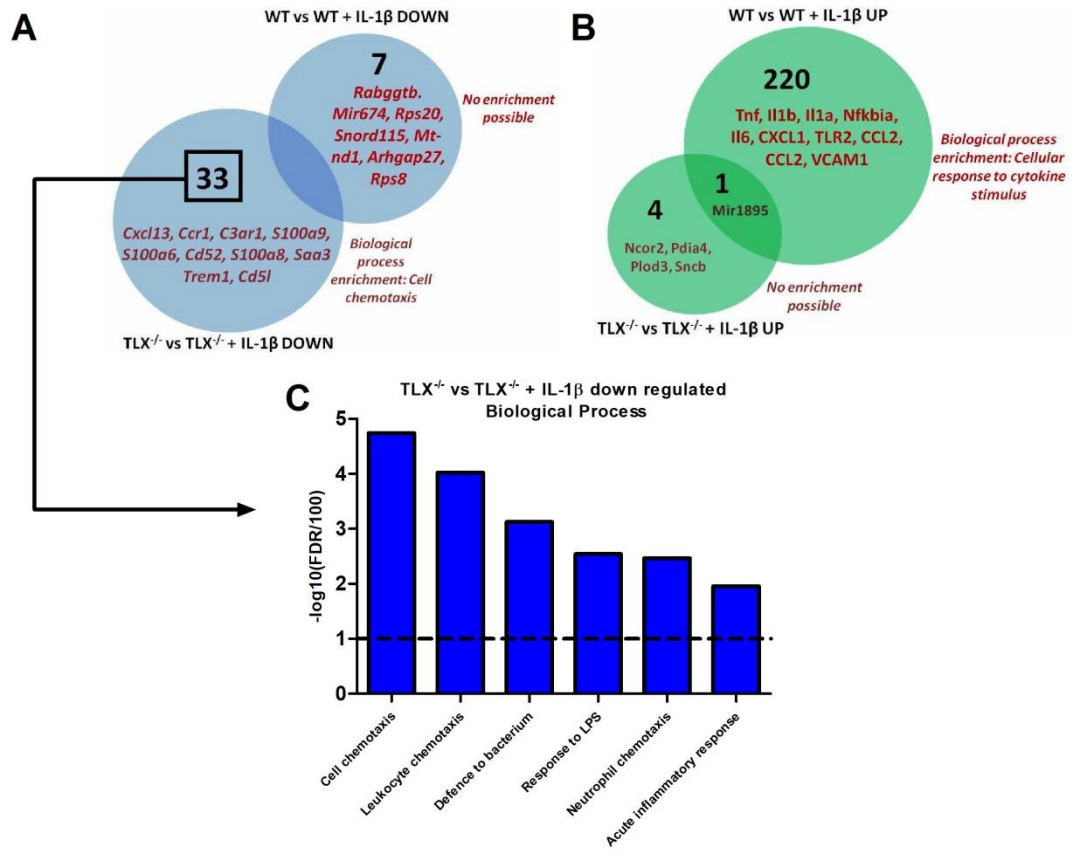


Figure 3.5: TLX$^{-/-}$ mice have a blunted transcriptional response to IL-1β compared to WT mice and an increased inflammatory response to cannulation surgery. **A)** Venn diagram showing the total number of DEGs down-regulated when comparing WT + IL-1β to TLX$^{-/-}$ + IL-1β mice. **B)** Venn diagram showing the total number of DEGs up-regulated when comparing WT + IL-1β to TLX$^{-/-}$ + IL-1β mice. **C)** GO enrichment in biological processes for down-regulated genes comparing the overlap of TLX$^{-/-}$ to TLX$^{-/-}$ + IL-1β-treated mice. The dotted line represents significant level with values above this line deemed significant.

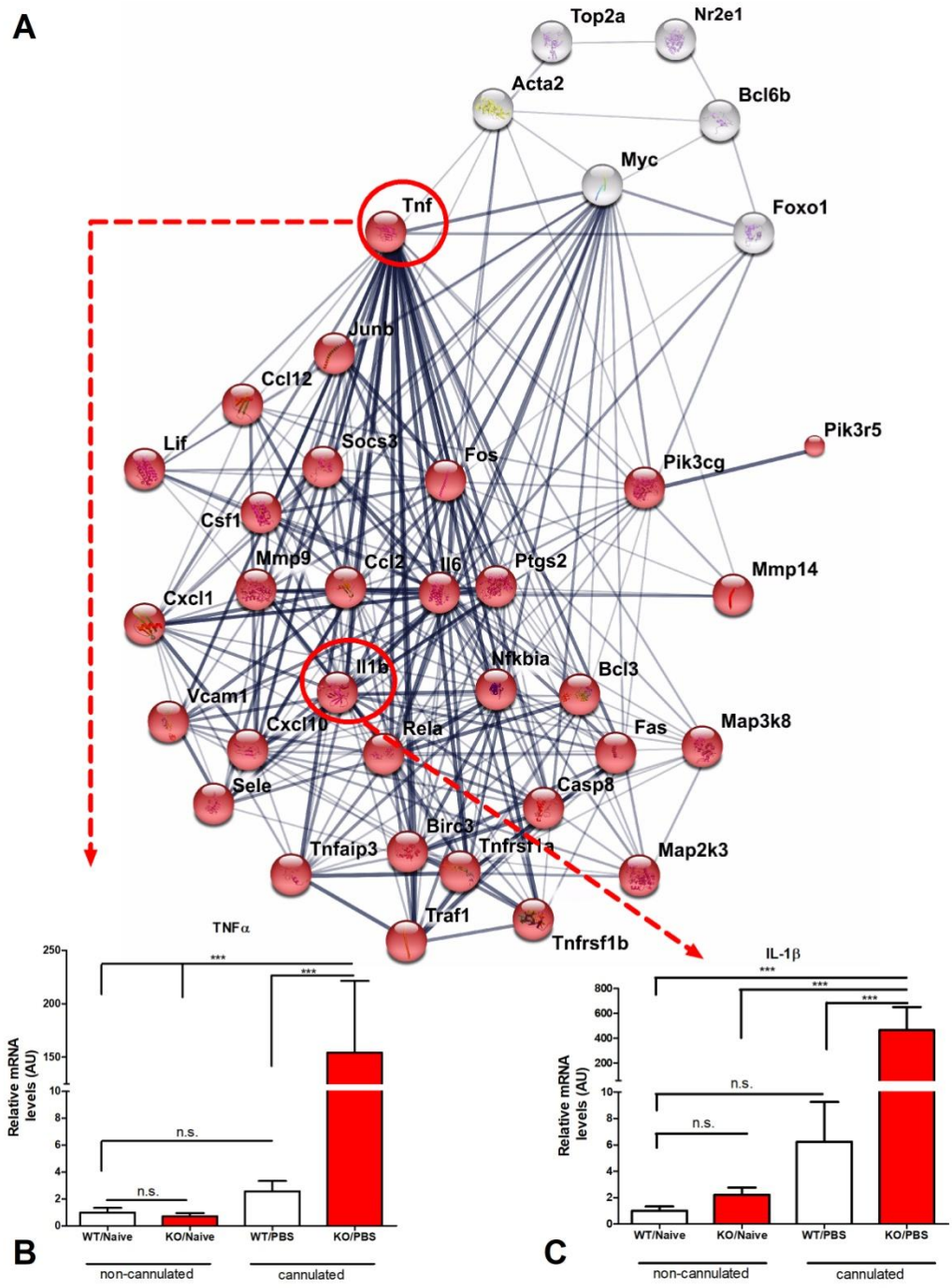


Figure 3.6: A) STRING analysis showing interactions between genes increased in $TLX^{-/-}$ mice compared to WT mice that are involved in TNF signalling, and how TLX interacts with TNF signalling. Thicker lines indicate stronger network connections between genes. Line thickness between nodes indicates the strength of the evidence to support the integration. **B)** and **C)** TNF α and IL-1β mRNA expression in non-cannulated and cannulated $TLX^{-/-}$ and WT mice, n.s = non-

significant (data expressed as mean ± SEM, ***=P<0.001 ANOVA and Tukey's *post-hoc* test). All data are presented as mean ± SEM.

Chapter 4

Enhanced hippocampal cell proliferation disrupts object recognition but not location recognition in rats

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Submitted to Neurobiology of Learning and Memory

Abstract

The orphan nuclear receptor tailless homolog (TLX) is an orphan nuclear receptor that is highly expressed in proliferating neural progenitor cells (NPCs) in the dentate gyrus (DG) of the hippocampus. It is a promoter of cell proliferation and therefore plays an important role in regulating hippocampal neurogenesis. However, research into the role of TLX in regulating hippocampal function is still in its infancy. Here, we describe behavioural and cellular effects of overexpressing TLX in the hippocampus of adult rats. We injected a lentivirus into the hippocampus of adult rats to induce the overexpression (OEX) of TLX and found that memory retrieval in a short term novel object recognition task was impaired. However, memory retrieval in a novel location task and hippocampal working memory (spontaneous alternation in the Y-maze) was unaffected in these rats. TLX OEX did not alter locomotor or anxiety-like behaviour. We also observed an increase in cell proliferation but not in cells survival or the birth of new neurons in response to TLX OEX. These data suggest that increased hippocampal cell proliferation via TLX OEX may impair perirhinal cortex-dependent object recognition and supports the concept that enhancement of cell proliferation alone does not promote hippocampal-dependent cognition.

1: Introduction

Hippocampal neurogenesis begins during the embryonic period and persists throughout adulthood in the dentate gyrus (DG) of the hippocampus (Gage, 2000). Successful hippocampal neurogenesis involves the proliferation of undifferentiated neural progenitor cells (NPCs), differentiation of the NPCs into neurons, and functional integration of those new neurons into the surrounding circuitry (Zhao et al., 2008). Hippocampal neurogenesis has been suggested to be involved in various hippocampal-associated memory functions including spatial learning and memory as well as emotional regulation (Snyder et al., 2005, Deng et al., 2010, Eisch and Petrik, 2012, Levone et al., 2015). Each stage of hippocampal neurogenesis is under strict regulation by a host of cell intrinsic and extrinsic factors (Zhao et al., 2008, O'Leime et al., 2017a). A key factor in the regulation of NPC proliferation is the orphan nuclear receptor tailless homolog (TLX; Islam and Zhang, 2014, Shi et al., 2004).

TLX (NR2E1) is an orphan nuclear receptor expressed within the mammalian central nervous system (CNS; Monaghan et al., 1995). Its expression is largely localised to the two neurogenic niches in the adult CNS; the DG and subventricular zone, where it is expressed at high levels in proliferating NPCs (Shi et al., 2004, Monaghan et al., 1997, Monaghan et al., 1995). TLX acts to maintain NPCs in a proliferative state and suppresses neuronal differentiation, thus it maintains the NPC pool within the DG. Specifically, TLX recruits histone deacetylases (HDACs 3 and 5), and histone demethylases (lysine-specific demethylase 1 (LSD1)) to repress the transcription of target genes involved in suppressing cell proliferation (Sun et al., 2007, Sun et al., 2010).

There is some evidence to suggest that TLX plays a role in certain hippocampal neurogenesis-associated cognitive processes including spatial memory in the Morris water maze (MWM) test (Murai et al., 2014, Zhang et al., 2008). For example, overexpression of TLX restricted to NPCs in adult mice has been shown to reduce the latency to find the hidden platform in the MWM (Murai et al., 2014). This suggests that enhancement of TLX function may induce improvements in certain forms of hippocampal-associated cognition. Conversely, spontaneous deletion of the TLX gene has negative effects on hippocampal-associated cognition such as spontaneous alternation in the Y-maze and contextual fear conditioning (O'Leary et al., 2016a). In the same strain of mice, absence of TLX also induced impairments in hippocampal synaptic plasticity and long-term potentiation (LTP) formation (Christie et al., 2006). Additionally, these mice exhibit impairments in non-hippocampal associated cued fear conditioning indicating that alterations in TLX expression may also impact upon non-hippocampal cognitive function (O'Leary et al., 2016a).

Most studies to date have investigating TLX gain of function by overexpression (OEX) using transgenic approaches in mice. In the current study, we performed a targeted delivery of the TLX gene to the hippocampus of rats using lentiviral technology. This allowed us to assess potential behavioural changes induced by increased TLX expression specifically during adulthood thus removing any effects observed as a result of increased TLX expression during development. Additionally, it allowed us to carry out behavioural tasks using rats and to rule out any non-specific effects that may occur with transgenic mouse models. Thus the aim of this study is to assess the effects of adult TLX OEX in adult rats on tasks that have been

associated with hippocampal and hippocampal neurogenesis-dependent function, namely novel object recognition, novel location recognition, and spontaneous alternation in the Y-maze (Jessberger et al., 2009b, Johnson et al., 1977, Hughes, 2004).

2: Methods

2.1: Animals

Adult male Sprague-Dawley rats obtained from Harlan UK (325-350 grams) were pair housed in a colony maintained at 22 ± 1°C, with a 12:12 hour light-dark cycle (lights on 0630-1830). All animal procedures were performed under licenses issued by the Health Products Regulatory Authority (HPRA, Ireland), in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Animal Experimentation Ethics Committee of University College Cork. All rats had *ad libitum* access to food and water for the duration of the study and animal weights were recorded weekly. See Fig. 4.1 for the schematic of the experimental design.

2.2 Lentiviral production

Production of plasmids and lentiviruses to overexpress GFP-tagged TLX (Nr2E1) or GFP was carried out by Genecopoeia (GeneCopoeia, MD, USA). Briefly, lentiviral particles pseudotyped with the vesicular stomatitis virus (VSV-G) glycoprotein and contained plasmids sequences for GFP or GFP-tagged TLX (Nr2e1) on an FIV backbone and driven by a cytomegalovirus promoter (CMV) promoter. These lentiviral particles were generated using standardised lentiviral production protocols and using EndoFectin-Lenti^{TM} and TitreBoost^{TM} reagents. The lentiviral transfer vector was co-transfected into 293Ta cells with Lenti-Pac^{TM} FIV packaging mix. Viral titres were determined via RT-PCR and were established as 1.63×10^7 copies/ml for the TLX lentiviral particles and 1.31×10^7 copies/ml for the GFP lentiviral particles. The lentivirus particles were then purified and stored at -80°C until use.

2.3: Stereotaxic Surgery and Lentiviral Injection

Rats were anaesthetised with isoflurane and placed into a Kopf stereotaxic frame. Lentiviruses to overexpress TLX or GFP (Genecopoeia, MD, USA) was injected into the dorsal hippocampus using the coordinates AP: -3.5 mm, ML: ± 2.4 mm, DV: -3.8 mm relative to Bregma (Barrientos et al., 2002) at a volume of 2µl and flow rate of 1µL/min followed by a 5 min diffusion. Rats were injected s.c. with carpofen and 5% glucose prior to anaesthetic recovery. All rats were allowed to recover for 3 weeks with *ad libitum* access to food and water prior to behavioural testing in order to allow viral uptake. Animals were administered bromodeoxyuridine (BrdU) (1x150mg/kg, i.p.) one week after surgery for immunohistochemical assessment of the survival of newly born cells.

2.4 Open field

Rats were placed in an open field arena (90 cm diameter and 45cm wall height) under bright lighting conditions for 10 minutes. Distance travelled and time in the centre of the arena was recorded and calculated using Ethovision software (Noldus). The arena was cleaned with a 50% ethanol solution between exposures of animals to the arena to remove odour cues.

2.5 Spontaneous Alternation in the Y-maze

Spontaneous alternation behaviour is used as a measure of hippocampal-dependent working memory (Hughes, 2004). The Y-maze consisted of three arms 120° from each other (16 cm x 6.5 cm; made in house) (adapted from (Senechal et al., 2007)). Each animal was placed into the first arm of the maze facing the wall, and allowed to explore the maze for five minutes. The number and order of arm entries were

recorded. An arm entry was defined as all four paws entering into the arm (four paw criteria). An alternation was determined as the number of consecutive entries into the three maze arms. Alternations were then divided by the total number of entries during the five minute test period to give the percentage alternation for each animal which was plotted as the mean for each experimental group ± standard error of the mean (SEM).

2.6 Novel Object Recognition

Novel object recognition was assessed as previously described (Bevins and Besheer, 2006). The objects used for this task were either a 250ml Fisherbrand^{TM} glass bottle (Thermo Fisher Scientific, MA, USA) or a coffee cup. On day 1 the animals were habituated to the testing arena (45cm x 80cm rectangle arena with 45cm wall height) for a 10 minute exploration period. On day 2, two identical objects were positioned on adjacent corners approximately 5 cm from each wall of the arena and each animal was placed in the arena for a 10 minute exploration period. Animals were then placed directly back into their home cages. After a 3 hour inter-trial interval, one familiar object was replaced with a novel object, and each animal was placed in the arena for a 5 minute exploration period. Object exploration was defined as a 2 cm radius between the object and the animal's nose. The testing arena and objects were cleaned with a 50% alcohol solution between exposures. Videos were manually scored for object exploration and total exploration times with the novel and familiar objects were graphed as time (seconds) ± SEM.

2.7 Location recognition task

The location recognition task was conducted in the open field arena, covered with bedding under dim light conditions (black plastic bags were used to cover the lights in the testing room) (adapted from Bekinschtein et al., 2013b, Bekinschtein et al., 2014, Kent et al., 2015). The objects used were Carlsberg^{TM} 330ml glass bottles. The testing room had three proximal spatial cues and distal standard furniture. Objects and the arena were cleaned with a 50% ethanol solution between exposures of each animal to the arena to remove odour cues. Rats were habituated to the arena for 10 minutes per day for 5 consecutive days before testing began. During the acquisition phase, three identical objects were placed 15 cm from the edge of the open field, and 30 cm from the centre of the arena, and rats were allowed to explore for 10 minutes. The three objects were separated by 120° angles during acquisition. The test phase was conducted 24 hours following acquisition where one of the objects was removed and the two other previously used objects were placed in the arena opposite each other. One was placed in the same position it was in during the acquisition phase and the other was placed directly opposite. Rats were allowed to explore for 5 minutes. The location of the objects used was counterbalanced within and between groups. Time spent exploring the objects was manually scored and total exploration times with the novel and familiar objects were graphed as time (seconds) ± SEM.

2.8 Immunohistochemistry and imaging

Rats were euthanized with an i.p. injection of Euthatal (pentibarbitol; 1.0mL/kg) and transcardially perfused using a 0.9% phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde in PBS. After perfusion, brains were removed and post-fixed in 4% formaldehyde in PBS overnight, then transferred to a 30% sucrose solution until they had sunk. Coronal sections through the hippocampus

were cut at 40μm onto slides and stored at -80°C. For analysis of the survival of new cells, sections were incubated in HCl (2M) for 45 minutes at 37°C and renatured in 0.1M sodium tetraborate, and then treated with 3% hydrogen peroxide for 45 minutes. Sections were blocked in 10% normal rabbit serum (Sigma) and incubated with rat anti-BrdU (Abcam ab6326). Ki67 (Genetex, GTX16667) and DCX (Santa Cruz, sc-8066) staining was carried out for analysis of cell proliferation and neurogenesis, respectively (see table 4.1 for a list of all primary antibodies and their dilutions used in this study). Sections were blocked in 10% normal rabbit serum and incubated with the appropriate primary antibody. Detection and visualisation was carried out using HRP-linked rabbit-anti-rat secondary and ABC complex (Vector) before being placed into DAB and coverslipped with DPX mounting medium. Brightfield images across a 1:12 series through the dorsal DG were obtained using an Olympus BX53 upright microscope at both 10x and 20x (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, UCC). The total number of positively labelled cells were counted and expressed as cells per section within the hilus, subgranular zone (SGZ), and granular cell layer (GCL) of the DG. Fluorescent images of autofluorescent GFP-tagged TLX transfected cells were taken using an Olympus BX53 upright microscope at 20x across a 1:12 series through the hippocampus of each animal to confirm successful viral transduction (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, UCC). The presence of auto-fluorescent cells was deemed to confirm viral transduction.

Target	Antibody	Source	Company	Dilution
BrdU	Anti-BrdU	Monoclonal Rat	Abcam	1:100
Ki67	Anti-Ki67	Monoclonal Rabbit	Genetex	1:200

DCX	Anti-DCX	Polyclonal Goat	Santa Cruz	1:100

Table 4.1: List of antibodies used in chapter 4.

2.9 Statistics

All data are expressed as mean ± SEM. Student's t-tests or Two-way ANOVA were carried out where appropriate to determine statistical significance and $p \leq 0.05$ was deemed significant.

3: Results

3.1 Hippocampal TLX OEX did not alter weight, locomotor activity, or thigmotaxis.

There was no difference in the weight of the animals between both experimental groups for the duration of the experiment (Fig. 4.2A). In the open field, both groups travelled similar distances and spent similar amounts of time within the centre of the arena indicating no differences in locomotor activity or anxiety-like behaviour (Fig. 4.2B, C). As the TLX overexpression plasmid was tagged with GFP, we confirmed the presence viral transduction by visualising auto-fluorescence if the GFP-tagged TLX of successfully transduced cells within the hippocampus for each animal (Fig. 4.2D). In each animal auto-fluorescence of GFP-tagged TLX was evident.

3.2 Hippocampal TLX OEX impairs short-term object recognition memory but does not affect long-term location recognition memory or working spatial memory.

Both control and hippocampal TLX OEX rats had similar exploration times during the acquisition phase of the novel object recognition task and neither group showed a preference for any particular object used during the test phase (bottle or cup) (Fig. 4.3A). Rats with hippocampal OEX of TLX showed impaired object recognition after a 3 hour delay between acquisition and testing whereas control animals successfully identified the novel object (Fig. 4.3A, $t(17)=3.393$, $p=0.0035$, $*P<0.05$). However, these same rats performed to a similar extent as control rats in a location discrimination task after a 24 hour delay as no impairment or enhancement was observed (Fig. 4.3B, Control novel versus familiar $t(16)=2.158$, $p=0.0464$; TLX novel versus familiar $t(18)=3.286$, $p=0.0041$ $*P<0.05$ and $**P<0.01$ compared to respective familiar object of respective lentiviral treatment group). There were no

differences in spatial working memory between the groups as determined by spontaneous alternation behaviour in the Y-maze (Fig. 4.3C).

3.3: Hippocampal TLX OEX increases the number of proliferating cells in the subgranular zone.

TLX OEX induced a significant increase in the number of proliferating cells in the subgranular zone (SGZ) of the DG (Fig. 4.4A, Ki67 positive cells in the SGZ comparing control versus TLX $t(6)=3.784$, $p=0.0091$). No change in the number of proliferating cells was observed in the granular cell layer (GCL) or the hilus. There was no significant difference in the survival of new cells (Fig. 4.4C) or in the number of newborn neurons (Fig. 4.4E) in the hippocampus of rats with TLX OEX compared to controls.

4: Discussion

In the present study, we utilised lentivirus technology to induce TLX OEX within the dorsal hippocampus of adult male rats and assessed changes in hippocampal neurogenesis-associated cognition. We did not observe any significant improvement in novel location recognition or spontaneous alternation in the Y-maze, however we did observe a significant impairment in novel object recognition in these rats. We also show that TLX OEX induced a significant increase in cell proliferation in the subgranular zone of the hippocampus. We did not observe any changes in cell survival or in birth of new neurons in the DG of TLX injected rats. Thus, our results suggest that enhancing cell proliferation alone within the neurogenic niche of the hippocampus may not be sufficient to promote hippocampal neurogenesis-associated cognition, and may in fact impair certain forms of hippocampal cognition.

It is important to bear in mind that the tasks assessed in this study each reflect a different aspect of hippocampal-associated cognition. Spatial working memory is assessed in the spontaneous alternation task with very little learning involved (Gerlai, 2001, Gerlai, 1998). Novel object recognition does involve a minor learning component in that the animals must successfully encode or learn the memory of the familiar object (Cohen and Stackman, 2015, Aggleton and Brown, 2005, Bevins and Besheer, 2006). However, the perihinal cortex is also said to be involved in object recognition and depending on the duration if the intertrial-interval, may be more responsible for successful completion of the task than the hippocampus (Cohen and Stackman, 2015). Location recognition is suggested to be more hippocampal dependent than object recognition and provide a greater indication of specific hippocampal function (Dupret et al., 2008)

Numerous studies have linked the function of adult hippocampal neurogenesis to spatial memory and cognition. However, this link is complex as some studies have demonstrated the beneficial effects of enhancing neurogenesis (Snyder et al., 2005, Deng et al., 2009, Jessberger et al., 2009b) while others have demonstrated that there is no effect of manipulating hippocampal neurogenesis on spatial learning and memory (Dupret et al., 2008, Zhang et al., 2008, Hernandez-Rabaza et al., 2009, Wojtowicz et al., 2008). We demonstrate that enhancing cell proliferation in the hippocampus through lentivirus-mediated TLX OEX impairs memory recall in an object recognition task. Additionally, neither controls nor TLX OEX group showed any bias in exploring any object used in this study (cup or bottle). Object recognition involves direct or indirect neuronal inputs the perirhinal cortex to the hippocampus (Aggleton and Brown, 2005, Witter et al., 1989, Burwell et al., 1995). In support, it has been demonstrated that rats who underwent a location recognition task display increased expression of immediate early genes in the hippocampus only whereas rats who underwent an object recognition task show increased expression of these genes in the perirhinal cortex and not the hippocampus (Aggleton and Brown, 2005). Lesion studies have demonstrated that when the perirhinal cortex is damaged, object recognition is impaired yet spatial memory performance remains intact and when the hippocampus is damaged, spatial memory is impaired but object recognition remains intact. However, if the object recognition task is conducted in an open area, salient spatial or contextual cues result in the hippocampus being recruited to the neuronal circuitry underlying the object recognition task (Aggleton and Brown, 1999, Cassaday and Rawlins, 1997, Gaffan, 1994, Zola et al., 2000). This could explain why disruption to hippocampal function can impair object recognition. In the context

of our results, it could be suggested that this relay of information between the hippocampal DG to the perirhinal cortex may be disrupted by the heightened proliferative state of the hippocampus as a result of TLX OEX. One important caveat to bear in mind when interpreting the results of this study is the intertrial intervals used. For the object recognition task a three hour interval was used whereas for the location recognition task a 24 hour interval was used. Therefore it is difficult to determine whether TLX OEX rats and controls would exhibit equivalent object recognition memory after a 24 hour interval or a deficit in place recognition memory after a 3 interval. Future studies will aim to address this confounding factor by utilising a common intertrial interval between novel object and location recognition tasks.

The flow of information between the perirhinal cortex and the hippocampus during object recognition has been proposed to be heavily dependent on the duration of time that the rodents spend exploring the objects, as well as the length of time between acquisition and testing phases. Cohen and Stackman propose that shorter exploration times and inter-trial intervals results in the perirhinal cortext being the predominant structure involved in processing the task and as these times increase, information processing gradually switches to the hippocampus (Cohen and Stackman, 2015). They further suggest that the shift from perirhinal cortex to hippocampal information processing starts after approximately 30 seconds of object exploration. This time frame is similar to the exploration time of the novel object by both the experimental groups during the test phase in the current study. Thus, it may be the case that the increased cell proliferation in the hippocampus may impair the flow of this information from the perirhinal cortex to the hippocampus and thus lead to impaired

performance in the object recognition task. However, both groups spent approximately 80 seconds exploring the objects during the acquisition phase therefore the hippocampus should be sufficiently recruited at that stage for encoding the object memory. Notwithstanding these results open an interesting avenue of research to assess the detrimental potential of heightened proliferation in the hippocampus on hippocampal cognition to determine the mechanism of such impairments.

As neurogenesis is a multifactorial process of cell proliferation, differentiation and neuronal integration, it may be possible that enhancing just one of these factors is not sufficient to enhance hippocampal-associated spatial cognition. Furthermore, enhancing one specific component of neurogenesis, such as cell proliferation, may dysregulate the entire neurogenic process or not translate to increased neuronal differentiation. Indeed it has been demonstrated that when the transcription factor sonic hedgehog (Shh) is overexpressed in NPCs, there is a significant increase in cell proliferation while there is no effect on differentiation (Lai et al., 2003). Likewise, we observed no effect of TLX OEX on the number of DCX cells indicating a lack of effect of TLX on neuronal differentiation. Indeed, TLX has previously been shown to prevent differentiation by maintaining NPCs in a proliferative state (Niu et al., 2011, Shi et al., 2004, Li et al., 2008). Our results using a lentivirus-mediated approach also show that the increase in cell proliferation induced by TLX occurred without altering the differentiation status of these cells. This is in line with the work on Shh by Lai et al. (2003) and could explain why we did not see any effect of TLX OEX in the novel location test or spontaneous alternation in the Y-maze. These data reinforce the notion that for any enhancement in hippocampal cognitive function to

be induced by neurogenesis, there must be a robust enhancement of all aspects of neurogenesis.

Most studies to date have assessed the functional role of TLX by assessing the behaviour of mice in which there is a spontaneous deletion of the TLX gene (Young et al., 2002, Roy et al., 2002, O'Leary et al., 2016a). These studies have shown that impairment in TLX expression lead to deficits in locomotor activity, contextual and cued conditioning, and reduced anxiety-like behaviour. The broad range of behavioural deficits exhibited by these mice is likely a result of the lack of TLX expression throughout critical periods of brain development. Thus, to truly assess the functional role of TLX in adulthood, targeted manipulation of TLX should be assessed specifically during adulthood such as we employed here. Some studies have previously attempted to address this issue by transgenic methods to alter TLX expression in adulthood (Zhang et al., 2008, Murai et al., 2014). Specifically, it has been demonstrated that targeted deletion of TLX in adulthood led to impaired spatial navigation in the Morris water maze (MWM) yet did not affect contextual fear conditioning (Zhang et al., 2008). Conversely, transgenic overexpression of TLX specifically within the hippocampus enhanced spatial memory performance in the MWM (Murai et al., 2014). The use of lentiviral vectors is a useful method to either enhance or repress the expression of specific genes (Kunitsyna et al., 2016, Escors and Breckpot, 2010, Naldini, 1998, van Hooijdonk et al., 2009) and immunohistochemical data in this study confirmed that injection of lentiviral particles designed to overexpress TLX resulted in increased cell proliferation in the DG of the hippocampus. However, as TLX is a nuclear receptor, pharmacological manipulation of TLX would ultimately allow for greater control of its activity.

Research is currently underway to establish a pharmacological ligand to regulate TLX function, and so with the discovery of such ligands it will be possible to probe deeper into the role of TLX in hippocampal-associated cognitive processes (Benod et al., 2014b, Benod et al., 2016).

In conclusion, we show that TLX overexpression in the hippocampus impaired performance in an object recognition task. As TLX functions to promote proliferation of NPCs, we also confirmed that lentiviral mediated overexpression of TLX had a functional effect by increasing the number of proliferating cells in the SGZ of the hippocampus, a region rich in NPCs. Our data suggest that enhanced hippocampal cell proliferation may disrupt perirhinal cortex-hippocampal neuronal communication and thus impair object recognition memory. However, further research is needed to confirm the potential detrimental effect of elevated levels of hippocampal cell proliferation on hippocampal cognitive function. Additionally, future studies should adopt standardised intertrial intervals to concretely compare novel location and novel object recognition tasks without differing intertrial intervals being a confounding factor. As we observed no effect of neuronal differentiation, our data also supports the concept that enhancement of cell proliferation without successful integration of new neurons does not promote hippocampal-dependent cognition.



Figure 4.1: Experimental timeline. Wk, week; OF, open field; NOR, novel object recognition; NLR, novel location recognition; SA, spontaneous alternation dH, dorsal hippocampus; OEX, overexpression.

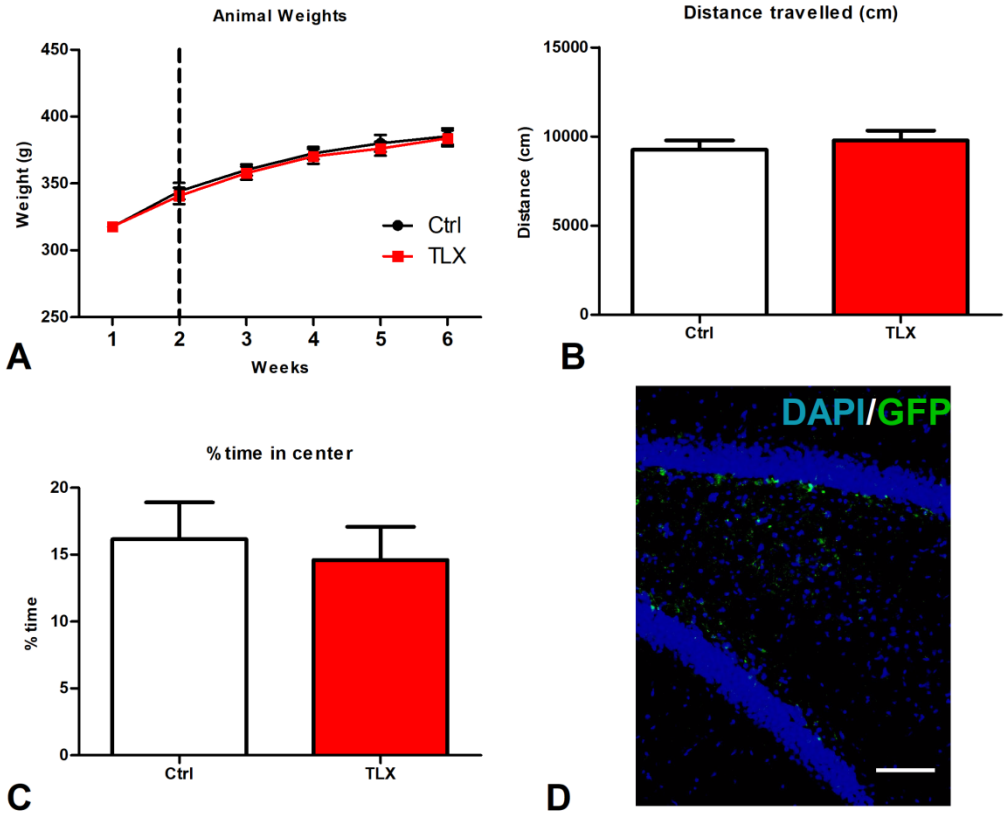


Figure 4.2: TLX overexpression in the hippocampus does not alter weight gain, locomotor behaviour, or thigmotaxis. **A)** Body weight of rats for the duration of the study. **B)** Distance travelled in the open field. **C)** Percentage time spent in the centre of the open field. **D)** Representative image of auto-fluorescence of GFP-tagged TLX in the dorsal hippocampus. Image taken at 20x and the scale bar represents 100µm. All data are depicted as mean ± SEM (n=9-10).

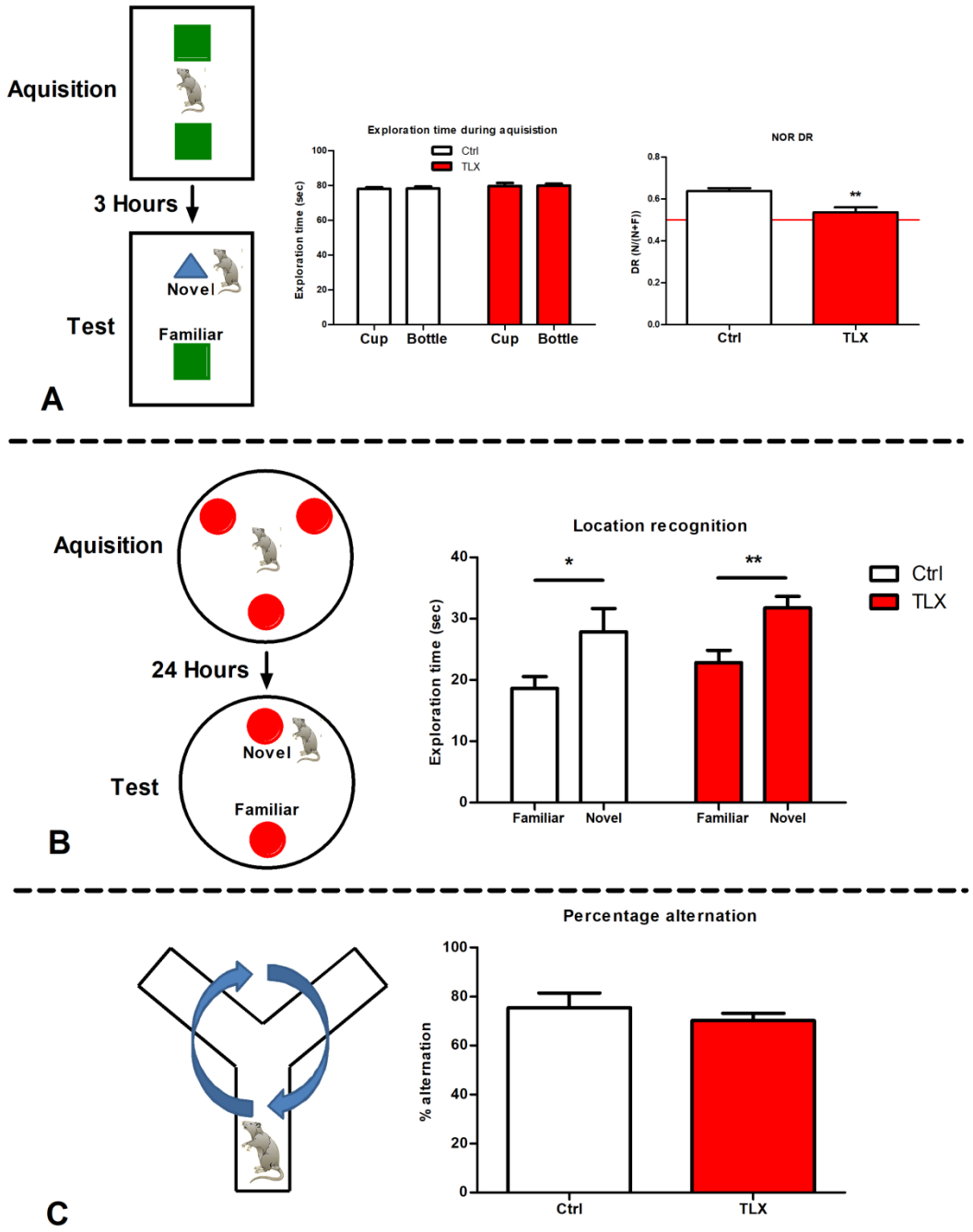


Figure 4.3: TLX overexpression in the hippocampus impairs novel object recognition but has no effect on novel location recognition or working spatial memory. **A)** Schematic of novel object recognition paradigm (left) and time spent with the familiar and novel objects (right). **B)** Schematic of novel location recognition paradigm (left) and time spent with the familiar and novel object locations (right). **C)** Schematic of spontaneous alternation in the Y-maze paradigm (left) and percentage alternations in the Y-maze (right). A student's t-test was used in to determine significance where *$p<0.05$ and **$p<0.01$ vs respective familiar object where n=9-10. Data are depicted as mean ± SEM.

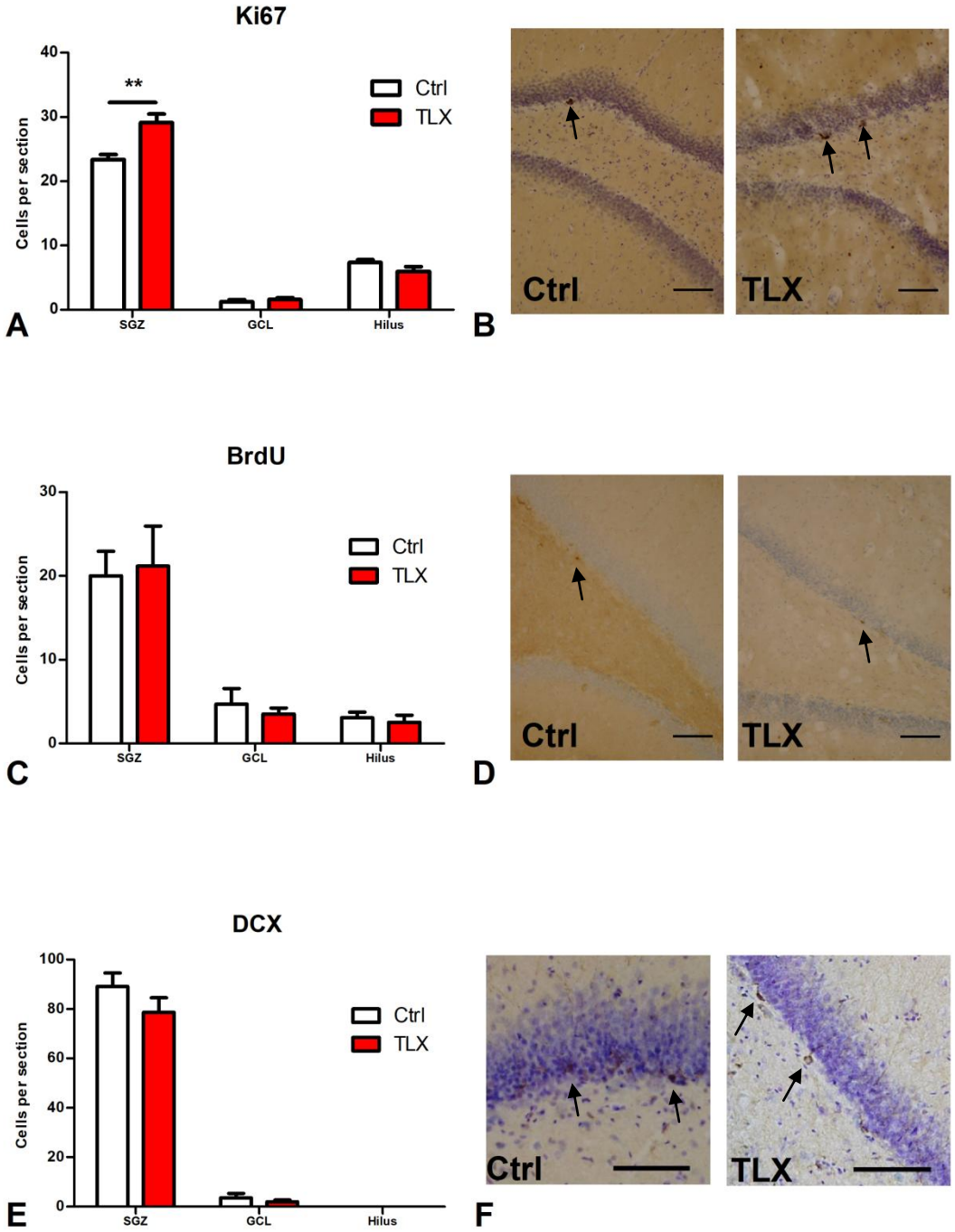


Figure 4.4: TLX overexpression in the hippocampus increases cell proliferation in the (SGZ) of the DG. Number of Ki67 **(A)**, BrdU **(C)** and DCX **(E)** positive cells per section in the SGZ, GCL and hilus of control and TLX OEX rats **p<0.01, n=4. Representative images of Ki67 **(B)** and BrdU **(D)**-positive cells in the DG. The scale bar represents 100μm. Representative images of DCX-positive cells in the DG **(F)**. The scale bar represents 75μm. Student's t-test used to determine significance p<0.05vs.ctrl.

Chapter 5

IL-1β but not adolescent cafeteria diet promotes fear learning in rats

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Manuscript in preparation

Abstract

The adolescent period is an important stage of maturation for various brain structures and it is during this time that effects of environmental factors such as diet can have a particular influence on mood and memory. Diets high in fat and sugar (termed a cafeteria diet) have been shown to negatively impact upon cognitive performance. It has been suggested that the negative effects of poor diet during adolescence on cognition can be reversed by switching to a normal diet during adulthood. However, it is unknown whether adolescent exposure to a poor diet potentiates the effects of known negative regulators of cognition during adulthood, such as inflammation. To address this, we allowed rats access to a cafeteria diet during the adolescent period after which time they were injected with a lentivirus in the hippocampus to induce chronic low-grade overexpression of the pro-inflammatory cytokine interleukin-1β (IL-1β). After viral integration, we assessed performance in a series of hippocampal-associated cognitive tasks. Our data suggests that adolescent exposure to a cafeteria diet does not induce any lasting cognitive deficits when the diet was replaced with a normal diet in adulthood. Additionally, IL-1β in the cafeteria diet fed rats did not alter this effect. IL-1β in the absence of a prior cafeteria diet impaired spatial working memory but enhanced contextual fear learning. In conclusion, we demonstrate a complex role of IL-1β in regulating hippocampal-associated behaviour.

1: Introduction

A major health concern in developed countries is the over consumption of high energy diets rich in saturated fat and sugar (Berthoud et al., 2011, Guyenet and Schwartz, 2012, Kearney, 2010). Overconsumption of these 'cafeteria' diets have led to a drastic increase in the prevalence of obesity and associated cardiovascular and metabolic disorders (Bruce-Keller et al., 2009, Haslam and James, 2005). Increased consumption of high fat and high sugar diets has also been shown to impair cognitive performance and the regulation of emotion (Jacka et al., 2010, Kanoski and Davidson, 2011). The hippocampus is a brain region involved in learning and memory as well as the regulation of emotional responses and its function is particularly affected by increased intake of high fat and/or high sugar diets (Heyward et al., 2012, McNay et al., 2010, Ross et al., 2009). For example, it has been demonstrated that daily access to sucrose induces impairments in hippocampal-associated pattern separation in adult rats (Reichelt et al., 2016) and a diet high in both fat and sugar has been shown to impair location recognition also in adult rats (Beilharz et al., 2014). However, some studies have shown no effect of high fat and/or high sugar diets on certain hippocampal dependent tasks in adulthood such as spatial navigation and object recognition indicating that the effects of diet may be task specific and/or diet specific (Beilharz et al., 2014, Reichelt et al., 2015, Valladolid-Acebes et al., 2013).

Increased consumption of cafeteria diets and obesity have been reported across all age groups however it is suggested that the adolescent period, a critical period for the development of normal cognitive function throughout life (Spear, 2000), is particularly susceptible to the cognitive impairments induced by poor diet intake

(Ogden et al., 2012, Vendruscolo et al., 2010, Boitard et al., 2014, Boitard et al., 2012, Reichelt et al., 2015). Consumption of high fat or high sugar in adolescent rats have been shown to result in impaired reward pathway regulation as well as motivational deficits and these effects are not evident in adult rats (Vendruscolo et al., 2010). Additionally, it has been demonstrated in rodents that adolescent consumption of a high fat diet impairs a variety of hippocampal-associated cognitive processes including spatial memory (Boitard et al., 2012, Boitard et al., 2014) and object location recognition (Valladolid-Acebes et al., 2013, Reichelt et al., 2015, Beilharz et al., 2014). Interestingly, these behavioural effects are not observed after consumption of the same diet in adult rats (Valladolid-Acebes et al., 2013, Reichelt et al., 2015, Beilharz et al., 2014). Similar effects have been demonstrated in humans where adolescents consuming high fat and high sugar diets have impaired visuospatial learning and memory performance which is associated with hippocampal function (Nyaradi et al., 2014).

Recent evidence has suggested that the negative effects of high fat diets on hippocampal-associated cognition during adolescence can be reversed in adulthood by simply reverting to a normal diet (Boitard et al., 2016). Thus, it may be that the effects of poor diet during adolescence on cognitive function are not permanent. However, it is unknown whether early exposure to a poor diet could exacerbate any potential impairment in cognition induced by a negative regulator of hippocampal function during adulthood. Interleukin-1β (IL-1β) is a pro-inflammtory cytokine which is prevalent in the hippocampus after infection and is responsible for mediating cellular processes associated with the cognitive and affective symptoms of neurodegenerative diseases and stress-induced major depression (Hauss-Wegrzyniak

et al., 1998, Ransohoff, 2016, Heppner et al., 2015, Müller, 2014, Salim et al., 2012). However, there are conflicting reports on the effects on hippocampal function with some studies demonstrating improved performance by rodents in certain hippocampal-associated tasks and others demonstrating an impaired performance (Goshen et al., 2007, Moore et al., 2009, Barrientos et al., 2002). Thus, the study described herein consisted of three main aims. Firstly, we aimed to determine whether adolescent exposure to a cafeteria diet high in both sugar and fat induces hippocampal-associated cognitive impairments in adulthood. Secondly, we aimed to determine the effects of lentiviral-mediated sustained overexpression (OEX) of IL-1β during adulthood on hippocampal associated cognition. Finally, we sought to determine whether adolescent cafeteria diet consumption could exacerbate IL-1β-induced effects on cognition during adulthood. Hippocampal cognitive performance was determined by assessing short-term spatial working memory, recognition memory, and fear memory tasks and associated neuroplasticity gene expression changes in the hippocampus were determined.

2: Methods

2.1: Animals

Male Sprague-Dawley rats were bred in-house (Biological Services Unit, UCC) and were weaned at postnatal day (PND) 21. Weaned rats were pair housed in a colony maintained at 22 ± 1°C with a 12:12 hour light-dark cycle (lights on 0639-1830). All animal procedures were performed under licenses issued by the Department of Health and Children (Ireland) and the Health Products Regulatory Authority (HPRA, Ireland), in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Animal Experimentation Ethics Committee of University College Cork.

2.2: Experimental design and diet

Rats were randomly divided into four experimental groups; control chow fed animals injected with an mCherry expressing lentivirus (Ctrl) (n=8), cafeteria diet fed animals injected with an mCherry expressing lentivirus (caf. diet) (n=10), control chow fed animals injected with an IL-1β expressing lentivirus (Ctrl + IL-1β) (n=10), and cafeteria diet fed animals injected with an IL-1β expressing lentivirus (caf. diet + IL-1β) (n=10). The cafeteria diet consisted of high fat and high sugar components and was given in addition to standard chow to diet fed rats (blue and green highlighted columns in figure 5.1 represent high fat and high sugar foods respectively). Each day, rats were given one high fat food and one high sugar food as well as regular chow. The combination of foods given was randomised such that rats had equal exposure to each food type across the length of the study. Foods were weighed prior being placed in the cages and were given in excess and the leftover amount of each component was weighed after 24 hours to determine the amount

consumed by rats in each cage. Standard chow was also weighed daily to determine consumption by rats in all cages (See table 5.1 for list of all foods given and nutritional information). Cafeteria diet began one week after weaning (PND28) and ended at PND56 when the animals underwent sterotaxic sugery. Standard chow was given for the remainder of the study. Behaviour testing started 3 weeks later to allow for lentiviral uptake. The animals were culled at PND98/99 and tissue collected for ex vivo analysis. Left and right hippocampus tissue was collected. Tailblood samples were taken before the start of the diet, immediately at the end of the diet, and truck blood was taken at cull to measure metabolic hormone levels. See fig. 5.1A for experimental schematic.

2.3 Lentiviral production

Production of plasmids and lentiviruses to overexpress mCherry or mCherry-tagged IL-1β was carried out by Genecopoeia (GeneCopoeia, MD, USA). Briefly, lentiviral particles pseudotyped with the vesicular stomatitis virus (VSV-G) glycoprotein and contained plasmids sequences for mCherry or mCherry-tagged IL-1β on an FIV backbone and driven by a cytomegalovirus promoter (CMV) promoter. These lentiviral particles were generated using standardised lentiviral production protocols and using EndoFectin-Lenti^{TM} and TitreBoost^{TM} reagents. The lentiviral transfer vector was co-transfected into 293Ta cells with Lenti-Pac^{TM} FIV packaging mix. Viral titres were determined via RT-PCR and were established as 1.93×10^{7} copies/ml for the IL-1β lentiviral particles and 2.42×10^{8} copies/ml for the GFP lentiviral particles. The lentivirus particles were then purified and stored at -80°C until use.

2.4: Sterotaxic surgery

Rats were anaesthetised with isoflurane and placed into a Kopf stereotaxic frame. Lentiviruses to overexpress mouse IL-1β or mCherry (Genecopoeia, MD, USA) were injected into the dorsal hippocampus using the coordinates AP: -3.5 mm, ML: ± 2.4 mm, DV: -3.8 mm relative to Bregma (Barrientos et al., 2002) at a volume of 2µl and a flow rate of 1µL/min followed by a 5 min diffusion. Rats were injected s.c. with carpofen and 5% glucose prior to anaesthetic recovery. All rats were allowed to recover for 3 weeks with *ad libitum* access to regular chow and water to allow viral uptake prior to beginning behavioural testing.

2.5: Open field

Rats were placed in an open field arena (90 cm diameter and 45cm wall height) under bright lighting conditions for 10 minutes. Behaviours were recorded, and distance travelled, velocity, and time in the centre of the arena were calculated using Ethovision software (Noldus). The arena was cleaned with a 50% ethanol solution between exposures of each animal to the arena to remove odour cues.

2.6: Spontaneous Alternation in the Y maze

Spontaneous alternation behaviour is the tendency of rodents to alternate their exploration of maze arms (such as those of the Y maze) and is used as a measure of hippocampal-dependent short-term working memory (Hughes, 2004). The Y maze consisted of three arms 120° from each other (16 cm x 6.5 cm; made in house). The protocol was adapted from (Senechal et al., 2007). Each animal was placed into the first arm of the maze facing the wall, and allowed to explore the maze for five minutes. The number and order of arm entries were recorded. An arm entry was

defined as all four paws entering into the arm (four paw criteria). An alternation was determined as the number of consecutive entries into the three maze arms. Alternations were then divided by the total number of entries during the five minute test period.

2.7 Novel Object Recognition

Novel object recognition was assessed as described (Bevins and Besheer, 2006). The objects used for this task were either a 250ml Fisherbrand^{TM} glass bottle (Thermo Fisher Scientific, MA, USA) and/or a coffee cup. On day 1, the animals were habituated to the rectangular testing arena (45cm x 80cm with 45cm wall height) for a 10 minute exploration period. On day 2, two identical objects were positioned on adjacent corners approximately 5 cm from each wall of the arena and each animal was introduced for a 10 minute exploration period. Animals were then placed directly back into their home cages. After a 3 hour inter-trial interval, one familiar object was replaced with a novel object, and each animal was introduced for a 5 minute exploration period. Object exploration was defined as when the animal's nose comes within a 2 cm radius of the object. The testing arena and objects were cleaned a 50% alcohol solution in between each exposure. Videos were then manually scored for object exploration.

2.8 Contextual and cued fear conditioning

We utilised contextual and cued fear conditioning paradigms to determine the effects of our experimental interventions on hippocampal and amygdala-associated function, respectively (Maren, 2001, Pattwell et al., 2011). During the acquisition phase, rats were placed into fear conditioning context A (Med Associates, VT, USA) which

consisted of a rectangular chamber with a grid floor and scented with a lemon and ginger tea bag (Twinings^{TM}). Animals were acclimatised to the chamber for 2 minutes after which they received three tone and shock pairs (30 s tone; 5 kHz; 70 dB; 1 s foot shock; 0.35 mA DC current) separated by 30 s intervals. Animals remained in context A for 1 minute after the final shock at which point they were returned to their home cage. Contextual fear memory was assessed 24 hours when animals were returned to context A chamber but did not receive a paired tone/shock. Time spent freezing was measured during a total 5.5 min protocol using specialized software (Video freeze, Med Associates, USA). Cued fear conditioning and memory extinction was measured 24 h after the contextual test in the same chambers but with contextual cues altered so that the animals experienced a novel context (context B). Context B consisted of a white floor, black wall insert at 60°, and scented with almond extract (1% in water). Animals were allowed 2 minutes to acclimatize to context B followed by 10 tone presentations (30 s; 5 kHz; 70 dB) separated 30 seconds apart. Freezing behaviour during the 30 second tone presentations was recorded (Video freeze,MedAssociates, USA) to assess cued memory recal and extinction of the cued memory.

2.9: Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from hippocampal tissue using the mirVana^{TM} total RNA extraction kit (Ambion/Life Technologies, Dublin, Ireland) and and DNase treated using Turbo DNA-free kit (Ambion/life technologies) as per the manufacturer's instructions. The total concentration of extracted RNA was quantified using a Nanodrop 2000 (Thermo Scientific, UK) and was stored at -80°C. cDNA was reversed transcribed using the high capacity cDNA reverse transcription kit (Applied

Biosystems) using the SureCycler® 8800 (Agilent Technologies) and diluted to a final concentration of 10ng/µl. All qRT-PCR was performed in 3 technical replicates for each biological sample on a LightCycler® 480 Instrument II (Roche) using the following primers: Synaptophysin F: CCCTTCAGGCTGCACCAA, R: TTGGTAGTGCCCCCTTTGAC; PSD95 F: ACGCCGAAGAGTCAGAGAAA, R: ACTGTTGGACCGAGTGAACC; R: CCCATTTGGGAACTTCTCCT, CXCL1: F: CTCCAGCCACACTCCAACAG, R: GACTTCGGTTTGGGTGCAGT and SHDA F: CCCACTAACTACAAGGGACAGG, R: TTGGCACCATGCACTGAG. The PCR consisted of the following steps: 95°C for 2 minutes to denature the cDNA followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. Each reaction consisted of 1µl of sample (5ng/ul), 5µl of Sybr MasterMix (KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX™ for ABI instruments, Sigma-Aldrich), 0.1µl of both forward and reverse primers, and 3.8µl of RNase free H_2O. Results were normalised to succinate dehydrogenase (SDHA). Quantification of gene expression was analysed using the $\Delta\Delta$ C_t method (Livak and Schmittgen, 2001).

2.10: Western Blot

Total protein was extracted from hippocampal tissue, separated using SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane as previously described (Green and Nolan, 2012a, Nolan et al., 2005). The membrane was then incubated with primary antibodies for IL-1β (R&D systems, goat anti-mouse IL-1β polyclonal, 1:500) and β-actin (Sigma, mouse polyclonal, 1:1000) and subsequently incubated with the appropriate secondary HRP tagged secondary antibodies. Proteins were then visualised on exposure film using ECL detection kit (GE healthcare). Densitometry analysis of the developed

immunoblot bands was carried out using ImageJ software. Intensity readings for each band were expressed as arbitrary units relative to normalised to controls (standard chow fed non-IL-1β injected rats).

2.11: ELISA

ELISA was carried out on serum collected from cafeteria diet fed animals before the start of the diet, immediately after the end of the diet, and at culling. Leptin and insulin concentrations were determined by following the manufacturers' guidelines (Millipore).

2.12: Statistics

One way or Two-way analysis of variance (ANOVA) tests were used where appropriate to determine statistical significance followed by the Bonferoni *post-hoc* test. Results were expressed as mean ± SEM and deamed significant when $P \leq 0.05$.

3: Results

3.1 Animals fed a cafeteria diet during adolescence consume foods with significantly larger energy content but no change in body weight.

Neither adolescent cafeteria diet consumption nor IL-1β (in combination or alone) induced any significant change in body weight of the rats throughout the study (Fig. 5.1B). Animals receiving the cafeteria diet consumed foods with a significantly larger energy content than control animals (Main diet effect during diet phase of the study $F(1,28)=474.004$, $P=0.0001$; main effect of time $F(1, 21)=353.8$, $P=0.0001$; Interation between diet and time $F(1,27)=9.211$, $P=0.0004$; ***$P<0.001$ diet vs. controls, Fig. 5.1C). Immediately after the end of the cafeteria diet, animals consumed foodstuffs amounting to lower energy content than control chow fed animals and this was mirrored by the amount of chow intake by animals that were previously fed the cafeteria diet (Main diet effect $F(3,120) = 31.53$, $P=0.0003$; main effect of time $F(9,120)=106.4$, $P=0.0001$; interaction between diet and time $F(27,120)=31.53$, $P=0.0001$; ***$P<0.001$ compared to controls, Fig. 5.1D, E). This effect normalised within two weeks of cessation of the cafeteria diet. The component of the cafeteria diet for the duration of the study predominantly consumed was high fat foodstuffs (Fig. 5.1F).

3.2 Lentivirus overexpression of IL-1β induced significantly increase levels of hippocampal IL-1β at 6 weeks post surgery.

Confirmation of lentiviral mediated overexpression of IL-1β was carried out by determining IL-1β protein expression in the hippocampus. There was a significantly elevated expression of IL-1β protein in the hippocampus 6 weeks after animals were injected with a lentivirus overexpressing IL-1β compared to rats injected with a

lentivirus overexpressing mCherry and this effect was not altered by the diet (Significant virus effect F(1,26)=28.319, *P*=0.0002; Diet effect F(1,26)=1.443, *P*=0.240; Interaction F(1,26)=1.451, *P*=0.239; ***$P<0.001$ vs ctrl, Fig. 5.2A).

3.3 Adolescent cafeteria diet consumption leads to sustained elevation in serum leptin and insulin concentrations

The serum concentration of the metabolic hormones leptin and insulin were measured in the cafeteria diet fed animals using ELISA prior to the start of the diet (baseline), immediately after the diet ended (T1), and again at the time of culling (cull). Consumption of the cafeteria diet induced a significant increase in both leptin and insulin serum concentrations at T1 and cull compared to baseline for both diet fed groups and this result was not affected by virus (Main effect of timepoint on leptin F(2,20)=35.85, *P*=0.0001; and insulin F(2,16)=8.66, *P*=0.0028; No effect of virus on leptin F(2,20)=0.041, *P*=0.949 or insulin F(2,16)=0.109, *P*=0.749 *$p<0.05$ compared to baseline counterparts, Fig. 5.2C, D). In animals in which IL-1β was overexpressed within the hippocampus, there was a higher insulin concentration maintained at cull compared to animals injected with a control lentivirus however after repeated-measures two-way ANOVA analysis this difference did not reach significance (Fig. 5.2D).

3.4 Adolescent cafeteria diet consumption and/or IL-1β does not alter anxiety-like behaviour

Animals fed the cafeteria diet during adolescence with or without hippocampal IL-1β overexpression or animals with IL-1β overexpression alone did not show any changes in anxiety-like behaviour as all groups of animals spent a similar percentage

of time within the center of the open field arena (Fig. 5.2E). Additionally, all animals travelled similar distances within the open field demonstrating no changes in locomotor activity across the groups (Fig. 5.2F).

3.5 IL-1β impairs spatial working memory but enhances contextual fear memory recall

Adolescent cafeteria diet consumption did not induce any changes in spontaneous alternation behaviour in the Y-maze, object recognition memory, contextual fear conditioning, or cued fear conditioning (Fig. 5.3A, D, F-H). There was a main effect of IL-1β observed on spontaneous alternation in the Y-maze (Main effect of IL-1β $F(1,31)=5.571$; no effect of diet $F(1,31)=0.135$, $P=0.716$; no interaction effect $F(1,31)=1.996$, $P=0.171$; *$p<0.05$, Fig. 5.3A) while it had no effect on novel object recognition (Fig. 5.3D). All animals successfully acquired a freezing response to the tone stimulus (Fig. 5.3F). IL-1β significantly enhanced contextual fear memory recall compared to controls (Main effect of IL-1β $F(3,12)=12.14$, $P=0.004$; no effect of diet $F(3,12)=2.9$, $P=0.24$; No interaction effect $F(3,34)=1.118$, $P=0.3505$; *$p<0.05$ vs Ctrl/mCherry and #$p<0.05$ vs Diet/IL-1β Fig. 5.3G) whereas no effect was observed on cued fear memory recall (IL-1β effect: $F(3,34)=0.1671$, $P=0.9178$; diet effect $F(3,34)=0.2164$, $P=0.8843$ Fig. 5.3H). All groups display similar memory extinction rates in response to multiple tones in the absence of shock stimuli (Fig. 5.3H).

3.6 IL-1β significantly increases post-synaptic synaptic but not pre-synaptic plasticity genes in the hippocampus.

After two-way ANOVA analysis, there was no significant difference in syanpatophysin (Diet effect $F(1,26)=1.966$, $P=0.173$; virus effect $F(1,26)=1.082$, $P=0.308$) or PSD95 (Diet effect $F(1,26)=0.01$, $P=0.986$; virus effect $F(1,26)=0.995$, $P=0.328$) mRNA expression across any of the groups (Fig. 5.4C, E). There was a significant effect of diet on CXCL1 expression ($F(1,26)=5.632$, $P=0.025$; #$p<0.05$ between Ctrl/IL-1β and Diet/IL-1β , Fig. 5.4B).

Discussion

There is a wealth of evidence to suggest that consumption of a high fat and/or high sugar diet during adolescence can have detrimental effects on hippocampal associated cognition (Vendruscolo et al., 2010, Boitard et al., 2014, Boitard et al., 2012, Reichelt et al., 2015). However, more recent data suggests that the effect of a high fat only diet on hippocampal cognition may be reversed by removal of the diet and replacement with normal chow in adulthood (Boitard et al., 2016). Our data supports the later finding. We demonstrate that three weeks of cafeteria diet consumption high in both sugar and fat during the adolescent period (PND28 – PND56), does not result in cognitive deficits in adulthood when the animals were switched to a normal chow diet at adulthood for three weeks prior to testing. However, the cafeteria diet fed animals still maintained altered levels of leptin and insulin in the serum six weeks after the diet had been replaced with standard chow. Additionally, we demonstrate that hippocampal overexpression of the proinflammatory cytokine IL-1β during adulthood does not induce any cognitive impairments in cafeteria diet fed animals. Surprisingly, we demonstrate that overexpression of IL-1β during adulthood after normal diet intake during adolescence, induces impairments in spatial working memory and an enhancement in fear memory. This behavioural effect was complemented with increases in markers of synaptic plasticity and chemokine expression in the hippocampus.

Previous work has shown that high fat/high sugar diets induce impairments in cognition during adolescence (Boitard et al., 2014, Boitard et al., 2012, Vendruscolo et al., 2010). These impairments have predominantly been demonstrated in hippocampal-associated spatial learning and memory tasks in rodents. For example,

Boitard et al demonstrated that after one or two months of high fat diet consumption starting at weaning, there was a significant impairment in long-term memory retention in the Morris water maze (despite no effect on acquisition or short-term memory retention) (Boitard et al., 2014). That study maintained the high fat diet throughout behavioural testing whereas in our study, the animals had been switched to a regular chow diet three weeks prior to the start of behavioural testing. This then may explain why we saw no effect on diet on any behavioural paradigm. Additionally, we assessed behaviour in different hippocampal-associated tasks than the ones used by Boitard et al and so adolescent cafeteria diet may only affect certain hippocampal-associated cognitive processes. Indeed this has been previously suggested as rats tested in a location recognition task after 20 days of high fat and high sugar diet consumption showed impairments in this task whereas no impairments were observed in an object recognition task (Beilharz et al., 2014) similar to what we observed. However, as with the work by Boitard et al, this study maintained the diet throughout behavioural testing. Therefore, the discrepancies in these studies compared to ours are most likely due to the fact that any effects that the diet may have had on hippocampal-associated cognition are likely reversed after a washout period. In this regard, that when rats that had consumed a high fat diet for three months starting at weaning, were switched to a regular chow diet and given a three month washout period before behavioural testing, no effect of adolescent high fat diet was observed in adulthood (Boitard et al., 2016).

It has previously been demonstrated that consumption of a high fat diet can induce an increase in the expression of pro-inflammatory cytokines such as IL-1β and TNFα as well as increased microglial activation in brain regions including the

hypothalamus and hippocampus (Hansen et al., 1998, Beilharz et al., 2014, Pistell et al., 2010, Boitard et al., 2014). Thus, we hypothesised that adolescent exposure to a high fat cafeteria diet could increase the susceptibility of the hippocampus to inflammation during adulthood even after the cafeteria diet has been switched to a regular chow. IL-1β itself has been demonstrated to have robust effects (both positive and negative) on hippocampal function such as impairments spatial navigation in the Morris water maze and increase contextual fear memory recall (Farrar et al., 1987, Yirmiya et al., 2002, Goshen et al., 2008, Goshen et al., 2007, Avital et al., 2003, Moore et al., 2009). However, in the current study, we found that overexpression of IL-1β during adulthood did not unmask any behavioural phenotype induced by adolescent cafeteria diet consumption. Despite this, IL-1β alone during adulthood impaired spontaneous alternation in the Y-maze and enhanced contextual recall in a fear conditioning paradigm. Thus, it would appear that adolescent diet consumption negated the cognitive effects of IL-1β-induced changes in fear learning.

A potential explanation is that alterations in factors induced by the cafeteria diet such as metabolic hormone and/or inflammatory markers may be impairing the ability of IL-1β to modulate cognition. For example, the expression of the IL-1RA has been shown to be significantly increased in the serum of obese patients (Meier et al., 2002). Additionally, mice fed a high fat diet (albeit for 35 weeks) have been shown to have elevated serum IL-1RA expression (Somm et al., 2006). As IL-1RA is known to cross the blood brain barrier (Gutierrez et al., 1994) this could impair IL-1β signalling within the hippocampus thus negating the IL-1β-induced changes in fear learning. However, this is rather simplistic view as high fat and high sugar diets

have been known to induce the expression of a plethora of inflammatory mediators such as IL-10, IL-6, and TNFα (Pistell et al., 2010, Boitard et al., 2014) as well as peripheral metabolic hormones that can regulate cognition. Indeed, we demonstrated significant main effect of diet on CXCL1 expression. CXCL1 is a chemoattractant (Johnson et al., 2011) and it may induce the migration of inflammatory cells such as astrocytes and microglia to the hippocampus which in turn can release inflammatory regulators (e.g. IL-1RA and IL-6) that could impact upon IL-1β-induced changes in hippocampal function.

Both spontaneous alternation in the Y-maze and contextual fear conditioning are reliant on some aspect of hippocampal function as studies where the hippocampus is lesioned or damaged demonstrated impairments in these tasks (Gerlai, 1998, Johnson et al., 1977, Saxe et al., 2006, Anagnostaras et al., 2001, Maren and Fanselow, 1997, Maren et al., 1997). Increased expression of IL-1β has been shown to impair performance in hippocampal-associated tasks such as spatial navigation in the water maze (Moore et al., 2009). However, there is also evidence available to suggest that IL-1β is required for certain forms of hippocampal-associated memory. Initially it was demonstrated that interleukin-1 receptor type 1 (IL-1R1) knockout (KO) mice have longer latencies to find the hidden platform in the Morris water maze test of spatial navigation and reduced contextual fear conditioning responses (Avital et al., 2003). IL-1R1 KO mice in that study also had reduced hippocampal long-term potentiation (LTP) formation however there were no deficits observed in hippocampal-independent tasks such as guided spatial navigation and cued fear conditioning. Subsequently it was demonstrated in rats that underwent fear conditioning based contextual learning that there is an increase in hippocampal IL-1β

expression during the learning phase (Goshen et al., 2007). Our data closely resembles the results found in these studies in that we see and enhancement in contextual fear conditioning as well as synaptic plasticity markers in rats exposed to IL-1β only. There is a strong argument that IL-1β at lower concentrations may enhance certain hippocampal-associated memory formation (Goshen et al., 2007, Avital et al., 2003), particularly in fear based learning paradigms, whereas it may impair spontaneous spatial memory performance as suggested from our data. However, determining the exact concentration and duration of IL-1β required to either enhance or impair cognition are largely unknown and would be an interesting avenue for future research.

It must also be acknowledged that we did not observe any difference induced by IL-1β on novel object recognition. Thus some, but not all, hippocampal associated tasks are sensitive to changes in hippocampal IL-1β expression. It is important to bear in mind that the tasks assessed in this study each reflect a different aspect of hippocampal-associated cognition. Spatial working memory is assessed in the spontaneous alternation task with very little learning involved (Gerlai, 2001, Gerlai, 1998). Fear conditioning involves a strong learning or acquisition aspect to successfully complete the task (Phillips and LeDoux, 1992, Anagnostaras et al., 2001). Novel object recognition does involve a minor learning component in that the animals must successfully encode or learn the memory of the familiar object (Cohen and Stackman, 2015, Aggleton and Brown, 2005, Bevins and Besheer, 2006). However, the perihinal cortex is also said to be involved in object recognition and depending on the duration if the intertrial-interval, may be more responsible for successful completion of the task than the hippocampus (Cohen and Stackman,

2015). Therefore, whether IL-1β negatively, positively or has any affect on hippocampal cognition depends on the neuronal circuitries recruited, the intertrial-interval used, or even the degree to which learning is involved in performing the task (Goshen et al., 2007, Barrientos et al., 2002, Gonzalez et al., 2013, Hein et al., 2010).

We assessed the expression of the pre-synaptic plasticity marker synaptophysin and the post-synaptic plasticity marker PSD95 in an attempt to determine whether the effects of IL-1β on cognition are associated with synaptic changes. Although we observed an increase in PSD95 expression it did not reach significance after two-ANOVA analysis. Additionally, we saw no significant effect on synaptophysin expression. Increased expression of these markers is considered an indication of increased synaptic plasticity (Janz et al., 1999, El-Husseini et al., 2000). For example, it has been demonstrated that after environmental enrichment, a process known in enhance synaptic plasticity rodents (Moser et al., 1994, Rampon et al., 2000), there is also an increase in both synaptophysin and PSD95 in the hippocampus (Nithianantharajah et al., 2004). It could be possible that PCR analysis is not sensitive to detect significant change in the mRNA expression of synaptophysin or PSD95 and so more work is needed to determine the role of lentiviral mediated enhancement of IL-1β expression on regulating hippocampal synaptic plasticity. As IL-1β at low concentrations has been suggested to promote LTP (a functional read-out of synaptic plasticity) (Di Filippo et al., 2008), it would be of value to determine the exact concentration of IL-1β is being produced in our lentiviral approach and whether other measures of synaptic plasticity such as LTP are affected.

In conclusion, we demonstrated that three weeks of cafeteria diet consumption during adolescence does not induce impairments in hippocampal-cognitive performance in adulthood and this is not affected by increased hippocampal IL-1β expression in adulthood. IL-1β in adulthood impairs spatial working memory and enhances fear memory coupled thus highlighting the complex role of IL-1β in regulating hippocampal-associated cognitive processes. Further studies should aim to address how adolescent cafeteria diet consumption may impair IL-1β-mediated regulation of hippocampal cognitive processes by determining the dietary-induced changes in expression of metabolic or inflammatory factors that can impact upon IL-1β signalling. Taken together, although poor diet consumption during adolescence may not induce lasting negative effects on cognition, it may negatively impact upon potential facilitators of learning and memory during adulthood.

Product	Brand	Energy (kJ/100g)	Fat (g/100g)	Sugar (g/100g)
Frosted Shreddies	Nestle	1556	1.5	27.0
Marshmallow Mateys	MaltOMeal	1611	3.5	41.0
Ripple Swiss Roll	Gateaux	1538	11.1	42.5
Jaffa Cakes	Sondey	1605	11.3	47.0
Custard Cream Biscuits	Tesco	2066	20.9	28.5
KitKat (Regular)	Nestle	2141	24.8	52.7
Digestive Biscuits	Tesco	2057	22.4	18.5
Toffee Cake	Tesco	1773	18.6	39.0
Peanut Butter Oreos	Mondelez	2014	21.0	34.0
Magic Stars	Milkyway	2330	34.7	53.6
Mini Rolls	Cadbury	1810	23.0	42.4
Battenberg Cake	Gateaux	1779	12.0	56.9
Plain M&Ms	Mars	2017	19.3	67.7
Jelly Babies	Sugarland	1445	0.1	69.3
Jelly Beans	Tesco	1548	0.7	60.2
Jelly Snakes	Natural Foods	1380	0.2	65.5
Cocoa Pops	SuperValu	1654	2.8	33
Buttersalt Popcorn	Perri	2252	36.4	0.1
Mild White Cheddar Cheese	Tesco	1725	34.9	0.1
Frankfurters	Dulano	1295	28.0	1.0
Everyday Value Ham	Tesco	1116	4.1	2.6
Sausage Rolls		1420	19.5	2.1
California Walnuts	Alesto	2938	69.1	3.0
BBQ Rib Flavour Doritos	Doritos	2060	25.6	3.4
Cheezit	Sunshine	2090	26.7	0.0
Jumbo Dry Roasted Peanuts	Tesco	2448	46.5	4.8
Chopped Pork and Ham	Dulano	1103	22.0	1.3
Ready Salted Crisps	Tesco	2270	33.2	0.4
Hula Hoops Cheese + Onion	KP Snacks	2137	27.0	2.0
Chow	Envigo	1300	6.2	

Table 5.1: Food list with nutritional information used for cafeteria diet and chow given to rats.

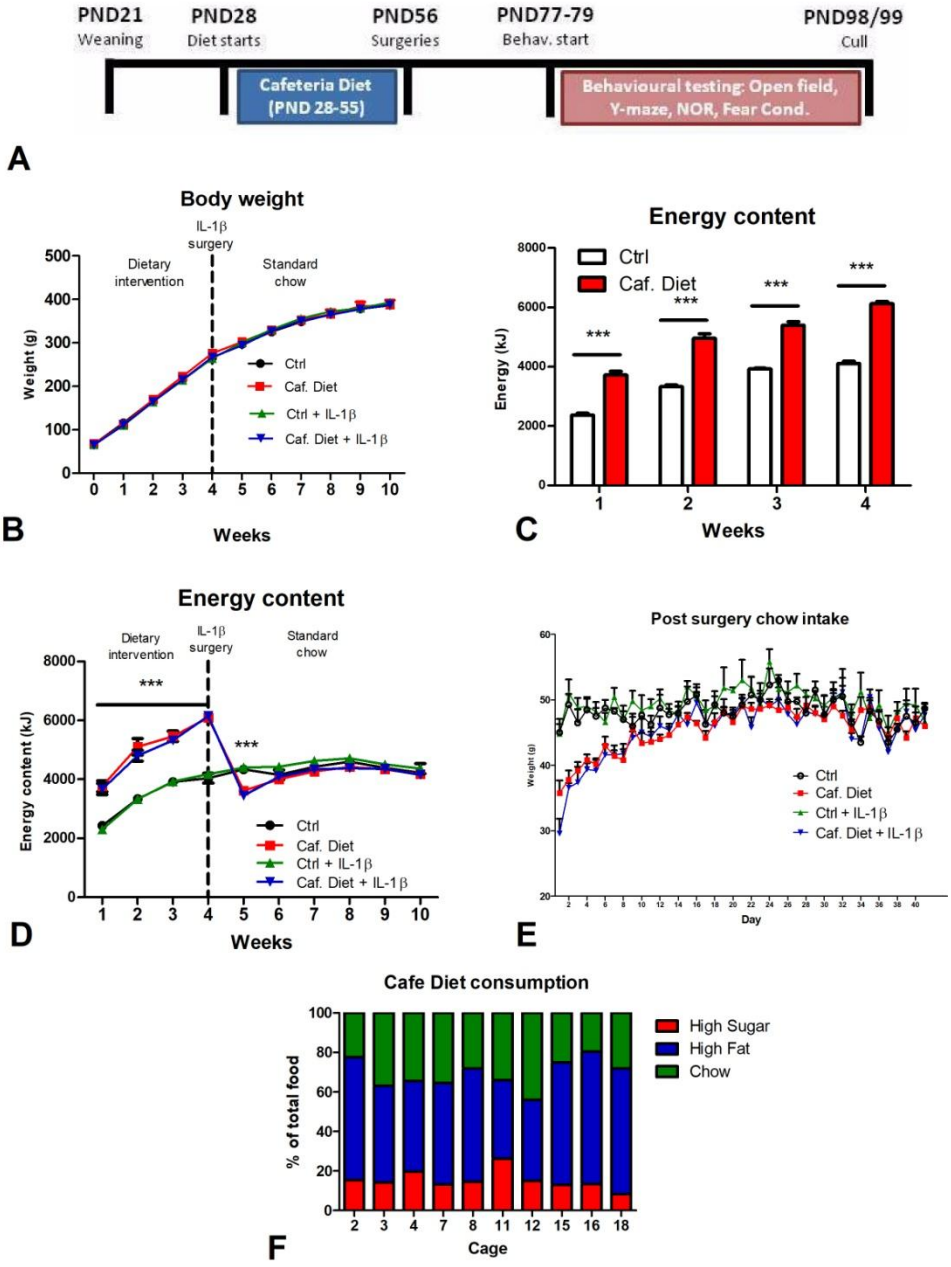


Figure 5.1: A) Experimental timeline. **B)** Body weight in of rats across the duration of the dietary intervention. **C)** Energy in kilojoules (kJ) of consumed foodstuffs across the duration of the dietary intervention. **D)** Energy in kilojoules (kJ) of consumed foodstuffs across the duration of the study. **E)** Regular chow intake by all animals averaged across each cage after surgery. **F)** Proportion of each cafeteria diet component consumed across the duration of the dietary intervention and as a percentage of total food eaten. PND, post-natal day. All data are expressed as mean

± SEM, n=8-10 for all groups, ***P<0.001 compared to controls, Two-way ANOVA and Bonferoni *post hoc*.

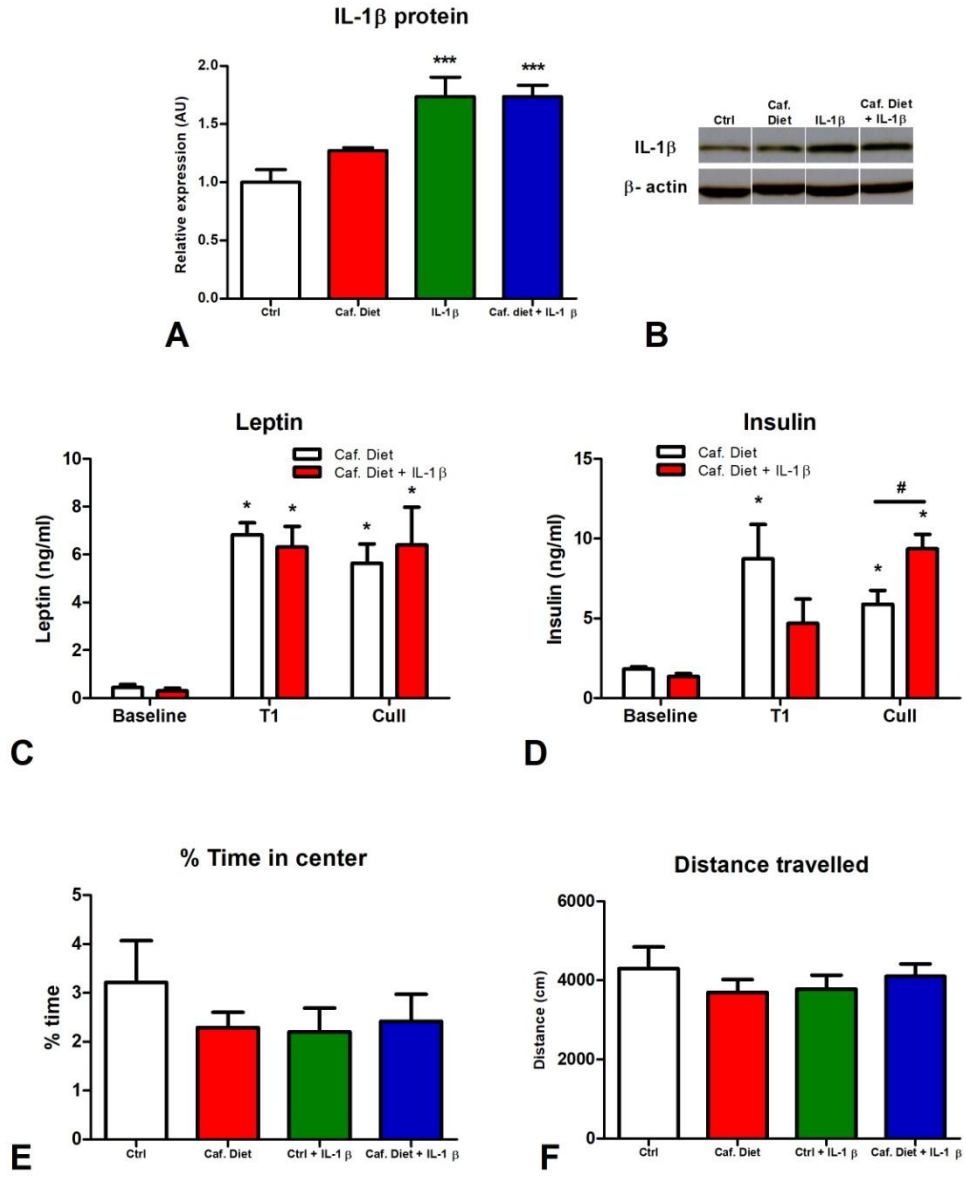


Figure 5.2: Administration of lentivirus induces significant increase in IL-1β expression and cafeteria diet consumption results in persistent elevated metabolic hormone concentration. **A)** Confirmation of IL-1β protein overexpression in the hippocampus (n=6-8). **B)** Representative immunoblot of mouse IL-1β protein expression from hippocampal tissue. **C)** Serum leptin concentration before (baseline) and immediately after (T2) diet and at cull (Cull) (n=5-7). **D)** Serum insulin concentration before (baseline) and immediately after (T2) diet and at cull (Cull) (n=5-7). **E)** Percentage time spent in the centre of the open field arena (n=8-10). **F)** Distance travelled in the open field (n=8-10). All data are expressed as mean ± SEM. Fig 2A, ***P<0.001 compared to controls; Fig 2C, D, *P<0.05 compared to

respective baseline, #$P<0.05$ compared to adolescent diet at cull, Two-way ANOVA and Bonferoni *post hoc*.

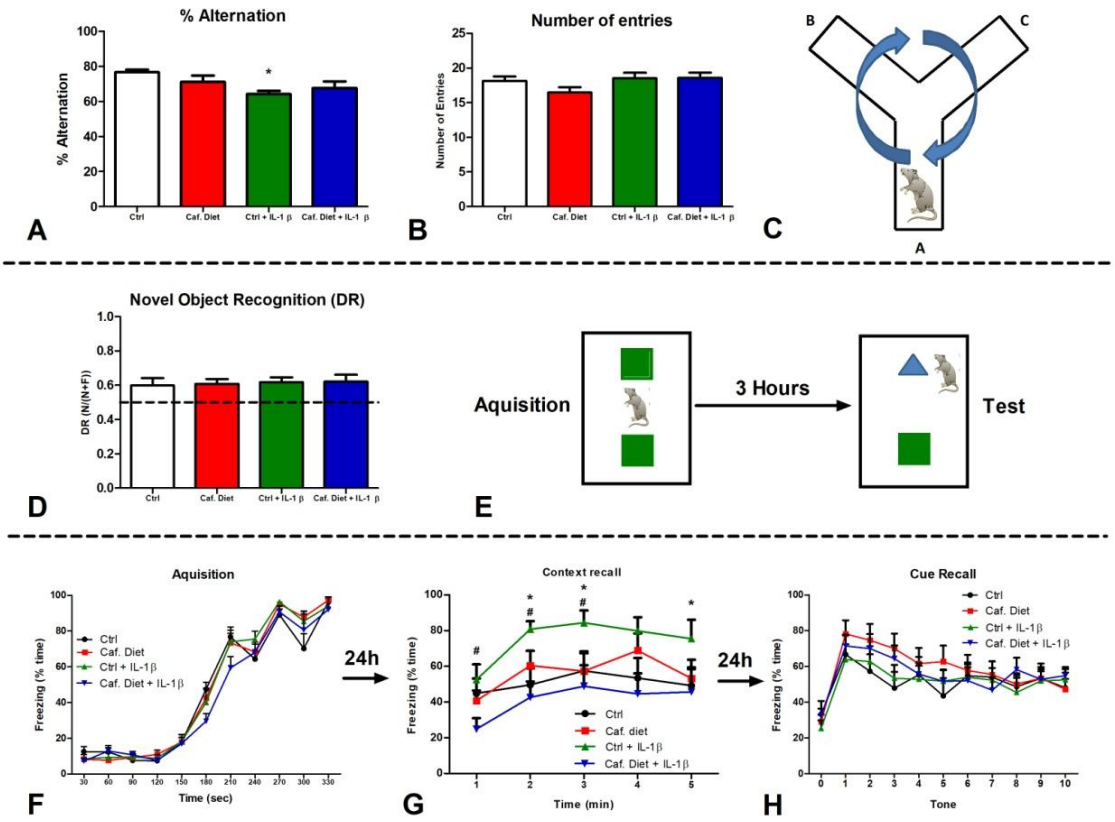


Figure 5.3: Cafeteria diet has no effect on hippocampal cognitive processes in adulthood whereas IL-1β impairs spatial working memory and enhances contextual fear conditioning. **A)** Percentage alternation in the Y-maze (*p<0.05 between ctrl and ctrl + IL-1β, Two-way ANOVA and Bonferoni *post hoc*). **B)** Number of arm entries in the Y-maze. **C)** Schematic of spontaneous alternation task. **D)** Novel object recognition depicted as discrimination ratio (DR) between novel (N) and familiar (F) objects. **E)** Schematic of novel object recognition task. **F)** Acquisition phase of fear conditioning paradigm. **G)** Freezing behaviour during contextual recall (*P<0.05 compared to control, and #P<0.05 compared to Diet/IL-1β, Two-way ANOVA and Bonferoni *post hoc*). **H)** Freezing behaviour during contextual recall. All data are expressed as mean ± SEM, n=8-10.

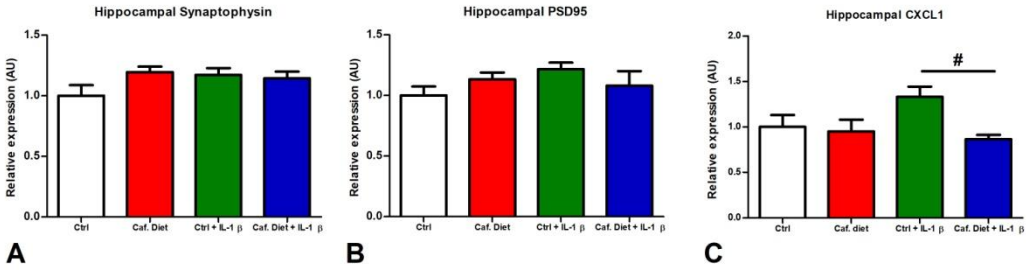


Figure 5.4: The effects of cafeteria diet and/or IL-1β overexpression and hippocampal gene expression. **A)** Relative expression of hippocampal synaptophysin. **B)** IL-1β induces a significant increase in PSD95 expression. **C)** Relative expression of hippocampal CXCL1 (#$P<0.05$ vs Caf. Diet + IL-1β). All data are expressed as mean ± SEM, n=6-8.

Chapter 6: General discussion

Over the past two decades there has been increasing evidence supporting the notion that hippocampal neurogenesis is involved in certain types of hippocampal cognitive function, particularly learning and memory and pattern separation (van Praag et al., 2002, Kempermann et al., 2004b, Zhao et al., 2008, Clelland et al., 2009). Indeed it has been suggesting that hippocampal neurogenesis may also interfere with the stability of existing memories and thus may have a function in the process of forgetting (Frankland et al., 2013, Akers et al., 2014, Epp et al., 2016). As our understanding of the functional roles of hippocampal neurogenesis in both health and disease continues to be refined (Groves et al., 2013, Glover et al., 2017, Levone et al., 2015, O'Leary and Cryan, 2014), a greater comprehension of the regulators of neurogenesis is required. Some of the most common positive regulators of neurogenesis include exercise (van Praag et al., 1999b), environmental enrichment (Kempermann et al., 1997b), learning (Gould et al., 1999), and healthy diet (Valente et al., 2009). Conversely, common negative regulators of neurogenesis include stress (Malberg and Duman, 2003), poor diet (Lindqvist et al., 2006), and neuroinflammation (Monje et al., 2003). It has been suggested that decreases in hippocampal neurogenesis could be responsible for some of the cognitive deficits observed in many neurodegenerative and psychiatric disorders (Balu and Lucki, 2009, Horgusluoglu et al., 2017, Winner et al., 2011, Lie et al., 2004, Lazarov et al., 2010) and a common factor in many of these disorders is the presence of hippocampal inflammation (Ransohoff, 2016, Heppner et al., 2015, Rao et al., 2010, Khansari and Sperlagh, 2012). However, whether intrinsic regulators of neurogenesis could be targeted therapeutically to mitigate cognitive deficits remains to be elucidated.

Neuroinflammation is a well established suppressor of hippocampal neurogenesis and a key component of neuroinflammation is the pro-inflammatory cytokine IL-1β (O'Leime et al., 2017a, Goshen et al., 2007, Yirmiya and Goshen, 2011). It is generally understood that IL-1β is detrimental to hippocampal neurogenesis however some studies have demonstrated that IL-1β can indeed enhance certain forms of hippocampal neurogenesis-associated cognition namely contextual fear conditioning (Yirmiya et al., 2002, Goshen et al., 2007). Whether IL-1β has positive or negative effects on hippocampal neurogenesis-associated cognition largely depends on the concentration present in the hippocampus. However, whether the negative effects of IL-1β could be exacerbated by pre-exposure to a negative regulator of neurogenesis such as poor diet merited further research.

TLX is a nuclear receptor expressed in NPCs within the hippocampus (Niu et al., 2011, Li et al., 2012, Shi et al., 2004, Islam and Zhang, 2014). As a regulator of gene expression, TLX acts to maintain NPCs in a proliferative state thus maintaining the NPC pool within the neurogenic niche of the hippocampus (Niu et al., 2011, Shi et al., 2004). It has previously been shown that enhancing TLX expression can promote certain hippocampal-associated cognitive processes in mice including spatial memory in the MWM (Zhang et al., 2008, Murai et al., 2014). TLX expression has been suggested to be negatively impacted by neuroinflammation. Specifically, the pro-inflammatory cytokine IL-1β has been shown to reduce proliferation and TLX expression in proliferating hippocampal NPCs (Ryan et al., 2013, Green and Nolan, 2012b). However, whether modulation of TLX expression could prevent IL-1β-induced effects on neurogenesis was not previously determined. Likewise, the effects

of modulating TLX expression on hippocampal-associated behaviours in animal models other than mice were not assessed prior to this thesis.

The present series of studies are divided into three main themes. Firstly, chapters 2 and 3 assessed the interactions between TLX and IL-β both in hippocampal NPCs *in vitro* and in the hippocampus using *in vivo* approaches. Specifically, these chapters aimed to determine the functional role of TLX in mitigating IL-1β-induced effects on neurosphere expansion and the transcriptional role of TLX in regulating IL-1β target genes, respectively. Secondly, chapter 4 aimed to determine the effects of over expressing TLX within the hippocampus of adult rats on hippocampal-associated cognitive tasks. Finally, chapter 5 deals with the impact of a diet high in fat and sugar during adolescence on IL-1β-induced changes in hippocampal-associated cognition.

Chapter 2 of this thesis described the inhibitory role of IL-1β on TLX expression in proliferating hippocampal NPCs and the role that NF-κB signalling had in mediating this effect. Additionally, this chapter outlines the potential protective capacity of TLX in mitigating the negative effects of IL-1β on neurosphere expansion. We ensured that our data was in line with previously published data (Green and Nolan, 2012b) by confirming that treatment of hippocampal neurosphere cultures with IL-1 β for 6 days produced a significant reduction in neurosphere expansion as well as TLX expression. We also demonstrated an increase in the expression of the TLX target gene p21 after IL-1β treatment for up to 6 days. p21 expression was suppressed by TLX and when its expression was increased it induced cell cycle arrest and reduced proliferation (Waga et al., 1994, Pechnick et al., 2011, Sun et al.,

2007). From this result, our data suggested that the increase in p21 expression may be involved in the impairment in neurosphere expansion, as expansion has been closely correlated with NPC proliferation (Widera et al., 2006, Murai et al., 2014).

In several cell types including rat hippocampal NPCs, mouse embryo fibroblasts, and human epithelial cells (Seitz et al., 2000, Chen et al., 2006, Koo and Duman, 2008), activation of the NF-κB pathway has been shown to suppress cell proliferation. As IL-1β has been shown to recruit this pathway in the hippocampus of rodents (Koo and Duman, 2008, Koo et al., 2010), we chose to determine whether it is activated in response to IL-1β specifically within hippocampal NPCs. We observed rapid translocation of the p65 subunit to the nucleus (an indicator of NF-κB activation (Xie et al., 2016, Zhu et al., 2015)) after IL-1β administration, which was coupled with activation of the NF-κB target gene, IκBα. JSH-23 is a well characterised inhibitor of NF-κB signalling (Koo and Duman, 2008, Arias-Salvatierra et al., 2011, de Vries et al., 2014, Kesanakurti et al., 2013), and in our study, co-treatment of NPCs with this compound and IL-β prevented the IL-1β-induced decrease in TLX expression and also reduced the increase in IκBα mRNA expression. Thus, the NF-κB pathway appeared to be a potential mediator of IL-1β-induced changes, not only on NPC proliferation as previously shown (Koo et al., 2010, Koo and Duman, 2008) but also on TLX expression. We did not observe any significant effect of JSH-23 on the TLX target gene, p21. JSH-23 has been established as a selective NF-κB inhibitor (Shin et al., 2004, Kumar et al., 2011, Arias-Salvatierra et al., 2011) however, as this compound had not previously been used specifically in hippocampal NPCs, we cannot rule out the fact that JSH-23 may have non-specific effects in these cells that could affect the expression of p21. That being said, as this compound attenuated

IκBα expression, our data are a proof of concept that NF-κB signalling is at least partially responsible for the effects of IL-1β on TLX expression.

Loss of TLX expression has been shown to result in both impaired NPC proliferation and neurogenesis (Islam and Zhang, 2014, Niu et al., 2011, Li et al., 2012, Shi et al., 2004). Conversely, it has been shown that over expressing or restoring TLX expression can rescue these proliferative defects (Murai et al., 2014, Zhang et al., 2008). From these studies we hypothesised that overexpression of TLX could rescue the effects of IL-1β on neurosphere expansion. We generated a lentivirus to over express TLX and used GFP as a control vector. In the GFP transfected neurospheres, expansion was impaired after IL-1β administration whereas TLX overexpression prevented this effect. Thus, these data suggest that an enhanced TLX function may have a protective effect against IL-1β on hippocampal NPC proliferation. However, these beneficial effects may only be evident when there is a potential insult such as neuroinflammation present as TLX was only maintained at a higher level in the cells co-treated with IL-1β and TLX overexpression compared to control NPCs treated with IL-1β.

Our data suggest that IL-1β acts at least in part via NF-κB to suppress TLX expression. However, it may be that TLX does not suppress NF-κB signalling to prevent the effects of IL-1β on neurosphere expansion. This suggestion comes from the fact that we did not observe any differences between GFP and TLX transfected NPCs with regards to IL-1β-induced increase in IκBα. If TLX were to suppress NF-κB signalling we would expect to see a differential response in NF-κB target genes in TLX OEX NPCs versus GFP OEX NPCs after IL-1β treatment. Thus, more

research is required to determine the exact signalling interaction between IL-1β and TLX. Additionally, it has been demonstrated that IL-1β can recruit signalling pathways other than NF-κB to exert its effects on neurogenesis (Wang et al., 2007, Zunszain et al., 2012a, O'Leime et al., 2017a). To follow on from our study, it may be beneficial to determine how TLX may associate with some of these IL-1β associated pathways to get a clearer picture as to how TLX and IL-1β interact.

RNASeq is a powerful tool to assess global transcriptome changes. In chapter 3 we took a sequencing approach to assess the regulatory role of TLX in IL-1β-induced changes within the hippocampus of mice. Specifically, we assessed these transcriptome changes in a mouse model in which there is a spontaneous deletion of the TLX gene (Young et al., 2002, Roy et al., 2002, O'Leary et al., 2016b). These mice lack TLX throughout development and as a result have impaired neurogenesis and associated cognition (Shi et al., 2004, O'Leary et al., 2016). In this chapter, we assessed the hippocampal transcriptome changes induced in these mice compared to WT control mice with normal expression of TLX. Our main finding from this study was the fact the TLX acts to maintain a normal inflammatory profile within the hippocampus as the absence of TLX leads to increased transcription of inflammatory related genes. We also highlighted the TNF pathway as a potential pathway through which TLX may act to assert its effects on hippocampal inflammatory gene transcription. Finally, we outline the vastly different transcription responses TLX KO mice have compared to WT controls in response to IL-1β.

Of the 1269 genes increased in TLX KO mice compared to WT mice, there was a strong enrichment in genes relating to inflammation. These genes included

transcripts for cytokines, cytokine receptors and markers of microglial activation. A recently published paper by our lab described that absence of TLX in a similar strain of mice induces a significant increase in hippocampal microglial activation and an increase in the level of pro-inflammatory cytokine IL-1β in the hippocampus (Kozareva et al., 2017a). The RNASeq data described in this thesis supports these cellular effects by confirming a robust transcriptional shift towards an inflammatory response within the hippocampus of TLX KO mice. Additionally, we identified a larger number of downregulated genes in TLX KO mice compared to WT mice, and the majority of these genes were involved in the regulation of synaptic signalling. Our data is again supported by previously published work where it was demonstrated in the same strain of TLX KO mice that there is a significant impairment in LTP formation within the DG (Christie et al., 2006).

As RNASeq projects generate vast amounts of data, we wished to utilise online network analysis platforms to gain an insight as to how TLX could be regulating inflammatory gene transcription. Using DAVID, we conducted KEGG pathway analysis on the upregulated genes identified in TLX KO mice to assess what cellular signalling pathways they are associated with. This analysis revealed that TNF signalling was strongly associated with the genes we identified as upregulated in TLX KO mice. Thus, from our data we hypothesise that TLX can regulate TNF signalling in some manner to repress the induction of an inflammatory profile. To further explore this hypothesis, we conducted STRING analysis which produces a network map of protein-protein interactions based on the differentially expressed genes identified from or sequencing data. STRING analysis highlights potential protein-protein interactions based on evidence from the literature and from online

protein databases. From this analysis, we identified some evidence that TLX may indirectly regulate TNF signalling via its protein interaction with *Top2a* and *Bcl6b*. However, STRING analysis highlighted that the evidence for an interaction between TLX and *Top2a* and *Bcl6b* is not strong as only structure homologs of these proteins have been shown to interact (Ku et al., 2009, Diner et al., 2015). As there is little research available into the role of TLX and the regulation of inflammation, STRING analysis software has little evidence to suggest potential interactions between TLX and inflammatory pathways. In this regard, more research is required to determine protein-protein interactions between TLX and inflammatory-related proteins. However, our data suggest that TNF signalling may be a potential candidate through which TLX represses inflammation.

A surprising finding from this study was the vastly different response to IL-1β between TLX KO mice and WT mice. TLX KO mice have a more blunted response with only 38 differentially expressed genes compared to 228 genes differentially regulated genes in the WT mice after IL-1β injection. We hypothesise that due to the fact the TLX KO mice have elevated transcription of inflammatory genes at baseline, these mice have reached an elevated inflammatory plateau and are unresponsive to further cytokine stimulus. Another consideration that must be made when discussing data generated from the strain of mice used in this study, is the fact that these mice are devoid of TLX throughout embryonic development and adulthood (Young et al., 2002). Thus, the alterations observed in inflammatory response to a cytokine stimulus are potentially due to the absence of TLX during critical stages in immune system and organ developmen (Monaghan et al., 1997, Roy et al., 2002). Therefore, future studies should aim to assess the regulatory role of TLX in models where TLX

is only absent in adulhood (Zhang et al., 2008). In conclusion, the data from this chapter positions TLX as a potential regulator of the inflammatory response with deficits in TLX expression leading to an elevated inflammatory response similar to that which occurs after a cytokine stimulus.

Previous work assessing the role of TLX in cognitive function utilised mouse models in which there was either a decrease or increase in TLX expression (Zhang et al., 2008, O'Leary et al., 2016b, Murai et al., 2014). The consensus from these studies is that TLX promotes neurogenesis-associated spatial learning and memory in the MWM (Murai et al., 2014, Zhang et al., 2008), while in a mouse strain where there is a spontaneous deletion in the TLX gene, impairments in contextual fear conditioning and spatial working memory were evident (O'Leary et al., 2016b). In chapter 4, we aimed to assess the role of enhanced TLX expression in promoting hippocampal-associated cognitive processes in rats. Firstly, we demonstrated that enhanced cell proliferation via OEX of TLX in the dorsal hippocampus does not induce anxiety-like behaviours. This is in line with some studies that also show that modulation of neurogenesis does not result in anxiety-like behaviours (Shors et al., 2002, Santarelli et al., 2003, Saxe et al., 2006, Leasure and Jones, 2008, Holick et al., 2008, Bessa et al., 2009). As TLX OEX was targeted at the dorsal hippocampus and it has been suggested that the ventral hippocampus is responsible for the regulation of emotion (Felice et al., 2012, O'Leary et al., 2012, Levone et al., 2015, O'Leary and Cryan, 2014), this then could explain why we did not observe any anxiety-like behaviours.

Secondly, we demonstrated that increased proliferation induced by TLX OEX impaired object recognition while it had no effect on location recognition or

spontaneous alternation in the Y-maze. Object recognition has previously been shown to rely on both the hippocampus and the perirhinal cortex with the 'flow' of information from one structure to the other being a critical factor in successful object recognition (Aggleton and Brown, 2005, Cohen and Stackman, 2015). Short term object exploration is thought to result in the perirhinal cortex being responsible for information processing during the object recognition task. It has been suggested that after approximately 30 seconds of object exploration, information processing begins to flow to the hippocampus with this region of the brain becoming increasingly involved in successful completion of the task (Cohen and Stackman, 2015). This 30 second exploration time is similar to the exploration time observed in our study and so it may be possible the flow of information from the perirhinal cortex to the hippocampus may be disrupted by the highly proliferative state of the hippocampus leading to impaired object recognition performance.

The involvement of neurogenesis in hippocampal-associated cognition is complex. However, it has been suggested that for neurogenesis to be implicated, the behavioural task being assessed needs to be sufficiently difficult (Curlik and Shors, 2011). From our work, it could be suggested that novel location recognition and spontaneous alternation do not sufficiently recruit hippocampal neurogenesis or are reliant upon it. Therefore, our TLX-induced enhancement in proliferation does not produce a behavioural phenotype that is detectable in the tasks we assessed. As the tasks we utilised for this current study rely on the natural preference for rats to explore novelty, there is very little learning involved in these tasks. As learning is a robust inducer of all aspects of neurogenesis, it may be more beneficial to assess the impact of TLX OEX in rats on other behavioural paradigms in which there is a

stronger role for learning or training such as spatial navigation tasks or contextual fear conditioning (Gould et al., 1999, Deng et al., 2010). Additionally, it should be noted that in our study, we only observed increased proliferation with no significant effect on neuronal differentiation or cell survival. As successful neurogenesis requires all stages of neurogenesis (proliferation, neuronal differentiation, and survival/integration) to be completed, the lack of improvement in location recognition or spatial working memory from our study may be due to the fact that there was no overall increase in neurogenesis.

Future *in vivo* TLX OEX studies must be carried out with extreme caution as overexpression of TLX can lead to hyperproliferation of NPCs and increased glioma formation (Liu et al., 2010, Park et al., 2010). However, efforts have been made to identify ligands for this orphan nuclear receptor in order to target and manipulate TLX function (Benod et al., 2014b). With the advent of selective TLX ligands, it may be possible to have more controlled regulation of TLX function and avoid the potential side-effects of enhanced TLX expression. Therefore, ligand based regulation of TLX function will vastly improve our understanding of its function *in vivo*.

IL-1β has been shown to have conflicting effects on hippocampal-associated cognition (Avital et al., 2003, Goshen et al., 2007, Ben Menachem-Zidon et al., 2008) and so in chapter 7 we aimed to determine whether the negative effects of IL-1β could be exacerbated by pre-exposure to another proposed negative regulator of hippocampal neurogenesis, namely poor diet intake. The adolescent period has previously been shown to be a particularly vulnerable period to the negative effects

of poor diet on cognition (Hueston et al., 2017, Boitard et al., 2014, Boitard et al., 2012, Vendruscolo et al., 2010). Indeed, it has been demonstrated that rats fed a high fat diet during adolescence show impairments in hippocampal-associated relational memory and neurogenesis whereas when the same diet is given in adulthood there was no significant impairments in these tasks (Boitard et al., 2012). Thus, we chose to expose rats to a diet high in both fat and sugar during adolescence and assess their performance in hippocampal-associated cognitive processes in adulthood. The diet mimics a cafeteria diet which is consumed in high levels in the western world and is associated with increased obesity as well as cardiovascular and metabolic disorders (Bruce-Keller et al., 2009, Haslam and James, 2005). Similar diets in rodent studies have been shown to induce hippocampal inflammation as well as impairments in hippocampal neurogenesis and associated cognitive processes (Hansen et al., 1998, Martire et al., 2014, Beilharz et al., 2014). However, from our data we did not observe any impairment in behaviours at adulthood induced by the adolescent cafeteria diet. The likely cause of this is the fact that the rats were switched to a regular chow diet three weeks prior to behavioural testing, thus any effects induced by the diet would have been normalised in that time. This theory is supported as it has been shown that switching from a high fat diet to a regular chow diet and allowing a 3 month washout period resulted in no changes in hippocampal-associated spatial navigation in the MWM (Boitard et al., 2016). However, we observed a lasting effect of increased levels of the metabolic hormones leptin and insulin in serum indicating that there may be lasting effects on metabolism in adolescent cafeteria diet fed rats.

High fat diets have been shown to induce hippocampal inflammation in the form of increasing expression of pro-inflammatory cytokines IL-1β and TNFα (Hansen et al., 1998, Beilharz et al., 2014, Pistell et al., 2010, Boitard et al., 2014). Thus, our main aim for this chapter was to determine whether pre-exposure to a cafeteria diet during adolescence could exacerbate any potential effects of IL-1β on cognition in adulthood. However, we did not observe any significant differences in hippocampal-associated behaviours induced by IL-1β in cafeteria diet fed rats compared to non-IL-1β injected cafeteria diet fed rats. Interestingly, IL-1β OEX alone impaired spatial working memory in the Y-maze and enhanced contextual fear conditioning both of which are hippocampal-associated. However, it has been shown that IL-1β at low expression levels is required for successful completion of certain hippocampal-associated tasks such as spatial navigation in the MWM and contextual fear conditioning (Avital et al., 2003, Goshen et al., 2007). From our data, it may be that spontaneous alternation is more sensitive to elevated levels of IL-1β in the hippocampus. However, contextual fear conditioning is enhanced by similar expression levels of IL-1β. Therefore, our data supports the complex role of IL-1β in regulating hippocampal-associated cognition.

Interestingly, the only effect that the adolescent cafeteria diet appeared to have was to attenuate the effects of IL-1β on hippocampal-associated cognition. As we observed similar levels of IL-1β in the hippocampus of both cafeteria diet and standard chow fed rats, viral uptake is not affected by the cafeteria diet. What is a more likely explanation is that alterations in factors induced by the cafeteria diet such as in metabolic hormone levels and/or inflammatory levels may be impairing the ability of IL-1β to modulate cognition. In support of this theory, IL-1RA has

been shown to be significantly increased in the serum of obese patients (Meier et al., 2002). Additionally, mice fed a high fat diet (albeit for 35 weeks) have been shown to have elevated serum levels of IL-1RA (Somm et al., 2006). As IL-1RA is known to cross the BBB (Gutierrez et al., 1994) it could impair IL-1β signalling in the hippocampus thus negating the IL-1β-induced cognitive changes we observed.

Despite the insights gained into the role of TLX and IL-1β in hippocampal neurogenesis and associated cognition, the limitations encountered throughout the course of this work are worth discussing. Firstly, although lentiviral technology is a valuable to enhance the expression of specific proteins, the lack of precise control as to exactly how much protein is expressed is a limitation. For example, although we could alter the concentration of the viruses we used in terms of transfection units (TU) which in theory should alter the amount of overexpressed protein is produced, variables such as subsequent inflammatory response to the virus, or stress levels of the animals after surgery can impact optimal viral uptake which would affect any results obtained using this technology (Escors and Breckpot, 2010, Naldini, 1998). The field of viral vector mediate gene transfer has advanced greatly and significant progress has been made to reduce inflammatory responses to viruses by minimising viral gene expression in these vectors (Escors and Breckpot, 2010). However, the lack of control of the expression or activation state of the overexpressed protein is still a factor that needs to be considered when designing studies using lentiviruses. Another limitation with the work described herein relates to our use of mice with a spontaneous deletion of TLX. These mice are devoid of TLX throughout development (Young et al., 2002) and so although our data strongly suggest a role for TLX in regulating the inflammatory transcriptional profile within the

hippocampus in adulthood, it may be that this effect is due to the fact that TLX is absent during critical stages of immune system development in early life. To address this limitation, future studies should aim to utilise rodents with a conditional knock-out of TLX in adulthood.

In conclusion, data in the present thesis adds to the current knowledge of how TLX and IL-1β interact and how these two factors independently or in combination can regulate hippocampal-associated cognition. We utilised hippocampal neurosphere cultures to identify the NF-κB pathway as a potential mediator of the effects of IL-1β on TLX expression, and also showed that restoration of TLX expression can prevent the suppressive effects of IL-1β on neurosphere expansion. Using RNASeq technology, we highlighted the potential a role for TLX in regulating and inflammatory transcriptional profile within the hippocampus both at baseline and in response to IL-1β stimulus. These data positions TLX as a potential transcriptional regulator of neuroinflammation within the hippocampus. We report that lentiviral mediated OEX of TLX did not promote hippocampal-associated cognition but rather impaired hippocampal-associated object recognition. We demonstrated that adolescent cafeteria diet consumption does not produce lasting effects on hippocampal-associated tasks. Finally, lentiviral-mediated overexpression of IL-1β represents a promising method to highlight the various effects of IL-1β on hippocampal-associated cognition. Specifically we outlined that this approach induced impairments in spontaneous alternation in the Y-maze and promoted contextual fear recall in adult rats. Interestingly, we demonstrated that these effects were attenuated by adolescent consumption of a cafeteria diet indicating that pre-exposure to a poor diet can impact upon the ability of IL-1β to modulate

hippocampal-associated cognition. From the *in vitro* and RNASeq evidence described herein, it appears that TLX represents a promising pharmaceutical target to mitigate the anti-neurogenic effects of IL-1β and regulate neuroinflammation within the hippocampus. However, more work is needed to ascertain the functional role of TLX in hippocampal-associated cognitive processes. Specifically, it should be addressed whether increased proliferation in the hippocampus can negatively impact upon neuronal signals entering the hippocampus from brain structures such as the perihinal cortex. Additionally, behavioural paradigms involving a strong learning component such as spatial navigation in the MWM or fear conditioning should be utilised to determine whether increase cell proliferation in the hippocampus of rats can enhance performance in these tasks. With the advent of pharmaceutical ligands which regulate TLX function, greater strides can be made to determine the protective role and therapeutic potential of TLX in IL-1β and neuroinflammatory-induced changes in hippocampal-associated cognition.

Chapter 7: Future Studies

***In vitro* studies**

1. a) Assess the interaction between TLX and various components of the NF-κB pathway in proliferating hippocampal NPCs after IL-1β stimulation.

   b) Determine which components of the NF-κB pathways are responsible for the effects of IL-1β on TLX expression by using different selective inhibitors of specific components of the NF-κB pathway.

2. Determine whether members of the mitogen activated protein kinase (MAPK) pathways are involved in IL-1β-induced suppression of TLX expression and/or neurosphere expansion.

3. Further characterise the effects of TLX overexpression on neuronal differentiation and survival of hippocampal NPCs.

4. Assess whether viral-mediated knockdown of TLX expression impairs neurosphere growth.

5. Determine the role of p21 in IL-1β-induced suppression of neurosphere expansion.

6. Assess whether a similar lentiviral transfection methodology can be employed to overexpress TLX in adult hippocampal neurosphere cultures.

7. Assess the interaction of TLX with TNF signalling in hippocampal NPCs *in vitro*.

***In vivo* studies**

1. Determine whether IL-1β (either lentiviral-mediated overexpression or injection of recombinant protein) can induce alterations in behaviour of TLX KO mice.

2. Utilise selective TLX ligands to modulate the activation state of TLX and assess its role on hippocampal-associated cognitive function.

3. a) Maintain a longer period of cafeteria diet consumption to assess whether cafeteria diet can induce impairments in fear conditioning

   b) Determine whether chronic lentiviral-mediated IL-1β overexpression impairs or protects performance.

4. Determine what factors are released within the hippocampus as a results of a cafeteria diet during adolescence that may impact upon the IL-1β-induced changes in cognition.

5. Assess whether TLX modulation by selective ligands can modulate the effects of a diet high in fat and sugar on hippocampal associated cognition.

6. Investigate whether TLX activation by a selective ligand can alter IL-1β-induced-induced changes in behaviour.

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Appendix A: Detailed materials & methods

1.1: Chapter 2 methods

Aseptic technique

All tissue culture procedures were carried out in a Class II Biological Safety Cabinet (Astec, AGB, Ireland). Gloves and specific tissue culture lab coats were worn at all times. All surfaces were sprayed with 70% ethanol prior to commencing work and all equipment and reagent bottles were sprayed with 70% ethanol before being put under the hood. All glassware, water, microcentrifuge tubes and pipette tips were autoclaved prior to use.

Epidermal growth factor

EGF (25µg) (Sigma, Ireland) was reconstituted using 0.2µm filtered sterile 10mM acetic acid (Sigma, Ireland) containing 0.01% Bovine serum albumin (BSA) (Sigma, Ireland) to give a stock of 20µg/ml. 50µl aliquots were stored at -20°C.

1, 4-Dithiothreitol (DTT) (100mM)

DTT (0.31g) (Sigma, Ireland) was dissolved in 20ml of sterile PBS (10mM). The solution was sterile filtered and stored at 4°C

Heparin (50mg/ml)

Heparin (50mg) (Sigma, Ireland) was dissolved in 1ml of sterile PBS (10mM). The solution was sterile filtered and stored at 4°C

FGF

bFGF (25µg) (Millipore) was reconstituted using 0.2µm filtered sterile PBS (10mM) containing 0.1% BSA, 1mM DTT, and heparin (5µg/ml), to give a stock solution of 20µg/ml. 50µl aliquots were stored at -20°C.

Complete tissue culture proliferation media for E18 rat hippocampal NPCs

A solution of Dulbeccos Modified Eagles Medium/F12 (Sigma Ireland) containing 200mM L-Glutamine (Sigma Ireland), 1% Penicillin/Streptomycin (Sigma Ireland), and 33mM D-Glucose (Sigma Ireland) was prepared and stored at 4°C. Complete tissue culture media was supplemented with 2% B27 without vitamin A (Invitrogen, UK) and 10ng/ml of both EGF (Sigma Ireland) and bFGF (Millipore) according to (Ray et al., 1993). This media was made up on the day of culture and kept at 37°C in an incubator (Binder, Germany).

IL-1β preparation

A stock solution of 10µg/ml of IL-1β (R&D systems) was made up by dissolving 50µg of IL-1β in 4.995ml sterile PBS (10mM) and 5µl of BSA (7.5%) (Sigma, Ireland). This solution was stored in 100µl aliquots at -80°C. This concentration was found to be lowest concentration of IL-1β to significantly alter NPC proliferation without affecting viability or cellular phenotype (Green et al. 2012).

JSH-23 preparation

A stock solution of 25mM of JSH-23 (Sigma, Ireland) was made up by dissolving 3.004mg of JSH-23 in 0.5ml dimethyl sulphoxide (DMSO) (Sigma, Ireland). This solution was store in 10µl aliquots at -20°C.

Poly-D-Lysine and coating of 13mm coverslips

0.1mg/ml poly-D-lysine (Sigma, Ireland) was prepared by dissolving 5mg of poly-D-lysine in 50ml of sterilised distilled water (dH_2O) and stored at -20°C in aliquots of 3.5ml (1 aliqout = 1x24 well plate). 13mm glass coverslips were placed in a glass petri dish and sterilised under UV radiation for 45mins. The sterilised coverslips were placed one per well in a 24 well plate under the laminar flow hood. 130µl of 0.1mg/ml poly-D-lysine was placed on each coverslips and the plate was left in the incubator at 37°C for 30mins. The 24 well plate with the coverslips was removed from the incubator and the coverslips were washed 3 times in dH_2O and left to dry for 1hr in the laminar flow hood. Coated coverslips were stored at -20°C until use.

Isolation of E18 rat hippocampal tissue

Time mated Sprague Dawley rats were provided by the Biological Services Unit (University College Cork, Ireland). Female rats were anaesthetised in a bell jar using isofluorane (Abbeyville Veterinary, Ireland) and then sacrificed by decapitation. An incision was made along the abdominal midline through the abdominal wall exposing the embryos. The embryos were removed from the female, placed in a petri dish and sacrificed by hypothermia on ice. The individual embryos were removed from their amniotic and chorionic sacks and decapitated. Under a dissecting stereo zoom microscope (Leica, Milton Keynes, UK), whole brains were then removed using two Dumont forceps and placed in a 20ml tube Hank's Balanced Salt Solution (HBSS) (Sigma, Irealnd) on ice. The hippocampi from each embryo were subsequently micro-dissected out and placed in a 20ml tube in HBSS on ice. All dissecting instruments were sterilised in 70% ethanol and rinsed in sterile HBSS. Hippocampi from one litter were pooled and represented an individual culture.

Preparation of E18 rat hippocampal neurosphere culture

All work from here was carried out under sterile conditions. Hippocampal tissue was transferred to a 35mm petri dish containing ice cold HBSS under a sterile microflow cabinet (Astec, AGB, Ireland). The tissue was then minced using a sterile scalpel blade. The tissue was then transferred to a 15ml tube and centrifuged for 5mins at 200rcf at room temperature (Beckman Coulter Allegra 21R). The supernatant was removed and pre-warmed 0.1% trypsin-EDTA (2ml trypsin-EDTA and 3ml HBSS) (Sigma, Ireland) was added for 5mins at 37°C. Soya bean trypsin inhibitor (0.5mg/ml, Sigma, Ireland) was added to inhibit the enzymatic reaction and the tube was inverted 3 times. Mechanical dissociation was carried out by triturating the tissue gently for approximately 1min using a flame polished Pasteur pipette (Sarstedt, Ireland) and followed once by a 23G needle and syringe (Sarstedt, Ireland). This suspension was centrifuged again at 200rcf for 5mins. The supernatant was removed and 1ml of pre-warmed proliferation media was added to the tube. The pellet was dissociated gently by brief trituration using a P1000 pipette. The solution was filtered by being passed through a 40µm cell strainer (BD Falcon, Ireland) into a new 15ml tube. 10µl of this cell suspension was placed in a 0.5ml microcentrifuge tube for cell counting. 10µl of trypan blue was added to the microcentrifuge tube and this mixture was added to a haemocytometer (Alkem, Ireland). The total number of live cells (cells which did not take up the trypan blue die) was counted under a microscope and a concentration of cells/ml was calculated using the following equation:

$$\text{Cells/ml} = \frac{\text{(Number of cells counted in 4 fields)}}{4} \times \text{(Dilution factor)} \times 10^4$$

Cells were seeded at a concentration of 2 million cells per flask in T25 flasks (Sarstedt, Ireland). Cells were cultures as neurospheres at 37°C in a humidified incubator containing 5% CO_2 and 95% air for up to 6 days. The growth factors EGF and FGF (both 20ng/ml) were added on the 2nd day and half the media in the flasks was replaced with fresh proliferation media on the 3rd and 5th days of culture.

IL-1β treatment timeline of E18 rat hippocampal neurospheres

Cell treatments were carried out after 6 days *in vitro* (6DIV). Prior to treatments, neurospheres were dissociated into a single cell suspension. Neurospheres were collected into a 15ml tube and centrifuged at 1100rpm for 5mins. The supernatant was removed and the neurospheres were resuspended in 1ml of 0.1% trypsin-EDTA for 5min at 37°C in an incubator. Neurospheres were removed from the incubator and 400µl of soya bean trypsin inhibitor was added to the tube. Mechanical dissociation, cell filtering, and cell counts were carried out as described above. Cells were then plated down onto 13mm poly-D-lysine coated coverslips. To do so, 40-60µl of cell suspension in proliferation media at a concentration of 50,000cells/coverslip was added to each well and left in the incubator for 1hr to adhere. After 1hr every well was flooded with proliferation media to give a final volume of 500µl per well. IL-1β was added to each well at specific timepoints (10min, 30min, 1h, 2h, or 4h) with a final concentration of 10ng/ml in each well. All wells were assigned at random. After the 4hrs (the final timepoint), the cells were washed in warmed HBSS then fixed for further immunocytochemistry analysis.

NPCs for PCR analysis were seeding in 24 well plates without poly-D-lysine coated coverslips and suspended in complete proliferation media with or without IL-1β treatments.

JSH-23 treatment of E18 rat hippocampal NPCs

2μl of stock solution of JSH-23 (25mM) was added to 2ml of culture media containing proliferating dissociated NPCs to give a final concentration of 25μM. This concentration had previously used to determine the effects of NF-κB signalling on cell proliferation within the hippocampus (Koo et al., 2010). Cells were incubated with IL-1β with and without JSH-23 or vehicle (0.001% DMSO) for 3 hours in 24 well plates before cell viability or TLX, p21 and IκBα mRNA expression was assessed. Cells were also attached to poly-D-lysine coated coverslips as described above to conduct immunocytochemical analysis of JSH-23 treated NPCs.

Lentiviral treatment of E18 rat hippocampal neurospheres

Mouse TLX (Nr2e1) was cloned into a pCDF-CMV-IRES-GFP lentiviral backbone. Transgene expression of TLX was driven by a cytomegalovirus (CMV) promoter. Lentiviral particles were produced by transfecting a HEK 293T producer cell line with the lentiviral Nr2e1 plasmid or lentiviral GFP control plasmid and pPACKF1^{TM} Lentivector Packaging Kit (System Biosciences, 2438 Embarcadero Way, Palo Alto, CA, 94303, USA). Harvested viral supernatant was aliquoted and stored at -80°C until use. Packaged viruses had titres of approximately $1x10^6$ TU/ml. On the first day of cultures, $5x10^5$ dissociated NPCs were plated in each well of a 6 well plate along with $7x10^4$ TU/ml of lentivirus overexpressing either TLX or GFP. After 24 hours, recombinant rat IL-1β (10ng/ml) or PBS was added. Cells were allowed to

proliferate as neurospheres and were kept under proliferative conditions for a further 6DIV.

Neurosphere expansion analysis

Floating neurospheres were observed under an Olympus IX70 inverted microscope at 10x magnification each day the cells were in culture. Phase contrast images were taken using the attached Olympus DP70 digital camera. For each flask five images were taken and saved. Within each image, the diameter of four spheres was measured using ImageJ.

Immunocytochemistry of treated NPCs

After cells were treated with IL-1β, they were washed in warmed in HBSS. The HBSS was removed and cells were fixed by adding ice cold methanol for 10mins at -20°C. The fixative was removed and cells were washed 3x5min in 0.02% triton-X in PBS. Following this, the cells were incubated overnight at 4°C in 5% donkey serum made in 0.02% triton-X 100 as a blocking agent. After an overnight incubation, the blocking solution was removed and the primary antibodies made in blocking solution were added overnight at 4°C. For each experiment, at least one well was designated as a negative control where only blocking solution without primary antibody was added. After an overnight incubation in primary antibody, 3x5min 0.02% triton-X washes were carried out followed by 2hr incubation in the dark at room temperature with secondary antibody. The secondary antibody was removed and 3x5min 0.02% triton-X washes were carried out followed by 5min incubation with the nuclear stain 4', 6-diamidino-2-phenylindole (DAPI). The cells were washed in PBS once for

10mins and the coverslips were mounted onto 76x26mm microscope slides using mounting media (DAKO).

Target	Antibody	Source	Company	Dilution
TLX	Anti-TLX	Polyclonal Rabbit	Santa Cruz	1:100
p65	Anti-p65	Polyclonal Rabbit	Santa Cruz	1:100

Table 1.1: List of primary antibodies used for *in vitro* study

Secondary antibodies	Company	Dilution
AlexaFluor 488-conjugated donkey anti-rabbit	Invitrogen, UK	1:2000

Table 1.2: Secondary antibody used for *in vitro* study

Imaging

Immunocytochemically stained cells were viewed using and Olympus Provis AX70 upright fluorescent microscope. Green and blue fluorescent images were taken at 40X magnification using the attached Olympus DP40 digital camera. Five random fields of view per coverslip were imaged and two pictomicrographs for each filter (green and blue) were taken per field of view. The results were merged and analysed using ImageJ.

Fluorescence measurements

The fluorescent intensity of TLX and p65 was calculated on ImageJ. Each TLX positive and p65 positive cell was highlighted using the draw tool on ImageJ and the fluorescent intensity was measured. For each pictomicrograph, ten immuno-positive cells were measured for their fluorescent intensity. Also for each pictomicrograph, three background measurements were taken. An average background value for each

image was calculated and subtracted from each cell value to obtain a corrected fluorescent intensity measurement.

Collection of NPCs for mRNA analysis

After IL-1β, JSH-23 or lentiviral treatments, proliferating NPCs were harvested as whole neurospheres (i.e. after expansion analysis with IL-1β and lentiviral treatments), or dissociated NPCs (i.e. after IL-1β timeline and JSH-23 treatments) as described above. Neurospheres or dissociated NPCs were collected into 15ml tubes and centrifuged at 200rcf for 5mins and room temperature. Neurospheres were then re-suspended in 1ml of warmed HBSS and transferred to a sterile 1.5ml microcentrifuge tube. These tubes were centrifuged (Eppendorf Minispin centrifuge) at 14,000rcf for 5mins. The supernatant was removed and the neurospheres or NPCs were flash frozen and stored at -80°C.

Ribonucleic Acid (RNA) extraction

Neurospheres used for PCR experiments were collected as described above. Cell pellets were defrosted on ice and total cellular RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Cell pellets were lysed by adding 250µl of lysis buffer and resuspending the cell pellet. This lysate was then passed through a filtration column within a 2ml collection tube and centrifuged at 14,000rcf for 2 minutes to remove cellular debris and shear DNA. Equal volume of 70% ethanol was added to the filtrate. The filtrate is then added to an RNA binding column and the RNA was washed by adding 500µl of Wash Buffer I and centrifuged at 14,000rcf for 30 seconds. The flow through was discarded and the binding column was placed in a new collection tube and 500µl of Wash Buffer II was added to the

column. The column was then centrifuged at 14,000rcf for 30 seconds. The flow through was discarded and a second 500µl of Wash Buffer II was added and the column was centrifuged at 14,000rcf for 1 minute to dry the column. The RNA was then eluted by adding 30µl of elution solution and centrifuged at 14,000 rcf for 1 minute. Any DNA left in the eluted RNA sample was removed using DNase I amplification grade kit (Sigma). RNA in 30 ml of elution solution was treated by adding 3µl of 10X Reaction Buffer and 5µl of DNase I Amplification Grade (Sigma), and incubating at room temperature for 15 minutes. Inactivate the DNase by adding 5µl of the Stop Solution and heating at 70 °C for 10 minute. The eluted RNA was then stored at -80°C until use.

RNA quantification

RNA was quantified using a Nano drop 3300 (Thermo Scientific, UK). 1µl of undiluted sample was placed on the nano drop electrode and the RNA concentration was calculated in ng/µl. The 260/280 and 260/230 ratio were also calculated to assess RNA purity.

cDNA Synthesis

RNA was reverse transcribed into cDNA using the ReadyScript® cDNA Synthesis Mix (Sigma). RNA concentration was normalised to 0.1µg/reaction by diluting in RNase free H_2O. In each 200µl cDNA reaction tube, 4µl of ReadyScript® cDNA Synthesis Mix was added along with varying volumes of RNA and RNase free H_2O to give a final volume of 20µl/tube with 0.1µg of RNA. The tubes were then placed in a thermal cycler (Sure Cycler 8800, Agilent Technologies) and underwent the following cycle: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and held

at 4°C. The samples were then diluted 1:4 to give 80µl of diluted sample within the 1pg threshold of the Sybr Master Mix used for RT-PCR. The cDNA samples were then either stored at -20°C or used immediately for RT-qPCR.

Real Time Quantitative PCR (RT-qPCR)

RT-qPCR was carried out using a StepOnePlus Real-Time PCR Machine (Applied Biosystems). Each run was carried on 96 well plates (Applied Biosystems). For each run, the samples were run in triplicate for each target gene and the housekeeping gene which was B-actin. Negative controls were also run for each target gene. Each reaction was carried out in 20µl volume containing 10µl of Sybr Master Mix (KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX™ for ABI instruments, Sigma), 0.2µl of both forward and reverse primers (Eurofins Genomics), 4.6µl of RNase-free H_2O, and 5µl of sample. Primers for the target genes were carried out using the primer designing tool on Pubmed (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The RT-PCR reaction was carried out as follows. The samples were heated to 95°C for 3mins to denature the cDNA. The samples then underwent 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The fluorescence data was collected at the end of each cycle. The raw data generated was exported as an excel file and the Cycle Threshold (CT) values were analysed using the Livak method (Livak & Schmittgen 2001).

Gene	Primers (From Pubmed Primer Blast)	NCBI Reference
SDHA	F cccactaactacaagggacagg	NM_130428.1
	R ttggcaccatgcactgag	
TLX	F gctttcttcacagcggtcac	NM_001113197.1
	R gcagacacagcggtcaact	
IkBa	F tgaagtgtggggctgatgtc	NM_001105720.2
	R agggcaactcatcttccgtg	

p21	F gtatgccgtcgtctgttcgg	U09793.1
	R gcagaagaccaatcggcgct	

Table 1.3: List of primers used for *in vitro* study

1.2: Chapter 3 methods

Preparation of WT and TLX$^{-/-}$ mouse hippocampal tissue for RNASeq

Anesthesia

All surgery to implant the guide cannula (*Plastics1*, Gauge:22, Pedestal Length:4mm, Projection:1.5mm) was conducted on WT and TLX$^{-/-}$ mice at p52 under general anaesthesia induced by a mixture of ketamine (0.25ml), xylazine (0.2ml) and sterile 0.9% saline (2.05ml) at a dose of 0.1mls/10g injected intraperitoneally (i.p).

Carprofen

A 50mg/ml stock solution of carprofen (Abbeyville Veterinary, Ireland) was diluted in sterile saline to give a final concentration of 1mg/ml. Mice were injected subcutaneously (SC) with carprofen at a volume of 0.1ml/30g post-surgery.

Glucose

Glucose (Sigma, Ireland) was dissolved in sterile saline to give a final concentration of 5%. Glucose was administered SC at a volume of 1.25ml/100g post-surgery.

Stereotaxic surgery for hippocampal cannulation

Animals were placed in a stereotaxic frame (Kopf, Tujunga, CA). The guide cannula was implanted unilaterally at the following co-ordinates for the dorsal hippocampus, AP -1.7, ML -1.2 and DV -1.5 relative to bregma (Haettig et al., 2013). Animals to be implanted on the left or right hemisphere were chosen at random within the WT

and $TLX^{-/-}$ groups. The cannula was then secured in place using superglue as a base layer followed by dental cement. After the dental cement had hardened sufficiently, recovery injections were administered (carprofen and glucose). Animals were returned to their home cage when recovered and 4 days later where taken for IL-1β or saline microinjections.

Intrahippocampal microinjections and hippocampal dissections

At p56, WT and $TLX^{-/-}$ mice received intrahippocampal injections of either IL-1β or PBS. The injection cannula used projected a further 0.5mm past the guide cannula to give a total depth of 2.0mm. Unilateral dorsal hippocampal injections of either recombinant mouse IL-1β or PBS at a volume and rate of 1ul at 0.5ul/min were performed using an automated microinjector 1h prior to sacrifice. The injection cannula was left in place for 2mins after the injection to allow diffusion of the injected volume. Mice were sacrificed by decapitation and hippocampal tissue from each mouse was isolated and snap frozen in 1.5ml microcentrifuge tubes on dry ice. The hippocampal tissue was then stored at -80°C.

RNA extraction

Hippocampal tissue was defrosted on ice and total RNA was extracted using mirVana™ total RNA extraction kit (Ambion/Life Technologies, Dublin, Ireland) as per the manufacturer's instructions. Hippocampal tissue was lysed by adding 300µl of lysis buffer and mechanically dissociated using pestles. Equal volume of phenol was added to the lysis buffer/tissue mix and left to incubate on ice for 10mins. Samples were then vortexed for 60 seconds and centrifuged for 5min at 10,000rcf. The upper aqueous solution was withdrawn and transferred to a fresh tube. 1.25

times volume of 100% ethanol was added to the aqueous solution which was then added to an RNA binding column and the RNA was washed by adding 700µl of Wash Buffer I and centrifuged at 14,000rcf for 30 seconds. The flow through was discarded and the binding column was placed in a new collection tube and 500µl of Wash Buffer 2/3 was added to the column. The column was then centrifuged at 14,000rcf for 30 seconds. The flow through was discarded and a second 500µl of Wash Buffer 2/3 was added and the column was centrifuged at 14,000rcf for 1 minute to dry the column. The RNA was then eluted by adding 100µl of elution solution at 95°C and centrifuged at 14,000rcf for 1 minute. Total extracted RNA was DNAse treated using Turbo DNA-free kit (Ambion/life technologies). The total concentration of extracted RNA was quantified using a Nanodrop 2000 (Thermo Scientific, UK) and was stored at -80°C until sent for sequencing or prepared for cDNA synthesis and PCR analysis.

mRNA sequencing

Sequencing was carried out as described in chapter 3.

1.3: Chapter 4 methods

Carprofen

Carprofen (50mg/ml) (Abbeyville Veterinary, Ireland) stock solution was diluted to a final concentration of 2.5mg/ml in sterile saline and administered SC in a volume of 1.5ml/kg post-surgery.

Glucose

Glucose (Sigma, Ireland) was dissolved in sterile saline to give a final concentration of 5%. Glucose was administered SC at a volume of 3ml/kg post-surgery.

Stereotaxic surgery and lentiviral injection

Rats were anaesthetised with isoflurane (Abbeyville Veterinary, Ireland) and placed into a Kopf stereotaxic frame (Kopf, Tujunga, CA). Lentiviruses to overexpress TLX (1.63×10^7 copies/ml) or GFP (1.31×10^7 copies/ml) (Genecopoeia, MD, USA) was injected into the dorsal hippocampus using the coordinates AP: -3.5 mm, ML: ± 2.4 mm, DV: -3.8 mm relative to Bregma (Barrientos et al., 2002) at a rate of 1µL/min followed by a 5 min diffusion. Rats were injected SC with carpofen and 5% glucose prior to anaesthetic recovery. All rats were allowed to recover for three weeks with *ad libitum* access to food and water prior to behavioural testing in order to allow viral uptake.

BrdU

A 30mg/ml solution of BrdU was prepared by dissolving 1.5g of BrdU (Sigma) in 50mls of sterile saline. The solution was incubated at 37°C and sonicated to dissolve the BrdU. BrdU was administered intraperitoneally (i.p.) at a volume of 5ml/kg. Animals were administered BrdU one week after surgery for immunohistochemical assessment of the survival of newly born cells.

Behavioural testing

Behavioural testing was carried out as described in chapter 4.

Sacrifce and perfusion

Rats were euthanized with an i.p. injection of Euthatal (pentibarbitol) (1.0ml/kg). Once anaesthetised, the thoracic cavity was opened and a 26G needled connected to tubing filled with ice cold 0.9% PBS was inserted into the left ventricle. The right atrium was cut and the PBS was pumped at a flow rate of 23ml/min to flush the blood out of the circulatory system. Once the out-flowing blood ran clear, the tubing was and transferred to pump 4.0% paraformaldehyde (PFA) until a sufficient level of fixed tissue was observed in the neck area. After perfusion, rats were decapitated and the brains were removed and post-fixed in 4% formaldehyde in PBS overnight, then transferred to a 30% sucrose solution until they had sunk. The brains were then snap frozen using isopentane as a freezing medium and liquid nitrogen and subsequently stored at -80°C until sectioned.

Brain sectioning and immunohistochemistry

Coronal sections through the hippocampus were cut at 40μm onto slides and stored at -80°C. For analysis of cellular survival with BrdU, sections were incubated in HCl (2M) for 45 minutes at 37°C and re-natured in 0.1M sodium tetraborate, and then treated with 3% hydrogen peroxide for 45 minutes. Sections were blocked in 10% normal rabbit serum and incubated with the appropriate primary antibody (See table 4.4). Detection and visualisation was carried out using HRP-linked rabbit-anti-rat, goat anti-rabbit, or rabbit anti-goat secondary antibodies from ABC complex kits (Vector) before being placed into DAB and coverslipped with DPX mounting medium. Brightfield images across a 1:12 series through the dorsal DG were obtained using an Olympus BX53 upright microscope at both 10x and 20x (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, UCC). The total number of positively labelled cells were counted and expressed as cells per

section within the hilus, subgranular zone (SGZ), and granular cell layer (GCL) of the DG. Images of autofluorescent TLX overexpressed as a result of the lentiviral injection were also captured across a 1:12 series throughout the hippocampus using an Olympus BX53 upright microscope at 20x (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, UCC).

1.4: Chapter 5 methods

Stereotaxic surgery and lentiviral injections

Rats were anaesthetised with isoflurane (Abbeyville Veterinary, Ireland) and placed into a Kopf stereotaxic frame (Kopf, Tujunga, CA). Lentiviruses to overexpress mouse IL-1β (1.93×10^7 copies/ml) or mCherry (2.42×10^8 copies/ml) (Genecopoeia, MD, USA) was injected into the dorsal hippocampus using the coordinates AP: -3.5 mm, ML: ± 2.4 mm, DV: -3.8 mm relative to Bregma (Barrientos et al., 2002) at a rate of 1µL/min followed by a 5 min diffusion. Rats were injected SC with carpofen and 5% glucose prior to anaesthetic recovery as per appendix section 1.3 above. All rats were allowed to recover for three weeks with *ad libitum* access to standard chow and water prior to beginning behavioural testing to allow viral uptake.

Behavioural testing

Behavioural testing was carried out as described in chapter 5.

Hippocampal RNA extractions

Total RNA was extracted as described in appendix section 1.2.

Quantitative real-time PCR (qRT-PCR)

cDNA was reversed transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the SureCycler® 8800 (Agilent Technologies) and diluted to a final concentration of 10ng/µl. All qRT-PCR was performed in 3 technical replicates for each biological sample on a LightCycler® 480 Instrument II (Roche). See table 1.5 for a list of primers used. The PCR consisted of the following steps: 95°C for 2 minutes to denature the cDNA followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. Each reaction consisted of 1µl of sample (5ng/ul), 5µl of Sybr MasterMix (KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX™ for ABI instruments, Sigma-Aldrich), 0.1µl of both forward and reverse primers, and 3.8µl of RNase free H_2O. Results were normalised to succinate dehydrogenase (SDHA) and quantification of gene expression was analysed using the $\Delta\Delta$ C_t method (Livak and Schmittgen, 2001).

Gene	Primers (From Pubmed Primer Blast)	NCBI Reference
SDHA	F cccactaactacaagggacagg	NM_130428.1
	R ttggcaccatgcactgag	
TLX	F gctttcttcacagcggtcac	NM_001113197.1
	R gcagacacagcggtcaact	
IL-1β	F aaagaagaagatggaaaagcggtt	NM_031512.2
	R gggaactgtgcagactcaaactc	
Synaptophysin	F cccttcaggctgcaccaa	NM_012664.3
	R ttggtagtgcccccttgac	
PSD95	F acgccgaagagtcagagaaa	NM_019621.1
	R actgttggaccgagtgaacc	
NeuroD1	F acaacaggaagtggaaacatgac	NM_019218.2
	R gctgggacaaacctttgcag	
TNFα	F gtctgtgcctcagcctcttc	X66539
	R cccatttgggaacttctcct	
CXCL1	F ctccagccacactccaacag	NM_030845.1
	R gacttcggtttgggtgcagt	

Table 1.5: List of primers used in chapter 5.

Protein extraction

Total protein was extracted from hippocampal tissue using a lysis solution of RIPA buffer (Thermo Fisher) with a protease and phophotase inhibitor tablet (Pierce, Thermo Fisher) along with mechanical dissociation using a pestle. The samples were centrifuged at 12,000 rpm for 12 minutes to remove tissue debris. The supernatant was then frozen at -80°C until required.

Protein concentration determination

The concentration of the extracted protein was determined by Bradford assay. A standard curve was prepared by making the following standards of bovine serum albumin (BSA) (Sigma) dissolved in distilled H_2O (dH_2O): 0.1, 1, 10, 100, 1000, 10000, 100000µg/ml. Standards and samples were vortexted prior to loading at 100µl onto the 96-well plate (Sarstedt). Bradford reagent was diluted 1:10 in dH_2O and loading at an equal volume to the standards and samples and incubated for 15 minutes and room temperature. The absorbance was measured at 600nm using a spectrophotometer plate reader (Multiskan, Thermo Fisher). The absorbance values of the standards were used to produce a log standard curve of known concentrations relative to absorbance values. This standard curve was then used to determine the concentrations of the samples.

Western blotting

Prior to western blotting the following buffer solutions were made as per table 1.5 below. Protein samples were mixed in a 1:1 ratio with sample buffer and incubated at 95°C for 5 minutes before being rapidly placed on ice. A 12% SDS resolving gel was first poured and left to solidify for 30mins prior to pouring a 4% SDS stacking

gel on top. Once the stacking gel had solidified, samples were loaded onto this gel. Gels were placed into a vertical western blot rig (Atto, Japan) and electrophoresis was started by applying 150V at room temperature (Bio-Rad power pack). Electrophoresis was ended just before the migrating protein line reached the bottom of the gel. The gels were then placed onto a transfer buffer soaked nitrocellulose membrane and the gel and membrane were sandwiched between layers of filter paper. The wet transfer was carried out for 75 minutes at 75 volts at 4°C (Trans Blot Cell, Bio-Rad). Once the transfer was complete, the membrane was then incubated with primary antibodies for IL-1β (R&D systems, goat polyclonal, 1:500) and β-actin (Sigma, mouse polyclonal, 1:1000) and subsequently incubated with the appropriate secondary HRP tagged secondary antibodies. Proteins were then visualised using ECL detection kit (GE healthcare). Densitometry for the developed immune blots was carried out using ImageJ software.

Buffer	Preparation
Sample buffer (2x in dH_2O) ***Made 1:1 ratio with samples***	12.5% Tris-HCl (Sigma) 20% Glycerol (Sigma) 2% Sodium dodecyl sulphate (SDS) (Sigma) 5% β-mercaptoethanol (Sigma) 0.05% w/v Bromophenol blue (Sigma)
Running buffer (10x in dH_2O) ***1x prepared for electrophoresis***	250mM Tris-base (Sigma) 1.92M Glycine (Sigma) 1% SDS (Sigma)
Transfer buffer ***Prepared in dH_2O on day of electrophoresis***	25mM Tris-base (Sigma) 192mM Glycine 5% v/v methanol
Tris-buffered saline (TBS) ***Prepared in dH_2O, pH 7.2-7.4***	200mM Tris-HCL 1.5mM NaCl
TBS-Tween (TBS-T)	0.05% Tween in 1L TBS
Ammonium persulphate (APS)	1g APS 10ml dH_2O
4% SDS stacking gel	1.87ml 0.5M Tris-HCL 975µl 30% acrylamide (Sigma) 4.5ml dH_2O 75µl 10% SDS 37.7µl 10% APS

	15µl Tetramethylethylenediamine (TEMED)
12% SDS separating gel	3.75ml 1.5M Tris-HCl 6ml 30% acrylamide (Sigma) 5.1ml dH_2O 150µl 10% SDS 75µl 10% APS 15µl TEMED

Table 1.6: List of buffers and details of preparation used for western blotting.

Appendix B: Published articles

The FASEB Journal article fj.201700495R. Published online September 27, 2017.

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TLX is an intrinsic regulator of the negative effects of IL-1β on proliferating hippocampal neural progenitor cells

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ABSTRACT: Hippocampal neurogenesis is a lifelong process whereby new neurons are produced and integrate into the host circuitry within the hippocampus. It is regulated by a multitude of extrinsic and intrinsic regulators and is believed to contribute to certain hippocampal-dependent cognitive tasks. Hippocampal neurogenesis and associated cognition have been demonstrated to be impaired after increases in the levels of proinflammatory cytokine IL-1β in the hippocampus, such as that which occurs in various neurodegenerative and psychiatric disorders. IL-1β also suppresses the expression of TLX (orphan nuclear receptor tailless homolog), which is an orphan nuclear receptor that functions to promote neural progenitor cell (NPC) proliferation and suppress neuronal differentiation; therefore, manipulation of TLX represents a potential strategy with which to prevent the antiproliferative effects of IL-1β. In this study, we assessed the mechanism that underlies IL-1β–induced changes in TLX expression and determined the protective capacity of TLX to mitigate the effects of IL-1β on embryonic rat hippocampal neurosphere expansion. We demonstrate that IL-1β activated the NF-κB pathway in proliferating NPCs and that this activation was responsible for IL-1β–induced changes in TLX expression. In addition, we report that enhancing TLX expression prevented the IL-1β–induced suppression of neurosphere expansion. Thus, we highlight TLX as a potential protective regulator of the antiproliferative effects of IL-1β on hippocampal neurogenesis.—Ó'Léime, C. S., Kozareva, D. A., Hoban, A. E., Long-Smith, C. M., Cryan, J. F., Nolan, Y. M. TLX is an intrinsic regulator of the negative effects of IL-1β on proliferating hippocampal neural progenitor cells. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: neurogenesis · nuclear receptor · inflammation · Nr2e1

Hippocampal neurogenesis is a process by which new neurons are born from neural progenitor cells (NPCs) in the hippocampus. This process begins during embryonic development and persists into adulthood, when it is thought to contribute to hippocampal-dependent cognitive function (1, 2). Evidence of the functional role of hippocampal neurogenesis in the adult has been shown *in vivo* as a result of its involvement in various cognitive tasks, such as the Morris water maze (3–6), novel object and novel location recognition (7–9), and pattern separation in rodents (10–13). In addition, adult hippocampal neurogenesis has been demonstrated to be impaired in conditions in which there is a subsequent impairment in hippocampal cognition, such as stress, depression, aging, and neurodegeneration (14–18). Many of these conditions have been associated with increased inflammation and expression of proinflammatory cytokine IL-1β in the brain (19–22).

Inflammation has been well established as having negative consequences on CNS function and hippocampal neurogenesis. Release of IL-1β occurs from activated microglia as a result of stress, infection, neurodegeneration, or, indeed, with aging (18, 21, 23, 24). IL-1 receptor type 1 (IL-1R1) is the cognate receptor for IL-1β and this receptor is highly expressed on glia, neurons, and NPCs in the hippocampus of the rodent brain (25–28). After binding to IL-R1, IL-1β induces an intracellular signaling cascade that leads to altered gene transcription and a heightened proinflammatory response (29–31). In most cell types, ligation of IL-1β to IL-1R1 activates the NF-κB signaling pathway (32). NF-κB is composed of homo- or heterodimeric combinations of the Rel family of proteins, which consists of p52, p65 (nuclear factor NF-κB p65 subunit; Rel A), c-Rel, Rel B, and p50. These proteins work in combination or with other cofactors, such as CREB binding protein, to regulate the transcription of NF-κB target genes (33). The consequences of the sustained

ABBREVIATIONS: DIV, day *in vitro*; GFP, green fluorescent protein; GSK-β, glycogen synthase β; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor α; IL-1R1, IL-1 receptor type 1; NPC, neural progenitor cell; Nr2e1, nuclear receptor subfamily 2 group E member 1; p21, cyclin-dependent kinase inhibitor 1; p65, nuclear factor NF-κB p65 subunit; TLX, orphan nuclear receptor tailless homolog

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doi: 10.1096/fj.201700495R

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elevation of IL-1β in the hippocampus include impaired synaptic plasticity and hippocampal-dependent learning and memory (28, 30, 34–36). Indeed, the NF-κB pathway has been suggested to mediate the effects of IL-1β on long-term potentiation (34, 37, 38). It has also been demonstrated that IL-1β can recruit the NF-κB pathway to suppress hippocampal neurogenesis. For example, it has been demonstrated that inhibition of the NF-κB pathway before the extrinsic administration of IL-1β to hippocampal NPCs *in vitro* prevented the IL-1β–induced suppression of proliferation in these cells (39); however, it is uncertain how the activation of this pathway can affect intrinsic regulators of hippocampal neurogenesis.

The study of the signaling mechanism within embryonic NPCs provides valuable insight into signaling mechanisms that may occur within adult NPCs (40–43). IL-1R1 is expressed on both embryonic and adult NPCs (27, 28), and IL-1β has been shown to negatively impact various aspects of hippocampal neurogenesis, such as proliferation, differentiation, and survival (27, 28, 39). TLX (orphan nuclear receptor tailless homolog)—or Nr2e1 (nuclear receptor subfamily 2 group E member 1)—is an orphan nuclear receptor and an intrinsic regulator of neurogenesis that is required to maintain the pool of NPCs within the hippocampus by promoting NPC proliferation and suppressing differentiation (44, 45). Studies that use transgenic models have shown that TLX can contribute to hippocampal-dependent learning and memory (44, 46, 47). For example, impairments in hippocampal-associated cognitive function and hippocampal neurogenesis have been reported in $TLX^{-/-}$ mice (48, 49). It has also been demonstrated that IL-1β negatively regulates the expression of TLX within both adult and embryonic NPCs, with subsequent impairment in NPC proliferation (28, 30, 50; however, the associated signaling remains largely unknown. Likewise, whether TLX manipulation can prevent the effects of IL-1β on NPC expansion has not been investigated. Here, we identify the NF-κB signaling pathway as a key mediator of the effects of IL-1β on TLX expression and demonstrate that the restoration of TLX expression is sufficient to prevent the negative effects of IL-1β on NPC expansion *in vitro*. These data, along with previous data from studies by us and others, highlight TLX as a potential regulator of IL-1β–induced impairment in hippocampal neurogenesis.

MATERIALS AND METHODS

Preparation and treatment of rat hippocampal NPCs

Embryonic day 18 rat hippocampi (Biologic Services Unit, UCC, Cork, Ireland) were cultured for 6 d *in vitro* (DIV) as previously described [Green *et al.* (27) and Keohane *et al.* (51)]. Neurospheres were kept under proliferative conditions in T25 flasks at a density of 2×10^6 cells/10 ml of proliferation media [DMEM:F12 (Sigma-Aldrich, St. Louis, MO, USA), 2% B27 supplement (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (Sigma-Aldrich), 10 ng/ml epidermal growth factor (Sigma-Aldrich), 10 ng/ml fibroblast growth factor (Millipore, Billerica, MA, USA), 200 mM L-glutamine (Sigma-Aldrich), and 33 mM D-glucose (Sigma-Aldrich). At 6 DIV, neurospheres were dissociated by using 0.1% trypsin-EDTA enzyme (Sigma-Aldrich) and treated as a single-cell suspension. Cells (5×10^4) were plated in proliferation media onto poly-D-lysine (Sigma-Aldrich)–coated coverslips for immunocytochemical analysis, and cells (5×10^5) were seeded per well in proliferation media in 6-well plates for PCR analysis. In the first set of experiments, recombinant rat IL-1β (10 ng/ml; R&D Systems, Minneapolis, MN, USA) was added to media for 10 and 30 min, 1, 2, and 4 h, or 6 DIV to measure the mRNA expression levels of TLX, p21 (cyclin-dependent kinase inhibitor 1), and IκBα (nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor α), and p65 and TLX protein levels. A second series of NPC cultures were treated with IL-1β (10 ng/ml) for 8, 16, and 24 h to assess the effects of longer-term treatment of IL-1β on TLX and p21 mRNA expression. In a subsequent series of experiments, IL-1β (10 ng/ml) was added to proliferation media with or without the NF-κB inhibitor, JSH-23 (25 μM; Sigma-Aldrich), for 3 h *in vitro*, and the mRNA expression levels of TLX, p21, and IκBα, as well as protein levels of TLX were assessed.

Lentiviral production and transduction of NPCs with TLX lentivirus

Mouse TLX (Nr2e1) was cloned into a pCDF–cytomegalovirus–IRES–green fluorescent protein (GFP) lentiviral backbone by using standard cloning methods. Transgene expression of TLX was driven by a cytomegalovirus promoter. Lentiviral particles were produced by transfecting an HEK293T producer cell line with the lentiviral Nr2e1 plasmid or lentiviral GFP control plasmid and pPACKF1 Lentivector Packaging Kit (System Biosciences, Palo Alto, CA, USA). Harvested viral supernatant was aliquoted and stored at −80°C until use. Packaged viruses had titers of approximately 1×10^6 transfection units/ml. On the day of seeding, 5×10^5 dissociated NPCs were plated in each well of a 6-well plate, along with 7×10^4 transfection units/ml of lentivirus that overexpressed either TLX or GFP. After 24 h, recombinant rat IL-1β (10 ng/ml) or PBS was added. Cells were allowed to proliferate as neurospheres and were kept under proliferative conditions for an additional 6 DIV.

PCR

Total cellular RNA was extracted from dissociated NPCs that were derived from neurospheres by using a Total Mammalian RNA extraction kit (Sigma-Aldrich) according to manufacturer instructions. Extracted RNA was treated with DNase (Sigma-Aldrich) to remove any DNA contamination. cDNA was prepared by using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). PCR was carried out on a 96-well plate by using a StepOnePlus Real-Time PCR machine (Applied Biosystems) using the following primers: TLX, (forward) GCTTTCTTCACAGCGGTCAC, (reverse) GCAGACACAGCGGTCAACT; IκBα, (forward) TGAAGTGTGGGGCTGATGTC, (reverse) AGGGCAACTCATCTTCCGTG; p21, (forward) GTATGCCGTCGTCTGTTCGG, (reverse) GCAGAAGACCAATCGGCGCT; and succinate dehydrogenase, (forward) CCCACTAACTACAAGGGACAGG, (reverse) TTGGCACCATGCACTGAG. PCR consisted of the following steps: 95°C for 3 min to denature the cDNA, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction consisted of 1 μl of sample (5 ng/μl), 10 μl of Sybr MasterMix (KiCqStart SYBR Green quantitative PCR ReadyMix with ROX reference dye for ABI instruments; Sigma-Aldrich), 0.1 μl of both forward and reverse primers, and 8.8 μl of RNase-free H_2O. RNA elution solution was used instead of samples to ensure that no amplification occurred as a result

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of self-complimentary primers. Results were normalized to succinate dehydrogenase and analyzed by using the $\Delta\Delta C_t$ method (52).

Immunocytochemistry

Cells were incubated in 5% donkey serum overnight at 4°C to block nonspecific Ab binding. Cells were then incubated with Abs against nestin (1:200, donkey anti-goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA), TLX (1:100, donkey anti-rabbit; Santa Cruz Biotechnology), and/or p65 (1:100, donkey anti-rabbit; Abcam, Cambridge, MA, USA) at 4°C overnight. Cells were subsequently incubated with the appropriate fluorescently tagged secondary Ab (Alexa Fluor; Thermo Fisher Scientific) for 2 h at room temperature in the dark. Cells were counterstained with DAPI (1:2500) to identify nuclei. For each protein, a sample of cells was incubated with blocking solution and secondary Ab, but without prior incubation with primary Ab, as a negative control.

Neurosphere diameter analysis, cell counts, and densitometry

Neurospheres were imaged by using an inverted microscope (IX70; Olympus, Tokyo, Japan) at ×10 magnification each day for 5 DIV. At 6 DIV, neurospheres were dissociated to assess changes in TLX and p21 expression as previously described. For diameter analysis, images of 5 individual neurospheres were captured per field of view from 4 fields of view per flask per time point. Neurosphere diameter was measured by using ImageJ (v.1.47; National Institutes of Health, Bethesda, MD, USA). At least 20 spheres were analyzed per condition. Each experiment was repeated at least 3 times. Immunolabeled cells were imaged by using an upright microscope (BX53; Olympus). Positively stained cells were counted from 5 random fields of view per coverslip. For each condition, cells from 4 coverslips were counted, and each experiment was repeated 3 times. Merged images of DAPI with TLX or p65 immunolabeled cells were used to analyze the densitometry of nuclear staining from 10 randomly selected TLX- or p65-positive cells per field of view by using ImageJ. Twenty micrographs per condition were analyzed from 3 independent experiments. Background fluorescence intensity was controlled for by thresholding. Data were expressed as arbitrary units of the intensity ratio of nuclear to nuclear+cytoplasmic TLX, and each data set that resulted from IL-1β treatment was normalized to its respective control.

Statistical analysis

An unpaired Student's *t* test and 1- or 2-way ANOVA, followed by Bonferroni *post hoc* test was performed, when appropriate, to determine which conditions were significantly different from each other. Results are displayed as means ± SEM and deemed significant at values of $P < 0.05$.

RESULTS

IL-1β suppresses neurosphere expansion

Two-way ANOVA revealed a main effect of IL-1β (10 ng/ml) treatment [$F(1, 20) = 15.805$; $P < 0.001$] and treatment duration [$F(4, 20) = 53.093$; $P < 0.001$]. *Post hoc* analysis demonstrated that IL-1β suppressed neurosphere expansion under proliferative conditions after 4 and 5 DIV ($P < 0.05$ *vs.* control; **Fig. 1*A*, *B***). Under the same conditions at 6 DIV, IL-1β induced a significant increase in the mRNA expression of the tumor suppressor gene, p21, in dissociated NPCs ($P < 0.01$ *vs.* control; Fig. 1*C*). IL-1β also induced a significant decrease in the mRNA expression of TLX—a known promoter of cell proliferation in NPCs—at this time point ($P < 0.01$; Fig. 1*D*). This is consistent with a previous report that demonstrated that IL-1β reduced protein expression levels of TLX in hippocampal NPCs after 7 DIV (30). IL-1β did not induce a reduction in cell viability at this dose and over this timeframe (data not shown), which is in agreement with previous data (27).

IL-1β induces rapid activation of NF-κB

To assess whether IL-1β administration induced the translocation of the NF-κB subunit, p65, to the nucleus in hippocampal NPCs—an indicator of NF-κB activation—we assessed the ratio of nuclear to cytoplasmic expression of p65 (Rel A), a key signaling component of the NF-κB pathway. As NF-κB signaling has been shown to be rapidly activated in human glial cells after IL-1β exposure (53), we assessed p65 translocation to the nucleus from 10 min to 4 h after IL-1β administration. IL-1β administration induced a significant increase in the nuclear translocation of p65 protein at 10 min after IL-1β, indicating increased NF-κB activation [$F(5, 114) = 19.21$; $P < 0.001$ *vs.* control; **Fig. 2*A*, *C***]. A negative feedback mechanism was evident because of the fact that mRNA expression of IκBα—a negative regulator and target gene of p65—was significantly increased 10 min after IL-1β [$F(5, 18) = 6.56$; $P < 0.001$; $P < 0.05$ *vs.* control; Fig. 2*B*)]. The increase in IκBα expression was maintained for up to 4 h, which coincided with a decrease in nuclear p65 protein expression at 4 h after IL-1β ($P < 0.01$ *vs.* control; Fig. 2*A*).

IL-1β induces suppression of TLX expression

As we observed NF-κB activation that was induced by IL-1β in hippocampal NPCs, we next assessed the effects of IL-1β on TLX expression in hippocampal NPCs over the same time period. After 30 min of IL-1β exposure, there was decreased mRNA expression of TLX in proliferating NPCs [$F(5, 12) = 3.17$; $P < 0.05$; **Fig. 3*A***]. This resulted in a significant decrease in TLX protein after 1 h of IL-1β exposure [$F(5, 114) = 20.75$; $P < 0.001$; Fig. 3*B*, *C*]. We assessed TLX mRNA expression after an extended treatment with IL-1β and demonstrated that it remained reduced after 8 h of IL-1β exposure, normalized at 16 h, and was significantly lower than control again at 24 h after IL-1β treatment [$F(3, 3) = 18.88$; $P < 0.001$; $P < 0.01$; Fig. 3*D*]. This corroborates previous data that show a reduction in TLX protein expression at 24 h in response to IL-1β (28). As TLX functions as a transcriptional suppressor, we assessed the effect of IL-1β on the expression of the TLX target gene and the suppressor of proliferation, p21. IL-1β induced a significant increase in p21 mRNA expression after 2 h [$F(5, 18) = 3.25$; $P < 0.01$ *vs.* control]; however, this effect was not significant after 4 h (Fig. 3*E*). Therefore, we extended our treatment times and observed that after 8 h

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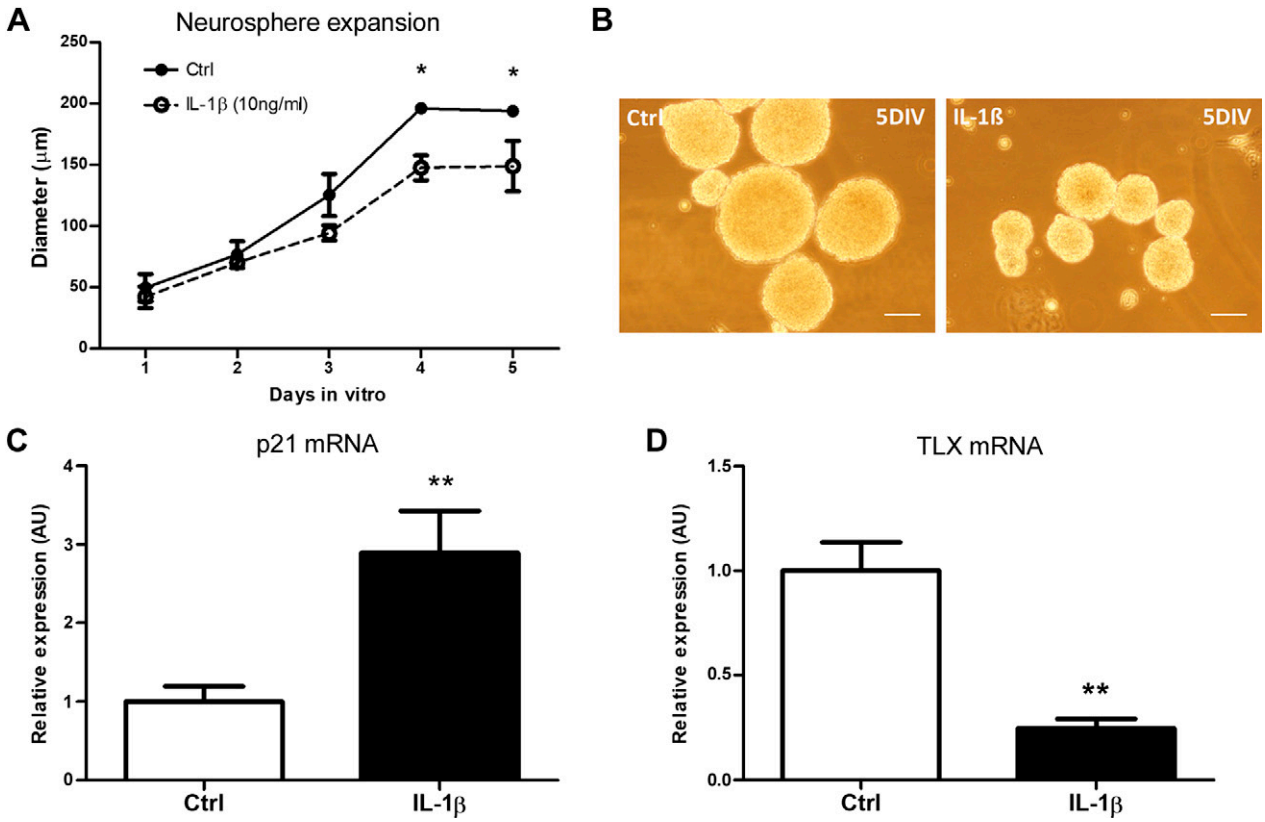


Figure 1. IL-1β–induced suppression of hippocampal neurosphere expansion is associated with increased p21 expression and reduced TLX expression. *A*) Neurosphere diameter from IL-1β–treated and untreated cultures (n = 3; 2-way ANOVA with Bonferroni *post hoc* test). *B*) Representative images of control and IL-1β–treated neurospheres at 5 DIV. Scale bars, 100 μm. *C*) Relative mRNA expression of p21 in untreated and IL-1β–treated cultures after 5 DIV under proliferative conditions (n = 8; Student's t test). *D*) Relative mRNA expression of TLX in untreated and IL-1β–treated cultures after 5 DIV under proliferative conditions (n = 8). AU, arbitrary units; Ctrl, control. Data are expressed as means ± SEM *$P < 0.05$, **$P < 0.01$ (Student's t test).

of IL-1β exposure, there was no change in p21 expression compared with control; however, after 16 and 24 h, p21 mRNA expression was significantly higher than controls [$F(3, 8) = 12.16$; $P < 0.01$; Fig. 3*F*].

IL-1β mediates its effects on TLX expression *via* NF-κB pathway activation

To assess whether the IL-1β–induced activation of the NF-κB pathway is responsible for the IL-1β–induced decrease in TLX expression, we pretreated hippocampal NPCs with JSH-23, an inhibitor of NF-κB activation (54). We demonstrated that when this pathway is inhibited, the IL-1β–induced reduction in TLX mRNA expression is attenuated [$F(3, 8) = 12.14$; $P < 0.001$ *vs.* control; $P < 0.05$ *vs.* IL-1β; **Fig. 4*A***]. Changes in TLX mRNA expression were mirrored by those in TLX protein expression [$F(3, 796) = 75.57$; $P < 0.001$ *vs.* control; $P < 0.001$ *vs.* IL-1β; Fig. 4*B, C*]. Attenuation of the IL-1β–induced decrease in TLX by JSH-23 was not a result of a reduction in total cell viability, as neither IL-1β, nor JSH-23 reduced cell viability compared with control during this acute incubation period (data not shown). Treatment of NPCs with JSH-23 had no effect on the IL-1β–induced increase in mRNA expression of the TLX target gene, p21 (Fig. 4*D*), but attenuated the IL-1β–induced activation of the NF-κB target gene, IκBα (Fig. 4*E*).

Increased TLX expression prevents IL-1β–induced suppression of neurosphere expansion

To assess whether TLX could prevent the IL-1β–induced decrease in neurosphere expansion, we transduced neurospheres with lentiviruses that expressed either GFP or TLX and demonstrated that they did not significantly impair neurosphere expansion compared with control nonlentiviral-transduced neurospheres across all time points (**Fig. 5*A, B***). Two-way ANOVA revealed a main effect of treatment with lentivirus with or without IL-1β [$F(5, 216) = 29.575$; $P < 0.001$] and treatment duration [$F(5, 216) = 120.422$; $P < 0.001$], and an interaction effect of treatment × treatment duration [$F(25, 216) = 1.780$; $P < 0.05$]. *Post hoc* analysis demonstrated that IL-1β treatment of GFP-transduced neurospheres impaired neurosphere expansion to an extent that was similar to nonlentiviral-transduced neurospheres that were treated with IL-1β at 4, 5, and 6 DIV ($P < 0.05$; Fig. 5*A*), whereas IL-1β treatment of TLX-transduced neurospheres did not (Fig. 5*B*). Moreover, neurospheres that were transduced with lentivirus that overexpressed TLX significantly attenuated IL-1β–induced impairment in neurosphere expansion at 4, 5, and 6 DIV ($P < 0.01$; Fig. 5*B, C*). IL-1β–treated GFP-transduced neurospheres demonstrated impaired expansion compared with GFP-transduced controls at 6 DIV

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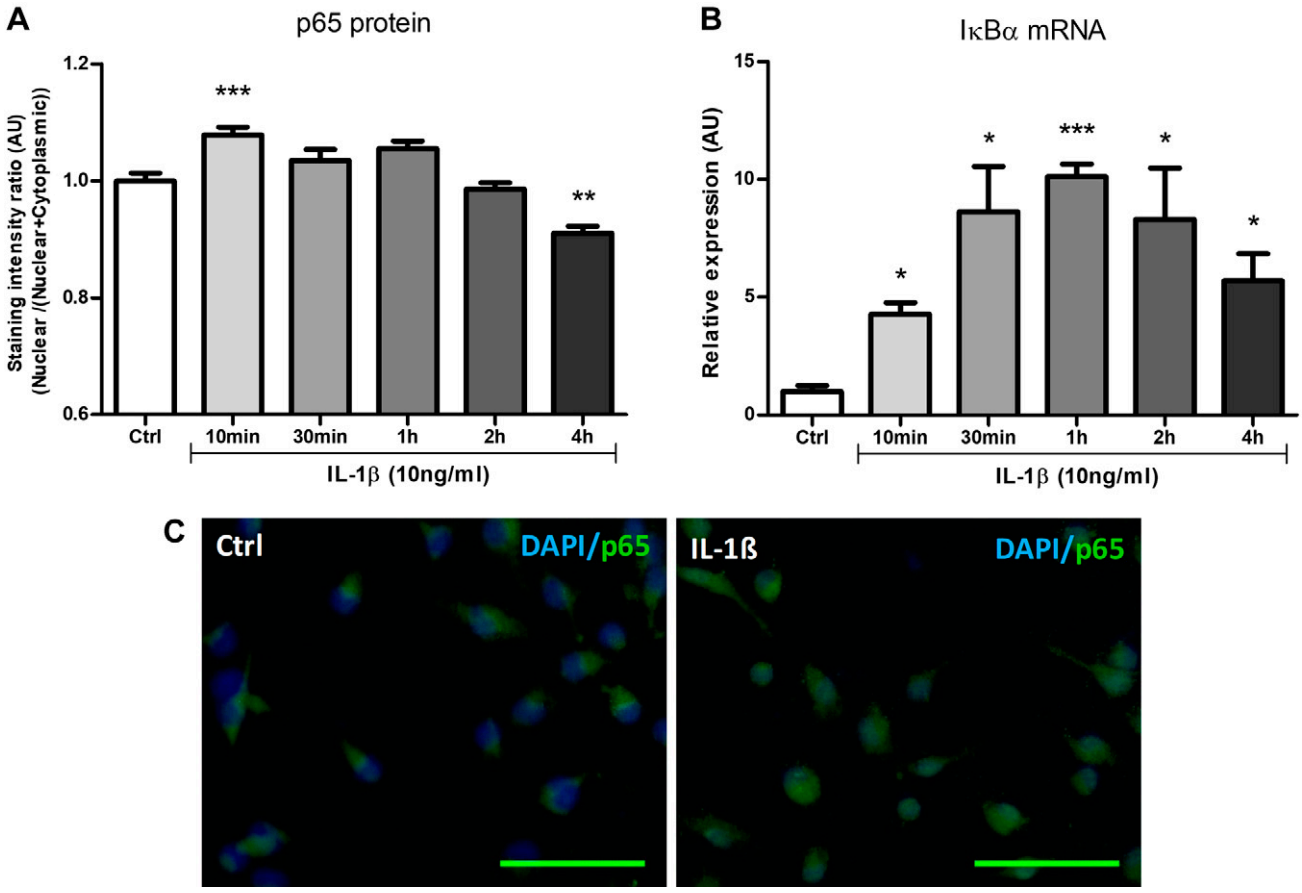


Figure 2. *A*, *B*) IL-1β induces the rapid activation of NF-κB signaling in proliferating hippocampal NPCs. Ratio of nuclear to cytoplasmic p65 staining intensity [arbitrary units (AU); $n = 3$; *A*] and relative mRNA expression of IκBα (*B*) in untreated and IL-1β–treated NPCs ($n = 3$). *C*) Representative images of p65 staining. Scale bars, 5 μm. Ctrl, control. Data are expressed as means ± SEM $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ (ANOVA with Bonferroni *post hoc* test).

($P < 0.001$; Fig. 5*D*), and this effect was not apparent in IL-1β–treated TLX-transduced neurospheres compared with TLX-transduced controls (Fig. 5*D, E*). To confirm the efficacy of lentiviral transduction, we assessed TLX mRNA expression in GFP- and TLX-transduced neurospheres at 6 DIV. In the absence of IL-1β, there was no significant difference in TLX mRNA expression between TLX- and GFP-transduced neurospheres (Fig. 5*F*); however, after treatment with IL-1β for 6 DIV, TLX-transduced neurospheres maintained a significantly higher expression of TLX mRNA compared with GFP-transduced neurospheres [$F(3, 25) = 7.73$; $P < 0.05$ between GFP + IL-1β and TLX + IL-1β; $P < 0.01$ between GFP and GFP + IL-1β[$P < 0.001$ between TLX and GFP + IL-1β; Fig. 5*F*]. Both GFP- and TLX-overexpressing cells display an increase in mRNA expression of IκBα in response to IL-1β treatment; however, the increase in expression only reaches significance in TLX-transduced neurospheres [$F(3, 19) = 5.71$; $P < 0.001$ *vs.* TLX; Fig 5*G*].

DISCUSSION

The present study demonstrates that TLX may protect against the IL-1β–induced suppression of hippocampal NPC proliferation, and, therefore, TLX is an important regulator of neuroinflammatory-induced changes in hippocampal neurogenesis. Specifically, we show that IL-1β induces a decrease in hippocampal NPC neurosphere expansion, which is consistent with previous reports (28, 30). This decrease is associated with a significant reduction in TLX expression—a regulator of NPC proliferation—and an increase in p21 expression, a mediator of cell-cycle arrest and reduced proliferation (55–57). In addition, we demonstrate that IL-1β recruits the NF-κB pathway in embryonic hippocampal NPC neurospheres and that pharmacologic inhibition of this pathway is sufficient to reverse the negative effects of IL-1β on TLX expression. Finally, we show that the restoration of TLX expression in the presence of IL-1β protects against the IL-1β–induced suppression of neurosphere expansion. This suggests that TLX can mitigate the antiproliferative effects of IL-1β on hippocampal NPCs.

We and others have previously established that IL-1β induces a decrease in hippocampal NPC proliferation in cultures of embryonic and adult neurospheres (28, 30, 39), and neurosphere expansion has been correlated with increased proliferation of NPCs from both the hippocampus and the subventricular zone of the lateral ventricles (46, 58). We also observed that IL-1β at the dosage and duration of treatment that were used in the current study does not affect cell viability; however, we did demonstrate that

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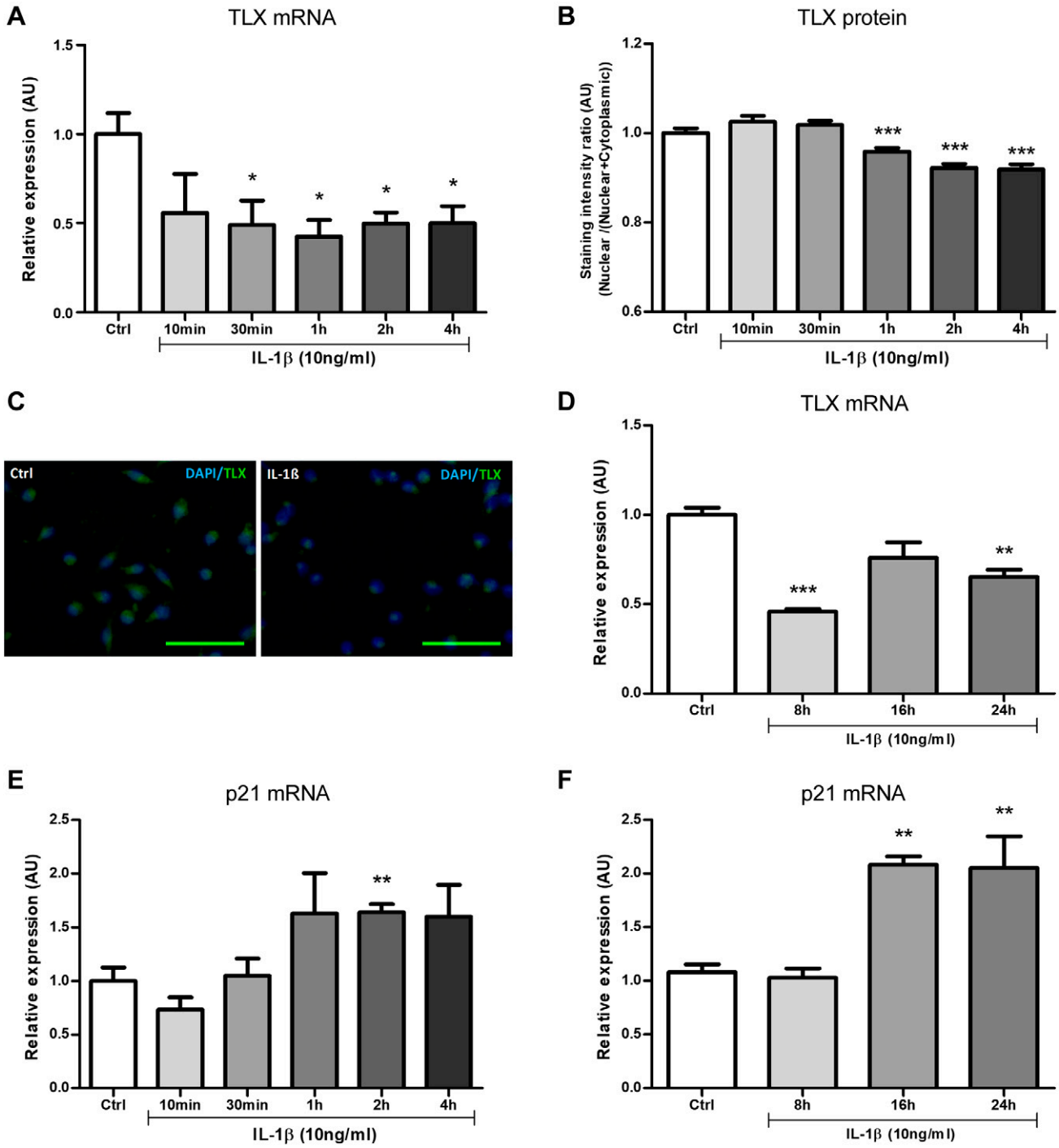


Figure 3. IL-1β suppresses TLX expression. *A*, *B*) Relative mRNA expression of TLX ($n = 3$; *A*) and ratio of nuclear to cytoplasmic TLX staining intensity [arbitrary units (AU); $n = 3$; *B*) in untreated and IL-1β–treated NPCs at 10 min, 30 min, 1 h, 2 h, and 4 h post–IL-1β treatment. *C*) Representative images of TLX staining. Scale bars, 5 μm. *D*) Relative mRNA expression of TLX in untreated and IL-1β–treated NPCs at 8 h, 16 h, and 24 h post–IL-1β treatment ($n = 3$). *E*) Relative mRNA expression of p21 in untreated and IL-1β–treated NPCs at 10 min, 30 min, 1 h, 2 h, and 4 h post–IL-1β treatment ($n = 3$). *F*) Relative mRNA expression of p21 in untreated and IL-1β–treated NPCs at 8 h, 16 h, and 24 h post—1β treatment ($n = 3$). Ctrl, control. Data are expressed as means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.0001$ compared with control (ANOVA with Bonferroni *post hoc* test).

IL-1β decreased mRNA expression of TLX after a short treatment duration for up to 24 h as well as after a longer treatment duration of 5 d. This supports previously published data that report an IL-1β– induced decrease in the protein expression of TLX at both 24 h (28) and 7 d (30). As TLX promotes cell proliferation, suppression of neurosphere expansion in response to IL-1β is potentially mediated *via* reduced expression levels of TLX. In addition, we observed an increase in mRNA expression of the TLX target gene, p21, after IL-1β treatment for up to 5 d. p21 expression in suppressed by TLX, and when its expression is increased, it induces cell-cycle

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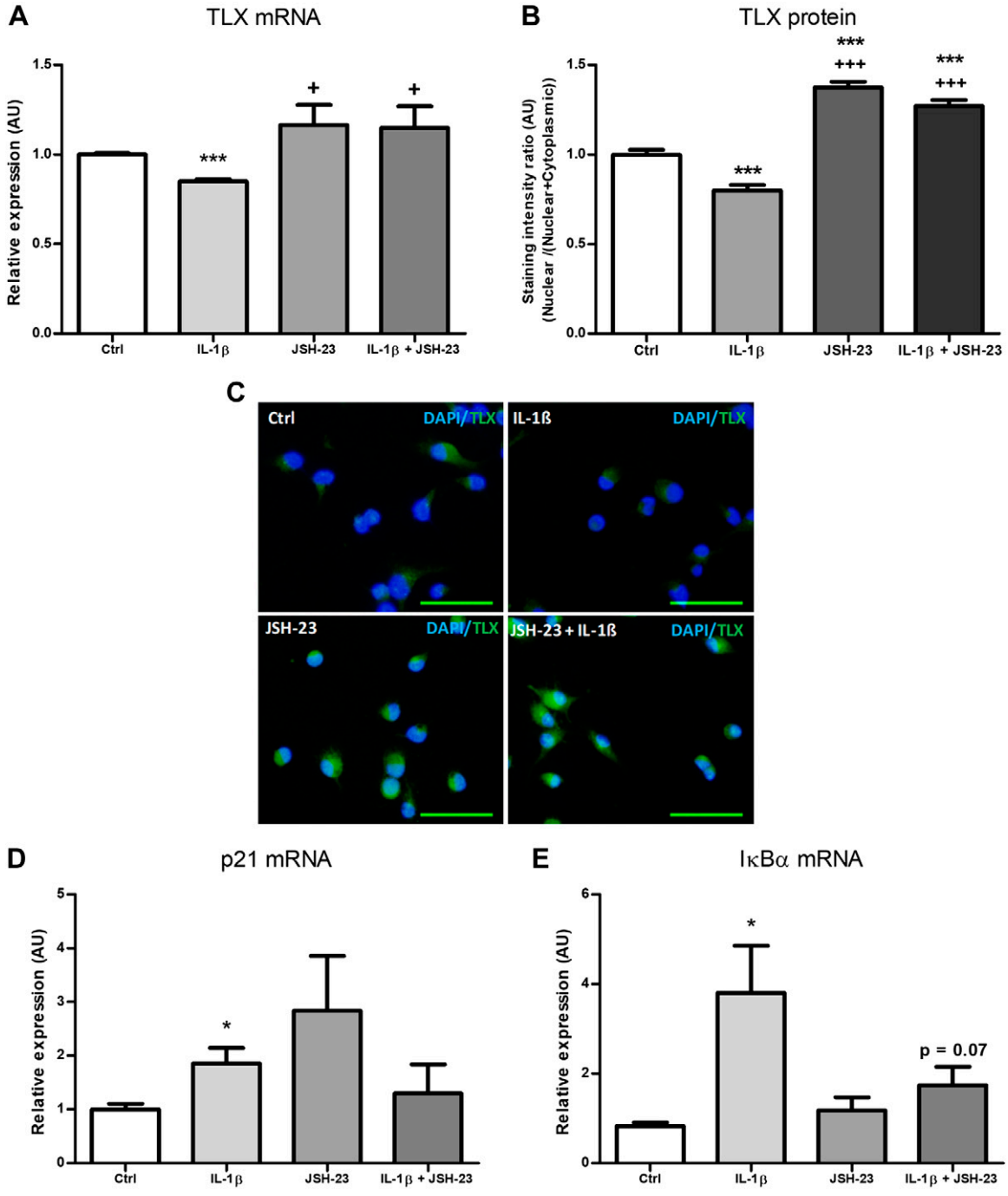


Figure 4. Inhibition of NF-κB signaling prevents the IL-1β–induced decrease in TLX expression. *A*, *B*) Relative mRNA expression of TLX (*A*) and nuclear TLX protein expression (*B*) in untreated, IL-1β–treated, JSH-23–treated, and IL-1β+JSH-23–treated hippocampal NPCs. *C*) Representative images of TLX staining. Scale bars, 5 μm. *D*, *E*) Relative mRNA expression of p21 (*D*) and IκBα (*E*) in untreated, IL-1β–treated, JSH-23–treated, and IL-1β+JSH-23–treated hippocampal NPCs. AU, arbitrary units; Ctrl, control. Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle; $^{+}P < 0.05$, $^{+++}P < 0.001$ compared with IL-1β (ANOVA with Bonferroni *post hoc* test).

arrest and reduces proliferation (55, 57, 59). Therefore, on the basis of the current study and previous reports (28, 30), the ability of IL-1β to reduce neurosphere expansion is likely a result of a reduction in NPC proliferation rather than an induction of cell death.

The NF-κB pathway has been shown to be responsible for the inhibition of cell proliferation *in vitro* and *in vivo* in several cell types, including rat hippocampal NPCs, mouse embryo fibroblasts, and human epithelial cells (39, 60, 61). IL-1β has previously been shown to recruit the NF-κB pathway to suppress hippocampal NPC proliferation *in vivo* (39). NF-κB is rapidly activated in response to IL-1β in human glial cells (53) and, therefore, we hypothesized that a rapid activation may also occur in hippocampal NPCs. Indeed, we observed that translocation of the p65 subunit to the nucleus [an

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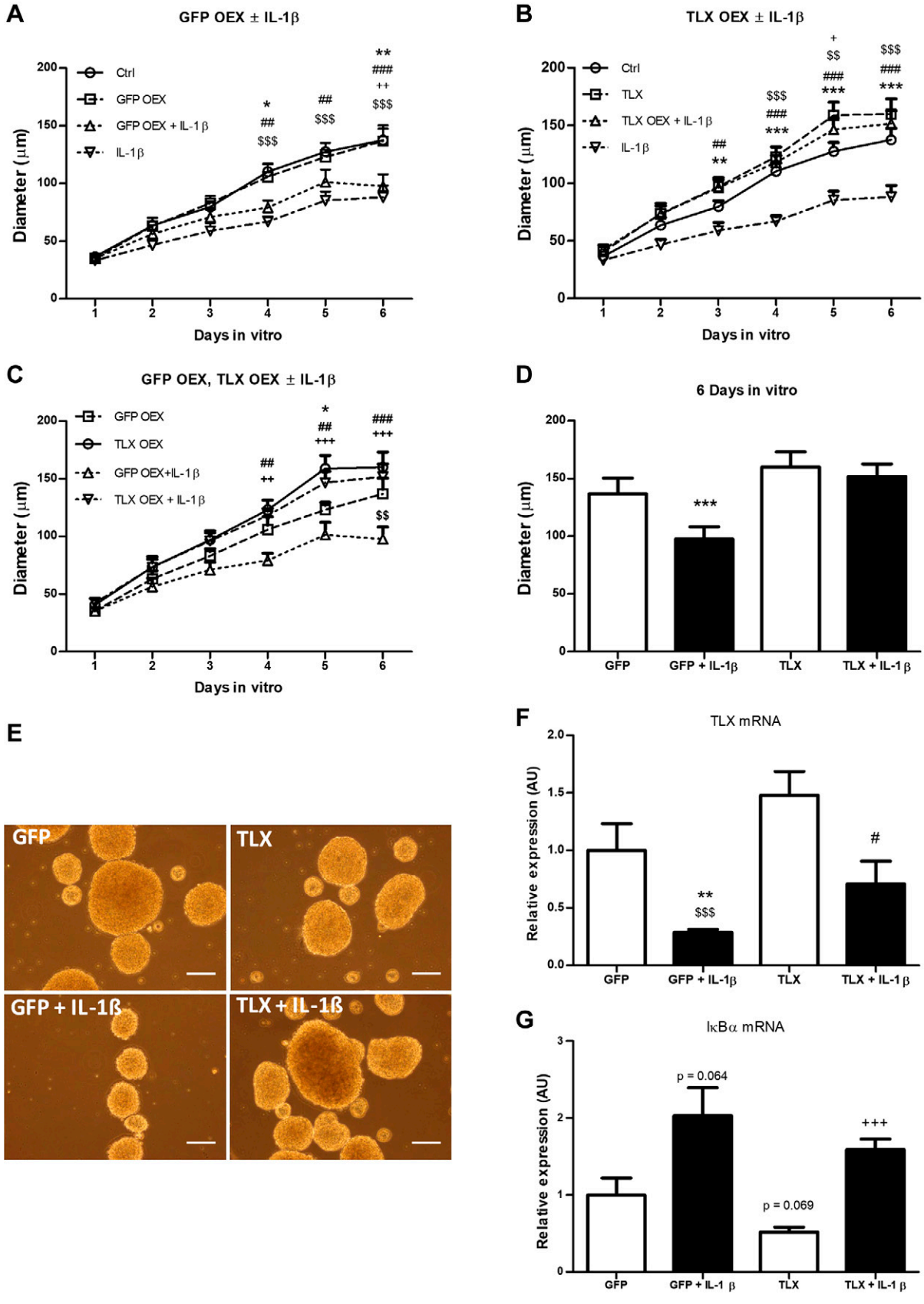


Figure 5. TLX overexpression (OEX) protects the IL-1β–induced suppression of neurosphere growth. *A*) Effect of GFP OEX on IL-1β–induced changes in neurosphere growth. $^{*}P < 0.05$; $^{**}P < 0.01$, control (Ctrl) compared with GFP+IL-1β; $^{\$\$\$}P < 0.001$, Ctrl compared with IL-1β; $^{++}P < 0.01$ GFP compared with GFP+IL-1β; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ GFP compared with IL-1β (2-way ANOVA with Bonferroni *post hoc* test; $n = 7$). *B*) Effect of TLX OEX on IL-1β–induced changes in neurosphere growth. $^{\$\$}P < 0.01$; $^{\$\$\$}P < 0.001$, Ctrl compared with IL-1β; $^{+}P < 0.05$ Ctrl compared with TLX; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ TLX compared with IL-1β;

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indicator of NF-κB activation (62, 63)] of hippocampal NPCs within 10 min of IL-1β administration. We also demonstrated the activation of the NF-κB target gene, IκBα, within this timeframe. Inhibition of NF-κB activation by using JSH-23 (39, 64–66) abrogated the IL-1β–induced decrease in TLX mRNA and protein expression in hippocampal NPCs after 3 h of exposure to IL-1β and also attenuated the IL-1β–induced increase in IκBα mRNA expression. Thus, the NF-κB pathway seems to be a potential mediator of IL-1β–induced changes, not only on NPC proliferation as previously shown (39, 54), but also on TLX expression. It is tempting to speculate that the suppression of TLX by IL-1β *via* enhanced NF-κB activation is the predominant mechanism that determines the outcome of IL-1β–induced changes in the proliferation of NPCs. The data herein would suggest that the IL-1β–induced activation of NF-κB results in the suppression of TLX gene expression, which results in a subsequent decline in TLX protein expression and NPC proliferation. This mechanistic pathway would be more likely than a direct protein interaction between TLX and NF-κB signaling because of the fact that changes in TLX and p65 expression occur at largely different time points and locations within NPCs.

We did not observe an effect of NF-κB inhibition on the expression of the TLX target gene, p21, in response to IL-1β. JSH-23 has been established as a selective NF-κB inhibitor, but any nonspecific signaling effects that JSH-23 treatment may have on hippocampal NPCs that may impact target genes cannot be ruled out (64, 67, 68). However, we observed that JSH-23 treatment attenuated the IL-1β–induced increase in the NF-κB target gene, IκBα; therefore, our data are a proof of concept that NF-κB signaling, at least in part, is responsible for the effects of IL-1β on TLX expression. It should be noted, though, that IL-1β does not exclusively recruit NF-κB signaling pathway components to mediate its effect on hippocampal NPC proliferation (69–71). Specifically, it has previously been demonstrated that IL-1β treatment for 48 h induced the activation of SAPK/JNK signaling in embryonic rat forebrain NPCs *in vitro* and that SAPK/JNK signaling, not the NF-κB pathway, mediated the IL-1β–induced decrease in the proliferation of these NPCs (69). Differences reported in that study may be related to brain region, embryonic age of NPCs, or treatment duration of IL-1β. Results also suggest that, at specific stages in development, IL-1β may recruit different pathways to exert its effects on NPCs, or, indeed, longer incubations with IL-1β may result in different signaling pathway activation. We have previously shown that IL-1β can increase the activity of glycogen synthase kinase 3 β (GSK-3β), a negative regulator of Wnt signaling pathway (72), in the proliferation of hippocampal embryonic NPCs with subsequent reductions in proliferation (30). In addition, that study demonstrated that when GSK-3β was inhibited, the IL-1β–induced suppression of NPC proliferation and TLX expression were ameliorated; however, that study assessed the effects of chronic IL-1β administration, and, hence, GSK-3β—and potentially Wnt signaling—may regulate the chronic effects of IL-1β on TLX and NPC proliferation. Of interest, GSK-3β can interact with NF-κB signaling to regulate cytokine production; therefore, the protective effects of the inhibition of GSK-3β after IL-1β exposure may be indirectly mediated by the subsequent effect on NF-κB signaling (73, 74). Assessment of the differential activation of specific pathways in response to individual activators as NPCs proliferate, differentiate, and integrate into the surrounding neuronal circuitry provides an interesting avenue for future research.

An interesting insight into the transcriptional effects of IL-1β in NPCs is the increase in p21 expression after IL-1β treatment of proliferating NPCs that is demonstrated in this study. p21 is a potent suppressor of the cell cycle, and, subsequently, is an important regulator of cell proliferation (56). TLX has previously been shown to suppress the expression of p21 in neural stem cell cultures (unknown source), which is correlated with an increase in NPC proliferation (55); however, the study did not attempt to manipulate the increase in p21 expression—either by knockdown or another method of inhibition—to assess the direct role of p21 on neural stem cell proliferation (55). The importance of p21 in regulating the proliferation of hippocampal NPCs has since been established in $p21^{-/-}$ mice for which there is a significant increase in NPC proliferation (59); therefore, it is reasonable to suggest that the mechanism by which TLX regulates proliferation involves the regulation of p21 expression. We demonstrate here that when TLX expression is suppressed by IL-1β, there is a concurrent increase in p21 expression, which suggests that IL-1β may suppress NPC proliferation by enhancing p21 expression. This theory is supported by findings that show an increase in hippocampal IL-1β expression, a subsequent increase in hippocampal p21 expression, and reduced hippocampal NPC proliferation and neuronal differentiation in a mouse model of inflammatory bowel disorder (75).

Deficits in neurogenesis in TLX-null mice have been reported by several research groups (46, 55, 76, 77). For

$^{**}P < 0.01$; $^{***}P < 0.001$ TLX+IL-1β compared with IL-1β (2-way ANOVA with Bonferroni *post hoc* test; $n = 7$). *C*) Comparison of GFP OEX and TLX OEX on IL-1β–induced changes in neurosphere growth. $^{*}P < 0.05$ GFP compared with TLX; $^{\$\$}P < 0.01$ GFP compared with GFP+IL-1β; $^{++}P < 0.01$; $^{+++}P < 0.001$ TLX compared with GFP+IL-1β; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ TLX+IL-1β compared with GFP+IL-1β (2-way ANOVA with Bonferroni *post hoc* test; $n = 7$). *D*) Comparison of GFP OEX and TLX OEX on IL-1β–induced changes in neurosphere growth at 6 DIV. $^{***}P < 0.001$ GFP compared with GFP+IL-1β (1-way ANOVA with Bonferroni *post hoc* test; $n = 7$). *E*) Representative images of GFP OEX and TLX OEX neurospheres, untreated or IL-1β treated. Scale bars, 100 μm. *F*, *G*) Relative mRNA expression of TLX (*F*) and IκBα (*G*) after lentiviral treatment of neurospheres with and without IL-1β treatment. AU, arbitrary units; Ctrl, control. Data are expressed as means ± SEM. $^{**}P < 0.01$ GFP compared with GFP+IL-1β; $^{\$\$\$}P < 0.001$ TLX compared with GFP+IL-1β; $^{\#}P < 0.05$ GFP+IL-1β compared with TLX+IL-1β (Student's *t* test; $n = 7$).

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example, Shi and colleagues have demonstrated that NPCs of $TLX^{-/-}$ mice have prolonged cell cycles and impaired proliferation during embryonic development (77). This was also demonstrated in adult hippocampal NPCs, in which there was impairment in NPC proliferation within the hippocampus of adult mice with an induced recombination of the TLX gene (78). Functionally, deficits in TLX expression and the subsequent suppression of hippocampal neurogenesis have been demonstrated to impair spatial memory in mice, which is a hippocampal-associated cognitive process (78). More recently, it has been shown that overexpressing TLX can rescue the proliferative defects in mice that lack TLX and improve hippocampal neurogenesis-associated cognition, as evidenced by improved performance in the Morris water maze (46). Taken together, these data show that TLX can be manipulated to positively regulate hippocampal neurogenesis and associated cognition; however, little has been done to assess whether TLX can mitigate the negative effects of an external insult, such as inflammation on hippocampal neurogenesis. In the current study, we show that the overexpression of TLX rescued the negative effects of IL-1β on hippocampal neurosphere expansion. Thus, TLX may act as a regulator of IL-1β–induced deficits in NPC expansion, but whether this effect translates *in vivo* remains to be determined. As we have demonstrated that NF-κB signaling may mediate the suppressive effects of IL-1β on TLX expression, the ability of TLX to counter the effects of IL-1β on neurosphere expansion may not be mediated by the suppression of NF-κB signaling. This is because of the fact that both GFP- and TLX-overexpressing neurospheres both demonstrate a similar increase in IκBα expression after IL-1β treatment. As previously discussed, IL-1β can recruit multiple pathways to regulate NPC proliferation; therefore, TLX may potentially interact with pathways other than NF-κB to mitigate the effects of IL-1β on neurosphere expression (69, 71, 79). Notwithstanding, our study demonstrates that TLX can prevent the functional effects of IL-1β on neurosphere expansion, and future studies will involve the identification of specific signaling interactions between these 2 regulators of NPC proliferation. It should be noted, however, that overexpression of TLX can lead to the hyperproliferation of NPCs and increased glioma formation *in vivo* (80, 81); however, this may be overcome with the advent of pharmacologic ligands for TLX. Indeed, efforts have been made to identify ligands for this orphan nuclear receptor to target and manipulate TLX function (82). Moreover, ligands may provide a controllable regulation of TLX function, thereby avoiding the potential adverse effects of enhanced TLX expression.

In conclusion, the current study provides evidence that IL-1β–induced down-regulation of TLX is mediated by the NF-κB pathway, and that enhanced TLX expression prevents IL-1β–induced reduction in NPC neurosphere expansion, potentially *via* an NF-κB–independent mechanism. We propose that the IL-1β–mediated suppression of TLX results in increased p21 expression, which leads to reduced cell proliferation. Additional research on the role of TLX in modulating neuroinflammatory-induced deficits in hippocampal neurogenesis will pave the way to the development of new therapeutic strategies for hippocampal-dependent cognitive disorders. FJ

ACKNOWLEDGMENTS

This work was supported by the Science Foundation Ireland (Grant 12/IA/1537). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

C. S. Ó'Léime, C. M. Long-Smith, J. F. Cryan, and Y. M. Nolan designed research; C. S. Ó'Léime, D. A. Kozareva, and A. E. Hoban performed the research; C. S. Ó'Léime, A. E. Hoban, and Y. M. Nolan analyzed the data; and C. S. Ó'Léime, and Y. M. Nolan wrote the paper.

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Received for publication May 30, 2017.
Accepted for publication September 11, 2017.

Downloaded from www.fasebj.org to IP 194.27.18.18. *The FASEB Journal* Vol., No. , pp:, October, 2017

Brain, Behavior, and Immunity xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Review Article

Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis

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ARTICLE INFO

Article history:
Received 3 May 2017
Received in revised form 15 July 2017
Accepted 23 July 2017
Available online xxxx

Keywords:
Neurogenesis
Neuroinflammation
Interleukin-1beta
Neural progenitor cells
Hippocampus nuclear receptors
Glucocorticoid receptors
Peroxisome proliferator activated receptors
TLX
NR2E1

ABSTRACT

Hippocampal neurogenesis, the process by which new neurons are born and develop into the host circuitry, begins during embryonic development and persists throughout adulthood. Over the last decade considerable insights have been made into the role of hippocampal neurogenesis in cognitive function and the cellular mechanisms behind this process. Additionally, an increasing amount of evidence exists on the impact of environmental factors, such as stress and neuroinflammation on hippocampal neurogenesis and subsequent impairments in cognition. Elevated expression of the pro-inflammatory cytokine interleukin-1β (IL-1β) in the hippocampus is established as a significant contributor to the neuronal demise evident in many neurological and psychiatric disorders and is now known to negatively regulate hippocampal neurogenesis. In order to prevent the deleterious effects of IL-1β on neurogenesis it is necessary to identify signalling pathways and regulators of neurogenesis within neural progenitor cells that can interact with IL-1β. Nuclear receptors are ligand regulated transcription factors that are involved in modulating a large number of cellular processes including neurogenesis. In this review we focus on the signalling mechanisms of specific nuclear receptors involved in regulating neurogenesis (glucocorticoid receptors, peroxisome proliferator activated receptors, estrogen receptors, and nuclear receptor subfamily 2 group E member 1 (NR2E1 or TLX)). We propose that these nuclear receptors could be targeted to inhibit neuroinflammatory signalling pathways associated with IL-1β. We discuss their potential to be therapeutic targets for neuroinflammatory disorders affecting hippocampal neurogenesis and associated cognitive function.

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http://dx.doi.org/10.1016/j.bbi.2017.07.153
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Please cite this article in press as: O'Léime, C.S., et al. Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis. Brain Behav. Immun. (2017), http://dx.doi.org/10.1016/j.bbi.2017.07.153

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1. Introduction

Hippocampal neurogenesis is a process by which neurons are born from neural progenitor cells (NPCs), and it is now known to occur from embryonic development throughout adulthood. Evidence of adult hippocampal neurogenesis first began to emerge as early as the 1960s when radiolabelling studies in adult rats showed that there was an increase in newborn cells within the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965). More recently, it has been estimated that approximately 700 new neurons are generated in the hippocampus of humans daily (Spalding et al., 2013). Functionally, hippocampal neurogenesis has been associated with hippocampal-dependent behaviours such as episodic and spatial learning and memory, as well as regulation of emotion (Deng et al., 2010; Aimone et al., 2014; Zhao et al., 2008; O'Leary and Cryan, 2014). Indeed, reductions in hippocampal neurogenesis have been associated with neurological and psychiatric disorders such as Alzheimer's disease and major depression, as well as with aging (Verret et al., 2007; Sahay and Hen, 2007; Kuhn et al., 1996). This then raises the question; can therapies aimed at protecting hippocampal neurogenesis be used to subsequently improve cognitive function in these diseases?

A common denominator in these conditions is the elevation in the expression of a key pro-inflammatory cytokine interleukin-1β (IL-1β) within the hippocampus (McGeer et al., 1994; Sluzewska et al., 1996; Leonard and Myint, 2006). IL-1β has been shown to negatively affect various stages of hippocampal neurogenesis from the proliferation of NPCs to their differentiation into neurons or astrocytes (Green and Nolan, 2012; Ben Menachem-Zidon et al., 2008; Goshen et al., 2008). Therefore, targeting regulators of these processes in NPCs could potentially mitigate the effects of IL-1β on hippocampal neurogenesis and associated cognition. In this regard, nuclear receptors (NR) are suitably poised as ideal therapeutic targets due to the fact that many are expressed within NPCs where they regulate aspects of neurogenesis. However, the question remains – can manipulating these regulators of neurogenesis prevent the negative effects of IL-1β on hippocampal neurogenesis and associated cognitive function? This review aims to address this question by firstly highlighting aspects of hippocampal neurogenesis that are regulated by both NRs and IL-1β, and secondly by exploring any signalling overlap between NRs and IL-1β with a view to identifying therapeutic targets for neurological or psychiatric disorders where deficits in hippocampal associated cognition is evident.

2. Hippocampal neurogenesis

Within the adult brain of many mammalian species including rodents and non-human primates, neurogenesis occurs in two specific regions known as neurogenic niches, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the DG in the hippocampus (Suh et al., 2009; Eriksson et al., 1998; Alvarez-Buylla and Lim, 2004). In these niches it has been shown that NPCs have the ability to replenish themselves via symmetrical division, or differentiate into specialized cells of the CNS such as mature neurons or astrocytes via asymmetrical division (Gage et al., 1995; Fisher, 1997; Zhao et al., 2008). In humans, while there is clear evidence to show ongoing neurogenesis in the DG of the hippocampus, there appears to be negligible levels of SVZ neurogenesis (Bergmann et al., 2012; Spalding et al., 2013). More recently, it has been proposed that neurogenesis occurs within the striatum of humans (Ernst et al., 2014). Hippocampal neurogenesis has been widely studied in terms of both its mechanism and functional role and will be the focus of this review. Multipotent neural stem cells (NSCs) reside within the embryonic and adult hippocampus and can give rise to NPCs (Gage, 1998; Kriegstein and Alvarez-Buylla, 2009; Eriksson et al., 1998). The distinctions between NPCs and NSCs are not yet clearly defined yet it is thought that NPCs are an intermediary between NSCs and differentiated cells and have a more limited capacity for self-renewal compared to NSCs (Gage, 1998, 2000; Sohur et al., 2006; Olynik and Rastegar, 2012). For simplicity, in this review NPCs will refer to all undifferentiated cells that have the ability to self-replicate and differentiate into various cellular lineages of the central nervous system (CNS) such as neurons, astrocytes or oligodendrocytes.

2.1. Developmental vs adult hippocampal neurogenesis

Development of the DG begins at embryonic day (E) 14 in the rat (Schlessinger et al., 1978; Laplagne et al., 2007). By E18, proliferating neuroblasts have formed the granular cell layer (GCL) with mature granule neurons evident 2–3 days before birth. The DG then continues to form until it is fully developed by postnatal day (P) 30. At this point NPCs are established in the SGZ where neurogenesis occurs throughout adulthood (Gage, 2000; Altman and Bayer, 1990). In the DG of the human brain, the granule neurons are identifiable at gestational week 11.5 (Humphrey, 1967). Using the proliferation marker Ki67 it has been shown that proliferating cells are widespread at the 14th week of gestation in various hippocampal regions, and as gestation progresses these proliferating cells become more restricted to the hilus region of the DG (Seress, 2007). It should be taken into account that humans have a much longer gestational period as well as having a larger hippocampal formation than rodents. Thus, taking rodent gestation to be approximately three weeks and human gestation to be on average 40 weeks, the appearance of granule neurons appear relatively much earlier in humans than rodents. However, this may also be due to the evolutionary differences between rodents and humans.

The cellular processes involved in embryonic and adult neurogenesis are very similar with the two main differences between each primarily arising from differences in the tissues that the NPCs are situated (Kempermann et al., 2004). Firstly, adult NPCs require a microenvironment conducive to neurogenesis (the neurogenic niche) within a larger non-neurogenic environment. In effect, adult NPCs need to be protected from the wide range of potential anti-neurogenic influences, which may reside within the surrounding

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brain tissue. In this regard, it has been shown that adult rat NPCs derived from the spinal cord, a non-neurogenic region, show self-renewal and multipotentiality properties once transplanted into the DG, indicating that the microenvironment in the DG is crucial for providing support for neurogenesis to occur (Shihabuddin et al., 2000). Secondly, adult neurogenesis is an individualistic process with cells at all stages of development present in the same microenvironment at the same time, whereas embryonic neurogenesis is a highly coordinated process with large groups of cells undergoing the same developmental process at the same time (Kempermann et al., 2004). Despite these differences, individual adult NPCs have been shown to undergo all of the same cellular processes as embryonic NPCs during developmental neurogenesis i.e. proliferation, differentiation, and functional integration of newborn neurons (Kempermann et al., 2004; Zhao et al., 2008). Additionally, the intracellular mechanistic features that regulate each stage of neurogenesis in the DG are suggested to be the same throughout all stages of development (Pleasure et al., 2000). Therefore, promoters and inhibitors of neurogenesis are likely to have similar effects on intracellular signalling mechanisms in both embryonic NPCs and adult derived NPCs.

2.2. Function of hippocampal neurogenesis

A growing body of evidence over the past two decades suggests that newly born neurons in the adult hippocampus contribute to hippocampal dependent learning and memory (Deng et al., 2010). Techniques to manipulate neurogenesis in rodents have provided direct evidence of the role of hippocampal neurogenesis in behaviour. These include irradiation of the hippocampus (Huo et al., 2012; Madsen et al., 2003; Saxe et al., 2006), as well as transgenic animal models (Snyder et al., 2016; Cummings et al., 2014; Saxe et al., 2006) and viral mediated techniques targeting neurogenic promoters specifically within NPCs (Murai et al., 2014; Jessberger et al., 2009; Taliaz et al., 2010) to ablate or enhance neurogenesis. The majority of these techniques have been used to ablate hippocampal neurogenesis and have demonstrated impairments in hippocampal associated behaviours, such as novel object and novel location recognition, fear conditioning, long-term spatial memory in a Morris water maze test, and pattern separation (an ability to distinguish two similar contexts) (Jessberger et al., 2009; Saxe et al., 2006; Clelland et al., 2009; Snyder et al., 2005; Dupret et al., 2008; Deng et al., 2009; Sahay et al., 2011; Winocur et al., 2006; Tronel et al., 2012). It should be noted that it has also been reported that alterations in hippocampal neurogenesis had no effect on some of these hippocampal-dependent tasks (Hernandez-Rabaza et al., 2009; Wojtowicz et al., 2008; Groves et al., 2013). Furthermore, a meta-analysis of the relevant literature revealed no significant effects of ablation of adult neurogenesis on spatial memory or cued fear conditioning (Groves et al., 2013), although the meta-analysis showed high levels of heterogeneity among the studies. Overall, while there is general consensus that hippocampal neurogenesis is required for pattern separation, the functional role of hippocampal neurogenesis has not yet been clearly demonstrated. It has been suggested that the heterogeneity in the findings to date may be due to differences in species, method of ablation, behavioural test used or in the variation in stress levels (Groves et al., 2013; Dranovsky and Leonardo, 2012; Glasper et al., 2012). For example, it has been suggested that the environment that the new neurons are born into will determine their functional significance (Dranovsky and Leonardo, 2012; Glasper et al., 2012) in that neurons born in conditions of low stress contribute to learning and memory while neurons born in conditions of high stress contribute to anxiety like behaviours (Glasper et al., 2012). Indeed, evidence now suggests that there is a critical role for hippocampal neurogenesis in the stress response and in the regulation of emotion (Cameron and Glover, 2015; Levone et al., 2015; Snyder et al., 2011). Moreover, recent evidence indicates that there is overlap between the role of neurogenesis in learning and emotion by demonstrating that inhibition of adult neurogenesis in the mouse DG reduces defensive behaviour to ambiguous threat cues but does not if the same negative experience is reliably predicted (Glover et al., 2017).

One proposed cellular mechanism for learning and memory is via changes in hippocampal synaptic plasticity. Long term potentiation (LTP) is an electrophysiological correlate of changes in synaptic plasticity and it has been shown that adult born neurons contribute to LTP formation within the DG of rats (Snyder et al., 2001). Ablation of neurogenesis by use of either transgenic mice or hippocampal irradiation inhibited LTP formation in the DG (Wang et al., 2008; Garthe et al., 2009). Interestingly, stress has been shown to impair DG LTP (Vereker et al., 2001) while LTP itself can increase the proliferation of NPCs in the DG if it is induced in the medial perforant pathway, which relays synaptic input to the DG (Bruel-Jungerman et al., 2006).

Another insight into the functional role of hippocampal neurogenesis emerged when it was shown that ablation of neurogenesis by irradiation blocked the behavioural effects of antidepressants in mice (Santarelli et al., 2003). This coupled with the discovery in the late 1990s of a pro-neurogenic effect of antidepressants (Malberg et al., 2000; Malberg and Duman, 2003; Gur et al., 2007) has led to the theory that some antidepressants work by altering neurogenesis (O'Leary and Cryan, 2014; Tanti and Belzung, 2013; Eisch and Petrik, 2012). It has been shown that neurogenesis and its functional consequences can be selectively enhanced by promoting cell survival. Specifically, a subsequent improvement in pattern separation has been reported in transgenic mice where NPC survival was enhanced by selectively ablating the pro-apoptotic gene *Bax* in NPCs (Sahay et al., 2011). It has also been established that increasing hippocampal neurogenesis via physical exercise induces an improvement in cognitive performance (Voss et al., 2013; van Praag et al., 2005; Ryan and Kelly, 2016). However, enhancing neurogenesis is complex due to its multifactorial process of proliferation, differentiation, and survival of the newborn neurons and thus it may be difficult to definitively uncover the role of enhanced neurogenesis by manipulating one aspect of the process.

2.3. Regulation of hippocampal neurogenesis

Hippocampal neurogenesis is regulated by a wide range of intrinsic signalling molecules as well as by external factors influenced by the surrounding environment (Aimone et al., 2014). Intrinsic regulators of hippocampal neurogenesis include intracellular signalling pathways within NPCs, such as Wnt, Notch, nuclear factor kappa B alpha (NF-κB) and mitogen activated protein kinase (MAPK) (Johnson et al., 2009; Aimone et al., 2014; Mu et al., 2010; Lie et al., 2005; Breunig et al., 2007; Faigle and Song, 2013). These intrinsic factors regulate gene expression within NPCs in order to control their proliferation, differentiation and maturation into neurons or glia (Mu et al., 2010). Expression and function of these intrinsic regulators can be altered by external environmental factors, which subsequently lead to altered hippocampal neurogenesis. The main external environmental factors that influence hippocampal neurogenesis include stress, exercise, diet, learning, environmental enrichment, and environmental toxins (Besnard and Sahay, 2016; van Praag et al., 1999a, Stangl and Thuret, 2009; Deng et al., 2010; Brown et al., 2003). Voluntary exercise, learning, environmental enrichment, and a healthy diet have all been shown to promote neurogenesis (van Praag et al., 1999a,b, Gould et al., 1999; An et al., 2008). However, the mechanism underlying their positive effects on neurogenesis is the subject of much research. For example, enhanced serum concentration of

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brain derived neurotrophic factor (BDNF) and increased levels of dietary nutrients, such as flavonoids (enriched in foods such as cocoa and blueberries) have been proposed as mediators of the pro-neurogenic effects of diet and exercise, respectively (Erickson et al., 2011; An et al., 2008). Environmental factors that negatively regulate neurogenesis include stress, an unhealthy diet, and environmental toxin exposure (Gould et al., 1998; Lindqvist et al., 2006; Sava et al., 2007; Desplats et al., 2012). Interestingly, these negative regulators are all associated with neuroinflammation as evidenced by increased microglial activation and increased pro-inflammatory cytokine release (Tynan et al., 2010; Goshen et al., 2008; Goshen and Yirmiya, 2009; Boitard et al., 2014; Purisai et al., 2007). Pro-inflammatory cytokines released from activated microglia can affect many aspects of hippocampal neurogenesis including the proliferation, differentiation, and maturation of NPCs, thus they are robust manipulators of hippocampal neurogenesis (Green et al., 2012; Green and Nolan, 2012; Ryan et al., 2013; Keohane et al., 2010; Yirmiya and Goshen, 2011; Borsini et al., 2015). While microglia are the predominant source of pro-inflammatory cytokines within the brain (Hanisch and Kettenmann, 2007), it should be noted that astrocytes are also susceptible to stimulation and can release cytokines. However, they produce relatively low levels of cytokines but have been shown to potentiate the release of cytokines from microglia in response to an inflammatory insult (Barbierato et al., 2013; Chen et al., 2015).

3. Regulation of hippocampal neurogenesis by IL-1β

It is now well established that neuroinflammation induced by injury, neurodegeneration, aging, as well as environmental factors, such as stress, unhealthy diet, and toxins (e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and paraquat) can negatively affect hippocampal neurogenesis, (Fig. 1) (Ekdahl et al., 2003; Monje et al., 2003; O'Leary et al., 2012; Heine et al., 2004; Green and Nolan, 2014; Hueston et al., 2017; Klein et al., 2016; Li et al., 2017). Neuroinflammation contributes to the pathophysiology of many psychiatric and neurodegenerative disorders where cognitive dysfunction and reductions in hippocampal neurogenesis are evident including Alzheimer's disease (AD), Parkinson's disease (PD), and depression as well as aging (Raison et al., 2006; Leonard, 2007; Tang et al., 2016; Höglinger et al., 2004). Indeed, in aged individuals and in both patients and animal models of AD, PD, and depression there is evidence of a heightened inflammatory state including increased pro-inflammatory cytokine expression within the hippocampus (Monson et al., 2014; Heppner et al., 2015; Heneka et al., 2015; Ryan and Kelly, 2016; Nolan et al., 2013, 2005). For example, heightened expression of pro-inflammatory cytokines in the cerebrospinal fluid of AD patients becomes evident in the preclinical stages of the disease concurrent with mild cognitive impairments (Monson et al., 2014). Additionally heightened expression of inflammatory cytokines have been demonstrated specifically within hippocampal tissue of AD patients and rodent models (Sheng et al., 1995; Hauss-Wegrzyniak et al., 1998). Levels fluctuate as the disease persists with elevated expression again evident in *post-mortem* hippocampal tissue (Monson et al., 2014; Heppner et al., 2015). Similarly in PD, pro-inflammatory cytokine expression is predominantly chronically elevated in brain tissue and it is proposed that a persistent inflammatory microenvironment around the susceptible neurons potentiates the disease progression (Herrero et al., 2015; Collins et al., 2012). Evidence from both human and animal studies show that both peripheral and hippocampal chronic low grade inflammation plays an important role in the pathology of depression, and it is particularly apparent in patients with treatment-resistant depression, and in patients who have experienced some form of early life trauma (Pariante, 2017; Kim et al., 2016) .

IL-1β is a pro-inflammatory cytokine that plays a role in hippocampal neuroinflammation due to its heightened receptor expression profile in the hippocampus (Farrar et al., 1987). While there is evidence to suggest that low expression levels of IL-1β are required for hippocampal-related learning and memory it is well established that chronically elevated IL-1β has detrimental effects on memory and cognition (Yirmiya and Goshen, 2011; Lynch, 2015). IL-1β is produced predominantly by microglia but also by pyramidal neurons and astrocytes in the hippocampus (Bandtlow et al., 1990; Lechan et al., 1990; Eriksson et al., 1999; Barbierato et al., 2013) and is released into the extracellular matrix, where it binds to its cognate receptor interleukin receptor type 1 (IL-1R1), which is expressed on NPCs in the hippocampus (Hanisch and Kettenmann, 2007; Rothwell et al., 1996; Green and Nolan, 2012). Thus, as well as influencing the function of mature neurons, IL-1β can directly influence NPCs via interaction with IL-1R1 on both embryonic and adult NPCs (Green and Nolan, 2012; Ryan et al., 2013). It should be noted that IL-1β may differentially influence NPCs or respond to changes in neurogenesis depending on the time during the lifespan at which changes in either inflammatory insult or neurogenesis occur. For example, an increase in hippocampal IL-1β was demonstrated in juvenile rats but not in adult rats subjected to whole brain irradiation (Blomstrand et al., 2014), which ablates neurogenesis, and thus suggests that different inflammatory mechanisms may be involved in regulating hippocampal neurogenesis at different ages. Likewise, inflammatory-induced changes in neurogenesis during embryonic or postnatal development may result in overall changes in the number of proliferating cells in the adult DG, which may consequently cause structural changes in the hippocampus, and ultimately lead to changes in behaviour (Green and Nolan, 2014). Development of anti-inflammatory interventions to circumvent inflammatory-induced impairments in neurogenesis and associated hippocampal functions should also take into consideration the timing during the lifespan of the intervention. Taken together, the evidence to date suggests that the mechanism underlying IL-1β-induced changes in neurogenesis during both development and in the adult hippocampus represents an important avenue for future research.

Upon binding to IL-1R1, IL-1β induces an intracellular signalling cascade, which ultimately leads to altered gene expression of IL-1β target genes and a heightened pro-inflammatory response (Sims et al., 1993). Alternatively, IL-1β can bind to IL-1R2, which is a decoy receptor and does not produce a physiological response (McMahan et al., 1991). The expression of IL-1R2 has not been demonstrated on NPCs. However, it has been shown to be mainly expressed on microglia, which suggests it plays a role in dampening cytokine-induced activation of microglia (Pinteaux et al., 2002). IL-1β signalling is inhibited by the IL-1 receptor antagonist (IL-1RA), which binds with almost equal affinity as IL-1β to IL-1R1 (Seckinger et al., 1987; Hannum et al., 1990). IL-1RA is expressed and produced by both pyramidal neurons and microglia within the hippocampus (Eriksson et al., 1999). IL-1β has been shown to negatively impact upon the proliferation, differentiation, and survival of hippocampal NPCs in cultures of rat hippocampal NPCs (Green et al., 2012; Ryan et al., 2013). There is further *in vitro* evidence to suggest that IL-1RA can reverse IL-1β-induced suppression of NPC proliferation as well as its promotion of astrogenesis in cultures of rat hippocampal neurospheres (Green and Nolan, 2012; Ryan et al., 2013; Koo and Duman, 2008).

It has also been shown that IL-1β can suppress neuronal differentiation in a human hippocampal progenitor cell line (Zunszain et al., 2012). While this study reported an increase in cell proliferation in response to IL-1β, which is contrary to what has been

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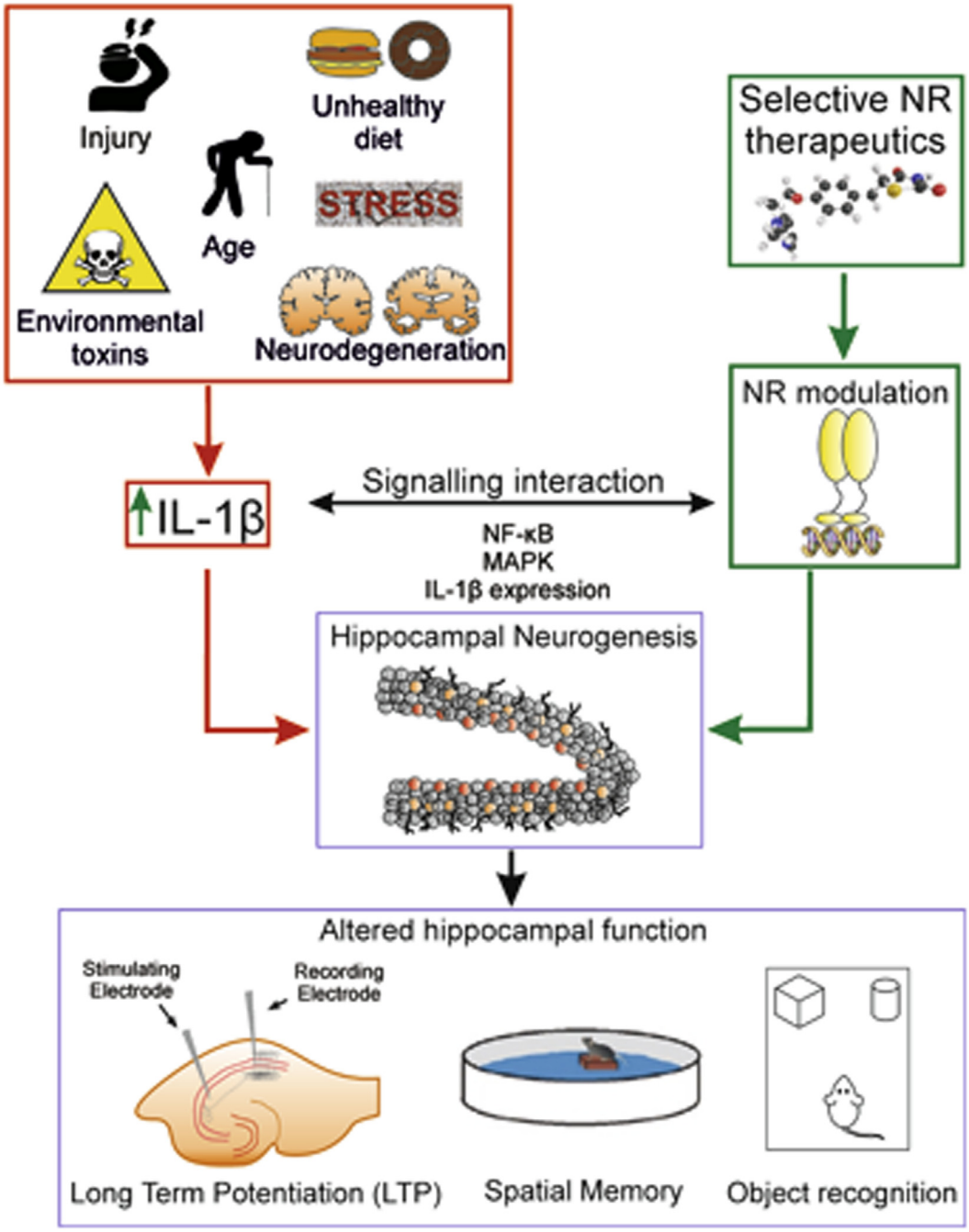


Fig. 1. IL-1β, nuclear receptors, and neurogenesis. IL-1β is released within the hippocampus in response to a host of factors, such as an unhealthy diet, increased age, brain injury, stress, environmental toxin exposure, and neurodegeneration. This can lead to suppression of neurogenesis and an impairment of hippocampal-dependent behaviours. Nuclear receptors (NRs) are known to regulate neurogenesis and are commonly targeted by small molecule therapeutics. As there is evidence to link NR signalling with signalling pathways activated by IL-1β, there is potential to therapeutically manipulate NRs to mitigate IL-1β-induced impairments in neurogenesis and hippocampal dependent cognition.

shown in rodent cell models, the increase in cell proliferation does not necessarily indicate enhanced neurogenesis. It is possible that an increase in cell proliferation in response to IL-1β leads to a phenotypic shift towards astrocyte rather than neuronal production, as has been previously demonstrated in response to IL-1β in primary cultures of rat NPCs (Green et al., 2012). It is also possible that the signalling mechanisms underlying IL-1β-induced changes in cell proliferation in human and rodent NPCs are not comparable but this has yet to be explored in more detail. In addition, it is likely that immortalised cells such as the human hippocampal progenitor cell line, may respond differently to cytokine stimulation than cultured primary cells or indeed than NPCs *in vivo*, due to genotypic and phenotypic variation which can come about due to serial passaging of cell lines over time (Pan et al., 2009). Notwithstanding, these discrepancies highlight the importance of assessing not only the proliferation status of NPCs but also their differentiation phenotype as only the complete progression from proliferating NPCs to integrated mature neurons represents successful neurogenesis.

In vivo studies have provided evidence that there are functional implications of neurogenesis as a mediator of IL-1β-induced changes in cognitive behaviours. For example, impairments in performance in the Morris water maze (MWM) and reduced

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neurogenesis due to elevated levels of hippocampal IL-1β in rodents can be rescued by administration by of IL-1RA (Ben Menachem-Zidon et al., 2014). Thus, IL-1RA is an important tool to examine the effects of IL-1β signalling on hippocampal neurogenesis and associated behavioural tasks. See Table 1 for a summary of evidence showing the effects of IL-1β on hippocampal neurogenesis and associated cognition.

3.1. IL-1β signalling pathways in NPCs

Downstream from IL-1R1, the NF-κB signalling pathway is one of the major mediators of the IL-1β intracellular response (Fig. 2). NF-κB is composed of homo- or heterodimeric combinations of the Rel family of proteins consisting of p52, p65 (Rel A), c-Rel, RelB, and p50. These proteins work in combination or with other co-factors, such as creb binding protein (CBP) to regulate the transcription of NF-κB target genes (Sheppard et al., 1999). The expression of p52, p65, p50, and CBP has been reported in NPCs from embryonic and rat forebrain where they have been shown to be involved in a range of processes, such as proliferation of NPCs and regulation of the effects of environmental enrichment on neurogenesis (Shingo et al., 2001; Lopez-Atalaya et al., 2011; Kaltschmidt and Kaltschmidt, 2009). RelB and c-Rel are expressed on migratory NPCs involved in SVZ neurogenesis and thus may play a role in the regulation of NPC migration (Denis-Donini et al., 2005). Evidence for the role of NF-κB signalling in neurogenesis comes from the fact that inhibition of NF-κB pathway by a specific pharmacological NF-κB signalling inhibitor impaired NPC neuronal differentiation in mouse NPCs *in vitro* (Rolls et al., 2007). Additionally, it has been shown *in vitro* that inhibition of NF-κB signalling impaired mouse NPC proliferation and neuronal differentiation (Zhang et al., 2012). A limited number of research studies have been carried out using human NPCs to highlight the signalling pathways employed by IL-1β. Zunszain and colleagues have shown that IL-1β increases the expression of enzymes involved in the kynurenine pathway in differentiating human hippocampal NPCs (Zunszain et al., 2012). This metabolic pathway is responsible for the enzymatic catabolism of tryptophan, the main amino acid involved in serotonin production, into kynurenine, which is known for its immunomodulatory effects (Nguyen et al., 2010). Dysregulation of this pathway has been associated with immune activation and it has been demonstrated to be activated during cytokine-induced depression (Dantzer et al., 2011; Davis and Liu, 2015). Interestingly, mice lacking the enzyme tryptophan-2,3-dioxygenase (TDO), which is involved in the catabolism of tryptophan, display enhanced hippocampal neurogenesis (Kanai et al., 2009). Moreover, it has been shown that the kynurenine pathway is activated by NF-κB in human placental cells (Dharane Nee Ligam et al., 2010). Thus, it is tempting to speculate that IL-1β may impair neurogenesis in the human hippocampus via NF-κB-mediated enhancement of kynurenine pathway activation.

Functional associations have also been made between impaired NF-κB signalling and deficits in hippocampal neurogenesis-associated cognition. It has been reported that reduced neuronal differentiation and impairments in spatial memory were evident in p50-deficient mice (Denis-Donini et al., 2008). Despite this evidence on the role of NF-κB signalling in neurogenesis, the direct interaction between IL-1β and NF-κB activation in NPCs has not been well studied. The main evidence for a role of NF-κB signalling in IL-1β-induced effects on hippocampal neurogenesis has shown

Table 1
IL-1β and neurogenesis. Summary of the effects of IL-1β on hippocampal neurogenesis, signalling mechanisms and function. Embryonic day (E), postnatal day (P), Morris water maze (MWM), overexpression (OEX), intracerebroventricular (ICV), Alzheimer's disease (AD).

Species	IL-1β Manipulation	IL-1β Signalling Mechanism	*Effects in vitro*	*Effects in vivo*	Gross effect on neurogenesis	Reference
E18 rat	10 ng/ml in culture media	Upregulation of GSK-3β	↓ NPC proliferation ↑ Astrocytic differentiation ↓ Neuronal differentiation	–	↓	Green and Nolan (2012) Green et al. (2012)
Adult rat	10 ng/ml in culture media	IL-1R1 binding	↓ NPC proliferation ↓ Neuronal differentiation	–	↓	Ryan et al. (2013)
Adult rat	Interferon alpha mediated increase in IL-1β	IL-1R1 binding	↓ NPC proliferation	–	↓	Kaneko et al. (2006)
1 rats	5, 10, and 10 ng/ml in culture media	IL-1R1 binding	↓ Neuronal differentiation (Serotonergic neurons)	–	↓	Zhang et al. (2013)
Human hippocampal progenitor cells	10 mg/ml in culture media	–	↑ NPC proliferation ↓ Neuronal differentiation	–	↓	Zunszain et al. (2012)
E16 rat	0.8–500 ng/ml in culture media	↑ SAPK/JNK	↓ NPC proliferation ↓ Neuronal differentiation	–	↓	Wang et al. (2007)
Aged adult mice	Inhibition of IL-1β activation by caspase-1 cleavage	–	–	↑ proliferation	↑	Gemma et al. (2007)
Adult mice	MyD88 knockout (disruption of IL-1R1 dependent signalling)	IL-1R1 independent	–	↓ NPC proliferation	↓	Wu et al. (2013)
Adult rat	20 ng/ml or 200 ng/ml ICV	↑NF-κB	–	↓ Cell proliferation	↓	Koo and Duman (2008)
Mice	OEX of IL-1RA	–	–	Impaired MWM performance	↓	Goshen et al. (2007)
Mice	Transplantation of NPC OEX IL-1RA	–	–	Prevention of stress induced impairments in fear conditioning	↑	Ben Menachem-Zidon et al. (2008)
Mice	Transplantation of NPC OEX IL-1RA	–	–	Improved MWM performance in model of AD		Ben Menachem-Zidon et al. (2014)
Rats	Intrahippocampal IL-1β infusion (5 ng/0.25 μl)	↑ MAPK (p-ERK)	–	Impaired fear conditioning Prevented by MAPK inhibition		Gonzalez et al. (2013)

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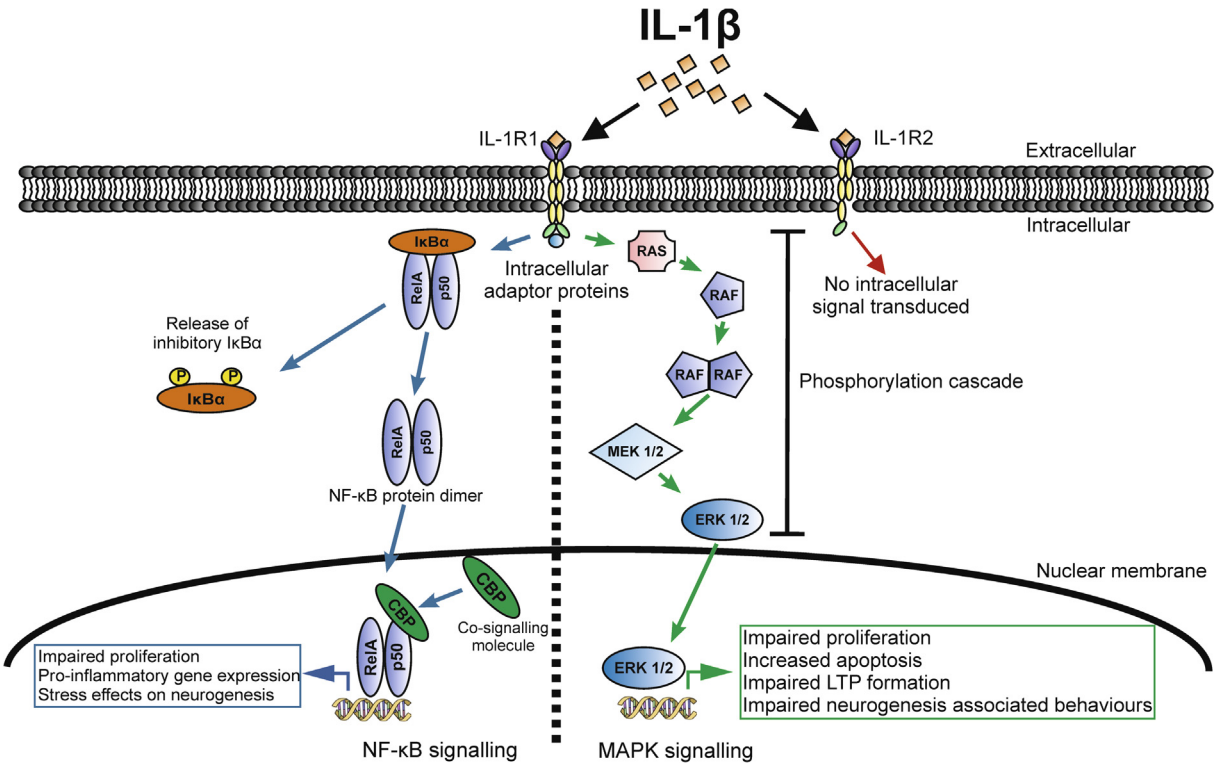


Fig. 2. Schematic of IL-1β signalling cascades. Within neurogenic cells, IL-1β signals via either NF-κB or MAPK pathways. interleukin-1 beta (IL-1β), interleukin receptor type 1 (IL-1R1), interleukin receptor type 2 (IL-1R2), creb binding protein (CBP), long term potentiation (LTP), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα).

that activation of the NF-κB pathway is responsible for the anti-neurogenic effects of IL-1β in adult rats *in vivo* and *in vitro* (Koo and Duman, 2008; Koo et al., 2010). In these studies, both stress and IL-1β induced a significant suppression of the proliferation of NPCs, which was prevented by NF-κB inhibition.

Another pathway that may mediate IL-1β-induced effects on hippocampal neurogenesis is the MAPK pathway (Fig. 2) (Tong et al., 2012; Huang et al., 2011). The MAPK pathway is a signal transduction pathway that can be activated by various cytokines and growth factors that act on cell surface kinase-linked receptors (Johnson and Lapadat, 2002). Once this pathway is activated there is a cascade of protein phosphorylation, which ultimately leads to altered gene expression and regulation of cell division, apoptosis, and tissue regeneration (Rang et al., 2012). IL-1β has been shown to regulate the MAPK pathway to produce various hippocampal neurogenesis associated cognitive effects. For example, in the hippocampus of rats, IL-1β decreases the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), a component of the MAPK pathway, which correlates with impairments in fear conditioning (Gonzalez et al., 2013). This study assessed MAPK signalling in hippocampal tissue as a whole rather than in any cell type in the hippocampus, and so the specific cell or cells mediating these effects remains unknown. A previous study employing neurosphere cultures prepared from embryonic rat forebrain demonstrated that the stress-activated protein kinase SAPK/JNK, a subfamily of MAPK signalling pathway, is activated in response to IL-1β, and that inhibition of JNK can prevent IL-1β-induced impairments in proliferation and apoptosis in these cultures of forebrain NPCs (Wang et al., 2007). This study also reported that IL-1β did not induce activation of p38MAPK in NPCs (Wang et al., 2007). However, a role for any of the MAPK subtypes in IL-1β-induced changes in proliferation or lineage fate specifically in hippocampal NPCs has yet to be determined.

It is worth noting that IL-1β-induced activation of either NF-κB or MAPK have different roles within the various cells types of the hippocampus (Srinivasan et al., 2004). For example, in microglia or astrocytes, IL-1β activates NF-κB and p38MAPK to promote further cytokine production (Pinteaux et al., 2002; McManus et al., 1998), whereas in hippocampal pyramidal glutamatergic neurons IL-1β regulates synaptic functions via activation of the p38MAPK pathway and promotes neuronal death via JNK activation (Katsuki et al., 1990; Bellinger et al., 1993; Murray and Lynch, 1998; Kelly et al., 2001). As hippocampal NPCs possess the ability to differentiate into either neuronal or glial cells, there may be differential pathway activation in response to IL-1β in these younger cells. Recent evidence into the mechanisms of IL-1β-induced changes in NPC proliferation has highlighted that the suppression of proliferation by IL-1β may be mediated by activation of the p53 tumour suppressor gene within NPCs (Guadagno et al., 2015). Interestingly, this gene has been shown to be regulated by both NF-κB and MAPK signalling in other cell types (Shi et al., 2014; Ryan et al., 2000; Fujioka et al., 2004) and thus provides a rational for research into IL-1β-induced activation of these pathways in NPCs. The general consensus is that elevated expression of IL-1β acts to impair hippocampal neurogenesis as shown by its ability to repress proliferation and impair neuronal differentiation (Ryan et al., 2013; Koo and Duman, 2008; Zunszain et al., 2012; Green et al., 2012). However, whether the effects of IL-1β on the various stages of neurogenesis are predominantly NF-κB or MAPK mediated is yet to be fully resolved and provides an interesting avenue for future research.

4. Nuclear receptors as modulators of IL-1β-induced changes in neurogenesis

NRs are a superfamily of ligand regulated transcription factors and are common drug targets: approximately 13% of all Food and Drug Administration (FDA) approved drugs target NRs

(Rask-Andersen et al., 2011). The NR superfamily shares a similar three domain structure. These domains include a highly conserved DNA binding domain, a ligand binding domain, and a variable N-terminal transactivation domain, which binds various co-activators and co-repressors. Functionally, NRs govern many aspects of development, reproduction, and metabolism (Sun and Shi, 2010). As transcriptional regulators, NRs may represent convenient targets to mitigate the inflammatory effects of IL-1β in the hippocampus. Moreover, research into the effects of NRs on neurogenesis and how they respond to IL-1β may highlight possible therapeutic intervention targets to prevent IL-1β-associated deficits in adult hippocampal neurogenesis and related cognitive impairments. Of particular note in this regard is the glucocorticoid receptor (GR), **peroxisome proliferator-activated** receptors (PPAR), estrogen receptor (ER), and NR2E1 receptor (Fitzsimons et al., 2013; Lehmann et al., 2013; Quintanilla et al., 2014; Wada et al., 2006; Li et al., 2011; Bowers et al., 2010; Green and Nolan, 2012; Islam and Zhang, 2014). We have selected to discuss the expression profile, regulation, and functional role of these four key NRs in adult hippocampal neurogenesis. We will highlight the potential signalling overlap between these NRs and IL-1β and whether this overlap could be exploited to regulate IL-1β-induced changes in hippocampal neurogenesis.

5. Glucocorticoid receptor

Glucocorticoid receptors (GRs) have a pivotal role to play in the stress response and immune function (Garcia et al., 2004; Busillo and Cidlowski, 2013). The main activators of GRs are adrenal glucocorticoid hormones or stress hormones. Corticosterone (CORT), or cortisol in humans, is a lipophilic glucocorticoid hormone produced from cholesterol that readily passes the blood brain barrier and can enter neuronal cells through the cell membrane. CORT is produced mainly by the adrenal glands however there is evidence to suggest that it is also produced within the hippocampus where there is an abundance of enzymes required for its production (Beyenburg et al., 2001; Gomez-Sanchez et al., 1996; Stromstedt and Waterman, 1995). Its release is classically triggered after psychological or physical stress (Sapolsky et al., 1986; Droste et al., 2003, 2007). Once inside the cell, CORT can bind to two types of receptor, the mineralocorticoid receptor (MR) and the GR. Activation of these receptors results in receptor dimerisation and translocation to the nucleus where they regulate gene transcription. This can lead to several physiological responses including repression of the immune system, neuronal death, and/or neuronal maturation and survival (Fig. 3) (Coutinho and Chapman, 2011; Reagan and McEwen, 1997). The GR is expressed in nearly all cell and tissue types of the body although the regulation of its expression can differ across different tissue types (Lu et al., 2006; Oakley et al., 1996; Lu and Cidlowski, 2005). In relation to its expression within the hippocampus, the GR has been reported to be highly expressed within the hippocampus of both rodents and humans, particularly within the CA1 and DG (Roland et al., 1995; Reul and De Kloet, 1985).

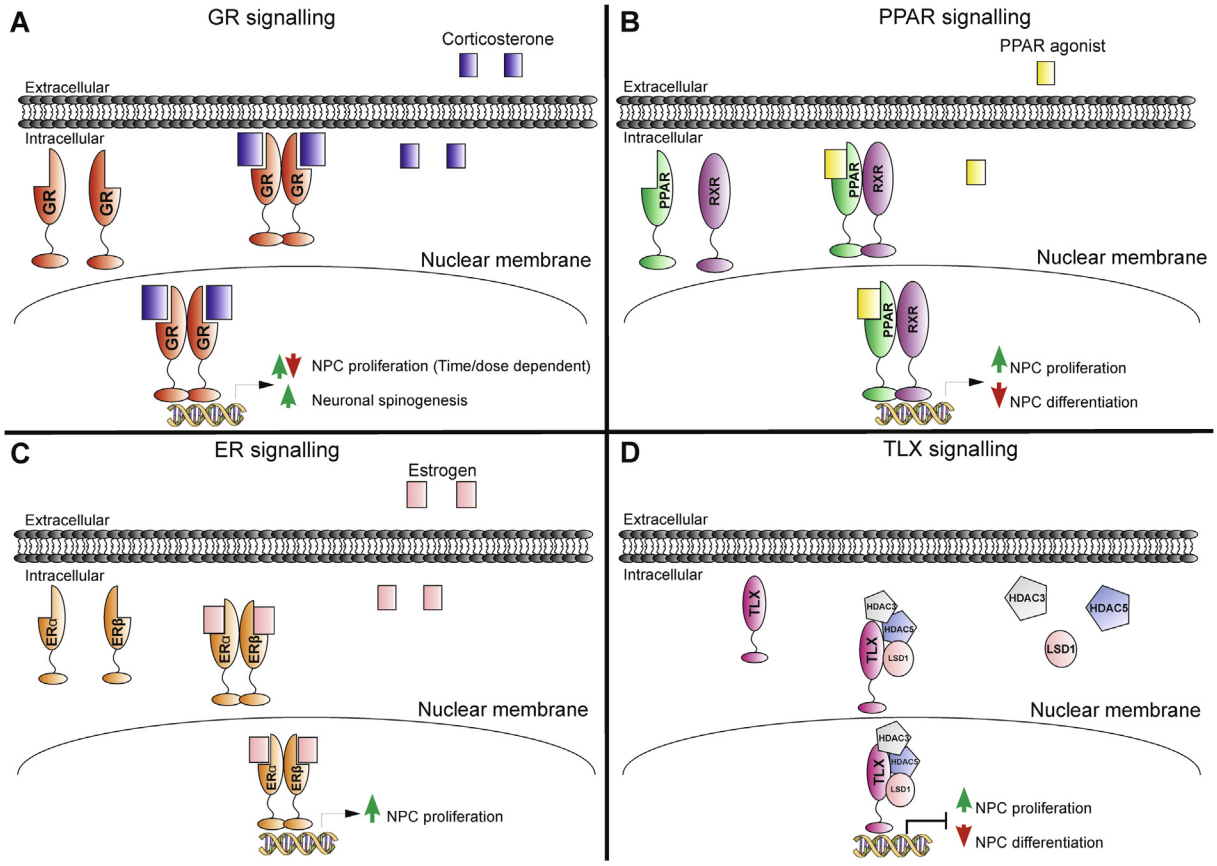


Fig. 3. Nuclear Receptor signalling. A) Glucocorticoid receptor (GR) signalling. B) **peroxisome proliferator-activated** receptor (PPAR) signalling. C) Estrogen receptor (ER) signalling. D) NR2E1 (TLX) receptor signalling. GRs are be activated by ligands such as corticosterone in rodents. Once activated they form homodimers and regulate gene transcription. PPARs are activated by a wide variety of synthetic and endogenous ligands and can form heterodimers with the retinoid x receptor (RXR) to regulate gene transcription. ERs form either homo or heterodimers with other ER subtypes when activated by ligands such as estradiol. NR2E1 is an orphan nuclear receptor and as such has no known ligand, yet it recruits various co-signalling molecules, such as HDAC3/5 and LSD1 to repress gene expression.

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5.1. GR and neurogenesis

In the hippocampus, CORT regulates information processing in neuronal networks associated with emotion, cognition, and memory (de Kloet et al., 2009; De Kloet et al., 1998). It has also been demonstrated that NPCs in the DG are located within close proximity to blood vessels and so are very susceptible to changes in circulating CORT levels (Wurmser et al., 2004). GRs are expressed in approximately 50% of proliferating NPCs in rodent hippocampus while MR expression is below detectable levels (Cameron et al., 1993; Boku et al., 2009; Garcia et al., 2004). As NPCs begin to express the immature neuronal marker doublecortin (DCX), the expression of GR within these cells is significantly reduced but is restored again once the NPCs mature into neurons (Garcia et al., 2004). This suggests that GRs initially mediate the effects of CORT on NPCs and are then subsequently involved in the regulation of NPC proliferation. Indeed, GR modulation has been shown to reduce the proliferation of hippocampal NPCs with subsequent alterations in differentiation and hippocampal network circuitry (Fitzsimons et al., 2013; Chetty et al., 2014). For example, it has been shown that administration of CORT to rats significantly reduced the number of proliferating cells in the DG (Cameron and Gould, 1994). Increased circulating CORT in rats also reduced neuronal differentiation of hippocampal NPCs (Wong and Herbert, 2006). Conversely, adrenalectomy, which reduces circulating CORT, significantly increased the number of proliferating cells in the rat DG (Cameron and Gould, 1994; Cameron et al., 1993). Suppression of GR function can also lead to impairments in hippocampal neurogenesis. For example, suppression of GR expression has been shown to reduce the proliferation of NPCs, accelerate their differentiation into neurons and induce abnormal neuronal integration and excitability in new born neurons (Fitzsimons et al., 2013). Moreover, in $GR^{+/-}$ mice where there is a reduction in GR expression by 50%, there is a reduction in the number of newborn neurons in the hippocampus, indicating that even partial suppression of GR expression can result in neurogenesis deficits (Kronenberg et al., 2009). Thus, the association between GR function and neurogenesis is complex (for an in-depth review please see (Egeland et al., 2015)). This is especially evident from studies showing that exercise, which is a potent promoter of hippocampal neurogenesis, also increases circulating CORT levels (Adlard and Cotman, 2004). However, a host of other pro-neurogenic factors are released after exercise. For example, BDNF, whose mRNA and protein expression has been shown to be increased within neurons of the DG and CA3 of the hippocampus after exercise (Cotman and Berchtold, 2002), may overcome the suppressive effects of CORT and enhance neurogenesis (Vaynman et al., 2004). Acute CORT administration has been shown to enhance plasma BDNF expression (Yau et al., 2012). This then may explain how acute stress, which elevates CORT, may subsequently either increase peripheral and/or DG neuronal BDNF expression, and in turn increase hippocampal NPC proliferation through the expression of BDNF receptor TrkB on NPCs (Horch and Katz, 2002; Choi et al., 2009; Bergami et al., 2008). Many of these discrepancies may also be explained by the type of stress induced. For example, social defeat stress negatively impacts upon hippocampal neurogenesis whereas environmental enrichment, which is known to increase adrenal activity in rodents (Moncek et al., 2004), positively affects hippocampal neurogenesis (Lehmann et al., 2013). In addition, the evidence to date suggests that acute stressors increase neurogenesis while chronic stress suppresses it (Pham et al., 2003; Kirby et al., 2013; Cheng et al., 2015). In summarising these complicated interactions between GR function and neurogenesis, it appears that strict regulation of GR activation is important for maintaining neurogenesis.

Mice lacking GR display impairments in hippocampal neurogenesis-associated behaviours. For example, deficits in contextual fear conditioning in GR knockdown mice is coupled with reduced NPC proliferation and accelerated neuronal differentiation (Fitzsimons et al., 2013). Interestingly, in this study no effects of GR knockdown were observed in amygdala-associated cued fear conditioning. This suggests that GR knockdown may lead to deficits in hippocampal function only due to the GR knockdown effects on neurogenesis. Conversely, activation of GR by systemic administration of CORT under baseline conditions has been shown to impair hippocampal neurogenesis-associated spatial memory in a water maze task (de Quervain et al., 1998). This reiterates that normal GR regulation is beneficial to neurogenesis and associated cognition while hyperactivation or suppression of GR activity leads to impairments in neurogenesis and cognitive function. In adrenalectomised rats, where there is reduced CORT and hippocampal GR activation, the impairment in hippocampal neurogenesis-associated spatial memory in the water maze was reversed by administration of CORT for 5–10 days prior to the start of behavioural testing (McCormick et al., 1997). However, chronic (5–6 weeks) CORT administration after adrenalectomy is not sufficient to restore spatial memory performance in the water maze and indeed it also impairs hippocampal NPC proliferation (Lee et al., 2011; Spanswick et al., 2007). This indicates that chronic disruptions in GR signalling may have long lasting effects on neurogenesis-associated cognition. Interestingly, it has been demonstrated that inhibition of GR signalling using the GR antagonist mefipristone in an AD mouse model rescued impairments in hippocampal neurogenesis associated novel object recognition (Lante et al., 2015; Dong et al., 2004). Indeed, mefipristone has been shown to reverse CORT-induced suppression of human hippocampal NPC proliferation (Anacker et al., 2013). Therefore, it may only be when deregulation of neurogenesis occurs, e.g. during neuroinflammation, that short-term GR manipulation may prove therapeutically viable to regain normal levels of neurogenesis.

5.2. GR and IL-1β signalling interaction in hippocampal NPCs

Due to the immunosuppressive consequences of GR activation, GR agonists, such as dexamethasone and prednisolone are well established anti-inflammatory agents (Sundahl et al., 2015). Despite a wealth of research outlining GR signalling interaction with IL-1β in post-mitotic cells, the interaction of these signalling cascades has not been explored in hippocampal NPCs. However, using the evidence to date, one can postulate as to how GR and IL-1β may interact in NPCs. Activation of GR signalling in mouse macrophages has been shown to interact with the IL-1β-associated NF-κB signalling pathway and to repress the expression of various inflammatory genes including IL-1β itself (Uhlenhaut et al., 2013). There are several mechanisms through which GR activation impairs NF-κB signalling. Specifically, GR can physically interact with p65, a member of the NF-κB family of proteins which is expressed in NPCs, and suppress its interaction with DNA in a monkey fibroblast cell line (Liden et al., 1997). GR activation has been shown to suppress the expression of NF-κB target genes by inducing the binding of p65 with interferon regulatory transcription factor 3 (IRF3) in a mouse macrophage cell line, thus preventing its transcriptional activity of p65 (Reily et al., 2006). Additionally, GR-mediated inhibition of IL-1β signalling can also occur through recruitment of histone deacetylases (HDACs) to NF-κB dependent promoters and consequent suppression of gene transcription in a human lung adenocarcinoma cell line (Ito et al., 2006). These studies, provide valuable insights into possible interactions between GRs and NF-κB signalling molecules and provide solid rational for further research into GR mediated regulation of NF-κB signalling in NPCs.

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There is little evidence for the relationship between GR activity, MAPK, and IL-1β within hippocampal NPCs and therefore this represents an open field of research. It has been demonstrated that in response to a prolonged increase in CORT-induced activation of GR, there is an exacerbation in both MAPK and NF-κB activation within the whole hippocampus of rats after lipopolysaccharide (LPS) exposure, a known inducer of inflammation and pro-inflammatory cytokine expression including IL-1β (Munhoz et al., 2010). Thus, in terms of IL-1β-mediated inflammation, chronic GR activation may elicit a stronger inflammatory response by IL-1β on NPCs by enhancing both MAPK and NF-κB pathway activation. This is in line with the overarching concept that chronic stress can induce a priming effect on immune activity such that exposure to stress can enhance pro-inflammatory cytokine expression and neuronal death after an inflammatory insult (for review see (Sorrells et al., 2009)) (de Pablos et al., 2006). Thus, the duration of GR activation in response to increased IL-1β expression is important in understanding the signalling connection between GR and IL-1β. It appears that prolonged activation of GR induces both MAPK and NF-κB activation and thus may potentiate IL-1β-induced changes, potentially on hippocampal neurogenesis.

6. PPAR receptors

Peroxisome Proliferator Activated Receptors (PPARs) consist of three main subtypes; PPARα, PPARβ/δ, and PPARγ. Functionally, PPARs are involved in the regulation of lipid metabolism and glucose homeostasis (Cimini and Cerù, 2008). They can be activated by endogenous lipid compounds, such as prostaglandins and other fatty acids, as well as synthetic compounds from the diabetic drug class thiazolidinediones (Ahmadian et al., 2013). Activated PPARs form a heterodimer complex with retinoic X receptors (RXRs) and then translocate to the nucleus where they modulate the expression of different target genes involved in processes, such as fatty acid metabolism (Fig. 3) (Forman et al., 1996; Cimini et al., 2007). RXRs, which are activated by retinoic acid, have been shown to be ubiquitously expressed within the hippocampus including in NPCs (Androutsellis-Theotokis et al., 2013; Zetterstrom et al., 1999). This heterodimer complex like most NR complexes can interact with various co-activators and co-repressors of gene expression (Quintanilla et al., 2014; Ahmadian et al., 2013; Forman et al., 1996). PPARα is highly expressed in the liver, heart, kidneys, and other metabolically active tissues (Rosen and Spiegelman, 2001; Forman et al., 1996). PPARβ is ubiquitously expressed but at different levels depending on the tissue (Coll et al., 2009). PPARγ is expressed mostly in fatty tissue and in the vasculature. However, it has been detected in the heart and brain tissue where its activation has been shown to be associated with reduced cardiovascular damage and reduced neurodegeneration, respectively (Barak et al., 1999; Tontonoz and Spiegelman, 2008).

6.1. PPARs and neurogenesis

Much of the focus on the role of PPARs in hippocampal neurogenesis to date has focused on PPARγ, although all PPAR subtypes have been shown to be expressed in the DG of the hippocampus of rodents (Bordet et al., 2006; Sun and Shi, 2010; Moreno et al., 2004; Cimini et al., 2007). Additionally, it should be noted that much of the research into PPAR function in neurogenesis has focused on embryonic neurogenesis rather than adult neurogenesis. However, many of the cellular processes of proliferation, differentiation, and survival are common to both embryonic and adult neurogenesis (Pleasure et al., 2000), and so these embryonic studies provide useful clues as to how PPARs may regulate these processes in adult hippocampal neurogenesis. PPARγ has been shown to play a role in cell proliferation and differentiation of NPCs derived from whole brain E13-14 mice embryos. Specifically, PPAR deficient NPCs failed to proliferate whereas treatment with the PPARγ agonist rosiglitazone induced cell growth and inhibited differentiation of NPCs into neuronal cells (Wada et al., 2006). Both PPARγ and PPARβ/δ activation in NPCs in the SVZ of adult mice leads to increased proliferation in these cells (Bernal et al., 2015). These authors also demonstrated that PPARγ activation increased the expression of PPARβ/δ, suggesting that these receptor isotypes work in tandem to promote proliferation of NPCs. Stimulation of PPARγ by pioglitazone, a synthetic PPARγ agonist, enhanced neurite outgrowth in SHY-SY5Y cells, which indicates its role in neuronal maturation (Miglio et al., 2009). Additionally, it has been demonstrated that PPARγ is important in the development of neuronal cells from a pluripotent embryonic stem cell line derived from mouse embryos. This indicates that PPARγ signalling supports preferential differentiation of neurons (Ghoochani et al., 2012). It should be noted however, that the variation of PPAR effects on neurogenesis may be due to the wide variety of compounds used to modulate their activity with each one having differing dosage and time dependent effects (Ghoochani et al., 2012; Wada et al., 2006). Indeed, it has been shown that with doses of the PPARγ agonists rosiglitazone or pioglitazone in excess of 30 μM, cell proliferation of embryonic NPCs is significantly reduced (Wada et al., 2006).

Synthetic PPAR agonists have been shown to have beneficial effects on hippocampal neurogenesis-associated cognitive function under inflammatory, stress and neurodegenerative conditions. For example, LPS-induced impairments in spatial memory in the Morris water maze as well as decreased levels of hippocampal neurogenesis were reversed by administration of rosiglitazone in female mice (Ormerod et al., 2013). Interestingly, there was no improvement in spatial memory performance or in neurogenesis induced by rosiglitazone in the absence of an LPS insult (Ormerod et al., 2013). Similarly, the protective effects of rosiglitazone have been reported in the Tg2576 and APP/PS1 mouse models of AD as assessed by radial arm maze and Morris water maze tasks (Pedersen et al., 2006; O'Reilly and Lynch, 2012). While impaired neurogenesis has been previously demonstrated in mouse models of AD (Ben Menachem-Zidon et al., 2014; Hsiao et al., 1996; Zeng et al., 2016), neurogenesis was not assessed in these studies. However, a direct effect of rosiglitazone on neurogenesis has been observed in that 3 weeks administration of rosiglitazone induces an increase in the number of immature neurons in the hippocampus (Cheng et al., 2015) although no cognitive behavioural correlates have been reported. Taken together, it is likely that, similar to studies on GR modulation, the beneficial effects of PPAR modulation are only evident in the presence of an insult that impairs neurogenesis and associated cognition.

6.2. PPARs and IL-1β signalling interaction in hippocampal NPCs

PPAR activation has been predominantly associated with anti-inflammatory effects (Blanquart et al., 2003). For example, activation of PPARα and PPARγ by the endogenous agonist 9-hydroxyoctadecadienoic acid (a major fatty acid in oxidized low density lipoprotein) in a mouse macrophage cell line inhibits IL-1β-induced NF-κB pathway activation (Chung et al., 2000). Additionally, activation of PPARα by fibrates (agonists of PPARα) has been shown to directly interact with p65 in mouse and human vasculatures after LPS or IL-1β induced inflammation and increases the expression of IkB, a negative regulator of NF-κB signalling (Delerive et al., 2000, 1999). PPARγ activation by rosiglitazone leads to reduced phosphorylation of p65 in mouse hippocampal neuronal cultures (Du et al., 2011). Thus, while it has not been

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specifically reported to date, it is likely that PPAR inhibition of IL-1β-induced effects on hippocampal neurogenesis involves suppression of the NF-κB pathway. PPAR activation by either pioglitazone or rosiglitazone has also been suggested to inhibit MAPK signalling within whole hippocampal tissue of rats in response to increased neuroinflammation induced by an ischemia model of stroke, which increases pro-inflammatory cytokine expression (Collino et al., 2006b,a) However the exact mechanism has not been definitively demonstrated, nor has the interaction between PPARs and MAPK signalling been explored within NPCs. Nevertheless, evidence that PPARs can suppress MAPK signalling after a neuroinflammatory insult, such as ischemic injury within the hippocampus as described above suggests that modulating PPARs in NPCs may have protective effects against neuroinflammation on these cells yet this requires further research. It should be noted that targeting PPAR signalling in NPCs to prevent the effects of IL-1β on hippocampal neurogenesis poses several problems. The first is the lack of specific ligands for PPARs as every currently available ligand is not specific for any individual PPAR (Grygiel-Górniak, 2014). This coupled with the ubiquitous nature of PPAR expression in the CNS leads to the potential of many side effects of PPAR modulation (Bordet et al., 2006). Nevertheless, PPAR activation possesses significant anti-inflammatory properties including an ability to impede IL-1β-associated signalling. With the advent of new ligands and technologies for modulating receptor expression (Niu et al., 2014; Brooks et al., 2015; Liu et al., 2016), assessing this interaction in NPCs warrants further research.

7. Estrogen receptor

The estrogen receptor (ER) family represents another group of nuclear receptors that can regulate neurogenesis and may interact with IL-1β signalling. Two types of ER exist; ERα and ERβ. Both are expressed within the hippocampus of rodents and humans with ERα displaying a slightly higher expression profile in rodents and ERβ being suggested to be the primary ER in humans (Mitterling et al., 2010; Pérez et al., 2003; Towart et al., 2003; Osterlund et al., 2000; González et al., 2007). Upon activation, these receptors form dimers of the ER subtypes and translocate to the nucleus to alter gene transcription (Fig. 3) (Hall et al., 2001). Cell surface G-protein coupled receptor ERs also exist within the hippocampus of rodents, however this review will focus on the nuclear receptor ERs (Brailoiu et al., 2007; Hazell et al., 2009). The sex or estrogen hormones estradiol, estrone, and estriol bind to ERs. Estradiol is the most potent and has been the focus of most research to date on the role of the ERs in hippocampal function (McEwen et al., 2012; McEwen and Milner, 2007; Vasudevan and Pfaff, 2008). Estradiol is synthesised from cholesterol mainly in the ovaries in females and estrogen has also been shown to be locally produced by hippocampal neurons in male rats (Hojo et al., 2004). Its level fluctuates during puberty and over the menstrual cycle and decreases after the menopause. It is also produced in bone, adipose, and nervous tissue in both females and males. The primary functions of estradiol in the CNS include regulation of stress, anxiety, feeding behaviour, aggression, and sexual behaviour (Arevalo et al., 2015; Do Rego et al., 2009).

7.1. ERs and neurogenesis

It has been shown in rats that ERs are expressed within adult and postnatal hippocampal NPCs (Mazzucco et al., 2006; Pérez et al., 2003). Administration of estradiol to female rats or meadow voles has been shown to increase NPC proliferation however this effect was only observed after 4 h of treatment whereas with 48 h of treatment there was a suppression of cell proliferation within the DG (Ormerod and Galea, 2001; Ormerod et al., 2003). One possible explanation for this effect is the immediate and latent side effects of estradiol. Estradiol induces a rapid increase in serotonin levels that has been suggested to mediate estradiol effects on NPC proliferation in the DG of rats (Banasr et al., 2001). With prolonged estradiol exposure, adrenal activity is increased and so suppression of the proliferation of NPCs within the DG ensues (Ormerod et al., 2003). In line with the role of ER in regulating sex hormones, there is evidence to suggest that there are sex differences in neurogenesis. Female rats have been shown to have higher levels of cell proliferation in the DG than males depending on which stage of the menstrual cycle they are in (Chow et al., 2013; Bowers et al., 2010; Marques et al., 2016; Tanapat et al., 1999), but this has not been observed in mice (Lagace et al., 2007). However, in this mouse study cell proliferation in females during the proestrus stage of the menstrual cycle were not compared with with males, which is when a difference in cell proliferation in rats was observed. There is also evidence to suggest that ER modulation after brain injury in mice enhances proliferation of cells in the DG (Li et al., 2011). Thus, it may be that ER-induced regulation of neurogenesis under baseline conditions is minimal but where there is an insult (such as in brain injury) and a consequent increase in pro-inflammatory cytokine expression, ERs play a role in recovery. Aging is associated with both decreased hippocampal neurogenesis and decreased circulating levels of estrogens (Lamberts et al., 1997; Kuhn et al., 1996; Kempermann et al., 1998). Indeed, there is some evidence to suggest that estrogen replacement in women can ameliorate age-related cognitive decline (Gibbs and Gabor, 2003; Sherwin, 2006). Thus, it would appear that the effect of estrogen signalling on neurogenesis is dose, time, sex and age-dependent.

Modulation of ERs by estrogen hormones, particularly estradiol, has been demonstrated to have beneficial effects on hippocampal neurogenesis-associated cognition. For example, estradiol induced significant improvements in spatial memory impairments in the radial arm maze and in the water maze in ovariectomised rats (which effectively reduces levels of circulating estrogens) (Luine et al., 1998; Daniel et al., 1997; Sandstrom and Williams, 2004), as well as in object location recognition in ovarectomised mice (Li et al., 2004). Similar improvements in spatial memory (object location recognition) have been demonstrated in male orchidectomised rats who received estradiol injections (Jacome et al., 2016). However, it has been demonstrated that ovarectomy itself does not appear to alter neurogenesis as no differences in cell proliferation were reported in rodents 3–4 weeks post ovarectomy (Green and Galea, 2008; Lagace et al., 2007; Tanapat et al., 1999). As estradiol can be produced locally within the hippocampus, there may be compensatory mechanisms at play to protect new neurons from any detrimental effect of ovarectomy (Hojo et al., 2004). It would be interesting to determine whether estradiol administration in the absence of ovarectomy or orchidectomy can rescue impairments in hippocampal-associated behaviours induced by suppression of neurogenesis (either transgenic models or hippocampal irradiation). It may be the case that estradiol treatment may only be beneficial for hippocampal neurogenesis-associated cognition when deficits in neurogenesis are present. Furthermore, little work has been carried out on the potential role of ER modulation in regulating inflammatory-induced changes in hippocampal neurogenesis-associated cognition, and so this represents another interesting avenue for future research.

7.2. ERs and IL-1β signalling interaction in hippocampal NPCs

It has largely been shown that estrogen acts antagonistically on IL-1β-induced changes in hippocampal function. For example, in mice that have been ovariectomised there is a significant increase

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in the expression of pro-inflammatory cytokines including IL-1β within the hippocampus (Benedusi et al., 2012). It has also been shown that estradiol treatment of rat hippocampal slice cultures reduced an ischemia-induced increase in cell death as well as IL-1β expression (Choi et al., 2008). The effect of estradiol on IL-1β may be due to the reduction in cell death however, estradiol also increased the expression of IL-1RA, which directly suppresses the potential negative effects of IL-1β in the hippocampus (Choi et al., 2008). Estradiol treatment has been shown to reduce IL-1β-induced apoptosis by inhibiting caspase 3 activation in mouse embryonic hippocampal NPC cultures *in vitro*, thus indicating that estrogen signalling may also have indirect effects on IL-1β-induced changes in NPC viability (Kajta et al., 2006). However, the mechanisms underlying this interaction have not been investigated to date, in NPCs or any other cell type. Understanding the mechanisms of interaction between ERs and IL-1β will not only provide better rationale for therapeutically targeting ERs to prevent IL-1β-associated impairments in neurogenesis and associated cognition, but it may also highlight any potential differences between the sexes in their response to inflammatory-induced changes in hippocampal neurogenesis.

8. NR2E1 (TLX)

NR2E1 is an orphan nuclear receptor, which (also known as TLX) is predominantly expressed in the eye and forebrain (Monaghan et al., 1995). Indeed, the expression of NR2E1 is mainly restricted to the mammalian CNS (Li et al., 2012). In the adult, NR2E1 is predominantly expressed in the DG in the hippocampus and in the SVZ zone of the lateral ventricles. Like most nuclear receptors, NR2E1 has at least three structural domains, a DNA binding domain, an N-terminal transactivation domain and a moderately conserved ligand-binding domain (LBD) (Yu et al., 1994; Bain et al., 2007; Benod et al., 2014). NR2E1 functions primarily as a transcriptional repressor of downstream target genes. To do so, NR2E1 recruits transcriptional co-repressors, such as the epigenetic modulators lysine-specific histone demethylase 1 (LSD1), and HDACs 3 and 5, which repress gene expression and in turn suppress the expression of anti-proliferative genes to regulate NPC proliferation (Sun et al., 2007, 2010, 2011). Genes regulated by NR2E1 include *Ascl1, Pou5f1, Pax2, Mir9, Mir137, Pten, p21, Cdkn1a, Sirt1, and Wnt7a* (Islam and Zhang, 2014). Importantly, repression of cyclin dependent kinase inhibitor *p21* and the tumour suppressor gene *Pten* by NR2E1 activation, promote the proliferation of NPCs (Sun et al., 2007).

8.1. NR2E1 and neurogenesis

At a cellular level, NR2E1 has been demonstrated to be critical for maintaining neural stem cell proliferation in both the developing embryonic forebrain and dorsal midbrain, and within the neurogenic niches of the adult brain (Monaghan et al., 1995; Shi et al., 2004; Sun et al., 2007). NR2E1 expressing NPCs from the SGZ, SVZ, and olfactory bulb proliferate and are capable of differentiating into all neural cell types whereas NPCs lacking NR2E1 fail to proliferate (Shi et al., 2004). The importance of NR2E1 was further demonstrated by the fact that reintroduction of NR2E1 into NPCs restored all of the deficits in proliferation observed in the cells lacking NR2E1 (Shi et al., 2004). Moreover, overexpression of NR2E1 within hippocampal NPCs has been shown to enhance the proliferation phase of neurogenesis and subsequently improve learning and memory processes in mice (Murai et al., 2014). NR2E1 knockout mice show deficits in hippocampal neurogenesis and impaired learning and memory as well as a heightened stress response (Monaghan et al., 1997; Young et al., 2002; O'Leary et al., 2016a). More recently, it has been demonstrated that mice with a spontaneous deletion of NR2E1 display impairments in hippocampal neurogenesis-associated cognitive tasks (O'Leary et al., 2016a). Specifically, this study demonstrated that NR2E1$^{-/-}$ mice displayed impaired short term spatial memory as tested by spontaneous alternations in the Y-maze. Furthermore, male adolescent mice displayed impaired contextual fear conditioning performance suggesting a role for NR2E1 in sex- and age-related effects on certain hippocampal neurogenesis-associated tasks (O'Leary et al., 2016a). It should be noted however that most work to date assessing the relevance of NR2E1 in hippocampal neurogenesis-associated cognition has focused on models of genetic reductions (germline knockouts or inducible knockouts) of NR2E1 expression (O'Leary et al., 2016b). For example, NR2E1 knockout in mice (cre-lox deletion) resulted in impaired performance in the Morris water maze spatial memory task (Zhang et al., 2008) which was associated with impaired hippocampal neurogenesis. Interestingly, it has been demonstrated that enhancing NR2E1 expression specifically in NPCs can promote hippocampal neurogenesis and enhance performance in the Morris water maze task (Murai et al., 2014). This study demonstrated the potential for NR2E1 as a therapeutic target to selectively enhance hippocampal neurogenesis and associated cognition. Thus, it would appear the NR2E1 is intrinsically linked with both the cellular mechanisms and functional outcomes of hippocampal neurogenesis and as such its role in mitigating neuroinflammation-induced deficits in neurogenesis will be of interest for future research.

8.2. NR2E1 and IL-1β signalling interaction in hippocampal NPCs

The link between IL-1β signalling and NR2E1 is only beginning to be studied. So far, it has been shown that IL-1β decreases NR2E1 expression in both adult and embryonic hippocampal NPCs (Green and Nolan, 2012; Ryan et al., 2013). Furthermore, co-treatment of hippocampal NPCs with IL-1β and IL-1RA or siRNA to inhibit IL-1R1 expression restored NR2E1 expression in hippocampal NPCs (Ryan et al., 2013). Little is currently known about the molecular interactions between IL-1β and NR2E1 signalling, however some theories can be postulated based on evidence from the literature. For example, many of the target genes of NR2E1, such as *Cdkn1a* and *Pten* are also regulated by NF-κB signalling in embryonic mouse fibroblasts. NF-κB targets these genes to enhance their expression while NR2E1 represses their expression (Xia et al., 2007). NR2E1 has also been shown to suppress the MAPK pathway. In NR2E1 null progenitor cells from mouse retina, there was a significant increase in the activation of p38MAPK (Zhang et al., 2006). This was also shown by signalling pathway analysis of RNA sequencing data of NR2E1 knockdown in NPCs from the SVZ (Niu et al., 2011). None of these signalling interactions have been demonstrated in hippocampal NPCs however and this represents an important area for future research.

Currently there is no known endogenous ligand for NR2E1. Therefore, drug discovery efforts are underway to identify selective synthetic ligands for this orphan nuclear receptor (Benod et al., 2014). Using medium throughput screening and thermal shift assays to assess the binding capabilities of a catalogue of compounds, Benod et al. demonstrated that the NR2E1 ligand binding domain (LBD) can be selectively targeted by three potential NR2E1-modulating synthetic ligands (Benod et al., 2014). Current research is establishing the efficacy of these ligands in modulating the function of the full length NR2E1 protein in cell lines (Benod et al., 2016). The discovery of ligands that can selectively modulate NR2E1 function will greatly enhance our understanding of the interaction of NR2E1 with IL-1β as well as the impact selective pharmacological manipulation of NR2E1 may have on neurogenesis-associated cognition. Moreover, the fact that NR2E1

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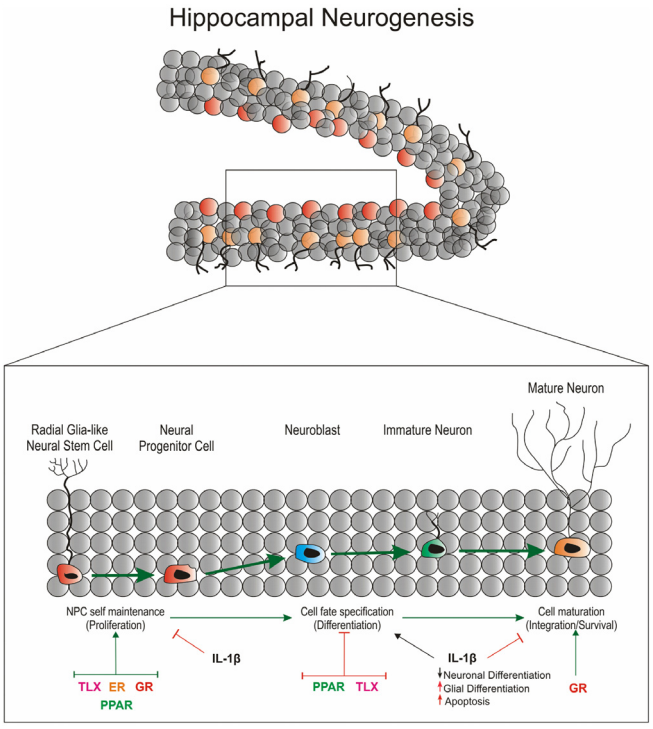


Fig. 4. A schematic of the overlap between the processes of hippocampal neurogenesis regulated by NRs and impaired by IL-1β.

is selectively expressed within proliferating NPCs of the neurogenic niches of the adult brain will serve to limit any potential side effects of a selective ligand. On the other hand, enhanced NR2E1 expression is associated with increased tumorigenesis (Zou et al., 2012; Park et al., 2010; Liu et al., 2010) and thus caution should be taken when manipulating NR2E1 within NPCs. Notwithstanding, although the data is still in its infancy, it is tempting to speculate that NR2E1 may be a potential therapeutic target for IL-1β-induced changes in hippocampal neurogenesis and related impairments in cognition.

9. Conclusions

Hippocampal neurogenesis has been shown to be functionally associated with hippocampal learning and memory processes and is known to be impaired in various neuropathologies affecting the hippocampus. Increased expression of IL-1β is evident in the hippocampus under neuropathological conditions and with age. Along with the high expression of its cognate receptor on hippocampal neurons and hippocampal NPCs, it is positioned as a major mediator of neuroinflammation and consequent decline in cognitive function. As such, determining the cellular signalling pathways responsible for mediating the detrimental effects of IL-1β on hippocampal neurogenesis is necessary. This review has highlighted two pathways that are activated downstream of IL-1β in NPCs; NF-κB and MAPK pathways. We speculate that IL-1β activation of NF-κB or MAPK pathways in NPCs may depend on the stage of neurogenesis that the cells are engaged in. In this regard we propose that NF-κB signalling may mediate IL-1β-induced changes on earlier stages of neurogenesis (e.g. during proliferation) with MAPK signalling mediating the IL-1β-induced changes in more mature NPCs and synaptic integration.

Evidence for IL-1β-induced NF-κB signalling in proliferating NPCs has been demonstrated due to the fact that inhibition of the NF-κB pathway, and not p38MAPK, in mice was sufficient to prevent the anti-proliferative effects of IL-1β in hippocampal NPCs *in vivo* (Koo and Duman, 2008; Koo et al., 2010). Other studies have demonstrated that IL-1β-mediated changes in NPC proliferation and differentiation are mediated in part by the kynurenine pathway and by GSK-3β activation (Green and Nolan, 2012; Zunszain et al., 2012). Interestingly, NF-κB activation has been suggested to be downstream of GSK-3β in the hippocampus (Bali and Jaggi, 2016) and moreover, the kynurenine pathway can be activated downstream of NF-κB activation, albeit in the human placenta (Dharane Nee Ligam et al., 2010). It has also been demonstrated that IL-1β activates both the NF-κB and p38MAPK pathways in hippocampal astrocytes but that only the p38MAPK pathway is activated in hippocampal neurons in response to IL-1β (Srinivasan et al., 2004; Huang et al., 2011). Therefore, as NPCs begin to differentiate into either glial cells or neuronal cells it appears that there is a diverging point at which they recruit either the MAPK or the NF-κB pathway in response to IL-1β depending on the cellular phenotype they adopt as they differentiate. It must been noted however that activation of SAPK/JNK (part of the MAPK signalling family but not encompassing p38MAPK) has also been demonstrated to mediate the effects of IL-1β on proliferation (albeit in whole embryonic forebrain tissue) (Wang et al., 2007) and so may play a contributing role to IL-1β-induced changes in hippocampal neurogenesis. Of course it is also possible that IL-1β may recruit either or both NF-κB and MAPK pathways in NPCs at any given stage of neurogenesis depending of the concentration of IL-1β, the duration of its exposure or indeed the time during the lifespan at which IL-1β is exposed to the hippocampal NPCs. These questions can only be answered with further research.

As IL-1β appears to regulate different signalling pathways within NPCs depending on what stage of neurogenesis they are in, mitigating these effects will require manipulating factors that can also regulate multiple processes and pathways within NPCs. It is clear that the four NRs discussed, namely the GRs, PPARs, ERs, and NR2E1, play a key role in a host of physiological processes including several aspects of neurogenesis (Fig. 4). However, the question remains as to whether these NRs can be targeted to prevent or reverse impairments in hippocampal neurogenesis induced by IL-1β. The NRs reviewed herein may be of value in exploring NPC based therapies for neuroinflammatory associated cognitive dysfunction. Due to their expression in hippocampal NPCs, their functional role in regulating neurogenesis, and their interaction with neuroinflammatory pathways, they position themselves as promising therapeutic targets. Moreover, as NRs function as ligand regulated transcription factors, they represent valuable targets for pharmacological manipulation. All except NR2E1 can currently be selectively targeted by numerous synthetic ligands to either activate or repress their activity. However, more work is needed to assess the effect of targeting these receptors on IL-1β-mediated suppression of neurogenesis. To this end, specific ligands for each NR discussed in this review and/or knockdown or overexpression studies will be invaluable in determining the potential of these receptors in mitigating the IL-1β-induced effects on neurogenesis.

Acknowledgments

This work was supported by Science Foundation Ireland (grant number 12/IA/1537). The authors have no conflicts of interest to declare.

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Please cite this article in press as: O'Léime, C.S., et al. Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis. Brain Behav. Immun. (2017), http://dx.doi.org/10.1016/j.bbi.2017.07.153

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Please cite this article in press as: O'Léime, C.S., et al. Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis. Brain Behav. Immun. (2017), http://dx.doi.org/10.1016/j.bbi.2017.07.153

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Please cite this article in press as: O'Léime, C.S., et al. Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis. Brain Behav. Immun. (2017), http://dx.doi.org/10.1016/j.bbi.2017.07.153

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Please cite this article in press as: O'Léime, C.S., et al. Nuclear deterrents: Intrinsic regulators of IL-1β-induced effects on hippocampal neurogenesis. Brain Behav. Immun. (2017), http://dx.doi.org/10.1016/j.bbi.2017.07.153