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

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6	The orphan nuclear receptor TLX regulates hippocampal transcriptome
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TLX knock-out and IL-1 β

22 Abbreviations: TLX, Orphan nuclear receptor tailless homolog; IL-1β, interleukin-1 beta;

- 23 TNF, tumor necrosis factor; NPCs, neural progenitor cells; DG, dentate gyrus; LTP, long
- term potentiation; P, post-natal day; KO, knockout; WT, wildtype; PBS, phosphate buffered
- saline; GO, gene ontology; FDR, false discovery rate; RNASeq, RNA sequencing; DAVID,
- 26 database for annotation visualisation and integrated discovery; STRING, search tool for the
- 27 retrieval of interacting genes/proteins.

28 Abstract

TLX is an orphan nuclear receptor highly expressed within neural progenitor cells (NPCs) in 29 the hippocampus where is regulates proliferation. Inflammation has been shown to have 30 negative effects on hippocampal function as well as on NPC proliferation. Specifically, the 31 pro-inflammatory cytokine IL-1^β has been shown to suppress NPC proliferation as well as 32 TLX expression in the hippocampus. However, it is unknown whether TLX itself is involved 33 in regulating the inflammatory response in the hippocampus. To explore the role of TLX in 34 inflammation, we assessed changes in the transcriptional landscape of the hippocampus of 35 TLX knockout mice (TLX^{-/-}) compared to wildtype (WT) littermate controls with and 36 without intrahippocampal injection of IL-1 β using a whole transcriptome RNA sequencing 37 approach. We demonstrated that there is an increase in the transcription of genes involved in 38 the promotion of inflammation and regulation of cell chemotaxis (Tnf, Illb, Cxcr1, Cxcr2, 39 Tlr4) and a decrease in the expression of genes relating to synaptic signalling (Lypd1, Syt4, 40 Cplx2) in cannulated TLX^{-/-} mice compared to WT controls. We demonstrate that mice 41 lacking in TLX share a similar increase in 176 genes involved in regulating inflammation 42 (e.g. Cxcl1, Tnf, Il1b) as WT mice injected with IL-1 β into the hippocampus. Moreover, 43 TLX^{-/-} mice injected with IL-1 β display a blunted transcriptional profile compared to WT 44 mice injected with IL-1 β . Thus, TLX^{-/-} mice, which already have an exaggerated 45 inflammatory profile after cannulation surgery, are primed to respond differently to an 46 inflammatory stimulus such as IL-1^β. Together, these results demonstrate that TLX regulates 47 hippocampal inflammatory transcriptome response to brain injury (in this case cannulation 48 surgery) and cytokine stimulation. 49

50

51 Keywords: RNA sequencing, TLX, interleukin-1 beta, hippocampus, neuroinflammation,

52 nuclear receptors,

53 1: Introduction

The hippocampus is one of two regions of the adult brain that neural progenitor cells (NPCs) 54 reside throughout the lifespan (Gage, 2000, Zhao et al., 2008). Specifically within the dentate 55 gyrus (DG) of the hippocampus, these NPCs are core components of neurogenesis (i.e. the 56 birth of new neurons) and this process is thought to contribute to hippocampal cognitive 57 functions such as spatial memory (Kempermann et al., 2004, Shors et al., 2002) as well as 58 playing a role in the regulation of emotion (O'Leary and Cryan, 2014). For hippocampal 59 neurogenesis to occur, NPCs must successfully progress from a proliferative state to fully 60 mature integrated neurons (Deng et al., 2010). This progression is under strict regulation by a 61 host of intrinsic and extrinsic factors (Suh et al., 2009). One of these regulators is the orphan 62 nuclear receptor subfamily 2 group E member 1 (Nr2e1 or TLX) (Niu et al., 2011, Li et al., 63 2012, Shi et al., 2004, Zhang et al., 2008). TLX is required to maintain NPCs in a 64 proliferative state and to prevent ectopic neural differentiation (Shi et al., 2004, Zhao et al., 65 2010). Its expression in the adult mouse brain is localised to the neurogenic niches and 66 specifically within the NPCs of these niches (Monaghan et al., 1995). Mice lacking TLX 67 display reduced hippocampal volume as well as impaired neurogenesis due to the fact that 68 NPCs fail to proliferate (Shi et al., 2004). TLX deficient mice also have impaired long-term 69 potentiation (LTP) in the DG and display deficits in hippocampal neurogenesis-associated 70 spatial memory cognition (O'Leary et al., 2016b, Christie et al., 2006, Roy et al., 2002, 71 72 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 73 transciptome may be affected by the loss of this NPC-specific transcription factor. Moreover, 74 75 whether intrinsic regulators of neurogenesis, such as TLX, can regulate wider hippocampal transcriptional responses to negative stimuli such as inflammation is not fully known. 76

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TLX knock-out and IL-1β

78	It has been established that hippocampal inflammation can induce deficits in hippocampal
79	neurogenesis and associated cognitive function (O'Leime et al., 2017a, Kohman and Rhodes,
80	2013, Yirmiya and Goshen, 2011). Interestingly, it has been demonstrated that NPCs
81	themselves can regulate the inflammatory environment when transplanted into a region of
82	tissue damage (Pluchino et al., 2005, Martino and Pluchino, 2006). Interleukin-1 β (IL-1 β) is a
83	pro-inflammatory cytokine whose receptor, IL-1 receptor type 1 (IL-1R1) is expressed in
84	proportionately higher levels in the hippocampus compared to other brain regions (Parnet et
85	al., 1994, Farrar et al., 1987). IL-1R1 is also expressed on NPCs in the hippocampus (Green
86	et al., 2012; Ryan et al., 2013). IL-1 β is predominantly responsible for the negative effects of
87	neuroinflammation on hippocampal neurogenesis and hippocampal-related impairments due
88	to its receptor expression profile (Ryan et al., 2013, Green and Nolan, 2012, Kelly et al.,
89	2003, Koo and Duman, 2008, Hein et al., 2010). It is noteworthy that IL-1 β has been
90	demonstrated to inhibit the expression of TLX in both embryonic and adult hippocampal
91	NPCs (Green and Nolan, 2012, Ryan et al., 2013). However, it is unknown whether TLX
92	itself can regulate the IL-1 β -induced inflammatory response within the hippocampus. To
93	address this, we compared the transcriptome changes in the hippocampus of TLX knockout
94	(TLX ^{-/-}) mice to wild type (WT) mice following an inflammatory stimulus with IL-1 β .
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96	

97 **2: Methods**

98 *2.1: Animals*

Postnatal day (P) 56 male TLX^{-/-} and wildtype (WT) littermate control mice (on a cross 99 BL6/129S1 background) were used in this study (n=4-8). Breeding pairs were kindly 100 provided by Prof. Elizabeth Simpson, University of British Colombia and were generated as 101 previously described (Wong et al., 2010). Briefly, male TLX heterozygous mice (TLX^{-/+}) on 102 a 129S1 background were crossbred with female BL6 TLX^{-/+} mice to generate TLX wildtype 103 controls $(TLX^{+/+})$, $TLX^{-/+}$, and TLX knockout $(TLX^{-/-})$ pups with offspring genotypes 104 followed Mendelian inheritance (See figure 1 for breeding summary and numbers of mice 105 used per group). All pups were weaned at P21 and tailsnips were taken for genotype analysis 106 which was carried out using an Extract N' Amp kit (Sigma-Aldrich) as per the 107 manufacturer's instructions. After genotyping, the animals were single housed under standard 108 housing conditions (temperature 21°C and relative humidity 55%), with food and water 109 available ad libitum. All experiments were conducted in accordance with the European 110 Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory 111 Authority Ireland and approved by the Animal Ethics Committee of University College Cork. 112 113

114 2.2: Experimental design

115For RNA sequencing analysis, TLX-/- and WT mice were injected with either IL-1β or116vehicle (phosphate buffered saline (PBS)) via cannulation, thus there were four experimental117groups for the RNA sequencing section of this study (Figure 1, Table 1). Hippocampal tissue118from an additional group of non-cannulated TLX-/- and WT mice was taken for PCR analysis119to assess the effect of cannulation on cytokine (IL-1β and TNFα) expression.

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121 2.3: Stereotaxic surgery for hippocampal cannulation

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122	At P52 (4 days prior to IL-1 β or PBS microinjection), TLX ^{-/-} and WT mice were
123	anaesthetised using a mixture of ketamine (0.25ml), xylazine (0.2ml) and sterile 0.9% NaCl
124	(2.05ml) at a dose of 0.1mls/10g (i.p.) and placed in a Kopf stereotaxic frame. A guide
125	cannula (Plastics1, Gauge: 22, Pedestal Length: 4mm, Projection: 1.5mm) was implanted
126	unilaterally into the dorsal hippocampus at the following co-ordinates: AP -1.7, ML -1.2 and
127	DV -1.5 relative to bregma (Haettig et al., 2013). Animals were randomly implanted on the
128	left or right hemisphere. The cannula was secured in place using superglue as a base layer
129	followed by dental cement. After the dental cement had hardened sufficiently, animals were
130	administered Carprofen (0.1ml/30g, i.p.) and 0.5ml of 5% Glucose (5g/100ml; i.p.), and
131	returned to their home cage.
132	
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2.4: Intrahippocampal microinjections 133

At P56 mice were unilaterally injected with 1μ of either recombinant mouse IL-1 β (10ng/ μ]; 134 R&D systems) or PBS (filtered using a 0.2µm sterile filter) and infused at a rate of 0.5ul/min 135 using an automated 'Pico Plus' microinjector (Harvard Apparatus, Kent, UK). The injection 136 cannula projected a further 0.5mm past the guide cannula to give a total depth of 2.0mm and 137 was left in place for an additional 2 min for diffusion before the needle was withdrawn. Mice 138 were sacrificed by decapitation one hour after injection. This timepoint was chosen as we 139 have previously demonstrated that IL-1 β can induce a significant reduction in TLX gene 140 expression in hippocampal NPCs at this timepoint (O'Leime et al., 2017b). Moreover, we 141 aimed to assess the immediate gene expression changes induced by IL-1ß rather than 142 secondary gene expression changes induced by the release of other regulators of gene 143 expression as a result of IL-1 β injection. The injection needle was attached to the guide 144 cannula and mice were allowed to move freely during the injection. After one hour, left or 145 right hippocampi were removed, flash frozen on dry ice and stored at -80°C. 146

147

148 2.5: Hippocampal RNA Extraction

149	Total RNA was extracted and DNase treated from hippocampal tissue from both WT and
150	TLX ^{-/-} animals using the mirVana TM total RNA extraction kit (Ambion/Life Technologies,
151	Dublin, Ireland) and Turbo DNA-free kit (Ambion/life technologies) as per the
152	manufacturer's instructions. The total concentration of extracted RNA was quantified using a
153	Nanodrop 2000 (Thermo Scientific, UK) and was stored at -80°C until sent for sequencing.
154	9
155	2.6: mRNA sequencing
156	Equal volumes of total hippocampal RNA from each animal was sent for sequencing by
157	Exiqon (Vedbaek, Denmark) and conducted on an Illumina NextSeq500 sequencer with an
158	average of 30 million reads with a 50 basepair paired-end read length. Annotation of the
159	obtained sequences was performed using the reference genome annotation: Mus musculus

160 (organism), GRCm38 (reference genome), Ensembl_70 (annotation reference).

161

162 2.7: Differential gene expression and functional enrichment analysis

Data analysis was conducted by Exigon using XploreRNA automated analysis software. The 163 data analysis pipeline used in this software is based on the Tuxedo software package which is 164 a combination of open-source software and makes use of peer-reviewed statistical methods. 165 Additionally, Exigon employs specialised software developed at Exigon to interpret and 166 enhance the readability of the finalised results. The components of the data analysis pipeline 167 used at Exiqon for RNA sequencing include Bowtie2 (v. 2.2.2.), Tophat (v. 2.0.11.), and 168 Cufflinks (v. 2.2.1.). Briefly, Bowtie2 is a sequence aligner used by Tophat to align the 169 sequencing reads to the reference genome (GRCm38, UCSC Genome brower and 170 Ensembl_70 (annotation reference)). Cufflinks uses the alignment results from Tophat and 171

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172 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 173 determines transcriptional start sites. Additionally, Exigon performs fragment bias correction 174 which corrects for sequence bias during library preparation. Cuffdiff is used when comparing 175 groups to calculate the number of fragments per kilobase of transcript per million mapped 176 fragments (FPKM) and to determine differential gene expression and regulation based on the 177 assembled transcripts from the submitted samples using the Cufflinks output. An adjusted p-178 value of ≤ 0.05 was considered significantly differentially regulated. Differentially regulated 179 genes were analysed for enrichment of Gene Ontology (GO) terms and KEGG pathways 180 using the DAVID Bioinformatics Resources (v6.8) (Huang da et al., 2009). 181 182 2.8 Quantitative real-time PCR (gRT-PCR) 183 Validation of RNA sequencing was performed using specific PrimerTime[®] qPCT assays 184 sourced from IDT (Integrated DNA Technologies) (Table 2). cDNA was reversed transcribed 185 using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the 186 SureCycler[®] 8800 (Agilent Technologies) and diluted to a final concentration of 10ng/µl. All 187 qRT-PCR was performed in 3 technical replicates for each biological sample on a 188 LightCycler[®] 480 Instrument II (Roche). Quantification of gene expression was analysed 189 using the $\Delta\Delta$ C_t method (Livak and Schmittgen, 2001). RNA sequencing data was validated 190 for four genes that contributed to the enrichment for TNF signalling in our KEGG pathway 191 analysis using the following primers from Integrated DNA Technologies (IDT): SDHA 192 (Mm.Pt.56a.12170577), IL-1β (Mm.PT.58.41616450), IL-6 (Mm.Pt.58.10005566), TNF 193 194 (Mm.PT.58.12575861). 195

196 2.9 STRING analysis

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197 The STRING database, which stems from computational predictions was used to investigate direct (physical) and indirect (functional) associations between differentially regulated genes. 198 An interaction network was created using a list of differentially regulated genes between WT 199 and TLX^{-/-} and genes that were commonly up-regulated in WT vs. TLX^{-/-} and WT vs. WT + 200 IL1B (172 genes). The string network was visualized using the following parameters: 201 excluded any genes that there was no evidence of association of connectivity, minimum 202 required interaction score was set at high confidence (0.007), and meaning of network edges 203 was set to line thickness indicating strength of data support. KEGG pathway analysis was 204 also conducted in STRING which indicated among the 172 up-regulated genes that TNF 205 signalling pathway was the most significantly enriched pathway p < 0.001 (red nodes). 206 207 208 2.10 Statistics An adjusted p value (q value, Benjamini-Hochberg method) of ≤ 0.05 for RNA sequencing 209

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.

215

216 **3: Results**

- 217 *3.1: TLX regulates inflammatory gene expression in the hippocampus of mice.*
- 218 By performing pairwise analysis of whole transcriptome RNA sequencing data, we compared
- 219 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 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 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
- 227 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 2E).

- 230
- 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 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 + IL1β. 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, 1l1b, Tnf, Ccl2*),
 regulation of inflammatory response (e.g. *Tlr2, Ccl4, Ccl7, Ier3*), and apoptotic processes
 (e.g. *Ier3, Cyr61, LgalsS3, Perp*) (Figure 3C, E). KEGG pathway analysis revealed a
- 240 significant enrichment for genes involved in inflammatory pathway signalling such as TNF

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241	signalling (Tnf, Fos, Jun, Il1b), cytokine-receptor interaction (Csf3, Il6, Ccl2, Il1b, Il1a), NF-
242	κB signalling (Nfkbia, Lbp, Ptgs2), and TLR signalling (Tlr2, Il1b, Nfkbia, Lbp) (Figure 3D,
243	E). This indicated that in TLX ^{-/-} mice, there is a similar dyresgulation of inflammatory gene
244	expression to that induced by IL-1 β within the hippocampus of WT mice. Additionally,
245	KEGG pathway analysis using two web based algorythims (DAVID and STRING) indicated
246	that TNF signalling was the most significant and prominent pathway to be enriched in this
247	subset of differentially up-regulated genes (172) (Figure 4A, B).
248	5
249	3.3: Cannulated TLX mice have a significantly different cytokine response to IL-1 β
250	WT + IL-1 β mice show a down-regulation in 7 genes and an up-regulation in 221 genes
251	within the hippocampus compared to cannulated WT mice (Figure 5A, B). TLX ^{-/-} + IL-1 β
252	mice display a much reduced transcriptional response as they display a differential
253	transcriptional response in 38 genes and only 5 of those were up-regulated comapred to TLX ⁻
254	^{/-} mice (Figure 5A, B). GO enrichment analysis only showed significant enrichment among
255	down-regulated genes (33 genes) between TLX ^{-/-} and TLX ^{-/-} + IL-1 β groups of mice (Figure
256	5A). Specifically, we observed enrichment in GO terms such as cell (including leukocytes
257	and neutrophils) chemotaxis, (Cxcl13, Ccr1, Trem1), defence to bacterium (Lyz2, Lyz1),
258	response to LPS (Lcn2, Cxcl13), and acute inflammatory response (S100a8, Saa3) (Figure
259	5C).
260	

261 3.4: TLX potentially regulates TNF signalling indirectly via two separate gene interactions 262 and cannulation induces a significant increase in IL-1 β and TNF α expression in TLX^{-/-} mice 263 As we identified TNF signalling as a possible mechanism through which TLX regulates 264 inflammation, we again used STRING analysis to identify the link between TLX (*Nr2e1*) and 265 TNF signalling. We identified *Top2a* and *Bcl6b* as two genes from all differentially regulated

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266	genes (1542 genes) in TLX ^{-/-} mice compared to WT mice that may interact with TLX and
267	potentially mediate the regulatory effects of TLX on TNF signalling (Figure 6A). We
268	confirmed our sequencing results using qRT-PCR that TNF α and IL-1 β , which have been
269	shown from our KEGG analysis to be highly involved in regulating TNF signalling, have
270	elevated expression levels in cannulated TLX ^{-/-} mice (Figure 6B, C). We also assessed the
271	expression of these genes in non-cannnulated mice. Non-cannulated TLX ^{-/-} mice have similar
272	hippocampal expression of IL-1 β and TNF α to that of non-cannulated WT mice (Figure 6B,
273	C). However, after hippocampal cannulation surgery, there is a significant increase in IL-1 β
274	$(F(3,24)=12.34, P=0.0001, ***=P<0.01)$ and TNF α $(F(3,24)=10.12, P=0.0003;$
275	***= P <0.01) expression in TLX ^{-/-} mice only (Figure 6B, C). There is a non-significant trend
276	towards an increase in these cytokines in WT mice after hippocampal cannulation surgery
277	(IL-1 β : t-test comparison between WT/Naive and WT/PBS indicates $P=0.0862$, TNF α : t-test
278	comparison between WT/Naive and WT/PBS indicates $P=0.0588$) which may be due to
279	limited samples numbers or to significant variations among individual mice. Likewise, PCR
280	analysis revealed that there was no statistically significant difference in IL-6
281	mRNAexpression between cannulated and non-cannulated mice for both WT and KO strains
282	(Figure 6D).
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290 Discussion

In the present study, we demonstrate that TLX acts to regulate the transcription of genes 291 involved in maintaining a normal inflammatory profile after cannulation surgery and in 292 293 response to an IL-1 β stimulus in the hippocampus of mice. Firstly, we demonstrated an orchestrated up-regulation in genes involved in inflammatory processes in the hippocampus 294 of TLX^{-/-} mice when compared to WT mice. WT mice injected with IL-1 β into the 295 hippocampus display a significant up-regulation in genes involved in cellular responses to 296 cytokines and inflammatory signalling pathways, and these genes overlap with the genes 297 increased in TLX^{-/-} mice. We showed that TLX^{-/-} mice have a blunted transcriptional response 298 to IL-1ß after cannulation surgery compared to cannulated WT mice and we highlight that 299 300 TLX may act to regulate inflammatory responses via TNF signalling. Finally, we outline that there was no difference in the expression levels of IL-1 β and TNF α between TLX^{-/-} and WT 301 naïve mice but that these cytokines were significantly increased in TLX^{-/-} mice after surgery. 302 Taken together, these data indicate that the absence of TLX results in disruption of the 303 304 hippocampal inflammatory transcriptome response.

305

There was a large dysregulation in the transcriptional landscape of the hippocampus of TLX^{-/-} 306 mice evident with 1542 genes differentially expressed compared to WT mice. The majority of 307 these genes (1272 in total) were up-regulated, which is in keeping with the current literature 308 describing TLX as a transcriptional repressor (Sun et al., 2007, Islam and Zhang, 2014). 309 Similarly, whole transcriptome analysis of another strain of TLX knockout mice revealed a 310 large number of differentially regulated genes (1721) in NPCs from the subventricular zone 311 of the lateral ventricles (Niu et al., 2011). Most likely these genes are not all targets of TLX 312 but rather are up-regulated as a knock-on effect due to a lack of regulation of TLX target 313 genes. These genes showed enrichment for GO terms such as the regulation of inflammatory 314

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315 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 316 (increased somal area) in the DG of TLX^{-/-} mice compared to WT counterparts under baseline 317 conditions, while there is no change in the number of apoptotic cells in the DG of these mice 318 (Kozareva et al., 2017a, Kozareva et al., 2017b). Thus, there is evidence to suggest that TLX 319 is intrinsically linked with inflammatory processes in the hippocampus as the absence of TLX 320 leads to increased microglial activation at baseline and a dysregulated inflammatory gene 321 expression profile. 322 323

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

As proliferating NPCs are the predominant source of TLX within the hippocampus (Shi et al.,

327 very low level within cerebral cortex microglia (EMBL gene expression atlas

328 (http://www.ebi.ac.uk/gxa/genes)), and thus it is likely that the regulatory role of TLX on

329 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

331 NPCs themselves have indirect beneficial effects on the surrounding tissue by modulating the

inflammatory environment (Pluchino et al., 2005, Martino and Pluchino, 2006).

333

324

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

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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).

347

Apart from genes involved in the regulation of inflammation, there are a large number of 348 genes involved in other processes such as intracellular signal transduction, cell chemotaxis, 349 and organ development that are differentially expressed in TLX^{-/-} mice. These mice have a 350 spontaneous deletion and are thus devoid of TLX from embryonic development through to 351 postnatal development and adulthood (Young et al., 2002). Therefore, the wide ranging 352 biological processes dysregulated in these mice are possibly due to the absence of TLX 353 354 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 355 inflammatory profile of mice with a conditional knockout of TLX in adulthood to address the 356 role that loss of TLX during embryonic development has on the development of the immune 357 system and its function in adulthood. 358

359

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-1 β , whose receptor is expressed on NPCs in the hippocampus (Green et al., 2012), has previously been shown to

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365 downregulate TLX expression in NPCs in vitro (Green and Nolan, 2012, Ryan et al., 2013, Farrar et al., 1987). In response to hippocampal IL-1ß injection in WT mice, we observed a 366 significant increase in inflammation and chemotaxis related genes such as Cxcl1, Illb, Tnf, 367 368 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 369 370 (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, 371 However, this may be due to the fact that TLX is only expressed in a subset of cells within 372 373 the hippocampus and as we extracted RNA from the whole hippocampus, any changes in specific TLX-expressing cells may be not be detected. 374

375

With regard to the similarity in transcriptome changes in TLX^{-/-} mice and IL-1 β -injected WT 376 mice compared to WT mice, there were 176 genes (of the 1272 genes up-regulated in TLX^{-/-} 377 mice) also up-regulated in WT mice in response to IL-1 β . It should be noted that RNA 378 379 sequencing analysis only highlighted similarly upregulated genes however, and did not compare the magnitude of gene expression change between the two groups. Notwithstanding, 380 these data suggest that the absence of TLX expression leads to an inflammatory state in the 381 hippocampus similar to that induced by IL-1 β in WT mice. KEGG pathway analysis of the 382 overlaping genes shared between TLX^{-/-} and WT mice injected with IL-1 β compared to WT 383 mice reveal that TNF signalling is a common pathway. This then suggests that TLX may act 384 to repress TNF signalling, which in turn is increased by knocking down TLX expression or 385 by increasing IL-1 β expression. It has been established that IL-1 β can induce the expression 386 of other cytokines such as TNF α , and that TNF α can itself suppress proliferation of TLX 387 expressing NPCs and subsequent neuronal differentiation (Monje et al., 2003, Ben-Hur et al., 388 2003, Keohane et al., 2010). Likewise, TNFa can induce the expression of other cytokines 389

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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.

396

We conducted STRING analysis on the up-regulated genes in TLX^{-/-} compared to WT mice 397 398 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 399 400 interconnecting lines depicted in Figure 6 is indicative of the amount of evidence supporting 401 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 402 potential interactions with Top2a and Bcl6b proteins. Both of these genes are up-regulated in 403 404 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 405 indicated by the thickness of the connecting lines in Figure 6A. Specifically, STRING 406 analysis highlighted these two proteins due to the fact that structural homologs of the proteins 407 (TLX, Top2a, and Bcl6b) have been shown to interact in human tissue and there is greater 408 409 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 410 TLX regulates inflammation (Glass and Saijo, 2010). Nuclear transrepression involves the 411 'tethering' of nuclear receptor to other signalling factors in order to prevent their signalling. 412 This is a common mechanism through which glucocorticoid receptors and PPARs act to 413 suppress inflammatory-related NF-kB signalling (Glass and Ogawa, 2006, Glass and Saijo, 414

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415 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. 416 However, to date no such transrepression mechanism has been described for TLX and any 417 component of TNF signalling. 418 419 Somewhat surprisingly, we observed a drastically different alteration in the transcriptional 420 landscape of TLX^{-/-} mice in response to IL-1β compared to WT mice injected with IL-1β. 421 TLX^{-/-} mice have a blunted response with only 38 differentially expressed genes compared to 422 228 genes differentially regulated in the WT mice after IL-1ß injection. Moreover, 423 enrichment could only be achieved for the down-regulated genes in the TLX^{-/-} mice in 424 425 response to IL-1 β , and this revealed an enrichment in GO terms such as cell chemotaxis 426 (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 427 TLX^{-/-} mice already have an elevated inflammatory response to the cannulation surgery. 428 Specifically, we observed a significant increase in the expression of IL-1 β and TNF α in TLX⁻ 429 ⁻ mice only after surgery. Thus, it could be suggested that these mice have reached an 430 inflammatory plateau after cannulation surgery and are resistant to further cytokine stimulus. 431 Additionally, these data indicate that $TLX^{-/-}$ mice may be more sensitive to brain injury 432 compared to WT mice. Despite the fact that we did not see any differences in IL-1 β or TNF α 433 expression levels between $TLX^{-/-}$ and WT mice at baseline, we have previously reported 434 increased microglial activation in TLX^{-/-} mice which indicates a heightened inflammatory 435 state in these mice at baseline (Kozareva et al., 2017a). With regards the role of TNF 436 signalling and its regulatory role on inflammation, it should also be noted that IL-1R1 is 437

438 expressed on endothelial cells throughout the brain (Matsuwaki et al., 2014). These cells also

439 express genes involved in the TNF signalling pathway and are thus critical in the regulation

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440 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 441 subsequently results in blunted responses to stimulation by inflammatory cytokines as well as 442 443 an enhanced inflammatory response to cannulation surgery. We assessed IL-6 mRNA expression via PCR and observed no significant differences between cannulated and non-444 cannulated mice. While IL-6 is a key marker of brain injury (Woiciechowsky et al., 2002, 445 Shohami et al., 1994), it may be the case that the TLX-/- mice used in the current study are 446 more susceptable to changes in IL-1 β and TNF α than to IL-6 as much greater differences in 447 448 the mRNA expression of IL-1 β and TNF α are observed. However, it should be noted that there was a large degree a variability in the PCR results for IL-6 which may be due to the 449 450 relatively limited number of samples available. This result highlights the facts that compared 451 to RNASeq, PCR analysis requires highly specific primer sequences and possibly larger sample sizes to accurately highlight smaller gene expression differences (Griffith et al., 452 2010). 453

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Disruption of TLX as a result of spontaneous deletion results in abnormalities in motor, 455 cognitive and anxiety-related behaviours. The most striking behavioural phenotype is 456 increased aggression (Young et al., 2002; O'Leary et al., 2016 review). The serotonin_{2A/C} 457 receptor has been shown to mediate the aggressive phenotype of $TLX^{-/-}$ mice (Juarez et al., 458 2013) and interestingly, from our GO enrichment of genes for biological processes we 459 460 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 461 also been consistently observed in TLX^{-/-} mice, and we have recently reported a progressive 462 decline in motor performance on the accelerating rotarod (O'Leary et al., 2016) which 463 implicate corticostriatal pathways. With regard to hippocampal-associated behaviours, TLX^{-/-} 464

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465	mice display poor performance in working memory as assessed by spontaneous alternations
466	in the Y-maze, as well as and contextual fear conditioning (O'Leary et al., 2016a). Extensive
467	evidence in the literature shows that hippocampal adminstration of IL-1 β results in
468	impairments in hippocampal-associated spatial learning and memory tasks and in LTP
469	(Moore et al., 2009, Kohman and Rhodes, 2013, Vereker et al., 2000). Although we did not
470	assess behavioural outcomes of IL-1 β administration to TLX ^{-/-} mice in the current study, it is
471	possible that IL-1 β may not exacerbate the already robust deficits in cognitive behaviours due
472	to the significant dysregulated and indeed pro-inflammatory transcriptional profile of the
473	TLX-/- mice in this study.
474	
475	In conclusion, this study provides valuable insights into the role of TLX as a regulator of
476	inflammation and lays the ground work for future studies assessing the effects of TLX on
477	inflammatory and cognitive processes. Moreover, as TLX is a nuclear receptor and thus has

the potential to be targeted therapeutically (Benod et al., 2014), future studies should aim to

identify selective ligands for TLX and determine the ability of modulating TLX activity to

480 mitigate the effects of extensive inflammation on hippocampal-associated cognition.

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- **Table 1:** Meaningful pairwise comparisons for gene expression changes conducted using
- 673 RNA sequencing (4 unique comparisons).

Experimental group	Abbreviation	n	Pairwise comparison
Wild type + PBS	WT	6	WT vs. WT + IL-1 β
TLX knockout + PBS	TLX ^{-/-}	4	WT vs. TLX ^{-/-}
Wild type + IL-1β	$WT + IL-1\beta$	7	WT + IL-1 β vs. TLX ^{-/-} + IL-1 β
TLX knockout + IL-1β	$TLX^{-/-} + IL-1\beta$	6	TLX ^{-/-} + IL-1 β vs. TLX ^{-/-}
Figure Legends		A	
Figure 1: Schematic of exp	perimental design and	timeline.	WT, wildtype; TLX ^{-/-} , TLX
knockout; PBS, Phosphate	buffered saline; IL-1β	8, Interleu	kin-1 beta; WT + PBS, Wildtype
PBS injected mice; WT + I	L-1β, Wildtype IL-1β	injected	mice; TLX ^{-/-} + PBS, TLX knockout
PBS injected mice; TLX ^{-/-} -	+ IL-1β, TLX knocko	ut IL-1β i	njected mice.

Figure 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

TLX knock-out and IL-1β

692	values above this line deemed significant. E) GO enrichment in biological processes for
693	down-regulated genes comparing TLX ^{-/-} to WT mice. Dotted line represents significance
694	level, with values above this line deemed significant.
695	
696	Figure 3: IL-1 β induces the transcription of inflammatory-related genes in WT mice similar
697	to that in TLX ^{-/-} mice. A) Volcano plot of all DEGs when comparing WT + IL-1 β and WT
698	mice vs. WT and TLX ^{-/-} mice. B) Venn diagram of all DEGs either up-regulated (green
699	circles) or downregulated (blue circles) and the overlap of these genes when comparing TLX
700	$^{/-}$ and WT mice. C) GO enrichment in KEGG pathway for up-regulated genes comparing the
701	overlap of WT vs. WT + IL-1 β and WT vs. TLX ^{-/-} mice. The dotted line represents
702	significance level, with values above this line deemed significant. D) GO enrichment in
703	biological processes for up-regulated genes comparing the overlap of WT vs. WT + IL-1 β
704	and WT vs. TLX ^{-/-} mice. The dotted line represents significance level, with values above this
705	line deemed significant. E) Venn diagram comparing the overlap in gene transcriptional

changes between WT vs. WT + IL-1 β and WT vs. TLX^{-/-} mice.

707

Figure 4: String analysis showing interactions between genes similarly increased in TLX^{-/-} mice and WT mice injected with IL-1 β compared to WT mice that are involved in TNF signalling. Line thickness between nodes indicates the strength of the evidence to support the interaction.

712

Figure 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.
B) Venn diagram showing the total number of DEGs up-regulated when comparing WT + IL-

TLX knock-out and IL-1 β

717	1 β to TLX ^{-/-} + IL-1 β mice. C) GO enrichment in biological processes for down-regulated
718	genes comparing the overlap of TLX ^{-/-} to TLX ^{-/-} + IL-1 β -treated mice. The dotted line
719	represents significant level with values above this line deemed significant.
720	
721	Figure 6: A) STRING analysis showing interactions between genes increased in TLX ^{-/-} mice
722	compared to WT mice that are involved in TNF signalling, and how TLX interacts with TNF
723	signalling. Thicker lines indicate stronger network connections between genes. Line thickness
724	between nodes indicates the strength of the evidence to support the integration. B), C) and D)
725	TNF α , IL-1 β and IL-6 mRNA expression in non-cannulated and cannulated TLX ^{-/-} and WT

- mice, n.s = non-significant (data expressed as mean \pm SEM, ***=P<0.01 ANOVA and
- Tukey's *post-hoc* test). All data are presented as mean \pm SEM.

Ó Léime et al. 2017: Highlights 729 TLX regulates inflammatory transcriptional profile within the hippocampus of mice. 730 IL-1 β induces inflammatory transcriptional changes in wildtype mice. 731 • TLX knockout mice have a blunted hippocampal transcriptional response to IL-1β. 732 • . infar TLX may interact with TNF signalling to regulate hippocampal inflammation. 733 734 735

Animal breeding and sterotaxic surgery

Tissue collection











