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

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Abbreviations: TLX, Orphan nuclear receptor tailless homolog; IL-1β, interleukin-1 beta; 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, database for annotation visualisation and integrated discovery; STRING, search tool for the retrieval of interacting genes/proteins.

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.

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

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., 2004, 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, 2012, 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, 2012, 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) 56 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 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°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 1, Table 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 (Haettig et al., 2013). 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 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.

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™ 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

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*

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

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 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 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).

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 + 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 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 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 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 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 comapred to $TLX^{-/-}$ mice (Figure 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 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 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 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 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 6B, C). However, after hippocampal cannulation surgery, there is a significant increase in IL-1β ($F(3,24)=12.34$, $P=0.0001$, ***=$P<0.01$) and TNFα ($F(3,24)=10.12$, $P=0.0003$; ***=$P<0.01$) expression in $TLX^{-/-}$ mice only (Figure 6B, C). There is a non-significant trend towards an increase in these cytokines in WT mice after hippocampal cannulation surgery (IL-1β: t-test comparison between WT/Naive and WT/PBS indicates $P=0.0862$, TNFα: t-test comparison between WT/Naive and WT/PBS indicates $P=0.0588$) which may be due to limited samples numbers or to significant variations among individual mice. Likewise, PCR analysis revealed that there was no statistically significant difference in IL-6 mRNAexpression between cannulated and non-cannulated mice for both WT and KO strains (Figure 6D).

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

365 downregulate TLX expression in NPCs *in vitro* (Green and Nolan, 2012, Ryan et al., 2013,
366 Farrar et al., 1987). In response to hippocampal IL-1β injection in WT mice, we observed a
367 significant increase in inflammation and chemotaxis related genes such as *Cxcl1, Il1b, Tnf,*
368 and *Tlr2*. This is supported by reports that increased cytokine expression or administration of
369 exogenous cytokines to the hippocampus can induce further endogenous cytokine expression
370 (Anisman et al., 2008, Moore et al., 2009, Shaftel et al., 2007, Skelly et al., 2013, Balschun et
371 al., 2004, del Rey et al., 2013). We did not observe a significant decrease in TLX expression,
372 However, this may be due to the fact that TLX is only expressed in a subset of cells within
373 the hippocampus and as we extracted RNA from the whole hippocampus, any changes in
374 specific TLX-expressing cells may be not be detected.
375
376 With regard to the similarity in transcriptome changes in $TLX^{-/-}$ mice and IL-1β-injected WT
377 mice compared to WT mice, there were 176 genes (of the 1272 genes up-regulated in $TLX^{-/-}$
378 mice) also up-regulated in WT mice in response to IL-1β. It should be noted that RNA
379 sequencing analysis only highlighted similarly upregulated genes however, and did not
380 compare the magnitude of gene expression change between the two groups. Notwithstanding,
381 these data suggest that the absence of TLX expression leads to an inflammatory state in the
382 hippocampus similar to that induced by IL-1β in WT mice. KEGG pathway analysis of the
383 overlaping genes shared between $TLX^{-/-}$ and WT mice injected with IL-1β compared to WT
384 mice reveal that TNF signalling is a common pathway. This then suggests that TLX may act
385 to repress TNF signalling, which in turn is increased by knocking down TLX expression or
386 by increasing IL-1β expression. It has been established that IL-1β can induce the expression
387 of other cytokines such as TNFα, and that TNFα can itself suppress proliferation of TLX
388 expressing NPCs and subsequent neuronal differentiation (Monje et al., 2003, Ben-Hur et al.,
389 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 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 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). With regards the role of TNF signalling and its regulatory role on inflammation, 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. We assessed IL-6 mRNA expression via PCR and observed no significant differences between cannulated and non-cannulated mice. While IL-6 is a key marker of brain injury (Woiciechowsky et al., 2002, Shohami et al., 1994), it may be the case that the TLX-/- mice used in the current study are more susceptable to changes in IL-1β and TNFα than to IL-6 as much greater differences in the mRNA expression of IL-1β and TNFα 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 relatively limited number of samples available. This result highlights the facts that compared to RNASeq, PCR analysis requires highly specific primer sequences and possibly larger sample sizes to accurately highlight smaller gene expression differences (Griffith et al., 2010).

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., 2014), 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.

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668 *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 1876-
669 81.

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672 **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-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. $TLX^{-/-}$

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676 **Figure Legends**
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679 **Figure 1:** Schematic of experimental design and timeline. WT, wildtype; $TLX^{-/-}$, TLX
680 knockout; PBS, Phosphate buffered saline; IL-1β, Interleukin-1 beta; WT + PBS, Wildtype
681 PBS injected mice; WT + IL-1β, Wildtype IL-1β injected mice; $TLX^{-/-}$ + PBS, TLX knockout
682 PBS injected mice; $TLX^{-/-}$ + IL-1β, TLX knockout IL-1β injected mice.
683
684 **Figure 2:** $TLX^{-/-}$ mice display elevated transcription of inflammatory genes in the
685 hippocampus compared to WT controls. **A)** The number of differentially expressed genes
686 (DEGs) between all pairwise comparisons across the groups. **B)** A volcano plot depicting the
687 number of differentially regulated genes between WT and $TLX^{-/-}$ mice. Each gene is graphed
688 as the fold change (log 2) versus q value (p adjusted value) for multiple comparisons. **C)**
689 Venn diagram outlining the number of DEGs either up-regulated (green circles) or down-
690 regulated (blue circles) across all groups. **D)** GO enrichment in biological processes for up-
691 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: 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 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 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-

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 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)**, **C)** and **D)** TNFα, IL-1β and IL-6 mRNA expression in non-cannulated and cannulated $TLX^{-/-}$ and WT mice, n.s = non-significant (data expressed as mean ± SEM, ***=$P<0.01$ ANOVA and Tukey's *post-hoc* test). All data are presented as mean ± SEM.

729 Ó Léime et al. 2017: Highlights

730 • TLX regulates inflammatory transcriptional profile within the hippocampus of mice.

731 • IL-1β induces inflammatory transcriptional changes in wildtype mice.

732 • TLX knockout mice have a blunted hippocampal transcriptional response to IL-1β.

733 • TLX may interact with TNF signalling to regulate hippocampal inflammation.

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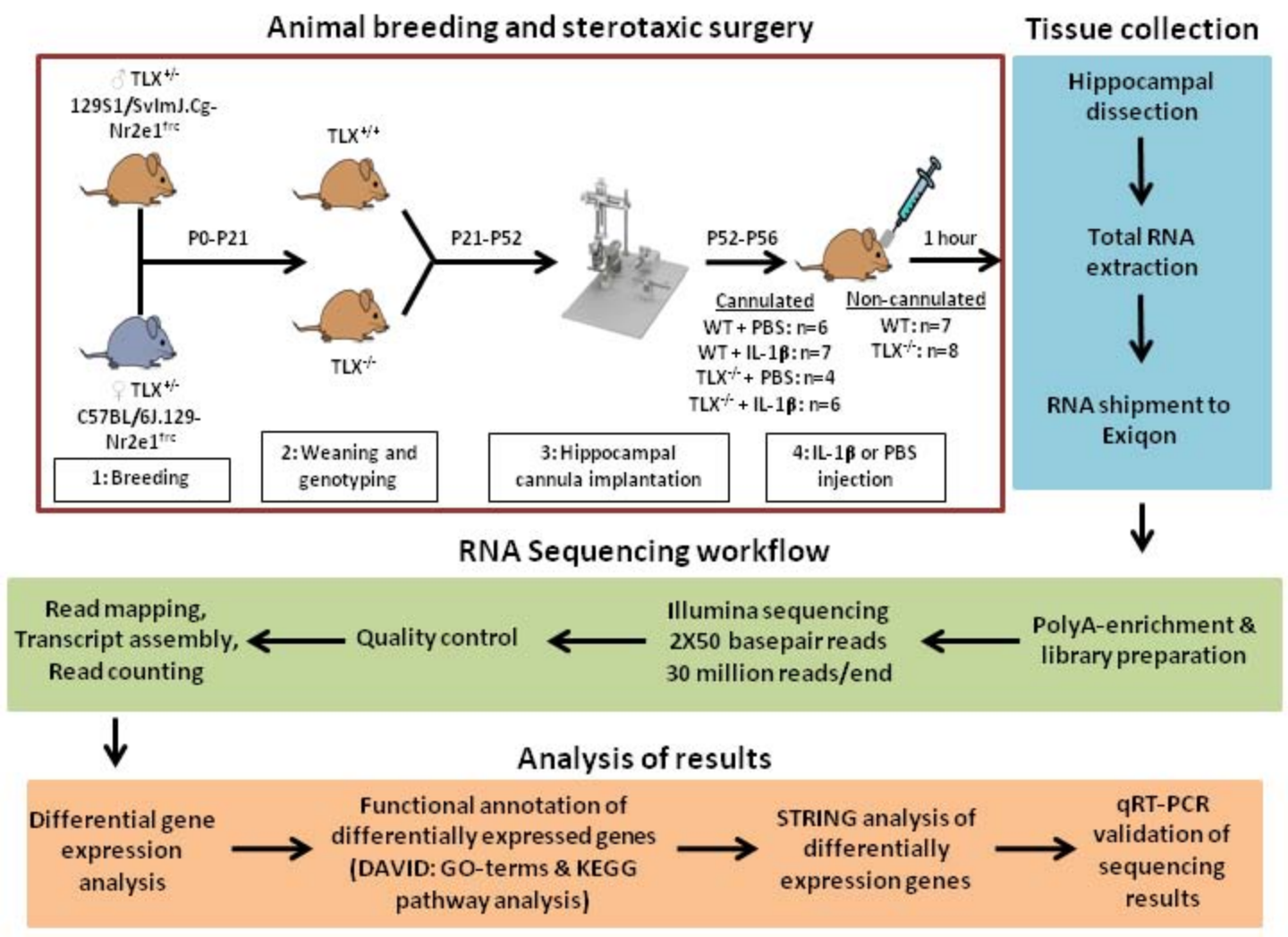
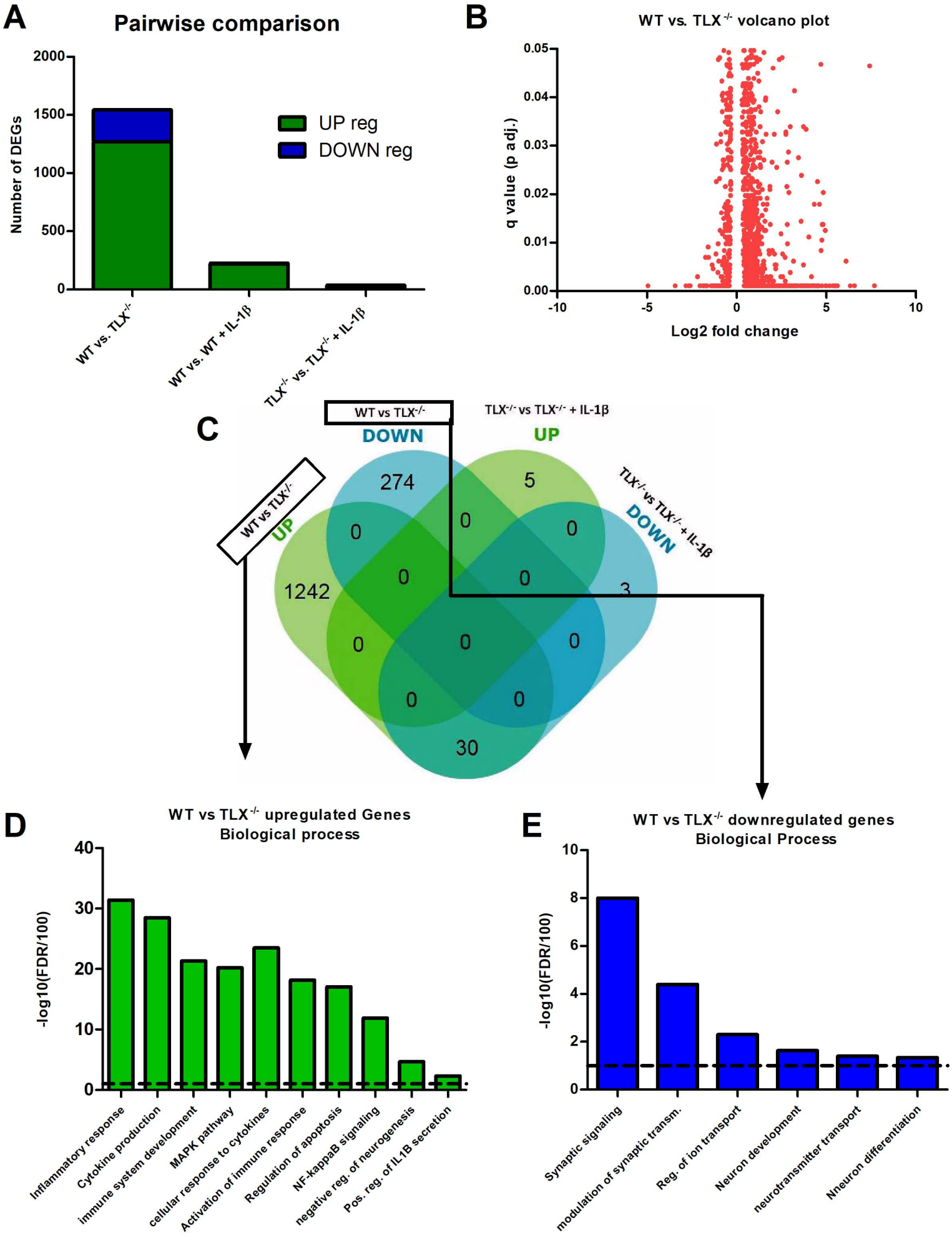
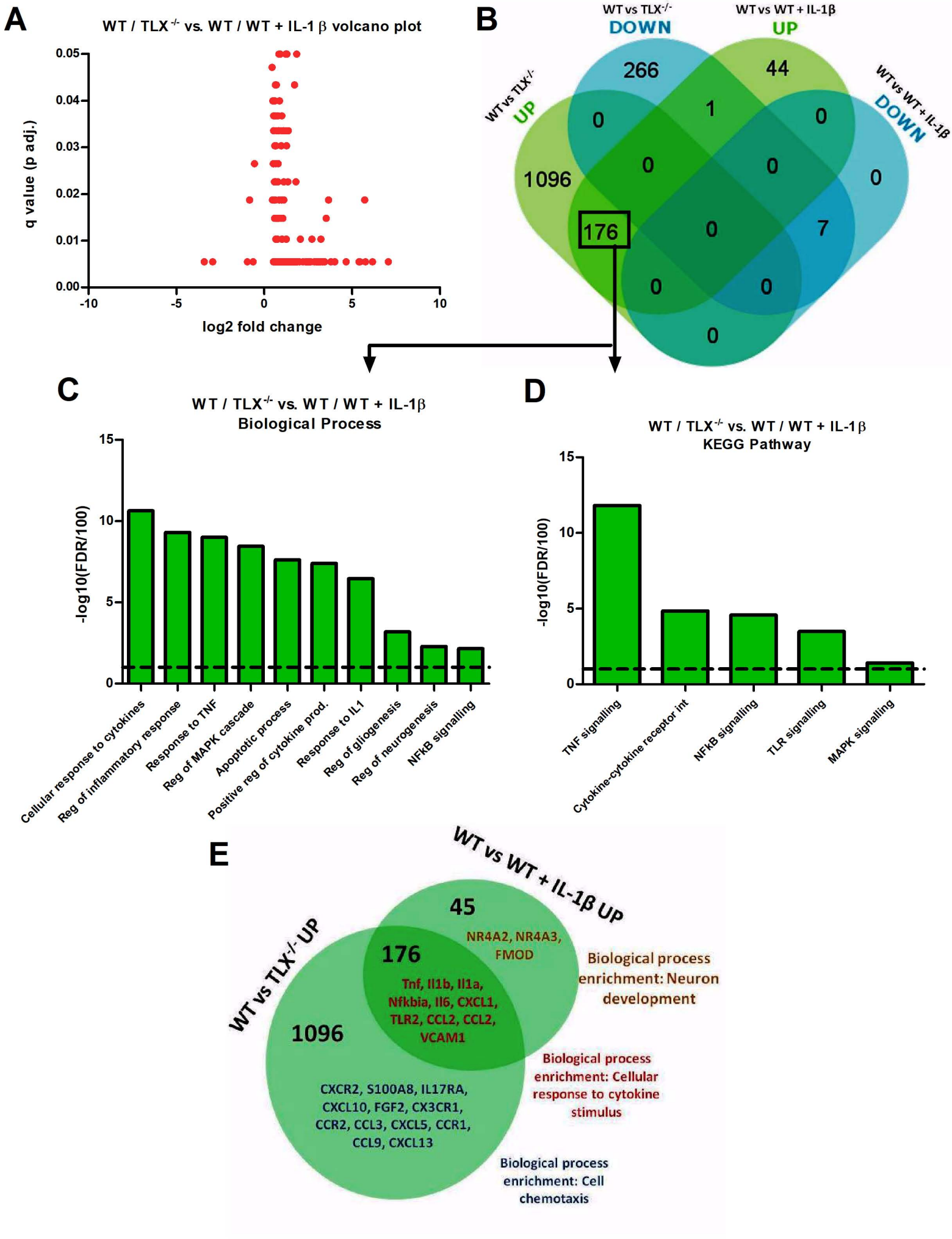
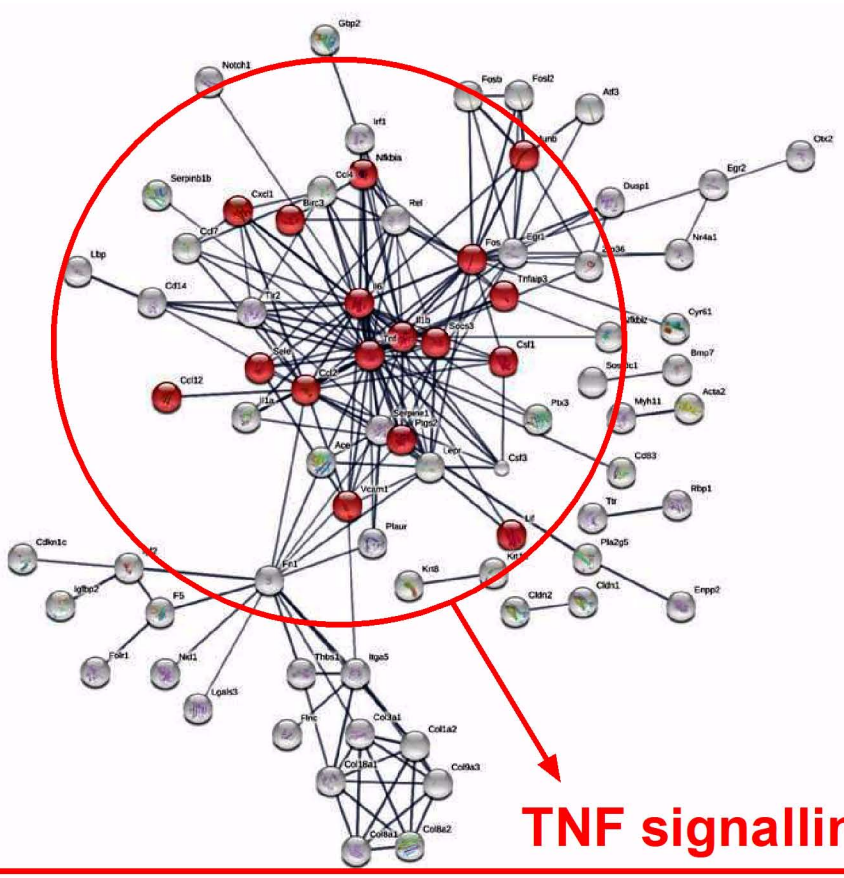

Animal breeding and sterotaxic surgery
Tissue collection
♂ TLX+/-
129S1/SvImJ.Cg-Nr2e1frc
♀ TLX+/-
C57BL/6J.129-Nr2e1frc
P0-P21
TLX+/+
TLX-/-
P21-P52
P52-P56
1 hour
Cannulated
WT + PBS: n=6
WT + IL-1β: n=7
TLX-/- + PBS: n=4
TLX-/- + IL-1β: n=6
Non-cannulated
WT: n=7
TLX-/-: n=8
1: Breeding
2: Weaning and genotyping
3: Hippocampal cannula implantation
4: IL-1β or PBS injection
Hippocampal dissection
Total RNA extraction
RNA shipment to Exiqon
RNA Sequencing workflow
PolyA-enrichment & library preparation
Illumina sequencing
2X50 basepair reads
30 million reads/end
Quality control
Read mapping,
Transcript assembly,
Read counting
Analysis of results
Differential gene expression analysis
Functional annotation of differentially expressed genes (DAVID: GO-terms & KEGG pathway analysis)
STRING analysis of differentially expression genes
qRT-PCR validation of sequencing results


A
Pairwise comparison
Number of DEGs
2000
1500
1000
500
0
UP reg
DOWN reg
WT vs. TLX-/-
WT vs. WT + IL-1β
TLX-/- vs. TLX-/- + IL-1β
B
WT vs. TLX-/- volcano plot
q value (p adj.)
0.05
0.04
0.03
0.02
0.01
0.00
-10
-5
0
5
10
Log2 fold change
C
WT vs TLX-/-
DOWN
TLX-/- vs TLX-/- + IL-1β
UP
WT vs TLX-/-
UP
TLX-/- vs TLX-/- + IL-1β
DOWN
274
5
1242
3
0
30
D
WT vs TLX-/- upregulated Genes
Biological process
-log10(FDR/100)
40
30
20
10
0
Inflammatory response
Cytokine production
immune system development
MAPK pathway
cellular response to cytokines
Activation of immune response
Regulation of apoptosis
NF-kappaB signaling
negative reg. of neurogenesis
Pos. reg. of IL1B secretion
E
WT vs TLX-/- downregulated genes
Biological Process
-log10(FDR/100)
10
8
6
4
2
0
Synaptic signaling
modulation of synaptic transm.
Reg. of ion transport
Neuron development
neurotransmitter transport
Nneuron differentiation

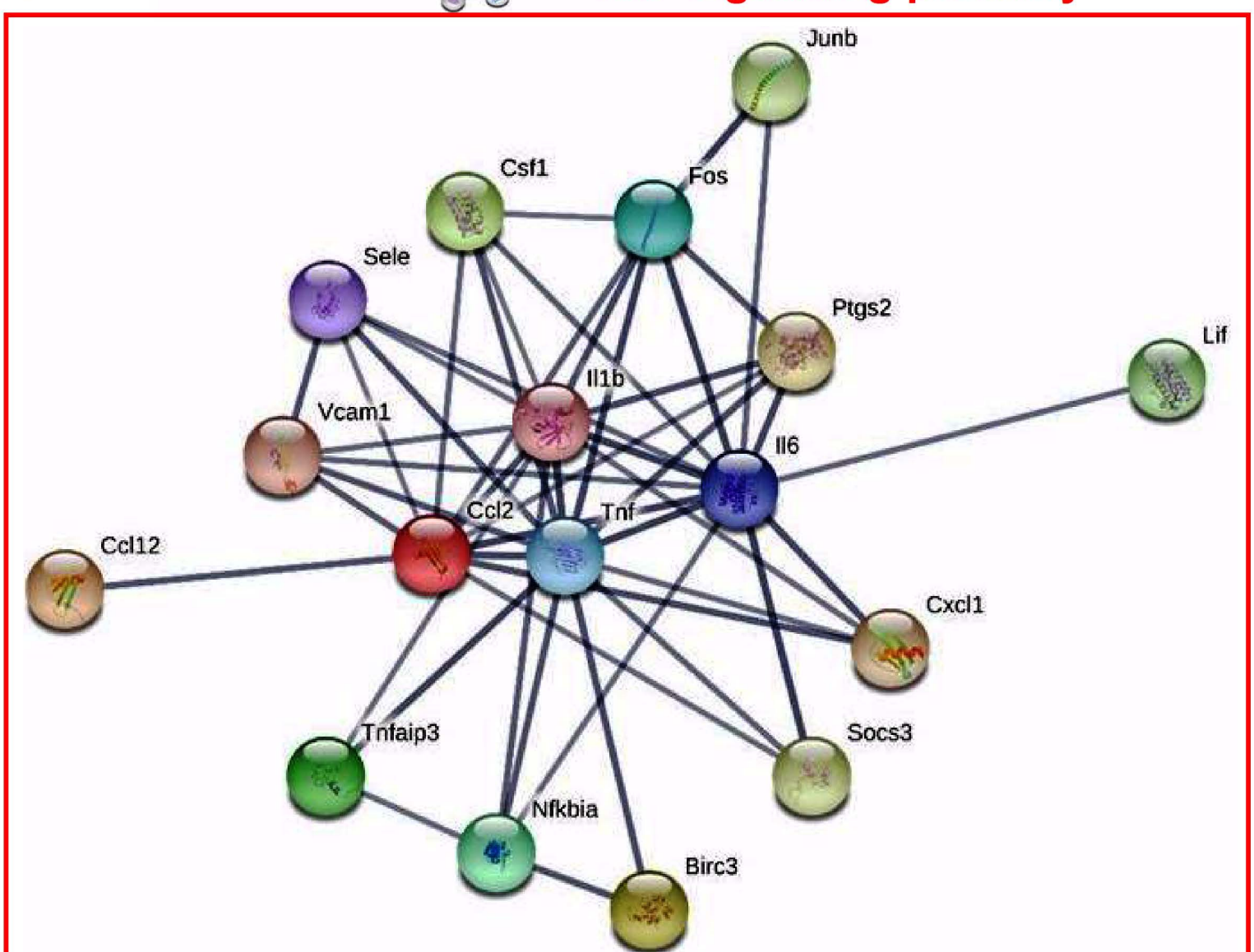

A
WT / TLX-/- vs. WT / WT + IL-1 β volcano plot
q value (p adj.)
0.05
0.04
0.03
0.02
0.01
0.00
-10
-5
0
5
10
log2 fold change
B
WT vs TLX-/- DOWN
WT vs WT + IL-1β UP
WT vs TLX-/- UP
WT vs WT + IL-1β DOWN
266
44
0
1
0
1096
0
0
0
176
0
7
0
0
0
C
WT / TLX-/- vs. WT / WT + IL-1β
Biological Process
-log10(FDR/100)
15
10
5
0
Cellular response to cytokines
Reg of inflammatory response
Response to TNF
Reg of MAPK cascade
Apoptotic process
Positive reg of cytokine prod.
Response to IL1
Reg of gliogenesis
Reg of neurogenesis
NFkB signalling
D
WT / TLX-/- vs. WT / WT + IL-1β
KEGG Pathway
-log10(FDR/100)
15
10
5
0
TNF signalling
Cytokine-cytokine receptor int
NFkB signalling
TLR signalling
MAPK signalling
E
WT vs WT + IL-1β UP
WT vs TLX-/- UP
45
NR4A2, NR4A3, FMOD
176
Tnf, Il1b, Il1a, Nfkbia, Il6, CXCL1, TLR2, CCL2, CCL2, VCAM1
1096
CXCR2, S100A8, IL17RA, CXCL10, FGF2, CX3CR1, CCR2, CCL3, CXCL5, CCR1, CCL9, CXCL13
Biological process enrichment: Neuron development
Biological process enrichment: Cellular response to cytokine stimulus
Biological process enrichment: Cell chemotaxis

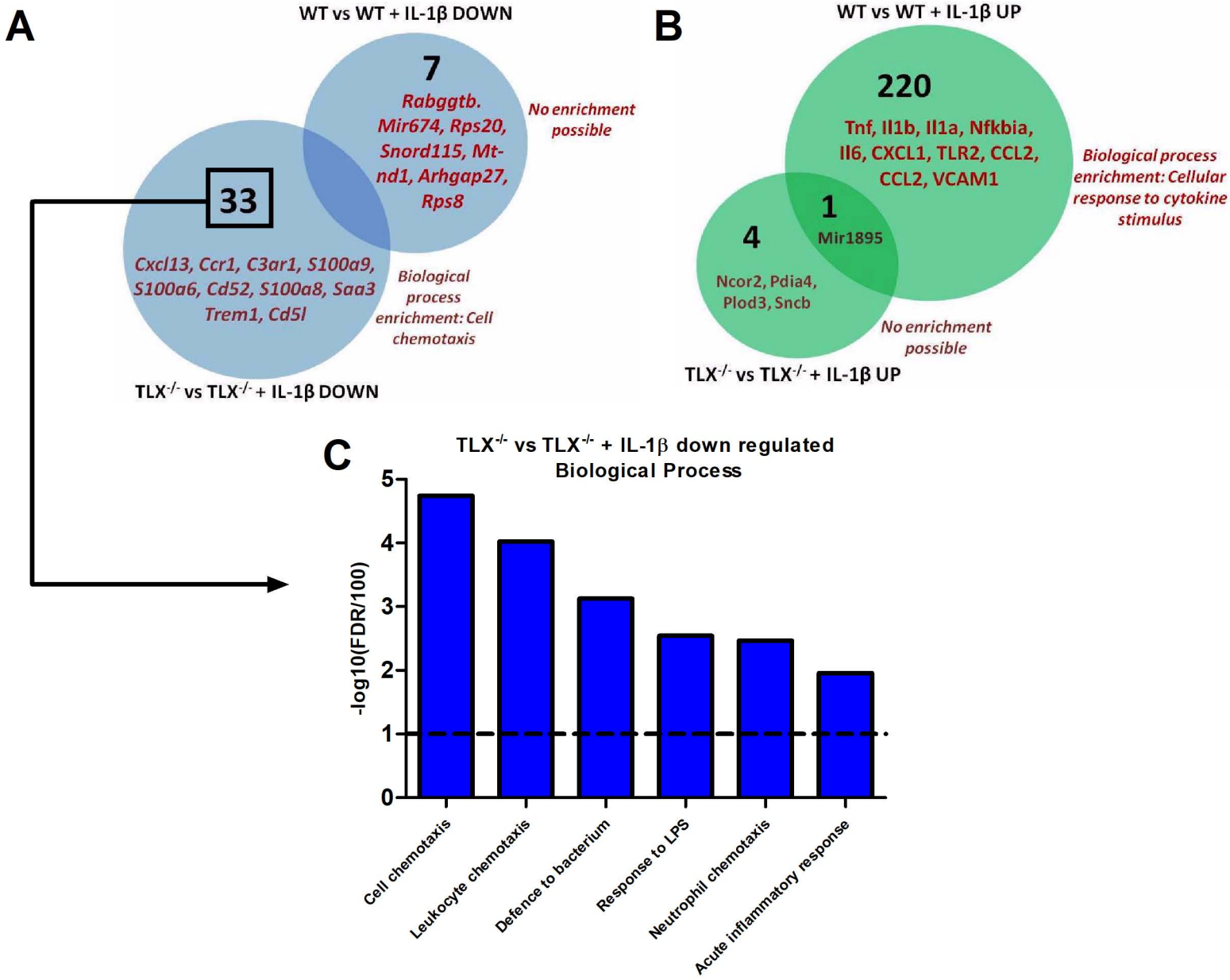
A

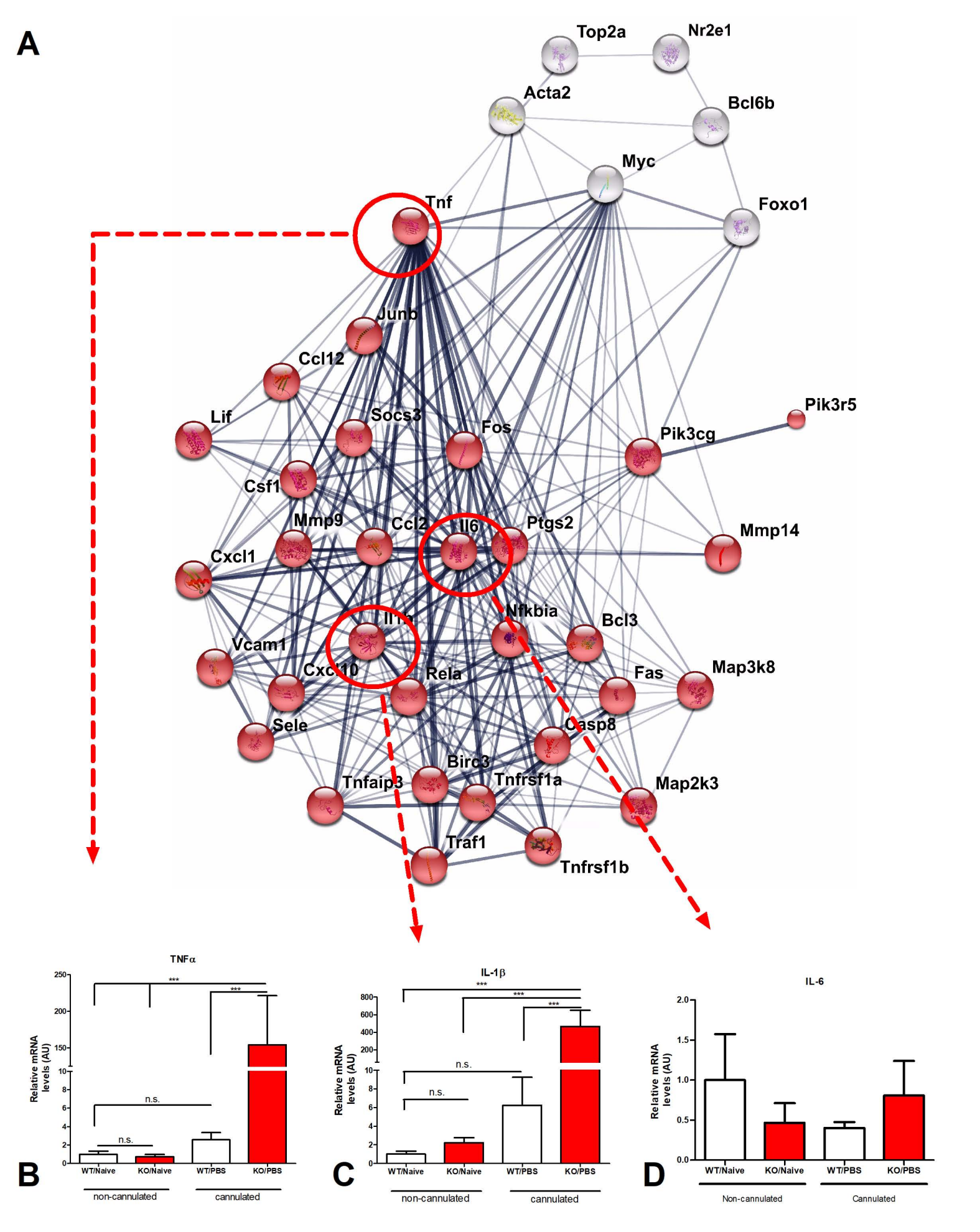


B

TNF signalling pathway




A
WT vs WT + IL-1β DOWN
7
Rabggtb. Mir674, Rps20, Snord115, Mt-nd1, Arhgap27, Rps8
No enrichment possible
33
Cxcl13, Ccr1, C3ar1, S100a9, S100a6, Cd52, S100a8, Saa3 Trem1, Cd5l
Biological process enrichment: Cell chemotaxis
TLX-/- vs TLX-/- + IL-1β DOWN
B
WT vs WT + IL-1β UP
220
Tnf, Il1b, Il1a, Nfkbia, Il6, CXCL1, TLR2, CCL2, CCL2, VCAM1
Biological process enrichment: Cellular response to cytokine stimulus
1
Mir1895
4
Ncor2, Pdia4, Plod3, Sncb
No enrichment possible
TLX-/- vs TLX-/- + IL-1β UP
C
TLX-/- vs TLX-/- + IL-1β down regulated
Biological Process
-log10(FDR/100)
0
1
2
3
4
5
Cell chemotaxis
Leukocyte chemotaxis
Defence to bacterium
Response to LPS
Neutrophil chemotaxis
Acute inflammatory response


A
Top2a
Nr2e1
Acta2
Bcl6b
Myc
Tnf
Foxo1
Junb
Ccl12
Lif
Socs3
Fos
Pik3r5
Pik3cg
Csf1
Mmp9
Ccl2
Il6
Ptgs2
Mmp14
Cxcl1
Nfkbia
Bcl3
Vcam1
Cxcl10
Rela
Fas
Map3k8
Sele
Casp8
Birc3
Tnfaip3
Tnfrsf1a
Map2k3
Traf1
Tnfrsf1b
TNFα
Relative mRNA levels (AU)
250
200
150
10
8
6
4
2
0
***
***
n.s.
n.s.
WT/Naive
KO/Naive
WT/PBS
KO/PBS
non-cannulated
cannulated
B
IL-1β
Relative mRNA levels (AU)
800
600
400
200
10
8
6
4
2
0
***
***
***
n.s.
n.s.
WT/Naive
KO/Naive
WT/PBS
KO/PBS
non-cannulated
cannulated
C
IL-6
Relative mRNA levels (AU)
2.0
1.5
1.0
0.5
0.0
WT/Naive
KO/Naive
WT/PBS
KO/PBS
Non-cannulated
Cannulated
D