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Supplemental information

TLR7 and IL-6 differentially regulate the effects of rotarod exercise on the transcriptomic profile and neurogenesis to influence anxiety and memory

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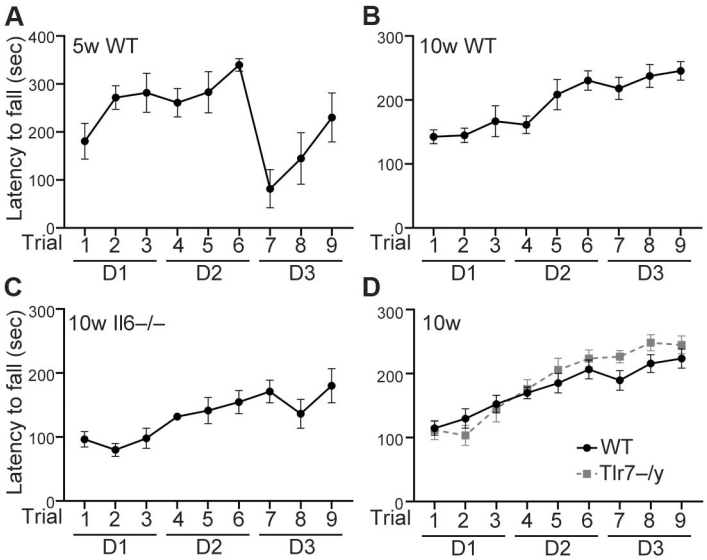
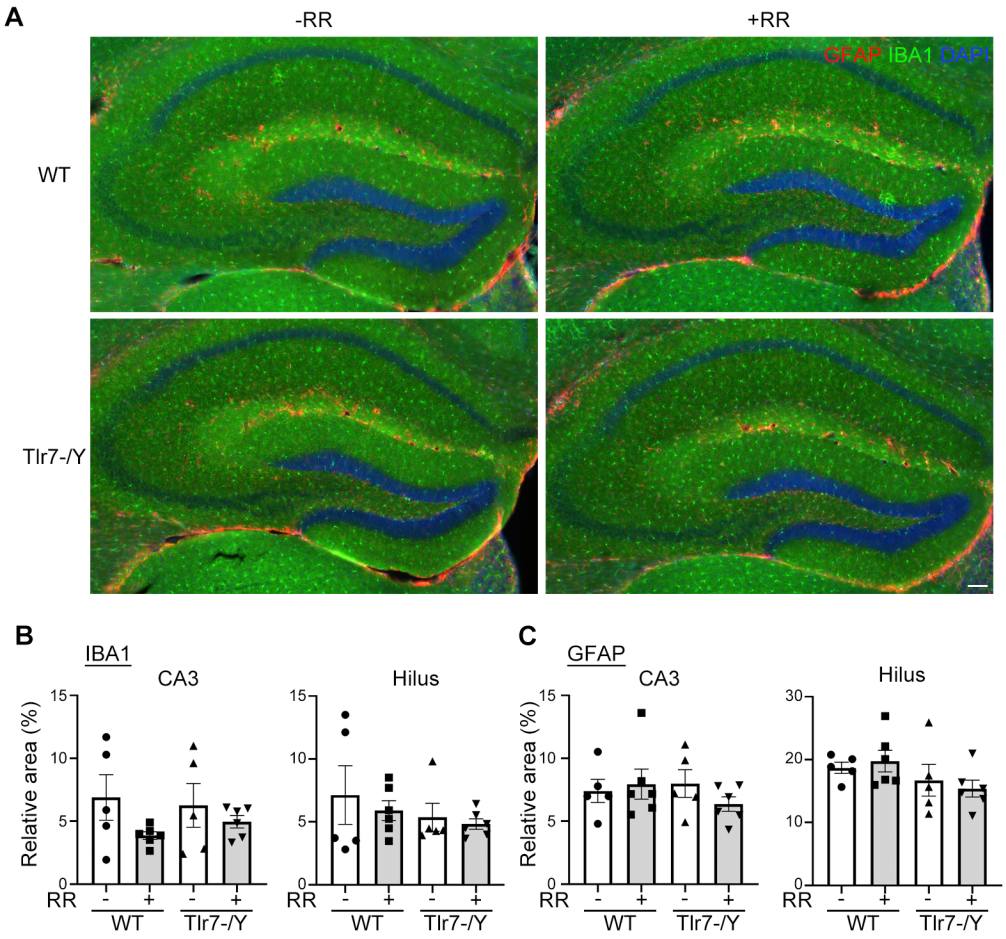
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



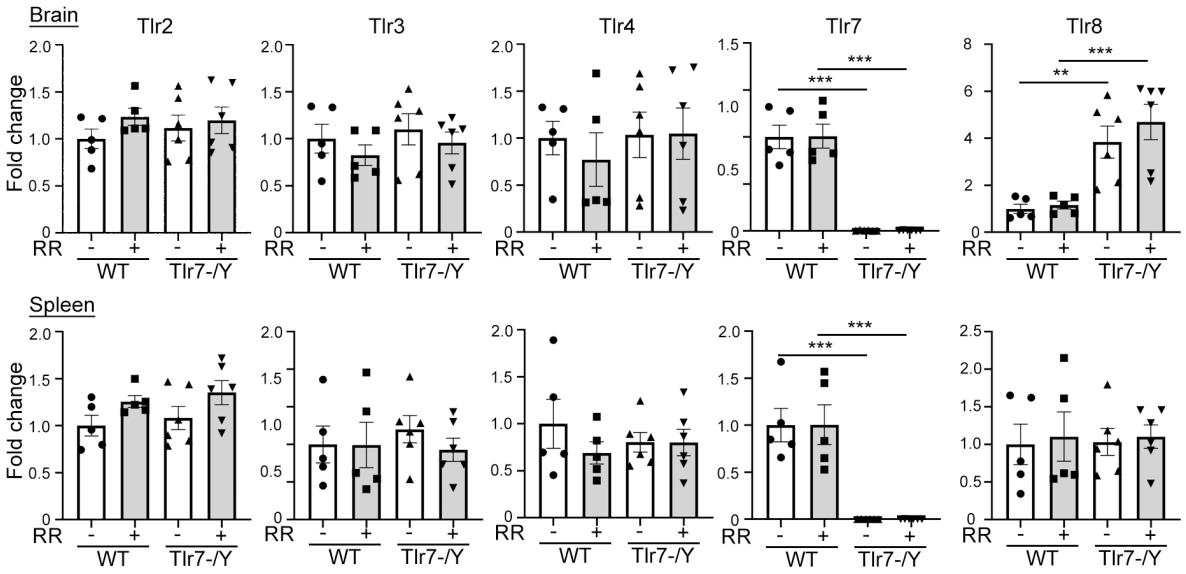
Supplemental Figure S1. The performance of wild-type, *Il6*$^{-/-}$ and *Tlr7*$^{-/y}$ mice on rotarod (related to Figures 1, 2 and 3).

Wild-type (WT) 5-week-old (**A**) and 10-week-old (**B**) mice, 10-week-old *Il6*$^{-/-}$ mice (**C**), and 10-week-old WT and *Tlr7*$^{-/y}$ mice (**D**) were subjected to accelerating rotarod test on three consecutive days. Latency to fall, i.e. the time period to stay on the rotarod, is shown. The curve in (**A**) is quite different from the curves in (**B**)-(**D**) because 5-week-old mice tended to jump away from the rotarod at Day 3. In (**D**), littermates were used to compare the effect of *Tlr7* knockout on rotarod. Similar to a previous report (Hung et al., 2018a), *Tlr7*$^{-/y}$ mice performed comparably to WT littermates in rotarod test. In conclusion, all tested mice at the age of 10 weeks can learn to stay on the rotarod and display better performance at Day 3 (D3). Data represent mean +/– SEM. Sample sizes have been shown in the legends of Figures 1, 2 and 3.



Supplemental Figure S2. Rotarod training does not alter the population of glial cells in either WT or *Tlr7*$^{-/y}$ mice (related to Figure 2).

A Representative images of GFAP (red) and IBA1 (green) dual immunostaining of hippocampus. Both WT and *Tlr7*$^{-/y}$ mice were subjected to rotarod training or mock control. **B-C** Quantification of relative area of IBA1 and GFAP immunoreactivities in CA3 and hilar regions. Data represent mean +/– SEM and the results of individual samples are shown. Two-way ANOVA test (B-C). Scale bar: 100 μm.



Supplemental Figure S3 Rotarod training does not change the expression of *Toll-like receptor* (*Tlr*) genes in mouse brain or spleen (related to Figure 2).

Quantitative PCR was performed to measure the expression levels of *Tlr2*, *Tlr3*, *Tlr4*, *Tlr7* and *Tlr8* in the brain and spleen of mice after undergoing rotarod training at 10 weeks of age. The results were normalized with internal control *Gapdh*. Rotarod training exerts no obvious change in expression of *Tlr* genes in brain or spleen of either WT or *Tlr7*$^{-/y}$ mice (n=5 for WT, n=6 for *Tlr7*$^{-/y}$). Data represent mean +/– SEM and the results of individual samples are shown. Two-way ANOVA with Bonferroni's multiple comparisons test. **, $P < 0.01$; *** $P < 0.001$.

SUPPLEMENTAL TABLES

Supplemental Table S1. Summary of animals used in this report (related to all Figures).

Supplemental Table S2. Q-PCR primers and UPL probes (related to Figures 2 and 6).

Supplemental Table S3. Key resource table (related to all Figures).

Supplemental Table S4. Quantitative analysis of differentially expressed genes influenced by rotarod training in WT and *Tlr7*$^{-/Y}$ mice (related to Figure 4, an Excel table)

Supplemental Table S5. List of differentially expressed genes in WT and *Tlr7*$^{-/y}$ mice with or without rotarod training (related to Figure 4).

Supplemental Table S6. Downregulated genes in WT mice that are less sensitive to rotarod training in *Tlr7*$^{-/Y}$ mice (related to Figure 7).

Supplemental Table S7. Selected genes from Supplemental Table S6 and their representative references relevant to physiological functions (related to Figure 7).

Supplemental Table S1. Summary of animals used in this report (related to all Figures).

Animal group number	Type of analysis	Rotarod treatment	Mouse line	Figures
I	Behavior	yes (5w)	WT	1B-1D
II	Behavior	no	WT	1B-1D
III	Behavior	yes (10w)	WT	1E-1G
IV	Behavior	no	WT	1E-1G
V	RNA collection	yes and no (10w)	WT, *Tlr7-/Y*	2A, 4, 5, 6, 7, S3
VI	Behavior	yes and no (10w)	*Il6–/–*	2B
VII	IBA1/GFAP staining	yes and no (10w)	*Il6–/–*	2C
VIII	Behavior	yes and no (10w)	WT, *Tlr7-/Y*	3C
IX	Behavior	yes and no (10w)	WT, *Tlr7-/Y*	3B, 3D
X	BrdU labeling	yes and no (10w)	WT, *Tlr7-/Y*	3E, 3F
XI	IBA1/GFAP staining	yes and no (10w)	WT, *Tlr7-/Y*	S2

Supplementary Table S2. Q-PCR primers and UPL probes (related to Figures 2 and 6).

Gene	Quantitative RT-PCR primer pairs	Probe
Il6	F: GCTACCAAACTGGATATAATCAGGA	#6
	R: CCAGGTAGCTATGGTACTCCAGAA	
Il1b	F: AGTTGACGGACCCCAAAAG	#38
	R: AGCTGGATGCTCTCATCAGG	
Tnfa	F: TTGTCTTAATAACGCTGATTTGGT	#64
	R: GGGAGCAGAGGTTCAGTGAT	
Ifnb	F: CACAGCCCTCTCCATCAACTA	#78
	R: CATTTCCGAATGTTCGTCCT	
Ccl5	F: TGCAGAGGACTCTGAGACAGC	#110
	R: GAGTGGTGTCCGAGCCATA	
Tlr2	F: GGGGCTTCACTTCTCTGCTT	#50
	R: AGCATCCTCTGAGATTTGACG	
Tlr3	F: GATACAGGGATTGCACCCATA	#26
	R: TCCCCCAAAGGAGTACATTAGA	
Tlr4	F: GGACTCTGATCATGGCACTG	#2
	R: CTGATCCATGCATTGGTAGGT	
Tlr7	F: TGATCCTGGCCTATCTCTGAC	#25
	R: CGTGTCCACATCGAAAACAC	
Tlr8	F: CAAACGTTTTACCTTCCTTTGTCT	#56
	R: ATGGAAGATGGCACTGGTTC	
Gapdh	F: AATGTGTCCGTCGTGGATCT	#80
	R: CCCAGCTCTCCCCATACATA	
Plin4	F: GCTGGAGTCAGTTACCGTCAA	#1
	R: CGCCTCCTTTTCCTCTCAT	
Nptx2	F: TCAAGGACCGCTTGGAGA	#78
	R: GCCCAGCGTTAGACACATTT	
Hspa5	F: CTGAGGCGTATTTGGGAAAG	#105
	R: TCATGACATTCAGTCCAGCAA	
Hsph1	F: AACCCCAGATGCTGACAAA	#75

	R: CCACCTTTATTTTAGGTTTCTTGG	
Calr	F: TGAAGCTGTTTCCGAGTGGT	#93
	R: GATGACATGAACCTTCTTGGTG	
Dcx	F: AGCTGACTCAGGTAACGACCA	#11
	R: GCTTTGACTTAGGTGTTGAGAGC	
Klf4	F: CGGGAAGGGAGAAGACACT	#62
	R: GAGTTCCTCACGCCAACG	
Fos	F: GGGACAGCCTTTCCTACTACC	#67
	R: AGATCTGCGCAAAAGTCCTG	
Egr2	F: CTACCCGGTGGAAGACCTC	#60
	R: AATGTTGATCATGCCATCTCC	
Npas4	F: AGGGTTTGCTGATGAGTTGC	#21
	R: TTCCCCTCCACTTCCATCTT	
Hprt	F: CCTCCTCAGACCGCTTTTT	#95
	R: AACCTGGTTCATCATCGCTAA	

Supplemental Table S3. Key resource table (related to all Figures).

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-IBA1	FUJIFILM Wako Pure Chemical Corporation	Cat# 019-19741, RRID:AB_839504
Mouse monoclonal anti-GFAP (clone GA5)	Millipore	Cat# MAB3402, RRID:AB_94844
Rat monoclonal anti-BrdU [BU1/75 (ICR1)]	Abcam	Cat# ab6326, RRID:AB_305426
Goat polyclonal anti-DCX (C-18)	Santa Cruz Biotechnology	Cat# sc-8066, RRID:AB_2088494
Mouse monoclonal anti-NeuN	Millipore	Cat# MAB377, RRID:AB_2298772
Experimental Models: Organisms/Strains		
Mouse: B6.129S1-Tlr7tm1Flv/J	The Jackson Laboratory	RRID:IMSR_JAX:008380
Mouse:B6;129S2-Il6tm1Kopf/J	The Jackson Laboratory	RRID:IMSR_JAX:002254
Mouse: B6	Taiwan National Laboratory Animal Center	N/A
Chemicals, Peptides, and Recombinant Proteins		
5-Bromo-2′-deoxyuridine	Sigma-Aldrich	B5002;CAS: 59-14-3
Oligonucleotides		
Primers for QPCR, see Table S5	This paper	N/A
Critical Commercial Assays		
Truseq Stranded mRNA kit	Illumina	1.48v
Transcriptor First Strand cDNA Synthesis Kit	Roche	N/A
LightCycler® 480 Probes Master	Roche	N/A
Universal Probe Library probes	Roche	N/A
Software and Algorithms		
ImageJ	https://imagej.nih.gov/ij/	1.48v
Smart Video Tracking System	Panlab, Barcelona, Spain	N/A
FreezeScan	CleverSys Inc., Reston, VA, USA	2.0
LightCycler480	Roche	1.5.0

UPL Assay Design Center Web Service	https://lifescience.roche.com/en_tw/brands/universal-probe-library.html#assay-design-center	N/A
CLC Genomics Workbench	https://www.qiagenbioinformatics.com/	v.10.1.1
Metascape	http://metascape.org/gp/index.html	N/A
IPA	QIAGEN	N/A
STRING	https://string-db.org/	N/A
Zen Blue	Zeiss	3.1
LSM700	Zeiss	N/A
Prism	GraphPad	8.0
Deposited Data		
Raw data	This paper	NCBI BioProject ID PRJNA702827

TRANSPARENT METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Wild type C57BL/6 male mice were purchased from the Taiwan National Laboratory Animal Center for behavioral analyses. *Tlr7*$^{-/y}$ mice (Lund et al., 2004) and *Il6*$^{-/-}$ mice (Kopf et al., 1994) in a C57BL/6 genetic background were originally imported from the Jackson Laboratory and bred and maintained in the animal facility of the Institute of Molecular Biology (IMB), Academia Sinica, with a 14 h light/10 h dark cycle and controlled temperature (20-23 °C) and humidity (48-55%) under pathogen-free conditions. For behavioral assays, mice were transferred to the behavioral room of IMB with a 12 h light/12 h dark cycle for at least 1 week before experiments. Only male mice were used for behavioral analyses. The mice were group-housed with their littermates and each cage contained 3 to 5 mice. All animal experiments were performed with the approval of the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 13-02-520) and in strict accordance with its guidelines and those of the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals.

To investigate the effect of forced rotarod exercise on mouse behaviors, we subjected mice to an accelerating rotarod test for three consecutive days before they underwent various behavioral assays at various later timeframes. The temperature (20-23 °C) and humidity (48-55%) of the behavioral room were controlled and it was equipped with a light intensity of 240 lumen/m2 (lux). All behavioral experiments were assayed during daytime from 10 am ~ 5 pm. All behavioral assays used in this manuscript were based on previous publications (Hsu et al., 2020; Hu et al., 2020; Huang et al., 2019; Huang et al., 2021; Hung et al., 2018a; Lin and Hsueh, 2014; Shih et al., 2020a; Shih et al., 2020b; Wu et al., 2017). The details are described below.

METHOD DETAILS

Behavioral tasks:

Accelerating rotarod

To ensure that mice could stay stably on the drum, mice were placed on the resting drum (3 cm in diameter) of a rotarod apparatus (RT-01, SINGA, Diagnostic & Research Instruments Co. Taoyuan, Taiwan) for at least 1 min. The speed of the rotarod was accelerated from 0 to 40 rpm over a 5-min period for mice at 10 weeks of age or a 6-min period for mice at 5 weeks of age. The mice were subjected to three trials per day, with 10-15 min intervals

between trails, for 3 consecutive days. Latency to fall in each trial was recorded. The apparatus was cleaned with 70% ethanol and air-dried between usages by different animals. Mock control mice were either placed on a non-moving rotarod for 5 min or without treatment. Since there was no difference between these two kinds of controls, it is not specified in this report.

Open field

The apparatus of open field was a transparent plastic box (40×40×32.5 cm). Before the assay, the experimental mouse was placed in a new cage for 10 min before being transferred to the center region of the transparent box. Their movements were recorded for 10 min by videotaping from above. The grooming, rearing, urine and stool number was counted manually. Rearing was defined as both forelimbs leaving the ground. The total moving distance, speed and the time spent in the four corners (10×10 cm for each corner) and the center (20×20 cm) were quantified with the Smart Video Tracking System (Panlab, Barcelona, Spain). The apparatus was cleaned with 70% ethanol and air-dried before being used for another mouse.

Elevated plus maze

The maze comprised two open-sided arms (30×5 cm) and two arms (30×5 cm) enclosed by a 14-cm-high wall. The central platform was a square of 5 cm and the entire apparatus was raised 45.5 cm above ground level. An individual mouse was placed into the central area facing one of the open-sided arms and allowed to freely explore the maze for 10 min. Mouse movements were recorded from above using a videocamera. The time spent in different areas was analyzed using the Smart Video Tracking System (Panlab, Barcelona, Spain). The maze was cleaned with 70% ethanol and air-dried before assaying another mouse.

Barnes maze

The maze was a white circular platform (100 cm in diameter) with 40 holes (5 cm in diameter) cut into the periphery. An escape box (black plastic box, 8×20×5 cm) was attached magnetically under one of the holes (target hole). At day 0, an individual mouse was put into a cylindrical start chamber in the middle of the maze and, upon removing the start chamber, it was guided to enter the target hole. Once the mouse had entered the target hole, the hole was covered and the mouse remained there for 2 min. In the training phase, mice were allowed to freely explore the maze individually until they had entered the target hole within 180 sec. If a

mouse had not escaped through the target hole within 180 sec, it was guided there by the experimenter and its escape latency was recorded as 180 sec. Experimental mice underwent four trials per day, with 15 min intervals between trials, for 4 consecutive days. Escape latency was measured as the time taken to enter the target hole from removing the start chamber. In a probe assay (day 5 and day 12), the target hole was covered and experimental mice were allowed to freely explore the maze for 90 sec. In all cases, mouse movements were recorded by videotaping from above using a wide-angle lens. The time taken to reach the target hole was quantified using the Smart Video Tracking System (Panlab, Barcelona, Spain). The apparatus was cleaned with 70% ethanol and air-dried between usages by different animals.

Fear conditioning

A fear conditioning chamber (Med Associates Inc., Fairfax, VT, USA) supported with the FreezeScan 2.0 analytical system (CleverSys Inc., Reston, VA, USA) was used to analyze the fear memory of mice. At day 1, an experimental mouse was habituated in Box A (a transparent plastic box) for 12 min. The freezing percentage during these 12 min was averaged to represent the "basal" freezing response. At day 2, an individual mouse was placed into Box B (a transparent colored box with slips of colored paper on the walls) containing scent of 1% acetic acid. After 4 min, three paired stimulations comprising a tone (2 kHz; 80 dB; 20 s) followed by an electronic foot shock (0.6 mA; 2 s) were applied with one min intervals. The freezing percentage within 1 minute directly after the third foot shock was used to represent the response "after stimulation (AS)", indicating the immediate response that is relevant to pain sensation of mice to foot shock. At day 3, mice were again placed in Box B for 12 min without experiencing tone or foot shock stimulations. The freezing responses from 3-6 min after entering Box B were averaged to indicate contextual fear memory. At day 4, the experimental mouse was placed into Box A for 4 min and then received twenty tones (2 kHz; 80 dB; 20 s) separated by 5-s intervals. The freezing responses of mice were averaged from the first six tones to indicate cued fear memory.

Quantitative RT-PCR (Q-PCR).

Three hours after rotarod training, mouse cortices and hippocampi were collected by removing the olfactory bulb and entire subcortical regions from whole brains. Cortical and hippocampal tissues were immediately lyzed in Trizol reagent by dounce homogenization and then stored at -80 °C until RNA extraction according to the manufacturer's instructions

(Invitrogen), followed by DNase I (New England BioLabs) digestion for 30 min at 37 °C to remove contaminating DNA. RNA isolated from mouse brain was then used for cDNA synthesis by the Transcriptor First Strand cDNA Synthesis Kit (Roche) with an oligo(dT)18 primer. A real-time PCR assay was performed using the LightCycler480 (Roche) and Universal Probe Library probes (UPL, Roche) system. The primers and their paired probes (see **Supplemental Table S2**) were designed using the Assay Design Center Web Service (Roche). The PCR thermal profile was set as follows: denaturation at 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 s; and a final cooling step at 40 °C for 30 s. Samples were assayed experimentally in triplicate and then averaged to represent the data of a single experiment. Data was analyzed and quantified in LightCycler 480 software.

RNA-seq and bioinformatic analysis

Mouse cortices and hippocampi were collected for RNA extraction as described in the previous paragraph. RNA quality and quantifications were determined using an Agilent 2100 Bioanalyzer. The mRNA sequencing libraries were prepared using a Truseq Stranded mRNA kit (Illumina) and 75-76 cycle single-read sequencing was performed using the 500 High-output v2 (75 cycle) sequencing kit on an Illumina NextSeq500 instrument. Sequenced reads were trimmed for adaptor sequence and low quality sequences, and then mapped to the GRCm38.p6 whole genome using CLC Genomics Workbench (v.10.1.1, https://www.qiagenbioinformatics.com/) with parameters: mismatches = 2, minimum fraction length = 0.9, minimum fraction similarity = 0.9, and maximum hits per read = 5. Genes differentially expressed in WT mice with or without rotarod testing, and between *Tlr7*$^{-/Y}$ mice with or without rotarod testing were analyzed using CLC Genomics Workbench. The output data included fold-change, p-value, false discovery rate (FDR), total counts, RPKM and TPM. Differentially expressed genes (DEG) were defined by the criteria of fold-change > 1.3, a false discovery rate < 0.1 and average transcripts per million (TPM) > 0 (in each group). Protein networks and functional enrichments were analyzed using ingenuity pathway analysis (IPA) software and STRING (https://string-db.org/). Gene ontology biological processes were assessed using Metascape (http://metascape.org/gp/index.html). For heatmap analysis, TPM values of each gene was first normalized with the group of WT mice without rotarod testing and subjected to GraphPad Prism 8.0 for heatmap generation. The raw RNA-seq dataset has been available online (NCBI BioProject ID PRJNA702827).

BrdU injection, immunohistochemistry and quantification

After rotarod assays, mice received a single intraperitoneal injection of BrdU at a dosage of 150 mg/kg bodyweight for short-term labeling (**Figure 2A (3)**). For long-term labeling, a single intraperitoneal injection of BrdU was carried out each day for four consecutive days at a dosage of 100 mg/kg body weight. Experimental mice were anesthetized and perfused with 4% PFA in PBS. After 4% PFA postfixation at 4 °C, brains were sliced into 50-µm-thick sections using a vibratome. For IBA1 and GFAP staining, the brain sections were permeabilized with 0.3% Triton-X 100 in PBS for 10 min, then blocked for 30 min with blocking solution (0.2% bovine serum albumin, 0.3% horse serum and 0.3% Triton-X 100 in PBS). The sections were incubated overnight with IBA1 antibody (1:500; Wako, 019-019741) and GFAP antibody (1:500; Chemicon, MAB3402) in blocking solution at 4 °C. Sections were then incubated with Alexa Fluor-488- and Alexa Fluor-594-conjugated secondary antibodies (1:500) with DAPI for 3 h at room temperature. After mounting with antifade solution (0.5% N-propyl gallate, 20 mM Tris (pH8.0), 90% glyserol), the images were captured with an Axio imager M2 microscope (Carl Zeiss) equipped with a 20×/NA 0.80 (Plan-Apochromat; Zeiss) objective lens. Images were captured using a cooled charge-coupled device camera (Rolera EM-C2, QImaging) driven by the digital image processing software Zen Blue (Zeiss). The images were quantified using ImageJ (NIH). All images were converted into 8-bit format and adjusted (manually) with the threshold function, and the areas of CA3 and hilus regions of the hippocampus were analyzed. For BrdU staining, the sections were incubated with 0.3% H_2O_2 for 30 min and then washed three times with PBS. The slices were incubated in 2N HCl for 30 min at 37 °C for DNA hydrolysis and then neutralized with 0.1 M sodium borate buffer pH 8.5 for 10 min at room temperature. After washing three times with PBS, the sections were blocked with blocking solution (3% horse serum and 0.1% Triton-X 100 in TBS) for 30 min. The slices were incubated overnight with BrdU antibody (1:200; Abcam, ab6326), together with Dcx antibody (1:100; Santa Cruz, sc-8066) or NeuN antibody (1:100; Millipore, MAB377) in blocking solution at 4 °C. Sections were incubated with secondary antibodies plus DAPI and mounted as described above. The images were captured using a confocal microscope (LSM 700, Zeiss) equipped with a transmitted light detector (Zeiss LSM, T-PMT) and a 20x/NA 0.80 (Plan-Apochromat) objective lens. Quantifications were carried out in ImageJ software.

Statistical analysis

Data are presented as mean plus s.e.m. GraphPad Prism 8.0 was used for analyses and to generate plots. No statistical method was applied to evaluate sample sizes, but our sample sizes are similar to those of previous publications (Hung et al., 2018a; Hung et al., 2018b) and follow previously promoted principles (Charan and Kantharia, 2013). Statistical analyses were performed using two-tailed nonparametric tests (Mann-Whitney test) and two-way ANOVA with Bonferroni's test as indicated in the figure legends. *P* values < 0.05 were considered significant.

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