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Nod-like receptors are critical for gut–brain axis signalling in mice

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Key points

- Nucleotide binding oligomerization domain (Nod)-like receptors regulate cognition, anxiety and hypothalamic–pituitary–adrenal axis activation.
- Nod-like receptors regulate central and peripheral serotonergic biology.
- Nod-like receptors are important for maintenance of gastrointestinal physiology.
- Intestinal epithelial cell expression of Nod1 receptors regulate behaviour.

Abstract Gut–brain axis signalling is critical for maintaining health and homeostasis. Stressful life events can impact gut–brain signalling, leading to altered mood, cognition and intestinal dysfunction. In the present study, we identified nucleotide binding oligomerization domain

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(Nod)-like receptors (NLR), Nod1 and Nod2, as novel regulators for gut–brain signalling. NLR are innate immune pattern recognition receptors expressed in the gut and brain, and are important in the regulation of gastrointestinal physiology. We found that mice deficient in both Nod1 and Nod2 (NodDKO) demonstrate signs of stress-induced anxiety, cognitive impairment and depression in the context of a hyperactive hypothalamic–pituitary–adrenal axis. These deficits were coupled with impairments in the serotonergic pathway in the brain, decreased hippocampal cell proliferation and immature neurons, as well as reduced neural activation. In addition, NodDKO mice had increased gastrointestinal permeability and altered serotonin signalling in the gut following exposure to acute stress. Administration of the selective serotonin reuptake inhibitor, fluoxetine, abrogated behavioural impairments and restored serotonin signalling. We also identified that intestinal epithelial cell-specific deletion of Nod1 (VilCre^{+}Nod1$^{f/f}$), but not Nod2, increased susceptibility to stress-induced anxiety-like behaviour and cognitive impairment following exposure to stress. Together, these data suggest that intestinal epithelial NLR are novel modulators of gut–brain communication and may serve as potential novel therapeutic targets for the treatment of gut–brain disorders.

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Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is a critical regulator of the stress response. It is one of the main pathways through which the gut–brain axis signals, with stress negatively impacting intestinal function, including permeability, motility (Huerta-Franco *et al.* 2012) and visceral sensitivity (Theodorou, 2013). Stress can also induce disease relapse in patients with inflammatory bowel disease (Mawdsley & Rampton, 2005; Martin *et al.* 2015) and cause symptom flare-ups in patients with irritable bowel syndrome (Qin *et al.* 2014). Stressful life events also represent risks factors for the development of psychiatric disorders, including anxiety and major depressive disorder (McEwen, 2000). Additionally, there is significant genetic pleiotropy for psychiatric and immune system disorders, suggesting an underlying common pathway of regulation (Wang *et al.* 2015). Although the precise mechanisms for this susceptibility to stress remain to be fully understood, it is considered to involve a complex interplay between environmental, biological and genetic risk factors.

The immune system plays a pivotal role in brain function and stress responses (Pruett, 2003; Pariante, 2016). Mice deficient in T- and B-cells display anxiety-like behaviour and cognitive deficits compared to wild-type (WT) controls (Smith *et al.* 2014). More recently, mice deficient for the innate immune pattern recognition receptor (PRR), peptidoglycan (PGN) recognition protein (PGLYRP) 2 gene, showed alterations in social behaviour in a sex-dependent manner (Arentsen *et al.* 2017). Together, these findings suggest a role for adaptive and innate immune pathways in regulating gut–brain communication. Nucleotide binding oligomerization domain (Nod)-like receptors (NLR), Nod1 and Nod2, are intracellular PRRs that recognize specific moieties of PGN and are important in maintaining gut homeostasis by eliciting protective immune responses to bacteria (Clarke *et al.* 2010; Claes *et al.* 2015). In addition to their well-established expression in the periphery, including in intestinal epithelial cells (IEC) and mucosal immune cells (Ogura *et al.* 2003; Franchi *et al.* 2009), Nod1 and Nod2 are expressed in several brain areas, including the hippocampus and diverse cell types, such as pyramidal neurons, astrocytes and microglia (Arentsen *et al.* 2017). Despite this evidence of CNS expression, the precise role of Nod1 and Nod2 in the brain remains to be fully clarified.

Given their physiological distribution, we hypothesized that NLR may represent a novel potential therapeutic target for the treatment of stress-related disorders and a novel modulator of gut–brain communication. Using mice deficient in both Nod1 and Nod2 (NodDKO), we assessed behaviour and brain function, as well as intestinal physiology, in the context of acute psychological stress.

Methods

Mice

Adult (6–8 weeks old) male and female mice (Jackson Labs, Sacramento, CA, USA; bred in-house) were used in the present study. Nod1/Nod2 double knockout (NodDKO; constitutive knockout; C57BL/6 background) mice and WT (C57BL/6) controls were maintained at the University of California Davis and in-bred for at least 11 generations (Keestra-Gounder *et al.* 2016). Nod1 floxed mice were created using a targeting construct for *Nod1* generated using C57BL/6-derived bacterial artificial chromosome clones. The construct was designed with *loxP* sites flanking

exons 2 and 3 of *Nod1* and an enhanced green fluorescent protein kanamycin/neomycin cassette was introduced for selection in *Escherichia coli* and embryonic stem (ES) cells, respectively. The targeting construct was confirmed by sequencing and electroporated into C57BL/6 ES cells. G418-resistant ES cell clones were picked and expanded. DNA was extracted and screened for targeting by PCR. ES cell clones that amplified a product of the expected size were further characterized by Southern blot analysis to confirm targeting at both the 5′ and 3′ ends of the targeting construct. Correctly targeted ES cell clones were karyotyped and two independently targeted clones with a normal chromosome count were prepared for micro-injection into blastocysts. Chimeric mice were prepared by microinjecting gene targeted ES cells into recipient blastocysts (Balb/c) and transferred to pseudo-pregnant recipients. The targeted ES cells were derived from C57BL/6 mice. Male chimeric mice were mated with WT C57BL/6 females to obtain germline transmission of the targeted allele. Animals that were heterozygous for the targeted allele (as determined by coat colour) were bred to generate *Nod1*$^{f/f}$ animals, which were bred and maintained under specific-pathogen-free conditions in the Monash Medical Centre Animal Facilities. Generation of floxed mice was performed in accordance with the guidelines of Monash University's Institutional Biosafety and Animal Ethics Committees. Nod2$^{f/f}$ mice were created as described previously (Kim *et al.* 2016). Once generated, both Nod1$^{f/f}$ and Nod2$^{f/f}$ mice were bred at the University of California Davis, where all of the experiments were performed.

Nod1$^{f/f}$ and Nod2$^{f/f}$ mice were crossed with IEC-specific Cre-expressing (VilCre) mice (Jackson Labs; bred in-house). Cages consisted of either VilCre^{-}Nod1$^{f/f}$ and VilCre^{+}Nod1$^{f/f}$ or VilCre^{-}Nod2$^{f/f}$ and VilCre^{+}Nod2$^{f/f}$ genotypes.

Mice were housed in cages lined with chip bedding and had free access to food and water throughout the study. The vivarium lighting schedule comprised a 12:12 h light/dark (L/D) cycle, with the temperature maintained at 21 ± 1°C. All behavioural tests were performed between 09.00 h and 18.00 h and all procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California Davis (IACUC protocol #20 072). Mice were killed by hypoxia via CO_2 followed by cervical dislocation in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines.

Fluoxetine treatment

Fluoxetine hydrochloride (18 mg kg^{-1} day^{-1}; Sigma-Aldrich, St Louis, MO, USA) was administered *ad libitum* in the drinking water for 4 weeks in opaque bottles to protect it from light. Selective serotonin (5-HT) reuptake inhibitors typically require several weeks to alleviate depressive symptoms as a result of unknown mechanisms (Perez-Caballero *et al.* 2014). The drinking water containing fluoxetine was changed every 3 days to prevent any possible degradation. Control animals received plain drinking water as vehicle. Animals were treated immediately starting after weaning for a period of 28 days. On day 28, animals underwent the 1 day behavioural testing protocol (as described below), plus the forced swim test (FST), 2 h later, to assess depressive-like behaviours.

Water avoidance stress (WAS)

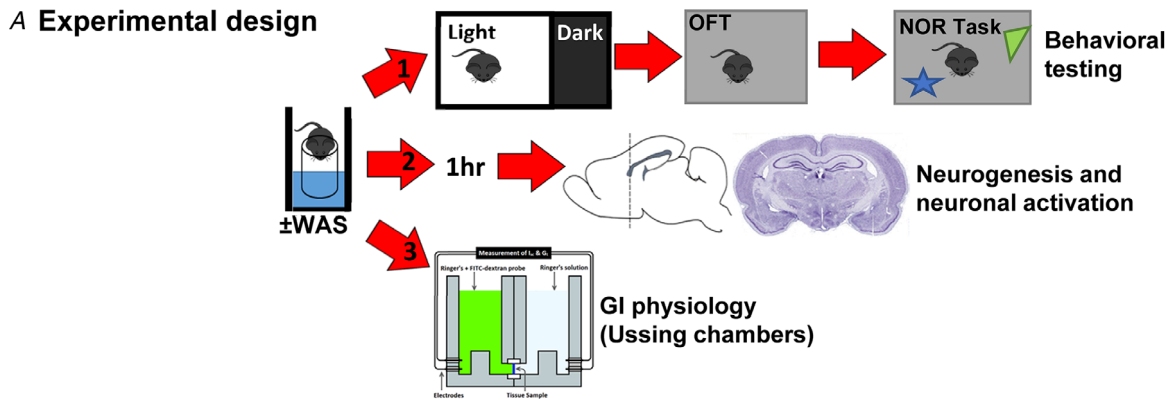
Mice were placed on small platforms (inverted 50 mL beakers) in a clean, standard housing cage filled with ∼2 cm of room temperature water for 1 h. WAS is a well-established model for inducing stress in rodents (Gareau *et al.* 2011), which can impact intestinal mucosal barrier function for up to 4 h (Gareau *et al.* 2008). After exposure to stress, mice immediately underwent (i) behavioural testing or were killed for (ii) perfusion (immunofluorescence) or (iii) gut physiology (Ussing chambers) (Fig. 1*A*).

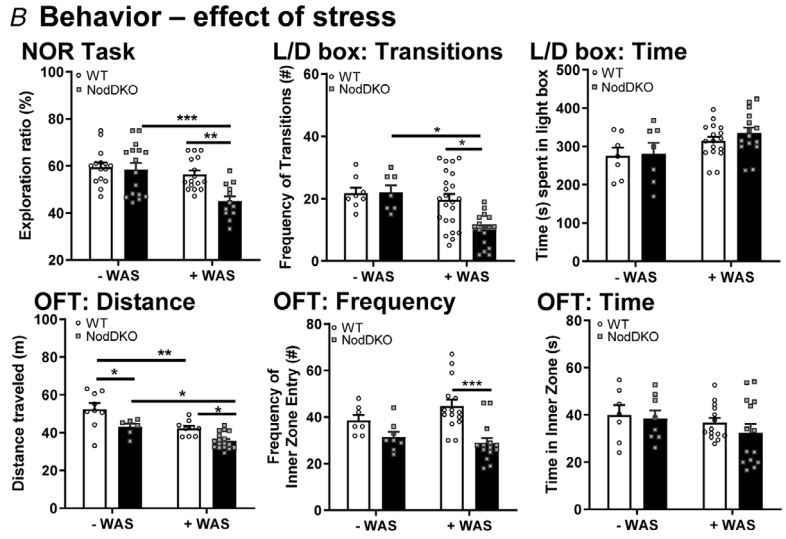
Behavioural testing

An L/D box, open field task (OFT) and novel object recognition (NOR) task were performed as described previously (Gareau *et al.* 2011; Smith *et al.* 2014; Emge *et al.* 2016) ± WAS (Fig. 1*A*). For mice exposed to WAS, behaviour was commenced immediately following exposure. All behaviour testing was completed within a 2 h block of time: 10 min L/D + 10 min OFT + 55 min NOR (5 min training + 45 min recovery + 5 min testing). Preliminary studies (performed by MS and MGG), coupled with published protocols (Crawley, 2008), confirmed that performing these tests alone or together in the same mouse does not affect outcome (data not shown).

L/D box test. To measure anxiety-like behaviour, the L/D box test was used as described previously (Gareau *et al.* 2011). Briefly, mice were placed in a box with a light (two-thirds) and a dark (one-third) compartment for 10 min, during which the behaviour of the mouse was video-recorded. These videos were analyzed using a digital tracking system (Ethovision; XT 8.5, Noldus, Leesburg, VA, USA) for the total time spent by the mouse in the lit portion of the box, with lower values ascribed to anxiety-like behaviour (Bourin & Hascoet, 2003). In addition, the number of times the mouse transitioned from the dark compartment to the light compartment of the box was quantified to indicate the mouse's activity and exploratory level.

OFT. Animal behaviour for the OFT was recorded for 10 min in the novel arena during acclimation for the NOR





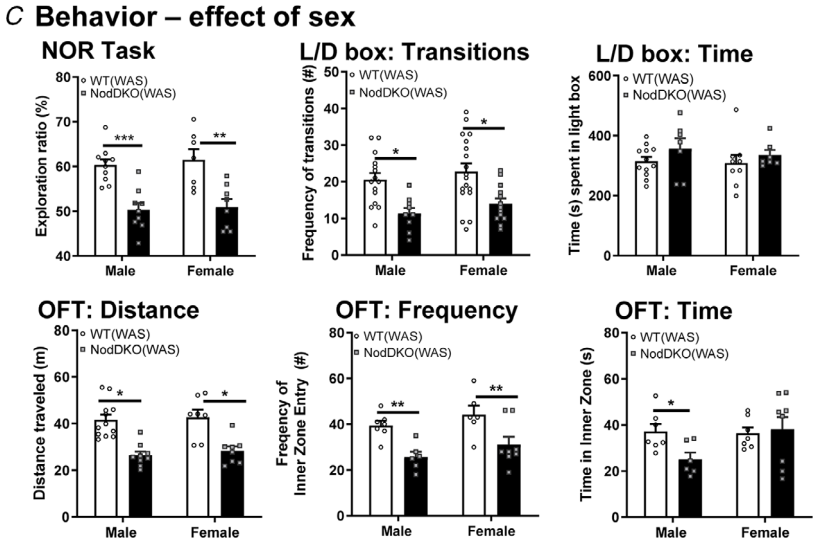


Figure 1. NodDKO(+WAS) mice display behavioural deficits
A, experimental timeline: adult C57BL/6 male and female WT and NodDKO mice ± water avoidance stress (WAS) underwent one of three study paradigms: (1) ± WAS +behavioural testing; (2) ± WAS + 1 hr wait followed by perfusion for hippocampal neurogenesis and neural activation analysis; or (3) ± WAS + gut physiology assessed by Ussing chambers. Behaviour: (*B*) stress effect on: novel object recognition (NOR) task, light/dark (L/D) box and open field test (OFT) (n = 10–16) and (*C*) sex effect on NOR task, L/D box, and OFT (n = 7–18). Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001, two-way ANOVA).

task. Both locomotor activity and anxiety-like behaviour were determined as quantification of total distance moved, time spent in the inner zone and frequency of inner zone entries (Ethovision).

NOR task. The NOR task was performed as previously described (Gareau *et al.* 2011; Smith *et al.* 2014). Briefly, mice were subjected to 10 min of acclimation to the novel arena (30 × 30 cm white plexiglass box) followed by 5 min of habituation to two identical objects (training phase). After a rest and recovery period (45 min), one of the two original objects was replaced with a novel object and interactions were monitored for an additional 5 min (testing phase). Direct contacts with the objects, including any contact with nose or paw, or an approach within 2 cm, were recorded and scored automatically (Ethovision) and confirmed manually. Any close contact in which the animal was not directly interacting with the object, but, instead, was exploring the chamber, was not interpreted as direct contact with the object and omitted. The results are expressed as a ratio quantifying preference for exploration of a novel object rather than a familiar object during the testing phase. An exploration ratio of greater than 50% indicates that the mouse investigated the novel object more than the familiar object, indicating memory recall for the latter (Gareau *et al.* 2011). Mice that did not pass the training phase [less than 40% or greater than 60% exploration ratio (suggesting bias towards one object)] were omitted.

FST. The FST was used to assess depressive-like behaviour in mice following fluoxetine or vehicle treatment. Briefly, mice were placed individually in Pyrex cylinders (height 40 cm, diameter 20 cm) filled with water (24.5°C) to a depth of 30 cm for 6 min. The last 4 min of the test were analyzed using a tracking system (Ethovision). During the behavioural analysis, mobility time, intended as any movements other than those necessary to balance the body and keep the head above the water, was measured. FST was performed after a 2 h rest in their home cage following completion of the NOR task.

Immunofluorescence

Brains [WT(+WAS) and NodDKO(+WAS)] were collected following anesthesia (5% isoflurane) and transcardial perfusion with 4% polyformaldehyde (PFA). Brains were post-fixed overnight at 4°C and placed in 30% (w/v) sucrose for 4 days before embedding in optimal cutting temperature medium in ice-cold isopentane followed by storage at −80°C. Samples were cut using a cryostat (Leica Microsystem, Wetzlar, Germany) into 20 µm thick coronal sections. Sections were serially collected and stored at −20°C until use. Immunofluorescence was performed as described previously (Murray *et al.* 2017). Briefly, sections underwent an antigen retrieval step using citrate buffer (10 mM, pH 6.0, 1 h, 95°C). After blocking in 5% bovine serum albumin normal goat serum (1 h at room temperature), samples were incubated with primary antibody overnight (16 h at 4°C). The primary antibodies used were: rabbit anti-c-Fos (2250S; Cell Signaling, Danvers, MA, USA), guinea pig anti-doublecortin (DCX) (AB2253; Millipore, Burlington, MA, USA) and rabbit anti-Ki67 (LS-C141898; Lifespan Biosciences, Seattle, WA, USA). Slides were washed (3 × 5 min) and incubated with appropriately labelled secondary antibodies (1 h at room temperature), then washed and mounted in Prolong diamond (Invitrogen, Carlsbad, CA, USA). The secondary antibodies used were Alexa 647 goat anti-rabbit (ab150155; Abcam, Cambridge, UK) for both c-Fos and Ki67, and Alexa 555 goat anti-guinea pig for DCX (A21435; Invitrogen). 4',6-Diamidino-2-phenylindole was used as a nuclear stain.

Image analysis and cell quantification

Confocal imaging was performed on a Leica SP8 STED 3X microscope. Immunofluorescence Z-stack images with a 1.04 µm step size were collected using a 20× objective. Systematic random sampling was used for the dorsal hippocampus by counting the cells in both hemispheres of each section in 1:6 series (120 µm apart). Every second section, for a total of three sections, was used either for DCX/Ki67 co-staining [dentate gyrus (DG) area] or for c-Fos staining [cornus ammonium (CA)3 and DG areas]. Cell quantification and calculation of the volume of the DG and CA3 were performed using the image processing software package Imaris x64_8.2.1 (Bitplane, Zurich, Switzerland). The ratio between the cell numbers and either the DG or CA3 volumes are expressed as an average of three sections per animal and presented as cells/$\mu M^3 \times 10^{-6}$. The dorsal hippocampus was defined as anteroposterior −0.94 to −2.30 in accordance with an atlas of the mouse brain (Franklin & Paxinos, 1996).

Serum corticosterone

Blood samples were collected via cardiac puncture following CO_2 exposure. Blood was centrifuged and serum aspirated and stored at −80°C. Corticosterone levels were assayed using a commercially-available enzyme immunoassay (EIA) kit (Corticosterone EIA Kit, ADI-900-097; Enzo Life Sciences, Farmingdale, NY, USA) in accordance with the manufacturer's instructions. Absorbance was read with a multimode plate reader (Synergy H1; BioTek Inc., Winooski, VT, USA) at 405 nm.

Quantitative PCR

Tissues [hippocampus, prefrontal cortex (PFC), colon and ileum] were collected and frozen at −80°C before

Table 1. Primer sequences

Gene		Primer sequences (5′ → 3′)
GR	Forward	TCCGATGAAGCTTCGGGATG
	Reverse	AATTGTGCTGTCCTTCCACTG
MR	Forward	TGGCCAAGGCAGCTATGGA
	Reverse	CATTGGTCCTCTCTGCAGGT
Arc	Forward	GGAGGGAGGTCTTCTACCGT
	Reverse	CTACAGAGACAGTGTGGCGG
Tph2	Forward	TCGAAATCTTCGTGGACTGC
	Reverse	CGGATTCAGGGTCACAATG
Tph1	Forward	AACAAAGACCATTCCTCCGA
	Reverse	TGTAACAGGCTCACATGATT
5HTr1a	Forward	CTAATGGGGCGGTGAGACAG
	Reverse	GGAGGTAGCTCCTGATTCGC
5HTr2c	Forward	GCATAGCCGGTTCAATTCGC
	Reverse	TTGCTTTCGTCCCTCAGTCC
SERT	Forward	GGCGCGAGGGTCGAG
	Reverse	ATCTGCCAAGGACCCTGACT
Bdnf	Forward	TGCAGGGGCATAGACAAAAGG
	Reverse	CTTATGAATCGCCAGCCAATTCTC
GABAA1A	Forward	TCCCAAGTCTCCTTCTGGCT
	Reverse	TTCGGGAGGGAATTTCTGGC
GABAA2A	Forward	GCCACTGGAGGAAAACATCTACT
	Reverse	GATGTTAGCCAGCACCAACCT
GABAB1A	Forward	CACTGCCAGGTGAATCGAAC
	Reverse	TTAACGTCCTCCAGCGCCAT
GABAB1B	Forward	GGCCTCTCACTCCCCTCATCT
	Reverse	TCATGGGAAACAGCGCCCC
GABAB2B	Forward	CTTCTTCGGAGTCACGGGTC
	Reverse	CGACCTTCACCTCTCTGCTG

homogenization in Trizol (Invitrogen). RNA was isolated in accordance with the manufacturer's protocol (Invitrogen), treated with DNase 1 (Invitrogen) and transcribed into cDNA (iSCRIPT cDNA Synthesis kit, Bio-Rad, Hercules, CA, USA; Proflex PCR System, Applied Biosystems, Foster City, CA, USA). qPCR was performed using SYBR green and β-actin used as a housekeeping gene on a QuantStudio 6 Flex Real time PCR machine (Applied Biosystems). Data were presented as $\Delta\Delta$CT. All primer sequences used in the present study are shown in Table 1.

Brain 5-HT and tryptophan (Trp) quantification

The hippocampus and brain stem were freshly dissected and stored at −80°C until analysis. Tissues were homogenized in 20 mM Hepes buffer (pH 7.5) containing 0.25 M sucrose and a cocktail of protease inhibitors (Calbiochem, Burlington, MA, USA) followed by ultracentrifugation at 100 000 *g* for 45 min. Membrane-free tissue supernatants (100 µL) were transferred to 96-well plates for analysis. Liquid chromatography-mass spectrometry (LC/MS) analysis was performed on an Agilent Infinity 1290 ultra-high-performance LC coupled to a triple quadropole MS. Chromatographic separation was carried out on an Agilent Pursuit 3 Pentafluorophenyl stationary phase (2 × 150 mm, 3 mm) column (Agilent Technologies Inc., Santa Clara, CA, USA). The mobile phases were 100% methanol (B) or water with 0.25% formic acid (A). The analytical gradient was: 2% to 60% B for 2 min, 100% B from 2 to 4 min, 2% B for 4 min. The flow rate was 300 µL min^{-1}. Samples were held at 4°C in the autosampler, and the column was operated at 40°C. The MS was operated in positive and selected reaction monitoring mode. A 5-HT standard was used for optimization and to generate the calibration curve for quantification. Data acquisition and processing was performed using Mass Hunter Qualitative and Quantitative Analysis, version B.06.00 (Agilent Technologies Inc.) and Quantitative Analysis, version B.08.00 (Agilent Technologies Inc.).

Serum Trp quantification

Serum proteins were removed by ultrafiltration using Ultra Centrifugal Filters (Amicon Corp., Danvers, MA, USA) (molecular weight cut-off 3 KDa). Briefly, the upper chambers of the centrifugal devices were rinsed with water (400 µL) twice and centrifuged at 10 000 *g* for 5 min before individual mouse serum samples (50 µL) were loaded. Water (150 µL) was added to the samples and they were centrifuged at 10 000 *g* for 5 min. Protein-free serum flow-throughs were collected in 1.5 mL tubes and aliquots (50 µL) were transferred into vials for MS analysis as described above.

Ussing chambers

Segments of gastrointestinal (GI) tract (distal ileum and proximal colon) were excised and cut along the mesenteric border and mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA), exposing 0.1 cm^2 of tissue area to 4 mL of circulating oxygenated Ringer solution maintained at 37°C. The buffer consisted of (in mM): 115 NaCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2.0 KH_2PO_4 and 25 $NaHCO_3$ at pH 7.35 ± 0.02. Additionally, glucose (10 mM) was added to the serosal buffer as a source of energy, which was balanced osmotically by mannitol (10 mM) in the mucosal buffer. Agar–salt bridges were used to monitor potential differences across the tissue and to inject the required short-circuit current (*Isc*) to maintain the potential difference at zero as registered by an automated voltage clamp. A computer connected to the voltage clamp system recorded *Isc* and voltage continuously and analysed using acquisition software (Acquire and Analyse; Physiologic Instruments). Baseline *Isc* values were obtained at equilibrium, ∼15 min after the tissues were mounted. *Isc*, an indicator of active ion transport, was expressed in µA cm^{-2}. Conductance (*G*) was used to assess tight

junction permeability and mucosal to serosal flux of 4 kDa fluorescein isothiocyanate (FITC)-labelled dextran (Sigma-Aldrich) over time (sampled every 30 min for 2 h) was used to assess macromolecular permeability. After completion of the FITC flux measurements, tissues were treated with forskolin (20 μM) to assess viability.

Study design

Three sets of animals were used for three parallel experiments.

(1) *Behavioural testing.* Mice (±WAS) were exposed to the L/D box, OFT and NOR task (Fig. 1*A*). Animals were habituated to the testing room by allowing them to acclimate in their home-cages in the testing room for at least 30 min prior to testing. Once the behavioural tests were completed, mice were killed by CO_2 inhalation followed by cervical dislocation. PFC, hippocampus, colon and ileum were collected for qPCR analysis. A subset of mice was used for the collection of serum, hippocampus and brain stem for 5-HT, and Trp analysis.
(2) *Brain imaging.* Mice (+WAS) were returned to their home-cages for 1 h followed by perfusion (4% PFA) and measurement of c-Fos, Ki67 and DCX expression levels in the hippocampus (Reichmann *et al.* 2013). The 1 h period is necessary to allow for c-Fos expression to occur without impacting cell proliferation or immature neuron expression, which takes multiple weeks, therefore allowing us to use the same animals for both imaging studies (Piatti *et al.* 2011).
(3) *Ussing chambers.* Mice (±WAS) were killed by CO_2 inhalation followed by cervical dislocation and both ileum and colon were collected to measure ion transport and permeability in Ussing chambers. Serum was also collected for corticosterone analysis.

Statistical analysis

Results are expressed as the mean ± SEM. An unpaired Student's *t* test or two-way ANOVA followed by Tukey's *post hoc* test were performed as appropriate using Prism, version 8 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was conisidered statistically significant (*n* represents a single mouse).

Results

NodDKO mice display stress-induced behavioural deficits

To study the role of NLR in regulating stress responses, behaviour was assessed in NodDKO mice ±WAS. The NOR task was used to assess recognition memory. All of the mice used for the NOR task successfully performed the training phase with no differences seen in success rate based on genotype, treatment, or sex (data not shown). Although baseline cognitive functions in NodDKO mice were intact, as determined by the ratio of interactions between a known and a novel object, exposure to a single session of acute WAS for 1 h led to a cognitive deficit compared to WT(+WAS) mice and NodDKO(–WAS) control mice (Fig. 1*B*). In the L/D box test, as used to assess anxiety-like behaviour, NodDKO(–WAS) mice did not demonstrate evidence of baseline anxiety-like behaviour, as indicated by total number of transitions between the L/D boxes and time spent in the light box compared to WT(–WAS) controls (Fig. 1*B*). By contrast, NodDKO(+WAS) mice displayed a significant decrease in the number of transitions between the L/D compartments compared to WT(+WAS) mice without an impact on time spent in the light box, indicative of anxiety-like behaviour. The OFT was used to measure exploratory behaviour and general well-being along with serving as a secondary assessment of anxiety-like behaviour. NodDKO(+WAS) mice displayed significantly reduced frequency of entries into the inner zone in the OFT compared to WT mice, without a difference in the total time spent in the inner zone (Fig. 1*B*). Exploratory behaviour, as determined by total distance travelled in the OFT, was significantly reduced in NodDKO(–WAS) mice compared to WT, which was further decreased in NodDKO(+WAS). Stress also caused decreased exploratory behaviour in WT(+WAS) mice. These findings suggest that deficiency in NLR increases susceptibility to acute stress, profoundly impacting behaviour.

Cognitive function and anxiety-like behaviours were similar in male and female mice for both WT(±WAS) and NodDKO(±WAS) groups; therefore, all subsequent data are derived from combined sexes, which were equally distributed (Fig. 2*C*).

NodDKO mice display hyperactivation of the HPA axis

Dysregulation of the HPA axis is associated with the development of anxiety-like behaviour (Kolber *et al.* 2008) and cognitive impairments (Avital *et al.* 2006). Given that exposure to WAS impaired behaviour in NodDKO mice, we measured serum corticosterone levels and expression of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in the hippocampus, which serves as the primary site for feedback inhibition of HPA axis signalling. As expected, WAS increased the concentration of serum corticosterone in WT mice, with this stress response being further increased in NodDKO(+WAS) compared to WT(+WAS) mice (Fig. 2*A*). Because basal corticosterone serum concentrations were similar between WT(–WAS)

and NodDKO(–WAS) mice, these data suggest NodDKO mice are susceptible to stress-induced hyperactivation of the HPA axis (Fig. 2*A*).

Corticosterone induces physiological and behavioural effects by binding to GR and MR, which are expressed in the hippocampus and serve to limit HPA axis activation. Glucocorticoids are proposed to cause stress-induced depression, with decreased GR function and expression implicated in the stress response, as well as being associated with depression (Zhu *et al.* 2014). Hippocampal mRNA expression of *GR* was significantly reduced in NodDKO(+WAS) mice compared to WT(+WAS) mice, whereas no differences in *MR* expression were observed (Fig. 2*B*). Decreases in *GR* and *MR* in NodDKO(+WAS) mice were also seen in the PFC region (Fig. 2*C*).

NodDKO mice have impaired stress-induced neural activation

Neural activation is critical for memory formation, with acute stress-induced increases in glucocorticoids necessary for hippocampal neuronal survival, memory acquisition and consolidation (Uchoa *et al.* 2014). To identify the neural circuitry underlying the differential stress susceptibility between NodDKO(+WAS) and WT(+WAS) mice, we measured expression of the immediate early genes (IEGs) *Arc* and c-Fos, indicative of neural activation (Tzingounis & Nicoll, 2006; Santos *et al.* 2018). IEG expression, associated with transcription factors, proteases, enzymes, etc., in neurons is extremely low at rest, generally below the limit of detection, although it can be rapidly induced following exposure to a stimulus, such as stress (Guzowski *et al.* 2005). *Arc* mRNA expression was significantly increased in the hippocampus of WT(+WAS) but not NodDKO(+WAS) mice compared to WT(–WAS) or NodDKO(–WAS) mice, respectively (Fig. 3*A*). This was specific for the hippocampus, with no changes in *Arc* seen in the PFC (data not shown). These findings suggest that Nod1/2 are required for stress-induced activation of hippocampal neurons and subsequent memory consolidation. By contrast, brain-derived neurotrophic factor (BDNF), which is important for maintaining neuronal survival, was not impacted in either the hippocampus or PFC in NodDKO(+WAS) mice (data not shown).

Neuronal activation was further assessed by quantification of c-Fos-positive cells in the DG and CA3 regions of the dorsal hippocampus by confocal microscopy in mice exposed to WAS. Given that the peak of c-Fos expression occurs 1–3 h following exposure to an acute stimulus (Reichmann *et al.* 2013), c-Fos positive cells were enumerated in hippocampal sections collected at 1 h post-WAS. The total number of c-Fos-positive cells, as quantified using Imaris, was significantly lower in NodDKO(+WAS) mice compared to WT(+WAS) in both the DG and CA3 region of the dorsal hippocampus (Fig. 3*A*). Taken together, these results suggest that neuronal activation following WAS is impaired in NodDKO(+WAS) mice, leading to a lack of consolidation of hippocampal-dependent memory, and subsequently leading to cognitive deficits compared to WT(+WAS) mice.

NodDKO(+WAS) mice exhibit reduced cell proliferation and decreased immature neurons in the dorsal hippocampus

Cell proliferation and the abundance of immature neurons in the adult hippocampus are involved in the process of neurogenesis, which is considered to play an important role in the stress response (Snyder *et al.* 2011), consolidation of memory (Jessberger *et al.* 2009) and regulation of mood (Hill *et al.* 2015). Given the impaired recognition memory and increased anxiety-like behaviour observed in NodDKO(+WAS) mice, we investigated whether hippocampal cell proliferation and the abundance of immature neurons were impacted by Nod1/2 deficiency. Cell proliferation was measured

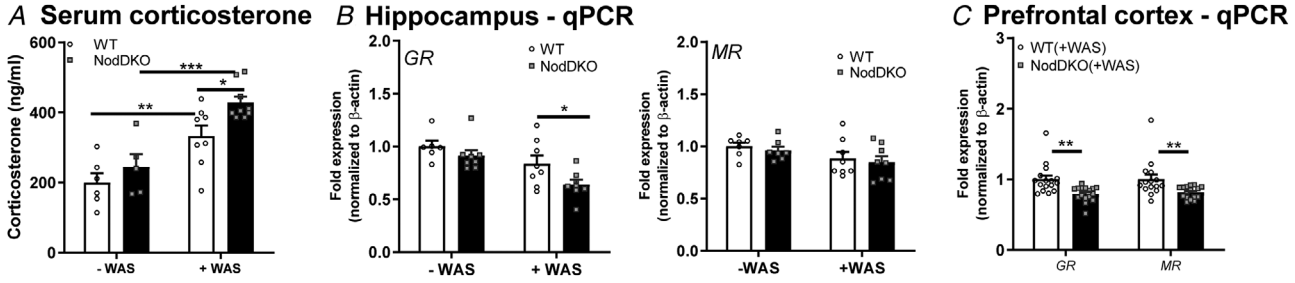


Figure 2. NodDKO(+WAS) mice display HPA axis hyperactivation
HPA axis: (*A*) serum corticosterone levels (n = 6–9), (*B*) hippocampal and (*C*) prefrontal cortex (PFC) glucocorticoid (*GR*) and mineralcorticoid (*MR*) receptor mRNA expression levels (n = 6–16) ± WAS. Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001, two-way ANOVA).

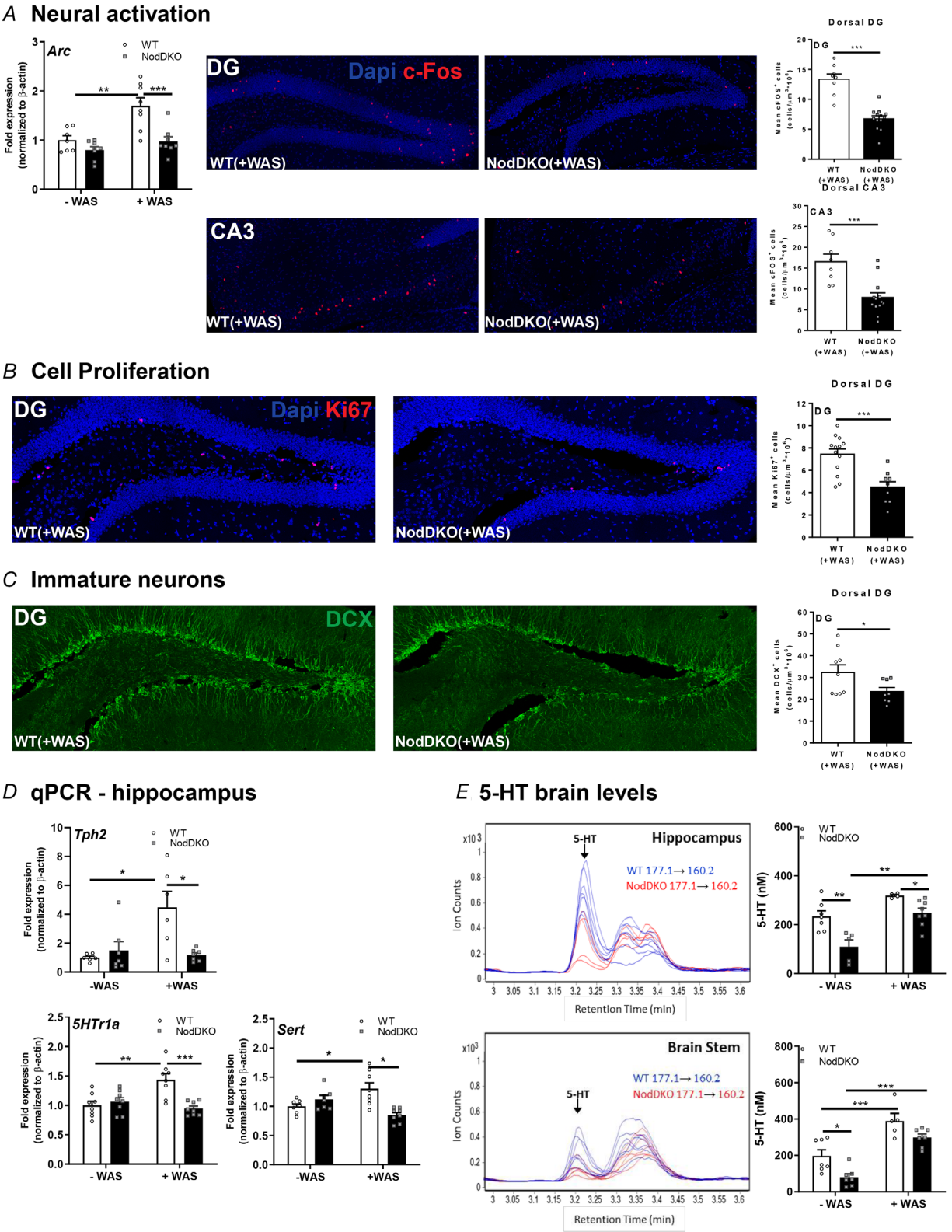


Figure 3. NodDKO(+WAS) mice display decreased neural activation, cell proliferation and immature neurons, with 5-HT signalling impairments
A, hippocampal *Arc* mRNA expression levels (n = 7–8), and c-Fos$^+$ cells in the dentate gyrus (DG) (n = 8–13) and the cornus ammonis (CA) 3 regions (n = 8–13) and representative images of c-Fos immunofluorescence. *B*, Ki67$^+$ cells (n = 8–13) and representative images of Ki67 immunofluorescence in the DG. *C*, DCX$^+$ cells (n = 6–7)

by staining for the proliferation marker Ki67 and the abundance of immature neurons was assessed by quantifying DCX^+ cells in the dorsal hippocampus. Confocal image analysis revealed significantly fewer Ki67-positive cells (Fig. 3*B*) and lower numbers of DCX-positive cells in the DG of NodDKO(+WAS) mice compared to WT(+WAS) controls (Fig. 3*C*). These findings suggest that altered hippocampal neurogenesis may be involved in the cognitive and emotional impairments found in NodDKO(+WAS) mice.

NodDKO mice exhibit down-regulation of the serotonergic system in the brain

Changes in the central 5-HT system can regulate adult hippocampal neurogenesis in rodents (Malberg *et al.* 2000; Song *et al.* 2017). To investigate the potential molecular mechanisms underlying the behavioural deficits observed in NodDKO(+WAS) mice, we first assessed components of the 5-HT signalling pathway by qPCR in the hippocampus and PFC. Expression of Trp hydroxylase (*Tph*)*2*, the rate limiting enzyme for 5-HT synthesis, the 5-HT receptor (*5HTr*)*1a*, which regulates 5-HT release in different brain areas including the hippocampus (Bravo *et al.* 2014), and the 5-HT transporter (*Sert*) was quantified. In the hippocampus but not the PFC (data not shown), increased expression of *Tph2*, *5HTr1a* and *Sert* was demonstrated in WT mice following exposure to WAS, which was impaired in NodDKO(+WAS) mice (Fig. 3*D*). By contrast, expression of *5HTr2c* was not impacted in either hippocampus or PFC (data not shown).

To determine the impact of reduced Tph2 expression on 5-HT concentrations in the hippocampus and brain stem, LC/MS was used. The brain stem was assessed because it represents the primary site of 5-HT synthesis in the brain (Charnay & Leger, 2010). Quantification of 5-HT revealed significantly reduced baseline concentrations in the hippocampus and brain stem of NodDKO(–WAS) mice compared to WT(–WAS) controls (Fig. 3*E*). 5-HT was increased by WAS in WT (brain stem) and NodDKO (hippocampus and brain stem) mice, with WT(+WAS) having higher hippocampal 5-HT levels compared to NodDKO(+WAS). These data demonstrate that Nod1/2 participate in an important control mechanism that regulates 5-HT concentration in the brain and impairs stress-induced 5-HT receptor expression.

Other neurotransmitters such as GABA are involved in the regulation of stress, anxiety, and cognition (O'Leary *et al.* 2014). Overall, no overt alterations were observed in GABA signalling either in the hippocampus or PFC, with only *Gabab1b* found to be increased in the PFC of NodDKO(+WAS) mice compared to WT(+WAS) (data not shown). These findings suggest a specific interaction between NLR and serotonergic signalling.

NodDKO mice exhibit stress-induced impairments in intestinal physiology

Psychological stress and the resulting HPA axis activation can detrimentally impact intestinal physiology, in part by causing elevated intestinal ion transport and increased intestinal permeability (Gareau *et al.* 2008). Given previous findings identifying a role for Nod1 and Nod2 in regulating intestinal mucosal barrier function (Natividad *et al.* 2012), we assessed intestinal physiology in ileal and colonic tissues from WT and NodDKO mice using Ussing chambers. Active ion transport was assessed by measuring *Isc* in ileal and colonic segments, with NodDKO(–WAS) mice displaying normal baseline values compared to WT(–WAS) mice (Fig. 4). Intestinal permeability was assessed by measuring *G* for tight junction permeability and flux of FITC-labelled dextran (4 kDa) for macromolecular permeability. Similar to ion transport, *G* and FITC flux were both normal in NodDKO(–WAS) mice compared to WT(–WAS) mice. By contrast, both ion transport [*Isc* (colon)] and permeability [*G* (colon) and FITC-dextran flux (colon/ileum)] were increased in in NodDKO(+WAS) mice compared to WT(+WAS) controls (Fig. 4).

Given the important role of 5-HT signalling in regulating gut physiology (Mawe & Hoffman, 2013), and our evidence of altered 5-HT signalling in the hippocampus in NodDKO(+WAS) mice, qPCR analysis for 5-HT signalling in the ileum and colon was performed. Increased *5HTr1a* and reduced *Tph1* expression were observed in the ileum of both NodDKO(±WAS) mice compared to WT(±WAS) controls (Fig. 4*A*). In the colon, baseline expression of *5HTr1a* and *5HTr2c* was increased and decreased, respectively, whereas *Tph1* was unaffected (Fig. 4*B*). Expression of the 5-HT reuptake transporter (*Sert*), which terminates 5-HT activity, was unaffected in both the ileum and colon (Fig. 4). Taken together, these findings identify that Nod1 and Nod2 deficiency leads to dysregulated peripheral 5-HT signalling, similar to the findings observed in the hippocampus.

and representative images of DCX immunofluorescence in the DG. *D*, *Tph2*, *5HTr1a* and *Sert* hippocampal mRNA expression levels (n = 6–16). *E*, 5-HT protein levels in the hippocampus (n = 5–8) and brain stem (n = 5–7) detected by LC/MS and multiple reaction monitoring chromatogram of 5-HT in cytoplasmic extracts of both the hippocampus and brain stem: *m/z* 177.1 corresponds to 5-HT precursor ion mass and *m/z* 160.2 corresponds to fragment ion mass. Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001, two-way ANOVA and unpaired Student's t test).

Stress-induced behavioural impairments in NodDKO mice are restored by chronic fluoxetine administration

The selective 5-HT reuptake inhibitor fluoxetine is one of the most commonly prescribed anti-depressant drugs that ameliorates cognitive impairments and depression both in rodents (David *et al.* 2009) and humans (Jaykaran *et al.* 2009), mainly by acting on the 5-HT system (Malagie *et al.* 2002; Taylor *et al.* 2005). Chronic fluoxetine administration is also known to attenuate

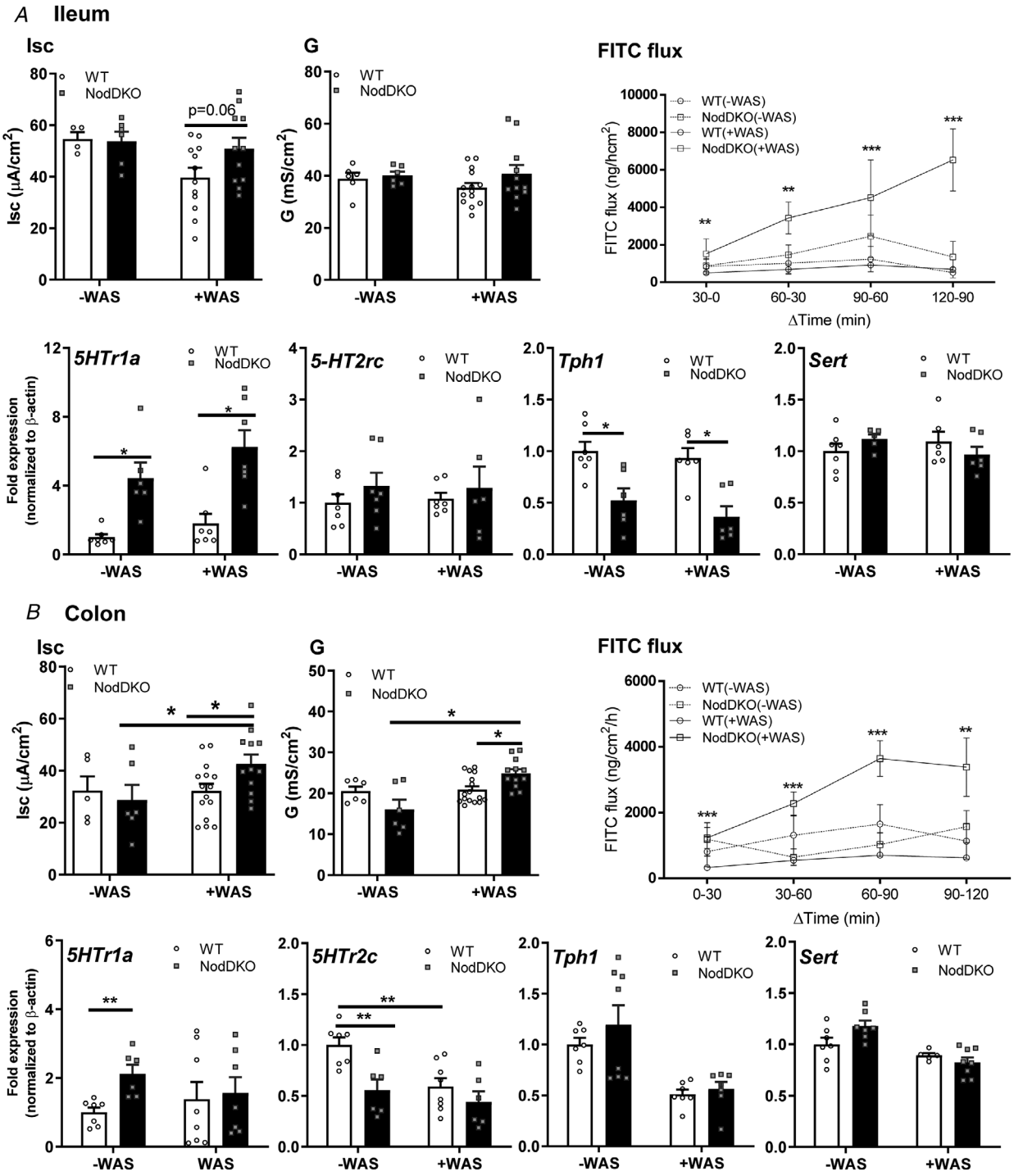


Figure 4. NodDKO(+WAS) mice exhibit altered GI physiology and impaired 5-HT signalling
Ileum (*A*) and colon (*B*) basal short circuit current (*Isc*), basal conductance (*G*) and FITC dextran flux assessment (n = 11–16), as well as *5HTr1a, 5HTr2c, Tph1* and *Sert* mRNA expression levels (n = 6–8). Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001, unpaired Student's t test and two-way ANOVA).

acute stress reactivity in mice (McVey Neufeld *et al.* 2018). In accordance with our observation of reduced 5-HT in the brain and down-regulation of the 5-HT signalling in NodDKO(+WAS) mice, mice were treated chronically with fluoxetine for 28 days starting at weaning to restore hippocampal 5-HT levels. Behaviour (L/D box, OFT and NOR) was performed as described previously (Fig. 1*A*) followed by a 2 h rest and, finally, the FST to assess depressive-like behaviour (Fig. 5*A*). Cognitive impairments seen in NodDKO(+WAS) mice in the NOR task relative to WT(+WAS) were reversed by fluoxetine administration compared to vehicle (water)-treated control NodDKO(+WAS) mice (Fig. 5*B*). Fluoxetine administration also improved anxiety-like behaviour by increasing the time spent in the L/D box, but not affecting the number of transitions, in NodDKO(+WAS) mice compared to WT(+WAS) fluoxetine-treated mice (Fig. 5*B*). Similarly, deficits in total distance traveled, time spent and frequency of entries into the inner zone of the OFT in NodDKO(+WAS) mice were reversed by fluoxetine treatment compared to vehicle controls (Fig. 5*B*). Given the anti-depressant properties of fluoxetine, depressive-like behaviour was assessed using the FST. The total time spent immobile was increased in NodDKO(+WAS) mice compared to WT(+WAS) mice administered vehicle, suggesting the presence of depressive-like behaviour. This was reversed by chronic fluoxetine administration to NodDKO(+WAS) mice (Fig. 5*B*). Chronic fluoxetine administration also normalized the decreased GR expression (Fig. 5*C*) but did not reduce the elevation of serum corticosterone levels that had previously been seen in NodDKO(+WAS) mice compared to WT(+WAS) mice (Fig. 5*C*), suggesting the ability of fluoxetine to partially modulate HPA axis activation. In addition, fluoxetine inhibited the decreased expression of hippocampal *Arc* seen in NodDKO(+WAS), suggesting restoration of neuronal activation (Fig. 5*D*).

Nod1/2 deficiency reduces serum and tissue Trp levels, which can be restored by chronic fluoxetine administration

Trp is the sole precursor of peripherally and centrally produced 5-HT (Richard *et al.* 2009). Trp exists in the circulation as both free or bound to albumin, with only free Trp able to cross the blood–brain barrier. However, given the high affinity for Trp to the transporter, *vs.* to albumin, ∼75% of bound Trp is assumed to be able to cross the blood–brain barrier, making almost all of it accessible for 5-HT synthesis within the CNS (Richard *et al.* 2009). Furthermore, given that reduced serum Trp levels correlate with alterations in mood and cognition (Young & Leyton, 2002; Toker *et al.* 2010), serum and hippocampal Trp concentrations were assessed by LC/MS. Lower free Trp levels were found in serum of both NodDKO(–WAS) (Fig. 5*E*) and NodDKO(+WAS) compared to their control WT(±WAS) counterparts (Fig. 5*E*), suggesting a role for Nod1/2 in regulating Trp transport. Interestingly, chronic fluoxetine treatment restored serum Trp concentration to WT levels in NodDKO(+WAS) mice (Fig. 5*E*). In the brain stem, Trp levels were decreased in NodDKO(+WAS) compared to WT(+WAS), which was restored by administration of fluoxetine (Fig. 5*E*). Although hippocampal Trp was not impaired in NodDKO(±WAS) (Fig. 5*E*), fluoxetine administration significantly increased hippocampal levels in both WT(+WAS) and NodDKO(+WAS) mice (Fig. 5*E*). Despite the ability to beneficially impact Trp levels, fluoxetine was not able to restore the decreased hippocampal *Tph2* or *Sert* expression levels in NodDKO(+WAS) mice, although it did normalize *5HTr1a* levels (Fig. 5*F*). Given that the brain stem is the major area of 5-HT synthesis in the brain, which can then be used by other regions, signalling in the brain stem may indirectly impact hippocampus function and behaviour.

Chronic fluoxetine administration did not restore intestinal physiology in NodDKO(+WAS)

Given the impairments in 5-HT signalling found in the ileum and colon of NodDKO(+WAS) mice, we investigated whether chronic fluoxetine administration could restore the altered intestinal physiology we observed in these animals (Fig. 6). By contrast to amelioration of behavioural deficits, the elevations in secretory state (*Isc*) and gut permeability (*G* and FITC flux) seen in NodDKO(+WAS) in colon were not reversed by chronic fluoxetine administration (Fig. 6*B*) and may even be slightly potentiated in the ileum (Fig. 6*A*). These findings suggest that, although intestinal alterations of selected genes of the 5-HT system were found, the mechanism by which fluoxetine normalizes behaviour is probably independent of changes in intestinal physiology.

Stress-induced behavioural deficits are dependent on intestinal epithelial Nod1 receptors

To determine whether intestinal NLR account for the behavioural and biochemical effects observed in NodDKO mice, we utilized a conditional knockout (cKO) strategy (Fig. 7*A*). Behaviour and intestinal physiology were assessed in IEC cKO mice ($VilCre^{+}Nod1^{f/f}$ and $VilCre^{+}Nod2^{f/f}$). $VilCre^{+}Nod1^{f/f}$(+WAS) mice demonstrated significantly reduced recognition of the novel object in the NOR task compared to control mice [$VilCre^{-}Nod1^{f/f}$(+WAS)] (Fig. 7*B*). Furthermore, $VilCre^{+}Nod1^{f/f}$(+WAS) mice displayed anxiety-like behaviour as determined by the L/D box (reduced time

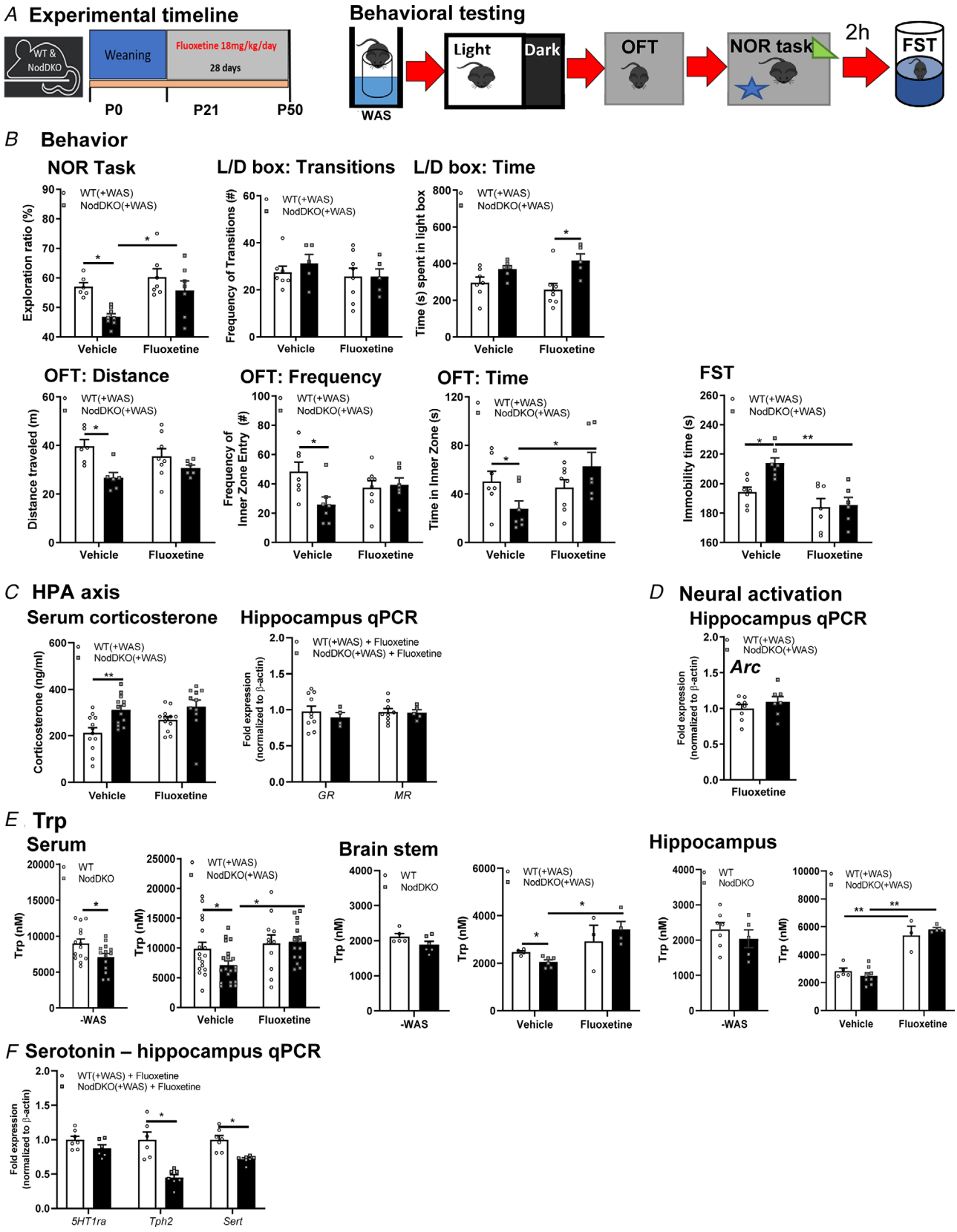


Figure 5. Chronic fluoxetine administration restored behavioural impairments, as well as HPA axis activity, Trp and 5HT signalling, in NodDKO(+WAS) mice

A, experimental timeline showing fluoxetine treatment and behavioural testing. Fluoxetine effect on (*B*) NOR task, L/D box, OFT and the FST (n = 6–8). *C*, corticosterone levels in serum (N = 11–13) and hippocampal GR and MR mRNA expression levels (N = 4–10). *E*, tryptophan levels in serum (n = 11–20), brain stem (n = 3–6) and hippocampus (n = 4–5) detected by LC/MS. *F*, hippocampal *5HT1ra*, *Tph2* and *Sert* mRNA expression levels (n = 6–7). Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01, unpaired Student's t test and two-way ANOVA).

spent in the light compartment) and OFT (reduced total distance traveled and time spent into the inner zone) (Fig. 7*B*). Similar to the findings in NodDKO mice, VilCre^{+}Nod1$^{f/f}$ mice also had increased serum corticosterone levels and decreased GR and MR expression in the hippocampus (Fig. 7*B*). These changes in HPA axis signalling were accompanied by decrease 5-HT signalling, with decreased expression of *5HTr1a*, *5HTr2c* and *Tph2* in the hippocampus (Fig. 7*B*). Taken together, these findings suggest that IEC Nod1 expression can regulate CNS mechanisms, including the HPA axis and 5-HT signalling, and impact behaviour.

In the GI tract, significantly increased ion transport (*Isc*) was found in the ileum of VilCre^{+}Nod1$^{f/f}$(+WAS) mice compared to their WT control littermates (Fig. 7*C*). By contrast, no changes in physiology were observed in the colon, with similar ion transport and permeability seen in both WT and VilCre^{+}Nod1$^{f/f}$(+WAS) mice, suggesting a differential role for Nod1 in the ileum *vs.* the colon in response to stress. qPCR analysis for 5-HT signalling in the ileum identified increased *5HTr1a* and *Sert* mRNA expression levels in VilCre^{+}Nod1$^{f/f}$(+WAS) mice compared to VilCre^{-}Nod1$^{f/f}$(+WAS) controls (Fig. 7*C*). In the colon, *5HTr1a* was increased, whereas *Tph1 m*RNA levels were decreased (Fig. 7*C*).

By contrast to VilCre^{+}Nod1$^{f/f}$(+WAS) mice, VilCre^{+}Nod2$^{f/f}$(+WAS) mice had normal cognitive function and anxiety-like behaviour compared to littermate controls (Fig. 8*A*). In the GI tract, VilCre^{+}Nod2$^{f/f}$(+WAS) mice displayed significantly increased *Isc* in the ileum, but not the colon, as well as increased *G* in both ileum and colon compared to their WT control littermates (Fig. 8*B*). These findings suggest a unique role for IEC Nod1 receptors in the regulation of behaviour through the GI tract with a possible involvement of the peripheral 5-HT system.

Discussion

Understanding the molecular mechanisms underlying stress susceptibility is key for the identification of novel pharmacological treatments for stress-related gut–brain

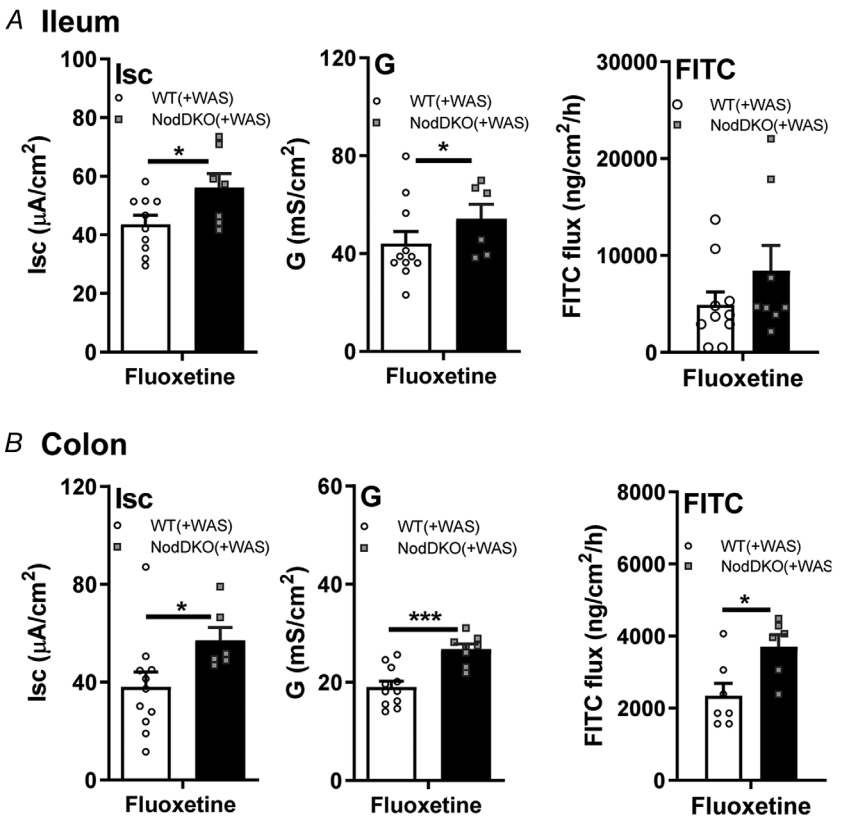


Figure 6. Chronic fluoxetine administration did not restore intestinal physiology in NodDKO(+WAS) mice
Ileum (*A*) and colon (*B*) basal short circuit current (*Isc*), basal conductance (*G*) and FITC dextran flux assessment (n = 6–8). Data are presented as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001, unpaired Student's t test).

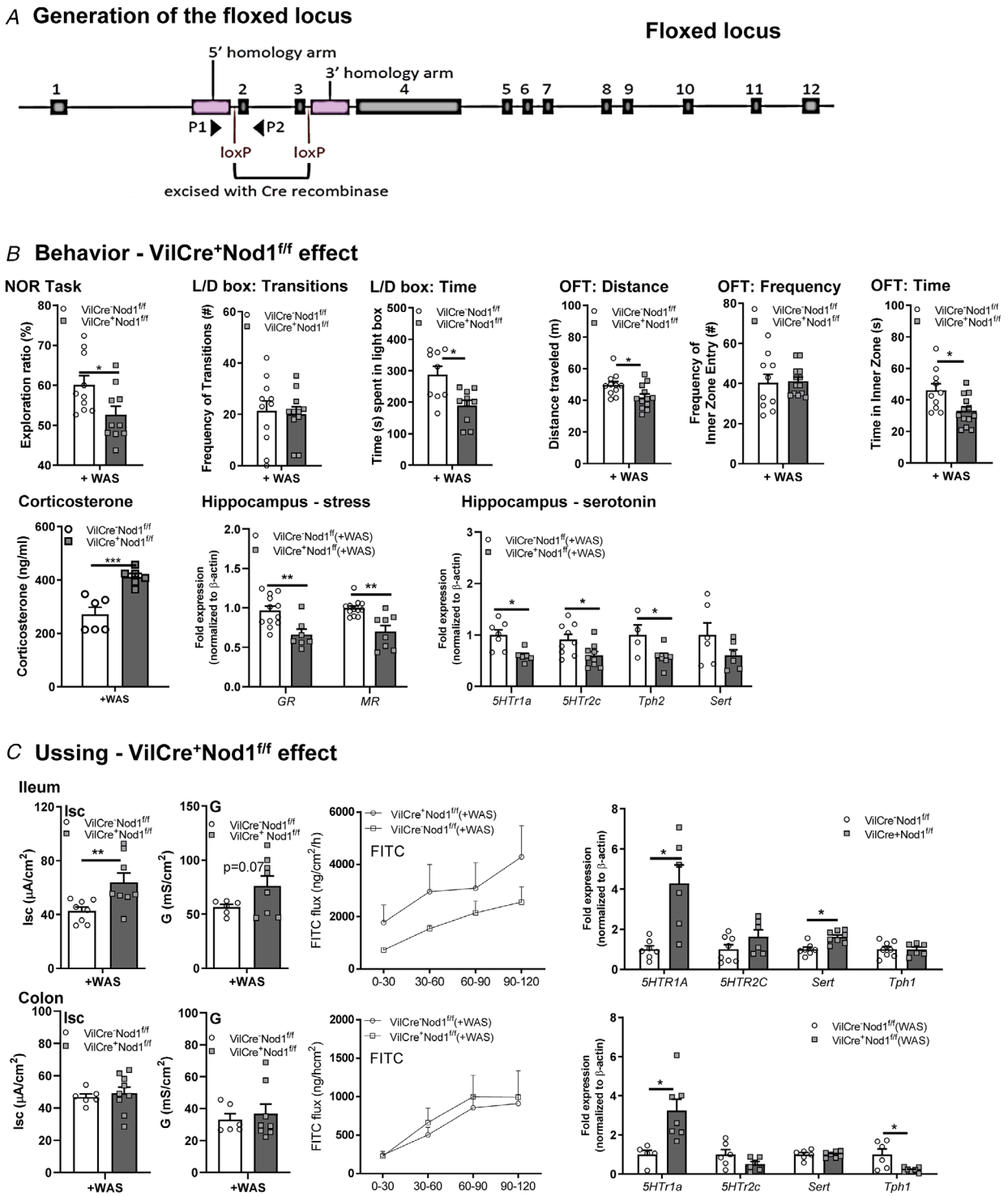


Figure 7. Behavioural deficits in NodDKO(+WAS) mice are dependent on intestinal epithelial Nod1 receptor expression
A, schematic representing the floxed locus for the generation of Nod1$^{f/f}$ mice. *B*, effect of intestinal epithelial Nod1 deletion on behaviour: NOR task, L/D box and OFT (n = 7–12), serum corticosterone and hippocampal mRNA expression levels of stress genes (*GR* and *MR)* and 5-HT signalling (*5HTr1a, 5HTr2c, Tph1* and *Sert)*. *C*, ileum and colon basal short circuit current (*Isc*), basal conductance (*G*) and FITC dextran flux assessment (n = 6–9), as well as *5HTr1a, 5HTr2c, SERT* and *Tph1 m*RNA expression levels (n = 6–8). Data are presented as the mean ± SEM. (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; unpaired Student's t test). [Colour figure can be viewed at wileyonlinelibrary.com]

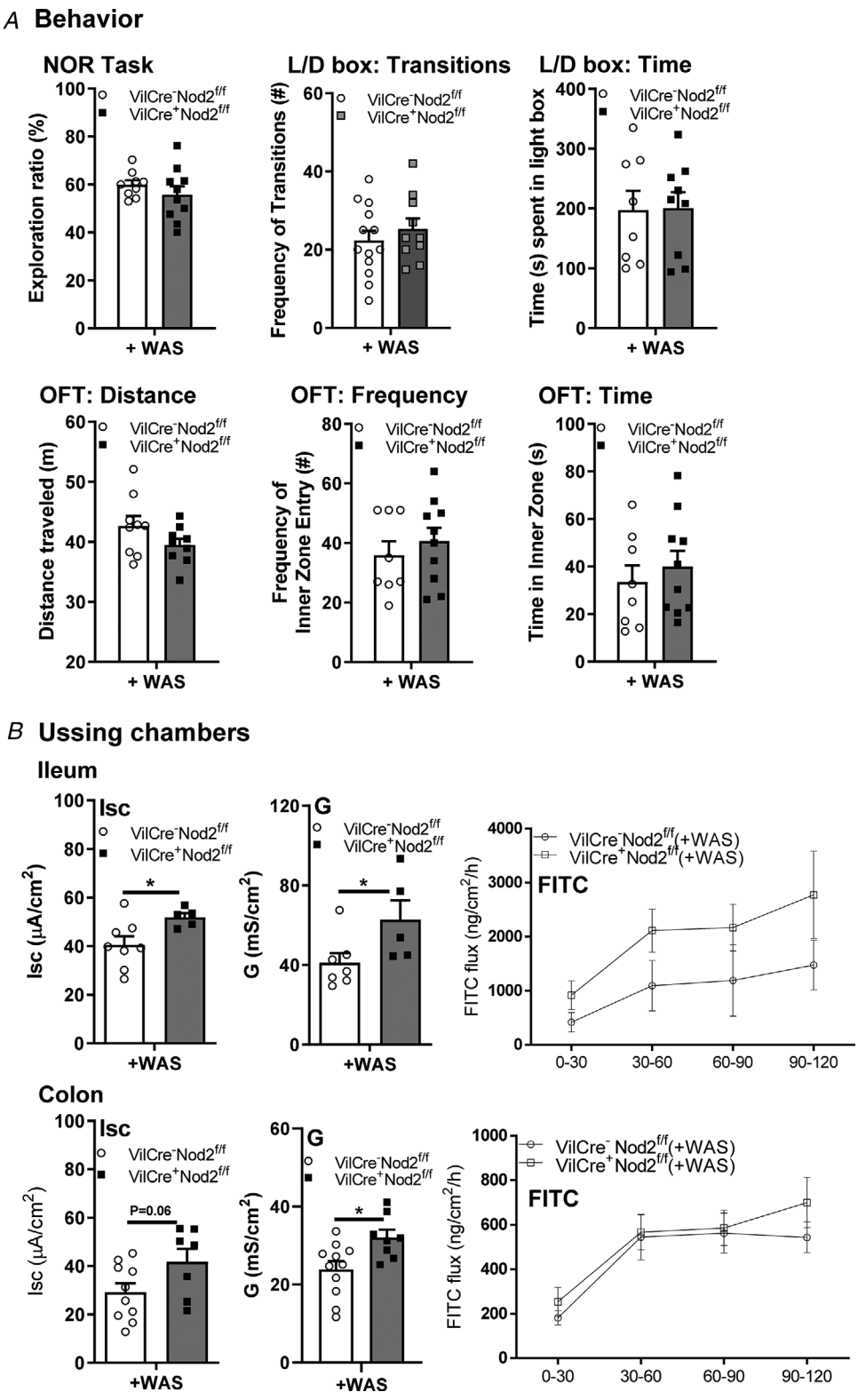


Figure 8. Behavioural, but not GI, deficits are independent of intestinal epithelial Nod2 receptor expression
A, effect of intestinal Nod2 deletion on NOR task, L/D box and OFT (n = 9–12) *B*, ileum and colon basal short circuit current (*Isc*), basal conductance (*G*) and FITC dextran flux assessment (n = 5–11). Data are presented as the mean ± SEM (unpaired Student's *t* test).

disorders. In the present study, we have demonstrated that mice deficient in Nod1 and Nod2 have altered serotonergic signalling in the gut and brain, which makes them susceptible to hyperactivation of the HPA axis following exposure to acute psychological stress (Fig. 9). This HPA axis activation leads to GI pathophysiology, cognitive deficits, anxiety-like behaviour and depressive-like behaviour. Highlighting a previously unappreciated role of Nod1/2 in regulating HPA axis activation and serotonergic signalling, behavioural deficits (but not abnormalities in GI physiology) in NodDKO(+WAS) mice were normalized by chronic fluoxetine administration. Using a cKO approach, we further identified that mice lacking Nod1 in IEC and exposed to WAS recapitulated the changes in behaviour and intestinal physiology seen in NodDKO(+WAS) mice. Deficiency of Nod1 in IEC in these mice resulted in stress-induced impairments in cognitive function and anxiety-like behaviour. These effects appear to reflect a specific function of Nod1 in IEC because there was no impact of loss of IEC Nod2 expression on behaviour or GI physiology. Together, these results identify Nod1 as a novel factor that regulates the stress response, 5-HT biosynthesis and signalling. These results further indicate that Nod1 may contribute to a previously unrecognized signalling pathway in the gut–brain axis.

NLR are expressed in both the brain and periphery, and function as receptors that detect bacterial PGN; therefore, we hypothesized that NLR could modulate gut–brain signalling. Highlighting this novel role, mice deficient in Nod1/2 subjected to WAS had pronounced cognitive dysfunction, as well as anxiety-like and depressive-like behaviours, compared to WT(+WAS) controls. These findings complement previous studies by Farzi *et al.* (2015), who showed a synergistic effect between agonists for Nod1/2 and the toll-like receptor 4 agonist lipopolysaccharide with respect to causing impairments in brain activity and sickness behaviour, demonstrating a role for NLR in regulating behaviour. In accordance, the present study identified a role for Nod1/2 in regulating mood and cognitive behaviour, which was uncovered after exposure to an acute stressor. This was associated with alterations in HPA axis signalling, as a result of increased serum corticosterone levels and decreased hippocampal GR expression in NodDKO(+WAS) mice. This finding may suggest a novel NLR-stress-dependent mechanism of action for the regulation of brain and behaviour.

Given the association between stress, hippocampal neuronal activation and behaviour (Finsterwald & Alberini, 2014; O'Leary *et al.* 2014; Kim *et al.* 2015), hippocampal neuronal activation was assessed by *Arc* expression and c-Fos activation. The increase in *Arc* expression that would otherwise be induced by stress was inhibited, and the numbers of c-Fos positive neurons were reduced in NodDKO(+WAS) mice. This indicates a lack of neuronal activation following exposure to WAS. Stress is also known to be detrimental for adult hippocampal neurogenesis (Baptista & Andrade, 2018), which has been shown to reduce memory recognition in rodents, as assessed by NOR tasks (Jessberger *et al.* 2009; Denny *et al.* 2012). Similarly, both the number of immature neurons and hippocampal cell proliferation were impaired in NodDKO(+WAS) mice. Taken together, these findings demonstrate that behavioural impairments driven by NLR appear to involve alterations in hippocampal neurons, including reduced neuronal activation and decreased numbers of immature neurons, which may lead to impaired consolidation of hippocampal-dependent memories and cognitive deficits. However, additional studies focused on the role of NLRs in regulating behaviour through neurogenesis-dependent and independent mechanisms are warranted.

Given the interaction between stress and hippocampal serotonergic signalling (Mahar *et al.* 2014), we aimed to assess 5-HT biology in NodDKO mice. In the hippocampus, a lack of Nod1/Nod2 receptors resulted in significantly decreased 5-HT at both baseline and following exposure to acute stress compared to WT controls. This finding was associated with significantly reduced expression of both *Tph2* (the rate-limiting enzyme for neuronal 5-HT synthesis) and the heteroreceptor *5HTr1a* in NodDKO(+WAS) mice. Although *Tph2* expression was not reduced in

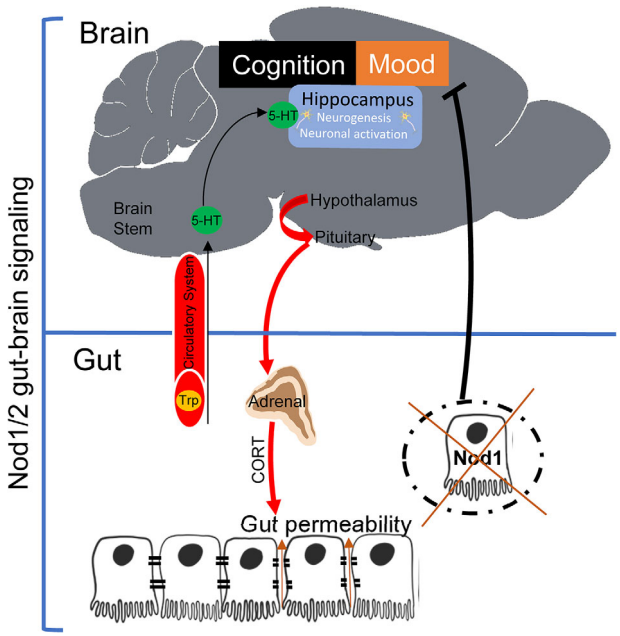


Figure 9. Graphical summary
Constitutive knockout of Nod1 and Nod2 induces gut dysbiosis and altered behaviour via the 5-HT system. Intestinal epithelial Nod1 receptor regulates susceptibility to cognitive impairment, anxiety-like and depressive-like behaviours.

NodDKO(–WAS) *vs.* WT(–WAS), significantly decreased hippocampal 5-HT concentrations in NodDKO(–WAS) mice compared to WT(–WAS) controls were detected. These data suggest that reduced *Tph2* expression may be sufficient to cause profound deficits in neurotransmitter production in the brain. Interestingly, both stress and elevated corticosterone levels have been shown to down-regulate the expression and the functionality of *5HTr1a* through the alteration of *GR* expression in the hippocampus of both rats and subjects with a history of depression (Meijer & de Kloet, 1994; Lopez *et al.* 1998). Highlighting a role for 5-HT in mediating cognitive function, administration of the selective post-synaptic *5HTr1a* agonist F15599 can ameliorate cognitive performance during the NOR task in a rat model of cognitive impairment (Horiguchi & Meltzer, 2012). Moreover, pharmacological blockade of *5HTr1a* can reduce hippocampal neurogenesis in adult rats (Radley & Jacobs, 2002) and regulate memory (Saxe *et al.* 2006), as well as emotional behaviour (Snyder *et al.* 2011). In addition to hippocampal changes, significantly lower 5-HT concentrations were observed in the brain stem of NodDKO(±WAS) mice, suggesting a lower release of 5-HT to the hippocampus (Charnay & Leger, 2010). Although reduced production of 5-HT was observed in both NodDKO(–WAS) and NodDKO(+WAS) mice, exposure to acute stress was necessary to uncover the behavioural deficits found in NodDKO(+WAS) mice. These data suggest a critical interaction between stress, NLR and the 5-HT system in the regulation of memory and emotional behaviour. Future studies characterizing the long-lasting effects of Nod1/Nod2 receptors on serotonergic signalling in response to chronic stress could support the concept that NLR play a crucial role in the pathophysiology of stress-related disorders, with implications for treatment.

To determine whether the behavioural deficits observed in NodDKO(+WAS) mice were dependent on the impaired serotonergic system seen in the hippocampus, behaviour and intestinal physiology were assessed in mice treated chronically with fluoxetine to increase 5-HT availability in the CNS. Restoration of cognitive impairment and reduced anxiety- and depressive-like behaviours were observed after chronic fluoxetine treatment, further supporting a role for 5-HT in mediating the behavioural deficits seen in NodDKO(+WAS) mice. In addition to increasing 5-HT concentration in the synaptic cleft by inhibiting 5-HT transporter (SERT), the anti-depressant effects of fluoxetine have been demonstrated to involve other mechanisms, such as neurogenesis (Malberg *et al.* 2000), increased BDNF levels (Nibuya *et al.* 1996), regulation of HPA axis activity (Pariante, 2004) and modulation of long-term potentiation (Rubio *et al.* 2013). Therefore, although NLR appear to regulate serotonergic signalling in the brain, the point in the signalling pathway at which they are critical for maintaining 5-HT levels in the brain and periphery remains to be determined.

Because NLR are important in maintaining intestinal physiology (Natividad *et al.* 2012) that can be detrimentally impacted by exposure to stress (Demaude *et al.* 2009; Hattay *et al.* 2017), we assessed ileal and colonic mucosal barrier function in WT(±WAS) and NodDKO(±WAS) mice. Although baseline ion transport (*Isc*) and permeability (*G*, FITC flux) were intact in NodDKO(–WAS), stress significantly increased *Isc*, *G* and FITC flux in the ileum and/or the colon of NodDKO(+WAS) mice *vs.* WT(+WAS) controls. These changes in GI physiology were associated with altered 5-HT signalling, with increases in *5HTr1a* and decreased *Tph1* expression being seen in the ileum. Mucosal barrier deficits in NodDKO(+WAS) mice were not restored by fluoxetine administration, even potentiating effects in the ileum, suggesting that fluoxetine ameliorates behavioural deficits via a mechanism independent of GI physiology. This may partly be a result of the dual function of 5-HT in the GI tract, depending on whether it is released from enteric nerves or enteroendocrine cells in the mucosa (Mawe & Hoffman, 2013), although future studies would be necessary to discern the specific contribution of each source in maintaining GI physiology when NLR are absent. Interestingly, activation of Nod1 has been shown to down-regulate the activity and expression of SERT in epithelial Caco-2/T7 cells (Layunta *et al.* 2018). Although these findings need to be confirmed *in vivo*, this suggests a potential interaction between NLR and the serotonergic system in the GI tract.

To assess whether Nod1/2 deficient mice have reduced peripheral availability of Trp, the biochemical precursor for 5-HT, the concentration of this amino acid was quantified in the serum. The concentration of serum Trp was significantly reduced in NodDKO(+WAS) mice but could be restored by chronic fluoxetine treatment. Because CNS concentrations of Trp are predominantly determined by availability in the periphery (Ruddick *et al.* 2006), our data suggest a reduced supply of this amino acid precursor of 5-HT to the CNS as a potential mechanism by which peripheral Nod1/Nod2 receptors can influence the CNS. Indeed, measurement of Trp in hippocampus and brain stem, with elevations in both regions following fluoxetine administration, confirm its ability to increase available Trp in the CNS for 5-HT synthesis.

Given our findings of altered physiology and 5-HT signalling in the GI tract in NodDKO mice, the role of IEC NLR expression in regulating gut–brain communication was assessed using cKO mice. Cognitive deficits and anxiety-like behaviour were observed in $VilCre^{+}Nod1^{f/f}$(+WAS), but not $VilCre^{+}Nod2^{f/f}$(+WAS) mice, suggesting that Nod1 expression in IEC can regulate cognition and mood in response to stress. Although

the anxiety-like behaviour parameters in our cKO mice did not identically phenocopy the changes seen in NodDKO(+WAS) mice, most probably partly as a result of slight changes in vivarium conditions over the timing of the multiple experiments, the presence of anxiety-like behaviour is consistent and supportive of a common behavioural feature between the two strains. Although our findings highlight a crucial role for IEC Nod1 receptors in maintaining behaviour, it also suggests a potential additional role of brain Nod1/2 receptors in the regulation of memory and emotional mood. Thus, the use of selective antagonists as well as cKO mice targeting Nod1/2 receptors in the brain would be valuable to further determine the role of NLR in regulating mood and cognition.

In conclusion, Nod1/Nod2 receptors represent novel mediators of gut–brain axis signalling. Deficiencies in NLR signalling in mice causes increased susceptibility to stress-induced behavioural deficits, including cognitive function, anxiety-like behaviour and depressive-like behaviour, as well as intestinal mucosal barrier defects. These effects were coupled with alterations in the serotonergic system that could be restored by chronic fluoxetine administration, suggesting a link between 5-HT signalling and NLR. Finally, these findings also indicate that Nod1/Nod2 receptors may be novel targets for the treatment of stress-related gut–brain disorders.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

MMP, MGG, KEB and CR designed research. MMP investigated animal behaviour. MMP, PS, GR and KAW performed RNA extraction and qRT-PCR. MMP, LRG, SEG, HJDK and IBM performed immunohistochemistry and confocal microscopy. MMP, GR and MB performed LC/MS analysis and data interpretation. CEK performed the corticosterone EIA. CL provided the LC/MS equipment. MMP, CTF and MGG performed the Ussing chamber experiments. MXB and AJB provided the NodDKO breeders. RF, CM and DJP designed the Nod1$^{f/f}$ mice. MS performed pilot animal behaviour experiments and pilot immunohistochemistry. MS, CEK, DJP, AJB, KEB and CR provided critical feedback on the manuscript. MMP and MGG analyzed and interpreted data. MMP and MGG wrote the paper. All authors have read and approved the manuscript submitted for publication. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Keywords

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