ORIGINAL RESEARCH ARTICLE

NOD1 downregulates intestinal serotonin transporter and interacts with other pattern recognition receptors†

Running title: Intestinal SERT downregulated by NOD1 activation

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Abstract

Serotonin (5-HT) is an essential gastrointestinal modulator whose effects regulate the intestinal physiology. 5-HT effects depend on extracellular 5-HT bioavailability, which is controlled by the serotonin transporter (SERT) expressed in both the apical and basolateral membranes of enterocytes. SERT is a critical target for regulating 5-HT levels and consequently, modulating the intestinal physiology. The deregulation of innate immune receptors has been extensively studied in inflammatory bowel diseases (IBD), where an exacerbated defense response to commensal microbiota is observed. Interestingly, many innate immune receptors seem to affect the serotonergic system, demonstrating a new way in which microbiota could modulate the intestinal physiology. Therefore, our aim was to analyze the effects of NOD1 activation on SERT function, as well as NOD1's interaction with other immune receptors such as TLR2 and TLR4. Our results showed that NOD1 activation inhibits SERT activity and expression in Caco-2/TC7 cells through the extracellular signal-regulated kinase (ERK) signaling pathway. A negative feedback between 5-HT and NOD1 expression was also described. The results showed that TLR2 and TLR4 activation seems to regulate NOD1 expression in Caco-2/TC7 cells. To assess the extend of cross-talk between NOD1 and TLRs, NOD1 expression was measured in the intestinal tract (ileum and colon) of wild type mice and mice with individual knockouts of TLR2, and TLR4 as well as double knockout TLR2/TLR4 mice. Hence, we demonstrate that NOD1 acts on the serotonergic system decreasing SERT activity and molecular expression. Additionally, NOD1 expression seems to be modulated by 5-HT and other immune receptors as TLR2 and TLR4. This study could clarify the relation between both the intestinal serotonergic system and innate immune system, and their implications in intestinal inflammation.

Keywords: 5-HT, SERT, ERK, TLR2, TLR4

Introduction

Serotonin (5-HT, 5-hydroxytryptamine) is a critical modulator for the gastrointestinal physiology as mediates numerous functions such as peristalsis and motility, secretion, absorption of nutrients or vasodilation (Mawe and Hoffman, 2013). Enterochromaffin cells synthesize 5-HT, which is released to apical and basal cell surfaces, activating specific 5-HT receptors and triggering different responses (Walther and Bader, 2003). Finally, serotonin transporter (SERT), expressed in both apical and basolateral membrane of intestinal epithelial cells, is responsible for removing 5-HT from the interstitial space into the cells, concluding 5-HT effects. Serotonin extracellular levels are determined by the balance between serotonin release (by enterochromaffin cells) and uptake (by SERT from enterocytes) and is critical for the development of 5-HT effects. Apart from the essential role of 5-HT in maintenance of intestinal homeostasis, serotonin also has important functions as the systemic level, participating in hematopoiesis and bone metabolism (Spohn and Mawe, 2017).

In recent years, alterations in the activity of the intestinal serotonergic system have been found to be involved in chronic gastrointestinal diseases such as inflammatory bowel diseases (IBD), which involves Crohn's disease and ulcerative colitis. In this context, recent studies have shown that 5-HT (Margolis and Gershon, 2009), as well as its receptors and SERT (Motavallian et al., 2013; Ravic et al., 2013) could play a critical role in IBD. In fact, studies based on animal models of IBD have shown there to be increases in 5-HT levels and enterochromaffin cells while SERT expression is decreased, leading to an increment of extracellular 5-HT bioavailability in the lumen and tissue of the intestinal tract (Linden et al., 2003; Linden et al., 2005). In this context, several works have analyzed the pro-inflammatory role of 5-HT in IBD (Bischoff et al., 2009; Ghia et al., 2009), suggesting the inhibition of mucosal serotonin as a new therapy for these pathologies (Levin and van den Brink, 2014).

Alterations of the intestinal serotonergic system have been described in IBD, but also changes in intestinal innate immunity have been reported in these pathologies (McGuckin et al., 2009). The innate immune system is crucial in the gastrointestinal tract, being the first line of defense against microorganisms. This immune system can detect and recognize microbial-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), triggering different responses to maintain intestinal homeostasis (Uematsu and Fujimoto, 2010). Since both TLRs and NLRs seem to be critical in the maintenance of intestinal homeostasis, several studies have pointed out their importance in the development of IBD as a consequence of aberrant over-activation in the presence of beneficial and non-pathogenic microbiota (Corridoni

et al., 2014). Several studies have shown the over-expression of TLR2 and TLR4 in the colonic mucosa of IBD patients (Tan et al., 2014b), and some TLR2 and TLR4 gene polymorphisms have been involved in the susceptibility to IBD (Cheng et al., 2015).

Regarding NLRs, NOD1 is a cytosolic receptor that is highly expressed in intestinal epithelial cells, which also plays an important role in maintaining gut homeostasis. NOD1 activates the innate immune system in response to the bacteria or antigens present in the cytoplasm (Chaves de Souza et al., 2016), but the involvement of NOD1 in relation to IBD has not been extensively studied. However, recent studies have determined strong evidence for NOD1 variants in IBD susceptibility, supporting its role in IBD pathogenesis (McGovern et al., 2005). In previous studies we have shown that activation of TLR2 or TLR4 inhibits intestinal SERT expression and activity (Latorre et al., 2016; Mendoza et al., 2009). Therefore, the aim of this work was to study the effects of NOD1 activation on SERT activity and expression in Caco-2/TC7 cells, as well as NOD1's interaction with other immune receptors (TLR2 and TLR4).

Materials and methods

Reagents and antibodies

The drugs and substances used (abbreviations and respective suppliers in parentheses) were as follows: serotonin (5-hydroxytryptamine, 5-HT) from Sigma–Aldrich (St. Louis, MO, USA) and [^{3}H]-5-HT (specific activity 28 Ci/mmol) from Perkin-Elmer (Boston, MA, USA), γ-D-glutamyl-meso-diaminopimelic acid (C-12-iE-DAP; DAP), lipopolysaccharide (LPS; Escherichiacoli O111:B4), N114 Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam3CSK4), S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 CF3COOH (Pam2CSK4) and Gefitinib RIP2 Tyrosine Kinase Inhibitor (gentamicin, receptor interacting protein-2 (RIP2) inhibitor) from InvivoGen (San Diego, CA, USA). 1,9-Pyrazoloanthrone (SP600125, c-Jun N-terminal kinases (JNK) inhibitor) and 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U0126, extracellular signal–regulated kinase (ERK) inhibitor) were acquired from Tocris (Madrid, Spain). In addition, the specific primary antibodies: goat polyclonal antibody anti-human-SERT (ab130130), rabbit polyclonal antibody anti-human/mouse NOD1 (ab105338), rabbit polyclonal antibody anti-human TLR2 (ab24192) and rabbit polyclonal antibody anti-human TLR4 (ab13556) from Abcam

(Cambridge, UK), as well as the goat polyclonal anti-human/mouse actin antibody (SC-1615) and secondary antibodies coupled to horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used. All generic reagents were purchased from Sigma-Aldrich and Roche Applied Sciences (Sant Cugat del Vallés, Barcelona, Spain).

Cell culture

Human enterocyte-like Caco-2/TC7 cells (Mesonero et al., 1994) were used to carried out this work. Previous studies have demonstrated that this cell line expresses SERT as well as innate immunity receptors, and it has been described as an ideal intestinal model for studying SERT and PRRs (Iceta et al., 2006; Latorre et al., 2014). Caco2/TC7 cells were cultured at 37°C in an atmosphere of 5% CO_2 with high glucose DMEM, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids, and 20% heat-inactivated fetal bovine serum (FBS) from Life Technologies (Carlsbad, CA, USA). The medium was changed two days after seeding and daily afterwards. The experiments were carried out nine days after reaching cell confluence (14 days after seeding) since SERT activity is maximal at this point (Iceta et al., 2006). The cell medium was free of FBS one day before the experiments. NOD1 agonist, C-12-iE-DAP (DAP) and other drugs were added to the culture medium at different concentrations and periods. Prior to the experiments, the cell monolayer was analyzed. None of the different conditions altered morphology, proliferation or monolayer integrity from the Caco-2/TC7 cells (data not shown).

Animals

C57BL/10 mice (wild type, WT) as well as knockout mice for TLR2 (*tlr2*$^{-/-}$), for TLR4 (*tlr4*$^{-/-}$) and double knockout mice for TLR2 and TLR4 (*tlr2,4*$^{-/-}$) were bred at the Centro de Investigación y Tecnología Agroalimentaria (CITA, Zaragoza, Spain). All of the mice genotypes were periodically tested. 10-12 week mice were maintained under pathogen-free conditions on a 12-hour light/dark cycle with food and water *ad libitum*. The Ethic Committee for Animal Experiments from the University of Zaragoza approved all experiments carried out in this study (P136/12). The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which meets the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Nº 123, Strasbourg 1985) and the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. Mice euthanasia was performed by using cervical dislocation, and immediately after that, the ileum and colon were removed and cleaned in an ice-solution of

NaCl (0.9%). Intestinal samples for RNA studies were collected in RNAlater from Qiagen (Hilden, Germany) and stored for one day at 4°C, being subsequently frozen at -80°C. The samples for protein assessments were immediately frozen in ice-cold isopropyl alcohol and stored at -80°C.

5-HT uptake studies

Uptake measurements were carried out in cell cultures in 24-well plates, as previously described (Iceta et al., 2006), either under a control condition or after treatment with a specific NOD1 ligand. The transport medium composition in mM, was: 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 HEPES pH 7.4, 4 glutamine, 1 ascorbic acid, 0.1% BSA, and both 0.2 µM 5-HT and [^{3}H]-5-HT (1.5 µCi/ml) as the substrate. Before uptake studies, cells were pre-incubated at 37°C in an atmosphere of 5% CO_2 with substrate-free transport medium for 30 minutes. The cells were washed with the substrate free transport medium at 37°C and then, incubated with the transport medium at 37°C for six minutes.

The uptake was stopped by removing the transport medium and washing the cells twice with an ice-cold transport medium containing 20 µM 5-HT. Finally, cells were solubilized in 0.5 ml of 0.1 N NaOH and a sample of 200 µl taken for radioactivity counting (Wallac Liquid Scintillation Counter, Perkin-Elmer). Protein concentration was calculated using the Bradford method from Bio-Rad (Hercules, CA) with BSA as the standard. All results obtained were considered in pmol 5-HT/mg protein and is expressed as a percentage of the control value (100%).

RNA extraction, reverse transcription and real-time PCR

Caco-2/TC7 cells were cultured in 6-well plates and total RNA was extracted using the RNeasy mini kit from Qiagen following the manufacturer's protocol. For intestinal RNA extraction, prior the use of the RNeasy mini kit, the samples were thawed in an ice-cold lysis RTL buffer (Qiagen) and homogenized using the Ultra-Turrax T25 from IKA (Staufen, Germany). The RNA extracted (1 µg) was used as a template for first-strand cDNA synthesis using oligo(dT) primers and a reverse transcriptase (Lucigen, Middleton, USA). A negative amplification was done without reverse transcriptase. The cDNA obtained was used to determine mRNA expression levels by real-time PCR using SYBR Green.

Quantification of the SERT, NOD1, TLR2 and TLR4 mRNA in Caco-2/TC7 cells and NOD1 mRNA in mice was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA), with GAPDH and HPRT1 as housekeeping. The following specific primers were used: hSERT sense (5' GGCCTGGAAGGTGTGATCA 3'), hSERT antisense (5'

GCGCTTGGCCCAGATGT 3') hNOD1 sense (5' GTACGTCACCAAAATCCTGGA 3'), hNOD1 antisense (5' CAGTCCCCTTAGCTGTGATC 3'), hTLR2 sense (5' GAAAGCTCCCAGCAGGAACATC 3'), hTLR2 antisense (5' GAATGAAGTCCCGCTTATGAAGACA 3'), hTLR4 sense (5' TTGAGCAGGTCTAGGGTGATTGAAC 3'), hTLR4 antisense (5' ATGCGGGACACACACACTTTCAAATA 3'), hHPRT1 sense (5' CTGACCTGCTGGATTACA 3'), hHPRT1 antisense (5' GCGACCTTGACCATCTTT 3'), hGAPDH sense (5' CATGACCACAGTCCATGCCATCACT 3'), hGAPDH antisense (5' TGAGGTCCACCACCCTGTTGCTGTA 3'), mNOD1 sense (5' CACAGCGCTCTTCACTTTTG 3'), mNOD1 antisense (5' GTTAGCCAGCAGGACCAGAG 3'), mHPRT1 sense (5' CTGGTGAAAAGGACCTCTCGAA 3'), mHPRT1 antisense (5' CTGAAGTACTCATTATAGTCAAGGGCAT 3'), mGAPDH sense (5' AACGACCCCTTCATTGAC 3') and mGAPDH antisense (5' TCCACGACATACTCAGCAC 3'). All of the samples analyzed were determined by triplicate, and the fluorescence raw data were analyzed by the Applied Biosystem StepOne Software v2.3 (Applied Biosystem). Thus, the mRNA relative expression was calculated as $\Delta\Delta Ct = \Delta Ct_{control} - \Delta Ct_{treatment}$ being $\Delta Ct = Ct_{gene} - Ct_{calibrator}$. GAPDH and HPRT1 were used as calibrators. Finally, the levels of relative gene expression were converted and expressed as fold difference ($=2^{-\Delta\Delta Ct}$).

Cell homogenates and brush border-enriched fraction for western blot analysis

Caco-2/TC7 cells were cultured in 75 cm^2 flasks and the cell brush border membrane-enriched fraction was obtained as described previously (Iceta et al., 2008). Briefly, cells were washed twice with PBS and re-suspended in an ice-cold Tris-mannitol buffer (2 mM Tris, 50 mM Mannitol, pH 7.1) with protease inhibitors and 0.02% sodium azide. The samples were disrupted using a Potter-Elvehjem homogenizer with a PTFE pestle and the obtained suspension was homogenized and disrupted by sonication (fifteen 1-s bursts, 60W). Brush border enriched fraction was obtained by addition of 20 mM $CaCl_2$ to the cell lysate and maintained for 10 minutes in ice. After this time, samples were centrifuged for 10 minutes at 950 g. The supernatant was centrifuged (30 minutes, 40,000 g) and the pellet re-suspended in a phosphate buffer (10 mM KH_2PO_4/K_2HPO_4 pH 6.8 to obtain the brush border membrane enriched fraction). Mice intestinal samples (ileum and colon) were cleaned and homogenized using Ultra-Turrax in Tris-mannitol buffer pH 7.1 (Latorre et al., 2016). Then, all of the samples were disrupted using a Potter-Elvehjem with a PTFE pestle and the obtained suspension was homogenized and disrupted by sonication (fifteen 1-s bursts, 60W).

All samples (cell lysate and brush border enriched fraction from Caco-2/TC7 cells, as well as ileum and colon homogenates of WT, *tlr2*$^{-/-}$, *tlr4*$^{-/-}$ and *tlr2,4*$^{-/-}$) were electrophoresed on 8% SDS-

PAGE gels and later transferred to PVDF membranes by electroblotting. Blots were blocked with 4% non-fat dried milk plus 1% BSA and probed with the following primary antibodies (1:1000): anti-human SERT, anti-human and anti-mouse NOD1, anti-human TLR2 and anti-human TLR4. Primary antibodies were detected using specific secondary antibodies coupled with horseradish peroxidase and the WesternBright Sirius HRP substrate from Advansta (Menlo Park, CA, USA). The signal was visualized using VersaDoc™ from Imagin System Bio-Rad. After stripping, membranes were re-probed with goat polyclonal anti-human or anti-mouse β-actin (1:10000) for the determination of differences in the sample loading. SERT, NOD1, TLR2 and TLR4/β-actin protein fraction was calculated in densitometric units by using Quantity One 1-D Analysis Software from Bio-Rad, and the results were expressed as a percentage of the control values (100%).

Statistical analyses

The results were expressed as the mean ± the standard error of the mean (SEM). Statistical comparisons were performed using either Student's unpaired t-test or one-way ANOVA. One-way ANOVA was followed by the Bonferroni post-test with a confidence interval of 95% ($p < 0.05$). Previously, normal distribution was confirmed with the D'Agostino-Pearson test. The statistical analysis is indicated in each figure and was developed with the computer-assisted Prism GraphPad Program (Prism version 5.03, GraphPad Software, San Diego, CA).

Results

NOD1 activation inhibits 5-HT uptake SERT expression in Caco-2/TC7 cells

Recent studies described NOD1 expression in different cell types, being mainly expressed in intestinal epithelial cells (Ting et al., 2008). Nevertheless, we considered critical to detect NOD1 expression in our enterocyte-like model. For this reason, we determined NOD1 mRNA and protein expression in Caco-2/TC7 cells by RT-PCR and western blot respectively (data not shown). After NOD1 expression was demonstrated, we studied the effect of NOD1 activation by C-12-iE-DAP (DAP) on SERT activity in Caco-2/TC7 cells. Thus, cells were treated with different DAP concentrations (0.1, 1 and 10 μg/ml) for a short (30 minutes) medium (6 hours) or long (1 day) term, and afterwards, 5-HT uptake was measured. The results showed that NOD1 activation with DAP diminishes 5-HT uptake, showing similar reductions in all periods of treatment used

(Figure 1A). Absolute control values were 7.27 ± 1.02, 4.28 ± 0.32 and 5.81 ± 0.26 pmol 5-HT/mg protein at 30 min, 6 hours and 1 day respectively.

Since NOD1 activation decreases SERT activity, we studied the influence of NOD1 on SERT molecular expression in Caco-2/TC7 cells. Thus, cells were treated for 1 day with DAP 10 µg/ml and the SERT mRNA and protein levels were analyzed. NOD1 activation over a period of 1 day significantly decreased SERT mRNA level (Figure 1B), and similarly, SERT protein expression was significantly reduced in cells treated with DAP in both the cell homogenate and apical membrane (Figure 1C). Therefore, NOD1 activation inhibits SERT activity in Caco-2/TC7 cells and induces a significant decrease of SERT molecular expression.

ERK Intracellular signaling pathway is involved in NOD1 inhibitory effect on SERT

Having demonstrated the NOD1 inhibitory effect on SERT activity and expression in Caco-2/TC7 cells, the intracellular signaling pathway involved was then studied. To do so, 1 hour before the cell treatment with DAP (10 µg/ml) for 1 day, Caco-2/TC7 cells were pre-treated with a specific pathway inhibitor (gentamicin 1 µM as RIP2 inhibitor, SP600125 1 µM as JNK inhibitor, or U0126 1 µM as ERK inhibitor) and then, the 5-HT uptake was measured.

We studied the RIP2 intracellular pathway because previous studies have described the NOD1 activation effects by this pathway (Irving et al., 2014). The results obtained showed that the RIP2 specific inhibitor did not reverse the inhibitory effect of NOD1 activation on SERT activity (Figure 2A). Following this, we analyzed whether the inhibitory effect of NOD1 could be through the JNK intracellular pathway, as JNK has been described as the main NOD1 intracellular pathway in different inflammatory processes (Girardin et al., 2001; Mercier et al., 2012). Figure 2B shows that the JNK inhibitor did not reverse the NOD1 effect on SERT activity. Finally, we studied ERK as a possible intracellular pathway of the NOD1 effect on SERT. The ERK/MAPK pathway has been previously implicated in several processes involving NOD1 (Berube et al., 2009). The results show that ERK/MAPK specific inhibitor reversed the NOD1 inhibitory effect on SERT activity in the Caco-2/TC7 cell line (Figure 2C). To confirm the role of the ERK/MAPK pathway in the long-term effect of NOD1 activation, SERT mRNA and protein expression were determined in the above same conditions. The results showed that the specific ERK inhibitor rescued the SERT mRNA reduction induced by DAP (Figure 2D). SERT protein expression was also studied in similar conditions. In accordance with the previous results, ERK inhibitor reversed the SERT protein level reduction due to DAP in both cell homogenate and apical membrane (Figure 2E).

5-HT negative feedback regulation of NOD1 expression

From the results above, we can conclude that NOD1 activation by DAP inhibits SERT activity in Caco-2/TC7 cells. In turn, SERT inhibition could lead to an increase of 5-HT extracellular availability, which has been demonstrated to be a common factor in IBD. Nonetheless, the effect of 5-HT in NOD1 molecular expression has not yet been demonstrated. For this purpose, cells were treated for 1 day with high (pathological) or physiological concentrations of 5-HT (10^{-4} M or 10^{-8} M respectively). Our results showed that NOD1 mRNA expression was significantly decreased in the treated cells with both 5-HT concentrations, with the decrease being more pronounced in the physiological than in the pathological concentration (Figure 3A). NOD1 protein expression was also analyzed, and a decrease in the NOD1 protein level was also found, however no differences were seen between both treatments (Figure 3B and 3C).

Interaction among TLR2, TLR4 and NOD1 in Caco-2/TC7 cells

Recent studies have established that TLR2 and TLR4 activation decrease 5-HT uptake in Caco-2/TC7 cells (Latorre et al., 2016; Mendoza et al., 2009). Moreover, over-expression of both receptors has been implicated in IBD (Cheng et al., 2015). Therefore, our next step was to analyze a possible relation between TLR2 and TLR4 activation with NOD1. For that, Caco-2/TC7 cells were treated with TLR4 agonist (LPS, 30 µg/ml), TLR2/1 agonist (Pam3CSK4, 10 µg/ml) and TLR2/6 agonist (Pam2CSK4, 100 ng/ml) for 1 day, after which the NOD1 mRNA and protein expression were analyzed. The results showed that the NOD1 mRNA level was decreased after LPS treatment, whereas TLR2/1 and TLR2/6 activation yielded a significant increase of NOD1 mRNA expression (Figure 4A). In line with mRNA results, NOD1 protein expression was decreased by TLR4 activation and increased by TLR2/1 and TLR2/6 activation (Figure 4B).

We also aimed to study the effect of NOD1 activation on TLR2 and TLR4 expression. Therefore, Caco-2/TC7 cells were treated with NOD1 ligand (DAP, 10 µg/ml) for 1 day, and then, TLR2 and TLR4 mRNA and protein expression were measured. The results showed that the TLR2 mRNA and protein levels decreased (Figure 5), whereas TLR4 mRNA expression did not seem to be significantly modified after NOD1 activation (Figure 5).

NOD1 expression in ileum and colon from tlr2$^{-/-}$, tlr4$^{-/-}$ and tlr2,4$^{-/-}$ mice

To corroborate our previous results, we analyzed NOD1 expression in the ileum and colon of *tlr2$^{-/-}$, tlr4$^{-/-}$ and tlr2,4$^{-/-}$* mice. In regard to the ileum, NOD1 mRNA and protein expression increased in both *tlr2$^{-/-}$* and *tlr4$^{-/-}$* mice (Figure 6A and 6B). Surprisingly, in the ileum from double

knockout mice *tlr2,4*$^{-/-}$, NOD1 mRNA and protein expression seemed not to be modified compared with WT mice (Figure 6A and 6B). Regarding the colon, NOD1 mRNA expression was diminished only in *tlr2,4*$^{-/-}$ mice, suggesting a possible collaborative action between TLR2 and TLR4 (Figure 6C). Nevertheless, the protein expression of NOD1 was unchanged in all deficient mice (*tlr2*$^{-/-}$, *tlr4*$^{-/-}$ and *tlr2,4*$^{-/-}$ mice) (Figure 6D).

Discussion

The gastrointestinal mucosa harbors the largest store of 5-HT in the body (approximately 95%). 5-HT is a signaling molecule implicated in a wide number of human physiological functions, being a key neuromodulator to the whole intestinal physiology (Mawe and Hoffman, 2013), and also can influence on the development and severity of gut inflammation, particularly in the setting of IBD (Coates et al., 2017). On the other hand, the innate immune system also plays an essential role in intestinal homeostasis, developing several crucial regulatory mechanisms and interactions; however deregulation of these mechanisms may be implicated in intestinal pathologies such as IBD (Uematsu and Fujimoto, 2010).

The results obtained in the present study demonstrate that NOD1 activation decreases SERT activity and molecular expression, mediated by the ERK/MAPK intracellular signaling pathway. This inhibitory effect on SERT activity would yield to an increment of extracellular 5-HT availability, which could increase NOD1 expression, resulting in a positive regulatory feedback. In agreement with our results, recent studies of our group showed that several TLRs downregulate 5-HT uptake with (Latorre et al., 2016; Mendoza et al., 2009) or without (Mendoza et al., 2012) the alteration of SERT expression. In this context, NOD1 not only inhibits SERT activity but also decreases SERT molecular expression, suggesting that long-term NOD1 activation may reduce SERT expression by transcriptional and/or post-transcriptional mechanisms. Thus, it seems that the detection of bacteria in the cytoplasm by NOD1 would cause an increase in serotonin levels, by inhibiting SERT, and contributing to a proinflammatory state for the destruction of a potential pathogen. At the same time, if the 5-HT increment levels were high, 5-HT would affect the intestinal functions, contributing to the symptoms of a gastrointestinal disorder as diarrhea, increase motility or abdominal pain.

Interestingly, results obtained at short stimulation periods (30 min) showed a more intense inhibition of SERT activity, suggesting that a soft short-stimulation would induce SERT inhibition and increasing 5-HT extracellular availability to promote a rapid inflammatory response;

however, if this low stimulation is maintained over time, the inflammatory involvement of serotonin would decrease or disappear, to avoid an undesired inflammatory response. Consistent with our results, previous studies regarding TLR3 and IL-10 effects on SERT activity have also shown effects dependent on the time and concentration used (Latorre et al., 2013; Mendoza et al., 2012).

NOD1 intracellular pathways involved in SERT downregulation at long-term were also studied. Our results suggest that neither RIP2 nor JNK intracellular pathway seem to be involved in the observed effects of NOD1, however, ERK inhibition neutralized NOD1's effect on SERT activity, suggesting the involvement of this downstream kinase. In agreement, the ERK/MAPK intracellular pathway has been implicated in SERT and 5-HT effects (Liu et al., 2004); however, another study, where ERK/MAPK downstream signaling was studied in neuronal SERT downregulation, showed that this pathway would not be involved in SERT modification, even if the same ERK inhibitor and concentration were used (Samuvel et al., 2005). Therefore, it seems that the involvement of the ERK pathway on SERT could depend on the cell model used.

Since NOD1 activation by DAP inhibits SERT activity and expression, 5-HT extracellular levels would be increased and might regulate NOD1 expression in the intestinal epithelium by a feedback mechanism. Our results have shown that 5-HT decreased NOD1 expression, suggesting a negative feedback regulation, that under physiological conditions (5-HT, 10^{-8} M) may maintain a low level of NOD1 expression. However, under elevated 5-HT concentration (10^{-4} M), that occurs in inflammatory pathologies (Mawe and Hoffman, 2013), the level of NOD1 expression increases, thus aggravating pathological NOD1 responses.

As discussed above, NOD1 activation could lead to a state of exacerbated or uncontrolled inflammation through a feedback mechanism with 5-HT, as it has been observed in prolonged and excessive activation of TLRs (Davies and Abreu, 2015). However, recent evidence have shown that NLRs and TLRs collaborate in mounting and balancing an efficient innate immune response to microbial pathogens (Foley et al., 2015). In this regard, and considering that TLR2 and TLR4 also inhibit SERT expression and activity (Latorre et al., 2016; Mendoza et al., 2009), we have analyzed the possible relation between TLR2 and TLR4 with NOD1 to clarify the role of NOD1 in homeostasis and inflammation. Recently, some works have studied the cooperation between NOD1 and TLR2 to enhance a defense response through inflammatory cells (Mercier et al., 2012), whereas other authors have suggested that NOD1 would not synergize with TLR2 in this process (Lee and Kim, 2007). Our results showed that TLR2 activation leads to an increment in NOD1 expression (mRNA and protein) in Caco-2/TC7 cells, and supporting this interdependence, NOD1 activation produced a decrement in TLR2 expression. Regarding TLR4, several authors have shown a synergic response with NOD1 (Huang et al., 2016), which agree

with our data where TLR4 activation by LPS decreased significantly NOD1 expression in Caco-2/TC7 cells. Furthermore, in the ileum of *tlr4*$^{-/-}$ mice, NOD1 expression increased, which corroborates the fact that TLR4 activation could downregulate NOD1 mRNA and protein expression. Surprisingly, *tlr2*$^{-/-}$ mice also showed an increased NOD1 expression in the ileum, although TLR2 activation upregulated NOD1 expression. Nevertheless, a recent analysis in a biological approach system revealed that TLR2 and TLR4 share the potential collaboration of crosstalk mechanisms to carry out cell responses (Muthukumar et al., 2014). Indeed, recently we have observed that *tlr2*$^{-/-}$ mice had an increased TLR4 expression and vice versa (Latorre et al., 2017), suggesting a compensatory mechanism between TLR2 and TLR4 expression that might explain our results.

Different PRRs could recognize the same fragment of a microorganism, thus developing a cooperative defense response. In fact, it has been shown that NOD1 could also recognize the same bacterial fragments as TLR2 heterodimers (Kawai and Akira, 2011). TLR4 is expressed, as TLR2, in the cell surface and recognizes LPS from Gram-negative bacteria. NOD1, able to detect DAP present in all bacteria membrane, could also recognize LPS, resulting in a collaborative response with TLR4 (Huang et al., 2016). Moreover, TLR4 activation seems to inhibit ERK/MAPK downstream kinase, which is the intracellular pathway involved in NOD1's effect on SERT (Shinohara et al., 2005). Colon analysis from TLR2 and TLR4 deficient mice showed no change in NOD1 expression. Unlike the ileum, in a previous work we observed that TLR2 and TLR4 did not appear to modulate their expression mutually in the colon (Latorre et al., 2017), suggesting that expression and regulation of TLRs and NOD1 may show a tissue-specific pattern. The diverse kind of cells presented in both ileum and colon could also explain these results; in addition, it is known that colon is more exposed to microbiota and presents different TLR expression (Davies and Abreu, 2015) since the levels of tolerance to bacteria are different from the ileum.

Interestingly, our results show a clear interaction between NOD1 and TLR2. Activation of both PRRs strongly decrease SERT expression, and therefore increase 5-HT bioavailability (Latorre et al., 2016). However, NOD1 activation would also decrease TLR2 expression, implying opposite effects on the extracellular levels of 5-HT, as a result to the activation of both PRRs. These data would be supported by the results obtained in intestinal tract of TLR2 deficient mice, which showed, in addition to the NOD1 increased expression, an increase in SERT expression (Latorre et al., 2016). At the same time, serotonin exerts counter-effects on the expression of NOD1 and TLR2; a pathological increment of 5-HT would increase NOD1 expression and decrease TLR2 levels, proving the negative crosstalk between NOD1 and TLR2.

The above results show the complicated network between PRRs, which seems to be controversial and unclear. Whereas several studies pointed to a synergic effect among several

PRRs (Santaolalla and Abreu, 2012), other results showed that TLRs could act in an antagonism crosstalk (Tan et al., 2014a).

In brief, our study determined that NOD1 activation inhibits SERT activity and expression in Caco-2/TC7 cells, being ERK/MAPK intracellular pathway involved. Moreover, the increment of extracellular 5-HT bioavailability, due to NOD1 activation, could also modify the receptor by itself in a feedback loop. Within this context, our work has also pointed out that the activation/depletion of other PRRs (as TLR2 and TLR4) would affect NOD1 and in turn, NOD1 activation could influence on TLR2, which also seems to modify SERT. The present work corroborate the impact of PRRs activation on serotonergic system, since previous studies in our laboratory have shown that activation of different TLRs also decrease the activity and expression of SERT (Latorre et al., 2016; Mendoza et al., 2009). All these data together suggest that intestinal serotonin is widely modulated by the innate immunity to maintain intestinal homeostasis and being also implicated in the control of intestinal inflammatory processes.

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Conflict of interest

The authors have no potential conflicts of interest to declare.

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Figure Legends

Figure 1. NOD1 activation decreases SERT activity and expression. A. 5-HT uptake was measured after 6 min incubation of 5-HT (0.2 µM). DAP concentrations assayed were 0.1, 1 and 10 µg/ml. The treatment periods were 30 min (short term), 6 h (medium term) and 1 day (long term). The results are expressed as the percentage of the uptake control (100%) and are the mean ± SEM of 10 independent experiments. Absolute control values were 7.27 ± 1.02, 4.28 ± 0.32 and 5.81 ± 0.26 pmol 5-HT/mg protein at 30 min, 6 hours and 1 day respectively. **B.** Real-time PCR analysis of the SERT mRNA expression level in cells treated for 1 day with DAP 10 µg/ml. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of three independent experiments. **C.** Immunodetection and quantification of SERT by western blot in cell lysate and apical membrane from Caco-2/TC7 cells treated with DAP (10 µg/ml) for 1 day. The quantitation of SERT protein in both cell lysate and apical membrane using β-actin as an internal control of the protein load (SERT/β-actin ratio) was also achieved. The results are expressed as a percentage of the control value and are the mean ± SEM of four independent experiments. **P<0.01 and ***P<0.001 compared with the corresponding control value (untreated cells).

Figure 2. Analyses of intracellular pathways involved in NOD1 effect on SERT. Cells were treated for 1 day with 10 µg/ml of DAP and/or gentamicin (1 µM, RIP2 intracellular pathway) (**A**), and/or SP600125 (1 µM, JNK intracellular pathway) (**B**), and/or U0126 (1 µM, ERK/MAPK intracellular pathway) (**C**). Uptake of 5-HT was measured after 6 min of incubation, and 5-HT concentration was 0.2 µM. The results were compared with untreated cells (control). The results, in all cases, are expressed as the percentage of the uptake control and are the mean ± SEM of four independent experiments. **P<0.01 and ***P<0.001 compared with the corresponding control value; $^{\#\#\#}$P<0.001 compared with treated cells with DAP. Analysis of SERT expression in cells treated with 10 µg/ml of DAP or/and U0126 (1 µM). **D.** SERT mRNA expression was analyzed by real-time PCR and relative quantification performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of four independent experiments. **E**. Immunodetection and quantification of SERT protein expression level by western blot in both cell lysate and apical membrane of Caco-2/TC7 cells. Results are expressed as SERT/β-actin ratio and are the mean ± SEM of five independent experiments. **P<0.01 and ***P<0.001 compared with the control value; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with treated cells with DAP.

Figure 3. 5-HT alters NOD1 expression. Caco-2/TC7 cells were treated with 5-HT 10^{-4} and 10^{-8} M for 1 day. **A**. Real-time PCR analysis of NOD1 mRNA expression level. Relative quantification was

performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of three independent experiments. **B.** Immunodetection of NOD1 by western blot in cell lysate of treated cells. **C.** Quantification of NOD1 protein relative expression in treated cells using β-actin as an internal control (NOD1/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of 3 independent experiments. **P<0.01 and ***P<0.001 compared with the control value; $^{\#\#}$P<0.01 compared among 5-HT treatments.

Figure 4. TLR2 and TLR4 modulate NOD1 expression. Cells were treated with LPS 30 µg/ml (TLR4 ligand), Pam3CSK4 10 µg/ml (TLR2/1 ligand) or Pam2CSK4 100 ng/ml (TLR2/6 ligand) for 1 day. **A**. Real-time PCR analysis of NOD1 mRNA expression in cells. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of four independent experiments. **B.** Immunodetection and quantification of NOD1 protein expression in cell lysate using β-actin as an internal control of the protein load (NOD1/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, and ***P<0.001 compared with the control value.

Figure 5. NOD1 modulates TLR expression. Cells were treated with DAP 10 µg/ml for 1 day. **A.** Real-time PCR analysis of TLR2 and TLR4 mRNA expression in treated cells treated. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SEM of five independent experiments. **B.** Immunodetection and quantification of TLR2 and TLR4 protein expression in cell lysate using β-actin as an internal control of the protein load (TLR2 and TLR4/β actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of five independent experiments. *P<0.05 and ***P<0.001 compared with the control value.

Figure 6. NOD1 expression in ileum (A, B) and colon (C, D) of *tlr2*$^{-/-}$ *tlr4*$^{-/-}$ and *tlr2,4*$^{-/-}$ mice. Real-time PCR analyses of NOD1 mRNA expression in ileum **(A)** and colon **(C)** of knockout mice. Relative quantification was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (WT = 1) and are the mean ± SEM of four animals. Immunodetection and quantification of NOD1 by western blot in ileum **(B)** and colon **(D)** of knockout mice, using β-actin as an internal control of the protein load (NOD1/β-actin ratio). The results are expressed as a percentage of the control value (100%) and are the mean ± SEM of four animals. *P<0.05 and ***P<0.001 compared with the control value.

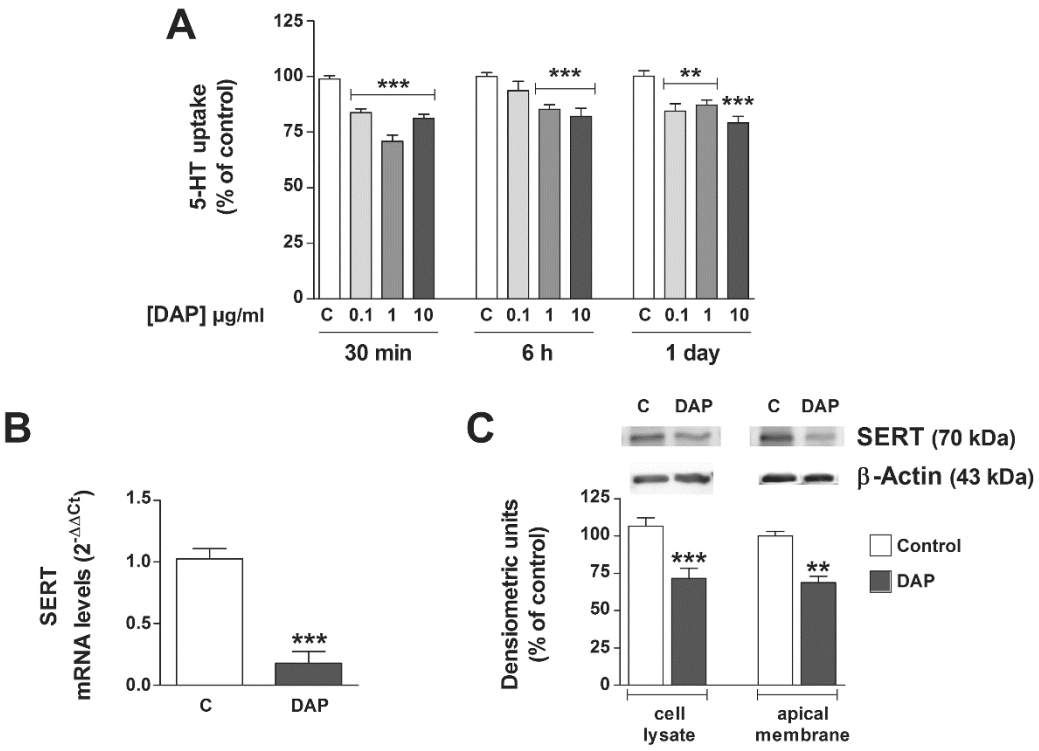

Figure 1

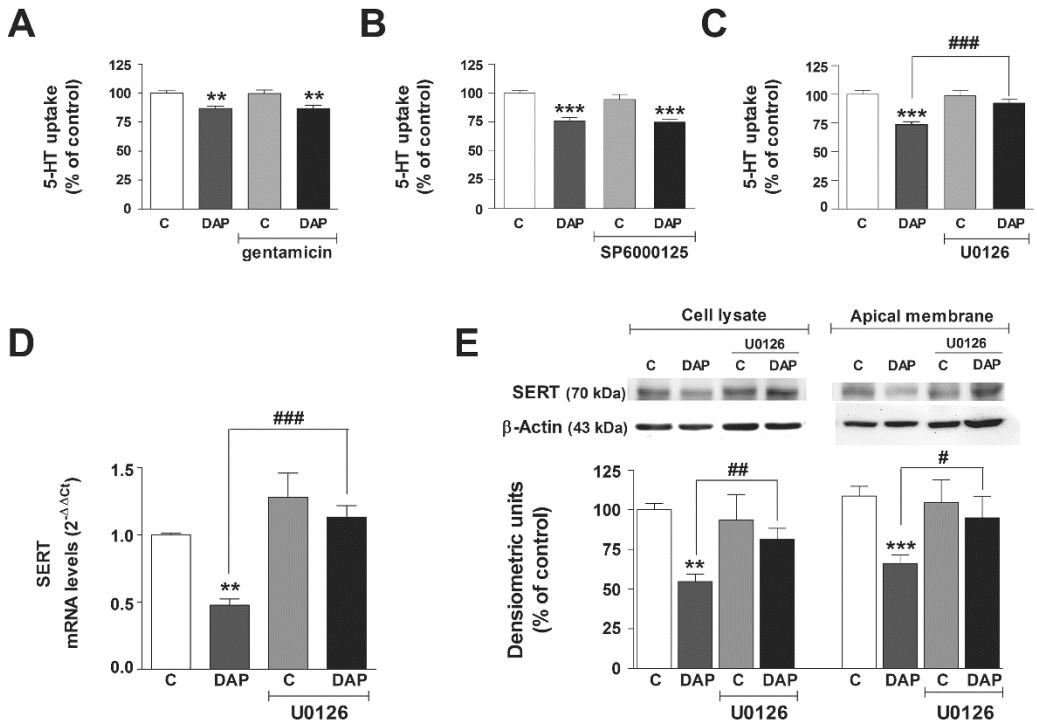

Figure 2

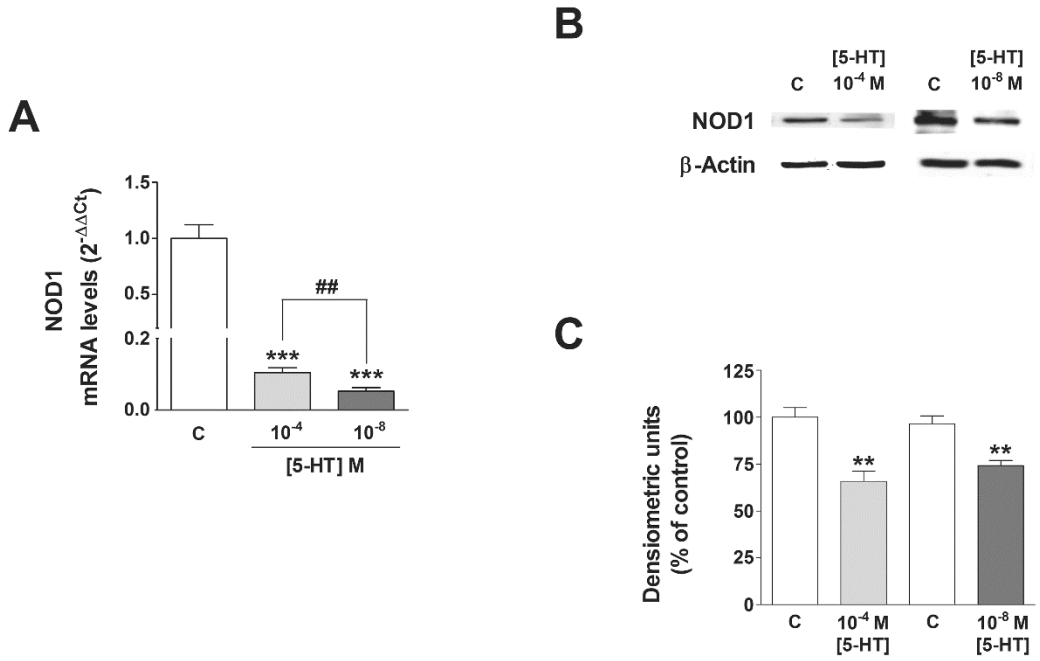

Figure 3

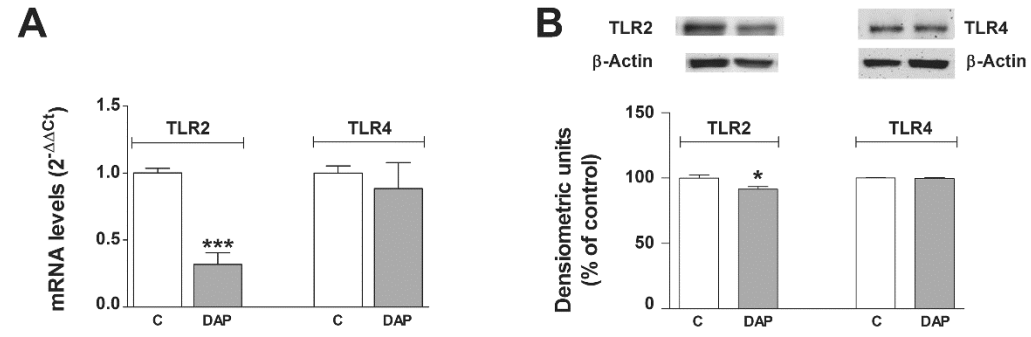

Figure 4

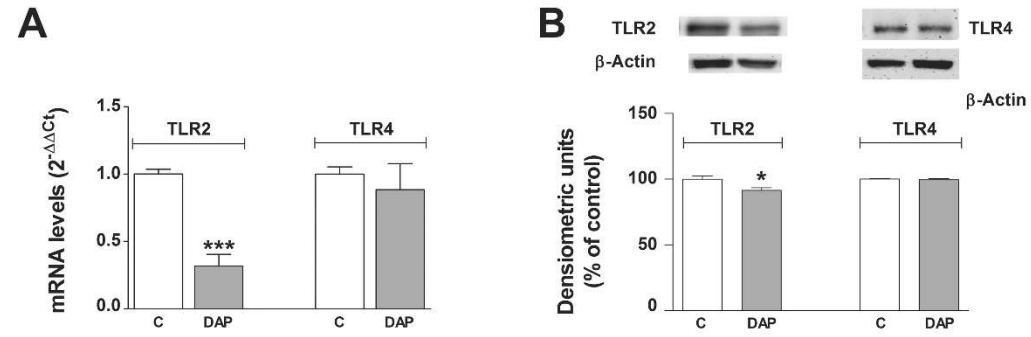

Figure 5

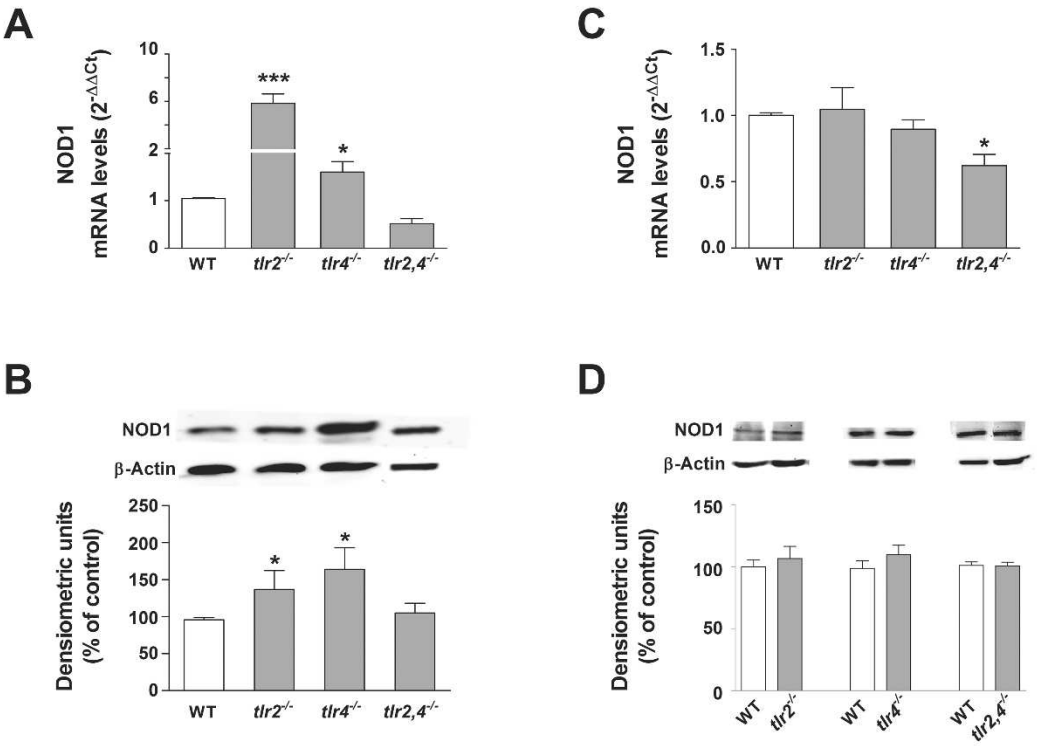

Figure 6