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Bacterial peptidoglycans as novel signaling molecules from microbiota to brain

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Mounting evidence indicates that gut microbiota exerts a broad range of effects on host physiology and development beyond the gastrointestinal tract, including the modulation of brain development. However, the mechanisms mediating the interactions between the microbiota and the developing brain are still poorly understood. Pattern recognition receptors of the innate immune system that recognize microbial products, such as peptidoglycans have emerged as potential key regulators of gut microbiome-brain interactions. Peptidoglycan-sensing molecules are expressed in the placenta and brain during specific time windows of development. Moreover, peptidoglycans are ubiquitously present in circulation and can cross the blood brain barrier. This review brings together the current evidence supporting a broad function of peptidoglycans well beyond host's immunity, extending to neurodevelopment and behavior.

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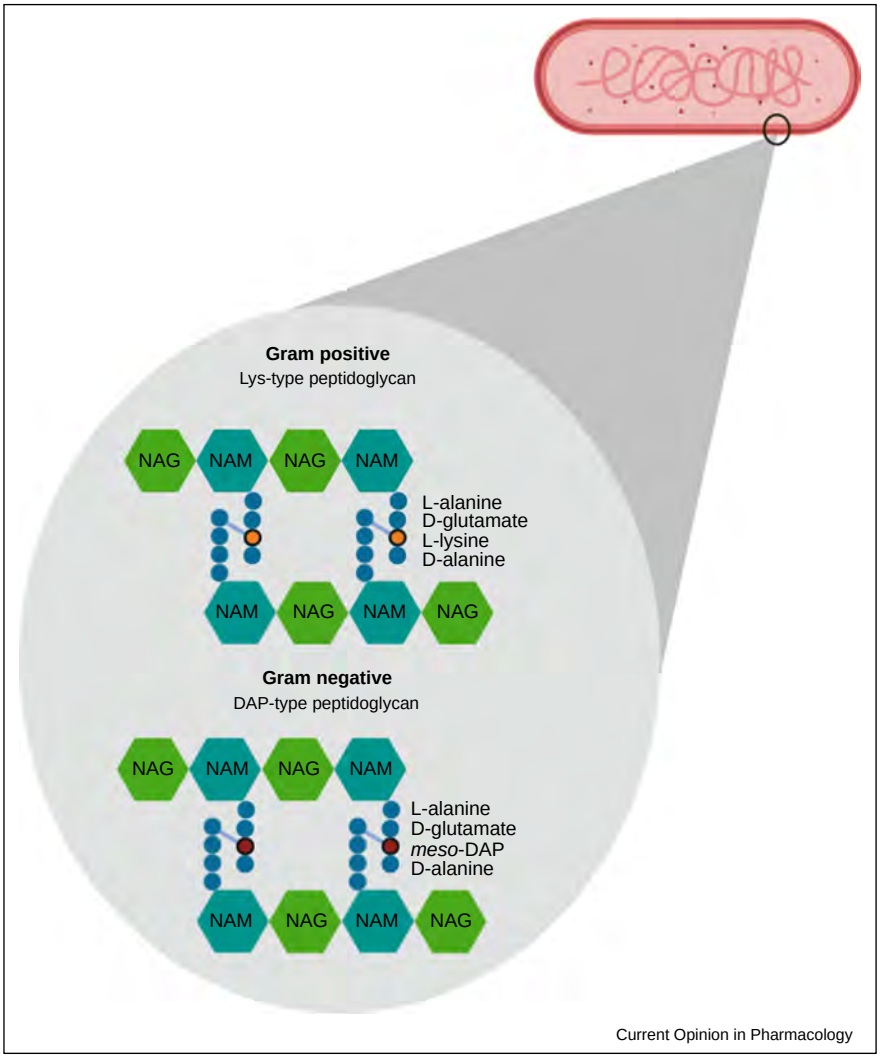
Introduction

It is now widely recognized that gut microbiota influences the development and function of the central nervous system (see Refs. [1,2]). Studies investigating the gut-brain axis have revealed a crucial role for the gut microbiota in a wide-range of neurodevelopmental processes, including blood-brain barrier (BBB) formation and integrity [3], microglial maturation and function [4,5], myelination [6–8], as well as several complex behaviors [9–11]. In addition, gut microbiota influences behavioral abnormalities observed in animal models of neurodevelopmental and psychiatric disorders [12,13]. The current challenge is to unravel the mechanisms mediating the early life gut microbiome–brain interactions to develop novel microbiota-modulating-based therapeutic interventions for infants and young children at risk for neurodevelopmental disorders. In this review, we provide a brief overview of the structure of peptidoglycan (PGN) and then highlight the emerging evidence supporting a novel role for PGN signaling from microbiota on normal brain development and behavior.

PGN structure and PGN-sensing molecules

Peptidoglycan (PGN, also called murein) is a unique and essential component of the bacterial cell wall that is absent in eukaryotic cells [14•,15]. It consists of glycan strands of two alternating β-1,4-linked sugars, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked by short peptides, containing two to five amino acids. The archetypical peptide structure is L-alanine, D-glutamate, a dibasic amino acid, D-alanine and D-alanine. Although the general glycan backbone is usually conserved in bacteria, the peptide moiety exhibits considerable diversity among Gram-positive and Gram-negative bacteria. Typically, the dibasic amino acid is L-lysine in Gram-positive bacteria, whereas Gram-negative bacteria contain *meso*-DAP (Figure 1). Remarkably, PGN is a highly dynamic structure that continuously undergoes remodeling during bacterial growth and maturation, causing PGN fragments to be shed from the cell wall into the environment, a process termed PGN turnover. The host innate immune system recognizes PGN fragments via a series of pattern recognition receptors (PRRs) that are highly conserved from insects to humans. Included among several important PGN-sensing molecules are the cytosolic NOD-like receptors (nucleotide-binding domain leucine-rich repeat containing receptors; Nod1 and Nod2) [16] and PGN recognition proteins (PGRPs, Pglyrp1-4) [17]. The structural requirements for PGN recognition by Nod1 and Nod2 have been extensively investigated [18–20]. The presence of the glycan moiety is not required for Nod1 recognition as the D-Glu-*meso*-DAP dipeptide (iE-DAP) is sufficient for the detection and innate immune activation of this PRR. In contrast, an intact NAM moiety is essential for Nod2 recognition, and this sugar must be attached to a dipeptide moiety (L-Ala-D-Glu or L-Ala-D-isoGln). Thus, Nod2 is strongly activated by muramyl dipeptides that are present in PGN of all Gram-positive and Gram-negative bacteria. Although all mammalian PGRPs are capable of binding PGN, only the Pglyrp 2 has *N*-acetylmuramyl-L-alanine amidase activity that

Figure 1



Bacterial PGN structure.
PGN consists of a carbohydrate backbone composed of repeated beta-1,4-linked sugars, cross-linked by short peptide chains. The sugar backbone is generally conserved among bacteria and is formed by *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) dimers. The common peptide structure is L-alanine, D-glutamate, a dibasic amino acid, which differs among Gram-positive (L-lysine) and Gram-negative bacteria (*meso*-diaminopimelic acid), and D-alanine.

hydrolyzes PGN between the sugar backbone and the peptide chain. Traditionally, the effects of PGN on host tissues have been extensively studied in the context of bacterial infection, gut inflammation, and as a target for antibiotic therapies. However, such a narrow perspective about the role of PGN-sensing molecules has been challenged by the emerging appreciation that PGN fragments are not exclusive to pathogens and are also abundantly produced by the indigenous gut microbiota, which normally does not trigger inflammation. In recent years, there has been an increasing recognition that PGN fragments (or so called muropeptides) and their sensing molecules may have adapted, in part, to mediate roles well beyond immunity, including the crosstalk between the gut microbiota and brain (see Ref. [21•]).

PGN from indigenous gut microbiota disseminate systemically

Traditionally, the translocation of bacterial products such as PGN has mainly been considered in the context of compromised intestinal epithelial barrier. In a landmark study, Clarke *et al.* provided experimental evidence that PGN fragments translocated from the lumen of the gut mucosa into the host circulation under basal conditions (in the absence of infection) [22••]. These authors colonized the guts of germ-free (GF) mice

with a non-pathogenic strain of *Escherichia coli* (labelled with [^{3}H] *meso*-DAP) via oral gavage and showed the presence of PGN systemically in sera and bone marrow cells. Critically, the translocated PGN fragments were bioactive as sera from stably colonized GF mice, but not from GF or antibiotic-treated mice, induced Nod-dependent NF-kB activation in HEK293 cell reporter assays. Moreover, they demonstrated that PGN recognition by Nod1 was sufficient to prime and restore neutrophils function in the absence of the microbiota. This elegant study shows a previously undescribed role for PGN in priming systemic innate immunity in the absence of infection, and a role for Nod1 receptor as a key homeostatic regulator *in vitro* and *in vivo*.

Recently, Huang *et al.* developed a monoclonal antibody (2E7) that targets MDP, the minimal bioactive PGN fragment common to all bacteria [23••]. Using a 2E7-based detection assay, MDP was found to be ubiquitously present in the serum of healthy humans, mice and monkeys. This was independently confirmed by the identification of muramyl acid in human serum using liquid chromatography-mass spectrometry. It is worth mentioning that the authors found a wide concentration range of this PGN fragment in healthy individuals (0.330–0.838 μg/ml). Moreover, patients suffering from systemic lupus erythematosus (SLS) and rheumatoid arthritis, two relatively common and severe autoimmune diseases, had higher levels of circulating PGN than healthy controls. These observations are consistent with earlier research showing the presence of antibodies specific for PGN in the CSF of patients with active multiple sclerosis, an inflammatory demyelinating disease of the CNS [24]. The presence of various PGN fragments has also been identified in cell culture fetal bovine serum and serum from healthy mice [25], suggesting that a homeostatic function of PGN signaling may have been previously underappreciated.

PGN fragments can be translocated into the brain and sensed by specific PRRs of the innate immune system

More than a century ago, it was suggested that bacterial products from gut microbiota may regulate sleep in mammals (see Refs. [26•,27]). In the 1980s and 90s, Krueger *et al.* detected PGN fragments in the CSF and urine of patients with sleep disorders [28,29], and also demonstrated that some PGN-derived muramyl peptides could regulate slow-wave sleep in rabbits [30,31]. The recent realization of the size and complexity of the human microbiome and its wide-ranging impact on host physiology and development has prompted a reevaluation of the possible role of PGN in the brain. In a recent study from our laboratory, we confirmed the presence of PGN fragments in the serum of healthy juvenile-specific-pathogen-free mice [32•]. As expected, the PGN levels in the serum of juvenile GF mice was very low or at the limits of detection, indicating that the microbiota is the main source of PGN fragments. The small amount of PGN found in the GF mice most likely is due to the detection of dead bacteria in their sterile diet. Importantly, we detected the presence of PGN fragments in the developing brain of healthy mice and showed that brain PGN levels increase in parallel with the postnatal bacterial colonization processes [32•]. However, we still need to identify the specific types of PGN fragments that can cross the BBB and determine how their structure and function relate to the composition of the gut microbiota during postnatal development. Critically, we found that all members of three families of PRRs that recognize PGN (i.e. PGRP 1–4, Nod-like receptors, and toll-like receptor 2) are expressed during specific time windows of postnatal brain development. Moreover, the expression of PGN-sensing molecules PGRP1-4 and Tlr2 is sensitive to perturbations of the gut microbiota (e.g. GF condition and perinatal antibiotic exposure) within the developing brain. In addition, we found brain region-dependent and sex-dependent differences in the expression of PGN-sensing molecules in the developing brain. Specifically, we found that three of the four PGRPs (i.e. *Pglyrp2*, *Pglyrp3* and *Pglyrp4*) are expressed at higher levels in females than in males, whereas Nod-like receptors (*Nod1* and *Nod2*) and *Pglyrp1* are expressed at higher levels in males. These differences were more pronounced in the developing prefrontal cortex, a key region implicated in a range of neurodevelopmental and psychiatric disorders including autism spectrum disorder (ASD) [33–35], that is often co-morbid with gastrointestinal problems and an altered gut microbiota composition [1]. These novel findings suggest that a highly dynamic and sensitive period exists, during which microbial colonization of the gut can influence brain development in an age-, region- and sex-specific manner via PGN signaling.

Putative mechanisms for PGN transport into neurons and glial cells

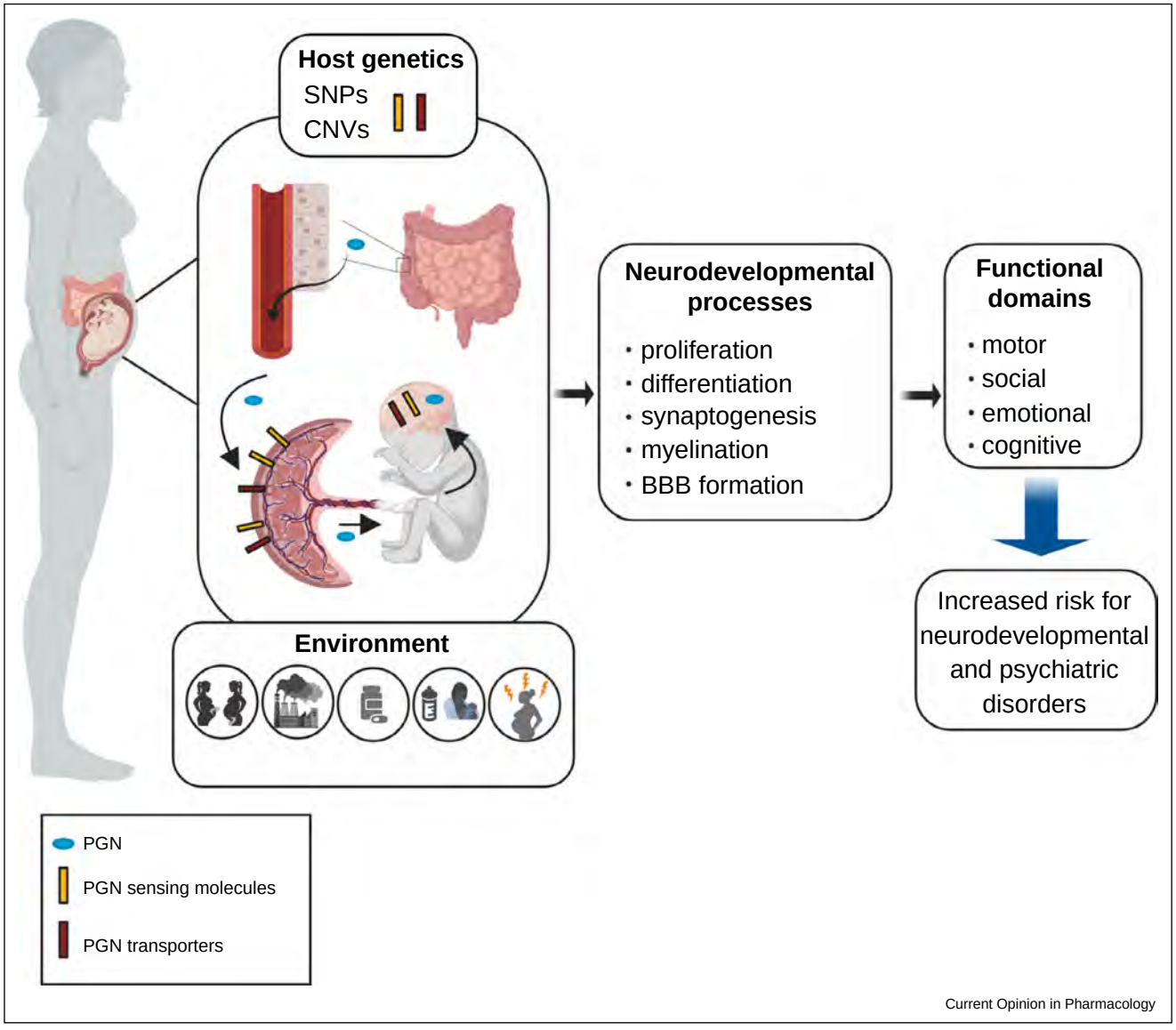
Although PGN-sensing molecules are expressed in the brain, the mechanisms whereby PGN fragments from indigenous microbiota enter brain cells (e.g. neurons, astrocytes, microglia and oligodendrocytes) and regulate developmental processes under physiological conditions remain poorly understood. In non-neuronal cells, three members of the proton-coupled oligopeptide transporter family (SLC15), PepT1 (SLC15A1), PepT2 (SLC15A2), and PhT1 (SLC15A4) have been implicated in the transport of PGN fragments [36]. These transporters are integral membrane proteins that mediate the cellular uptake of di-peptides and tripeptides and peptidomimetics. For example, in human intestinal epithelial cells, PepT1 is involved in the transport MDP and triDAP [37–39], while in lung epithelial cells, PepT2 has been implicated in the transport of γ-iE-DAP, which stimulates NOD1 [40]. Recently, we examined the expression profile of PepT1 in various brain regions (e.g. prefrontal cortex, striatum, cerebellum and hippocampus) during normal postnatal development in mice. In all brain regions examined, PepT1 mRNA and protein levels were higher during the first postnatal days of life than in adults [32•].

Consistent with a previous study [41], we also found a similar pattern of expression profile for PepT2. However, the expression of PhT1 remains similar throughout postnatal development (Diaz Heijtz Lab, unpublished results).

Emerging roles of PGN-sensing molecules on brain development and behavior

There is growing evidence that in addition to the traditional immune functions, PGN-sensing molecules can also regulate host developmental processes under normal physiological conditions (see Ref. [15]). For instance, PGN derived from Gram-negative bacteria is sufficient and necessary for the genesis of gut-associated lymphoid tissue (i.e. isolated lymphoid follicles) through recognition by Nod1 in epithelial cells [42]. Previous studies have demonstrated that several classical immune molecules, including members of the Toll-like receptor family are able to modulate

Figure 2



Schematic representation of the proposed PGN dissemination from maternal microbiota to the fetal brain.
PGN fragments derived from indigenous maternal gut microbiota translocate from the lumen of the gut mucosa into the bloodstream, reaching the placenta. PGN sensing molecules and transporters expressed in the placenta and in the fetal brain recognize PGN fragments, which can then cross the placenta and enter the fetal brain. PGN trafficking is affected by both host genetics, for example, single nucleotide polymorphisms (SNPs), and copy number variants (CNVs) in genes coding for PGN sensing molecules and/or transporters and external factors such as mode of delivery, environment, antibiotics infant feeding practices and maternal stress. In the fetal brain, PGN fragments affect key neurodevelopmental processes that may lead to atypical motor, social, emotional and cognitive development and increased risk for neurodevelopmental and psychiatric disorders.

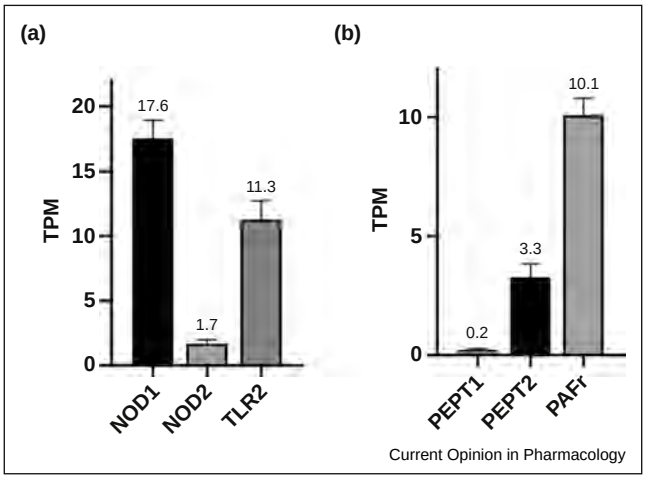
key neurodevelopmental processes such as neurite outgrowth, neuronal proliferation and differentiation [43–47]. In addition, a member of the PGRP family (PGRP-LC) is required for the induction and sustained expression of homeostatic synaptic plasticity in the nervous system of fruit flies [48]. Recently, work from our laboratory has shown that the absence of PGN-recognition protein 2 (Pglyrp2) leads to alterations in the expression of the autism risk gene *c-Met* and brain-derived neurotrophic factor, both implicated in the formation and modulation of brain circuits [32•]. Moreover, juvenile Pglyrp2 knock-out (KO) mice exhibit sex-dependent changes in social behavior, without any changes in motor activity or anxiety-like behavior. This phenotype is more pronounced in male offspring. More recently, we discovered that the absence of Pglyrp2 leads to major sex-dependent alterations in motor and anxiety-like behavior later in adulthood [49]. Interestingly, there is also evidence supporting a role for Pglyrp1 (also called tag7) in the homeostatic regulation of sleep [50]. Taken together, these findings support a novel role for PGRPs as potential key regulators of normal brain development, function and behavior. However, further studies, using advanced transgenic mouse models (e.g. brain-specific conditional KO) are required to clarify the role of brain-specific expressed Pglyrp2 and other PGRPs on motor, socio-emotional and cognitive development.

Emerging roles of maternal derived circulating PGN on placenta and fetal brain development

The maternal-fetal environment plays a crucial role in the development and long-term function of the fetal brain, including later-life susceptibility to mental disorders. Indeed, several studies have linked maternal infection and inflammation during pregnancy with ASD and schizophrenia [51–53]. It is increasingly recognized that the indigenous maternal gut microbiota is a common denominator by which a wide range of environmental risk factors such as diet, preterm birth, and stress can have long lasting effects on the neurodevelopment of the offspring (see Refs. [54,55]). During prenatal life, the developing fetus receives oxygen and nutrients along with other bioactive compounds from the maternal circulation. Therefore, alterations in the composition and metabolic activity of maternal gut microbiota could have a major impact on fetal brain development. The importance of the maternal microbiome for fetal brain development is exemplified by two studies in GF mice [3,4] that showed that the absence of microbiota leads to perturbations in BBB formation, and maturation of microglia (the resident macrophages of the brain and major contributors to brain circuits formation) already observed during prenatal life.

Recently, it has been proposed that PGN could be an important mediator for bacteria-host communication at the maternal-fetal interface, since PGN reaches maternal circulation not only during bacterial infections, but also from the maternal gut microbiota in healthy pregnancy [22••] (see Figure 2). Consistent with this notion, Humann *et al.* demonstrated that components of the bacterial cell wall can cross the placenta and affect the developing brain leading to long-term postnatal behavioral consequences [56]. Specifically, they showed that the PGN-teichoic acid complex of *Streptococcus pneumoniae* (a major pathogen causing infections such as meningitis) can cross the mouse placental barrier and fetal BBB through the platelet activating factor receptor (PAFr). Interestingly, this complex induces the transcription factor *FoxG1* in the fetal cortex via activation of Tlr2, leading to neuroproliferation in the ventricular zone of the cortex that results in a 50% greater density of neurons in the cortical plate. This abnormal neuroproliferation was associated with altered cognitive development [56]. Previous studies have implicated Tlr2 in immune response to infection, stress and injury [57]. However, our laboratory has recently shown that the expression of Tlr2 in the developing brain is highly sensitive to manipulations of the indigenous gut microbiota [32•]. Taken together, these observations support the hypothesis that Tlr2 and other PGN-sensing molecules may have a dual role in immunity and in neural development. However, little is known about the expression of PRRs and putative PGN transporters in the developing human brain and placenta. Such information is essential to better understand signaling pathways through which the maternal gut microbiota can influence human fetal brain development. Taking advantage of an existing public database [i.e. the Human

Figure 3



Expression of PGN sensing molecules and putative PGN transporters in the placenta.
mRNA expression data for **(a)** PGN sensing molecules NOD1, NOD2, TLR2 and **(b)** putative PGN transporters PEPT1, PEPT2 and PAFr were downloaded from The Human Protein Atlas, HPA RNA-seq database (https://www.proteinatlas.org/). For each gene, the mRNA amount is indicated as 'Transcript Per Million' (TPM). Data are presented as mean ± SEM (n = 8).

Protein Atlas; (https://www.proteinatlas.org/)] [58]. Containing transcriptomic datasets from different human tissues, we investigated whether PGN-sensing molecules are expressed in the human placenta. We have ascertained that NOD1, NOD2, TLR2, as well as the putative transporters PepT2 and PAFr are abundantly expressed in the placenta (Figure 3). Collectively, these observations raise the possibility that PGN fragments from the maternal indigenous microbiota may also be capable of influencing the developing fetal brain, and that disruption of components of this signaling pathway could lead to abnormal motor, social, and cognitive development.

Conclusions and future perspectives

In the last decade, our understanding of the PGN biology has been rapidly expanding from innate immunity to an unprecedented role in neurodevelopment, by the growing appreciation that animals, including humans harbor a diverse and complex indigenous microbiota which can communicate with distal organs such as the brain. How indigenous bacteria influence the developing brain and later-life behavior remains to be fully understood. The gut microbiota contains trillions of bacteria producing a diverse 'peptidoglycome' [15] which can disseminate systematically and reach the brain. PGN sensing molecules are abundantly expressed in the placenta and developing brain during specific time windows of perinatal development. Detailed characterization of the specific types of PGN that can cross the BBB under physiological conditions, and how they are transported into brain cells will provide key mechanistic insights into how indigenous bacteria can directly influence brain development, function and behavior. This knowledge may provide novel biological mechanisms contributing to neurodevelopmental and psychiatric disorders such ASD, as well as new therapeutic targets for pharmacological intervention.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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