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Abstract

It is becoming increasingly recognized that the gut microbiota profoundly influences many aspects of host development and physiology, including the modulation of brain development and behavior. However, the precise molecular mechanisms and signaling pathways involved in the communication between the microbiota and the developing brain remain to be fully elucidated. Germline-encoded pattern recognition receptors that recognize conserved microbial molecular signatures, such as bacterial surface molecules (*e.g.*, peptidoglycans) have emerged as potential key regulators of gut microbiota-brain interactions. In this review, we highlight current evidence supporting multiple and essential roles for peptidoglycans and their sensing molecules well beyond innate immunity, extending to neurodevelopment and behavior. In addition, the possible implications of the peptidoglycan signaling pathway for the pathogenesis of neurodevelopmental disorders such as autism spectrum disorder is considered.

The Gut Microbiota: A Key Regulator of Neurodevelopment and Behavior

The mammalian gastrointestinal tract harbors a dynamic and complex community of microorganisms, including bacteria, archaea, fungi, and viruses–known collectively as the gut microbiota. Microbial colonization is an evolution-driven process that has provided mutual benefits to both microbes and the mammalian host. The first major exposure to a complex microbiota occurs during birth via vertical mother-to-infant microbial transmission (i.e., when infants are exposed to their maternal vaginal, fecal and perineal microbes), and is a pivotal event early in life determining the initial assembly of the infant gut microbiota [1] (Box 1). The bacterial population is currently the most well characterized. Although the ratio between bacteria to human cells is close to one [2], the total number of bacterial genes in the collective human microbiome is estimated to be 2-20 million, exceeding the human genome by at least a factor of 100 [3, 4]. Many of these bacterial genes encode enzymes that can perform metabolic functions not achievable by the host and produce metabolites that can impact host health and disease. Over the past decades, studies have demonstrated that gut bacteria interact and perform numerous critical functions for their host, including dietary energy extraction, the production of vitamins, protection against invading gut pathogens [5, 6], as well as promoting the development and function of the immune system [1, 7]. Recent investigations have revealed that the gut microbiota exerts a broader range of effects on host physiology and development beyond the gastrointestinal tract, including the modulation of brain development and behavior [8, 9].

In a landmark study, Sudo and colleagues in 2004 showed that postnatal bacterial colonization affects the early life programming of the hypothalamic–pituitary–adrenal (HPA) axis, the central stress response system [10]. More specifically, they took advantage of germ-free (GF; devoid of bacteria throughout life) mice to assess how the absence of microbiota affects the HPA-axis response to various stress modalities. In adult GF mice, exposure to a mild restraint stress induced an exaggerated release of adrenocorticotropic hormone (ACTH) and corticosterone compared with control mice with a normal composition of microbiota and no specific pathogens (known as specific-pathogen-free mice; SPF). The exaggerated HPA stress responses of GF mice were fully reversed through colonization with *Bifidobacterium infantis*. Several years later, Diaz Heijtz and colleagues demonstrated that the absence of microbiota profoundly affects motor activity and anxiety-like behavior, the

expression of four canonical pathways (i.e., Krebs cycle, synaptic long-term potentiation, steroid hormone metabolism and cAMP-mediated signaling) and the turnover of neurotransmitters, including dopamine and serotonin [11]. Importantly, microbiota reconstitution of GF mice in early life partially normalized their behavioral phenotypes, whereas colonization of adult GF mice had no effect. Together, these observations led to the concept of a sensitive period early in life, in which microbial colonization of the gut can influence brain development, function and behavior. Around the same time other groups independently published similar findings ([12, 13]; see online Milestone 18: The microbiota-gut-brain axis in Nature Milestones in Human Microbiota Research: https://www.nature.com/immersive/d42859-019-00041-z/index.html). Since then, several studies have demonstrated that the gut microbiota modulate a wide-range of neurodevelopmental processes, including blood-brain barrier (BBB) formation and integrity [14], microglial maturation and function [15, 16], and myelination [17-19], as well several complex behaviors (see Refs. [20, 21]). One important concept that has emerged from these preclinical studies is that certain influences of the gut microbiota on the brain are sex specific [22]. Other studies showed that gut bacteria influence behavioral abnormalities and associated brain pathology in animal models of neurodevelopmental and psychiatric disorders [23-25]. These preclinical and other clinical findings lent support to the hypothesis that gut microbiota may also influence the development of the human brain, and that its disruption could contribute to the pathogenesis of these disorders [25, 26].

It is now increasingly recognized that neurodevelopmental disorders such as autism spectrum disorder (ASD) are often co-morbid with gastrointestinal (GI) problems. For example, children with ASD have been reported to be six to eight times more likely to suffer from one or more chronic GI problems than typically developing children [27]. These GI issues include frequent abdominal pain, diarrhea, constipation, gaseousness, and painful stooling. Moreover, some studies have found a strong positive association of autism severity with GI dysfunction [28, 29]. These associations of ASD with increased prevalence of GI issues and reports of altered gut bacterial composition in this population motivate further explorations into the precise molecular mechanisms mediating the crosstalk between the gut microbiota and the developing brain [26, 30]. The gut microbiota contains trillions of bacteria which are source of a diverse peptidoglycome, bacterial peptidoglycan motifs that

are shed from the cell wall as bacteria divide [31, 32]. In this review, we highlight the emerging evidence supporting novel roles for bacterial peptidoglycans (PGN; a unique and essential component of the bacterial cell wall that is absent in eukaryotic cells) in neurodevelopment and behavior, after a general overview of key biological signaling pathways implicated in the microbiota-gut-brain axis. Finally, we discuss how alterations in the PGN signaling pathways early in life could impact neurodevelopmental trajectories and increase risk for neurodevelopmental disorders.

Biological signaling pathways in the microbiota-gut-brain axis

It has long been known that a complex bidirectional communication network exits between the gut and the central nervous system (CNS), referred to as the gut–brain axis. This network includes the sympathetic and parasympathetic branches of the autonomic nervous system, the enteric nervous system, neuroendocrine and neuroimmune pathways. In recent decades, the gut microbiota has been recognized as an important “third component” to the gut-brain axis, thus leading to the new concept of the microbiota-gut-brain axis. However, the precise molecular mechanisms mediating interactions between gut microbes and the brain have yet to be clearly defined. Emerging evidence suggests the involvement of multiple potential direct and indirect pathways (Figure 1), including signals carried by neuronal circuits (e.g., bidirectional vagus nerve-to-brain communication, and the enteric nervous system), activation of immune responses (e.g., cytokine and chemokine release within the gut or elsewhere that subsequently influence the brain), gut hormone signaling, tryptophan metabolism, and the production of microbial metabolites that indirectly or directly influence the brain, including bacterial fermentation metabolic by-products, such as short chain fatty acids (SCFAs; propionate, butyrate and acetate) (see Ref. [33]).

Undoubtedly, one of the most direct routes for the microbiota to communicate with the brain is through the vagus nerve [34]. Studies have shown that several probiotics (beneficial bacteria that produce health outcomes such as *Bifidobacterium* and *Lactobacillus* families, which do not possess pro-inflammatory lipopolysaccharide chains) utilize the vagus nerve signaling to communicate with the brain and influence behavior [35]. For example, the beneficial effects of *Lactobacillus (L.) rhamnosus* JB1 on stress-induced anxiety- and depression-related behavior are abolished by vagotomy [36]. Similarly, the vagus nerve is a

central gut-brain communication pathway by which *L. reuteri MM4-1A* rescue social deficits in several mouse models of ASD, including a genetic model (Shank3B mutant mice) [37], three environmental models (valproic acid, GF and maternal high-fat diet) [37, 38], and an idiopathic model (BTBR T+ Itpr3tf/J) of ASD [37]. However, the precise mechanisms by which *L. reuteri* interacts with the vagus nerve is not fully understood. It has recently been discovered that sensory cells from the GI tract with a primary endocrine function (enteroendocrine cells; referred to as neuropods) possess axon-like basal processes that form synapses with vagal afferents [39]. Therefore, it will be interesting to explore whether the actions of probiotics such as *L. reuteri* involve signaling through the neuropods. A recent optogenetic study showed that activation of gut-innervating vagal sensory ganglion neurons can also modulate reward-relating behavior (*e.g.*, self-stimulation behavior and conditioned place preference) and dopamine release from the substantial nigra in mice, indicating that the vagal-gut-to brain axis is also an important component of the brain reward system [40].

In recent years, there has been a keen interest in the role of SCFAs since they exert many beneficial effects on host physiology (e.g., they enhance the integrity of the intestinal epithelial barrier and protect against inflammation) [41]. As SCFAs can interact with the gut–brain axis through several different mechanisms including the modulation of host chromatin structure and gene transcription as well as the activation of G-protein-couple receptors, exposure to these bacterial metabolites during critical time windows of development might have important implications for early-life programming of the brain and behavior. So far, there is evidence that acetate can cross the BBB at physiological levels [42], but it is unclear to what extend propionate and butyrate cross into the brain. Nevertheless, some studies have demonstrated that SCFA butyrate can restore the integrity of the BBB and that a mix of SCFAs can reverse aberrant microglia phenotypes in adult GF mice [14], indicating that SCFAs may be one of the signaling pathways by which bacteria modulate the brain. Several commensal bacteria have also been shown to produce neurotransmitters such as dopamine, serotonin, and metabolites that enter circulation and exert a range of consequences outside the GI tract [43]. With regard to the brain, a new study has shown that direct metabolites of the gut microbiome (e.g., imidazole propionate) or products of the combinatorial metabolism between the microbiome and host (e.g., 3-indoxyl-sulfate, trimethylamine-N-oxide, and phenylacetylglycine) are present in the forebrains of healthy mice as early as the

168 neonatal period and remain into adulthood [44]. However, the role of these metabolites in
169 neurodevelopment remain poorly understood. Recently, studies have raised the possibility
170 that bacterial-derived products such as PGN might directly affect the developing brain and
171 behavior through central activation of pattern recognition receptors (PRRs) of the innate
172 immune system [45].
173

174 Pattern recognition receptors as potential key regulators of gut microbiota-brain 175 interactions

176 There is growing recognition that PRRs have been adapted to mediate roles far beyond
177 innate immunity, including host homeostasis and development [46]. PRRs are a series of
178 germline-encoded host sensors that recognize highly conserved microbial molecular
179 signatures or "motifs" such as bacterial surface molecules (e.g., PGN), often referred to as
180 microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). Included among
181 several important PRRs of the innate immune system are membrane-bound Toll-like
182 receptors (TLRs; [47, 48]), cytosolic NOD-like receptors (nucleotide-binding domain leucine-
183 rich repeat containing receptors; Nod1 and Nod2 receptors, [49, 50]) and PGN recognition
184 proteins (PGRPs, Pglyrp1-4; [51, 52]). Traditionally, the expression and function of PRRs have
185 been investigated in the context of pathogenic microorganisms. However, the microbial
186 motifs that activate PRRs are not restricted to pathogens, since they include structural
187 molecules such as lipopolysaccharide, a component of the outer membrane of Gram-
188 negative bacteria, and PGN, common to almost all bacterial cell walls ([46]; Figure 2).
189

190 There is evidence from both invertebrates and vertebrates that motifs derived from
191 commensal bacteria interact with PRRs to promote developmental processes and maintain
192 homeostasis of the host [53]. For example, bacterial motifs have been implicated in the
193 regression of a juvenile-specific epithelium that facilitates the colonization of the Hawaiian
194 squid by the marine symbiotic bacterium *Vibrio fischeri*, which provides luminescence to the
195 squid (Figure 3A) [54]. Moreover, the maturation of mammalian gut associated lymphoid
196 tissues (i.e., isolated lymphoid follicles; ILFs) is triggered by the exposure of intestinal
197 epithelial cells to PGN motifs derived from commensal Gram-negative bacteria during
198 postnatal life, thus leading to the activation of the cytoplasmic PRR Nod1 and secretion of
199 factors that stimulate the proliferation of isolated ILFs (Figure 3B) [7, 55] . Furthermore,

200 accumulating data indicate that PGN motifs from commensal bacteria can also diffuse
201 through the epithelial barrier in the intestine and reach the systemic circulation. A seminal
202 study by Clarke and colleagues showed that gut microbiota is a source of PGN that
203 systemically primes the innate immune system [56]. Specifically, these authors showed
204 translocation of the meso-diaminopimelic acid (meso-DAP)-type PGN from non-pathogenic
205 Gram-negative bacteria from the intestinal mucosa to neutrophils in the bone marrow of
206 mice. The activation of the cytoplasmic PRR Nod1 by meso-DAP-type PGN was sufficient to
207 prime and restore neutrophil function in the bone marrow of mice with a manipulated
208 microbiota (i.e., GF condition or antibiotic treated mice), thus revealing a previously
209 undescribed role for PGN in priming systemic innate immunity in the absence of infection
210 (Figure 3C). Other groups have recently detected traces of various PGN fragments in fetal
211 bovine serum (the most widely used serum-supplement for *in vitro* cell culture) and serum of
212 healthy mice [57], suggesting that a homeostatic function of PGN signaling may have been
213 previously underappreciated.
214
215 A new antibody-based detection assay for the PGN muramyl-L-alanyl-D-isoglutamine (MDP)
216 was described recently. The authors showed that this PGN monomer is ubiquitously present
217 in serum or plasma of healthy humans, rodents and non-human primates [58]. It should be
218 noted that a wide concentration range of MDP levels were found in healthy individuals from
219 European or Asian ancestry. Approximately 75 % of all individuals had a range between 0.16-
220 1 µg/ml, 22.5% between 1-4 ug/ml and at the extreme end of the spectrum either very high
221 levels (4-5 ug/ml) or below the level of detection (see Figure 2d in Ref. [58]). However,
222 patients suffering from autoimmune diseases (i.e., rheumatoid arthritis and systemic lupus
223 erythematosus) had higher levels of circulating MDP than healthy controls, thus raising the
224 possibility that disturbance of equilibria between PGN motifs and their sensing PRRs could
225 contribute to host pathologic states. This finding is consistent with earlier work from Laman
226 and colleagues showing the presence of antibodies specific for PGN in the cerebral spinal
227 fluid (CSF) of patients with active multiple sclerosis (MS), and inflammatory demyelinating
228 disease of the CNS [59]. For further details about the roles of PGN as driver of chronic brain
229 inflammation in MS and its experimental autoimmune encephalomyelitis models, see a
230 recent review by the latter authors [60] .
231

232 **Bacterial PGN motifs can be translocated into the brain and sensed by innate immune**
233 **PRRs**

234 The notion that bacterial products derived from microbiota could influence brain functions is
235 not new [61, 62]. In fact, several decades ago, it was shown that some MDP-type PGN induce
236 prolonged (6-12 h) increases in slow-wave sleep (SWS) in rabbits, rats and monkeys, and
237 enhanced amplitude of EEG slow waves (0.5-4.0 Hz) during bouts of SWS. Of note, the
238 somnogenic properties of muramyl peptides are dependent upon the precise biochemical
239 structure (see Refs. [63, 64]), and can be distinguished from other activities of muramyl
240 peptides such as induction of fever and adjuvant activity (see Ref. [61]). Moreover, PGN
241 fragments were detected in the CSF and urine of patients with sleep disorders [65, 66], thus
242 implicating PGN in the pathophysiology of sleep disorders. New scientific discoveries
243 showing a previously unappreciated complexity of the human microbiome and its wide-
244 ranging impact on host health and disease has triggered a re-evaluation of the possible role
245 of microbial molecules and metabolites on host tissues, including the brain. Recently,
246 Arentsen and colleagues showed the presence of PGN fragments in the serum of healthy
247 juvenile mice (*i.e.*, specific-pathogen-free mice; see also Figure 2e in Ref. [58]). PGN levels in
248 the serum of juvenile GF mice were found to be very low or at the limits of detection,
249 confirming that microbiota is the main source of PGN in the circulation. The same authors
250 showed the presence of PGN fragments in the normal developing mouse brain, which
251 increases in parallel with the postnatal bacterial colonization processes ([45]; Figure 4A). It
252 was also demonstrated that two families of PRRs that specifically detect PGN [i.e., PGN-
253 recognition proteins (PGRPs) and NOD-like receptors] and the PGN transporter PepT1 are
254 highly expressed in the developing brain during distinct windows of postnatal mouse
255 development. These findings raise the possibility that PGN motifs (for example, muramyl
256 dipeptides and mesoDAP-type PGN) may differentially affect brain development depending
257 upon the postnatal age. At least two PGN-sensing molecules (Pglyrp2 and Nod1) have been
258 found to be highly expressed in neurons in several brain regions including the prefrontal
259 cortex, hippocampus and cerebellum, thus indicating potential direct effects of PGN on
260 neurons ([45]; Figure 4B). Interestingly, the peak expression of Nod1 receptors coincides
261 with the period of synaptogenesis in rodents. These molecules are also expressed in
262 astrocytes and microglia (albeit at lower levels than in neurons). In addition, oligodendrocyte
263 development and maturation may be affected by PGN since they express some PGN-sensing

264 molecules such as Nod1, according to an open access database containing transcriptomic
265 datasets from oligodendrocytes from different developmental stages
266 (https://ki.se/en/mbb/oligointernode). It is worthwhile pointing out that the expression of
267 PGN-sensing molecules (PGRP1-4, Nod1, Nod2, Tlr2, PepT1) is highly sensitive to
268 manipulations of the gut microbiota (*i.e.*, GF condition and perinatal antibiotic exposure)
269 within the developing brain [45]. Moreover, there are brain region-dependent and sex-
270 dependent differences in the expression of PGN-sensing molecules during postnatal brain
271 development. Interestingly, these differences appear to be more pronounced in the
272 prefrontal cortex, a key region implicated on a broad range of neurodevelopmental and
273 psychiatric disorders [67]. Taken together, these findings suggest that a highly dynamic and
274 sensitive period exists, during which postnatal microbial gut colonization can influence
275 developmental brain trajectories in an age-, region- and sex-specific manner via PGN
276 signaling.
277

278 **Emerging roles of PGN-sensing molecules on brain development, function and behavior**

279 There is a growing body of literature demonstrating that classical peripheral immune
280 molecules can have important roles in the CNS under normal physiological conditions. For
281 instance, the major histocompatibility complex (MHC) class I and the paired-
282 immunoglobulin-like receptor B (PirB; an MHCI-binding receptor) have been implicated in
283 synaptic plasticity, learning and memory [68-70]. Moreover, some members of the TLR
284 family have been shown to modulate key neurodevelopmental processes (e.g., neurite
285 outgrowth, neuronal proliferation/differentiation, and neuronal cell death) and behavior
286 [71-77]. Emerging evidence also points to novel roles for PGN-sensing molecules (that is
287 PGRPs and NOD-like receptors) in the CNS [78]. The first known function of a PGN-sensing
288 molecule in the CNS came from the *Drosophila* model system [79]. The authors
289 demonstrated that a member of the PGRP family (PGRP-LC) is expressed at the presynaptic
290 terminal of *Drosophila* motor neurons. This PGN-sensing molecule plays a key role in the
291 induction and sustained expression of presynaptic homeostatic plasticity; thus, it controls
292 the homeostatic modulation of the readily releasable pool of synaptic vesicles following
293 inhibition of postsynaptic glutamate receptor function.
294

295 In mammals, there are four PGRPs including a “long” isoform (Pglyrp2 or PGRP-L) that is a N-
296 acetylmuramoyl-l-alanine amidase that hydrolyzes the amide bond between MurNAc and l-
297 Ala of bacterial PGN (which is unique among the mammalian PGRPs) [52]. In mice, Pglyrp2 is
298 highly expressed in the brain (e.g., prefrontal cortex, striatum, hippocampus and cerebellum)
299 during the first few days of life [45]. In line with this, human PGLYRP2 protein has also been
300 detected in postmortem cerebral cortex tissue of a young child, but not in most adult
301 cortical tissues [i.e., moderate to strong labelling in the neuropil and glial cells, respectively;
302 the Human Protein Atlas; (https://www.proteinatlas.org/) [80]]. In a recent study, it was
303 demonstrated that Pglyrp2 knockout (KO) mice have an altered expression of the ASD risk
304 gene c-Met, and BDNF (brain-derived neurotrophic factor) [45], both implicated in the
305 formation and modulation of brain circuits [81, 82]. Moreover, juvenile Pglyrp2 KO mice
306 display a significant increase in social interaction levels toward an unfamiliar stimuli mouse,
307 without any alterations in anxiety-like behavior or motor activity [45]. Interestingly, this
308 phenotype was more pronounced in male offspring. In another study, the same authors
309 demonstrated that the absence of Pglyrp2 leads to major sex-dependent alterations in
310 motor and anxiety-like behavior later in adulthood (i.e., 15 month-old mice) [83]. In this
311 case, Pglyrp2-deficient female mice, but not males, show better motor performance.
312 However, they display increased levels of anxiety-like behavior, suggesting that the
313 modulatory effects of Pglyrp2 in the brain are highly dependent upon multiple host factors
314 including age, sex and type of neuronal circuits. Peptidoglycan recognition protein 1
315 (Pglyrp1, also known as tag7 protein or PGRP-S) is another member of the mammalian PGRP
316 family that is expressed in the brain. Unlike Pglyrp2, the expression of Pglyrp1 peaks around
317 the second week of postnatal life and continues to be expressed into adulthood [45].
318 Previous work has shown that PGRP1 mRNA levels increase in brain stem and hypothalamus
319 after sleep deprivation in rodents [84], suggesting a possible role for this PGN-sensing
320 molecule in homeostatic regulation of sleep. However, studies using more advanced
321 transgenic mouse models (e.g., conditional brain-specific knockdown of Pglyrp2) are
322 fundamental to unravel the specific role of PGRPs in the CNS.
323
324 Recently, Pusceddu and colleagues provided the first experimental evidence implicating the
325 NOD-like receptors in the regulation of the stress response, serotonin (5-HT) signaling and
326 behavior [85]. Specifically, these authors showed that NodDKO mice deficient in both Nod1

327 and Nod2 exhibit signs of stress-induced anxiety-like behavior, cognitive impairment and
328 depressive-like behavior under conditions of a hyperactive HPA axis, but not under baseline
329 conditions. Surprisingly, no major sex differences were found. Moreover, these authors
330 identified the central 5-HT system as one potential neural substrate mediating the
331 behavioral phenotype of NodDKO mice. In fact, NodDKO mice had lower levels of 5-HT in
332 both the hippocampus and brain stem under basal conditions. In addition, the expression
333 profile of several key components of the 5-HT signaling pathways such as tryptophan
334 hydroxylase 2 (the rate limiting enzyme for 5-HT synthesis), the 5-HT receptor 1a (which
335 regulates 5-HT release) and the 5-HT transporter were impaired after exposure to stress.
336 Importantly, these authors showed that the stress-induced behavioral impairments
337 observed in NodDKO mice could be restored by chronic treatment with a 5-HT reuptake
338 inhibitor (fluoxetine). Another important finding of this study was that intestinal epithelial
339 cell (IEC)-specific ablation of Nod1 (VilCre+Nod1f/f), but not Nod2 receptors, resulted in
340 stress-induced impairments in cognitive function and anxiety-like behavior. The latter
341 observations highlight a novel role for the IEC Nod1 receptors in behavioral responses to
342 stress and identify this PGN-sensing molecule as potential new target for the treatment of
343 stress-associated gut-brain disorders. However, the specific roles of NOD-like receptors
344 within the brain remain to be further clarified. Previous studies have shown that murine
345 microglia express robust levels of Nod2 mRNA and protein, but little or no expression of
346 Nod1 [86, 87]. On the other hand, Nod1 protein is highly expressed in developing neurons
347 across multiple brain regions including the prefrontal cortex, hippocampus and cerebellum
348 (see supplementary figure 3 in Ref. [45]). Both Nod1 and Nod2 are moderately expressed in
349 astrocytes [45, 87]. These findings raise the possibility that Nod1 and Nod2 may have distinct
350 functions in glia and neurons of the adult and developing brain.
351

352 **Potential role of PGN in the Pathophysiology of Neurodevelopmental Disorders**

353 It is now widely recognized that the maternal-fetal environment plays a crucial role in the
354 development of the fetal brain and long-term neurodevelopmental trajectories, including
355 susceptibility to mental health disorders in childhood and adulthood [88]. Indeed,
356 epidemiological studies have shown an association between maternal infection/
357 inflammation during pregnancy and increased risk for neurodevelopmental and psychiatric
358 disorders such as ASD [89-92]. These findings are supported by experimental mouse studies

359 demonstrating that maternal immune activation during pregnancy (MIA; through exposure
360 to microbial pathogens or pro-inflammatory PAMPs) induces ASD-like behavioral traits in
361 their offspring [93, 94]. Recent work by Choi, Huh and colleagues have demonstrated that
362 MIA promotes abnormal cortical development and ASD-like behavioral phenotypes in mouse
363 offspring through the activation of the maternal T helper 17 cell-interleukin-17a (IL-17a)
364 pathway [95]. These authors discovered that MIA-induced phenotypes in mouse offspring
365 require commensal maternal gut bacteria (*i.e.*, segmented filamentous bacteria; SFB) that
366 promote Th17 cell differentiation [96]. For instance, pregnant C57BL/6 mice, which are
367 colonized with SFB and contain higher numbers of gut Th17 cells were more susceptible to
368 MIA-induced behavioral and morphological pathology. Conversely, pregnant C57BL/6 mice
369 which are not colonized by SFB and lack gut Th17 cells were not susceptible. These findings
370 suggest that the composition of the maternal gut microbiota is an important contributing
371 risk factor for MIA-induced behavioral abnormalities in offspring. Indeed, mounting evidence
372 identifies the maternal gut microbiota as a common denominator by which a broad range of
373 environmental risk factors such as diet (e.g., high-fat diet), infections, antibiotic exposure
374 and stress can affect both the maternal health and fetal development [9, 97, 98]. The
375 developing fetus receives oxygen and nutrients along other bioactive compounds from the
376 maternal circulation, and therefore, alterations in the composition and metabolic activity of
377 maternal gut microbiota could profoundly influence prenatal brain development processes
378 (e.g., neurogenesis, migration, and differentiation) and subsequent neurodevelopmental
379 outcomes. In line with this notion, a new study in mice demonstrated that during normal
380 pregnancy, SCFAs from the commensal maternal gut microbiota can reach the developing
381 embryo and promote the differentiation of sympathetic neurons via activation of GPR41
382 [99]. Moreover, studies demonstrating disrupted BBB maturation and an altered microglial
383 transcriptome profile in GF mice during prenatal life underscores the importance of the
384 commensal maternal microbiome during fetal brain development [14, 16].
385
386 Recently, it has been proposed that MAMPs such as PGN could be an important mediator of
387 host-microbiome interactions at the maternal-fetal interface since PGN reaches the systemic
388 circulation not only during bacterial infections, but also during normal physiological
389 conditions [78]. In line with this notion, a recent study showed that components of the
390 bacterial cell wall (*i.e.*, PGN-teichoic acid complex of *Streptococcus pneumoniae*, a major

391 pathogen causing infections such as meningitis) can traverse the placental barrier through
392 the platelet activating factor receptor (PAFr) and affect the developing mouse fetal brain
393 [100]. This PGN-teichoic acid complex induces the transcription factor FoxG1 in the fetal
394 neocortex via the activation of the Tlr2, resulting in a 50% greater density of neurons in the
395 cortical plate. This abnormal neuroproliferation was associated with abnormal cognitive
396 development. Intriguingly, a Tlr-2-dependent response to this PAM did not induce
397 inflammation in the fetal brain. Recently, it was reported that NOD1, NOD2, TLR2, as well as
398 the putative PGN transporters PEPT2 and PAFr are abundantly expressed in the human
399 placenta [78]. These findings suggest that bacterial PGN motifs from the maternal
400 commensal gut microbiota may also be capable of influencing the developing brain, and that
401 disruption of components of this signaling pathway could lead to abnormal motor, social,
402 and cognitive development (Figure 5).
403
404 In humans, accelerated brain growth in infancy has been associated with a broad range of
405 developmental delays in motor, language, and cognitive functions [101, 102]. For instance,
406 neurodevelopmental disorders, including ASD, have been associated with atypical brain
407 connectivity patterns involving higher-order association neocortical regions [103-105]. A
408 large proportion of high-confidence ASD-associated risk genes are pleiotropic, and affect
409 proliferation/differentiation and/or synapse development in the neocortex, amygdala,
410 hippocampus, striatum and cerebellum, supporting the notion that ASD begins during
411 prenatal life [106]. It will therefore be of great interest to investigate the potential link
412 between maternal circulating PGN levels during pregnancy, and social-cognitive
413 development of infants, as well as potential novel associations between genetic variants in
414 PGN-sensing molecules and ASD ([107]; see Clinician's Corner).
415

416 **Concluding Remarks**

417 Recent discoveries showing an unprecedented role for the commensal gut microbiota in the
418 regulation of brain development and behavior has triggered a paradigm shift in our
419 conceptualization of the origin of human brain disorders. The current focus of this new
420 microbiome-gut-brain axis field is to delineate the precise molecular mechanisms and
421 signaling pathways employed by commensal gut bacteria to influence the brain. PRRs that
422 recognize bacterial surface molecules, such as PGN, have emerged as potential novel

423 mediators of microbiota-gut–brain axis signaling. The gut microbiota contains trillions of
424 commensal bacteria producing a diverse peptidoglycome that can disseminate systemically
425 and reach peripheral organs such as the brain. PRRs that specifically recognize PGN (i.e.,
426 PGN-sensing molecules; NOD-like receptors and PGN-recognition proteins), and the PGN
427 transporter PepT1 are highly expressed in the developing brain during specific windows of
428 postnatal development. Emerging evidence suggests crucial roles for PGN-sensing molecules
429 in the regulation of social behavior and anxiety, and behavioral responses to stress. Despite
430 recent advances, many questions remain to be answered, including the characterization of
431 the specific types of PGN that can cross the BBB under physiological conditions, and how
432 they are transported into brain cells (neurons, astrocytes, microglia and oligodendrocytes
433 (see Outstanding Questions). It is worthwhile acknowledging that the laboratory of
434 Gomperts Boneca at the Institut Pasteur in Paris has been advancing some of these
435 questions by combining different PGN labelling methods with advanced imaging and genetic
436 approaches (see abstracts from the Great Wall Symposium 2019, September 25-27, 2019).
437 From a clinical perspective, it would be of great importance to determine whether serum or
438 plasma levels of PGN motifs during pregnancy and/ or early postnatal life could help to
439 identify infants and children at risk for atypical social and cognitive development. It would be
440 equally important to identify potential genetic risk variants involved in the sensing of
441 bacterial-derived products (e.g., by creating a polygene risk scores of key genes involved in
442 PGN-sensing molecules) that are associated with atypical neurodevelopmental trajectories.
443 This could be accomplished in a multicenter, prospective, longitudinal study of infants at
444 high- and low-risk for atypical brain development such as ASD. In parallel, investigate
445 potential effects of PGN motifs on early aspects of human brain prenatal development by
446 taking advantage of human induced pluripotent stem cells technology, which allows the
447 generation of personalized neurons or glial cells. This information will provide key
448 mechanistic insight into how PGN can influence brain development, function and behavior,
449 as well as novel potential therapeutic targets for the treatment of neurodevelopmental and
450 psychiatric disorders, often associated with co-morbid gastrointestinal problems and altered
451 gut microbiota composition.
452

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674 ## Figure legends

675

676 **Figure 1. Biological signaling pathways and molecules involved in the microbiota-gut-brain**
677 **axis.** There are multiple direct and indirect pathways through which gut microbiota may
678 influence the brain, including signals carried by neuronal circuits [e.g., bidirectional vagus
679 nerve-to-brain communication, the enteric nervous system and neuropods; (1)], the
680 production of bacterial fermentation metabolic by-products, such as short chain fatty acids
681 [SCFAs; propionate, butyrate and acetate; (2)], tryptophan metabolites and
682 neurotransmitters (3), release of cytokines by immune cells [A. Dendritic cell; B. B cell; C.
683 Mast cell; D. T cell; (4)], and gut hormone signaling (5). Some of these molecules can activate
684 the vagus nerve or reach the brain via systemic circulation, and directly affect brain
685 functions. Activation of the hypothalamic-pituitary-adrenal (HPA) axis is characterized by the
686 release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus, which
687 then stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior
688 pituitary gland. ACTH then acts on the adrenal cortex to stimulate the production and
689 release of glucocorticoids (corticosterone in rodents and cortisol in humans), which can have
690 a major impact on gut physiology (e.g., modulating the intestinal epithelial barrier and
691 immune responses), and gut microbiota composition (6). The gut microbiota has been
692 shown to influence various neurodevelopmental processes such as microglial maturation
693 and function, blood brain barrier formation and integrity, myelination and neurogenesis (7).

694

695 Figure 2. Bacterial PGN location and structure. **A.** Gram-positive bacteria display a multilayered PGN
696 cell wall, which contains teichoic acids and often other polysaccharides, proteins and lipoproteins
697 (not shown). In contrast, Gram-negative bacteria have a relatively thin PGN cell wall, surrounded by
698 an outer membrane containing lipopolysaccharides (LPS). **B.** PGN is a polymer consisting of β (1-4)-
699 linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), with all lactyl groups of
700 MurNAc substituted with stem peptides, typically containing four alternating L- and D-amino acids.
701 The stem peptides from adjacent strands are often crosslinked, either directly or through short
702 peptides. Shown are two examples of the general types of PGN, lysine-type PGN characteristic for
703 cell walls in most Gram-positive bacteria and DAP-type PGN present in Gram-negative bacteria and a
704 subset of Gram-positive bacteria. PGN motifs are recognized via a series of PRRs such as NOD-like
705 receptors. For example, an intact MurNAc moiety is essential for Nod2 recognition, which is strongly
706 activated by muramyl dipeptide (MDP). In contrast, the presence of the glycan moiety is not required
707 for Nod1 recognition as iE-DAP or Tri-DAP are sufficient for the activation of this PRR. However, Nod1
708 is also activated to meso-DAP-type PGN containing muramyl tripeptides (M-TriDAP) or tetrapeptides
709 (M-TetraDAP). In addition, M-TriDAP modified by amidation of the meso-DAP residue (DAPNH2) have
710 been shown activates Nod2 and not recognized by Nod1 receptor [31].

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717 Figure 3. Evidence from invertebrate and vertebrate models supporting a role of PGN in the
718 crosstalk between microbial symbionts and their hosts. **A.** Beneficial symbiotic relationship
719 between the squid Euprymna scolopes and the luminescent bacteria Vibrio fischeri.
720 Epithelial cells of the light organ of E. scolopes secrete mucus-containing peptidoglycan
721 recognition protein 2 (PGRP2) that select V. fischeri from other bacterial species present in
722 the water. V. fischeri enters through the pores into the ducts of the light organ and colonize
723 the deep crypts, where they induce apoptosis and morphogenesis of the light organ (figure

724 modified from [52]). **B.** Development of isolated lymphoid follicles (ILFs) by commensal gut
725 microbiota. Nod1 receptors in intestinal epithelial cells are activated by PGN released by the
726 gut microbiota inducing the expression of CCL20 and β-defensin-3. These factors are CC
727 chemokine receptor 6 (CCR6) agonists expressed on intestinal B cells and lymphoid tissue
728 inducer (LTi) cells in cryptopatches. Activated CCR6 cause the recruitment of CCR6+ B cells to
729 form immature ILFs. The development of immature ILFs into mature follicles require the
730 tumor necrosis factor-α (TNF-α) produced by dendritic cells. B cells from mature ILFs
731 produce IgA that modulates the composition of the gut microbiota (figure modified from
732 [108]). **C.** Gut microbiota primes the bone marrow-derived neutrophils. Meso-DAP-type PGN
733 released by Gram-negative bacteria in the gut can be translocated into the systemic
734 circulation and reach the neutrophils in the bone marrow. The activation of Nod1 receptor in
735 the neutrophils result in their increased capacity to react against microorganisms (figure
736 modified from [56]).

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746 Figure 4. Translocation of bacterial PGN from gut microbiota to the brain. **A.** PGN motifs
747 from the commensal gut microbiota disseminate systemically and can reach the brain. **B.**
748 Schematic representation of blood brain barrier composed by endothelial cells of the brain
749 capillary wall, astrocyte end-feet surrounding the capillary and pericytes embedded in the
750 capillary basement membrane. PGN motifs can cross blood brain barrier and are sensed by

751 PRRs expressed in brain cells (neurons, oligodendrocytes and microglia). See main text for
752 further details.

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770 Figure 5. Schematic representation of the proposed PGN dissemination from maternal
771 microbiota to the fetal brain. **A.** PGN fragments derived from commensal maternal gut
772 microbiota translocate from the gut lumen into the bloodstream and reach the placenta.
773 PGN can transverse the placenta and enter the fetal brain, where it can affect key
774 neurodevelopmental processes (e.g., proliferation, differentiation, synaptogenesis and
775 myelination) via activation of PGN-sensing molecules of the innate immune system. **B.** PGN

776 signaling can be affected by host genetics (e.g., single nucleotide polymorphisms (SNP) and
777 copy number variations (CNV) in genes coding for PGN sensing molecules and/or
778 transporters) and external factors such as antibiotic exposure, maternal stress and mode of
779 delivery. Optimal low PGN levels are needed for the typical brain development. However,
780 too low or high PGN levels gives rise to atypical brain development.

781

782

783 ## Box 1. Factors influencing the development of the infant microbiota

784 During early postnatal life, the development of the infant gut microbiota follows successive
785 waves of microbial exposures and colonization that is influenced by age-associated events
786 such as dietary transitions (e.g., cessation of breastfeeding and the introduction of solid
787 foods into the diet), leading to a more complex and diverse adult-like ecosystem around the
788 first 2-3 years of age (see Ref. [109]). However, the exact age at which the toddler gut
789 microbiota comes to resemble the adult pattern may vary depending upon the prenatal and
790 neonatal history.

791 Several factors play an important role in shaping the development of the infant microbiota
792 and potential functional outcomes including the mode of delivery (cesarean delivery vs.
793 vaginal delivery), breastfeeding practices (breast milk vs. formula feeding) and use of
794 antibiotic (see Refs. [8, 9]). In addition, other factors such as length of gestation (i.e. full-
795 term vs. pre-term birth), nutritional status (under- and overnutrition), hospitalization and
796 genetics can have a profound impact on neonatal microbial colonization trajectories. The
797 fact that the assembly and maturation of the infant gut microbiota co-occurs in parallel with
798 key neurodevelopmental process (e.g., myelination, synaptogenesis, synaptic refinement
799 and acquisition of brain functions) has led to the realization that the early postnatal period
800 represent not only a critical time-window in brain and microbial development, but also a key
801 determinant period of health in later-life [8, 9].

802 ## Clinician's Corner

803 Traditionally, the translocation of bacterial products such as peptidoglycans (PGN) to the
804 brain has mainly been considered in the context of infection and inflammation. However,
805 the recent realization of the size and complexity of the human microbiome and its wide
806 ranging impact on host physiology and development has triggered a reevaluation of the
807 possible role of bacterial-derived products and metabolites from the non-pathogenic
808 microbiota on host tissues, including brain development and behavior.
809
810 PGN from commensal microbiota are ubiquitously present in circulation and can cross the
811 blood brain barrier. PGN-sensing molecules are highly expressed in the normal developing
812 brain and their expression is highly sensitive to manipulations of the gut microbiota,
813 including perinatal antibiotic exposure. Work from transgenic animal models implicate PGN-
814 sensing molecules in the control of social behavior and anxiety.
815
816 PGN sensing molecules are expressed in the human placenta and represent an important
817 mediator for microbiota-host communication at the maternal-fetal interface. Studies in
818 rodents suggest that circulating PGN fragments from maternal gut microbiota can influence
819 the fetal brain, and subsequent cognitive development.
820
821 From a clinical point of view, detection of PGN levels during the perinatal period could
822 represent a new strategy to screen and identify infants and children at risk of
823 neurodevelopmental and psychiatric disorders such as autism spectrum disorder.
824
825 At the genetic level, there is a need to explore potential associations between genetic
826 polymorphisms in PGN-sensing molecules (e.g., peptidoglycan recognition proteins and Nod-
827 like receptors) and neurodevelopmental disorders.
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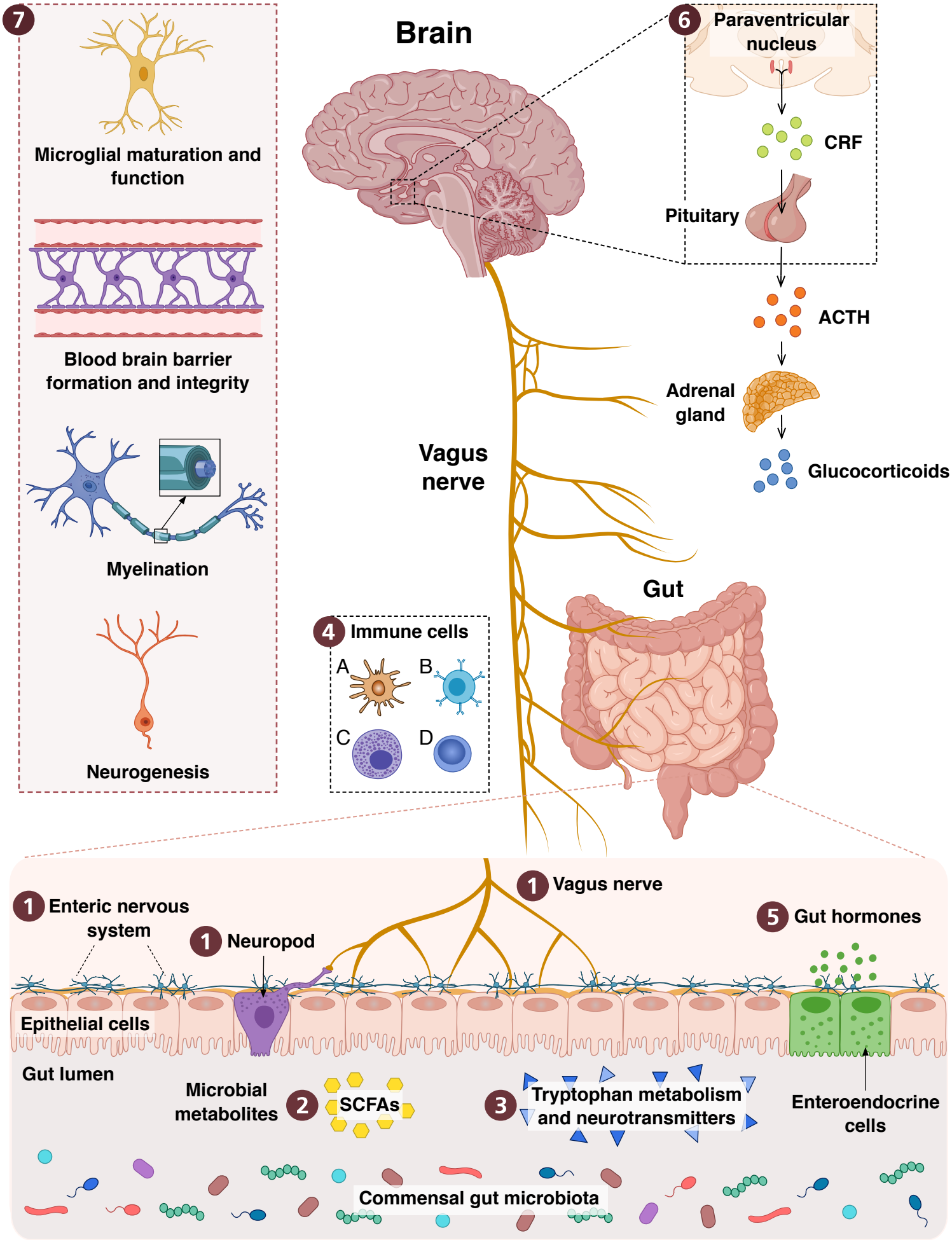
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Microglial maturation and function
Blood brain barrier formation and integrity
Myelination
Neurogenesis
Brain
6
Paraventricular nucleus
CRF
Pituitary
ACTH
Adrenal gland
Glucocorticoids
Vagus nerve
Gut
4
Immune cells
A
B
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D
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Vagus nerve
1
Enteric nervous system
1
Neuropod
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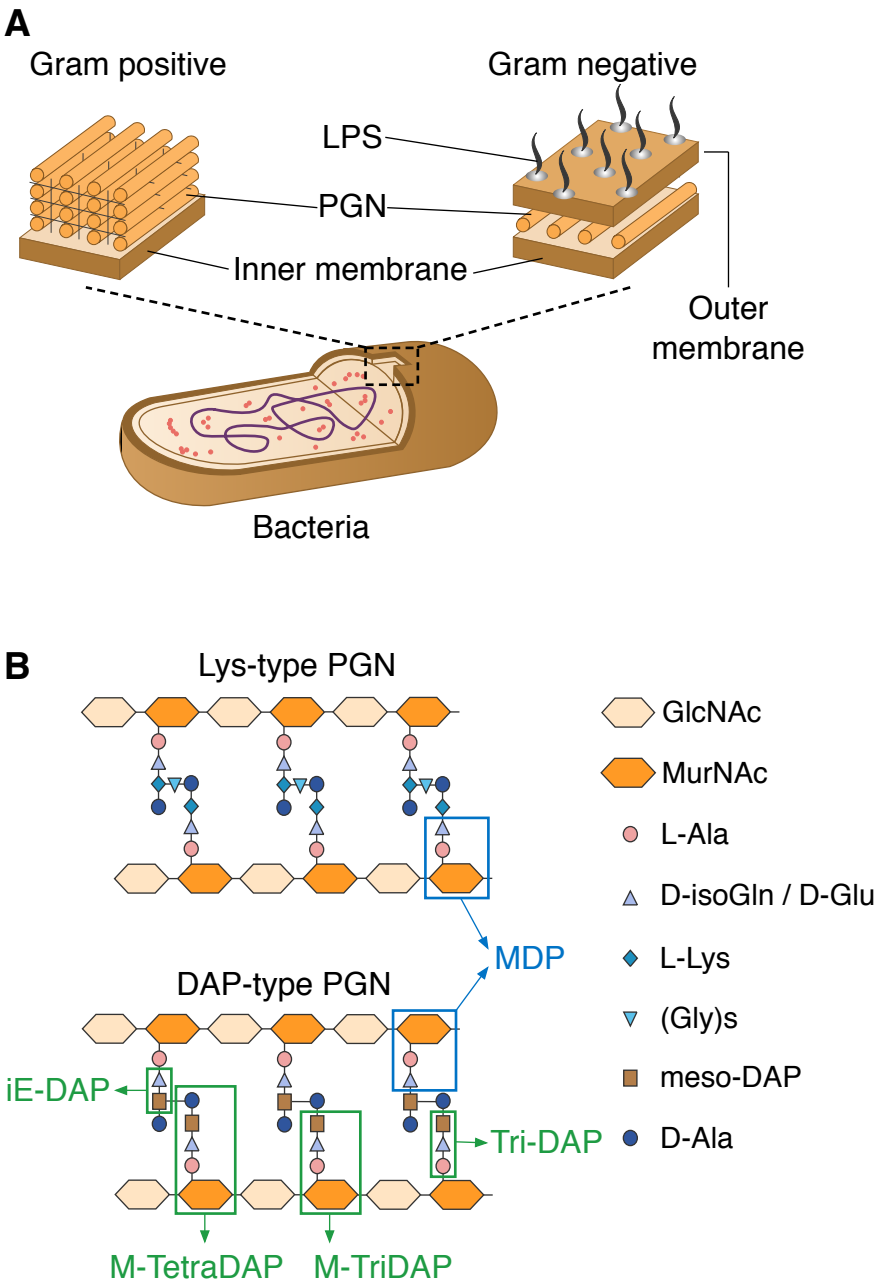
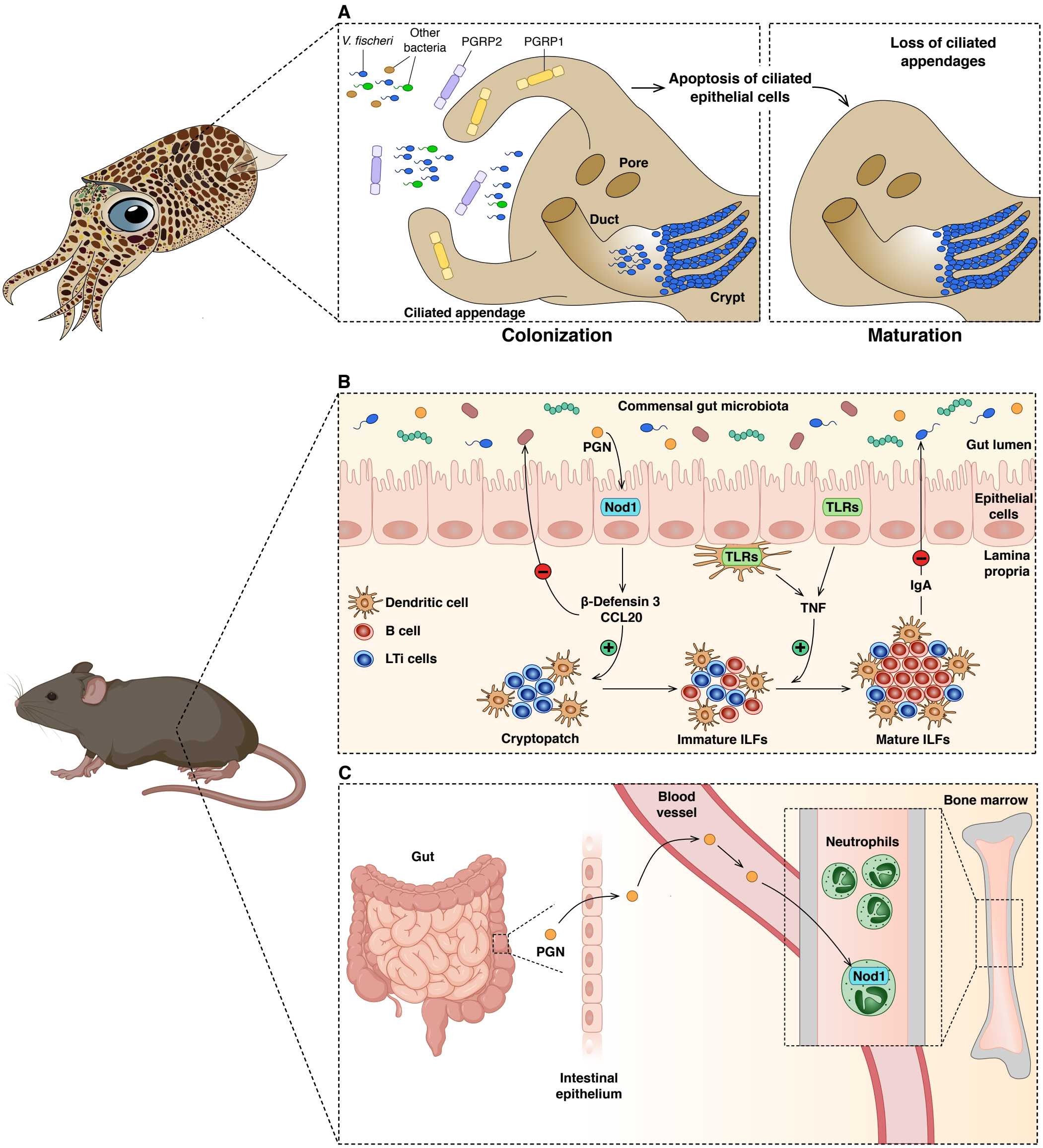
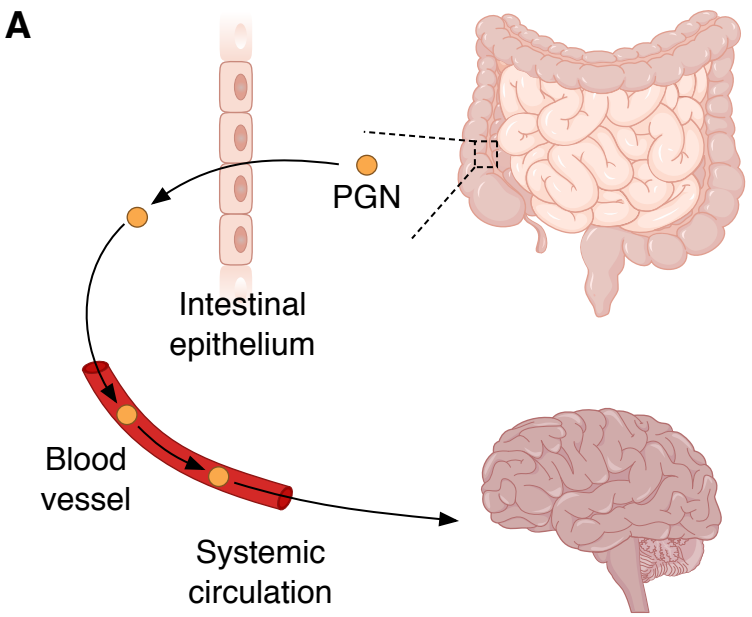
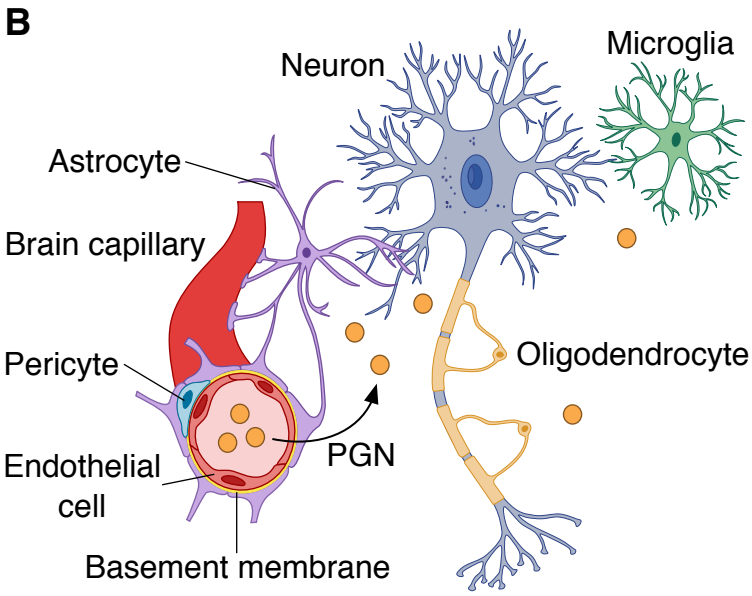
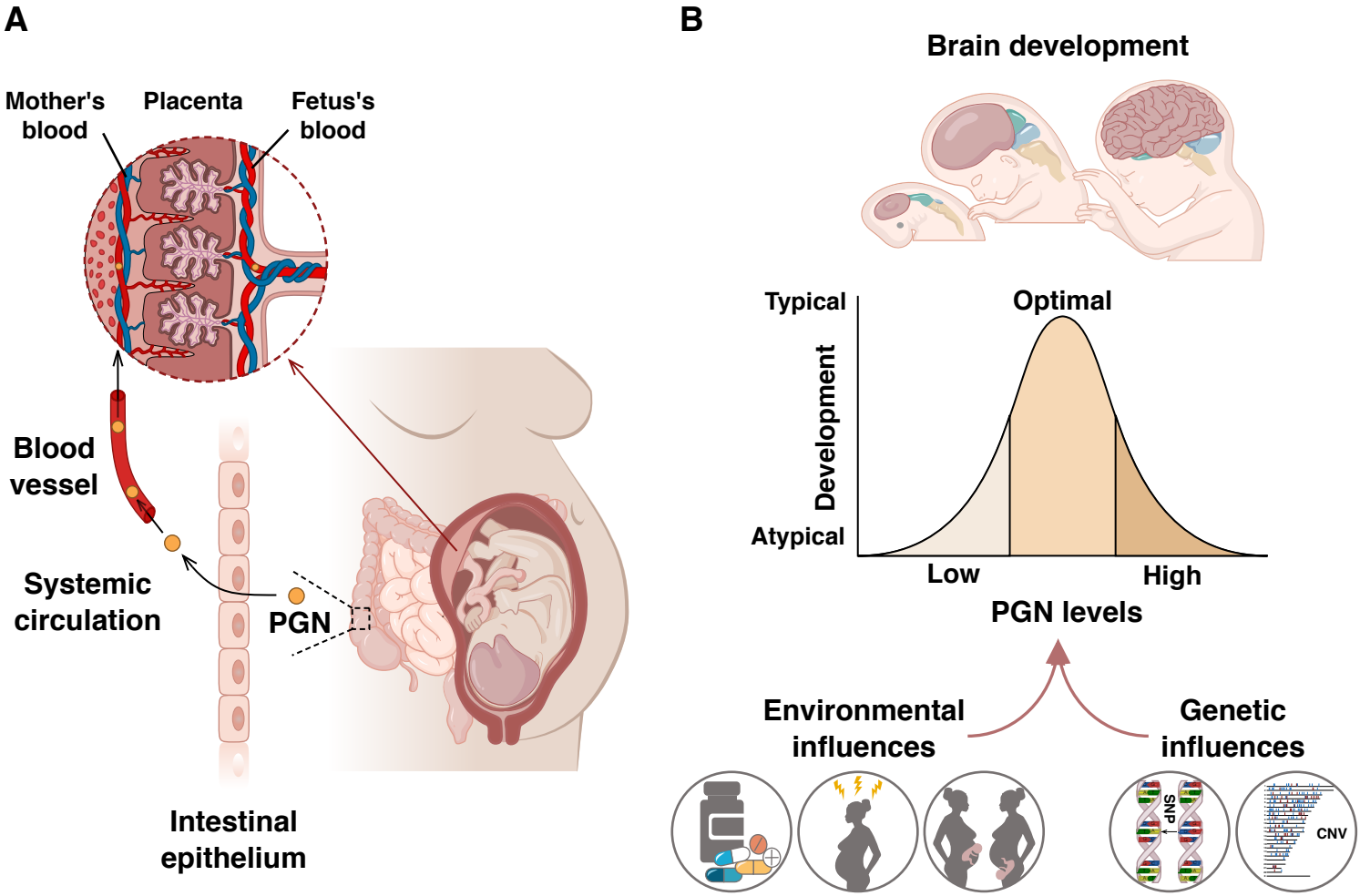
Gut hormones
Epithelial cells
Gut lumen
Microbial metabolites
2
SCFAs
3
Tryptophan metabolism and neurotransmitters
Enteroendocrine cells
Commensal gut microbiota


A
Gram positive
Gram negative
LPS
PGN
Inner membrane
Outer membrane
Bacteria
B
Lys-type PGN
DAP-type PGN
MDP
iE-DAP
Tri-DAP
M-TetraDAP
M-TriDAP
GlcNAc
MurNAc
L-Ala
D-isoGln / D-Glu
L-Lys
(Gly)s
meso-DAP
D-Ala


A
V. fischeri
Other bacteria
PGRP2
PGRP1
Apoptosis of ciliated epithelial cells
Loss of ciliated appendages
Pore
Duct
Crypt
Ciliated appendage
Colonization
Maturation
B
Commensal gut microbiota
PGN
Gut lumen
Nod1
TLRs
Epithelial cells
TLRs
Lamina propria
IgA
Dendritic cell
B cell
LTi cells
β-Defensin 3
CCL20
TNF
Cryptopatch
Immature ILFs
Mature ILFs
C
Blood vessel
Bone marrow
Neutrophils
Gut
PGN
Nod1
Intestinal epithelium


A
PGN
Intestinal
epithelium
Blood
vessel
Systemic
circulation


B
Neuron
Microglia
Astrocyte
Brain capillary
Pericyte
Oligodendrocyte
PGN
Endothelial
cell
Basement membrane


A
Mother's blood
Placenta
Fetus's blood
Blood vessel
Systemic circulation
PGN
Intestinal epithelium
B
Brain development
Typical
Optimal
Development
Atypical
Low
High
PGN levels
Environmental influences
Genetic influences
SNP
CNV