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Critical Windows: The Microbiota-Gut-Brain Axis in Neurocognitive Development

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Running Head: Critical Windows in the Microbiota-Gut-Brain Axis

Conflict of Interest Statement

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1. Introduction

"Can the brain understand the brain? Can it understand the mind?

Is it a giant computer, or some other kind of giant machine, or something more?"

– David H. Hubel

This past decade has seen this idea of "something more" expand to the trillions of bacteria within the gut, the microbiota (Cryan et al., in press; Rhee et al., 2009). Indeed, there has been an explosion of research investigating the microbiota and its contribution to all aspects of human health and disease including psychological well-being and neurodevelopment. But what exactly is the microbiota? It is defined as the collection of micro-organisms, including bacteria, viruses, fungi, and archaea, which reside in a particular niche (note that the 'microbiome', although often used interchangeably with 'microbiota', refers to both the community and its collective genome). It has been estimated that the human microbiota contains 3.8 $x10^{13}$ bacteria, more than the number of human cells in the body (Sender et al., 2016), with its genome outnumbering human genes by up to 150:1 (Qin et al., 2010). The largest of these microbial communities resides within the gastrointestinal tract, with the gut microbiota playing a key role in extracting nutrients from the diet and training the immune response at the body's largest immune interface (Gensollen et al., 2016). The gut microbiota is therefore the subject of the vast majority of research regarding the impact of the microbiota on brain and behavior. In this review we focus on the gut microbiota as a key regulator of brain and behavior in early life. We will first examine what is known about the developmental trajectory of the microbiota and propose that there are sensitive periods in the microbiota-gut-brain axis with consequences for neurocognitive development. We will then provide evidence for this hypothesis based on studies of both humans and non-human

animals, examine potential mechanisms for interactions between the developing microbiota and neurodevelopment, and finally discuss challenges for translational research in this area.

1.1 The Development of the Microbiota

Using the latest sequencing technologies, a clearer picture is beginning to emerge of how the gut microbiota develops over the lifespan and how this influences the host (see Box 1 for further information on how the microbiota is measured). Whether microbial colonization occurs *in utero* is currently the subject of heated debate (Perez-Muñoz et al., 2017; Walker et al., 2017). What is clear is that the microbiome is rapidly populated at birth (Ferretti et al., 2018). This initial seeding and the subsequent development of the microbiota is dependent on many factors (see Box 2 and Figure 1), but there are some clear trends across maturation. At least in vaginally-delivered babies, the majority of early gut colonizers are transmitted from the maternal microbiota (from various sites, but predominantly the maternal vaginal and stool microbiota; Ferretti et al., 2018). After the first few days, there is a slow increase in the number and diversity of species represented, shifting from mostly aerobic or facultative species (that can survive in oxygenated environments) towards more anaerobic species (Ferretti et al., 2018; Timmerman et al., 2017). The early microbiota seems to be geared towards extracting nutrients in order to support the rapid development of the brain and body of the host (Hollister et al., 2015; Koenig et al., 2011). There is another rapid burst in development of the microbiota at weaning, as the infant shifts from exclusive breastfeeding or formula intake to a solid diet (a pattern observed across species, including humans and rodents; Al Nabhani et al., 2019; Guevarra et al., 2018; Koenig et al., 2011; Stewart et al., 2018; Yatsunenko et al., 2012). According to some studies, weaning is followed by a period of relative stability in the microbiota (Stewart et al., 2018; Yatsunenko et al., 2012). However, other results suggest there is ongoing change and plasticity across middle

childhood and adolescence (for a review, see Derrien et al., 2019). These investigations have shown that developing individuals continue to exhibit microbiota differences from adults well into adolescence, in a pattern that seems to indicate gradual development towards an adult-like profile (Agans et al., 2011; Hollister et al., 2015).

Box 1. Measuring the Microbiota

The most commonly used techniques for measuring the microbiota are high-throughput sequencing methods such as 16S rRNA sequencing and shotgun metagenomics. Using these methods, we can identify the composition of the microbiota (i.e., who is in there, based on the genetic read-out) and the functional potential of the community (i.e., what are they doing?; see Bastiaanssen et al., 2019; Claesson et al., 2017 for summaries of these techniques, written for clinicians). Different measures of diversity are usually used to describe and compare the output of these analyses, with the two main metrics being alpha and beta diversity.

Alpha diversity. Refers to diversity within a given microbial community. Counting the number of species present is one simple way to estimate diversity (more species = more "richness" ≈ more diversity). Some measures of alpha diversity also take into account the "evenness" of a community, which refers to the balance of different species (similar numbers of each species = more evenness ≈ more diversity). Other measures take into account the phylogenetic relatedness of the community members (more closely related species ≈ less diversity). Examples of common alpha diversity metrics include the Chao1, Shannon, and Simpson's Indices.

Increased alpha diversity is commonly interpreted as an indicator of a healthier microbiota. This is based on the assumption that diversity increases the capacity of the community to

perform a broader range of functions and resist invasion from pathogens. However, more diversity is not always better and we should be particularly wary of such simplistic interpretations in the context of development.

Beta diversity. Refers to the differences between two (or more) microbiota. As for alpha diversity, different metrics may take into account different factors such as phylogenetic relatedness. Beta diversity is usually shown using a principal component analysis (PCA) plot or similar data simplification method to represent the complex microbial communities in just 2-3 dimensions. In these plots, the further apart two communities are, the more different they are and thus the higher their beta diversity.

Box 2. Factors that Shape the Developing Microbiota

Starting even before birth, there are many factors that shape the developing microbiota. These include, but are not limited to, genetics, stress, mode of birth, diet, infection or disease, medication (including antibiotics), and environmental factors. Overall, the responsive nature of the microbiota offers a potential advantage when it comes to targeting disease.

Genetic Factors. Studies of the relationship between host genetics and the microbiota have discovered and validated many heritable gut bacteria in humans (Goodrich et al., 2016; Goodrich et al., 2014; Lim et al., 2017; Turpin et al., 2016). However, a large proportion of the microbiota is not heritable and environmental factors explain far more of the variation in the human microbiota (Rothschild et al., 2018).

Stress. Stress influences all levels of the microbiota-gut-brain axis, particularly in the early stages of development. Both pre- and post-natal stress are established risk factors for the development of psychological and neurodevelopmental disorders (Kessler et al., 2010; Kim et al., 2015), as well as gastrointestinal problems and/or microbiota alterations (Callaghan et al., in press; Michels et al., in press; Pohl et al., 2015). These observations in humans are reflected in experimental animal models of early-life stress, which have long been used to model both psychological and gastrointestinal disorders (O'Mahony et al., 2011). Moreover, a number of recent studies have demonstrated links between prenatal stress (including maternal anxiety) or early-life adversity and alterations in the human microbiota across the lifespan (Callaghan et al., in press; D'Agata et al., 2019; Hantsoo et al., 2019; Hemmings et al., 2017; Hu et al., 2019; Labus et al., 2017; Michels et al., in press; Zijlmans et al., 2015).

Mode of delivery at birth. Parturition acts as the first large-scale exposure to environmental microbes and mode of delivery has a profound effect on the initial composition of the infant microbiota (Stewart et al., 2018). Vaginally-delivered babies are exposed to microbes resident in the maternal birth canal, whereas babies born via Caesarean section exhibit a microbiota profile that more closely resembles adult skin and/or hospital microbiota (Dominguez-Bello et al., 2010). This effect of delivery mode emerges in the transitional stool rather than the meconium (i.e., the first stool after birth), but dissipates within a few weeks/months of birth (Chu et al., 2017; Hill et al., 2017; Stewart et al., 2018).

Diet. During infancy, one of the most robust microbiota findings is that formula- and breast-fed infants exhibit divergent profiles, with cessation of breastfeeding accelerating the development of the microbiota (Stewart et al., 2018). In cases of childhood malnutrition, the maturation of the microbiota is delayed, an effect that is only partially or temporarily restored

by dietary intervention (Smith et al., 2013; Subramanian et al., 2014). Diet continues to play an important role in determining microbiota composition throughout life; even short manipulations of diet in adulthood can have rapid effects on the microbiota (David et al., 2014; Johnson et al., 2019). This is intriguing given that diet tends to change over the lifespan, with adolescents being particularly prone to poor food choices, in combination with drug and alcohol experimentation, at a time when their microbiota and brains are still developing (Flannery et al., in press; McVey Neufeld et al., 2016).

Medical factors. Bacterial or viral infections, gastrointestinal problems (e.g., diarrhea), and antibiotic use are obvious sources of variation in the microbiota (Mortensen et al., 2018). However, other non-antibiotic medications (including many targeting neurological or psychological problems) have also been shown to alter the growth of certain gut bacteria *in vitro* as well as overall microbiota composition in rodents (Cussotto et al., 2018; Maier et al., 2018). In adult humans, bariatric surgery results in substantial changes to the microbiota (Guo et al., 2018). There is also a rich literature showing that premature infants, who typically undergo multiple medical procedures in the early stages of life, exhibit microbiota alterations (Chernikova et al., 2018; La Rosa et al., 2014; but see also, Stewart et al., 2018).

Environmental factors. Many facets of day-to-day life contribute to microbial exposure and therefore to the microbiota. Urbanization (encompassing less contact with the natural environment and accompanying changes in diet), pet ownership, and increasing use of antibiotics and disinfectants are all factors that alter microbial exposure and have been associated with altered microbiota (Ayeni et al., 2018; De Filippo et al., 2017; Stewart et al., 2018; Tun et al., 2017; Tun et al., 2018).

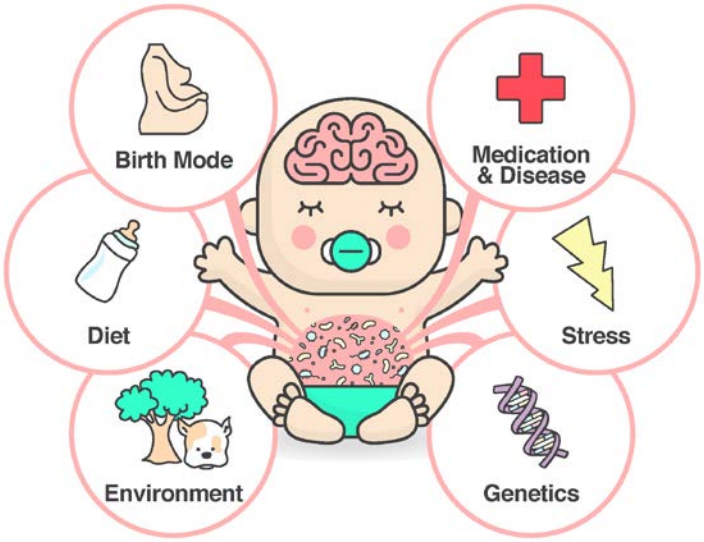

Figure 1. Many factors affect the developing microbiota and may therefore alter the developmental trajectory of the microbiota-gut-brain axis. See Box 2 for further discussion.

Intriguingly, periods of change in the microbiota coincide with times of rapid development in other bodily systems and particularly the brain (Borre et al., 2014). This parallel development is likely to be biologically relevant, and we hypothesize that these developmental windows correspond to sensitive periods in the microbiota-gut-brain axis.

1.2 Sensitive Periods

Sensitive periods (often used synonymously with the terms “critical periods” and “critical windows”) are defined as specific developmental windows during which a system exhibits heightened plasticity and is particularly responsive to certain environmental cues. These cues (also known as “expected inputs”) are used to tune the system in a highly efficient manner. The classic experiments by Nobel prize winners Hubel and Wiesel showed that deprivation of light input into one eye permanently changes the activity of neurons in the cerebral cortex of cats, but only during a narrow developmental time window (Hubel & Wiesel, 1970). Later exposure to light was insufficient to reverse the changes that occurred during the sensitive period, while depriving an eye of light outside the sensitive period had little effect on

neuronal activity or vision. The existence of such sensitive periods optimizes systems development when certain experiences (like exposure to light) reliably occur during certain stages of development (during the time of first eye opening).

Sensitive periods have now been described for other, more complex brain functions and behaviors such as language (Werker & Hensch, 2015) and emotional learning (Alberini & Travaglia, 2017; Gogolla et al., 2009; Hartley & Lee, 2015). Particularly for these higher order functions, there is not just one critical window but a cascading series of sensitive periods with intricately linked timing, each dependent on the preceding experiences. Furthermore, the timing of onset and offset of the sensitive period is less well-defined for these higher order functions, at least in part due to the extended development of the supporting brain regions (Tottenham, 2017). Even the most well-defined sensitive periods exhibit a gradual decline in plasticity, subthreshold responses to the expected inputs into adulthood, and potential to be re-opened by pharmacological or environmental manipulations (Morishita & Hensch, 2008). This latter property has great therapeutic application, offering hope for effective intervention even after the closure of a sensitive period.

1.3 Sensitive Periods in Microbiota-Gut-Brain Axis Development

We hypothesize that signals from the microbiota are required for certain functions, acting like an expected input (similar to the role of the light in the original Hubel and Wiesel experiments) to calibrate the microbiota-gut-brain axis. The absence or disruption of the microbiota during specific developmental windows would therefore have a disproportionate effect on specific functions that are relevant to the time window of disruption and potentially for regulation of the system as a whole. In addition, external cues such as changes in diet and exposure to environmental microbes may be considered expected inputs for the developing

microbiota (see Box 2). Multiple sensitive periods likely occur across the development of the microbiota-gut-brain axis, just as there are multiple sensitive periods within each domain of neurodevelopment (see Figure 2). While these ideas are still in a preliminary phase of testing, the current evidence points to their validity, as we will endeavor to show throughout this review.

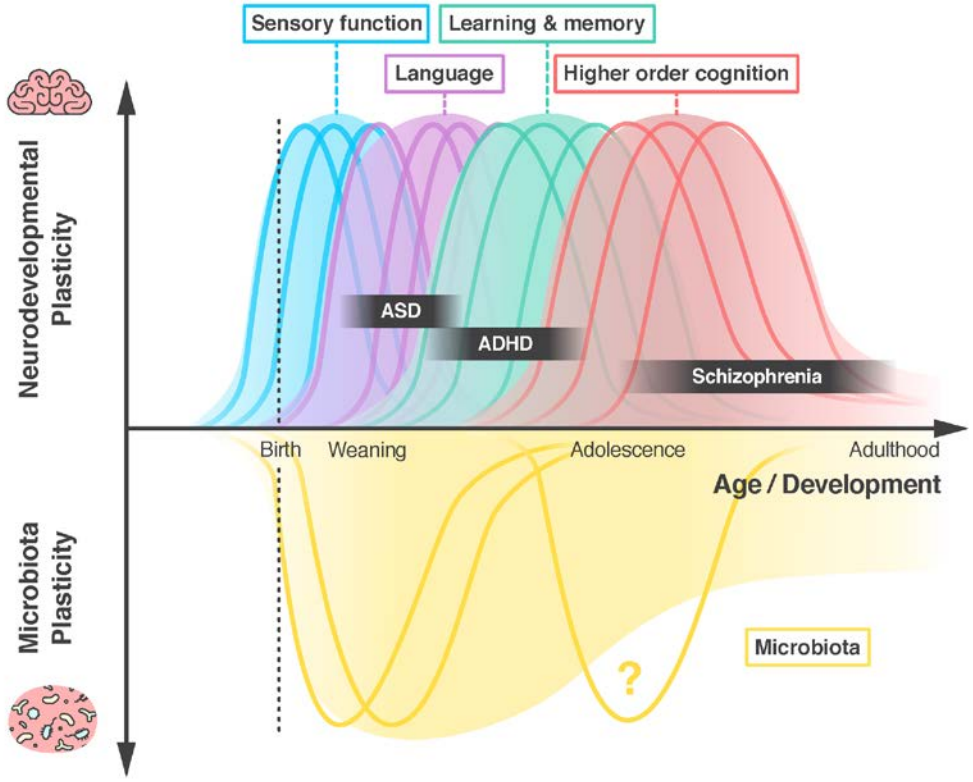

Figure 2. Sensitive periods, or critical windows of heightened plasticity, occur in a cascading fashion across human development, with multiple critical windows for each functional domain (shown on the upper axis). The timeframes for peak plasticity in the microbiota (in the early post-natal period, at weaning, and possibly again at adolescence; lower axis) overlap with these trajectories and also coincide with peaks in the onset of neurodevelopmental disorders (shown in black). Note that, compared to the brain, the microbiota exhibits a relatively high level of ongoing plasticity beyond the developmental period.

The microbiota itself seems to be especially sensitive to disruption during early life. Although its composition continues to be malleable to some extent throughout life, it has been hypothesized that early colonizers, or “founder species”, in the gut have a disproportionate

influence on the ultimate steady-state composition of the microbiota (Litvak & Baumler, 2019). The nature of the maturational trajectories of the microbiota also makes it more responsive to external influences during development. The postnatal microbiota is relatively volatile, gaining stability across maturation (Koenig et al., 2011; Stewart et al., 2018; Yee et al., 2019), perhaps due to the increasing diversity described in Section 1.1 above. Periods of microbiota instability also occur during later phases of development, with a clear shift at weaning and an understudied transition considered likely during the adolescent period (Figure 2). Given the dearth of longitudinal studies examining patterns of microbiota development beyond infancy, there may very well be other sensitive periods in between (see Derrien et al., 2019 for a discussion of the pre-school and primary school years).

There is additional evidence that the microbiota is particularly influential on the gut-brain axis during development. It has recently been shown that the natural course of microbiota maturation at weaning is crucial for development of the immune system, one of the key gut-brain pathways (Al Nabhani et al., 2019). Weaning induced a sudden change in the composition of the microbiota, with an associated spike in inflammatory activation that was termed the “weaning reaction”. Using several elegant methods to alter the timing of weaning or suppress the weaning reaction, it was demonstrated that such disruptions to the developmentally appropriate timing of the weaning reaction led to pathological imprinting of the immune system, increasing risk for various inflammatory disorders in response to later immune challenges.

Taking a further step into the microbiota-gut-brain axis, there are now several examples that support the idea of critical windows for microbiota modulation of brain and behavior in animals. Studies where germ-free rodents have been re-colonized with “normal” microbiota

(i.e., from specific pathogen-free animals) at different ages have shown that post-weaning re-colonization is more effective at restoring germ-free deficits than re-colonization later in life, at least for specific aspects of brain or immune function and behavior (Buffington et al., 2016; Diaz Heijtz et al., 2011; Olszak et al., 2012; Sudo et al., 2004). For example, social deficits in germ-free mice were reversed by re-colonization at weaning but not by re-colonization 4 weeks later in adulthood (Buffington et al., 2016). Still other functions in germ-free animals cannot be restored by re-colonization even at the time of weaning, hinting that the window for microbial influence on these functions is already closed by the age of weaning (e.g., Clarke et al., 2013). In support of the translational value of the sensitive periods hypothesis, two studies of childhood antibiotic exposure have reported that exposures in the first year of life, but not at later time-points, have a negative impact on cognitive development (Slykerman et al., in press; Slykerman et al., 2017; although see Section 2.1 for a discussion of limitations in these studies).

2. Evidence for Microbial Modulation of Neurocognitive Development

2.1 Behavioral and Cognitive Development

Human Studies. Two studies, including a recent investigation of a large Finnish cohort, have observed complex, sex-specific relationships between the microbiota composition and very early temperament, including extraversion, regulation, and fear reactivity (Aatsinki et al., in press; Christian et al., 2015). Gastrointestinal distress, which is associated with disruption of the microbiota, has been shown to predict future anxiety across childhood, even when accounting for initial anxiety (Callaghan et al., in press). The evidence for microbiota modulation of psychological or behavioral outcomes in pediatric populations with a physical illness is limited and mixed (Kan et al., in press). However, there are some promising studies

suggesting that modulation of the microbiota can improve behavioral symptoms of neurodevelopmental disorders (see Section 4.1).

With respect to cognitive development in humans, we are aware of just one study that has directly measured both the microbiota and cognitive performance, which is part of the ongoing University of North Carolina Early Brain Development Study (Carlson et al., 2018; Gao et al., 2019). In that cohort of 89 infants, the community structure (i.e., beta diversity) of the fecal microbiota at one year of age predicted cognitive performance on the Early Learning Composite (a global cognitive index) of the Mullen scale at 2 years. This effect was driven by differences in the receptive and expressive language domains. Alpha diversity at 1 year of age was also predictive of cognitive performance, as indicated by negative correlations between alpha diversity and the overall Early Learning Composite, expressive language scale, and visual reception scale at 2 years of age.

Finally, there have been some preliminary human developmental studies examining both neural/cognitive and behavioral outcomes in relation to early-life microbiota manipulations. It has been reported that antibiotic use in the first year of life has a detrimental effect on overall IQ and reading ability during the primary school years (Slykerman et al., 2017). Such early-life antibiotic exposure was also associated with greater behavioral difficulties, more oppositional behavior, and more symptoms of ADHD and depression. However, the strength of these proposed microbiota-cognition and microbiota-behavior relationships is difficult to ascertain as the researchers did not directly examine the microbiota nor collect data on the reasons for antibiotic use. The same research group later conducted a placebo-controlled trial of two probiotic strains administered throughout pregnancy and the first two years of life. In that sample, which exhibited high rates of antibiotic use (>80% in the first 2 years, similar

across treatment groups), there were no significant effects of either probiotic on cognitive or behavioral outcomes at 11 years of age (Slykerman et al., 2018), although the effect of early-life antibiotics was replicated (Slykerman et al., in press). However, again, this study had several limitations; there was no justification provided for the choice of probiotic strains, the doses, or the methods of administration, nor was there a measure of the microbiota across treatment (problems that are common in this area of research). Finally, a recent study of premature infants in a Neonatal Intensive Care Unit (NICU) also examined the effect of perinatal antibiotics administered either to the mother during gestation or to the infant during the NICU stay (Firestein et al., 2019). In this study, which excluded cases of confirmed sepsis to reduce the confounding effect of infection, perinatal antibiotic exposure was associated with increased attentional problems at 4-5 years of age. Antibiotic-exposed children also exhibited higher delta power on an electroencephalogram (EEG), a pattern observed in ADHD and interpreted as a delay in maturation. Finally, an intervention that encouraged physical contact (and therefore transmission of microbes) between parent and infant during the NICU stay reduced the risk for both behavioral and neural alterations (Firestein et al., 2019). In addition to this preliminary evidence for microbiota modulation of developmental outcomes in humans, there have been many more studies using animal models to explore different aspects of cognitive and behavioral development.

Animals - Social Behavior & Cognition. Perhaps the strongest evidence for microbial modulation of cognition and behavior in animal models comes from studies of social interactions (Sherwin et al., in press). Germ-free rodents typically exhibit deficits in both sociability and memory for social stimuli (indexed by preference for a novel social partner; Buffington et al., 2016; Desbonnet et al., 2014; Sgritta et al., 2019; Stilling et al., 2018) (but see also Arentsen et al., 2015). Similar effects are observed following antibiotic depletion of

the microbiota during development (Degroote et al., 2016; Desbonnet et al., 2015), while administration of certain probiotic species can enhance social behavior in various murine models of autism (Buffington et al., 2016; Hsiao et al., 2013; Sgritta et al., 2019) and inflammation-induced social withdrawal (D'Mello et al., 2015). In fruit flies (*Drosophila melanogaster*), both developing and mature individuals rely on microbiota-derived volatile compounds as social signals, influencing food and mating preference (Sharon et al., 2010; Venu et al., 2014). This is reminiscent of the associations between microbiota composition and extraversion observed in human infants, as described above (Aatsinki et al., in press; Christian et al., 2015). Thus, there is a robust relationship between social behavior and the microbiota observed across species. It has been hypothesized that this relationship has an evolutionary basis, driven by the mutual benefits of social interaction for both host and microbiota (Stilling et al., 2014). That is, social interaction encourages propagation and transfer of microbes to different hosts and simultaneously increases diversity in the microbiota of any particular individual, with potential benefits for satisfying the nutritional and health needs of the host.

Animals - Learning and Memory. Germ-free studies have demonstrated that rodents reared without a commensal microbiota exhibit exaggerated conditioned fear responding (Hoban et al., 2018), but impairments in object recognition memory and working memory on a spontaneous alternation test in adulthood (Gareau et al., 2011; Luk et al., 2018). Antibiotic administration from adolescence or adulthood has a similar effect on the novel object task, impairing recognition memory (Desbonnet et al., 2015; Fröhlich et al., 2016). The effects of antibiotic administration on spatial memory performance are more mixed, with one developmental study finding that early-life antibiotic treatment did not alter spatial memory in adulthood (O'Mahony et al., 2014), despite other evidence that chronic antibiotics impair

spatial memory in adult rats (Hoban et al., 2016a; Wang et al., 2015). These differences may be attributable to differences in the antibiotic regimen and rodent species; further developmental studies would be useful to understand the discrepancy.

Dietary interventions have shown promise in reducing the impact of stress or other microbiota perturbations on learning and memory outcomes across the lifespan. In adult rodents, probiotic supplementation has been used to reverse spatial memory deficits following stress, infection, or antibiotic treatment (Gareau et al., 2011; Liang et al., 2015; Wang et al., 2015), and even to provide benefits in healthy animals in terms of spatial memory, object recognition memory, and long-term fear memory (Savignac et al., 2014). During adolescence, dietary supplementation with omega-3 polyunsaturated fatty acids and vitamin A restored cecal microbiota composition and novel object recognition impairments following chronic social instability stress (Provensi et al., in press). Earlier in development, specific probiotic strains (*Lactobacillus rhamnosus* and *Lactobacillus helveticus*) rescued the expected developmental patterns of conditioned fear behavior in infant rats exposed to early-life maternal separation stress (Callaghan et al., 2016; Cowan et al., 2016b). Stressed infants exhibit more persistent fear memories and are more vulnerable to relapse following fear extinction, but both these behavioral abnormalities are reversed by probiotic treatment (an effect demonstrated in two separate labs; Cowan et al., 2016a; Peng et al., 2019). Remarkably, the probiotic treatment is even effective to prevent the generational transmission of these stress-induced behavior changes (Callaghan et al., 2016).

2.2 Brain Development

Morphology. Studies of the microbiota's role in human structural brain development are still rare. In adults, a cluster analysis identified different microbiota compositions that were

associated with different white matter and gray matter signatures, including regional volume differences in the right hippocampus, left nucleus accumbens, right anterior occipital sulcus, and cerebellum (Tillisch et al., 2017). Alpha diversity in the microbiota has also been linked to microstructure of the hypothalamus, caudate nucleus, and hippocampus of obese adults, while the relative abundance of a specific phylum, Actinobacteria, correlated with microstructure variables in the hypothalamus, thalamus, and amygdala (Fernandez-Real et al., 2015). In children, there has been just one study examining brain structure and gut microbiota, which reported positive associations between alpha diversity at 1 year of age and volumes of the left precentral gyrus, left amygdala, and right angular gyrus at 2 years (Carlson et al., 2018). The same study also reported specific regional brain volume differences based on a cluster analysis of the microbiota.

A more experimental approach was taken in a fecal microbiota transplant study from pre-term babies in the Neonatal Intensive Care Unit (NICU; Lu et al., 2018). Germ-free mice colonized by the microbiota of babies who showed poor growth in the NICU exhibited indicators of delayed brain development with respect to markers of neuronal differentiation, oligodendrocyte development, and myelination in the cerebral cortex compared to recipients of the high-growth microbiota. The microbiota from low-growth babies also affected various neurotransmission pathways and increased neuroinflammation while lowering circulating levels of growth hormones.

Such extensive changes in the structural development of the brain are similarly observed in animal models of microbiota depletion. Germ-free mice exhibit alterations in gross morphology, including expansion of the amygdala and hippocampus (Luczynski et al., 2016). Morphology of germ-free mice also differs at the neuronal level in a region-specific manner,

with hypertrophy of neurons in the amygdala and periaqueductal gray but shorter, less complex neurons in the anterior cingulate cortex and hippocampus (Luczynski et al., 2017; Luczynski et al., 2016). These structural differences, at least in the hippocampus, are likely related to observations of increased rates of hippocampal neurogenesis in both germ-free (Ogbonnaya et al., 2015) and antibiotic-treated mice (Möhle et al., 2016). Microbiota-related structural brain changes are not limited to the neuronal architecture; germ-free and antibiotic-induced microbiota depletion also leads to changes in microglia maturation (more immature microglia; Erny et al., 2015; Thion et al., 2018b) and levels of myelination (hypermyelination of the prefrontal cortex; Gacias et al., 2016; Hoban et al., 2016b), while white-matter integrity was associated with diet-induced changes in the gut microbiota in a diffusion tensor imaging study of rats (Ong et al., 2018).

Function and Activity. In adult humans, several brain imaging studies have shown that probiotic bacteria can alter brain activity in response to emotional stimuli (Pinto-Sanchez et al., 2017; Tillisch et al., 2013; Wang et al., in press), while certain microbiota compositions are associated with different patterns of brain activity (Tillisch et al., 2017). Metabolic function of the microbiota (specifically synthesis of phenylalanine, a dopamine precursor) was associated with decreased ventral striatal activity during reward anticipation in a group of young adults with or without ADHD (Aarts et al., 2017).

To our knowledge, there are only two published studies examining microbial modulation of brain function or activity in children, both of which were published in the last year. In the first, functional connectivity of various brain networks was assessed in sleeping 1-year-olds (Gao et al., 2019). Alpha diversity of the fecal microbiota correlated with functional connectivity in three separate networks: amygdala-thalamic, anterior cingulate cortex-right

anterior insula, and supplemental motor area-left parietal cortex. Further, this latter cluster was associated with performance on a cognitive assessment at 2 years of age, perhaps providing a partial mechanism for the previously described association between the microbiota and cognitive performance (Carlson et al., 2018). The second study was a recent pilot conducted in 5-11 year-old children exposed to early adversity (orphanage rearing) and age-matched controls (Callaghan et al., in press). In this cohort, levels of certain bacterial taxa correlated with prefrontal cortex activation to emotional faces. Some of these taxa were less prevalent in the children with a history of early adversity, supporting the idea that the microbiota acts as a link between early traumatic experiences and alterations in both neurodevelopment and psychological risk.

This study fits nicely with observations in animal models of early-life stress. Following maternal separation, a rodent model of early-life stress known to disturb the microbiota (O'Mahony et al., 2009), rat pups exhibit accelerated maturation of conditioned fear responding (Callaghan & Richardson, 2013) and a corresponding increase in activation of the prefrontal cortex during expression and inhibition of conditioned fear (Cowan et al., 2019). Probiotic supplementation during the stressful period was sufficient to reverse the effects on both behavior and prefrontal cortex activation (Cowan et al., 2016b; Cowan et al., 2019). Outside the context of stress, there is a strong and growing evidence base for microbial modulation of brain function or activity in various animal models (for a comprehensive review, see Cryan et al., in press). For example, transcriptomic analysis of the amygdala of germ-free animals revealed differential gene expression, exon usage, and RNA editing (Stilling et al., 2015). In addition, there are a number of reports of alterations in various neurotransmitter systems by manipulations of the microbiota, notably in regards to serotonin and BDNF (Bercik et al., 2011; Clarke et al., 2013; Desbonnet et al., 2015; Diaz Heijtz et al.,

2011; Gareau et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). Together with the studies of brain morphology, this body of work highlights the far-reaching influence of the microbiota when it comes to brain development.

3. Digging Deeper into Mechanism: Pathways for a Microbial Influence on Neurodevelopment

There are now a number of known communication pathways within the microbiota-gut-brain axis, including the vagus nerve, HPA axis, spinal cord, immune system, and peripheral transport of metabolites, amongst others. These have been reviewed in detail elsewhere (e.g., Cryan et al., in press). Here, we will provide a brief and simplified overview of just some of these pathways with a specific developmental focus (see Figure 3).

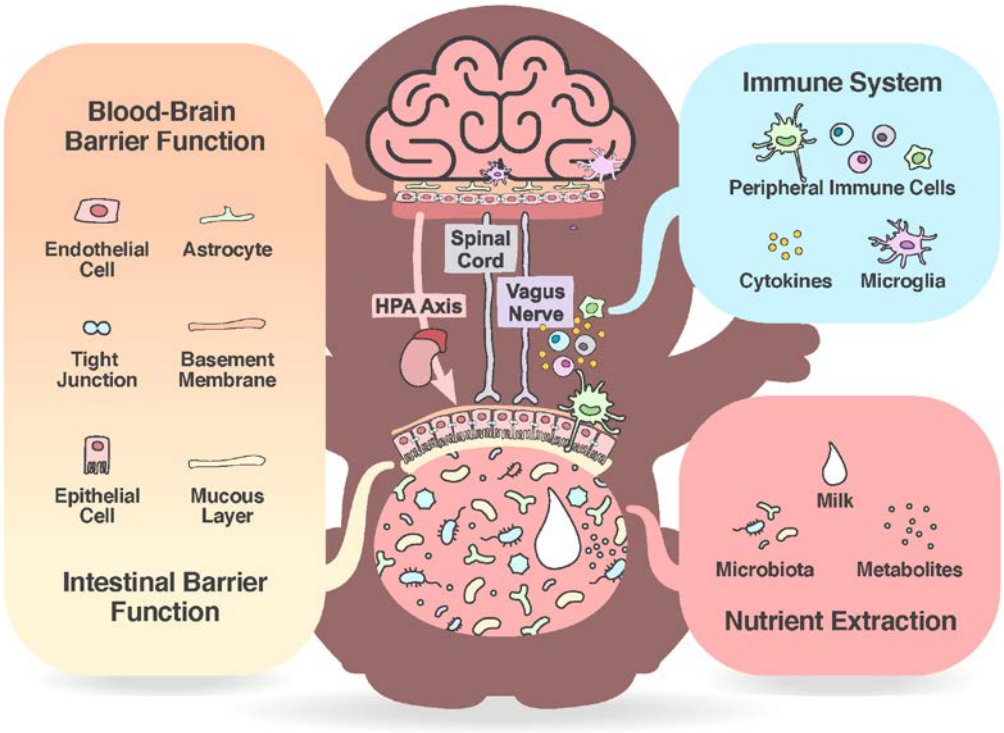

Figure 3. The microbiota-gut-brain axis consists of multiple pathways that allow bidirectional communication between the microbiota and the brain. During development, some of the key pathways include nutrient extraction, immune signaling, and barrier function, as well as neuronal and hormonal signaling along the spinal cord, vagus nerve, and hypothalamic-pituitary-adrenal (HPA) axis.

3.1 Metabolism and Nutrient Availability

Perhaps the most parsimonious hypothesis to explain microbial modulation of neurodevelopment is that the microbiota is a key source of essential nutrients and energy for the growing brain. Certainly, the microbiota is known to convert food components that would otherwise be indigestible into products with nutritional or biological value (see Rowland et al., 2018; Sela & Mills, 2010, for reviews on this topic, the latter in infants), and nutrition itself is a well-established modulator of cognitive outcomes. Breastfeeding, a dietary factor that influences microbiota maturation (Ho et al., 2018), has long been purported to improve cognitive outcomes (Johnstone et al., 1999), although more recent data indicates that this effect is largely accounted for by confounding variables such as maternal intelligence or education levels (Walfisch et al., 2013). On the other hand, both specific nutrient deficiencies and low overall diet quality are associated with negative long-term impacts on cognitive development (Freeman et al., 1980; Tandon et al., 2016), and nutritional interventions in vulnerable populations have been shown to improve cognitive outcomes during childhood (e.g., Freeman et al., 1980; Isaacs et al., 2009; Lucas et al., 1998). It has been argued that these effects need to be considered in terms of the developing microbiota (Goyal et al., 2015). In support of this argument, a recent cohort study in Dutch primary school children found that the strength of the relationship between pre-school diet and metabolic phenotype was dependent on microbiota composition (Zhong et al., 2019). Childhood malnutrition delays maturation of the microbiome (Smith et al., 2013; Subramanian et al., 2014), and fecal microbiota transplant from undernourished children into rodents or pigs indicates that this altered microbiota plays a causal role in the stunting and metabolic problems associated with undernutrition (Charbonneau et al., 2016; Smith et al., 2013). These microbiota-dependent problems were resolved in both animal models by supplementation with sialylated milk

oligosaccharides (Charbonneau et al., 2016). These compounds are typically found in breastmilk, act as prebiotics for the microbiota, and were low in the breastmilk of mothers with malnourished children. Another recent trial of "microbiota-directed complementary food" found promising effects compared to conventional food treatments in both animal models and malnourished children (Gehrig et al., 2019). The microbiota-targeted intervention supported microbiota maturation as well as biomarkers of growth, neurodevelopment, and immune function in malnourished children. Thus, there appears to be an important interaction between nutrient availability, the microbiota, and metabolic development whereby feeding the microbiota allows the microbiota to feed the developing body and brain.

3.2 Barrier Function

Between the microbiota and the brain, there are two major barriers: the gastrointestinal barrier and the blood-brain barrier (BBB). The permeability of these barriers is particularly relevant for transmission of microbiota-derived metabolites and neurotransmitters as one conveyor of signals along the microbiota-gut-brain axis; the more permeable the membrane, the more signals will be transmitted. There are striking similarities between the two barriers (Daneman & Rescigno, 2009). First, the main function of both is to protect against invading pathogens and toxins. Second, they share some broad structural similarities, being composed of a cellular layer that forms the main physical barrier alongside immune cells (particularly T cells at the gut barrier, microglia in the brain) that guard against pathogens (although there are also obvious structural differences, including the presence of a mucosal layer in the gastrointestinal tract). Third, despite these structural boundaries and the importance of separating the inner and outer environments at these critical interfaces, neither barrier is completely impenetrable. Rather, both are selectively permeable to certain biological and chemical elements. In a healthy state, this allows nutrients or signaling molecules from the

gastrointestinal milieu to flow into the bloodstream and/or pass between the brain and body. However, if these tightly regulated systems break down, increased barrier permeability can cause vulnerabilities to various forms of pathology. Fourth, both barriers continue to develop in the post-natal period and are considered most vulnerable during this early developmental stage (Saunders et al., 2012; van Elburg et al., 2003).

Finally, both the gastrointestinal and blood-brain barriers are known to be regulated by the microbiota, at least in animal models. The microbiota closely regulates development of the gastrointestinal tract, with germ-free animals exhibiting profound structural and functional alterations in the intestinal barrier (for a review, see Sommer & Bäckhed, 2013). These include alterations in expression of tight junction proteins and mRNA (claudin-1 and occludin), elongated microvilli, and loss of the mucous layer, all of which may contribute to an observed increase in intestinal permeability (Hayes et al., 2018). More surprisingly, the microbiota has also been implicated in the development of the BBB (Braniste et al., 2014). Germ-free animals exhibit a striking increase in BBB permeability, starting *in utero* and continuing into adulthood. Loss of BBB integrity in GF animals was accompanied by reduced expression of the tight junction proteins occludin and claudin-5. Both intestinal and BBB permeability can be restored in GF animals by re-colonization in adulthood, pointing to a life-long connection between barrier function and the gut microbiota (Braniste et al., 2014; Hayes et al., 2018).

3.3 Immune System

A growing literature is exploring the “neuro-immune axis” as its own bidirectional network or as a component of the microbiota-gut-brain axis (Irwin & Cole, 2011; Kraneveld et al., 2014). Apart from regulating barrier function, inflammatory signals alter neural activity

through several mechanisms, including interactions with the HPA axis and vagus nerve as well as the direct action of cytokines in the brain. Cytokines are produced by many different cell types, including glia and neurons in the brain, and can also be actively transported across the blood-brain barrier following peripheral secretion. The levels of cytokines in the central nervous system can alter metabolism of various neurotransmitter systems (including serotonin, dopamine, and glutamate; Miller et al., 2013). In this manner, aberrant cytokine levels can disrupt the function of many important neurocircuits, including those involved in motivation and emotion (Miller et al., 2013). Certain cytokines can also act as growth factors and/or activate gene pathways involved in various basic cell functions (survival, migration, proliferation, differentiation, apoptosis), which are particularly vulnerable to disruption during development (Deverman & Patterson, 2009). It is therefore unsurprising that abnormal cytokine production (e.g., following maternal infection during pregnancy or in the case of allergy) is a risk factor for neurodevelopmental disorders (Garay & McAllister, 2010; van Sadelhoff et al., 2019). Thus, we can conclude that appropriate cytokine signaling and immune function is essential for neurodevelopment.

Extending the neuro-immune axis to the gut microbiota, there is a growing understanding that the microbiota plays a critical role in development of the immune system. The gastrointestinal tract represents the largest immune interface in the body, and exposure to gut microbes is an important part of the process for training the immune system to distinguish harmful and innocuous stimuli and to subsequently mount appropriate responses to these different elements (Gensollen et al., 2016). Germ-free animals exhibit profound alterations in both innate and adaptive immunity (Strauch et al., 2005), while specific commensal bacteria can regulate the maturation of and balance between different types of T cells *in vitro* and *in vivo* (Baba et al., 2008; Ivanov et al., 2009; Sudo et al., 2002). Microbiota manipulations can

alter both circulating and central levels of cytokines (Burokas et al., 2017; Luczynski et al., 2017; O'Mahony et al., 2005; Sudo et al., 2004) as well as microglia development (Erny et al., 2015; Thion et al., 2018b). Moreover, there is a growing realization of a link between immune/allergic sensitivity and alterations in the microbiota-gut-brain axis (Berni Canani et al., 2019). Finally, in keeping with the sensitive periods hypothesis, there also seem to be critical windows for both microbiota-immune interactions (as described in Section 1.2; Al Nabhani et al., 2019) and neural-immune interactions (for a review, see Thion et al., 2018a).

3.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis

One of the seminal studies to investigate the role of the microbiota in brain development focused on the hypothalamic-pituitary-adrenal (HPA) axis stress response (Sudo et al., 2004). The study demonstrated that adult germ-free mice have an exaggerated HPA response to restraint stress, including elevated levels of ACTH and corticosterone (since replicated by Clarke et al., 2013). This effect could be attenuated or exacerbated by colonization with specific probiotic or pathogenic bacteria, respectively (Sudo et al., 2004). Re-colonization via fecal microbiota transfer from healthy mice also attenuated the HPA stress response. In keeping with our sensitive periods hypothesis, this strategy was only effective when the re-colonization occurred by 6 weeks of age (Sudo et al., 2004). In terms of stress experienced during development, the microbiota has been shown to mediate the effects of early-life stress in the maternal separation model. Whereas conventionally colonized mice exhibit exaggerated anxiety- and depressive-like behaviors following maternal separation stress, germ-free mice are not affected by maternal separation with regards to these behaviors (De Palma et al., 2015). Treatment with specific bacteria (*Lactobacillus rhamnosus* and *Lactobacillus helveticus*) was also effective at reversing the effects of maternal separation

stress on glucocorticoid production during development, both under basal conditions and in response to an acute stressor (Gareau et al., 2007; Peng et al., 2019).

3.5 The Vagus Nerve

The vagus nerve is considered to be the most direct route for signals from the microbiota to reach the brain (Fülling et al., 2019). It is the longest cranial nerve and consists of many branches that innervate the visceral organs, including the gastrointestinal tract, with both afferent (sensory) and efferent (motor) neurons. These fibers continue to develop across the first year of life in humans, with an increase in myelinated fibers (Pereyra et al., 1992). Signals from gastrointestinal vagal afferents reach the brain at the nucleus stria terminalis, which acts as a "relay station" through its projections to other brain regions, including many relevant to behavior (e.g., hypothalamus, amygdala, ventral tegmental area). Lesion of the vagus nerve prevents the behavioral effects of certain probiotics (Bravo et al., 2011; Sgritta et al., 2019). Most recently, it was shown that vagotomy at weaning prevented the rescue of social behavior by probiotic treatment in a genetic mouse model of autism (Sgritta et al., 2019).

4. Clinical Significance

The above indications of the sensitivity of the microbiota-gut-brain axis during early development, with long-term effects on host health, have implications for various clinical conditions. Here, we will focus on neurodevelopmental disorders, although there is also substantial evidence that many other psychological disorders have developmental origins (Kessler et al., 2007; Lee et al., 2014) and are modulated by the microbiota (for reviews, see Bastiaanssen et al., 2019; Foster & McVey Neufeld, 2013).

4.1 Microbiota and Neurodevelopmental Disorders

The most concrete evidence for a role of the microbiota in any neurodevelopmental disorder is found in the study of autism spectrum disorders (ASD). A large proportion of individuals with ASD report comorbid gastrointestinal problems (Chaidez et al., 2014; Parracho et al., 2005) and several observational studies have reported differences in the microbiota between children with ASD and neurotypical controls (Adams et al., 2011; Finegold et al., 2010; Kang et al., 2018; Kang et al., 2013; Parracho et al., 2005). Emerging experimental studies corroborate a more causal link between ASD symptoms and the microbiota, although all have been small studies and caution must therefore be taken in their interpretation. Fecal microbiota transplant from a small number of autistic individuals into germ free mice led to an emulation of aspects of autism (social deficits and increased repetitive behavior; Sharon et al., 2019). Three open-label pilot studies have demonstrated promising effects of antibiotics (Sandler et al., 2000), probiotics (Shaaban et al., 2017), or fecal microbiota transplants from healthy donors (Kang et al., 2019; Kang et al., 2017) as strategies to reduce symptom severity in ASD. Another three studies have used double-blind, placebo-controlled designs to assess different microbiota interventions. The initial trial of a 12-week probiotic treatment for 3-16 year old children with ASD was plagued by a high drop-out rate, but still indicated some benefit of the treatment for bowel function and perhaps for behavioral symptoms as well (Parracho et al., 2010). Similar benefits for gastrointestinal and behavioral symptoms were observed in a dietary intervention that assessed an exclusion diet with or without a prebiotic supplement (Grimaldi et al., 2018). Both the diet and the prebiotic led to alterations in microbiota composition that correlated with fecal metabolite content. Interestingly, the behavioral changes were only observed in the group that received both the exclusion diet and the prebiotic, whereas the changes in gastrointestinal symptoms were driven by the exclusion diet. Finally, a preliminary trial (N = 75) of post-natal probiotic supplementation for the first

6 months of life found that treatment had a prophylactic effect, reducing the risk for ASD at 13 years of age (Pärtty et al., 2015).

In addition to reducing risk for ASD, postnatal probiotic treatment (*Lactobacillus rhamnosus* GG) also reduced risk for attention deficit hyperactivity disorder (ADHD; Pärtty et al., 2015). Diet has long been considered a contributing factor to ADHD symptomatology and a Western-style diet (high in processed meat, refined grains, fats and sugars) during adolescence has been linked to the disorder (Howard et al., 2011). Many dietary interventions have been trialed in ADHD with varying degrees of success, the most effective being elimination diets that exclude artificial food dyes (Jacobson & Schardt, 1999; Pelsser et al., 2011). More direct examinations of the microbiota have shown that individuals with ADHD have a different microbiota profile compared to age-matched healthy controls (Aarts et al., 2017; Prehn-Kristensen et al., 2018). Using a hypothesis-driven approach, it was identified that alterations in the predicted functionality of the microbiota along a dopamine pathway were associated with activity during a reward-based task (Aarts et al., 2017). These findings suggest that it is worthwhile to further evaluate the links between microbiota, diet and symptomatology in ADHD, especially in childhood.

Finally, there has been much speculation about the role of the microbiota in schizophrenia. This relationship is particularly difficult to disentangle due to the almost ubiquitous use of antipsychotic medications, which are known to alter the microbiota. Nonetheless, some studies have identified differences in the oral and fecal microbiota of patients with schizophrenia, including particular taxa that correlated with symptom severity (Castro-Nallar et al., 2015; Zheng et al., 2019). Transplant of the fecal microbiota from patients into germ-free mice induced hyperactivity and exaggerated startle responses as well as changes in

hippocampal glutamatergic function, but no changes in social behavior or pre-pulse inhibition (Zheng et al., 2019). In humans, probiotic interventions have thus far not shown any effect on symptom severity in this complex neurodevelopmental disorder (see Ng et al., 2019, for a systematic review of the 3 available studies, all with negative results).

4.2 Challenges for Translation from Animals to Humans

The state of the research has not yet reached the stage where recommendations can be made for the use of microbiota-based medicines or diagnostics in clinical settings. One of the chief limiting factors is the relative dearth of studies in humans and particularly in children. As discussed above, the evidence points to a link between the microbiota and both neurocognitive and psychological development but, with few exceptions, this evidence has been generated from animal models and/or correlational studies. While this is a common problem across many scientific fields, there are a few challenges that become acutely evident when considering the study of the microbiota-gut-brain axis during development.

The study of such a complex whole-body system that incorporates multiple organs presents a number of challenges in itself. First, the complexity of interactions demands collaboration between experts in each system. There is a need for input from gastroenterologists, child psychologists/psychiatrists, microbiologists, and bioinformaticians to design appropriate experiments and ensure that the data is correctly analyzed. Second, this multiple-systems problem compounds the species differences within each individual system, which raises the barrier for translation of results from animal models. While rodents are the most common model system partly because they exhibit many similarities to humans in terms of both the brain and the gastrointestinal system, there are also clear differences in each component of the microbiota-gut-brain axis across species (Nguyen et al., 2015). For instance, the brain and

gastrointestinal tract are both simpler at a gross morphological level in rodents, while humans also have several distinct features such as the relative dominance of the cortical regions in the brain and the relatively small size of the cecum.

Considering these systems across development introduces additional complexity due to variation in the developmental timelines across species. The trajectory of neurodevelopment is very different for humans and rodents; the rodent brain at birth is similar to that of a very premature infant (approximately the start of the third trimester; Clancy et al., 2007). Another clear example is the timing of weaning, a key event that we have discussed as relevant to potential critical windows of microbiota-gut-brain development but which happens at different stages of brain development in rodents and humans. Young rodents begin to ingest solid food around postnatal day (P) 16 and are fully weaned by P21 (Londei et al., 1988). For human children, the World Health Organization recommends introduction of semi-solid foods from 6 months of age, with a completely solid diet at 18-24 months (World Health Organization, 2005). However, equating P16 in a rodent to 6 months in a human does not necessarily match on to estimates of neurodevelopment, which can vary wildly depending on the metric of interest. For example, some place a P16 rodent at the equivalent of 2-3 human months based on physical characteristics (Clancy et al., 2007), or more than 2 human years based on development of attachment relationships and fear neurocircuitry (Callaghan et al., 2019). Thus, it will be important to consider the specific developmental trajectories of different aspects of neurodevelopment when attempting to predict how studies of the microbiota in model species will translate to humans.

Another factor that warrants further attention is the impact of sex. Certainly, there are clear sex differences in the prevalence of neurodevelopmental disorders, with ASD, ADHD, and

schizophrenia being more common in males. The microbiota contribution to such sex differences in neuropsychological function is largely unknown, despite strong arguments to support further investigation and some intriguing preliminary findings (Audet, 2019; Jašarević et al., 2016). In rodents, there are now several examples where more pronounced effects of early microbiota disruption have been observed in males compared to females (Clarke et al., 2013; Desbonnet et al., 2014; Hoban et al., 2016b; Jašarević et al., 2017; but see also complex sex-specific effects described by Luk et al., 2018). For example, prenatal stress has more profound effects on the developing male microbiota (Jašarević et al., 2017; Jašarević et al., 2015), while germ-free males but not females exhibit hypermyelination of the prefrontal cortex (Hoban et al., 2016b) and disruption of the hippocampal serotonergic system (Clarke et al., 2013). Similarly, maternal germ-free status had a substantially greater impact on the microglia of males during embryonic development (>1,000 genes differentially expressed in male microglia vs 20 genes in females), although this sex difference was reversed in adulthood (Thion et al., 2018b). Following a fecal microbiota transplant from children with ASD, stronger behavioral deficits were observed in male recipient mice than in females (Sharon et al., 2019). In healthy children, two studies tested for but did not detect sex differences in the composition of the developing microbiota (Carlson et al., 2018; Stewart et al., 2018). However, interactions between sex and microbiota-relevant outcomes have been reported in at least two observational studies. In the first, the effects of prematurity on cognitive performance was stronger in boys, who also showed greater improvement with a dietary intervention (Lucas et al., 1998). In the other (Aatsinki et al., in press), associations between microbiota composition and temperament were stronger in boys (Aatsinki et al., in press; although this sex effect was not observed in a smaller, less well-powered study examining the same variables; Christian et al., 2015). Thus, the preliminary findings hint that males may be more vulnerable to the effects of microbiota manipulations on

neurodevelopment, in keeping with the pattern of sex differences observed for neurodevelopmental disorders.

The sheer size of the datasets generated by sequencing technologies can be bewildering to most experimental researchers. The vast majority of microbiota members are not well-characterized at the species or strain level, leaving large gaps in our knowledge regarding their function and relative importance. Databases are developing at a rapid rate in an attempt to close these gaps, but this requires vigilant updating and maintenance of the databases and techniques used to analyze them, impeding direct comparison between studies. When choosing methods to manipulate the microbiota in humans, more consideration needs to be given to choices of probiotic, dosage titration (particularly across development), method of administration, and target population (with some evidence that microbiota-based interventions are more useful when there is a pre-existing imbalance in the microbiota-gut-brain axis; Ng et al., 2018).

5. Conclusions

The field is beginning to narrow the gaps in terms of our understanding of the pathways that allow communication along the microbiota-gut-brain axis. More work is needed to fine-tune this knowledge and understand the boundary conditions for when specific pathways are most important. This mechanistic approach needs to be complemented with more translational studies to test the relevance of evidence produced by animal models for humans. In particular, studies examining sex differences, clinical populations, and prospective longitudinal cohorts are needed to assess the dynamic contribution of the microbiota to neuropsychological and clinical outcomes. Based on the evidence for potential sensitive periods in the microbiota-gut-brain axis, investigations focused on early development could

be a fruitful approach for gaining traction in harnessing the potential of the microbiota for treatment of psychological or cognitive problems.

Key Points

- The gut microbiota, or the collection of microorganisms that inhabit the gastrointestinal tract, have been shown to affect cognitive and behavioral outcomes across species
- The microbiota-gut-brain axis is made up of many pathways through which the microbiota and brain can communicate in a bidirectional manner
- We propose that developmental transitions in the microbiota may represent key sensitive periods during which the microbiota-gut-brain axis is most vulnerable, but also most malleable to intervention
- More work is needed to test the translational value of evidence gathered from animal models regarding the potential for targeting the microbiota in neurodevelopmental and psychological disorders, particularly in developing populations

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