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Altered gut microbiota and activity in a murine model of autism spectrum disorders

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ABSTRACT

Autism spectrum disorder (ASD) is a heterogeneous group of complex neurodevelopmental disorders with evidence of genetic predisposition. Intestinal disturbances are reported in ASD patients and compositional changes in gut microbiota are described. However, the role of microbiota in brain disorders is poorly documented. Here, we used a murine model of ASD to investigate the relation between gut microbiota and autism-like behaviour. Using next generation sequencing technology, microbiota composition was investigated in mice *in utero* exposed to valproic acid (VPA). Moreover, levels of short chain fatty acids (SCFA) and lactic acid in caecal content were determined. Our data demonstrate a transgenerational impact of *in utero* VPA exposure on gut microbiota in the offspring. Prenatal VPA exposure affected operational taxonomic units (OTUs) assigned to genera within the main phyla of Bacteroidetes and Firmicutes and the order of *Desulfovibrionales*, corroborating human ASD studies. In addition, OTUs assigned to genera of *Alistipes*, *Enterorhabdus*, *Mollicutes* and *Erysipelotrichalis* were especially associated with male VPA-exposed offspring. The microbial differences of VPA *in utero*-exposed males deviated from those observed in females and was (i) positively associated with increased levels of caecal butyrate as well as ileal neutrophil infiltration and (ii) inversely associated with intestinal levels of serotonin and social behaviour scores. These findings show that autism-like behaviour and its intestinal phenotype is associated with altered microbial colonization and activity in a murine model for ASD, with preponderance in male offspring. These results open new avenues in the scientific trajectory of managing neurodevelopmental disorders by gut microbiome modulation.

1. Introduction

The critical role commensal microbes play in health and disease, by influencing physiological homeostasis in the intestines and periphery, is well documented (Kinross et al., 2011; Sebat et al., 2007). It has been shown that gut microbiota contribute to maintaining resistance to infections and stimulate immunological as well as metabolic development. The brain is closely connected to the gut via 200–600 million neurons (Furness, 2006). Currently, an increasing number of studies investigate the bidirectional gut–brain axis. The communication network between the brain and the gut mainly describes how signals from the brain can influence intestinal physiology, and the other way around, how visceral messages can impact brain functions (Diaz Heijtz et al., 2011). The specific role of the gut microbiome and the immune system in the gut–brain axis remains to be further explored.

Several animal models have been used to explore the link between the gut microbiota and the enteric and central nervous systems. Gut microbiota differences have been identified in rodents exposed to early life stress such as depression and anxiety-like behaviour (Bangsgaard Bendtsen et al., 2012; O'Mahony et al., 2011). Maternal separation of rat pups led to alterations in intestinal permeability and a disruption of gut microbiota that persisted into adulthood (O'Mahony et al., 2009). In rhesus monkeys, maternal separation led to a significant decrease in the level of lactobacilli in faeces as well as an increased susceptibility to opportunistic bacterial infections (Bailey and Coe, 1999). Germ free (GF) mice provide a useful tool to investigate the influence of gut microbiota on the gut–brain axis and have been used for studying different brain disorders such as depression and stress. For instance, behavioural studies revealed that GF mice show reduced anxiety-like behaviour and increased motor activity, compared to specific conventional mice (Diaz Heijtz et al., 2011). Increased endocrine

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responses to stress were reported in GF mice, which could be reversed by mono-association with *Bifidobacterium infantis* and not with enteropathogenic *Escherichia coli* (Sudo et al., 2004). In order to correlate significant alterations of the gut microbiota with behavioural parameters, Bercik et al., showed that antibiotic treatment in specific pathogen free mice led to increased motor activities (Bercik et al., 2011). Interestingly, infection with *Campylobacter jejuni* was correlated with increased anxiety-like behaviour while intervention with a probiotic *Bifidobacterium* strain was associated with decreased anxiety and reduced depressive behaviour (Bercik et al., 2012; Goehler et al., 2005). The effect of the probiotic strain was shown to be mediated by the vagus nerve. Overall, accumulating evidence is suggesting that microbial colonization initiates signalling mechanisms affecting neuronal circuits involved in motor control and anxiety-like behaviour.

An interesting avenue of research concerns the potential role of intestinal microbiota in the pathophysiology of autism spectrum disorders (ASD). ASD is one of the fastest growing neurodevelopmental disorders in the industrialized world and has been linked to several environmental triggers including pre- or postnatal exposure to chemicals and drugs, air pollution, stress, maternal infection, and dietary factors (Dietert et al., 2011). Intriguingly, gastrointestinal disturbances, such as abdominal pain, diarrhoea and gas retention, are frequently reported in infants with ASD, which may correlate with the severity of the disorder (Adams et al., 2011; Buie et al., 2010; de Theije et al., 2011). Intestinal permeability is also increased in ASD patients when compared to healthy subjects (de Magistris et al., 2010; de Theije et al., 2011). It is hypothesized that such gastrointestinal deficits may be associated with compositional changes and metabolic activities of intestinal bacteria (Bolte, 1998). Indeed microbial composition differences have been reported as well as altered levels of bacterial metabolites derived from the fermentation of undigested food components. More specifically, levels of SCFA, including butyric, propionic, acetic and valeric acid, were significantly increased in children with ASD when compared with controls (Wang et al., 2012). Also by-products of microbial protein fermentation, such as ammonia and free amino acids were shown to be increased in children with ASD (De Angelis et al., 2013; Wang et al., 2012). Additionally, numerous species within the main bacterial phyla, Bacteroidetes and Firmicutes, have been identified to be differentially abundant in faecal samples. Most consistently, *Clostridium* species are reported to be higher in patients with ASD compared to controls (De Angelis et al., 2013; Finegold et al., 2002; Parracho et al., 2005; Song et al., 2004; Williams et al., 2011). Furthermore, *Bifidobacterium* species were frequently reported to be lower (Adams et al., 2011; De Angelis et al., 2013; Wang et al., 2011), while *Sutterella* and *Desulfovibrio* species were reported to be increased in ASD patients (Finegold et al., 2010; Williams et al., 2011). However, these observations have not been consistent (Adams et al., 2011; Parracho et al., 2005) and one study reported no clinically meaningful differences between groups (Gondalia et al., 2012). Limitations of comparing these results are heterogeneity in age and presence of gastrointestinal problems, as well as in family bonds between subject and control groups. Moreover, different microbiota detection methods have been used, based on molecular methods or bacterial culture. The latter is known to suffer from the inability to culture the majority of species from the gut. Next generation sequencing technologies, like pyrosequencing, were proven to be more powerful tools to study the true complexity of the intestinal microbiota (Sim et al., 2012).

In addition to aberrant microbial composition, treatment with vancomycin, a minimally absorbed oral antibiotic targeting gram-positive anaerobes, provided transient improvement in gastrointestinal symptoms in patients with regressive-onset autism. Interestingly, cognitive skills were also improved in these children, which undergo typical development until a clear deterioration in behaviour is observed (Sandler et al., 2000). These outcomes clearly indicate an imperative role of the gut microbiota in these autistic patients. As antibiotic therapy is not a long-term solution, nutritional concepts selectively modulating the gut microbiota may be a promising avenue for therapeutic targeting in specific groups of ASD patients.

Prenatal exposure to teratogens, such as the anticonvulsant valproic acid (VPA), is a significant risk factor for the development of ASD (Christensen et al., 2013; Dufour-Rainfray et al., 2011; Moore et al., 2000; Rasalam et al., 2005). The mechanism for VPA-induced symptoms of ASD is still unclear, but preclinical studies suggest involvement of folic acid metabolism, histone deacetylation, oxidative stress, synaptic plasticity and neuronal apoptosis (Ornoy, 2009). In rodents, *in utero* exposure to VPA induces developmental and behavioural deficits that persist into adulthood and are comparable to those observed in ASD patients. Behavioural abnormalities include deficits in social (Kataoka et al., 2013; Roullet et al., 2010; Schneider et al., 2008) and repetitive behaviour (Gandal et al., 2010; Schneider et al., 2008) and communication (Gandal et al., 2010; Roullet et al., 2010). Developmental deficits include abnormalities in neuroanatomy and neuronal morphology and molecular dysregulation of monoamines and neuropeptides in various brain regions important for emotional, social and repetitive behaviour (reviewed by Roullet et al., 2013). Interestingly, observations were more prominent in male than in female offspring (Kataoka et al., 2013; Kim et al., 2013), which is a representative reflection of the human situation where a marked male preponderance is observed in ASD patients (Fombonne, 2005; Lord et al., 1982). Although the exact mechanism for VPA-induced behavioural deficits remains unclear, we hypothesize here that VPA-treatment of the dams may affect postnatal development of gut microbiota of the offspring. Alteration of early microbial colonization may interfere with brain development triggering or enhancing autistic-like behaviour in the offspring. The effect of *in utero* VPA exposure on gut microbiota of the offspring has, to our knowledge, never been investigated before, neither in humans nor in animals. In the present study, we investigated the microbial composition of the offspring of VPA-exposed pregnant mice along with the levels of microbial derived metabolites, namely SCFA and lactic acid. Microbial parameters described correlated to ileal levels of serotonin and intestinal neutrophil infiltration as well as measurements of social behaviour that are described in detail elsewhere (de Theije et al., co-submitted). The aim of this study was to identify specific links and further contribute to the understanding of the role of the gut microbiota in early life development of brain and behaviour.

2. Material and methods

2.1. Animals and experimental design

Male and female BALB/C mice from Charles River laboratories, the Netherlands, were housed together in plastic cages with standard chip bedding and free access to food ('Rat and mouse breeder and grower' from SDS special diet services, the Netherlands) and water. Lights were set on a 12 h on:12 h off-cycle and temperature was maintained at 25 °C. All females were mated until a vaginal plug was detected, recorded as gestational day 0 (G0), and females were housed separately. Pregnant females were treated subcutaneously on gestational day 11 (G11) with 600 mg/kg VPA (100 mg/ml). Control females were treated with phosphate buffered saline (PBS) also on G11. Day of birth was recorded as P0 and mother and pups were housed in one cage per litter, resulting in a total of 4 cages per group. This yielded 8 VPA exposed pups ($n = 4$ females and $n = 4$ males) and 11 control pups ($n = 6$ females and