Neuroimmunomodulation of the young brain

NUTRITION, A GUT FEELING

Caroline de Theije

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(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER ONE

General introduction

Brain development is a prolonged process that extends at least through late adolescence and arguably throughout lifespan (1). Human brain development begins in the third week of gestation, with differentiation of neural progenitor cells and subsequent formation of the neural tube (1). The mouse brain develops in a similar but more rapid manner and neural tube formation occurs at gestational day 8.5 (2). General mechanisms involved in neurodevelopment include cell induction, proliferation, migration, differentiation, and synapse formation (1). These complex and precisely regulated processes are dependent on transient expression patterns of numerous genes. Mutations in these genes lead to neurodevelopmental disorders. For example autism spectrum disorder (ASD) is a highly heritable disorder, as indicated by the high recurrence risk in families with an affected child and the common genetic variants observed in approximately 20 % of children with ASD (3, 4).

For the majority of individuals the cause of ASD onset remains unknown, implying that besides genetic predispositions, also environmental factors play an important role. This implication instigated research on the non-genetic causes of ASD, which is now believed to be a multi-factorial disorder. Environmental insults during pre- and postnatal neurodevelopment can have permanent effects on brain functioning and consequently behaviour across lifespan. In recent years, preclinical and clinical studies have shown that prenatal environmental factors such as maternal immune activation, stress, nutrition and drugs use during pregnancy increase the risk of neurodevelopmental disorders in the newborn (5). Early life programming is thought to be a crucial mechanism by which maternal risk factors may predispose offspring to develop psychiatric disorders including ASD, schizophrenia, and depression (6), but also immune diseases such as allergy (7). This may be of interest, as neurodevelopmental disorders are frequently reported to be associated with immune dysfunction, particularly in the intestinal tract (8). Because conflicting results have been published on the prevalence of intestinal dysfunction in neurodevelopmental disorders such as ASD, the existence of the so called gut-brain axis in ASD is still under debate.

Not exclusively *in utero*, but also during early life, environmental factors can have a persistent impact on brain development (9). It is hypothesized that immune activation contributes to the behavioural impairments in neurodevelopmental disorders. In particular allergic immune activation has been associated with ASD and attention-deficit hyperactivity disorders (ADHD) (10). Multiple studies observed increased immunoglobulin levels or T helper 2 (Th2)-type cytokines in serum of patients with ASD. Moreover, increased behavioural impairments were observed in ASD patients

after challenge with cow's milk (10, 11). However, there is need for fundamental studies supporting or rejecting the hypothesis that allergic immune activation affects brain regions and behaviours relevant to ASD.

Despite the inconclusive evidence, a considerable amount of patients with ASD are on specific diets, aimed to improve intestinal dysfunction and behaviour. These nutritional interventions include vitamin, mineral, and n-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) supplementations, as well as gluten-free and milk-free diets (12). Nutritional opportunities for the treatment of behavioural and brain dysfunction have recently received increased interest, and efficacy is currently under investigation in disorders of neurodevelopment and neurodegeneration (13). Although still based on preliminary evidence, some patients with neurodevelopmental disorders may benefit from nutritional supplementation. Safety and efficacy of nutritional supplementation should be ascertained in preclinical models.

AIM AND OUTLINE OF THIS THESIS

This thesis aims to gain insight in the importance of intestinal inflammation in ASD and, vice versa, in the behavioural and neurochemical consequences of (allergic) intestinal immune activation in a preclinical setting. In the first part of this thesis, the inflammatory state of the intestinal tract was investigated in a well-defined mouse model for ASD, induced by *in utero* exposure to valproic acid (VPA). The second part of this thesis describes the impact of induction of food allergy in mice, using sensitization to whey protein, on behaviour and neurochemical changes in the brain. The interplay between impaired neurodevelopment and food allergy was examined in a preliminary study described in the last part of this thesis.

Chapter 2 describes the scientific background regarding the gut-brain axis in ASD and provides an in depth review on the pathways that may underlie this connection and how they could be targeted for disease management. **Chapter 3** demonstrates that intestinal inflammation is present in a mouse model for ASD. Moreover, it shows that behavioural and intestinal impairments are only observed in male offspring and are accompanied by decreased serotonin (5-HT) levels in the brain as well as in the intestinal tract. **Chapter 4** subsequently describes the alterations in microbiota composition and activity observed in these male mice exposed to VPA *in utero*. **Chapter 5** demonstrates that a multi-nutrient diet, containing specific anti-inflammatory and neuroprotective ingredients, provided during pre- and postnatal development, improves social and anxiety-like behaviour and restores levels of 5-HT and its metabolite in the brain and intestinal tract in a mouse model for ASD.

The second part of this thesis focuses on the effects of food allergy on the developing brain and on behaviour. **Chapter 6** reviews the preclinical and clinical background on the involvement of food allergy in neurodevelopmental disorders and includes an evaluation of the food-based therapies that have been studied. **Chapter 7** demonstrates that food allergy reduces social behaviour and increases repetitive behaviour in a mouse model for cow's milk allergy. Neuronal activation is increased in the prefrontal cortex and reduced in the paraventricular nucleus of the hypothalamus. Moreover, dopaminergic activity is reduced in the prefrontal cortex and increased in the amygdala of food allergic mice. While no changes are observed in the serotonergic system in the brain, increased levels of 5-HT are present in the intestinal tract after an allergic response. **Chapter 8** shows that a diet high in n-3 LCPUFA prevents impairments in social behaviour, in prefrontal dopaminergic activity, and in intestinal 5-HT levels of food allergic mice. Social and repetitive behaviour are also prevented in food allergic mice by a multi-nutrient diet containing specific anti-inflammatory and neuroprotective ingredients (**chapter 9**). **Chapter 10** further demonstrates that *in utero* exposure to VPA alters allergic sensitization to cow's milk protein, characterized by an increased polarization of the immune response towards Th2, which is prevented by dietary intervention with the specific multi-nutrient diet. Finally, the findings described in this thesis are summarized, discussed, and concluded in **chapter 11**.

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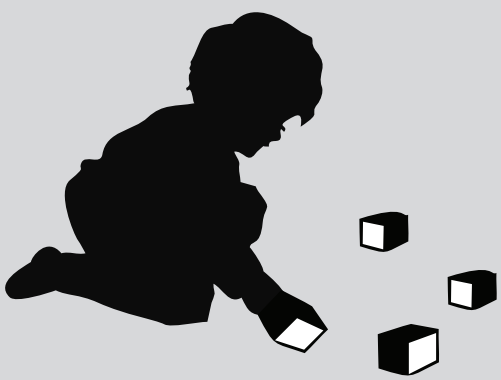
CHAPTER TWO



Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management

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ABSTRACT

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder, characterized by impairments in social interaction and communication and the presence of limited, repetitive and stereotyped interests and behaviour. Bowel symptoms are frequently reported in children with ASD and a potential role for gastrointestinal disturbances in ASD has been suggested. This review focuses on the importance of (allergic) gastrointestinal problems in ASD. We provide an overview of the possible gut-to-brain pathways and discuss opportunities for pharmaceutical and/or nutritional approaches for therapy.

Autism spectrum disorder (ASD) comprises autism, pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger's disorder. These pervasive neurodevelopmental disorders are characterized by impairments in social interaction and communication and the presence of limited, repetitive and stereotyped interests and behaviours (1, 2). So far, no biomarkers for ASD have been identified. Therefore, diagnosis of ASD is entirely dependent on behavioural observations, according to the DSM-IV criteria (3). Literature suggests that the prevalence of ASD has increased 20 times, from a rate around 1:2500 in the mid 1980s to a rate of 9:1000 at present (4, 5). Nowadays, early diagnostic tools are available (6) and diagnostic stability has been established (7). Although many believe that the ASD escalation is a consequence of better and earlier diagnosis, improved awareness and expanding criteria to fulfil the diagnosis, some believe that these changes do not adequately account for the rapid rise (8).

Current interest in research on ASD is boosted, but the underlying pathophysiology of the disorder remains unknown. Despite the importance of genetic factors, as indicated by the high concordance rates among twins (9), ASD is most likely a multi-factorial disease, in which a combination of genetic disturbances and environmental factors play a role in the expression of the autistic phenotype. Currently, many environmental factors, both pre- and postnatal, are found to be associated with ASD, including gastrointestinal disturbances. Although data are conflicting and more studies are required to establish the prevalence of gastrointestinal disorders in the autistic population, bowel symptoms in autistic patients are repeatedly reported. This review focuses on the importance of (allergic) gastrointestinal disturbances in ASD. We provide an overview of the possible gut-to-brain pathways and discuss opportunities for pharmaceutical and/or nutritional approaches for therapy.

GASTROINTESTINAL DISTURBANCES IN ASD

Due to social and communicative impairments, identifying gastrointestinal problems in patients with ASD is challenging. Many autistic patients have verbal impairments, which makes it difficult for them to express their discomfort. Even autistic individuals who are verbally skilled may be less able to express their feelings, because of their social disabilities. Therefore, it is challenging to determine the true prevalence of gastrointestinal disturbances in the autistic population. The reported prevalence ranged from 10 % to 90 % (10-16), an immense dispersion that is partially due to different interpretations of 'gastrointestinal problems'. Frequently observed symptoms among autistic patients include chronic constipation or diarrhoea, abdominal pain and pathological observations such as food allergy, gastroesophageal reflux disease (GERD), enteric colitis, lymphoid hyperplasia and oesophagitis (17, 18). Pang *et al.* (2010) determined the incidence of ASD among patients presented to their Paediatric Surgical Constipation clinic (19). ASD

appeared to be almost 10 times more common in the constipation clinic (8.5 %) than in the general population (0.9 %). Even more recently, Peeters *et al.* (2011) performed a similar study, determining the prevalence of ASD in children presented at their clinic with functional constipation or functional non-retentive faecal incontinence (20). Remarkably, 18 % of the children had scores indicative for ASD. The study of Pang et al. (2010) also showed that the onset of constipation was earlier in patients suffering from autism and moreover, earlier than the average onset of autism in a different study (13). From this, they suggested that constipation is an intrinsic rather than secondary factor in the development of ASD. Ibrahim *et al.* (2009) were unable to find significant differences between the overall prevalence of gastrointestinal problems in ASD compared to controls, but they did identify a higher prevalence of constipation (ASD: 33.9 % vs. controls: 17.6 %; $P = 0.003$). In a different study, diarrhoea was linked to ASD as well (21). At 30 and 42 months of age, children with ASD were more likely to have two or more stools a day and the incidence of diarrhoea was significantly enhanced in the autistic group compared to controls (ASD: 58 % vs. controls: 44 %; $P = 0.039$).

GASTROINTESTINAL PATHOLOGY IN ASD

Three studies investigated enteric lymphocyte infiltration in biopsies of children with ASD and found remarkable results (22-24). Compared to histologically non-inflamed controls, there was a higher number of infiltrated helper and cytotoxic T cells and $CD19^+$ B cells in biopsies of the duodenum, terminal ileum and colon of autistic patients with gastrointestinal disturbances (22-24). Furthermore, even compared to histologically inflamed controls, there was more infiltration of helper T cells and $CD19^+$ B cells in all three intestinal compartments of these autistic children (22). Even more surprisingly, helper T cell infiltration was also more enhanced in the terminal ileum and colon of these children with autism, compared to children suffering from inflammatory bowel disease (22). In a different study, enhanced density of dendritic T cells was observed in the colon of ASD children with gastrointestinal disturbances compared to histologically non-inflamed controls, and even compared to controls suffering from lymphoid nodular hyperplasia, Crohn's disease and ulcerative colitis. Basement membrane thickness was enhanced as well, compared to all other groups. However, histopathology demonstrated that lymphocytic colitis was less severe in autistic children than in classical inflammatory bowel disease (23). Furthermore, on the basolateral enterocyte membrane of autistic children with gastrointestinal disturbances, deposition of IgG1 and IgG4 was shown to be accumulated compared to normal controls and celiac patients (24).

A factor that might contribute to the gastrointestinal disturbances among autistic individuals is an abnormal composition of gut microbiota. Several groups have studied the intestinal microbiota of the autistic population and found a different composition of several microbial species compared to healthy controls. These ASD-related microbial species mainly comprise species within the phyla of Bacteriodetes, Firmicutes, and Proteobacteria, and often belong to genera of *Bacteroides, Ruminococcus,* and *Desulfovibrio* (12, 25-27). A recent paper by Adams *et al.* (2011) demonstrated lower levels of *Bifidobacterium* and higher levels of *Lactobacillus* in ASD, both considered to be beneficial bacteria (28). Colonization of *Clostridium* species to the expense of *Bifidobacterium* have been associated with higher risks of food allergy in children and with the development of (paediatric) inflammatory bowel diseases as well (29-32). Interestingly, antibiotic treatment of ASD children did not only lead to gastrointestinal improvements, but also improvements in cognitive skills (33).

The data on gastrointestinal disturbances, such as changes in gut microbiota and T cell infiltration, indicate an altered immune status in the intestine of autistic individuals. It is unknown whether the association between autistic behaviour and gastrointestinal disturbances is a cause-and-effect relationship and what factor could be intrinsic. Given the fact that gastrointestinal disturbances are strongly correlated with the severity of autistic behaviour (28), we hypothesize that the presence of gastrointestinal inflammation makes a child with a genetic predisposition for ASD more prone to express the autistic phenotype or that it increases the severity of autistic behaviour. In **Fig. 1**, the possible pathways are depicted in which immune factors of gastrointestinal origin can influence neuronal functioning and thereby behaviour.

NEUROIMMUNE INTERACTIONS AT THE SIDE OF INTESTINAL INFLAMMATION

Pathway of intestinal inflammation

The gastrointestinal tract continuously encounters dietary antigens as well as bacteria and their products. Therefore, it is a crucial site of innate and adaptive immune regulation. Ingested antigens enter the gut mucosa through the microfold (M) cells in the Peyer's patch or through damaged epithelium, from where they are transferred to or directly taken up by antigen presenting cells (APCs). APCs, most likely dendritic cells (DCs), move to T cell areas, such as the Peyer's patch or mesenteric lymph node (MLN), where they interact with naive lymphocytes to initiate an adaptive immune response. Upon repeated encounter of the antigen, memory T and B cells are activated, resulting in a proliferative response and cytokine release, leading to gastrointestinal inflammation (34). Chronic inflammation in the gut can damage the epithelial cell layer and thereby increase intestinal permeability, resulting in a higher antigenic load. Intestinal permeability was found to be enhanced in autistic patients (35). Recently, de Magistris

et al. (2010) confirmed these findings by demonstrating significantly increased intestinal permeability in children with ASD and their first-degree relatives (36). Abnormal high intestinal permeability was observed in 36.7 % of the patients with ASD, compared with none of the age-matched controls. Among the first-degree relatives, 21.2 % showed abnormal high intestinal permeability compared with 4.8 % of the adult controls. The enhanced intestinal permeability observed in the autistic population could be both the cause and the result of inflammation in the gastrointestinal tract of these children. Nevertheless, high intestinal permeability enhances gastrointestinal inflammation and thereby worsens gastrointestinal discomfort.

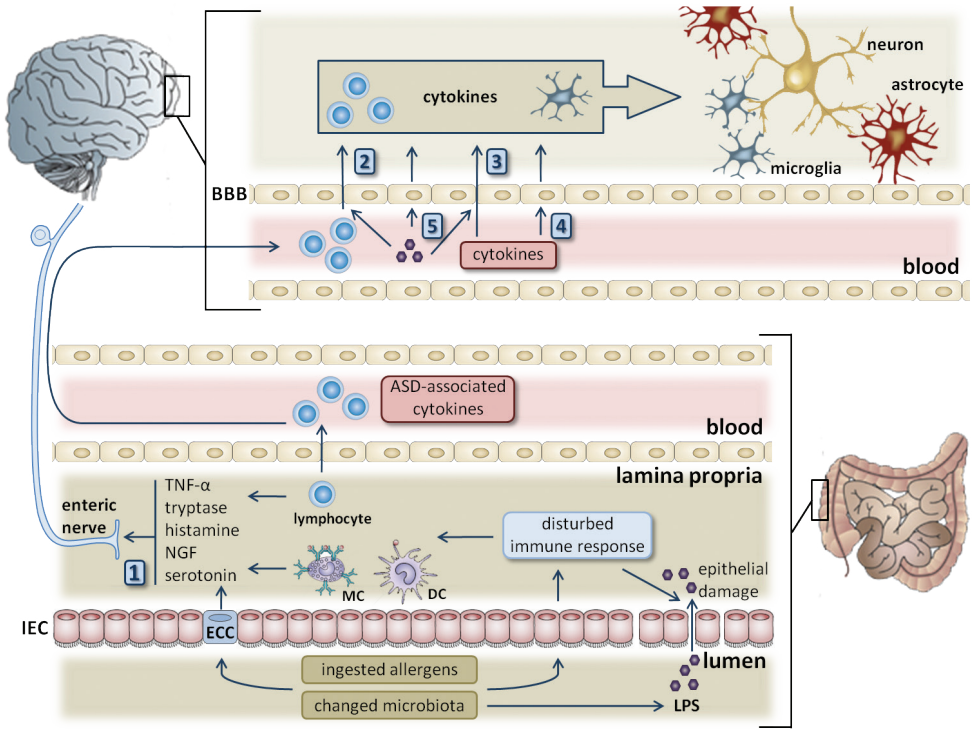

Fig. 1. Possible pathways involved in neuroimmune interactions in ASD. Upon immune disturbance in the gastrointestinal tract, intestinal epithelial cells (IECs) become more permeable and enterochromaffin cells (ECCs), lymphocytes, mast cells (MCs) and dendritic cells (DCs) secrete neuroimmune factors that can stimulate enteric nerves (**1**). In addition, ASD-associated cytokines (IL-1β, IL-4, IL-5, IL-6, IL-12, IL-13, IFN-γ, TNF-α) and lymphocytes are present in the circulation. Subsequently, lymphocytes can pass the blood-brain barrier (BBB) (**2**), serum cytokines (IL-1β, IL-6, IFN-γ, TNF-α) can pass the blood-brain barrier (**3**) and cytokines (IL-1β, TNF-α) can bind to brain endothelial cells inducing an immune response at the brain side (**4**). Lipopolysaccharides (LPS) can increase the permeability of the blood-brain barrier, enhancing cytokine and lymphocyte infiltration, or bind to brain endothelial cells inducing an immune response at the brain side (**5**). The immune response in the brain can consist of an increased number of lymphocytes and cytokines (IL-1β, IL-6, CXCL-8, IL-10, IFN-γ, TNF-α, CCL-2 and GM-CSF), produced also by neuroglia, resulting in changed neuronal homeostasis.

Serotonin: neurotransmitter and mediator of inflammation

The serotonergic system has been implicated in the pathogenesis of ASD since increased levels of blood serotonin (5-hydroxytryptamine; 5-HT) were first described in children with autism (37). Subsequent studies demonstrated that about one-third of the patients with ASD has blood hyperserotonemia (38, 39). On the other hand, the capacity of 5-HT synthesis in the global brain was decreased in children with autism (40), indicating a lower brain 5-HT availability. The cause of ASD-related hyperserotonemia is thought to arise from genetic (41), gastrointestinal (42, 43) or immune (44, 45) changes. Based on intestinal low-grade inflammation, blood hyperserotonemia and low 5-HT synthesis in the brain, we propose the following hypothesis. During an inflammatory response in the gut, 5-HT is produced and released by enterochromaffin cells and intestinal inflammatory cells such as mast cells and platelets, resulting in a faster moving gut and an increase in secretion, vasodilatation and vascular permeability. This, in turn, leads to problems in functional dysmotility, stool consistency (diarrhoea or constipation) and infiltration of leukocytes in the intestinal wall. Because of the increased utilization of dietary tryptophan by the gut, there will be less tryptophan available for passage through the blood-brain barrier. As a result, brain 5-HT levels are reduced and this may lead to mood and cognitive dysfunctions found in ASD. Indeed, the availability of tryptophan was demonstrated to be important, since depletion of tryptophan from the diet increased autistic behaviour in affected adults (46). More research is required to establish whether 5-HT metabolism can be a therapeutic target in ASD, either by providing dietary tryptophan or by pharmaceutical treatments such as selective serotonin reuptake inhibitors (SSRIs). Recently, it was reported that there is no evidence for a beneficial effect of treatment with SSRIs in autistic children and only limited evidence exists for the effectiveness of SSRIs in adults suffering from ASD (47). Perhaps, targeting the ASD-associated low-grade intestinal inflammation might be more successful in restoring the availability of tryptophan for 5-HT synthesis in the brain.

Food allergy in ASD

A disturbed intestinal immune reaction can be directed against food particles, initiating an allergic response. Food allergy has often been suggested to be present among autistic individuals. Parental reports indicate that food allergy is more common in the autistic population compared with healthy controls (48, 49). It is important to take into account that ASD children are likely under-diagnosed for food allergies, because of their impaired ability to express their discomfort. Lucarelli *et al.* (1995) observed that an oral challenge with cow's milk protein led to worsening of some of the behavioural symptoms specific for ASD. They also found significantly higher serum levels of IgA, IgG and IgM for casein and IgA for lactalbumin and β-lactoglobulin in children with ASD compared with healthy controls (50). Furthermore, the intake of milk protein was a significant predictor of constipation in the autistic population (51). Therefore, patients with ASD often exclude gluten and milk protein from their diet, better known as gluten free, casein

free diets. Some publications on gastrointestinal disturbances in ASD compared ASD patients on a gluten and milk free diet with ASD patients on an unrestricted diet. For instance, eosinophil infiltration in intestinal biopsies of children with regressive autism and gastrointestinal disturbances was significantly less abundant in those on a gluten and milk free diet compared with those on an unrestricted diet (22). Moreover, the ASD patients that excluded gluten and milk proteins, showed a significant reduction in the enhanced intestinal permeability compared with ASD patients on an unrestricted diet (36). In addition to the beneficial effects on gastrointestinal disturbances, a gluten and milk free diet was claimed to improve autistic behaviour as well. Indeed, parents reported improvements in social behaviour and linguistic skills (52). Few studies have been performed on the efficacy of a gluten and casein elimination diet in autistic individuals, showing improvements in rituals, verbal communication, interpersonal relations and learning (53-56). Unfortunately, these studies comprised either small cohort studies or case reports and could therefore not confirm the beneficial outcome of a gluten free, casein free diet. More research is necessary to strengthen these findings.

The majority of allergies is characterized by a T helper (Th) 2-type immune reaction. Th2 effector cells produce Th2 cytokines (interleukin (IL)-4, IL-5 and IL-13) and can activate memory B cells to secrete immunoglobulins (57). Supporting the suspected role of allergy in ASD, there seems to be an imbalance in Th1 and Th2 cytokines in these patients. Indeed, peripheral blood mononuclear cells (PBMCs) of children with ASD produced significantly higher levels of IL-4, IL-5 and IL-13 than their matched controls (58). In blood of ASD children, interferon (IFN)-γ and IL-2 positive helper and cytotoxic T cells were less abundant than in blood of healthy controls. In contrast, IL-4 positive helper and cytotoxic T cell numbers were enhanced (59). In addition to these data on a disturbed Th1/Th2 balance, a lower IFN-γ/IL-10 ratio was observed in male rats prenatally exposed to valproic acid, a well-characterized animal model for autism (60). In response to cow's milk protein, PBMCs from ASD children with and without gastrointestinal disturbances produced more tumor necrosis factor (TNF)-α and IL-12 than those from control subjects (61).

Furthermore, there were less IL-10 positive T cells present in both the periphery and the gut mucosa of ASD children with gastrointestinal symptoms, compared with non-inflamed controls and children with Crohn's disease (62). T cells that produce the anti-inflammatory cytokine IL-10 are mainly inducible T regulatory cells (Treg). Allergen-specific Tregs are predominantly present in healthy individuals to suppress an allergic response. Less IL-10 positive T cells are therefore associated with enhanced Th2 responses. Plasma levels of another T regulatory cytokine transforming growth factor (TGF)-β, were decreased as well, as observed by two groups (63, 64). Low TGF-β levels

were inversely correlated with behavioural scores (64). This indicates that Treg responses are decreased in individuals with ASD and that the lack of suppressive capabilities of the immune system could be involved in the expression of autistic behaviour.

During an allergic reaction, immunoglobulins activate mast cells and basophils, causing the release of various mediators, including histamine and cytokines. Mast cell activation has been suggested to play a role in autistic disorders as well. This hypothesis is supported by a preliminary report, indicating that ASD is more prevalent in patients with mastocytosis than in the general population (65). Not only immunoglobulins, but also several neuropeptides can trigger mast cell activation, including substance P, nerve growth factor (NGF), vasoactive intestinal peptide (VIP) and neurotensin (66). Neurotensin was significantly increased in serum of children with ASD (67). Upon activation, mast cells can express various substances that can trigger enteric neurons, such as tryptase, histamine, 5-HT, NGF and TNF-α (68) (**Fig. 1**, pathway 1). Mast cell-neuron interactions occur in the gastrointestinal tract, for instance in inflammatory bowel disease and irritable bowel syndrome (68). Therefore, an allergic reaction in the gut might influence behaviour via mast cells or other immune cells, which are able to trigger enteric neurons to convey information through afferent pathways in vagal and spinal nerves to the central nervous system (CNS).

Association between ASD and maternal allergic diseases

Cumulating to the importance of allergy in the pathophysiology of ASD is the finding that mothers diagnosed for asthma or allergies (such as atopic eczema and rhinitis) during the second trimester of their pregnancy had a greater than two fold elevated risk for ASD in their offspring (69). In addition, there was an enhanced association observed between allergic conditions and autism in families with more than one ASD-affected child. This observation suggests that genes underlying atopy may be related to the aetiology of ASD. (69). Recently, King (2011) hypothesized that epigenetic disruption of brain development is caused by gestational exposure to allergy-associated inflammatory mediators (for example IL-6 and histamine) (70). These mediators promote retinoic acid and oestradiol gene transcription, resulting in overexposure of the foetus to retonic acid and oestradiol. Retinoic acid (a vitamin A metabolite) is required for growth and development. An excess in vitamin A or retinoic acid is associated with brain abnormalities reminiscent of those present in ASD, such as cerebellar malformations, cranial nerve abnormalities and abnormalities of the dopaminergic system (71). Oestradiol is known to defeminize the foetal brain, playing an important role in sexual differentiation. Overexposure to oestrogen affects a wide range of cognitive functions which are characteristic for autistic individuals such as anxiety, motor deficits, stereotype and repetitive movements, hyperactivity and attention deficits (70, 72).

Other immune processes in ASD

Although many studies support the hypothesis that ASD is associated with a Th2-skewed immune response, there are also studies that indicate the involvement of other immune pathways. For instance, plasma levels of IL-12 and IFN-γ were shown to be increased in autistic individuals, suggesting rather an enhanced Th1 response instead of Th2 (73). Reduced cytotoxic activity of natural killer (NK) cells was also suggested (74). Recently, Ashwood *et al.* (2011) reported increased plasma levels of a heterogeneous group of cytokines, including IL-1β, IL-6, CXCL8 and IL-12p40 (75), making it even more difficult to identify a specific type of immune response. Furthermore, macrophage migration inhibitory factor (MIF), which is also constitutively expressed in brain tissues (76), was enhanced in peripheral blood of autistic individuals compared to typically developing controls. The high plasma MIF levels were positively correlated to autistic behaviour (77). Chemokines CCL2, CCL5 and CCL11 were also enhanced in plasma of children with ASD, compared with healthy controls. The increased chemokine levels were associated with higher aberrant behaviour scores (78). The heterogeneity of autistic disorders may be the reason behind these conflicting data.

NEUROIMMUNE INTERACTIONS AT THE SIDE OF THE BLOOD-BRAIN BARRIER

Immune cells produce all kinds of substances upon gastrointestinal inflammation, such as cytokines and chemokines. These immune cells and their substances are not restricted to the gut, but enter the circulation and will therefore pass all organs in the body, including the brain. The brain is a highly vascularized organ, but brain cells are protected from harmful compounds in the blood by means of the blood-brain barrier. This barrier is a layer of endothelial cells, cemented together with tight junctions. The cells lack intracellular fenestrations and have very little ability to undergo pinocytosis (79). The uniquely modified endothelial cells prevent free transport of most soluble substances between blood and brain. However, cytokines are still able to cross the barrier by active transport and even immune cells can pass through tight junctions by diapedesis (80). Therefore, gastrointestinal inflammation in autistic patients may influence the brain and thus behaviour through many different pathways, as indicated in **Fig. 1**.

Although ASD are considered neurodevelopmental disorders, the neuropathology remains poorly understood. Brain growth abnormalities are the most prominent findings in the neuropathology of ASD. The brain undergoes a period of rapid growth, followed by slow growth later in development (81). In addition to the abnormal growth patterns of the brain, one of the most consistent findings of neuroimaging studies in autistic individuals is the presence of abnormalities in the cerebellum, increased cerebral white matter and thickening of cerebral cortex (82-84).

Lymphocytes enter the brain and influence neurons via the production of immune factors

The endothelial cell layer of the blood-brain barrier is surrounded by a basal lamina that is in direct contact with pericytes and astrocytes, as well as with microglia in close attendance. Physiological changes in neuroglial cells can influence the blood-brain barrier integrity and make it more permeable for lymphocytes (80). Immune factors can also alter blood-brain barrier permeability. TNF-α, for instance, can disrupt the barrier by increasing P-glycoprotein expression (85) and by altering brain endothelial cell cytoskeletal architecture (86). Lymphocyte migration over the blood-brain barrier occurs under healthy circumstances and lymphocytes are consistently present in the brain. However, infiltration is highly increased upon immune activation (**Fig. 1**, pathway 2). After infiltration into the brain, lymphocytes secrete cytokines and chemokines that can activate microglia and thereby alter neuronal functioning. One group studied the presence of lymphocytes in postmortem brains of autistic children, but could not identify lymphocyte infiltration or immunoglobulin deposition (87). Therefore, it could be rather cytokines than lymphocytes initiating an immune response.

Cytokines enter the brain and influence neurons via neuroglia

Numerous cytokines are able to cross the blood-brain barrier, for example IL-1β, IL-6, and TNF-α (88-91). As mentioned, IL-1β and IL-6 plasma levels were shown to be enhanced in autistic patients and PBMC stimulation with cow's milk resulted in an enhanced TNF-α response (61, 75). Because these cytokines are able to cross the blood-brain barrier, they are important in neuroimmune interactions (**Fig. 1**, pathway 3). In the brain, these cytokines can interact with neuroglial cells to induce neuroinflammation. In the healthy CNS, astrocytes and microglia play important roles in neuronal function and homeostasis, as they are both fundamentally involved in cortical organization, neuroaxonal guidance and synaptic transmission (92). Furthermore, astrocytes and microglia are also crucial for the regulation of immune responses in the CNS. Microglia are the macrophages of the brain and therefore involved in immune surveillance (93). Astrocytes and microglia are able to produce neurotrophic factors, cytokines, and chemokines (94, 95) and are important in regulating the integrity of the blood-brain barrier (96). In response to an immune challenge, activated astrocytes and microglia can induce neuronal and synaptic changes, which modify CNS homeostasis and contribute to neuronal dysfunction during disease processes.

In postmortem brains of autistic patients, enhanced activation of astrocytes and microglia was observed (87). Astrocyte activation was identified in the subcortical white matter of the midfrontal and anterior cingulate gyrus as well as in the granular and Purkinje cell layers, and in the white matter of the cerebellum. In addition, enhanced astrocyte activation was observed in the striatum, hippocampus and cerebral cortex of mice with fragile X syndrome (highly related to autism) (97). Microglia activation was predominantly

observed in granular cell layer and white matter of the cerebellum of ASD brains (87). It is unclear when or how neuroglia become activated in the brain of autistic patients. To investigate this, Vargas *et al.* (2005) additionally characterized cytokine and chemokine profiles in the midfrontal gyrus, the anterior cingulate gyrus and the cerebellum of ASD brains and cerebrospinal fluid. Enhanced levels of IL-6, IFN-γ, CCL2, CCL4, CXCL8 and CXCL10 were found in the cerebrospinal fluid of autistic children. In postmortem brains of autistic individuals, TGF-β levels were increased in all three brain regions (midfrontal gyrus, anterior cingulate gyrus and cerebellum) and pro-inflammatory chemokines CCL2 and thymus and activation-regulated chemokine (TARC) levels were increased in the anterior cingulate gyrus and the cerebellum. Furthermore, the anterior cingulate gyrus showed increased levels of a wide range of pro-inflammatory cytokines and chemokines, including IL-6, IL-10, CCL7, CCL22, CCL23, CXCL9, and CXCL13 (87). Another group measured cytokine profiles in the frontal cerebral cortex of ASD brains and observed enhanced levels of pro-inflammatory cytokines IL-6, TNFα and granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ and chemokine CXCL8 (98). Because no enhanced lymphocyte infiltration was observed in the brains of autistic individuals, it may be more likely that neuroglia become activated upon stimulation by infiltrated cytokines. The activated neuroglia can produce immune factors modifying neuronal homeostasis and functioning,

Immune factors influence neurons by binding to brain endothelial cells

Brain endothelial cells function as a barrier between blood and brain and regulate the infiltration of immune factors. In addition to this barrier function, brain endothelial cells are known to be activated by cytokines and to produce cytokines themselves (**Fig. 1**, pathway 4). IL-1β (99) and TNF-α (100), two cytokines that are also relevant in ASD, can bind to brain endothelial cells and induce an immune response (101). In turn, brain endothelial cells are important sources of pro-inflammatory mediators, such as prostaglandins, leukotrienes, cytokines and chemokines (101-103). The factors that they produce, including cytokines such as IL-6, GM-CSF and TNF-α and chemokines like CCL2 and CXCL8 (103, 104), can be released both at the side of the brain and the blood vessel. When brain endothelial cells secrete immune factors at the brain side, astrocytes and microglia become activated and consequently influence neuronal functioning.

LPS influences neurons via the blood-brain barrier

Lipopolysaccharide (LPS) plasma levels were shown to be enhanced in patients with severe autism. Moreover, LPS levels correlated with the severity of behaviour in this subset of patients (105). LPS, the known toll-like receptor-4 (TLR-4) ligand, is a major component of gram-negative bacteria and high plasma levels of LPS are likely due to enhanced intestinal permeability. LPS is an important player in neuroinflammation, because of its influence on brain endothelial cells, which express TLR-4 (106). LPS can increase blood-brain barrier permeability through many different pathways (107-109) (**Fig. 1**, pathway 5).

It can enhance endocytosis by brain endothelial cells (110) and facilitate immune cell trafficking (111, 112). Furthermore, LPS can stimulate brain endothelial cells to secrete cytokines (103, 113). Enhanced LPS levels in severe autistic patients may stimulate brain endothelial cells to secrete cytokines and can make the blood-brain barrier more permeable. This would enhance neuroinflammation and might therefore exacerbate behavioural deficits.

MTOR AS A POSSIBLE LINK BETWEEN ASD-ASSOCIATED DISTURBANCES IN IMMUNE SYSTEM AND CNS

The mammalian target of rapamycin (mTOR) is a highly conserved, intracellular serine/threonine kinase that regulates cell growth and metabolism in response to a wide variety of signals, including growth factors, nutrients, energy and inflammatory factors (114-116). mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and serves as the catalytic subunit of two structurally and functionally distinct multi-protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). **Fig. 2** depicts a schematic illustration of the major upstream and downstream mTOR signalling pathways. Rapamycin, which is a macrolide produced by soil bacterium *Streptomyces hygroscopicus* (117), disrupts mTORC1 complex formation (118). mTORC2 shares several proteins with mTORC1 and was originally described as a rapamycin-insensitive complex, as acute rapamycin treatment is unable to inhibit mTORC2 (119). However, subsequent studies have shown that, in some cell types, prolonged rapamycin treatment inhibits the assembly of mTORC2 (120).

mTORC1 responds to growth factors such as insulin, through PI3K-AKT pathway, to regulate various cellular processes that are involved in cell growth and metabolism. The binding of insulin to its receptor on the cell membrane leads to the recruitment and phosphorylation of the insulin receptor substrate (IRS), which in turn, via a complex signal transduction, results in activation of Ras-like small GTP-ase, Rheb. Rheb was shown to directly bind to mTOR in mTORC1 and stimulate the catalytic activity of mTOR, inducing phosphorylation of specific targets that regulate protein synthesis and many other growth-related processes (121). Other upstream signalling cues of mTORC1 are nutrients, energy and inflammatory stress, such as cytokines and cross-linking of immunoglobulin receptors (114). In contrast to mTORC1, relatively little is known about the signalling upstream of mTORC2. However, mTORC2 can indirectly activate mTORC1.

In patients with ASD, several mutations in genes are found that are strongly linked to the mTOR signalling pathway. Tuberous sclerosis is a genetic disorder caused by heterozygous mutations in the mTOR pathway related *Tuberous sclerosis complex* (*Tsc)1* or *Tsc2* genes and is commonly associated with the autistic phenotype. Mice with a

heterozygous mutation in the *Tsc2* gene (*Tsc2*$^{+/-}$ mice) demonstrate enhanced mTOR signalling in the hippocampus, which contributes to learning and memory impairments in *Tsc2*$^{+/-}$ mice. Treatment of adult *Tsc2*$^{+/-}$ mice with rapamycin reversed the learning and memory impairments (122). In addition, *Tsc1*$^{+/-}$ mice also displayed reduced levels of social behaviour and cognitive function (123). PTEN (phosphatase and tensin homolog) acts as a phosphatase that dephosphorylates one of the upstream TSC/mTOR -associated signal transduction molecules, resulting in reduced activity of mTOR. Mutations in PTEN are associated with a wide variety of human neurological disorders, including ASD (124). PTEN gene mutation analysis has been suggested for patients with macrocephaly, a condition that is observed in 20 % of patients with ASD (125). *Pten* knock-out mice with deletion of *Pten* in neurons in the cortex and hippocampus develop autistic phenotypes such as macrocephaly and reduced social behaviour. Moreover, changes in cell morphology have been observed, including neuronal hypertrophy and loss of neuronal polarity, which means that the establishment of axons and dendrites in these neurons is disrupted. Treatment with rapamycin in *Pten* knock-out mice reversed neuronal hypertrophy and macrocephaly and ameliorated ASD-related, abnormal behaviours (126). Furthermore, mTOR is involved in synaptogenesis. Activation of the mTOR pathway can increase the production of synaptic signalling proteins and the formation of new spine synapses in the prefrontal cortex of rats. mTOR inhibition with rapamycin blocked synaptic protein synthesis and antidepressant behavioural responses in rats (127).

Currently, it is becoming more and more evident that mTOR also plays a central role in directing immune responses. A recent study suggests that Th1 and Th17 differentiation are specifically regulated by mTORC1 signalling. In contrast, Th2 differentiation is dependent on mTORC2 signalling, as T cells in which mTORC2 activity is eliminated failed to differentiate into Th2 cell both *in vitro* and *in vivo* but were able to differentiate into Th1 and Th17 cells (127). Furthermore, it was shown that T cells differentiated into Tregs in the presence of a conventional dose of rapamycin, which inhibits mTORC1 and mTORC2 (127). Indeed, rapamycin-induced mTOR inhibition resulted in elevated Tregs in tissue culture of nasal polyps obtained from patients suffering from chronic allergic rhinitis (128). Furthermore, mTORC1 activation in mast cells is associated with survival, differentiation, migration and cytokine production (129). Finally, increased mTOR activity is shown to attenuate autophagy (130). This finding could explain the reduced clearance and maintenance of inflammatory cells at sites of allergic inflammation. In conclusion, because of its function in immune and neuronal pathways, mTOR may be a possible target for treatment in ASD.

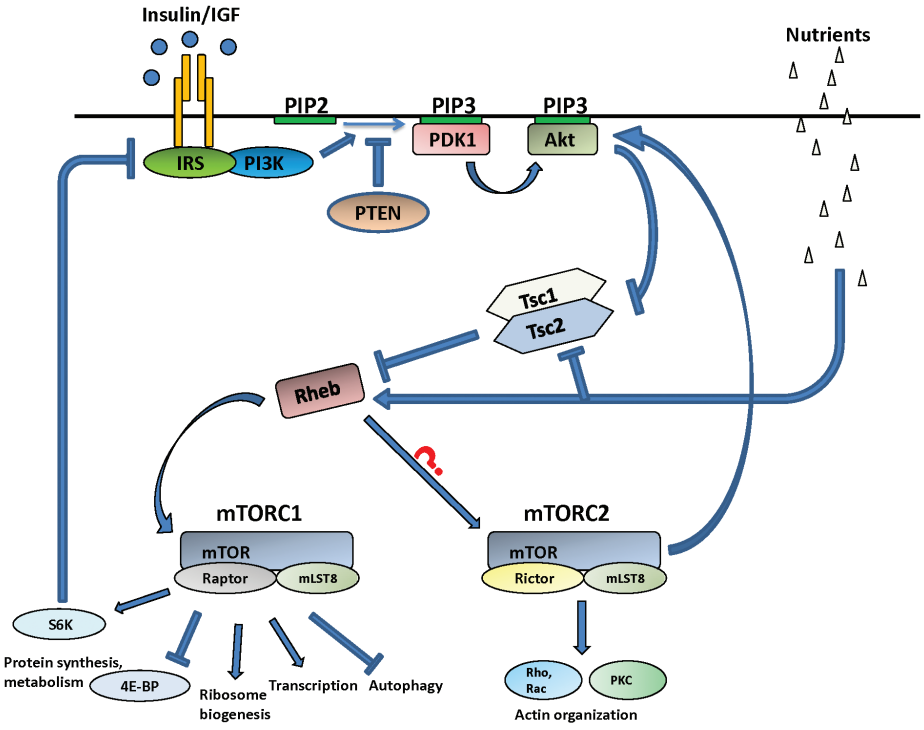

Fig. 2. Schematic illustration of mTOR signalling pathway. Two multi-protein complexes, mTORC1 and mTORC2, are centrally involved in the mTOR signalling network. mTORC1, which is rapamycin sensitive, is activated by growth factors through the PI3K/Akt signalling pathway and by nutrients, energy, stress, leading to the phosphorylation of S6K and 4E-BP1 and thereby regulating protein synthesis and cell growth. In contrast to mTORC1, the upstream signalling of rapamycin insensitive mTORC2 is currently unknown. mTORC2 can directly phosphorylate Akt upstream of mTORC1 and thereby indirectly activate mTORC1. mTORC2 has also been involved in regulating cytoskeletal organization through the activation of PKC and RhoA and Rac1.

TARGETING THE GASTROINTESTINAL TRACT IN ASD

Many parents report that their autistic child suffers from gastrointestinal symptoms. This has led to research on the prevalence and characteristics of gastrointestinal disturbances in the autistic population. The conflicting results on the prevalence of gastrointestinal disturbances are possibly due to different facts; interpretation of gastrointestinal symptoms, social and communicative impairments of patients and the heterogeneity of ASD. The severity of autistic behaviour was shown to correlate with gastrointestinal disturbances, increased intestinal permeability, and enhanced serum levels of LPS, cytokines and chemokines. Therefore, we hypothesize that children with a genetic predisposition are more susceptible for developing ASD when they suffer from immune disturbance or that the presence of gastrointestinal inflammation worsens behaviour in children with ASD. This would mean that immunomodulatory dietary interventions, allergen-free diets and pharmaceuticals (mast cell stabilizers and anti-inflammatory or immunosuppressive drugs) for the treatment of gastrointestinal inflammation may also be beneficial for the treatment of autistic behaviour.

The use of Complementary and Alternative Medicine (CAM) practices for children with ASD is often reported (131-137). Examples of such treatments include the use of vitamin and mineral supplements, secretin, melatonin and gluten-free, casein-free diets (135). At this moment, approximately 50 % of parents with an ASD child have tried CAM (134), and half of these are using a gluten-free, casein-free diet (135). Results from a recent study indicate that gluten and milk free diets improve behaviour in children with ASD (56). This result suggests the presence of food hypersensitivity or allergy in the autistic population. In addition to the elimination diet, dietary ingredients such as n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) and pre- and probiotics might be beneficial to the dietary management of autistic behaviour and the associated gastrointestinal symptoms, because of their effects on CNS, immune system and/or on microbiota profile.

There is increasing evidence for prebiotics to have effects not only on enteric mucosa but also on systemic immunity. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of *Bifidobacteria* and lactic acid bacteria in the colon, which are important markers of a healthy gut microbiota (138-140). The expertise on prebiotics originates primarily from the efforts to simulate the beneficial effects of breastmilk (141-143). Human milk favors the growth of *Bifidobacteria*, which activates the immune system and defends from pathogens. A recent study shows that prebiotics have long bifidogenic effects in the intestines of infants (144). Non-digestible oligosaccharides are examples of prebiotics and consist of naturally occurring sugar base units (e.g. glucose, fructose and galactose). These oligosaccharides are not hydrolyzed in the upper small intestine and reach the large intestine intact to serve as substrates for bacterial metabolism (145). Non-digestible oligosaccharides were shown

to be beneficial for disease progression and immune status in various studies, including murine models for allergy (146) and clinical trials for treatment of allergy (143). The immunomodulatory effects and potential working mechanism of orally applied non-digestible carbohydrates are reviewed (147). Since the microbiota profile of ASD patients is enriched in pathogenic bacteria species (12, 25-27), possibly exacerbating impaired behaviour (33), these patients may benefit from dietary supplementation with a prebiotic mixture.

Also probiotics may be beneficial in the treatment of gastrointestinal problems observed in patients with ASD. Although no reliable conclusion can be drawn from the results on functional studies with probiotics, some evidence suggests that supplementation with probiotics is associated with a reduction in the risk of nonspecific gastrointestinal infections and lower frequency of colic or irritability (148). Lower levels of beneficial *Bifidobacteria* were observed in ASD patients. This bacterium has also been associated with food allergy and inflammatory bowel disease, suggesting that it is associated with intestinal inflammation. Moreover, increased intestinal permeability was also observed in patients with ASD. Because probiotics were thought to reduce intestinal permeability and restore a 'healthy' gut (149-151), probiotics may improve gastrointestinal health and possibly behaviour.

Another dietary intervention that may be beneficial for the ASD population is supplementation with n-3 LCPUFA. Decreased levels of incorporated n-3 LCPUFA have been observed in peripheral blood cells of ASD patients repeatedly (152, 153). After treatment with n-3 rich fish oil, LCPUFA levels were enhanced and a decreased ratio of n-6/n-3 was observed (154). Moreover, a significant improvement of behaviour was observed after treatment of ASD patients with fish oil. The effect of LCPUFA on autistic behaviour may work via two different mechanisms. LCPUFA are present in neuronal membranous phospholipids in the myelin sheath (155), where they modulate membrane fluidity and hence neuronal functioning, including receptor function and neurotransmitter release and uptake (156). Indeed, deficiencies of n-3 LCPUFA lead to learning disabilities and memory loss (157). Besides effects on the brain, n-3 LCPUFA have also been claimed to have a function in modulating the immune response. N-3 LCPUFA can be incorporated in the membrane of immune cells, where they modulate intracellular pathways leading to an anti-inflammatory response (158). This anti-inflammatory response is mediated by a number of independent mechanisms. First, the effect of n-3 is caused by replacing the pro-inflammatory n-6 arachidonic acid. Second, n-3 fatty acids give rise to the production of resolvins that can resolve inflammation (159). Third, n-3 fatty acids decrease the expression of adhesion molecules and prevent adherence of monocytes and macrophages (160, 161). Finally, n-3 fatty acids have been shown to decrease the production of inflammatory cytokines (162). This means that supplementation of n-3 LCPUFA could be beneficial for patients with ASD, because n-3 LCPUFA can act either directly on neuronal responses or indirectly via the immune system and gastrointestinal tract.

Nowadays, ASD treatment includes behavioural, educational and pharmacological therapy. No single drug has been proven to be effective for treating symptoms associated with autism. However, because many of the behavioural features are similar to serotonin-related disorders and because plasma hyperserotonemia is observed in about one third of the autistic population, SSRIs are often prescribed to ASD patients. It is hypothesized that gastrointestinal disturbances in ASD patients lead to high serotonin levels in the gut. This can be reflected by blood hyperserotonemia and consequently lead to reduced tryptophan availability for the brain, resulting in decreased serotonin synthesis in the brain. This hypothesis would suggest that it might be more effective to combine SSRIs with dietary interventions that reduce gastrointestinal disturbances. The strong gut-to-brain connection described in this review provides a compelling opportunity to target the brain via the gut and the immune system by using nutritional interventions.

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CHAPTER THREE

Intestinal inflammation in a murine model of autism spectrum disorders

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ABSTRACT

Autism spectrum disorder (ASD) is a cluster of neurodevelopmental disorders characterized by impairments in communication, social interest and stereotypical behaviour. Dysfunction of the intestinal tract is reported in patients with ASD and implicated in the development and severity of ASD symptoms. However, more research is required to investigate the association of intestinal problems with ASD and the potential underlying mechanisms. The purpose of this study was to investigate co-morbid symptoms of intestinal inflammation in a murine model of ASD induced by prenatal exposure to valproic acid (VPA). Pregnant BALB/c females were treated subcutaneously with 600 mg/kg VPA or phosphate buffered saline on gestational day 11. Offspring were housed with their mother until weaning on postnatal day 21 (P21). All pups were exposed to a social behaviour test on P28. Inflammatory correlates and activity of the serotonergic system were measured in brain and intestinal tissue. Here we demonstrate, in addition to reduced social behaviour and increased expression of neuroinflammatory markers in the brain, that VPA *in utero*-exposed male offspring showed epithelial cell loss and neutrophil infiltration in the intestinal tract. Furthermore, reduced levels of serotonin were not only observed in the prefrontal cortex and amygdala of VPA *in utero*-exposed males, but also in the small intestine. Overall, we demonstrate that gender-specific inflammatory conditions are present in the small intestines of VPA *in utero*-exposed mice and are accompanied by a disturbed serotonergic system in the brain as well as in the intestinal tract.

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous cluster of severe neurodevelopmental disorders. It is characterized by impairments in social interaction and communication and by the presence of stereotyped behaviours (1). Although the aetiology of ASD is unknown, it is thought that ASD is a multifactorial disorder with a strong genetic component (2, 3). A variety of environmental factors is suggested to contribute to ASD development. For example, prenatal exposure to teratogens has been shown to be a significant risk factor for ASD (4). Indeed, maternal use of the anticonvulsant valproic acid (VPA) is associated with the development of ASD in the offspring (5-7). The mechanism for VPA-induced symptoms of ASD is still unclear, but several pathways have been proposed. These include attenuation of folic acid metabolism, inhibition of histone deacetylases and increased oxidative stress (8). In search of underlying mechanisms, animal models of VPA-induced autism-like behaviours have been established in rats and mice. In a well-characterized murine model, VPA *in utero*-exposed mice exhibit developmental and behavioural deficits comparable to those observed in ASD patients, including deficits in social behaviour (9, 10), stereotyped behaviour (11), anxiety and impairments in cognition (10). Furthermore, observations were more prominent in male offspring compared to female offspring (10, 12), which reflects the human situation where a marked male preponderance is observed in ASD patients (13, 14).

Disturbances in the immune system are repeatedly reported in various organs of ASD patients. Since ASD is primarily a disorder of the central nervous system, the brain is a major target for immunological research. In post-mortem brains of patients with ASD, marked activation of astroglia and microglia is observed when compared to controls (15-19), indicative of neuroinflammation. Enhanced activation of neuroglia was also observed in various murine models of autism (20-22). In addition, enhanced levels of a wide range of cytokines and chemokines were found in the brain (23) and in the cerebrospinal fluid (16) of autistic children, compared to healthy children. This supports the presence of neuroinflammatory conditions in the brain of ASD patients. Peripheral immune abnormalities in autistic individuals have also been reported, including differential monocyte responses to in vitro stimulation (24, 25), dysfunctional natural killer (NK) cells (26, 27) and altered serum levels of immunoglobulins (28-30), cytokines (31-33) and chemokines (34).

Immune disturbances have also been observed in the gastrointestinal tract of ASD patients. The presence of gastrointestinal problems in these patients is repeatedly reported in literature and include chronic constipation, diarrhoea and abdominal pain (35). These symptoms have been attributed to changes in gut microflora (36, 37), increased intestinal permeability (38) and intestinal inflammation (39). Although evidence is emerging, there is still much debate about the presence of gastrointestinal

disturbances in ASD. Indeed, the reported prevalence of gastrointestinal symptoms ranges from 10 % to 90 %, an immense range probably due to varying interpretations of gastrointestinal problems and inability of ASD patients to express their discomfort (40). Moreover, diagnosis of ASD is based on behavioural observations, gathering a heterogeneous cluster of patients with different aetiologies. Deficits in the intestinal tract could therefore be specific for a subgroup of ASD patients. Since gastrointestinal deficits have been suggested to contribute to the development or severity of autistic behaviour (41), a considerable number of ASD patients is on a specific diet to improve gastrointestinal function and behaviour (42). Nevertheless, more research is required to clarify the importance of gastrointestinal disturbances in ASD patients and to understand possible underlying mechanisms. The aim of this study was to investigate the effects of prenatal exposure to VPA on immune activation in the gut and brain. We also investigated the serotonergic system in the gut and brain as a putative neuroimmune modulator and a potential mechanism underlying the effects of prenatal VPA exposure on behaviour and intestinal phenotype.

MATERIALS AND METHODS

Animals and experimental design

Specific pathogen-free BALB/c breeding pairs from Charles River laboratories (Maastricht, The Netherlands) were housed under a 12 h light/dark cycle with access to food and water. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands. All females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G11, after neural tube closure (43), pregnant females were treated subcutaneously with 600 mg/kg VPA (Sigma, Zwijndrecht, The Netherlands; 100 mg/mL, $n = 5$) or phosphate buffered saline (PBS, $n = 5$). Offspring (males: max $n = 2$ per litter, females max $n = 3$ per litter) were housed with their mother until weaning on postnatal day 21 (P21). All pups (PBS group: $n = 9$ males and $n = 13$ females, VPA group: $n = 8$ males and $n = 11$ females) were exposed to a social behaviour test on P28 and subsequently euthanized by decapitation to collect brain and intestinal tissue (PBS group: $n = 4$ males and $n = 4$ females, VPA group: $n = 4$ males and $n = 4$ females). A second set of male pups from different mothers exposed to 500 mg/kg VPA or PBS underwent the same protocol and were used to detect serotonin levels in the brain and water content in stool (PBS group: $n = 5$, VPA group: $n = 7$). Mothers were exposed to 500 mg/kg VPA because this was less harmful for the pregnant dam and sufficient to initiate the same behavioural abnormalities and crooked tail formation in offspring, as compared to 600 mg/kg (data not shown).

Social behaviour test

The behavioural assessment used was adapted from a previous description (44, 45). On P28, mice were placed in a 45 x 45 cm open field, with two small perforated Plexiglas cages (10 cm diameter) located against opposite walls allowing visual, olfactory, and minimal tactile interaction (**Fig. 1A**). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), zones around the cages were digitally determined. Time spent in the interaction zone near the cage of the target mouse and total distance moved were measured. The ratio of time in interaction zone in presence to absence of a target mouse was presented (time in zone with target mouse/ time in zone without target mouse).

Real-time PCR analysis to assess neuroinflammation

Since morphological and immunological changes are observed in the dorsal hippocampus, prefrontal cortex and amygdala of VPA-exposed rats and patients with ASD (15, 46, 47), these regions were dissected from brain for mRNA expression level analysis. Expression of glial fibrillary acidic protein (*Gfap*) and *CD11b* are markers for activation of astroglia and microglia, respectively. Pro-inflammatory cytokine interleukin (*Il*)*-1b* and cyclooxygenase (*Cox*)*-2* are associated with inflammatory conditions in the brain. Total RNA was isolated using the RNeasy mini kit (Qiagen, Germantown, MD, USA) and cDNA was produced using the iScript cDNA synthesis kit (BioRad, Veenendaal, The Netherlands). Quantative real-time PCR analysis was performed on a CFX96 real-time PCR detection system (BioRad) using iQ SYBR green supermix (BioRad) and qPCR primers (Qiagen). mRNA expression levels were calculated with CFX Manager software (version 1.6) and corrected for the expression of the housekeeping gene *Rps13*.

HPLC for analysis of 5-HT and 5-HIAA in brain and intestine

Serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured in brain and intestinal tissue by HPLC with electrochemical detection as previously described (48). The tissue samples were sonicated in 50 - 100 µL ice-cold solution containing 5 µM clorgyline, 5 µg/mL glutathione, and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL homogenate, 12.5 µL of 2 M $HClO_4$ was added and mixed. Then, 20 µL of 2.5 M potassium acetate was added and again mixed. After 15 min in ice water, the homogenates were centrifuged for 15 min at 15,000 x g (4 °C). The HPLC system consisted of a pump model P100, an autosampler model AS300 (both from Thermo Separation Products, Waltham, MA, USA), an ERC-3113 degasser (Erma CR. Inc. Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential + 450 mV (ESA Inc. Bedford, MA, USA), a BD 41 chart recorder (Kipp & zn, The Netherlands),

and a column (150 mm x 4.6 mm i.d.) packed with Hypersil BDS C18, 5-µm particle size (Alltech Associates, Deerfield, IL, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutylamine, and 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM.

Histology of intestine

Small intestinal tissue was removed, opened longitudinally, divided in a 7 cm proximal part (jejunum) and 7 cm distal part (ileum), rolled and fixated with 10 % formalin for at least 24 h. After paraffin embedding, 5 µm sections were cut and stained with haematoxylin/eosin (H & E) according to standard methods. These intestinal samples were used to blindly determine the morphology.

Myeloperoxidase (MPO) ELISA

Small intestinal tissue was homogenized using Precellys 24 homogenizer (Bertin Technologies, France) and centrifuged for 10 min at 14,000 rpm. Protein concentration of supernatants was assayed using Pierce BCA protein assay kit (Thermo Scientific, Breda, The Netherlands) and samples were diluted to a protein concentration of 2 mg/mL. MPO levels were measured in homogenates by ELISA using the Mouse MPO ELISA kit (Hycult Biotechnology, The Netherlands) according to manufacturer's instructions.

Immunohistochemistry for analysis of 5-HT positive cells in ileum

Formalin-fixed, paraffin-embedded tissue sections (5 µm) of ileum were used for detection of 5-HT positive cells. Sections were incubated with 0.3 % H_2O_2 in methanol for 30 min, rehydrated and incubated for 5 min with proteinase K solution (Dako, Enschede, The Netherlands). Aspecific background was blocked with 5 % goat serum and sections were incubated overnight at 4 °C with rabbit anti-5-HT (1:8000 Sigma). Next day, sections were incubated with biotinylated goat anti–rabbit (1:200, Dako), followed by ABC-HRP complex (Vector Laboratories, Peterborough, UK). Staining was visualized using 0.05 % DAB solution for 10 min and sections were counterstained with Mayer's haematoxylin (Merck Millipore, Amsterdam, The Netherlands). Digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera, at a magnification of 20 times. 5-HT positive cells in the epithelial layer of the intestinal mucosa were counted in 15 consecutive villi at three different places in the intestinal swiss roll. Data were expressed as the percentage of villi that contained no, one, or more than one 5-HT positive cell.

Analysis of CXCL1 in ileum

Multiplex cytokine and chemokine analysis in ileal tissue homogenate supernatants was conducted using the Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore), according to the manufacturer's instructions.

Water percentage in stool

Fresh faeces were collected from individual mice during the social behaviour test. Faeces were weighed, heated at 37 °C for 3 days and weighed again. The percentage of water was calculated from the difference in faecal weight before and after drying.

Statistical analysis

Differences between groups were statistically determined using two-way ANOVA with Bonferroni's multiple comparisons test to compare PBS and VPA groups in male and female offspring. When data were not normally distributed, a one-way ANOVA was used with Kruskal-Wallis test. To compare differences between male PBS and VPA offspring in brain serotonergic system and water content in stool, an unpaired two-tailed Student's *t*-test was used. Analyses were performed using GraphPad Prism (version 5.03) and results were considered statistically significant when $P < 0.05$. Experimental results are expressed as mean ± S.E.M for parametric data and median with 5 - 95 percentiles for non-parametric data.

RESULTS

Prenatal VPA exposure causes reduced social interaction in male offspring

Control PBS *in utero*-exposed males and females spent on average 135.2 s in the interaction zone when a target mouse was present, which was 2.6 times longer than in absence of a target mouse. Prenatal exposure to VPA on G11 decreased social interaction with an unfamiliar target mouse ($F_{1,35} = 4.82$, $P < 0.05$, **Fig. 1B**). More specifically, male VPA *in utero*-exposed mice spent less time in the interaction zone compared to male PBS-control mice ($P < 0.05$), while this effect was not observed in female offspring. Locomotor activity in absence of a target mouse was not altered in VPA *in utero*-exposed offspring compared to controls (**Fig. 1C**).

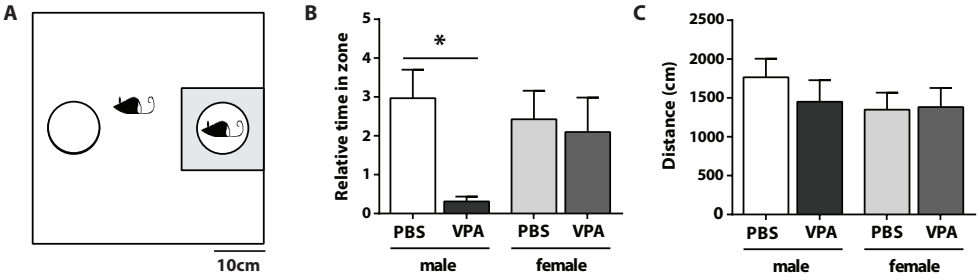

Fig. 1. Social interaction is reduced in male valproic acid (VPA)-exposed offspring. (**A**) Schematic representation of the social interaction test, illustrating location and size of the cages (white circles) and the interaction zone (grey rectangle). (**B**) Compared to males exposed to PBS, VPA *in utero*-exposed males spent significantly less time in the interaction zone. The female offspring of VPA-treated dams did not show deficits in social interaction when compared to female controls. Data was presented as relative time in zone (time in zone in presence of target mouse/ time in zone in absence of target mouse) (**C**) Locomotor activity, measured by total distance moved, was similar in all groups. * $P < 0.05$, $n = 8$ - 13 per group.

Markers for neuroinflammation are increased in VPA *in utero*-exposed offspring

Expression of markers for neuroinflammation was measured in the dorsal hippocampus, prefrontal cortex and amygdala. In the prefrontal cortex and amygdala, mRNA expression levels of *Gfap*, *CD11c*, *Il-1b* and *Cox-2* were not altered (data not shown). In the dorsal hippocampus, a significant effect of VPA exposure was observed on levels of neuroinflammatory markers in both males ($F_{1,24} = 8.48$, $P < 0.01$) and females ($F_{1,24} = 7.55$, $P < 0.05$) in a two-way ANOVA (**Fig. 2**). Bonferroni's multiple comparisons test did not reveal single markers to be significantly elevated. When comparing single neuroinflammatory markers in separate two-way ANOVAs, no effect of gender was observed on any of the neuroinflammatory markers.

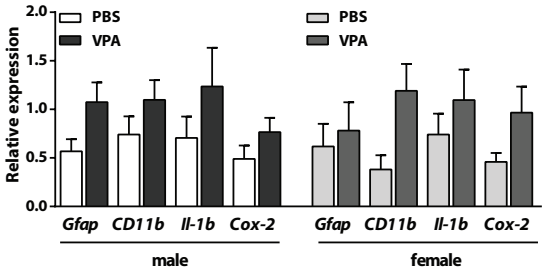

Fig. 2. Markers for neuroinflammation are increased in the dorsal hippocampus of offspring of valproic acid (VPA)-treated dams. A significant effect of VPA exposure was observed by two-way ANOVA on neuroinflammation markers in males ($F_{1,24}$ = 8.48, $P < 0.01$) and females ($F_{1,24}$ = 7.55, $P < 0.05$). Bonferroni's multiple comparisons test did not reveal any significant differences between VPA and PBS exposure on levels of single neuroinflammatory markers. $n = 4$ per group.

VPA *in utero* exposure in males causes reduced serotonin levels and increased turnover in the brain

To determine the effect of prenatal exposure to VPA on the serotonergic system in the brain, levels of 5-HT, its metabolite 5-HIAA, and the ratio 5-HIAA/5-HT were measured in homogenates of the dorsal hippocampus, prefrontal cortex, and amygdala of a separate set of male offspring prenatally exposed to VPA or PBS (**Fig. 3**). Female offspring was not analysed in this set of animals, since behaviour was not altered upon *in utero* VPA exposure in females. Levels of 5-HT were significantly decreased in VPA *in utero*-exposed males compared to controls in prefrontal cortex (t_{10} = 2.24, $P < 0.05$) and amygdala (t_{10} = 3.28, $P < 0.01$). Furthermore, a strong trend towards an increase in 5-HIAA concentration was observed in the amygdala (t_{10} = 2.13, $P = 0.06$). Turnover of serotonin was assessed by calculating the ratio of 5-HIAA/5-HT. Increased serotonin turnover was observed in the prefrontal cortex (t_{10} = 2.60, $P < 0.05$) and amygdala (t_{10} = 3.60, $P < 0.01$) and a trend was found in the dorsal hippocampus (t_{10} = 1.87, $P = 0.09$).

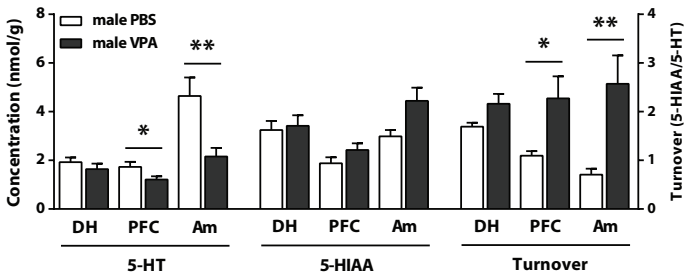

Fig. 3. Prenatal exposure to valproic acid (VPA) causes decreased 5-hydroxytryptamine (5-HT) levels and increased breakdown in male offspring. 5-HT levels were decreased in prefrontal cortex (PFC) and amygdala (Am) of male offspring of VPA-treated dams compared to PBS-treated controls. In Am, a trend towards increased 5-hydroxyindoleacetic acid (5-HIAA) levels was observed upon prenatal exposure to VPA in male offspring ($P = 0.06$). Turnover of 5-HT was increased in the PFC and Am and a trend was observed in the dorsal hippocampus (DH) ($P = 0.09$) of male offspring of VPA-treated dams compared to controls. * $P < 0.05$, ** $P < 0.01$, n = 5-7 per group.

Intestinal inflammation and immune activation is present in VPA *in utero*-exposed male offspring

Examination of the intestinal tract revealed loss of the epithelial barrier and cellular infiltration in ileum of male offspring prenatally exposed to VPA (**Fig. 4B**) compared to PBS-control males (**Fig. 4A**). No epithelial damage was present in ileum of VPA *in utero*-exposed females (**Fig. 4D**) and control females (**Fig. 4C**). To quantify neutrophilic inflammation, MPO levels were assessed in jejunum and ileum. Significantly increased levels of MPO were found in ileum of VPA compared to PBS *in utero*-exposed offspring ($F_{1,12} = 10.83$, $P < 0.05$) and this effect was only observed in males ($P < 0.05$, **Fig. 4E**). Next, we measured tissue levels of CXCL1, a chemokine with profound neutrophil chemoattractant activity. Although no statistical differences were observed, concentration of CXCL1 was higher in the jejunum of VPA *in utero*-exposed male offspring (**Fig. 4F**). As an indication of diarrhoea or constipation, the percentage of water in faeces was measured in a different subset of male offspring of VPA or PBS-treated dams. A significant increase in water content was observed in faeces of VPA *in utero*-exposed male offspring compared to controls ($P < 0.05$, **Fig. 4G**).

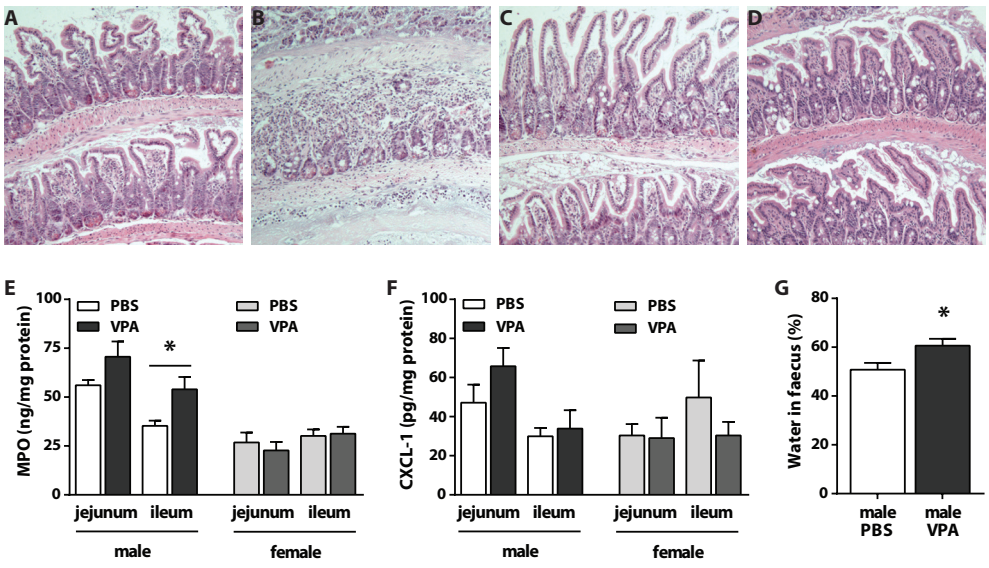

Fig. 4. Prenatal exposure to valproic acid (VPA) induces epithelial loss and intestinal inflammation in ileum of male offspring. (**B**) Typical example of epithelial loss in ileum of male VPA *in utero*-exposed mice, compared to (**A**) phosphate buffered saline (PBS)-exposed males as well as (**D**) VPA and (**C**) PBS-exposed females. (**E**) Myeloperoxidase (MPO) as a marker of neutrophil infiltration was significantly increased in ileum of VPA *in utero*-exposed males compared to PBS-control male offspring. (**F**) Although not significant, higher levels chemokine CXC ligand-1 (CXCL1) were observed in jejunum of male VPA-exposed offspring compared to male controls. (**G**) Increased water content was observed in faeces of a different set of VPA *in utero*-exposed males compared to PBS-control males. * $P < 0.05$, A-F: $n = 4$ per group, G: $n = 5$-7 per group.

Prenatal VPA-exposure causes reduced 5-HT levels and number of 5-HT positive cells in ileum of male offspring

Similar to brain samples, *in utero* exposure to VPA caused an overall significant decrease in 5-HT levels in ileum compared to PBS exposure ($F_{1,12} = 9.22$, $P < 0.05$). Furthermore, this decrease was only observed in male offspring ($P < 0.05$, **Fig. 5A**). Measurements of 5-HIAA and turnover did not result in significant differences in male nor female offspring of VPA-treated dams compared to their relative controls (data not shown), indicating a decrease in 5-HT production instead of an increase in 5-HT turnover. Moreover, 5-HT positive cells were detected in intestinal sections by immunohistochemistry. A significant interaction was observed between the groups and the number of 5-HT positive cells per villus ($F_{6,27} = 3.16$, $P < 0.05$), and *post-hoc* analysis revealed a significant increase in the percentage of villi without 5-HT positive cells in the epithelial layer of VPA *in utero*-exposed males ($P < 0.05$, **Fig. 5B**, **D**) compared to PBS control males ($P < 0.05$, **Fig. 5B**, **C**), indicating less enterochromaffin (EC) cells. The percentage of 5-HT positive cells in the epithelial layer of VPA *in utero*-exposed females (**Fig. 5F**) was not different compared to control females (**Fig. 5E**).

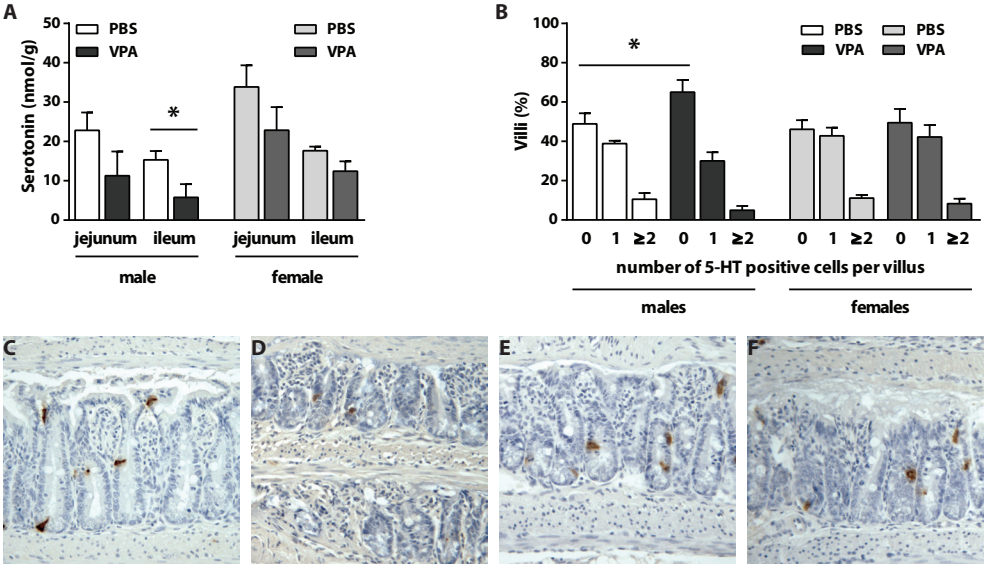

Fig. 5. Prenatal exposure to valproic acid (VPA) causes reduced 5-hydroxytryptamine (5-HT) levels and numbers of 5-HT positive cells in ileum of male offspring. Both (**A**) 5-HT levels and (**B**) number of 5-HT positive cells were reduced in (**D**) VPA *in utero*-exposed males compared to (**C**) phosphate buffered saline (PBS)-treated control males. No differences were observed in (**F**) VPA *in utero*-exposed females, compared with (**E**) PBS *in utero*- exposed females. * $P < 0.05$, $n = 4$ per group.

DISCUSSION

In this study, we demonstrate that prenatal exposure to VPA on gestational day 11 not only leads to reduced social interaction, increased expression of neuroinflammatory markers in the dorsal hippocampus and abnormalities in the serotonergic system of the amygdala and prefrontal cortex, but is also associated with intestinal inflammation in male offspring. More specifically, our results show that epithelial cell loss and increased neutrophil infiltration is present in the ileum of VPA *in utero*-exposed male offspring, compared to controls. Furthermore, these signs of intestinal inflammation are accompanied by decreased serotonin levels and number of serotonin positive EC cells in the ileum. Although pathological signs of inflammation were restricted to the small intestine and not the colon, *in utero* VPA exposure resulted in soft stools that are indicative for disturbed colonic water absorption and diarrhoea in male offspring. Female VPA *in utero*-exposed mice, on the contrary, did not display behavioural or intestinal alterations when compared to control females.

The enigma of the male bias in ASD remains unsolved. Not only in humans, but also in preclinical rodent studies, the effects of prenatal immune activation and maternal stress on neurodevelopment and behaviour are strongly sex dependent (49). Programming of the sexually dimorphic brain is determined by exposure to testosterone and oestrogen that affect cell differentiation, migration, survival and connectivity (50-52). VPA inhibits the conversion of testosterone to oestradiol (53), which could explain the sex-specific neurodevelopmental effects of VPA. Furthermore, it is suggested that oestrogen and progesterone provide protection against the neurotoxic effects of VPA by enhancing anti-oxidant mechanisms, making females less vulnerable (50). We observed in this study that female offspring are protected from the teratogenic effects of VPA in terms of social behaviour and intestinal abnormalities. However, *in utero* exposure to VPA significantly increased mRNA expression of neuroinflammatory markers in both male and female offspring and both genders develop tail malformation (data not shown), indicating that VPA does affect development of female offspring. Our results suggest that neuroinflammation is not the sole determinant of aberrant social behaviour and associated intestinal phenotype observed in VPA-exposed male offspring. Rather, we hypothesize that vertebral and neuroinflammatory abnormalities are derived from distinct underlying pathophysiological mechanisms than the behavioural and intestinal abnormalities.

The serotonergic system is implicated in the effects of VPA on brain and behaviour during development. Prenatal exposure to VPA caused disturbed distribution of serotonergic neurons in rat embryos (54, 55), which persisted into adulthood (56). Moreover, *in vitro* it was shown that differentiation of progenitor cells into serotonergic neurons was decreased when exposed to VPA (56). Measurements of total 5-HT levels in the hippocampus of VPA

in utero-exposed rats have not been consistent, as both increased (57) and decreased (58) levels have been observed. In our study, we observed little difference in 5-HT levels in the dorsal hippocampus of VPA and PBS *in utero*-exposed male mice. The most pronounced decrease in 5-HT levels was found in prefrontal cortex and amygdala of male offspring of VPA-treated dams compared to controls. It is thought that prenatal VPA exposure impairs normal development of serotonergic neurons via downregulation of sonic hedgehoc (SHH), a signalling molecule required for 5-HT neuronal differentiation. Indeed, prenatal exposure to VPA caused lower expression of sonic hedgehog around the isthmus of rat embryos (54, 55). Furthermore, *in vitro* it was shown that recombinant SHH partially compensated the VPA-induced differentiation defects of serotonergic neurons (56).

We demonstrate that VPA exposure during embryonic development not only affects serotonergic transmission in the brain, but also disturbs the serotonergic system in the intestines. Decreased 5-HT levels were accompanied by reduced EC cell numbers. It remains to be explored whether the reduced number of EC cells is a direct developmental deficit caused by VPA or a result of the inflammatory state of the intestine. In addition to its function as an enteric nervous system neurotransmitter (59), enteric 5-HT is also important in intestinal motility (60) and it regulates intestinal epithelial homeostasis. Crypt epithelial cells express 5-HT2A receptors (61) and 5-HT antagonists were shown to impede crypt cell proliferation (62, 63). Although EC cells are located within the epithelial layer, it was recently shown that 5-HT from enteric neurons, rather than from EC cells, promotes growth and turnover of the intestinal mucosal epithelium (64). Deficits in development of enteric serotonergic neurons could therefore lead to disturbances in mucosal homeostasis, including epithelial and EC cell loss and reducing 5-HT levels in the small intestine. Since *in utero* VPA exposure affects serotonergic differentiation and migration in the CNS, it is likely that enteric serotonergic neurons are also affected. As demonstrated in the brain, SHH is also crucial for normal differentiation of enteric neural crest cells and consequently, development of the enteric nervous system (65, 66). Moreover, *Shh* mutant mice showed abnormal migration of enteric neurons into the villi throughout the small intestine (65). Intestinal epithelial homeostasis may also be affected directly by *in utero* VPA exposure, since *Shh* mutant mice display disturbed development of intestinal villi (65).

Loss in epithelial barrier function is expected to lead to immune activation and intestinal inflammation, as observed in VPA *in utero*-exposed males by a significant increase in MPO levels in the small intestine, as a marker for neutrophil infiltration, accompanied by higher levels of CXCL1, a chemokine involved in neutrophil attraction. Disturbed immune responses have been observed previously in prenatally VPA-exposed rats, including lower thymus weight, decreased proliferative activity of and decreased IFN-γ/IL-10 ratio produced by stimulated splenocytes, compared to controls. Moreover, disturbed immune responses were specifically observed in male offspring (50).

Defects in the serotonin system have been implicated in the pathogenesis of ASD ever since hyperserotonemia was first described in these patients in 1961 (67). Subsequent studies observed that increased serotonin levels in platelets are observed in about one third of the patients with ASD (68, 69). Various mechanisms have been proposed underlying the serotonergic deficits in ASD patients, including altered serotonin synthesis (70-72), reuptake (73-76) and receptor binding (77). Considerable studies indicate the serotonin system to be involved in the pathogenesis of ASD, but a common mechanism of action remains elusive.

In summary, in this study we demonstrated that inflammation was present in the intestinal tract of male mice prenatally exposed to VPA, as a mouse model for autism. These results support the association of intestinal deficits with autism-related behaviours and calls for additional research on the translation to humans. It is important to examine whether intestinal dysfunction is restricted to a subpopulation of ASD patients, for example to those prenatally exposed to teratogens, or to the male population. Furthermore, this study emphasizes the serotonergic system as a possible common pathology leading to dysfunction of the brain and the intestinal tract in mice prenatally exposed to VPA. All together, these findings provide potential targets for new approaches in the treatment of gastrointestinal dysfunction and behaviour in patients with ASD.

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CHAPTER FOUR

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.

INTRODUCTION

The critical role commensal microbes play in health and disease, by influencing physiological homeostasis in the intestines and periphery, is well documented (1, 2). 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 (3). 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 (4). 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 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 (5, 6). Maternal separation of rat pups led to alterations in intestinal permeability and a disruption of gut microbiota that persisted into adulthood (7). 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 (8). 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 (4). Increased endocrine responses to stress were reported in GF mice, which could be reversed by mono-association with *Bifidobacterium infantis* and not with enteropathogenic *Escherichia coli* (9). 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 (10). 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 (11, 12). 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 disorder (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 (13). 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 (14-16). Intestinal permeability is also increased in ASD patients when compared to healthy subjects (15, 17). It is hypothesized that such gastrointestinal deficits may be associated with compositional changes and metabolic activities of intestinal bacteria (18). 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 short chain fatty acids (SCFA), including butyric, propionic, acetic, and valeric acid, were significantly increased in children with ASD when compared with controls (19). Also by-products of microbial protein fermentation, such as ammonia and free amino acids were shown to be increased in children with ASD (19, 20). 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 more pronounced in patients with ASD compared to controls (20-24). Furthermore, *Bifidobacterium* species were frequently reported to be lower (16, 20, 25), while *Sutterella* and *Desulfovibrio* species were reported to be increased in ASD patients (24, 26). However, these observations have not been consistent (16, 23) and one study reported no clinically meaningful differences between groups (27). 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 (28).

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 (29). 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 (30-33). 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 (34). 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 (35-37) and repetitive behaviour (35, 38) and in communication (37, 38). 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* (39)). Interestingly, observations were more prominent in male than in female offspring (36, 40), which is a representative reflection of the human situation where a marked male preponderance is observed in ASD patients (41, 42). 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 in 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 (5-hydroxytryptamine; 5-HT) and intestinal neutrophil infiltration as well as measurements of social behaviour that are described in detail elsewhere (43). 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.

MATERIALS AND METHODS

Animals and experimental design

Male and female BALB/c mice from Charles River laboratories (Maastricht, 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 light/dark 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 (Sigma, Zwijndrecht, The Netherlands; 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 n = 5 males). Pups were weaned at P21 and a social behaviour test was conducted at P28 after which they were sacrificed. All animal experimental procedures were carried out according to the governmental guidelines and approved by the Ethical Committee for Animal Research of the Utrecht University, The Netherlands.

Caecal sample preparation

Caeca were removed from the sacrificed mice and immediately frozen at -80 ºC until further analyses. Caeca were defrosted on ice and its content was isolated under sterilized conditions and divided into three aliquots. Two aliquots were diluted 10 times with ice-cold PBS and subsequently stored at -20 °C until analysis of SCFA and lactic acid. Total genomic DNA was extracted from the third aliquot combining bead-beating with phenol-chloroform extraction as previously described (44) and DNA extracts were stored at -20 ºC until use for pyrosequencing.

Short chain fatty acids and lactic acids

The ceacal SCFA levels of acetic, propionic, butyric, isobutyric and valeric acids were quantitatively determined as well as levels of lactic acids, as described previously (45, 46). The SCFA were captured using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector. SCFA concentrations were determined using 2-ethylbutyric acid as an internal standard. Lactic acids were determined enzymatically using a D/L-lactic acid detection kit with D- and L-lactate dehydrogenase (EnzyPlus, BioControl Systems, Bellevue, WA, USA).

Pyrosequencing analysis

Total genomic DNA concentration was measured using the Quant-iT™ dsDNA BR Assay kit (Invitrogen) and purity was checked using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc.). The V3–V5 regions of the bacterial 16S rRNA gene were amplified, using primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 926Rb (5'-CCGTCAATTYMTTTRAGT-3'), and pyrosequenced using a 454 FLX Sequencer (454 Life Sciences, Branford, CT, USA) as described previously (28).

Bioinformatic analysis

Sequencing data was analysed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline (47). Quality control filters retained sequences with a length between 200 and 1000 bases and a mean sequence quality score of at least 25. Presence of homopolymers or ambiguous bases of more than 6 bases, and sequences with mismatched primers were omitted. The sequences were grouped into operational taxonomic units (OTUs) by UCLUST (48) *de novo* at 97 % sequence identity to approximate species-level phylotypes. The sequences were aligned using release 108 of the SILVA ribosomal RNA database project as reference (49) and representative sequences (most abundant) for each OTU were selected for taxonomy assignment against the SILVA ribosomal database, applying the Ribosomal Database Project Classifier (50). ChimeraSlayer was applied, as part of QIIME, to filter for chimeric sequences. Rarefaction was applied to the OTUs so that the number of reads per sample would be identical and subsequently used to

conduct alpha and beta diversity calculations. Alpha diversity was assessed using the metrics Phylogenetic Diversity, Chao1, Shannon and Observed species. Weighted and unweighted UniFrac distances were used to assess the (dis-)similarities between the samples (51, 52).

Statistical analysis

Principal coordinate analysis and distance based redundancy analysis (db-RDA) were used to explain the (dis)similarity in species composition (using weighted and unweighted UniFrac distances) using the following explanatory variables; treatment, gender and microbial metabolites levels (caecal SCFA and lactic acid). The significance of separation in db-RDA was assessed with the Monte Carlo Permutation Procedure (MCPP) (53). Analyses were performed using Canoco 5 software for multivariate data exploration (54).

To identify differential abundant taxa from pyrosequencing data, the linear discriminant analysis effect size (LEfSe) method was applied on taxonomic read abundances (55). Both treatment and gender were used as classification in this analysis. Spearman correlations were applied to associate differential abundant taxa with caecal levels of SCFA and lactic acid, as well as with measures described by Theije *et al.* (43), namely social behaviour scores, and ileal levels of 5-HT and neutrophil infiltration (myeloperoxidase), using the add-in XLSTAT (version 2013.3.05, Addinsoft™) developed for Microsoft Excel. Direct comparisons of caecal levels of SCFA and lactic acid for treatment and gender were performed by Mann-Whitney U tests using statistical software Analyse-it for Microsoft Excel (version 2.20).

RESULTS

Description of most abundant bacterial phyla and orders in VPA *in utero*-exposed and control mice

The pyrosequencing dataset contained a total of 195,757 sequences passing the quality filters, resulting in an average of 10,303 reads per sample (range 6,057- 15,622). The Bacteriodetes (19.7 % in VPA-exposed mice, 25.8 % in control mice, on average) and Firmicutes (76.4 % in VPA-exposed mice, 69.4 % in control mice, on average) encompass the majority of the bacterial phyla identified in the caecal samples of both groups, as depicted in **Fig. 1**. Moreover, the majority of bacteria of the Bacteroidetes phylum were assigned to genera within the order of *Bacteroidales* (18.8 % in VPA-exposed mice, 25.1 % in control mice, on average). Most bacterial groups within the Firmicutes belong to several genera within the order of *Clostridiales* (73.6 % in VPA-exposed mice, 68.4 % in control mice, on average).

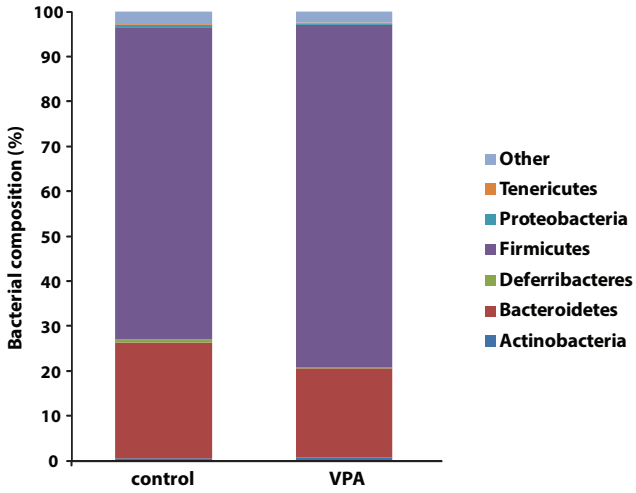

Fig. 1. Bar chart summarizing overall microbial composition of control and valproic acid (VPA) *in utero*-exposed offspring. Microbial taxa, identified at phylum level, greater than 0.1 % average read abundance are displayed, else designated as "Other".

Impact of *in utero* VPA exposure on microbial activities in the offspring

The following SCFA were not quantifiable in the ceacal content of all sampled pups: propionic acid, iso-butyric acid, valeric acid, and iso-valeric acid. The results for acetic, lactic acid and butyric acid are shown in **Fig. 2**. No differences were found in levels of acetic acid (**Fig. 2A**) in VPA *in utero*-exposed mice (median = 53.50 mmol/kg), when compared to control mice (median = 33.00 mmol/kg) nor when taking gender into account (data not shown). Butyric acid (**Fig. 2B**) was found to be significantly increased in VPA *in utero*-exposed offspring (median = 18.69 mmol/kg), compared to control mice (median = 12.64 mmol/kg, MWU test $P = 0.02$). Moreover, this increase in butyric acid levels was only observed in VPA-exposed males (median = 24.23 mmol/kg) and significantly different compared to control male offspring (median = 9.48 mmol/kg, MWU test $P = 0.02$, **Fig. 2D**). No significant differences was observed in female offspring (median = 14.17 and 12.80 mmol/kg for VPA and PBS-exposed females, respectively). Furthermore, a trend towards a decrease in caecal levels of lactic acid (sum of D- and L-lactic acid, **Fig. 2C**) was observed in VPA *in utero*-exposed offspring (median = 0.04 mmol/kg) when compared to control offspring (median = 4.62 mmol/kg, MWU, $P = 0.10$), while no effect of gender was observed (data not shown).

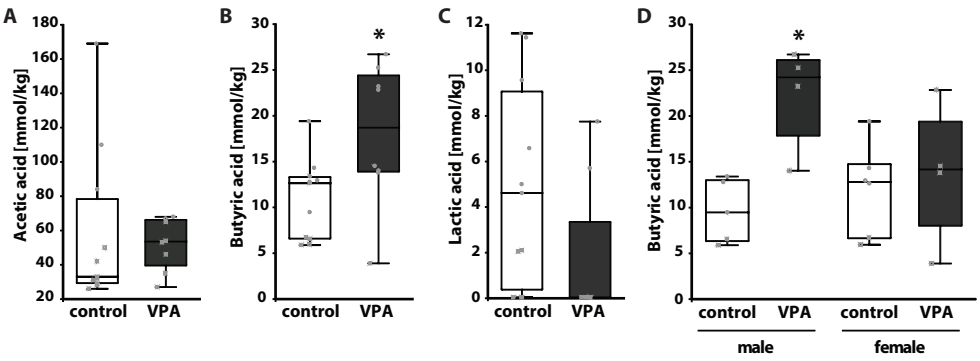

Fig. 2. Skeletal boxplots with caecal concentrations of short chain fatty acids (SCFA) and lactic acid in control versus valproic acid (VPA) *in utero*-exposed offspring in mmol per kg of caecal content. Results of (**A**) acetic acid, (**B**) butyric acid, (**C**) lactic acid for treatment, and (**D**) butyric acid for treatment and gender. *$P < 0.05$ for comparisons of VPA-exposed versus control mice using Mann-Whitney U tests.

Association of microbial diversity with VPA exposure and gender

The microbial diversity and species richness as assessed with alpha diversity metrics on the OTU data did not show differences between VPA-exposed and control mice, nor when taking gender into account (data not shown). When comparing (dis)similarities in microbial composition using weighted UniFrac distances, taking relative abundances into account, no clustering of samples was observed by treatment or gender (data not shown). However, when comparing unweighted UniFrac distances, rather taking presence or absence into account, clustering of samples by treatment and gender was observed. Principal coordinate analysis revealed that the majority (7 out of 11) of control mice clustered in the lower-left part of the plot, while 3 out of 4 VPA-exposed male mice clustered in the upper-left part of the plot and 3 out of 4 VPA females clustered in the lower right part of the ordination (**Fig. 3A**). Using the classifying variables (i) control, (ii) VPA male and (iii) VPA female in a distance-based redundancy analysis (db-RDA) explained the observed clustering well (**Fig. 3B**), and accounted for 13.3 % of the total variation. The MCPP, testing the relation between the microbiota composition and the 3 classifying variables, were significant for both the first axis ($P = 0.044$) and the 2 axes generated ($P = 0.008$, DF = 2) in this constrained model.

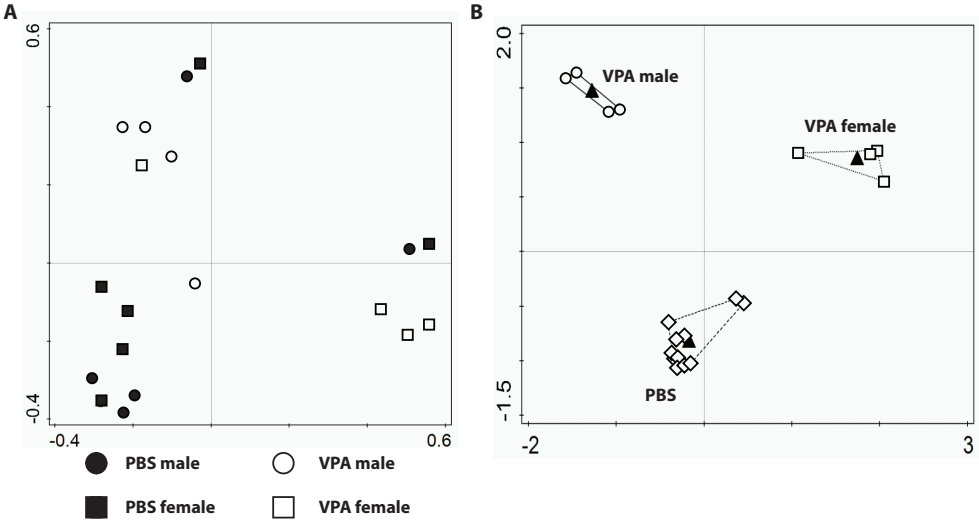

Fig. 3. Multivariate analysis of unweighted UniFrac distances. (**A**) Results of principal coordinate analysis showing the first two axes (gender and treatment). Clustering of samples, based on microbiota species composition, by treatment and gender was observed. The first (horizontal) axis explains 9.98 % and the second (vertical) axis explains 7.87 % of variation in species composition. (**B**) Results of distance-based redundancy analysis using control (PBS), VPA male and VPA female as classifying variables to explain the patterns observed in the PCoA. The explanatory variables accounted for 13.3 % of the total variation observed. Monte Carlo Permutation Procedure (MCPP) applied to test the relation between the species composition and the explanatory variables used in the analysis were significant for both the first axis ($P = 0.044$) and the 2 axes generated ($P = 0.008$).

Additionally, levels of caecal SCFA (acetic and butyric acid) and lactic acid were used as environmental variables in a db-RDA (**Fig. 4**), to further explain the patterns observed. Together these 6 explanatory variables accounted for 30.2 % of the total variation observed. The MCPP was significant for both the first axis (P = 0.042) and all 5 axes generated (P = 0.012, DF = 5) in the analysis. This analysis further revealed that the composition of particularly VPA exposed males were positively associated with high caecal butyrate levels, while control mice were associated with both lactic and acetic acid. Separate clustering as observed for the VPA females from both VPA males and control mice is mainly explained by gender. Differences observed between VPA-exposed male and female offspring is in accordance with observations of autistic-like behaviour, increased intestinal inflammation, as well as decreased serotonin levels in VPA *in utero*-exposed males, and not in female offspring (43).

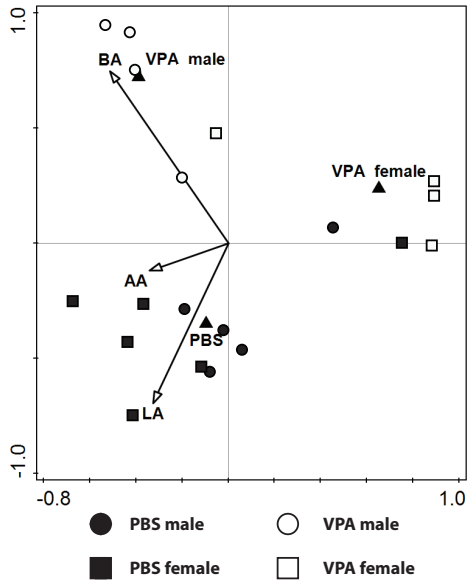

Fig. 4. Distance based redundancy analysis based on unweighted UniFrac distances with the following explanatory variables: treatment and gender (VPA male, VPA female and PBS), and levels of caecal Acetic Acid (AA), Butyric Acid (BA) and Lactic Acid (LA). Together these 6 explanatory variables accounted for 30.2 % of total variation in species composition. The first axis (horizontal) was associated with gender and the second with treatment and levels of SCFA and lactic acid. Monte Carlo Permutation Procedure (MCPP) applied to test the relation between the species composition and the explanatory variables used in the analysis were significant for both the first axis (P = 0.042) and all 5 axes generated (P = 0.012).

Association of bacterial taxa abundances with VPA exposure and gender

Microbial composition of VPA and PBS *in utero*-exposed offspring was compared by applying the LEfSe algorithm on relative taxonomic abundances at different phylogenetic levels (from phylum until genus level). When compared to controls (Kruskal Wallis sum-rank test, **Fig. 5A**), VPA-exposed offspring showed decreased presence of an abundant uncultured genus (designated S24-7) of *Bacteroidales* (9.7 % and 17.8 % on average for VPA and PBS-exposed offspring, respectively, $P = 0.026$) and of a low abundant group of *Deltaproteobacteria*, mainly consisting of *Desulfovibrionales,* (0.3 % and 0.6 % on average for VPA and PBS-exposed offspring, respectively, $P = 0.046$). Moreover, a low abundant uncultured genus of *Erysipelotrichales* was significantly increased in VPA-exposed mice (0.3 % on average), compared to control mice (0.0 % on average, $P = 0.011$). Next, sex-dependent taxa were identified by directly comparing VPA *in utero*-exposed males with VPA *in utero*-exposed females (**Fig. 5B**). This revealed a specific association of an uncultured genus of *Erysipelotrichales* with VPA males (0.5 % on average), compared to VPA females (0.0 % on average, $P = 0.021$). Furthermore, VPA *in utero*-exposed males were associated with the genus *Alistipes* within the order of *Bacteroidales* (12.1 % and 1.6 % on average for VPA males and VPA females, respectively, $P = 0.021$), the genus *Enterorhabdus* within the order of *Coriobacteriales* (1.1 % and 0.2 % on average for VPA males and VPA females, respectively, $P = 0.021$), an unknown genus of *Lactobacillales* (0.5 % and 0.1 % on average for VPA males and VPA females, respectively, $P = 0.043$) and an uncultured genus (designated Rf9) of *Mollicutes* (0.3 % and 0.0 % on average for VPA males and VPA females, respectively, $P = 0.018$).

Correlation of intestinal microbiota with social behaviour and ileal levels of serotonin and neutrophil infiltration

Spearman correlations (**Table 1**) were applied to associate the differentially abundant taxa of VPA *in utero*-exposed and control offspring, with caecal levels of SCFA and lactic acid, as well as with social behaviour scores (time spent near unfamiliar gender-matched mouse) and ileal levels of serotonin and neutrophil infiltration, as described by de Theije et. al. (43). The uncultured genus of *Erysipelothrichales*, increased under VPA and especially males, was positively associated with ileal levels of neutrophil infiltration ($rs = 0.533$, $P = 0.035$) and caecal butyric acid ($rs = 0.466$, $P = 0.046$), and inversely correlated with ileal levels of serotonin ($rs = -0.513$, $P = 0.045$). The uncultured genus of *Bacteroidales*, significantly decreased in VPA-exposed mice, was inversely correlated with butyric acid ($rs = -0.519$, $P = 0.024$). Moreover, the genera *Rikenella* ($rs = -0.531$, $P = 0.021$) and *Mucispirillum* ($rs = -0.559$, $P = 0.014$), associated with VPA females, were also inversely correlated with butyric acid. The latter genus was inversely correlated to actic acid as well ($rs = -0.517$, $P = 0.025$). The genus *Alistipes*, associated with VPA males, inversely correlated with ileal levels of serotonin ($rs = -0.531$, $P = 0.037$), while the genus *Moryella*, associated with VPA females, positively correlated with serotonin ($rs = 0.509$, $P = 0.046$). Furthermore, the uncultured genus of *Mollicutes*, associated with VPA *in utero*-exposed

males, was positively associated with caecal levels of acetic acid (rs = 0.503, *P* = 0.030). The genus of *Enterorhabdus*, showed no significant correlation, however, at the order level of *Coriobacteriales,* a positive correlation with butyrate was observed (rs = 0.472, *P* = 0.043, data not shown). No differentially abundant taxa were directly correlated with social behaviour scores, however caecal levels of lactic acid were positively associated with social behaviour (rs = 0.480, *P* = 0.045) and inversely associated with caecal levels of acetate (rs = -0.525, *P* = 0.023) and butyrate (rs = -0493, *P* = 0.034).

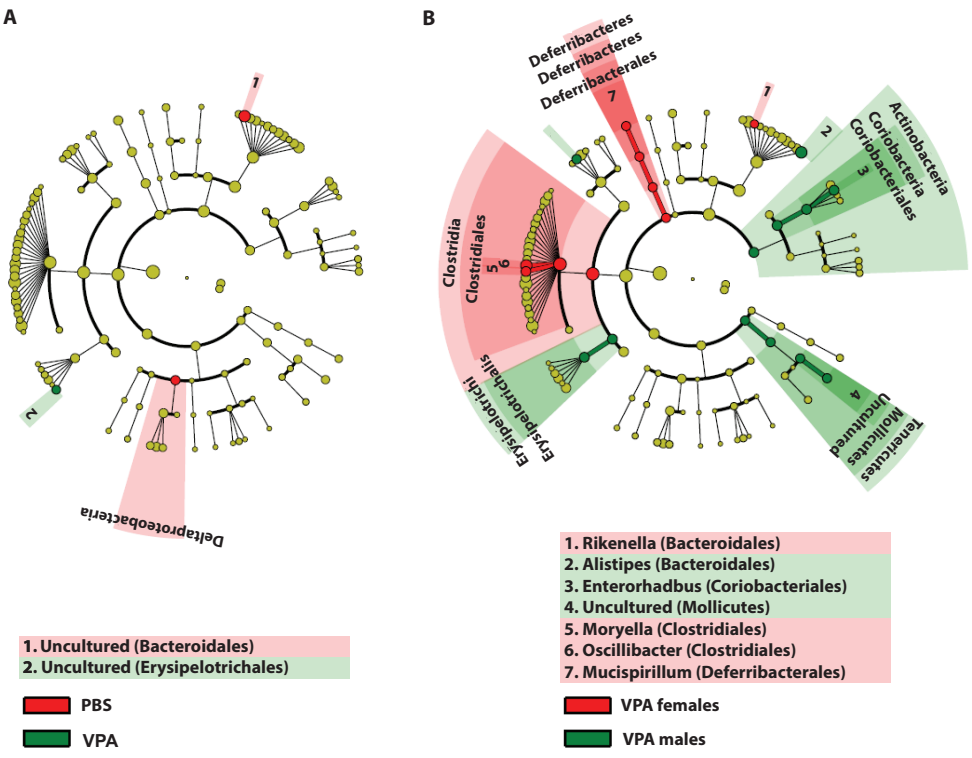

Fig. 5. Taxonomic representation of statistical differences in relative abundances between (**A**) PBS and VPA-exposed offspring or (**B**) female and male VPA-exposed offspring. Linear discriminant analysis Effect Size (LEfSe) was conducted on relative taxonomic abundances from phylum until genus level. Differences are represented in the color of the most abundant class (red: PBS or VPA-female, green: VPA or VPA-male, yellow: non-significant). Each circle's diameter is proportional to the taxon's abundance.

Table 1. Correlation matrix (Spearman) of differentially abundant taxa with social behaviour, ileal levels of myeloperoxidase (MPO) and serotonin, as well as caecal levels of acetic, butyric, and lactic acid.

Variables	SB	MPO	5-HT	AA	BA	LA
Social behaviour (SB)	x					
Myeloperoxidase (MPO)	-0.036	x				
Serotonin (5-HT)	0.041	-0.350	x			
Acetic acid (AA)	-0.447	0.035	0.112	x		
Butyric acid (BA)	-0.393	0.306	-0.338	**0.786**	x	
Lactic acid (LA)	**0.480**	-0.237	0.224	**-0.525**	**-0.493**	x
Coriobacteriales | Enterorhabdus	0.141	-0.059	-0.372	0.354	0.451	-0.134
Bacteroidales | Alistipes	0.123	0.324	**-0.531**	0.002	0.226	0.328
Bacteroidales | Rikenella	0.027	-0.481	0.305	-0.349	**-0.531**	0.448
Bacteroidales | Uncultured	0.393	0.156	0.233	-0.391	**-0.519**	0.436
Deferribacterales | Mucispirillum	0.259	-0.177	0.120	**-0.517**	**-0.559**	-0.034
Lactobacillales | Other	0.181	0.479	-0.469	-0.175	0.146	0.030
Clostridiales | Moryella	0.112	-0.315	**0.509**	-0.126	-0.356	-0.022
Clostridiales | Oscillibacter	0.098	-0.318	0.131	-0.151	-0.275	0.315
Erysipelotrichi | Erysipelotrichales	0.158	**0.533**	**-0.513**	0.183	**0.466**	0.002
Deltaproteobacteria | Desulfovibrionales	0.082	-0.041	0.302	-0.068	-0.240	0.015
Mollicutes | Uncultured	-0.038	0.003	-0.191	**0.503**	0.354	-0.002

Values in bold are different from 0 with a significance level alpha = 0.05

DISCUSSION

The gut-brain axis, encompassing the enteric nervous system (ENS) with an estimated 200 - 600 million neurons, provides a bidirectional communication between epithelial and immune cells in the gastrointestinal tract and the CNS (56). Since gut bacteria continuously challenge the intestinal immune system, via pathogen associated molecular patterns (PAMPs) on the microbial side (57) and pathogen recognition receptor (PRR) on the host side (58), it is compelling to implicate the gut microbiome in the gut-brain axis and thus in brain functioning (56). Indeed, several studies in animal models have proven that the gut microbiota in extreme conditions, such as GF or when altered under antibiotic treatment, correlate with changes in levels of various neurotrophins and monoamine neurotransmitters involved in brain development and plasticity (59). The exact signalling mechanism of how the microbiome interferes with brain functioning and behaviour or vice-versa, how the CNS influences the microbial composition, remains speculative. Here we aimed to investigate how postnatal microbial colonization may be associated with intestinal and behavioural deficits related to ASD. To reach this objective, we combined the well-characterized VPA murine model for ASD with in-depth characterization of the microbiota composition as well as its major products of fermentation, the SCFA and lactic acids.

We have reported the development of disturbed social interaction and increased expression of neuroinflammatory markers as well as deficits in the serotonergic system in the brain of VPA *in utero*-exposed male offspring (43). Interestingly, these neurological and behavioural changes were associated with intestinal inflammation as indicated by an increased neutrophil infiltration, accompanied with decreased serotonin levels and serotonin positive cells in the small intestine of VPA *in utero*-exposed male offspring, compared to controls (43). In this study, we demonstrate for the first time, that prenatal exposure to VPA has a transgenerational impact on the gut microbiota of both male and female offspring, which leads to increased levels of caecal butyrate predominantly in VPA-exposed males.

Maternal influence on the development of the intestinal microbiome of newborns is well documented in animals and humans (60, 61). Overall, the bacterial phyla recovered in our sequencing pool fits with recent descriptions of intestinal microbiota of BALB/c mice, dominated by the phyla Bacteroidetes and Firmicutes (5). Our data shows an aberrant microbial composition in the gut of VPA-exposed offspring, with shifts observed in the main bacterial phyla. Generally, a decrease was observed of Bacteroidetes, mainly consisting of *Bacteroidales*, and an increase of Firmicutes, mainly consisting of *Clostridiales*. Significant effects were found on the abundance of an uncultured genus of *Bacteriodales,* as well as a class of *Deltaproteobacteria*, mainly consisting of *Desulfovibrionales*. Interestingly, these bacterial groups are also associated with ASD in children (62, 63), although no clear trend has been identified yet. In addition, we observed an effect of gender on the gut microbiota composition of VPA *in utero*-exposed offspring, which was attributed to increased levels of OTUs assigned to genera of *Alistipes*, *Enterorhabdus*, *Mollicutes, Lactobacillales* and *Erysipelotrichalis* in VPA *in utero*-exposed males.

In order to connect microbial activity to the intestinal inflammatory phenotype and development of autism-like behaviour, as observed in VPA *in utero*-exposed males, we investigated the caecal levels of bacterial fermentation products. Lactic acid levels tended to be decreased in the VPA-exposed pups without reaching significance. A significant increase of caecal butyrate levels were observed in VPA-exposed offspring, which was more pronounced in males. Whether increased butyrate levels are due to less absorption or more production is questionable. More likely, it is a net result of the overall alteration observed in the gut microbial composition of VPA-exposed males. When looking at the correlations, four genera were indentified to correlate with butyrate; *Rikenella* (*Bacteroidales*), uncultured *Bacteroidales*, *Mucispirillum* (*Deferribacterales*) and uncultured *Erysipelotrichales*. From these genera, decreased abundance of *Bacteriodales* was observed in VPA-exposed offspring and increased abundance of *Erysipelotrichales* was observed in VPA-exposed male offspring. The uncultured members of *Erysipelotrichales* are associated with known butyrate producers (64), supporting a possible role for this bacterial group in increased levels of butyrate in VPA-exposed male offspring.

In addition, male-specific increases of the genera of *Erysopelitrichales* and *Alistipes* significantly correlated with decreased levels of ileal serotonin, which were shown to be accompanied by decreased number of enterochromaffin cells in the intestinal epithelial cell layer of VPA *in utero*-exposed males (43). Enterochromaffin cells (EC) are a subset of enteroendocrine cells located in the epithelial lining of the intestine that produce about 90 % of the body's serotonin. In patients with colorectal adenoma, faecal butyrate levels were inversely related to enteroendocrine cell numbers in colon tissue (65). Therefore, it is possible that the observed changes in microbiome composition of VPA-exposed males affect serotonin production via the inhibitory effect of increased butyrate levels on enterochromaffin cell development. De Angelis *et. al.*, recently reported that increased abundance of *Alistipes* in ASD children was accompanied by increased intestinal levels of indole, a bacterial metabolite of tryptophan (20). Bacterial species of the genus *Alistipes* are described to be indole-positive and may thus influence tryptophan availability. As the amino acid tryptophan is also the precursor of serotonin, increased abundance of genera of *Alistipes* in VPA-exposed males might therefore contribute to the male-specific disturbance in the intestinal serotonergic system upon VPA exposure. Sex-dependent effects of the microbiota on the serotonergic system have been observed in GF studies, and suggest critical roles of oestrogen in CNS serotonergic neurotransmission. In addition, many of the observed deficits in VPA *in utero*-exposed offspring, behavioural as well as immune, are specific to males, which is a representative reflection of the human situation where a marked male preponderance is observed in ASD patients (41, 42).

SCFA (principally acetate, propionate and butyrate) are considered to be neuroactive microbial metabolites that can cross the blood-brain barrier and modulate CNS functions, brain development and behaviour (66-68). Propionate and butyrate have been shown to elicit behavioural changes in rodents, with propionate inducing changes similar to autism (67). Hence, the observed increased butyrate levels in VPA *in utero*-exposed male offspring may contribute to deficits in social behaviour. Increased levels of SCFA have been associated with ASD in children, both for total levels as well as individual levels of SCFA (19). Valproate and butyrate have similar bioactive effects and both have been associated with impaired carnitine pathway for transporting fatty acids, which may cause mitochondrial dysfunction, thereby influencing host physiology and behaviour (66). Moreover, high levels of butyrate in the gut of VPA *in utero*-exposed male offspring can influence epigenetic processes via the inhibition of histone deacetylase (HDAC) (36). The inhibitory activity of butyrate on histone deacetylase (H3/H4) in the gut is implicated in the mechanism of modulating intestinal mucin via MUC-2 gene expression (69). Since butyrate affects MUC genes expression and thus mucus composition, it can regulate epithelial protection and gut morphology (70) and may thereby contribute to an intestinal inflammatory phenotype as observed in male VPA *in utero*-exposed offspring (43). Moreover, if butyrate levels are also increased systemically, it can inhibit activity of

HDAC11 in various neuronal cells and thereby affect maturation of oligodendrocytes and hippocampal neuronal cells in the brain during postnatal development (71, 72). This mechanism can have an effect on the vital process of postnatal brain development which can be strongly implicated in the aetiology of autism-like behaviour in mice.

Overall, we demonstrate that prenatal exposure to VPA induces a rearrangement of early microbial colonization, leading to an increase of butyrate levels in the gastrointestinal tract of male offspring. Consequently, the increased levels of butyrate in the gut may interfere directly with gene expression in intestinal cells and if butyrate enters the circulation, it can affect gene expression in neuronal cells. This could lead to changes in intestinal and CNS physiology and functioning. These results open new avenues in the scientific trajectory of managing neurodevelopmental disorders including ASD. The approach of microbiome manipulation is considered a novel strategy for the management of specific CNS-related disorders. Thus, administration of live organisms or non-digestible oligosaccharides and proteins may become an adjuvant therapy with negligible toxicity profile to the present conventional pharmacological approaches for improving mental health in children and adults.

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CHAPTER FIVE

A diet containing specific anti-inflammatory and neuroprotective ingredients ameliorates behavioural and serotonergic deficits in a murine model of autism spectrum disorders

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ABSTRACT

Prenatal exposure to valproic acid (VPA) is associated with teratogenic effects, including increased risk of autism spectrum disorder (ASD). VPA exposure *in utero* in male mice was previously shown to induce abnormalities in social behaviour, accompanied by reduced serotonin (5-HT) levels in brain and intestine. Gastrointestinal problems in ASD patients are reported and have been attributed to changes in gut microbiota, increased intestinal permeability and inflammation. The purpose of the present study was to investigate the effects of a specific multi-nutrient supplementation diet, containing anti-inflammatory and neuroprotective ingredients, provided during pre- and postnatal development, on VPA-induced behavioural and serotonergic deficits. Pregnant BALB/c females, fed either the control or the active diet, were treated subcutaneously with 500 mg/kg VPA or PBS on gestational day 11. Social and anxiety-like behaviour were assessed on postnatal day 28 and 5-HT metabolism was measured in brain and intestine. Here, we demonstrate that the active diet was able to prevent impaired social and anxiety-like behaviour in male mice exposed to VPA *in utero*. Furthermore, the active diet normalized VPA-induced reductions in 5-HT levels and restored increased levels of its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in brain and intestine. Estimates of 5-HT turnover in brain and intestine were highly correlated, indicating that a common aetiology may underlie VPA-induced serotonergic disturbances. Overall, this study provides new insights into the pathophysiological relevance of intestinal disturbances in patients with ASD and suggests that a multi-targeted diet may be beneficial in the prevention or early treatment of VPA-induced disturbances in serotonin metabolism, social behaviour and anxiety.

INTRODUCTION

Valproic acid (VPA) is clinically used as an antiepileptic drug and mood stabilizer (1). When used during pregnancy, it is associated with an increased risk of adverse effects to the foetus, including congenital malformation, reduced cognitive function, and neurodevelopmental disorders such as autism spectrum disorder (ASD) (2-4). ASD is a heterogeneous cluster of severe neurodevelopmental disorders. It is characterized by impairments in social interaction and communication and the presence of stereotyped behaviours (5). The mechanism for VPA-induced symptoms of ASD is still unclear, but proposed pathways include inhibition of histone deacetylases, attenuation of folic acid metabolism, and increased oxidative stress (2). In search of underlying mechanisms, animal models of VPA-induced ASD-like behaviours have been established in rats and mice. *In utero* exposure to VPA in mice results in developmental and behavioural deficits comparable to those observed in ASD patients, including deficits in social behaviour (6, 7), stereotyped behaviour (8), anxiety, and cognition (7). In line with the observed male preponderance in ASD patients (9, 10), behavioural and developmental deficits were more prominent in VPA-exposed male offspring compared to female offspring (7, 11, 12).

In addition to behavioural deficits, it was recently shown that *in utero* exposure to VPA in mice also caused reduced tissue levels of serotonin (5-hydroxytryptamine; 5-HT) in both brain and intestine (12). Disturbances in development of the serotonergic system are implied in the pathophysiology of VPA-induced behavioural deficits relevant to ASD. Prenatal exposure to VPA caused impaired distribution of serotonergic neurons in rats (13-15). This may be caused by downregulation of *sonic hedgehog* gene expression (14, 15), or the pro-neural gene *Ascl1b* (16), but this remains to be further explored. In patients with ASD, 5-HT abnormalities are frequently reported. An extensive number of studies observed hyperserotonemia, or elevated platelet 5-HT, in approximately 30 % of individuals with ASD (17). Possible explanations for these elevated levels result from successive studies in ASD patients reporting age- and brain region-dependent changes in 5-HT synthesis (18, 19) as well as altered 5-HT receptor binding (20) and increased 5-HT reuptake (21). Thus, considerable studies indicate involvement of the 5-HT system in the pathogenesis of ASD, but mechanisms of action remain elusive.

The serotonergic deficits in mice *in utero* exposed to VPA are associated with an intestinal inflammatory phenotype (12). In a different report, reduced thickness of the mucosa and muscularis as well as reduced gastrointestinal motility was observed in male VPA-exposed rats (22). Presence of gastrointestinal problems in ASD patients is repeatedly reported in literature and includes chronic constipation, diarrhoea and abdominal pain (23). These symptoms have been attributed to changes in gut microbiota (24), increased intestinal permeability (25), and intestinal inflammation (26). As gastrointestinal deficits are suggested to exacerbate autistic behaviour (27), a considerable number of ASD patients

are on specific diets to improve gastrointestinal function and potentially behaviour (28). These diets include gluten and milk elimination diets (29) and supplementations of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) (30), vitamins and minerals (31). A few clinical trials have been conducted to determine the efficacy and safety of elimination or supplementation diets, but these studies are subject to limitations in sample size or study design, warranting further research (32).

Previously, it was observed that *in utero* exposure to VPA in mice caused gender-specific reductions in social behaviour in male offspring, which was associated with intestinal inflammation and reduced serotonin levels in both brain and intestine (12). In the present study, it was assessed whether nutritional intervention with a multi-nutrient diet containing anti-inflammatory and neuroprotective ingredients (33-43), provided during pre- and postnatal development, was able to ameliorate behavioural and serotonergic abnormalities in male mice exposed to VPA *in utero*.

MATERIALS AND METHODS

Diets

The iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on standard animal food for laboratory rodents AIN-93G (44). Compositions of active and control diets are listed in **Table 1**. The active diet consisted of low-glycemic index carbohydrates, dietary fibres, high tryptophan content and a lipid profile that predominantly differed in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content. Diets were stored at -20 °C prior to use to prevent fatty acid oxidation.

Animals and experimental design

Specific pathogen-free BALB/c breeding pairs from Charles River laboratories (Maastricht, The Netherlands) were housed under a 12 h light/dark cycle with access to food and water *ad libitum*. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands (DEC2012.I.06.079). From two weeks preconception, females were fed either the control or the active diet and diets were fed to mothers and offspring throughout the experiment. All females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G11, pregnant females were treated subcutaneously with 500 mg/kg VPA (Sigma-Aldrich, Zwijndrecht, The Netherlands; 100 mg/mL, $n = 4$ per diet) or phosphate buffered saline (PBS, $n = 4$ per diet). Offspring were housed with their mother until weaning on postnatal day 21 (P21). A maximum of 2 male pups per litter was used for subsequent testing (control diet: $n = 5$ for PBS, $n = 7$ for VPA, and active diet: $n = 8$ for PBS and $n = 8$ for VPA). Male offspring were exposed to a social behaviour and open field test on P28 and subsequently euthanized by decapitation to collect brain and intestinal tissue.

Table 1. Diet composition

active compared to control (per kg diet)	supplier
carbohydrates (ref 39)	
dextrinized cornstarch and sucrose substituted by:	
41.5 wt% maltodextrin (DE6)	Roquette (Lestrem, France)
15.0 wt% free galactose	Inalco (Milan, Italy)
42.5 wt% isomaltulose	Beneo-Palatinit (Mannheim, Germany)
1 wt% fructose	Brenntag (Dordrecht, The Netherlands)
fibres (ref 30- 33, 40)	
2.8% cellulose substituted by:	
2% rice fiber RemyLiVe200	Beneo Orafti (Oreye, Belgium)
0.72% GOS*	FrieslandCampina (Amersfoort, The Netherlands)
0.08% Beneo Raftiline HP FOS^	Beneo (Leuven, Belgium)
protein (ref 35)	
soy protein isolate 770LN substituted by:	
1:1 soy protein isolate 770LN and α-lac enanced whey	Solae company (St. Louis, MO, USA) Arla Food ingrediets (Wageningen, The Netherlands)
addition of:	
2.3 g tryptophan	
lipids (ref 34, 36- 38)	
to obtain 0.53% DHA$^{<}$ and 0.92% EPA$^{>}$, part of lipid fraction substituted by:	
27.5 g Nissui anchovy oil	Nippon Suisan Kaisha (Tokyo, Japan)
6.5 g Biopure DHA IF tuna oil	Bioriginal (Den Bommel, The Netherlands)
7.6 g soy lecithin Emulpur	Cargill (Mechelen, Belgium)
vitamins	
extra vitamins (reaching 200 % value): vitamin A, B6, B12, D2, folic acid	

Social behaviour test

The behavioural assessment was adapted from a previous description (12, 45). Mice were placed in a 45 x 45 cm open field, with two small perforated Plexiglas cages (10 cm diameter) located against opposite walls allowing visual, olfactory and minimal tactile interaction (**Fig. 1A**). Mice were habituated to the field for 5 min and an age- and gender-matched unfamiliar mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), zones around the cages were digitally determined. Latency and frequency of entrance and time spent in the interaction zone, near the cage of the target mouse, as well as total distance moved were measured.

Open field test

Rodents naturally tend to explore the environment while avoiding open spaces. Mice that express anxiety-like behaviour tend to spend less time in the centre of the open field (46). The open field apparatus consisted of a 45 x 45 cm arena with a solid floor. After the mouse was placed in the centre of the arena, it was allowed to explore the environment for 5 min. By using Ethovision software (Noldus), an inner zone was digitally determined at 10 cm from the walls. Post-acquisition analysis allowed measurement of latency of first occurrence, frequency of entrance, and time spent in the inner zone, as well as total distance moved.

HPLC analysis of 5-HT and 5-HIAA in brain and intestine

After decapitation, brains were rapidly removed, frozen in isopentane (Sigma-Aldrich) and brains as well as intestinal tissues were stored at -70 °C until further analysis. Prefrontal cortex (PFC), amygdala and dorsal hippocampus were isolated with 500 µm coronal sections using a cryostat (Model 700, Laméris Instruments, Utrecht, The Netherlands). Tissue samples were sonicated in 50-100 µL ice-cold solution containing 15 µM clorgyline and 0.6 µM or 2 µM N-methylserotonin (NMET, internal standard) for brain or intestinal tissue, respectively. To 50 µL homogenate, 12.5 µL of 2 M $HClO_4$ was added. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000 × g (4 °C). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutylamine, and 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol (pH = 3.3). Separation was performed at 40 °C using a flow rate of 0.8 mL/min. The limit of detection (signal/noise ratio 3:1) was 0.9 nM for brain tissue and 0.5 nM for intestinal tissue. The concentration of each compound was calculated as nmol per gram tissue weight by comparison with both the internal and the external standards. Settings of HPLC with electrochemical detection were described elsewhere (12).

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Differences between groups were statistically determined with a two-way ANOVA followed by a Bonferroni's multiple comparisons test. Log transferred data were used to obtain normality for two-way ANOVAs in case of the following data: time in zone (social behaviour and open field) and latency (open field) and intestinal 5-HT turnover. As frequency in zone in the open field did not obtain normality, data were analysed with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test and presented as box-and-whisker Tukey plot. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

RESULTS

The active diet ameliorates impaired social behaviour of male mice exposed to VPA *in utero*

To assess the effect of the active diet on behaviour of male offspring exposed to VPA or PBS *in utero*, mothers and male offspring were fed either the control or the active diet. A social interaction test was conducted at P28 (**Fig. 1A**). In absence of a gender- and age-matched unfamiliar target mouse, no difference was observed in the time spent in the interaction zone (**Fig. 1B**). When a target mouse was placed in the cage, VPA *in utero*-exposed male offspring fed the control diet spent significantly less time in the interaction zone compared to offspring *in utero* exposed to PBS ($P < 0.05$, **Fig. 1C**). The active diet was able to prevent reduced social interaction observed in VPA-exposed offspring ($P < 0.01$).

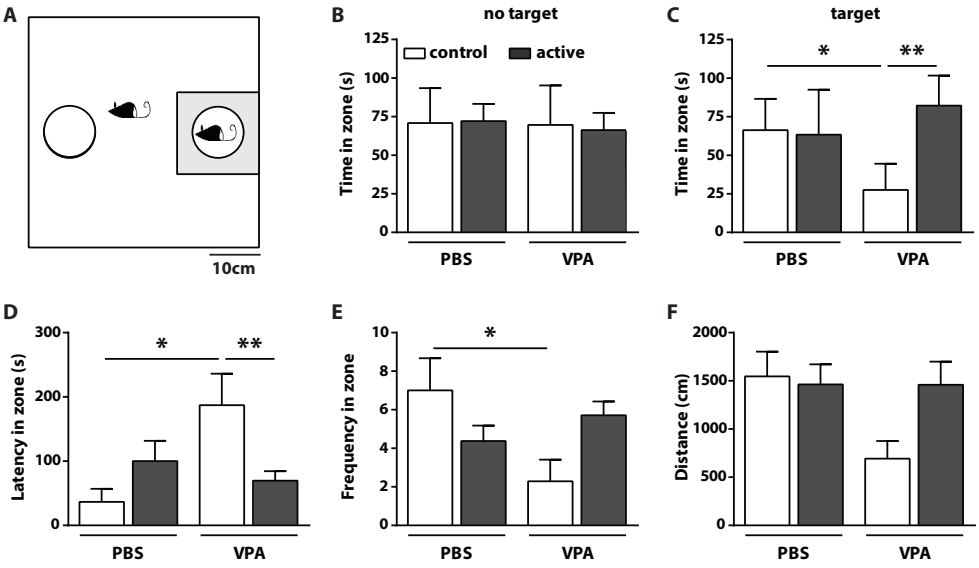

Fig. 1. Social interaction of VPA or PBS *in utero*-exposed male offspring fed the control or the active diet. (**A**) Schematic representation of the social interaction test, illustrating the interaction zone (grey rectangle) and two cages (white circles) on opposite sides of the open field. Time spent in the interaction zone was measured in (**B**) absence (treatment: ns, diet: ns, interaction: ns) and (**C**) presence of a social target (treatment: ns, diet: $P = 0.06$, interaction: $P < 0.05$). (**D**) Latency of first occurrence (treatment: $P = 0.09$, diet: ns, interaction: $P < 0.05$) and (**E**) frequency of entries (treatment: ns, diet: ns, interaction: $P < 0.01$) in the interaction zone in presence of a social target were also assessed. (**F**) Total distance moved was determined as a measure for locomotor activity (treatment: $P = 0.07$, diet: ns, interaction: $P = 0.06$). Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, ns: not significant.

Furthermore, it took longer for VPA-exposed mice to first approach the social target ($P < 0.05$, **Fig. 1D**) and they entered the interaction zone less frequently ($P < 0.05$, **Fig. 1E**), when compared to PBS-exposed offspring fed the control diet ($P < 0.01$). When pre- and postnatally subjected to the active diet, latency of first approach was significantly reduced in VPA *in utero*-exposed offspring, compared to VPA-exposed offspring fed the control diet ($P < 0.01$). Without reaching significance in a two-way ANOVA, it was observed that locomotor activity tended to be reduced in VPA-exposed offspring compared to PBS-exposed offspring when fed the control diet, but not when fed the active diet (**Fig. 1F**).

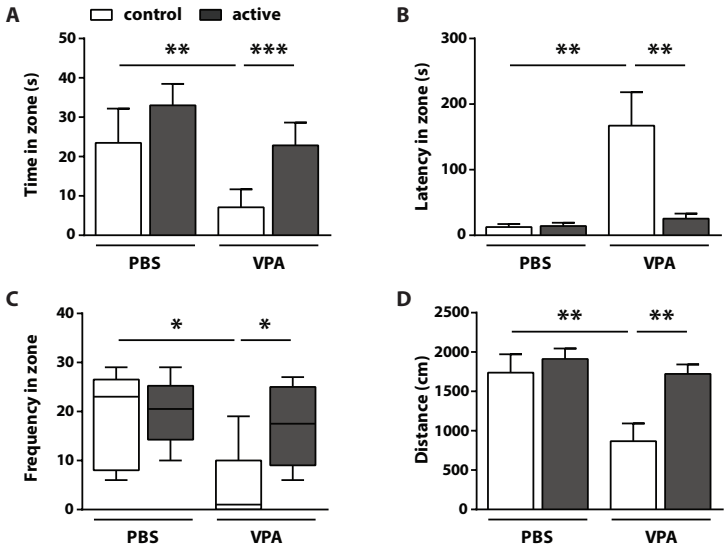

Fig. 2. Anxiety-like behaviour of VPA or PBS *in utero*-exposed male offspring fed the control or the active diet. (**A**) Time spent in the inner zone (treatment: $P < 0.01$, diet: $P < 0.01$, interaction: $P < 0.05$), (**B**) latency until first occurrence (treatment: $P < 0.01$, diet: $P < 0.05$, interaction: $P < 0.05$), (**C**) frequency of entries (Kruskal-Wallis test: $P < 0.05$), and (**D**) total distance moved (treatment: $P < 0.01$, diet: $P < 0.01$, interaction: $P = 0.06$) were assessed in the open field. Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. For frequency in zone, Kruskal-Wallis test was conducted followed by Dunn's multiple comparisons test and data are presented as box-and-whisker Tukey plot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant.

The active diet normalizes anxiety-like behaviour of male VPA-exposed mice

Male offspring were placed in an open field at P28 for 5 min to assess anxiety-like behaviour. *In utero* exposure to VPA reduced time spent in the inner zone ($P < 0.01$, **Fig. 2A**) and reduced latency of first occurrence in the inner zone ($P < 0.01$, **Fig. 2B**), when compared to *in utero* exposure to PBS. The active diet was able to significantly normalize both time ($P < 0.001$) and latency ($P < 0.01$) in VPA-exposed offspring. Furthermore, the frequency of entries in the inner zone was significantly reduced in offspring *in utero* exposed to VPA,

compared to PBS-exposed offspring, when fed the control diet ($P < 0.05$, **Fig. 2C**). This reduction was prevented when VPA-exposed mice were pre- and postnatally subjected to the active diet ($P < 0.05$). *In utero* VPA exposure also affected locomotor activity in the open field, as total distance moved was significantly decreased in VPA-exposed offspring fed the control diet ($P < 0.01$, **Fig. 2D**). The active diet normalized locomotor activity in offspring *in utero* exposed to VPA ($P < 0.01$).

The active diet normalizes levels of 5-HT and 5-HIAA in prefrontal cortex and amygdala of male offspring exposed to VPA *in utero*

Because we previously observed that *in utero* exposure to VPA caused impairments in the serotonergic system of brain and intestines, 5-HT and 5-HIAA levels were measured in the PFC (**Fig. 3A**), amygdala (**Fig. 3E**), and dorsal hippocampus (data not shown) of male mice *in utero* exposed to PBS or VPA pre- and postnatally subjected to the control or active diet. In line with previous observations (12), tissue levels of 5-HT and 5-HIAA were not different between groups in the dorsal hippocampus (data not shown). Analysis of 5-HT in the PFC, however, revealed a significant reduction in 5-HT levels in offspring exposed to VPA *in utero* compared to PBS-exposed offspring, when fed the control diet ($P < 0.05$, **Fig. 3B**). The active diet was able to normalize 5-HT levels in PFC of VPA-exposed mice, as levels were significantly increased when compared to VPA-exposed mice fed the control diet ($P < 0.001$). Levels of 5-HIAA in the PFC were not significantly altered in the PFC of offspring exposed to VPA *in utero* compared to PBS-exposed offspring (**Fig. 3C**). Turnover of 5-HT, estimated by the ratio of 5-HIAA over 5-HT, was more than doubled in offspring exposed to VPA *in utero* compared to PBS-exposed offspring ($P < 0.05$, **Fig. 3D**). The turnover was restored to control levels in VPA-exposed male offspring pre- and postnatally subjected to the active diet ($P < 0.01$).

Similar to the observation in the PFC, 5-HT levels were decreased in the amygdala of mice exposed to VPA *in utero* compared to PBS-exposed mice, when fed the control diet ($P < 0.01$, **Fig. 3F**). When VPA-exposed mice were pre- and postnatally subjected to the active diet, 5-HT levels were normalized ($P < 0.001$). A trend toward an increase in 5-HIAA levels was observed in VPA-exposed mice compared to PBS-exposed mice fed the control diet ($P = 0.07$) and 5-HIAA levels tended to be decreased when mice exposed to VPA *in utero* were fed the active diet ($P = 0.06$). The 5-HT turnover was more than three times increased in offspring *in utero*-exposed to VPA when compared to PBS-exposed mice ($P < 0.01$, **Fig. 3H**) and 5-HT turnover was restored to control levels when VPA-exposed offspring were pre- and postnatally exposed to the active diet ($P < 0.001$).

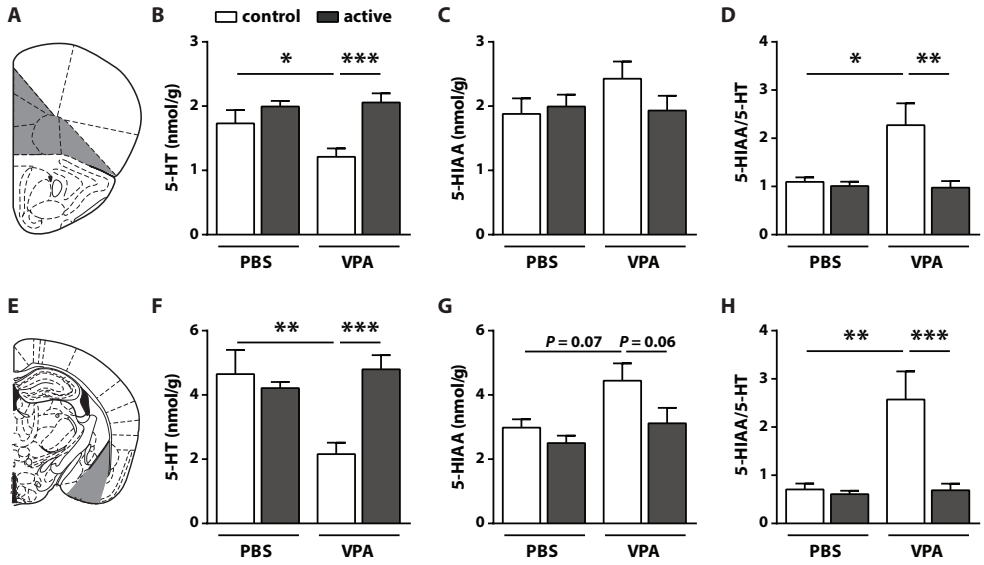

Fig. 3. Levels of 5-HT and 5-HIAA in (**A**-**D**) prefrontal cortex and (**E**-**H**) amygdala of VPA or PBS *in utero*-exposed male offspring fed the control or the active diet. Levels of (**B**) 5-HT (treatment: ns, diet: $P < 0.001$, interaction: $P < 0.05$), (**C**) 5-HIAA (treatment: ns, diet: ns, interaction: ns) and (**D**) turnover (treatment: $P < 0.05$, diet: $P < 0.05$, interaction: $P < 0.05$) in the prefrontal cortex. Levels of (**F**) 5-HT (treatment: $P < 0.05$, diet: $P < 0.05$, interaction: $P < 0.01$), (**G**) 5-HIAA (treatment: $P < 0.05$, diet: $P < 0.05$, interaction: ns) and (**H**) turnover (treatment: $P < 0.01$, diet: $P < 0.01$, interaction: $P < 0.05$) in the amygdala. Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant.

The active diet normalizes levels of 5-HT and 5-HIAA in the intestinal tract of male offspring exposed to VPA *in utero*

Next, the effect of the active diet on the VPA-induced changes of the intestinal serotonergic system was assessed by measurements of 5-HT and 5-HIAA levels in four regions of the intestinal tract; proximal (**Fig. 4A**) and distal jejunum (**Fig. 4E**), distal ileum (**Fig. 4I**), and proximal colon (**Fig. 4M**). *In utero* VPA exposure and dietary intervention caused the same pattern of changes in 5-HT and 5-HIAA levels in all intestinal regions as observed in the amygdala and PFC. More specifically, levels of 5-HT were decreased in proximal jejunum (**Fig. 4B**) and distal ileum (**Fig. 4J**) of VPA-exposed mice compared to PBS-exposed mice when fed the control diet ($P < 0.05$), while reduced 5-HT levels were not observed when VPA-exposed mice were pre- and postnatally subjected to the active diet. In distal jejunum, distal ileum, and colon, increased levels of 5-HT were observed in offspring exposed to VPA *in utero* when they were fed the active diet, compared to the control diet ($P < 0.05$, **Fig. 4F**; $P < 0.01$, **Fig. 4J**; and $P < 0.01$, **Fig. 4N** for distal jejunum, distal ileum and colon, respectively). Overall, this indicates that active diet normalized VPA-induced reductions in intestinal 5-HT levels.

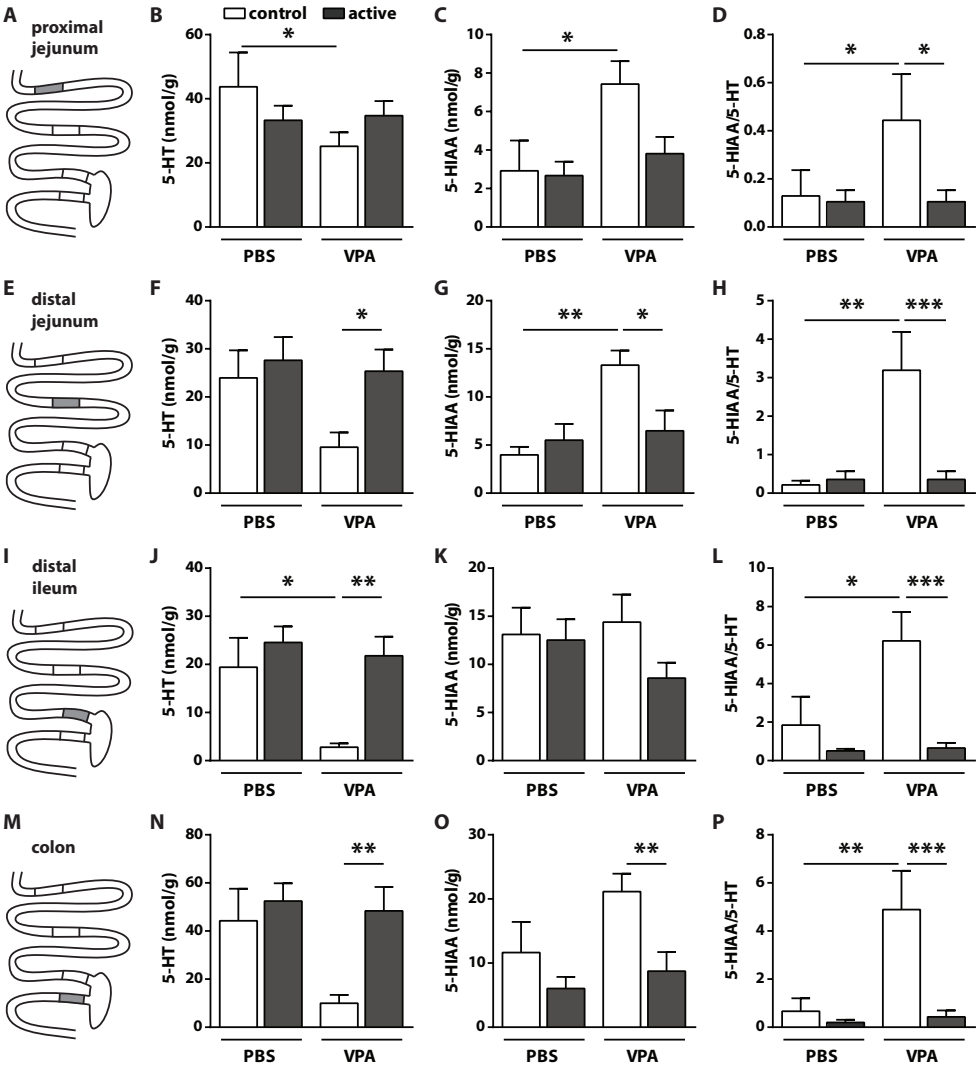

Fig. 4. Levels of 5-HT and 5-HIAA in (**A**-**D**) proximal jejunum, (**E**-**H**) distal jejunum, (**I**-**L**) distal ileum, and (**M**-**P**) colon of VPA or PBS *in utero*-exposed offspring fed the control or the active diet. Levels of (**B**) 5-HT (treatment: $P < 0.05$, diet: ns, interaction: $P < 0.05$), (**C**) 5-HIAA (treatment: $P < 0.05$, diet: $P = 0.08$, interaction: ns) and (**D**) turnover (treatment: $P < 0.05$, diet: ns, interaction: $P < 0.05$) in proximal jejunum. Levels of (**F**) 5-HT (treatment: $P = 0.09$, diet: $P < 0.05$, interaction: ns), (**G**) 5-HIAA (treatment: $P < 0.05$, diet: ns, interaction: $P < 0.05$) and (**H**) turnover (treatment: $P < 0.01$, diet: $P < 0.01$, interaction: $P < 0.01$) in distal jejunum. Levels of (**J**) 5-HT (treatment: $P < 0.05$, diet: $P < 0.01$, interaction: $P = 0.07$), (**K**) 5-HIAA (treatment: ns, diet: ns, interaction: ns), (**L**) turnover (treatment: $P < 0.05$, diet: $P < 0.001$, interaction: $P < 0.05$) in distal ileum. Levels of (**N**) 5-HT (treatment: ns, diet: $P < 0.05$, interaction: $P = 0.07$), (**O**) 5-HIAA (treatment: ns, diet: $P < 0.01$, interaction: $P < 0.05$), (**P**) turnover (treatment: $P < 0.01$, diet: $P < 0.001$, interaction: $P < 0.05$) in colon. Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant.

In utero exposure to VPA in male offspring increased 5-HIAA levels in proximal ($P < 0.05$, **Fig. 4C**) and distal ($P < 0.01$, **Fig. 4G**) jejunum when compared to PBS exposure, while elevated 5-HIAA levels were not observed when VPA-exposed mice were pre- and postnatally subjected to the active diet. Dietary intervention with the active diet reduced levels of 5-HIAA in distal jejunum ($P < 0.05$, **Fig. 4G**) and colon ($P < 0.01$, **Fig. 4O**) of offspring exposed to VPA *in utero*.

5-HT turnover was significantly increased in all four intestinal regions examined of mice exposed to VPA *in utero*, when compared to mice exposed to PBS ($P < 0.05$, **Fig. 4D**; $P < 0.01$, **Fig. 4H**; $P < 0.05$, **Fig. 4L**; and $P < 0.01$, **Fig. 4P**, for proximal jejunum, distal jejunum, distal ileum, and colon, respectively). Dietary intervention with the active diet normalized VPA-induced increase in 5-HT turnover in tissue of all four intestinal regions ($P < 0.05$ for proximal jejunum and $P < 0.001$, for distal jejunum, distal ileum and colon).

Correlation between 5-HT turnover in brain and intestinal tract

To investigate the relation between brain and intestinal serotonergic disturbances, Pearson's correlations were conducted between 5-HT turnover in PFC or amygdala and turnover in the four regions of the intestinal tract. The observed degree of catabolism of 5-HT in 5-HIAA in the amygdala and PFC highly correlated with those observed in all four regions of the intestinal tract (**Fig. 5**). Most significant correlations with the brain were observed for distal jejunum ($P < 0.0001$, r = 0.76 and r = 0.73, for PFC and amygdala, respectively) and for colon ($P < 0.0001$, r = 0.75 and r = 0.74 for PFC and amygdala, respectively). Significant correlations between brain and proximal jejunum ($P < 0.001$, r = 0.62 and r = 0.67, for PFC and amygdala, respectively) and distal ileum ($P < 0.01$ and $P < 0.001$, r = 0.60 and r = 0.63 for PFC and amygdala, respectively) were also observed.

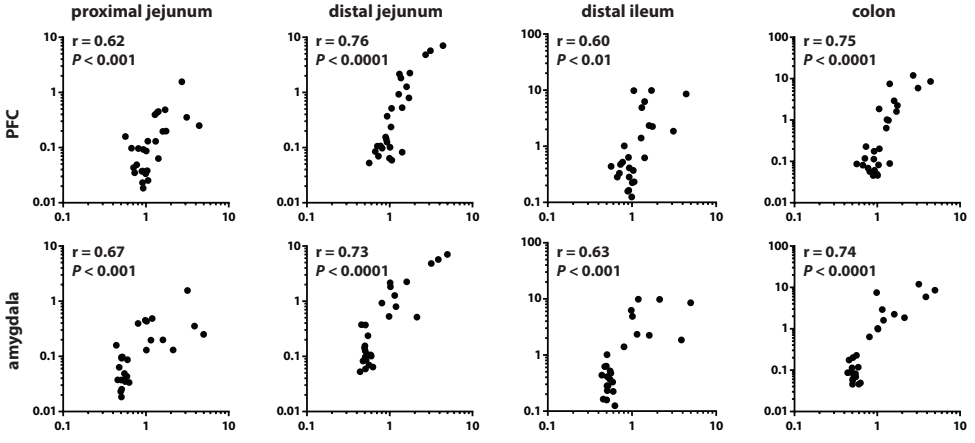

Fig. 5. Correlations between 5-HT turnovers in different regions of the brain and the intestinal tract. Pearson's correlation analysis was conducted to analyse correlations between PFC or amygdala and proximal jejunum, distal jejunum, distal ileum, or colon. r: Pearson's correlation coefficient.

DISCUSSION

We previously showed that prenatal VPA exposure induced behavioural deficits, accompanied by an impaired serotonergic phenotype in brain and intestines of male mice (12). In the present study, we demonstrated that behavioural and serotonergic abnormalities induced by *in utero* VPA exposure in male mice were prevented by a pre- and postnatal dietary intervention with a specific multi-nutrient diet containing anti-inflammatory and neuroprotective ingredients (**Table 1**), aimed to normalize introvert behaviour. More specifically, both reduced social behaviour and increased anxiety-like behaviour in male offspring exposed to VPA *in utero* were reversed by the active diet. Of note is the reduced locomotor activity of VPA-exposed mice on control diet, which may confound the time spent in the interaction zone or in the centre of the open field. However, as time spent in the interaction zone was not different between groups in absence of a target mouse, it is most likely that reduced social behaviour in VPA-exposed mice was not the result of reduced locomotor activity.

In addition to the beneficial effects on behaviour, the active diet also normalized the increased turnover of 5-HT in specific brain regions as well as in the intestinal tract. In agreement with previous observations (12), 5-HT levels were reduced and 5-HIAA levels increased in male mice exposed to VPA *in utero*. The active diet restored 5-HT and 5-HIAA levels in brain and intestines. These results support the hypothesis that an impaired serotonergic system in the brain is involved in the behavioural deficits in offspring exposed to VPA *in utero*. 5-HT in the PFC and amygdala is involved in the regulation of social behaviours (47) and anxiety (48). In patients with ASD, decreased 5-HT synthesis was observed in various regions of the frontal cortex, including the prefrontal cortex (49). Moreover, 5-HT transporter gene polymorphisms that enhance transporter activity are associated with ASD (50) and limited evidence suggests that some ASD patients may respond well to selective serotonin reuptake inhibitors (SSRIs) (51).

5-HT turnovers in all intestinal regions highly correlate with 5-HT turnovers in PFC and amygdala. This implicates that a common aetiology may underlie the serotonergic disturbance in the intestine and in the brain. *In utero* VPA exposure at E11 was shown to be associated with abnormal migration of serotonergic neurons in the brain (13) and *in vitro* it was shown that differentiation of progenitor cells into serotonergic neurons was decreased when exposed to VPA (14). Therefore, VPA may impair serotonergic development, affecting both the central and enteric serotonergic neurons, thereby disturbing central and intestinal physiology. Because of the epigenetic effect of VPA and the observed alterations in 5-HT and 5-HIAA levels, VPA may also affect functioning of enzymes involved in 5-HT production and metabolism, such as tryptophan hydroxylase (TPH), SERT and monoamine oxidase (MAO). This would not only affect serotonergic neurons and thereby behaviour, but all cells containing 5-HT, including enterochromaffin

cells, immune cells, and platelets, disturbing the immune system and intestinal physiology. As intestinal 5-HT imbalance is associated with inflammatory conditions (52), the serotonergic system may be a promising target in the improvement of intestinal dysfunction associated with ASD. Tryptophan, precursor of 5-HT and component of the multi-nutrient diet, may be a tool in targeting intestinal 5-HT. Moreover, maternal supplementation of 5-HT precursor tryptophan was shown to reduce maternal immune responses and reduce infection-induced abortion in mice (53). Therefore, tryptophan may beneficially affect the *in utero* environment of the developing foetus, which may attenuate VPA-induced teratogenic effects.

During pregnancy, VPA is transported across the placenta and accumulates in the foetal circulation, reaching higher concentrations than in maternal blood (54). VPA can affect development of the embryo by inhibiting histone deacetylase (HDAC) and thus altering gene expression. The effects of nutrition on epigenetical processes such as DNA methylation and histone acetylation have recently been extensively reviewed (55, 56). Components of the multi-nutrient 'active' diet such as vitamins and n-3 LCPUFAs (eicosapentaenoic acid; EPA and docosahexaenoic acid; DHA) are suggested to modulate epigenetic processes (57, 58). Dietary salmon, aimed to enhance dietary n-3 LCPUFA intake, during pregnancy resulted in reduced surface expression of intercellular adhesion molecule 1 (ICAM-1) on cultured endothelial cells from umbilical cord veins after stimulation with lipopolysaccharides (LPS). ICAM-1 is involved in the binding of white blood cells to endothelial cells, allowing them to transmigrate into tissues. This implicates an epigenetic anti-inflammatory effect of maternal dietary n-3 LCPUFA on the newborn. Both maternal LCPUFA and folic acid supplementation are suggested to beneficially affect neurodevelopmental outcomes in the newborn (59-63). VPA is suggested to inhibit methionine-mediated DNA methylation, possibly by interference with vitamin B12 and folic acid (64-66). Nevertheless, recent meta-analysis failed to identify conclusive evidence for a long-term benefit of maternal LCPUFA or folic acid supplementation on neurodevelopment (67, 68). In addition to n-3 LCPUFA and vitamin supplementation, administration of galacto-oligosaccharides (GOS) and fructo-oligosacchardes (FOS) to rats was recently shown to increase levels of brain-derived neurotrophic factor (BDNF) in the dentate gyrus of the hippocampus (43). As BDNF signalling is critical for neuronal protection, survival and plasticity (69), this study may evoke further investigation of GOS and FOS supplementation on brain development and neuroprotection.

LCPUFAs and dietary fibres are thought to modulate gut microbiota composition as well as intestinal physiology and the immune system (35, 70). For example, a pre- and postnatal dietary intervention with GOS/FOS significantly increased colon length (71) and enhanced tolerance-related immunoglobulins as well as gut barrier functions (72) in healthy mice. Moreover, the rice fibre used in the multi-nutrient diet was previously shown to reduce inflammation and restore 5-HT levels in a mouse model for colitis (34). Therefore, LCPUFA and dietary fibres in the active multi-nutrient diet may improve intestinal physiology and thereby improve the intestinal serotonergic system.

As the active diet is a multi-nutrient supplementation diet, it is likely to target multiple pathways in which VPA may disturb neurodevelopment. Identifying the individual effects of the active components would require further extensive research subdividing components and multiple combinations to identify synergistic effects. Furthermore, future research on the timing of dietary intervention, more specifically during gestation, lactation, or possibly even later in life, could provide important information on the mechanism by which the active diet may beneficially affect VPA-induced behavioural and serotonergic deficits.

In conclusion, the results of this study imply that social and anxiety-like behavioural deficits induced by *in utero* exposure to VPA can be reversed by a pre- and postnatal nutritional intervention with a multi-nutrient diet containing specific anti-inflammatory and neuroprotective ingredients. Moreover, the active diet beneficially affected the brain and the intestines by restoring VPA-induced impaired serotonergic activity in both organs. Overall, these results show that the active diet may be beneficial in the prevention or early treatment of introvert behaviour and anxiety-like behaviour and associated impaired serotonergic activity induced by *in utero* exposure to VPA.

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CHAPTER SIX

Food allergy and food-based therapies in neurodevelopmental disorders

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ABSTRACT

Autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD) are neurodevelopmental disorders which occur in childhood and often persist into adulthood. Although the aetiology of these disorders is largely unknown, genetic and environmental factors are thought to play a role in the development of ASD and ADHD. Allergic immune reactions, in prenatal and postnatal phases, are examples of these environmental factors and adverse reactions to foods are reported in these children. In this review, we address the clinical and preclinical findings of (food) allergy in ASD and ADHD and suggest possible underlying mechanisms. Furthermore, opportunities for nutritional interventions in neurodevelopmental disorders are provided.

Already in 400 B. C., Hippocrates described the importance of the gastrointestinal (GI) tract in health and disease, by stating 'bad digestion is the root of all evil'. Although written in a period long before the major developments of modern medicine, current understanding of the physiology of the GI tract proves him right in many ways. The intestines have a profound effect on the entire body, including the brain (1). Discovery of the enteric nervous system (ENS) around 1900 was pivotal in the field of gut-brain interactions. Consisting of a complexity comparable to the central nervous system (CNS), the ENS is often described as the 'second brain' (2). Bidirectional communications between the brain and the gut occur via various pathways, involving the vagus nerve, autonomic nervous system and neuroimmune interactions both in the GI tract and in the brain (3). Over the past few decades, strong correlations have been observed between the occurrence of GI problems and psychiatric disorders, augmenting the interest in the gut-brain connection (4). Food allergy is suggested to be one of the GI triggers for various psychological and psychiatric conditions. Allergic reactions to food are primarily observed in children and an association with neurodevelopmental disorders has therefore been proposed. Although the significance of this link is still under debate, a considerable amount of literature has been published on food allergy or food-based therapies both in the fields of autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD). However, no review has thus far combined the knowledge on food allergy, derived from both fields. In this review, we discuss the clinical and preclinical findings on (food) allergic reactions and food-based therapies in neurodevelopmental disorders of the autism and attention-deficit hyperactivity spectra and we describe possible mechanisms of action.

FOOD ALLERGY

Under physiological conditions, the body develops a tolerogenic reaction to harmless food antigens. Oral tolerance is a consequence of complex immune cell interactions, among which dendritic cells (DCs) and various subsets of T cells are involved. Oral tolerance is characterized by immunosuppressive conditions, in which regulatory T cells (Tregs) produce transforming growth factor (TGF)-β and interleukin (IL)-10 to suppress the inflammatory response (5). Loss of tolerance is most often observed towards high allergenic food proteins, derived from for example cow's milk, peanut, egg, soy, and wheat, resulting in an allergic response. Food allergy can be defined as an immune-mediated hypersensitivity reaction against food proteins. Although different immunological mechanisms can underlie the pathology, food allergy is generally characterized by T helper (Th) 2 skewing of the immune system and is accompanied by reduced Treg function (5).

The majority of food allergic reactions is mediated by immunoglobulin (Ig) E, secreted by plasma cells upon antigen presentation by Th2 cells and stimulation with Th2-associated cytokines (interleukin (IL)-4, -5 and -13). IgE binds to its receptor on mast cells or basophils and after a successive food exposure, the allergen can bind conjugated IgE, inducing mast cell degranulation and secretion of inflammatory mediators such as cytokines, histamine, leukotrienes and prostaglandin. As the onset of symptoms evolves within several minutes to several hours after contact with the allergen, IgE-mediated allergic reactions are also referred to as 'immediate-type hypersensitivities'. During non-IgE-mediated reactions, the allergic response can be mediated by Ig-free light chains (6) or by cells. Cell-mediated food allergy does not involve immunoglobulins and onset of symptoms is observed from one hour to many days after ingestion of the food protein. Cell-mediated food allergy is therefore classified as 'delayed-type hypersensitivity' and is mediated by Th1 and Th17 cells. IgE-mediated food allergy can be diagnosed by skin prick test and allergen-specific serum IgE levels. No standardized test is available for detection of non-IgE-mediated food allergy and diagnosis is based on elimination of the suspected allergenic food for several weeks. A challenge test can be used to confirm the diagnosis.

Clinical symptoms of food allergic reactions are not restricted to the GI tract, but also involve the skin and airways. In addition to these non-GI symptoms, it has also been suggested that behavioural and psychological symptoms can be evoked by allergic reactions (7). Food allergy has been implicated in anxiety, depression (8), migraine (9), schizophrenia (10), ASD (11) and ADHD (12). Also allergic asthma has been associated with behavioural dysfunction (13). A meta-analysis revealed that internalizing behaviours were more frequently reported in children with asthma and asthma severity correlated with behavioural scores (14). Furthermore, the prevalence of developmental and behavioural dysfunction, including ADHD, was shown to be twice as high in children with, compared to children without asthma and was associated with disease severity (15). It is unknown whether this association has a direct cause-effect relationship or an underlying common aetiology. A meta-analysis not only demonstrated a positive association between overall allergic diseases and psychosocial abnormalities, but also claimed that psychosocial factors and neurodevelopment can contribute to the development of allergic disorders (16). Recently, Meldrum and colleagues observed that children suffering from allergic diseases in the first year of life scored lower in behavioural outcomes compared with controls, indicating that allergic diseases during infancy predict later neurodevelopmental scores. More specifically, children with eczema in the first year of life had relatively poorer gross motor skills, whereas food allergy was predominantly associated with lower social emotional scores and internalizing behaviours (17). We hypothesize that both prenatal and postnatal allergic immune activation regulates complex, but critical, neuroimmune interactions that affect neurodevelopment and brain function. Together with a genetic predisposition and multiple environmental factors, these effects of allergic immune activation might contribute to the development or severity of ASD and ADHD (**Fig. 1**).

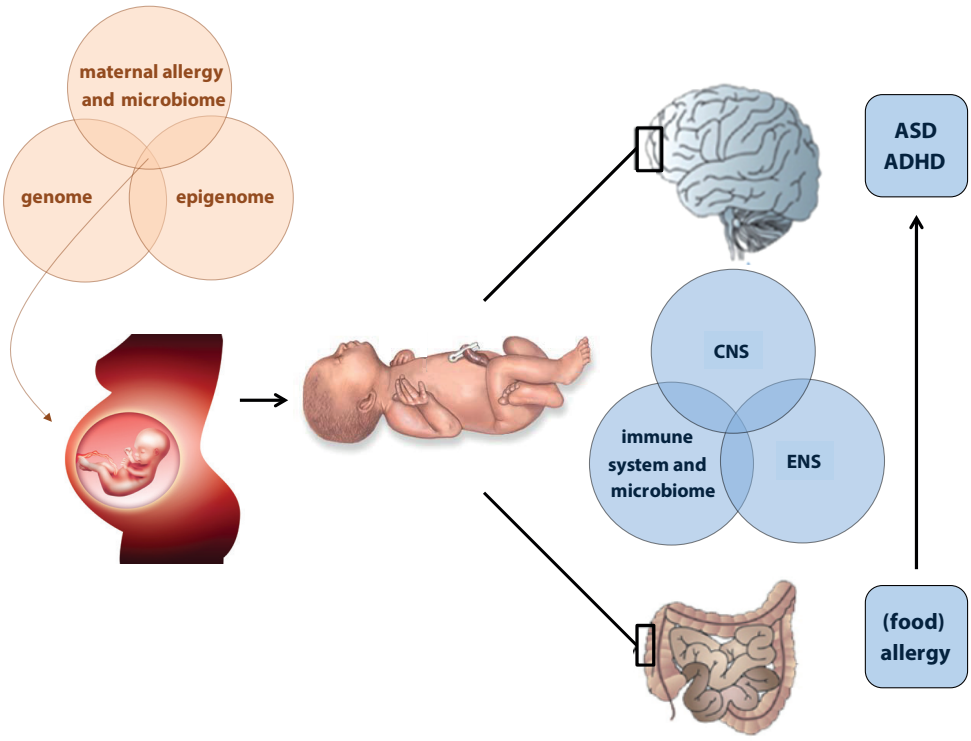

Fig. 1. Multifactorial aetiology of neurodevelopmental disorders. Genetic predisposition via genetic mutations (genome) and epigenetic changes by means of DNA methylation and histone modification (epigenome) can cause deficits in neurodevelopment leading to autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD). Maternal allergic immune reactions may enhance such epigenetic changes. Also during postnatal development, (food) allergic immune activation is thought to affect brain function via neuroimmune interactions that affect the enteric nervous system (ENS) and central nervous system (CNS). As food allergy changes microbial composition in the gastrointestinal (GI) tract of the mother and new born, it is compelling to consider the microbiota-gut-brain axis as a mediator in these neuroimmune interactions in the GI tract.

FOOD ALLERGY IN AUTISM SPECTRUM DISORDER

ASD is a heterogeneous cluster of neurodevelopmental disorders characterized by impairments in communication and social interaction and by repetitive behaviour (18). The gut-brain axis is an emerging field of research on ASD. Accumulating evidence strongly suggests that GI dysfunctions and abdominal pain are often present in children with ASD (19). The reported prevalence of GI symptoms ranges from 10 % to 90 %, an immense range probably due to varying interpretations of gastrointestinal problems, inability of ASD children to express their discomfort and the heterogeneity of the disorder. The cause of these GI symptoms has been attributed to increased intestinal permeability (20), intestinal inflammation (21), changes in microbial composition (22), and allergic reactions (11). Parents of autistic children reported more often that their child suffered from food allergies than parents of healthy children or children with special education needs (23, 24). Indeed, serum levels of immunoglobulins IgA, IgG and IgM specific for cow's milk-derived allergens and total IgE were shown to be increased in children with ASD compared with healthy controls (25). In addition, peripheral blood mononuclear cells (PBMCs) of ASD children produced more tumour necrosis factor (TNF)-α and IL-12 in response to cow's milk-derived allergens than those from control subjects (26). Furthermore, milk intake by autistic patients was a significant predictor of constipation (27) and led to the worsening of some of the behavioural symptoms specific for ASD (25). Gluten intake has also been suggested to exacerbate autistic behaviour (28, 29), although no association between gluten sensitivity or celiac disease and ASD has been reported (30).

Supporting the assumed role of allergy in ASD, an imbalance in T cell subsets was observed in these patients. Low numbers of Th1-type interferon (INF)-y and IL-2 positive cells were observed in blood of ASD children compared with healthy controls (31). Moreover, significant differences were observed between Th2 cytokine production by PBMC from autistic children when compared to age-matched controls regarding IL-4, IL-5 and IL-13 (32). Concerning regulatory T cell responses, reduced levels of IL-10 and TGF-β were observed in children with ASD (33, 34), indicating reduced tolerance. Moreover, these low TGF-β levels correlated with worse behavioural scores (33), suggesting that reduced regulatory T cell responses could be involved in the regulation of autistic behaviour.

FOOD ALLERGY IN ATTENTION-DEFICIT HYPERACTIVITY DISORDER

ADHD is a neurodevelopmental disorder that is characterized by inappropriate symptoms of inattention, impulsiveness and hyperactivity (18). Although little research in the field of ADHD is focused on the involvement of the gut-brain axis, the importance of allergic diseases and the influence of dietary factors have received considerable attention. The relationship between allergic diseases and ADHD has been a matter of debate ever since Geschwind and Behan hypothesized on an association between left-handedness, immune disease and developmental learning disorders (35). Subsequent studies provided conflicting results, where some groups found no significant association between prevalence of ADHD and allergic diseases (36-39), but others did (40). From a systemic review in 2010, it was concluded that ADHD was positively associated with eczema and asthma, but not with allergic rhinitis and total serum IgE levels (40). On the contrary, a successive study showed that only the prevalence of allergic rhinitis, but not other allergic diseases, was associated with ADHD. In this study, the frequency of any positive skin prick test, however, was higher in ADHD patients compared with controls (41). More recent data revealed that paediatric patients with allergic disorders had a substantially increased risk of developing ADHD (42, 43) and conversely, patients with ADHD had an elevated risk of developing allergic diseases (44, 45).

Research on atopic diseases in ADHD patients is predominately focused on asthma, eczema and rhinitis, but findings regarding food allergy are limited. One case study showed that a 7-year-old boy with ADHD and severe IgG-mediated food allergy received a dietary supplement intervention that lowered IgG antibodies, which was accompanied by improvement in behaviour (46). However, in a small study of 40 patients with ADHD, no significant increase of a positive skin prick test to food allergens was observed compared with controls (41). A different approach to test the importance of food allergens in ADHD children is a food challenge. Hyperactive children reacted badly to allergenic foods like cow's milk, wheat and eggs (47-49). This reaction was not associated with a positive skin prick test, although total serum IgE levels were higher in responders compared with non-responders (48). Because of the mixed results on the involvement of a food allergic reaction, Pelsser and colleagues postulated a new intriguing hypothesis. They suggested that ADHD is not caused by the allergic response, but that ADHD is a (non-) allergic hypersensitivity disorder itself. Food-derived allergens trigger a hypersensitivity reaction that causes ADHD symptoms, possibly via an IgE or non-IgE allergic response or a non-allergic mechanism (12). According to the hypothesis, ADHD can be subdivided in hypersensitive and non-hypersensitive ADHD, derived from different pathophysiologies. This would provide opportunities for nutritional interventions or elimination diets for a subset of children with ADHD.

MECHANISMS

Maternal factors

Maternal allergy has been shown to be a stronger determinant of allergy in offspring than paternal allergy, indicating that the maternal allergic response affects the immune system of the developing foetus *in utero* (50, 51). Development of the foetal immune system is affected not only by the maternal immune system, but also by maternal psycholocial factors. Chronic maternal stress not only affects behavioural and cognitive functions later in life (52), it also affects immune system development. High blood levels of cortisol have been shown to shift the cytokine balance to a Th2-type profile (53). As high maternal cortisol levels reflect those of the foetus, it is likely that this Th2-type profile is also provoked in the developing foetus. Indeed, adult women who were exposed to chronic stress during prenatal development established a Th2-type dominated immunity (54), which increases the risk of allergic sensitization.

In addition to prenatal development of the immune system, development of the brain is also highly sensitive to maternal immunological and psychological challenges. Accumulating evidence indicates that maternal infection during pregnancy is a significant risk factor for the development of schizophrenia in offspring (55). Also in patients with ASD, prenatal immune activation is suggested as to be environmental risk factor. Maternal viral infection in the first trimester and bacterial infection in the second trimester were found to increase the risk of ASD in the offspring (56). In addition, animals prenatally exposed to infections, display features relevant to autism in humans, suggesting a causal link between maternal immune activation and autistic behaviour (57). Maternal allergies are also implicated to put the offspring at risk of developing ASD. In a large study population, a greater than twofold elevated risk of ASD was observed for maternal asthma and allergy diagnosed during the second trimester, but not for maternal autoimmune diseases (58). Furthermore, a preliminary report indicated that mothers with mastocytosis during pregnancy, characterized by increased number of mast cells in many organs, had a higher risk of delivering a child with ASD (59). Although little research is conducted on the importance of prenatal immune activation in patients with ADHD, one study suggested that viral rashes during pregnancy increased the risk of developing ADHD in the offspring (60).

Elevated levels of pro-inflammatory cytokine IL-6 have been claimed as a mechanism underlying the effect of maternal immune activation on neurodevelopment. Indeed, prenatal exposure to IL-6 alone is sufficient to initiate abnormal behaviour and neurodegeneration in the hippocampus of rodents (61). IL-6, which is able to cross the placenta and the blood-brain barrier (62), has been shown to affect developing serotonergic and dopaminergic neurons *in vitro* (63). Cytokines in the CNS can activate monoamine transporters (64), resulting in reduced neurotransmission, which may lead

to symptoms observed in ADHD and ASD (65). In patients with autism, increased levels of deficits in the serotonergic system have been repeatedly described and it is hypothesized that this phenomenon is due to an enhanced serotonin transporter (SERT) activity (66, 67). Furthermore, associations with important genes in the dopaminergic pathway have also been reported in both ASD and ADHD (68), including the dopamine transporter gene DAT1. Interestingly, maternal immune activation in animal models caused defects in dopaminergic development in the offspring (69-71) and neutralization of IL-6 in pregnant dams prevented the dopaminergic and behavioural alterations induced by maternal immune activation (72). These findings suggest that prenatal immune activation disturbs neurodevelopment via IL-6, leading to behavioural abnormalities as found in ADHD and ASD.

Mast cells

One of the most crucial cells in allergy is the mast cell. Several neuropeptides can trigger mast cell activation, including substance P, nerve growth factor, vasoactive intestinal peptide and neurotensin (73). Mast cells express various substances that can trigger enteric neurons, such as tryptase, histamine, 5-HT, NGF and TNF-α (74). Mast cell–neuron interactions occur in the GI tract, for instance in inflammatory bowel disease and irritable bowel syndrome (74). In theory, a food allergic reaction might therefore influence behaviour via intestinal mast cells that trigger enteric neurons to convey information through afferent sensory pathways to the CNS. Although there is very little direct evidence suggesting that mast cells are involved in neurodevelopmental disorders, a preliminary report indicated ASD to be more prevalent among children with mastocytosis (59).

Activated Th2 and mast cells produce IL-6, which promotes B cell differentiation and production of immunoglobulins (75) and inhibits Tregs (76), leading to an enhanced allergic reaction. The production of IL-6 by activated mast cells is mediated by the mammalian target of rapamycin (mTOR) pathway (77). Deficiency of PTEN, a negative regulatory molecule of mTOR, caused mastocytosis in mice and increased mast cell degranulation and IL-6 production, leading to a higher allergic response (78). Interestingly, besides the effect on the allergic response, PTEN-deficient mice also developed autistic features in behaviour (79). Indeed, increased mTOR signalling has been suggested to be involved in neurodevelopmental disorders. For example, tuberous sclerosis, a genetic disorder of mutations in tuberous sclerosis complex (Tsc) 1 or Tsc2 genes that negatively regulate mTOR, is associated with ASD (80) and ADHD (81). In conclusion, increased activity of the mTOR-signalling pathway causes higher IL-6 production by mast cells and mastocytosis in mice, leading to a higher allergic response. Furthermore, it is associated with neurodevelopmental disorders and causes behavioural deficits in mice. Possibly, the effects of mTOR activity on behaviour are partially mediated by IL-6, as enhanced mTOR activation results in IL-6 production and IL-6 has been shown to induce behavioural deficits and is enhanced in post-mortem brains of patients with ASD (82, 83).

Microbiota

The microbiota has been a major point of attention in research on the gut-brain axis. Recently, it has been shown that gut bacteria influence CNS development and therefore behavioural and cognitive responses. For example, germ-free mice expressed decreased anxiety-like behaviour, while colonization of these mice early in life, but not as adults, normalized these behavioural deficits (84). This indicates that the microbiota contributes to neurodevelopment and it is compelling to consider its influence on ASD and ADHD. Several groups have studied the intestinal microbiota of the autistic human population and found differences in composition of *Ruminococcus*, *Bacteriodes* and *Desulfovibrio* genera from the phyla of Firmicutes, Bacteriodetes and Proteobacteria, respectively (85-88). A recent study demonstrated lower levels of *Bifidobacterium* and higher levels of *Lactobacillus* in ASD, both considered to be beneficial bacteria (89). Interestingly, antibiotic treatment of children with ASD not only led to GI improvements, but improvements in cognitive skills as well (90). However, more research on the microbiota of children with ASD is required to understand the role of a microbial factor in ASD. To our knowledge, no studies have been published on the microbial status of children with ADHD. Investigating the microbial composition of children with ADHD could lead to new insights on the effects of food or food allergy on behaviour in these patients.

Interestingly and very recent, the first proof in humans showing that the microbiota-gut-brain axis exists has been reported (91). In this study in healthy women, chronic ingestion of several strains of gut bacteria, including *Bifidobacterium* and *Lactobacillus*, reduced activity of brain regions involved in processing of emotion and sensation. Several possible mechanisms have been proposed in which the microbiota can modulate brain activity. Preclinical models indicate that the microbiota can signal to the brain via vagal afferent nerves and the nucleus tractus solitarius (NTS) (1). This signalling could be mediated by direct communication of microbiota with the intestinal epithelium and serotonin-producing enterochromaffin cells, affecting the ENS and immune system. The microbiota can also target epithelium and enteric nerves via the production of short chain fatty acids and neuroactive signalling molecules (1, 92). Nevertheless, more (pre)clinical research is needed to identify the signalling pathways by which the microbiota communicates with the brain in health and in neurodevelopmental disorders.

Food-based therapies

If children with a genetic predisposition are more susceptible to develop ASD or ADHD when exposed to an allergic immune challenge, allergen-free diets and immunomodulatory dietary interventions could be beneficial for the treatment of behaviour. Indeed, approximately 30 % of ASD patients (93) and 12 % of ADHD patients (94) are using complementary and alternative medicine (CAM). Examples of such treatments include the exclusion of hyperallergenic foods and use of long chain polyunsaturated fatty acids (LCPUFA), vitamins and mineral supplements.

Both in ASD and ADHD, elimination diets have received most attention. Although the efficacy of an elimination diet in children with both disorders is still under debate, exclusion of hyperallergenic foods was claimed to be beneficial in multiple studies. Considering ASD, studies indicate that gluten and milk free diets improved behaviour in children with ASD (95, 96). In addition to these behavioural improvements, gluten and milk-free diets were also shown to reduce the enhanced intestinal permeability observed in ASD patients (20). However, large double-blind and well-controlled trials are required to strengthen these observations. Considering ADHD, foods that are often eliminated are cow's milk, wheat, egg, chocolate, cheese, nuts and citrus fruits. Two early studies reported that behavioural symptoms improved in children with ADHD (47, 48). More recently, Pelsser and colleagues observed improvements in behaviour in 62 % of ADHD patients using an elimination diet (97). The effectiveness of this diet was further investigated in a randomized controlled 'INCA' trial (98). In this successive study, 64 % of children with ADHD on the elimination diet responded by showing improvement in behaviour after 5 weeks. Serum levels of food-specific IgE and total IgE did not differ between responders and non-responders. These findings suggest that ingested foods can affect behaviour in a subset of children with ADHD through a mechanism that involves a non-IgE-mediated, cell-mediated or non-allergic response.

In addition to the use of an elimination diet in ASD or ADHD patients, dietary ingredients such as n-3 LCPUFA, non-digestible oligosaccharides and beneficial bacterial strains might also be beneficial to the dietary management of behaviour, because of their effects on CNS, immune system and microbiota profile. Nutritional supplementation with n-3 LCPUFA was observed to be beneficial in both ASD (99) and ADHD (100). Decreased levels of incorporated n-3 LCPUFA have been observed in peripheral blood cells of ASD and ADHD (100) patients repeatedly. After treatment with fish oil, n-3 LCPUFA levels and ratio of n-6/n-3 were restored and a significant improvement of behaviour was observed in patients with ASD (99) and ADHD (100). LCPUFA are present in neuronal membranous phospholipids, where they modulate membrane fluidity and thereby affect receptor function, resulting in changes in neurotransmitter release and uptake (101). In addition to effects on the brain, n-3 LCPUFA have also been claimed to modulate the immune response. N-3 LCPUFA can be incorporated in the membrane of immune cells, where they modulate intracellular pathways, such as nuclear factor κB (NFκB) and peroxisome proliferator activated receptor (PPAR)-γ, leading to an anti-inflammatory response through the production of resolvins and inhibition of leukotrienes, prostaglandins, pro-inflammatory cytokines and adhesion molecules (102) . Therefore, supplementation of n-3 LCPUFA could be beneficial for patients with ASD and ADHD, either by acting directly on neuronal responses or indirectly via the immune system and GI tract (103).

The effects of non-digestible oligosaccharides and beneficial bacterial strains in the treatment of neurodevelopmental disorders have not been studied. Regarding allergic diseases, non-digestible oligosaccharides were shown to be beneficial for disease progression and immune status (104, 105). Non-digestible oligosaccharides consist of naturally occurring sugar base units that are not hydrolyzed in the upper small intestine and reach the large intestine intact to serve as substrates for bacterial metabolism. Non-digestible oligosaccharides are suggested to selectively stimulate growth and/or activity of Bifidobacteria and lactic acid bacteria in the colon, which are important markers of a healthy gut microbiota (106, 107). Considering the increased recognition of a microbial role in the regulation of behavioural responses and neurodevelopment, children with ASD and ADHD may benefit from dietary supplementation with non-digestible oligosaccharides or beneficial bacterial strains. Beneficial bacterial strains were thought to reduce intestinal permeability and restore a 'healthy' gut (108), which could be of specific importance in the treatment of GI disturbances and behavioural deficits of ASD patients. However, preclinical studies in disease models of ASD and ADHD are required to study the benefits and safety of supplementation with non-digestible oligosaccharides and beneficial bacterial strains in the treatment of behaviour.

As prenatal (immune) factors are thought to have detrimental effects on behaviour of the offspring later in life, it may be even more interesting to provide genetically or environmentally predisposed mothers with beneficial diets during pregnancy. Preclinical murine studies showed that a maternal diet rich in n-6 LCPUFA during gestation and lactation induced autism-like behaviour in offspring (109). N-3 LCPUFA rich fish consumption during pregnancy was shown to be protective of ADHD-related behaviour in children (110). Furthermore, a traditional Dutch diet during pregnancy was positively associated with externalizing behaviours in children, while a Mediterranean diet was negatively associated (111). Folic acid supplementation during pregnancy protected offspring from internalizing and externalizing behaviours and the development of ASD (112, 113). These results indicate that the maternal diet during pregnancy affects neurodevelopment and thus the risk of developing ASD or ADHD. Therefore, future research should not exclusively be focused on nutritional interventions in postnatal development, but should also aim at beneficially directing neurodevelopment via maternal diets.

CONCLUSION

In this review, we addressed the findings of (food) allergic diseases and food-based therapies in ASD and ADHD. Based on clinical and preclinical observations, it seems feasible to hypothesize that allergic reactions to foods indeed trigger or worsen the manifestation of neurodevelopmental disorders in at least a subset of paediatric patients. In children with ASD, these allergic reactions could be both IgE as non-IgE-mediated, while in ADHD, it is more likely that non-IgE-mediated, cell-mediated or non-allergic responses to foods are involved. We hypothesize that both prenatal and postnatal allergic immune activation regulate complex, but critical, neuroimmune interactions and therefore affect neurodevelopment and brain function. Pathways involved in allergy-mediated effects on neurodevelopment and behaviour could include neuroimmune interactions induced by mast cells and cytokines such as IL-6, as these mediators have been shown to induce behavioural deficits in preclinical models and are enhanced in ASD patients. Changes in the microbiota and subsequent signalling to the brain could also be involved in the behavioural consequences of an allergic response. Nevertheless, very little preclinical research is conducted on the pre- and postnatal effect of an allergic response on neurodevelopment and behaviour related to ASD and ADHD. Untangling the mechanisms underlying the effects of hyperallergenic foods and food allergic reactions on neurodevelopmental impairments could lead to new targets for the treatment of children with ASD or ADHD.

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CHAPTER SEVEN

Autistic-like behavioural and neurochemical changes in a mouse model of food allergy

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ABSTRACT

Food allergy has been suggested to contribute to the expression of psychological and psychiatric traits, including disturbed social behaviour and repetitive behaviour inherent in autism spectrum disorder (ASD). Most research in this field receives little attention, since fundamental evidence showing direct effects of food allergic immune responses on social behaviour is very limited. In the present study, we show that a food allergic reaction to cow's milk protein, induced shortly after weaning, reduced social behaviour and increased repetitive behaviour in mice. This food allergic reaction increased levels of serotonin (5-hydroxytryptamine; 5-HT) and the number of 5-HT positive cells, and decreased levels of 5-hydroxyindoleacetic acid (5-HIAA) in the intestine. Behavioural changes in food allergic mice were accompanied by reduced dopaminergic activity in the prefrontal cortex. Furthermore, neuronal activation (c-Fos expression) was increased in the prefrontal cortex and reduced in the paraventricular nucleus of the hypothalamus after exposure to a social target. We hypothesize that an intestinal allergic response regulates complex, but critical, neuroimmune interactions, thereby affecting brain circuits involved in social interaction, repetitive behaviour and cognition. Together with a genetic predisposition and multiple environmental factors, these effects of allergic immune activation may exacerbate behavioural abnormalities in patients with ASD.

INTRODUCTION

The intestinal tract continuously encounters foreign antigens and is therefore the most complex organ of the immune system. The majority of these antigens are harmless food antigens to which the body has formed a tolerogenic reaction. Genetic predisposition and environmental factors, however, can abrogate tolerance towards food allergens, leading to a Th2-directed immune response characterized by production of allergen-specific immunoglobulins during sensitization, and mast cell degranulation upon a second exposure to the allergen (1).

The intestinal tract is not only distinguished by its crucial immune function, but also exerts an important neurological function and is called 'the second brain' because of its abundant amount of enteric nerves. Evidence is emerging that intestinal immune disturbances can signal to the brain via various pathways, affecting behaviour and emotion (2). Food allergy has been suggested to be one of the intestinal triggers that contribute to the expression of various psychological and psychiatric traits, including anxiety, depression, migraine, schizophrenia, attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) (3-6). Supporting the hypothesis that food allergy can affect mental disorders of psychosocial relevance, Meldrum *et al.* observed social neurodevelopmental abnormalities in food allergic children at 18 months of age (7). Diagnosis of food allergy was associated with enhanced internalizing behaviour and a trend towards low social emotional scores. Intestinal problems are often reported in children with ASD (8, 9) and milk intake was found to be a predictor of constipation (10). Furthermore, a (gluten and) milk protein free diet was suggested to improve autistic behaviours (11-13) and to restore the increased intestinal permeability observed in these children (14). Preclinical studies on neurological effects of food allergy are limited. Mice immunized to ovalbumin (OVA) displayed increased anxiety 1 h after oral challenge with OVA (15). Moreover, c-Fos staining in the paraventricular nucleus (PVN) of the hypothalamus and central nucleus of the amygdala was observed in these mice 90 min after OVA challenge, accompanied by increased serum levels of corticosterone.

Also other allergic diseases have been associated with neuropsychological sequelae (7, 16, 17). Symptoms of developmental and behavioural dysfunction were more frequent in children with asthma compared to control children and asthma severity was shown to correlate with greater behavioural difficulties (16, 18). In addition, ADHD was positively associated with eczema and asthma (19) and a preliminary report indicated that ASD was more prevalent among children with mastocytosis (20), suggesting a role for mast cell activation in triggering neurological manifestations. Preclinical studies showed that OVA-immunized mice challenged via the airways displayed comparable brain activation as mice challenged via the oral route (15). Furthermore, allergic rhinitis increased anxiety and reduced social interaction in rats and mice, one day after allergen challenge (21).

Despite these clinical and preclinical indications, there is still much debate on the existence of food allergy-enhanced psychosocial disabilities and the question whether food allergy in mice affects social and repetitive behaviour has never been explored. Therefore, this study investigated the effects of a food allergic immune response on social interactions, repetitive behaviour and spontaneous alternation in mice and examined associated region-specific neuronal activation and monoamine levels.

MATERIALS AND METHODS

Cow's milk allergy mouse model

Three-week-old, specific pathogen free, male C3H/HeOuJ mice were purchased at Charles River Laboratories (L'Arbresle Cedex, France) and housed at the animal facility of the Utrecht University on a 12 h light/dark cycle with access to food and water *ad libitum*. Mice were bred and raised on a cow's milk protein free diet (Special Diet Services, Witham, UK). All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2009.I.12.112, DEC2011.I.08.082). After one week habituation, mice were sensitized intragastrically (i.g.) with 20 mg whey/0.5 mL PBS containing 10 µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized control mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks as previously described by Schouten *et al.* (22) (**Fig 1**). One week after the last sensitization, mice were challenged i.g. with 50 mg whey/0.5 mL PBS. The day after challenge, mice were exposed to a social behaviour test. To further exploit behaviour and avoid multiple behavioural testing on one day, self-grooming and T-maze alternation was assessed one and two days after the last sensitization, respectively. An intestinal allergic response to the fifth sensitization is confirmed by elevated serum levels of mMCP-1 in whey-sensitized mice (data not shown).

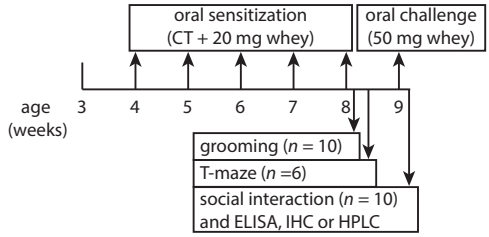

Fig 1. A schematic overview of the sensitization and challenge protocol and the behavioural tests conducted. Mice were exposed to one of the behavioural tests; self-grooming (one day after last sensitization), spontaneous alternation in the T-maze (two days after last sensitization) or social interaction (one day after challenge). Serum, brain and intestinal tissues of mice that were exposed to the social interaction test were used for further analysis by ELISA, IHC and HPLC.

Social interaction test

The behavioural assessment used was adapted from a previous description (23, 24). The morning after oral challenge, mice were exposed to a social interaction test (n = 10 per group). Mice were placed in a 45 x 45 cm open field, with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (**Fig. 3a**). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands), an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved was measured.

Self-grooming

The morning after the last sensitization, mice (n = 10 per group) were scored for spontaneous grooming behaviours as described earlier (25, 26). Each mouse was placed individually in an empty home cage (35 cm x 20 cm) without bedding and video recordings were used for behavioural scorings of frequency and cumulative time spent grooming all body regions. Open field was cleaned with water followed by 70 % ethanol after each test. After a 5 min habituation period in the cage, each mouse was scored blindly for 5 min by two independent researchers. Inter-rater reliability was 97.8 %.

T-maze spontaneous alternation

Two days after the last sensitization, spontaneous alternation was tested in a T-maze set-up. The T-maze comprised one start arm (49 cm long x 10 cm wide x 19 cm high) and two lateral arms (32 cm long x 10 cm wide x 18 cm high). A trial consisted of 2 runs, with a time interval of 2 min. After the mouse (n = 6 per group) had been released from the start arm, the animal was free to choose between both goal arms. As soon as the animal had entered one goal arm, the other arm was closed and the animal was confined for 30 s in the goal arm by lowering the door. The animal was then returned to his home cage. After thoroughly cleaning the T-maze with 70 % ethanol, the mouse was put back to the start arm and was free to choose one of the goal arms. Total of 5 trials was performed. The alternation ratio was defined as the amount of trials in which an animal alternated divided by the total amount of trials.

Measurements of serum mMCP-1 and whey-specific immunoglobulins

Blood was collected 16 h after oral challenge and centrifuged for 15 min at 14,000 rpm (*n* = 10 per group). Serum was stored at -70 °C until analysis. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 µg/mL whey in carbonate/ bicarbonate buffer (0.05 M, pH = 9.6; Sigma-Aldrich, Zwijndrecht, The Netherlands) overnight at 4 °C. Plates were blocked for 1 h in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05 % Tween-20 and 0.5 % BSA in PBS). Serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 µg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h. Plates were subsequently incubated with streptavidin-horse radish peroxidase (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h and developed using *o*-phenylendiamine (Sigma-Aldrich). Reaction was stopped after 10 min with 4 M H_2SO_4 and absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU), composed using a titration curve of pooled sera from whey-alum i.p. immunized mice serving as an internal standard.

Immunohistochemistry for c-Fos

Since maximum c-Fos expression occurs between 1 and 3 h following exposure to a stimulus (27), mice were sacrificed 1.5 h after the social interaction test. Mice (*n* = 5 per group) were deeply anaesthetized with pentobarbital and perfused transcardially with PBS, followed by buffered 4 % paraformaldehyde (pH = 7.2). Brains were removed and post-fixed in the same fixative overnight, following cryoprotection with 30 % sucrose (Sigma-Aldrich) in PBS. Coronal slices of 40 µm were sectioned using a cryostat (CM3050, Leica Microsystems, Rijswijk, The Netherlands), collecting 5 parallel series in PBS. Sections were incubated with 0.3 % H_2O_2 in PBS for 30 min and blocked using PBS-BT (0.1 % BSA and 0.3 % Triton-X-100 in PBS) for 30 min. Sections were incubated overnight with rabbit anti-c-Fos (Santa Cruz Biotechnology, Heidelberg, Germany) 1:5,000 in PBS-BT. Next day, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, UK) 1:1500 in PBS-BT for 90 min and subsequently incubated for 90 min with ABC-HRP complex (Vector Laboratories, Peterborough, UK) in PBS-BT. Reaction was visualized by incubation for 10 min in a chromogen solution consisting of 0.02 % DAB (Sigma-Aldrich) and 0.03 % ammonium-nickel-sulphate (Sigma-Aldrich) dissolved in 50 mM Tris buffer (pH 7.6) and subsequently 10 min in chromogen solution with H_2O_2. Sections were washed, mounted on adhesive microscope slides, dehydrated and coverslipped.

Initial screening of c-Fos patterns resulted in selection of 3 brain areas where potential changes were detected; prefrontal cortex (PFC), amygdala and hypothalamic paraventricular nucleus (PVN). Digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera, at a magnification of 10 or 20 times for PFC and PVN respectively. Image J software allowed us to mark c-Fos immunoreactive nuclei. Numbers of c-Fos positive neurons were counted bilaterally from a single section and the sum was used as the outcome for a single animal.

HPLC for analysis of L-tryptophan, monoamines and metabolites in brain and intestine

After decapitation, brains (*n* = 8 per group) were rapidly removed, frozen in isopentane (Sigma-Aldrich) and brain and intestinal tissue were stored at -70 °C. Bilateral regions of PFC, amygdala and PVN were dissected out with 500 µm coronal sections using a cryostat (Model 700, Laméris Instruments, Utrecht, The Netherlands). L-tryptophan (TRP), dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT) and their metabolites (3,4-dihydroxyphenylacetic acid; DOPAC, 3-methoxytyramine; 3-MT, and homovanillic acid; HVA and 5-hydroxyindoleacetic acid; 5-HIAA, respectively) were measured simultaneously in brain and intestinal tissue by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Lelystad, The Netherlands) as previously described (28). Tissue was sonicated in 50-100 µl ice-cold solution containing 5 µM clorgyline, 5 µg/mL glutathione and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL homogenate, 12.5 µL 2 M $HClO_4$ was added and subsequently, 10 µL 2.5 M potassium acetate. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000 x g (4 °C). The HPLC system consisted of a pump model P100, an autosampler model AS300 (both from Thermo Separation Products, Waltham, MA, USA), an ERC-3113 degasser (Erma CR. Inc., Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +450 mV (ESA Inc., Bedford, MA, USA), a data acquisition system (Atlas 2003, Thermo Electron Corporation, Brussels, Belgium) and a column (150 mm x 4.6 mm i.d.) packed with Hypersil BDS C18, 5 µm particle size (Alltech Associates, Deerfield, IL, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM. Turnovers of DA and 5-HT were calculated by dividing metabolite concentrations by monoamine concentrations ((DOPAC+3-MT+HVA)/DA and 5-HIAA/5-HT, respectively).

Immunohistochemistry for 5-HT

Ileal and colonic tissue was removed, opened longitudinally, rolled and fixated with 10 % formalin for at least 24 h. Formalin-fixed, paraffin-embedded tissue sections (5 µm) were incubated with 0.3 % H_2O_2 in methanol for 30 min, rehydrated and incubated for 5 min with proteinase K solution (Dako, Enschede, The Netherlands). Non-specific background was blocked with 5 % goat serum and sections were incubated overnight at 4 °C with rabbit anti-5-HT (Sigma-Aldrich) 1:8,000. Next day, sections were incubated with biotinylated goat anti–rabbit (Dako) 1:200, followed by ABC-HRP complex (Vector Laboratories). Staining was visualized using 0.05 % DAB solution for 10 min and sections were counterstained with Mayer's haematoxylin (Merck Millipore, Amsterdam, The Netherlands). Digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera, at a magnification of 20 times. 5-HT positive cells in the epithelial layer of the intestinal mucosa were counted in 15 consecutive villi or crypts, for ileum and colon respectively, at three different places (proximal, middle and distal) in the intestinal swiss roll. Data were expressed as the number of 5-HT positive cells per 10 villi or crypts for ileum and colon, respectively.

Statistical analysis

Differences between groups were statistically determined with an unpaired two-tailed Student's *t*-test and experimental results are expressed as mean ± S.E.M. As data on the humoral response and latency until first occurrence in the interaction zone were not normally distributed, differences between groups were statistically determined using a Mann-Whitney test and presented in a Box-and-Whisker Tukey plot. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 5.03.

RESULTS

Allergic response to oral whey challenge in whey-sensitized mice

The allergic response was assessed by measuring mMCP-1 levels as a marker for mucosal mast cell degranulation in serum collected 16 h after challenge. Serum mMCP-1 concentrations were increased in allergic whey-sensitized mice compared to control sham-sensitized mice ($P < 0.001$, **Fig. 2A**). Furthermore, levels of whey-specific IgE ($P < 0.01$, **Fig. 2B**), IgG1 ($P < 0.001$, **Fig. 2C**) and IgG2a ($P < 0.001$, **Fig. 2D**) were increased in serum of allergic mice compared to control mice.

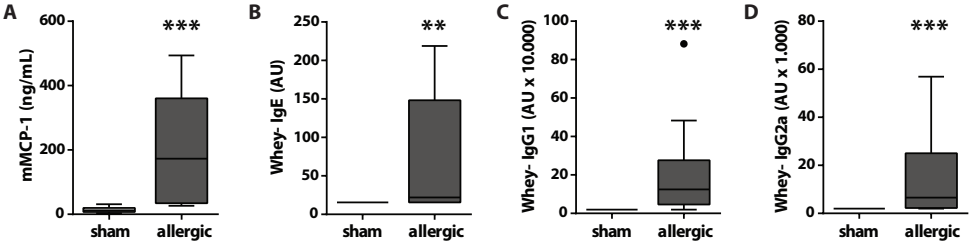

Fig 2. Humoral response to oral whey challenge in whey-sensitized allergic mice and sham-sensitized control mice. Serum levels of (**A**) mouse mast cell protease-1 (mMCP-1) and arbitrary units (AU) of whey-specific immunoglobulins (**B**) IgE, (**C**) IgG1 and (**D**) IgG2a were significantly increased in whey-sensitized allergic mice compared to sham-sensitized control mice. Mann-Whitney test was conducted and data are presented as box-and-whisker Tukey plots. ** $P < 0.01$, *** $P < 0.001$, $n = 10$ per group.

Food allergic mice display reduced social interaction and increased repetitive behaviour

To assess the effect of the allergic response on social behaviour, mice were exposed to a social interaction test, 14 h after challenge with the food allergen (**Fig. 3A**). Whey-sensitized allergic mice spent as much time in the interaction zone in absence of a social target, compared to sham-sensitized control mice ($P = 0.83$, **Fig. 3B**). However, in presence of a social target, allergic mice spent significantly less time in the interaction zone, compared to control mice ($P < 0.05$). Furthermore, latency of first approach to the social target was significantly increased in allergic mice compared to control mice ($P < 0.05$, **Fig. 3C**), while latency was not affected in absence of a social target ($P = 0.92$). Locomotor activity was not altered in allergic mice compared to control mice (no target: $P = 0.65$, target: $P = 0.98$, **Fig. 3D**). Moreover, reduced social interaction was not observed in a dextran sodium sulphate (DSS)-induced mouse model for colitis (**Supplementary data**), suggesting that reduced social interaction is not a general result of intestinal inflammation. DSS treatment, however, did reduce locomotor activity, compared to controls.

To further investigate behaviour of whey-sensitized allergic mice, self-grooming was scored as a measure of repetitive behaviour. Food allergic mice placed individually in a novel empty cage spent more time self-grooming than controls. Both cumulative time ($P < 0.01$, **Fig. 3E**) and frequency ($P < 0.01$, **Fig. 3F**) of grooming was significantly increased in allergic mice compared to controls.

Spontaneous alternation was tested in a T-maze set-up, as a measure for spatial memory (29) and/or to strengthen repetitive behaviour (30, 31) in food allergic mice. In contrast to control mice that tend to choose the other arm after a successive introduction in the T-maze, food allergic mice alternated to a significantly lower rate ($P < 0.01$, **Fig. 3G**).

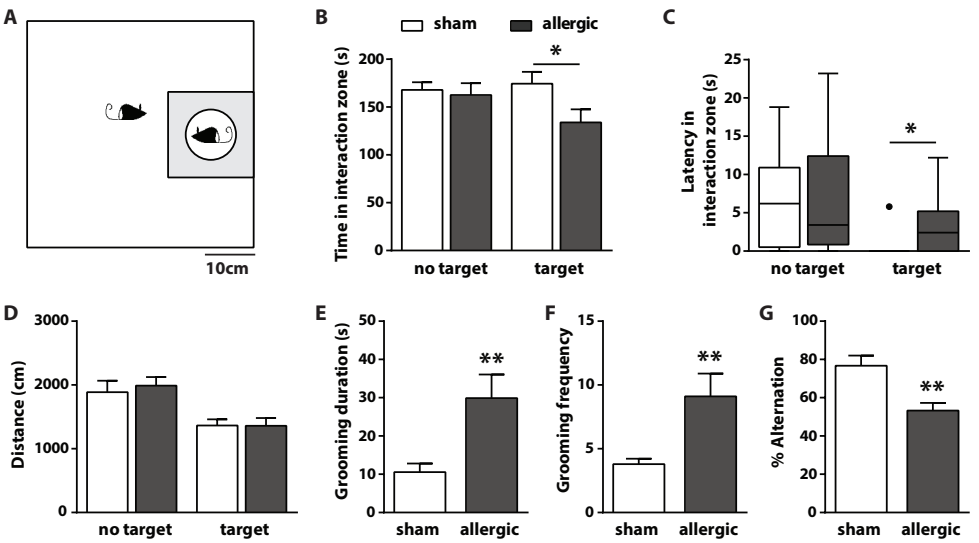

Fig 3. Behaviour of food allergic mice compared to control mice. (**A**) Schematic representation of the social interaction test, illustrating location and size of the interaction zone (grey rectangle) and the cage (white circle) in which an unfamiliar mouse (target) was placed. (**B**) Compared to sham-sensitized control mice, whey-sensitized allergic mice spent significantly less time in the interaction zone and (**C**) it took longer for them to approach the target mouse for the first time. One allergic animal was excluded from C as a significant outlier (181 s, Grubbs' test). (**D**) Total distance moved was similar in both groups. (**E**) Cumulative time spent grooming and (**F**) frequency of grooming, during a 5 min session in an empty home cage without bedding, were significantly increased in food allergic mice compared to controls. (**G**) Spontaneous alternation ratio in the T-maze, defined by the amount of trials in which an animal alternated divided by the total amount of trials, was significantly decreased in food allergic mice compared to controls. B and D-G: Student's *t*-test was conducted and data are presented as mean ± S.E.M. C: Mann-Whitney test was conducted and data are presented as Box-and-Whisker Tukey plots. * $P < 0.05$, ** $P < 0.01$, B-F: $n = 10$ per group, G: $n = 6$ per group.

Neuronal activation is increased in the PFC and reduced in the PVN of allergic mice

Next, c-Fos expression was measured in the brain as a marker of neuronal activation, 2 h after social interaction. Compared to control mice, robust c-Fos induction was observed in the lateral and ventral orbital PFC (oPFC) of food allergic mice after exposure to a social target ($P < 0.05$, **Fig. 4A**,**C**). This induction was restricted to these orbital areas, since no change in neuronal activation was observed in the medial orbital (MO), prelimbic (PrL) and cingulate (Cg1) areas of the PFC. Furthermore, allergic mice showed a blunted c-Fos response in the PVN compared to control mice ($P < 0.05$ **Fig. 4B**,**D**). No differences in neuronal activation were observed in nuclei of the amygdala (data not shown).

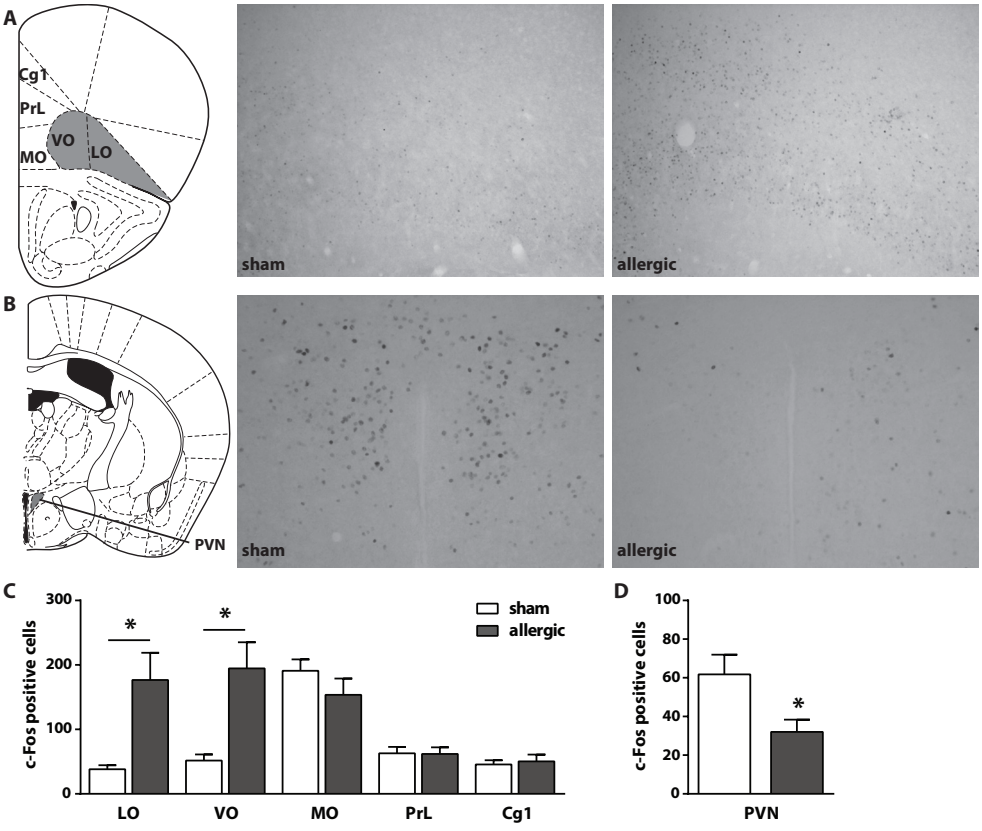

Fig 4. Neuronal activation in the prefrontal cortex (PFC) and hypothalamic paraventricular nucleus (PVN) of food allergic mice, 2 h after the social interaction test. (**A** and **C**) Expression of c-Fos was significantly increased in whey-sensitized allergic mice compared to sham-sensitized control mice in the lateral (LO) and ventral (VO) orbital PFC, but not in the medial orbital (MO), prelimbic (PrL) and cingulate area 1 (Cg1) regions of the PFC. (**B** and **D**). Decreased c-Fos expression was observed in the PVN of allergic mice, compared to control mice. Student's *t*-test was conducted and data are presented as mean ± S.E.M. * $P < 0.05$, $n = 5$ per group.

Dopaminergic activity is decreased in the prefrontal cortex and increased in the amygdala of allergic mice

To determine the effect of the allergic immune response on the dopaminergic and serotonergic systems in the brain, monoamine and metabolite levels were measured in tissue homogenates of PFC, PVN and amygdala, 16 h after oral challenge. A significant decrease in levels of DA ($P < 0.05$, **Fig. 5A**) and its metabolites DOPAC, 3-MT and HVA ($P < 0.01$) were observed in the PFC of allergic mice compared to control mice. Although levels of 5-HT were not changed in the PFC ($P = 0.78$, **Fig. 5B**) the concentration of 5-HIAA was significantly decreased in allergic mice compared to controls ($P < 0.05$). However, the proportion of change was marginal compared to the DA changes. Food allergy did not induce alterations in the dopaminergic (**Fig. 5C**) or the serotonergic (**Fig. 5D**) systems in the PVN. In the amygdala, a tendency towards an increase in DA levels ($P = 0.06$, **Fig. 5E**) and a significant increase in metabolite levels of DOPAC ($P < 0.05$), but not 3-MT ($P = 0.36$) and HVA ($P = 0.23$), were observed in allergic mice compared to control mice. Levels of 5-HT ($P < 0.05$, **Fig. 5F**), but not 5-HIAA ($P = 0.35$), were increased in the amygdala of allergic mice compared to controls. Levels of tryptophan and turnover rates of 5-HT and DA in all three brain areas remained unaffected by a food allergic response in the intestine (data not shown).

Increased levels of 5-HT and number of 5-HT positive cells in the intestinal tract of food allergic mice

Considering the importance of the 5-HT system in intestinal neuroimmune interactions, levels of 5-HT and 5-HIAA were also measured in ileum and colon, 16 h after oral challenge. In the ileum of allergic mice, a significant increase in 5-HT levels was observed ($P < 0.05$, **Fig. 6A**), accompanied by decreased 5-HIAA levels ($P < 0.01$, **Fig. 6B**), leading to a reduced 5-HT turnover ($P < 0.01$, **Fig. 6C**). However, no significant changes were observed in 5-HT and 5-HIAA levels in colon of food allergic mice. Immunohistochemistry of 5-HT revealed that an increased number of 5-HT positive cells was present in the epithelial layer of both ileum ($P < 0.05$ **Fig. 6D** and **E**) and colon ($P < 0.05$, **Fig. 6D** and **F**) of allergic mice, compared to control mice. In contrast, number of 5-HT positive cells in the lamina propria remained unaffected (data not shown), suggesting that there is an increase in EC cells, but not in other 5-HT positive cells.

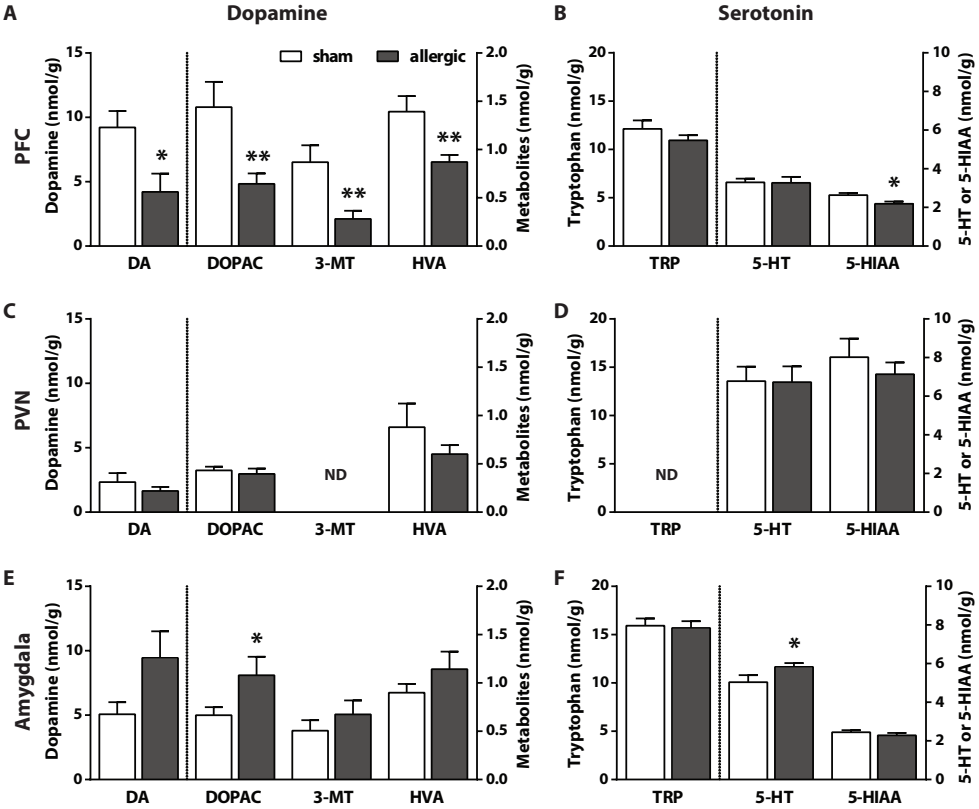

Fig 5. Monoamine and metabolite levels in prefrontal cortex (PFC), hypothalamic paraventricular nucleus (PVN) and amygdala of food allergic mice. Concentrations of dopamine (DA), serotonin (5-HT; 5-hydroxytryptamine), their metabolites (3,4-dihydroxyphenylacetic acid; DOPAC, 3-methoxytyramine; 3-MT, homovanillic acid; HVA, and 5-hydroxyindoleacetic acid; 5-HIAA, respectively) and tryptophan (TRP) in brain tissue homogenates of the (**A** and **B**) PFC, (**C** and **D**) PVN, and (**E** and **F**) amygdala of whey-sensitized allergic mice compared to sham-sensitized control mice. Student's *t*-test was conducted and data are presented as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, ND = not detectable, $n = 8$ per group.

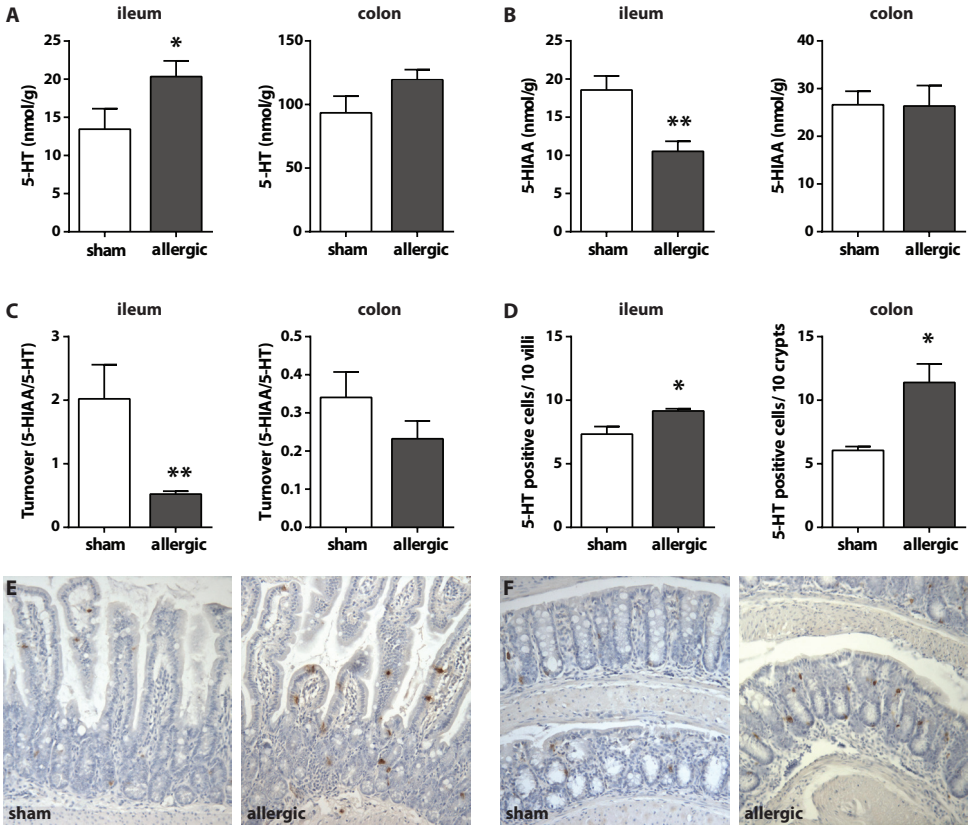

Fig 6. Levels of serotonin (5-HT; 5-hydroxytryptamine) and 5-HIAA (5-hydroxyindoleacetic acid) and number of 5-HT positive cells in ileum and colon of food allergic mice. In ileum of whey-sensitized allergic mice, (**A**) significantly increased concentrations of 5-HT and (**B**) decreased concentrations of 5-HIAA were observed, (**C**) resulting in reduced 5-HT turnover rate, compared to sham-sensitized control mice. No significant differences were found in colon of allergic compared to control mice. The number of 5-HT positive cells in the epithelial layer was increased both (**E**) in ileum and (**F**) in colon of allergic mice compared to control mice. (**D**) Quantification was presented as number of 5-HT positive cells per 10 villi or crypts for ileum or colon, respectively. Student's *t*-test was conducted and data are presented as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, A - C: $n = 8$ per group, D - F: $n = 4$ per group.

DISCUSSION

There is little fundamental evidence showing direct effects of food allergic immune responses on social and repetitive behaviour. In the present study, we showed that an IgE-mediated allergic immune response in the intestinal tract of mice, induced shortly after weaning, is associated with disturbed social interaction, without any overt signs of sickness. This effect was specific for food allergy, as social behaviour was not changed in DSS-induced colitis mice. Notably, the social interaction data may be confounded by the observation that DSS-treated mice had reduced locomotor activity, meaning that DSS treatment may actually stimulate social interaction. In addition to social behavioural deficits, food allergic mice showed increased self-grooming, indicative for increased repetitive behaviour, and reduced alternation in the T-maze, which is not exclusively a test for spatial memory, but also involves the willingness to explore novel environmental stimuli (32, 33) and repetitive behaviour (31, 34).

A food allergic reaction in the intestine increased levels of 5-HT in the ileum of food allergic mice compared to controls. This increase in 5-HT levels may be explained by an increase in the number of enterochromaffin (EC) cells that was observed in food allergic mice, as the vast majority of intestinal 5-HT is stored in EC cells (35). In colon of food allergic mice, the number of EC cells was doubled when compared to controls, while 5-HT was not significantly increased, suggesting that EC cells produce less 5-HT per cell. Mast cells are also important sources of 5-HT, in particular during an allergic reaction. Serum levels of mMCP-1 were significantly increased in food allergic mice and previous studies showed that this was accompanied by decreased mMCP-1 positive mast cells in the intestines (36), indicating that intestinal mast cells are degranulated after oral challenge. Although no increase in the number of lamina propria 5-HT positive cells in ileum and colon was observed in this model, increased numbers of intestinal mast cells have been described in food allergic mice (37). Therefore, additional to the increased number of EC cells, mast cells may also contribute to the increased 5-HT levels observed in the intestines of allergic mice. Increased 5-HT levels were accompanied by decreased levels of 5-HIAA in ileum of food allergic mice. Intestinal 5-HT is taken up by the serotonin transporter (SERT) on epithelial cells, where most intestinal 5-HT is degraded into 5-HIAA (35). Therefore, reduced levels of 5-HIAA may be explained by decreased SERT activity, or reduced availability of extracellular 5-HT. A decrease in SERT activity would predict lower 5-HT uptake and metabolism in the mucosa, decreasing intestinal 5-HIAA/5-HT ratio and diverting 5-HT to metabolism in the liver or lung or uptake by platelets. A pro-inflammatory environment in the intestine is thought to decrease SERT activity, as activity was reduced in intestinal epithelial cells and immune cells *in vitro* upon exposure to lipopolysaccharides and cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-4 (IL-4) (38-40). EC cell hyperplasia and increased levels of intestinal 5-HT accompanied

by depressed levels of 5-HIAA, and thus a reduced turnover rate, were also observed in intestinal biopsies of patients with coeliac disease (41) and IBS (42). Interestingly, this disturbance in intestinal 5-HT metabolism in both diseases is associated with increased platelet 5-HT, a repeated finding in approximately 30 % of patients with ASD (43).

Signalling molecules produced by the intestinal mucosal and immune cells, including cytokines, neuropeptides and neurotransmitters, can activate their respective receptors on extrinsic vagal and spinal afferents to signal to the brain stem and spinal cord, respectively (44). 5-HT produced by EC cells and mast cells is an example of such a signalling molecule. Extrinsic vagal afferents are in sufficient proximity to the epithelial layer where EC cells are located (45) and to mast cells (46). Vagal afferents respond to secreted 5-HT via 5-HT_3 receptors by signalling to the nucleus tractus solitarius (NTS) and PVN (47). Food allergy-induced c-Fos expression in rat brains was reduced after blockade of 5-HT_3 receptors and vagotomy (48). This suggests that 5-HT released during a food allergic response acts through 5-HT_3 receptors on vagal afferents to affect neuronal activation in the brain. However, administration of a 5-HT_3 receptor antagonist partially reversed food allergy-induced aversion to food (49), indicating that signalling to the brain is not solely mediated by release of 5-HT. Moreover, DSS-induced colitis mice did not show impaired social behaviour, while increased 5-HT release has been reported in these mice (50). Therefore, we hypothesize that immune signalling to relevant brain regions involved in the regulation of social behaviour is mediated by a combination of various allergy-induced signalling molecules, including 5-HT. Indeed, for example mast cell release of histamines and prostaglandins were also shown to stimulate enteric neurons (51). Furthermore, IgE can bind to the functional high-affinity FcεRI on neurons (52), resulting in an antigen-specific neuronal stimulation (53). Unravelling which allergy-induced signalling molecules are responsible for impaired social behaviour would require further investigation of immune-nerve interactions in the intestines of food allergic mice.

In contrast to the profound effects of food allergy on the 5-HT system in the intestinal tract, changes in central 5-HT systems were less pronounced than changes observed in the DA system. Of note is the practical limitation of measuring monoamines in tissue homogenates, omitting information about monoamine levels in the neuronal synaptic cleft. Microdialysis studies in this murine model could provide more insight into synaptic transmission of 5-HT in the brain. Nevertheless, pronounced changes in the mesocorticolimbic DA system were observed. Results suggest that DA and metabolite levels were decreased in the PFC and increased in amygdala of allergic mice compared to controls. While multiple neural systems undoubtedly underlie social-emotional behaviours, the mesocorticolimbic DA system seems a crucial pathway involved (54). This mesocorticolimbic DA system originates in the ventral tegmental area (VTA) of the midbrain and projects to various forebrain regions including PFC, amygdala and nucleus accumbens. Socially rewarding stimuli activate the mesocorticolimbic DA system,

resulting in feelings of desire, wanting and excitement (55, 56). Evidence for involvement of the mesocorticolimbic DA system in the pathophysiology of ASD is limited, but it is reported that children with ASD display reduced levels of DA in the medial PFC, as assessed with PET (57). Furthermore, dampening of the DA system in the PFC was also observed in a fragile X mouse model for ASD (58). Multiple studies revealed that social interaction in rodents is accompanied by increased DA levels in the PFC (59, 60) and depletion of DA in the PFC markedly reduces social interactions (61). Moreover, abnormal vagal functioning inhibits the DA system, but not 5-HT, in various brain regions, including PFC (62), indicating that vagal input can affect the mesocorticolimbic DA system. Overall, this suggests that the observed dampening of the DA system in the PFC of food allergic mice may underlie their reduced social behaviour and that this attenuation of the DA system in the PFC could be mediated by intestinal signalling through the vagus nerve.

Marked neuronal activation was observed in the LO and VO regions of the PFC of food allergic mice, after exposure to a social target. The oPFC is involved in cognitive processing of decision making in response to emotional stimuli that can have a rewarding or punishing value. Therefore, it is also important in guiding social-emotional behaviour (63, 64). In line with our observations, patients with ASD showed increased oPFC activation in response to tasks involving facial recognition (65), motor function (66) and attention (67). Furthermore, patients with orbitofrontal lesions have impaired abilities to recognize and interpret emotional expressions and to respond in a socially proper manner (68-71). Since the oPFC guides social-emotional behaviour, enhanced neuronal activation in the oPFC of food allergic mice, as observed in the present study, may be implicated in their reduced social behaviour. However, based on the c-Fos staining we cannot speculate about the subtype of these neurons that are activated in the oPFC of allergic mice, as many different subtypes exist in the oPFC, and whether these are excitatory or inhibitory neurons.

Basso *et al.* (72) reported increased neuronal activation of the PVN after sensitization and challenge with ovalbumin allergen. However, c-Fos expression was measured 90 min after oral challenge, whereas in our study, c-Fos expression was determined after a social interaction test. Exposure to a social target elicits a specific neuronal activation pattern, making it impossible to compare these studies. In our study, decreased neuronal activation was observed in the hypothalamic PVN of food allergic mice after exposure to a social target. PVN neurons release oxytocin, a hormone that has numerous effects in the body, but also acts as neuromodulation in the brain. Oxytocin is released upon positive social interactions and enables individuals to overcome their natural avoidance of social approach (73). Deficits in oxytocin may partly underlie social impairments found in patients with ASD, since decreased levels of peripheral oxytocin in ASD patients have been observed (74) and oxytocin administration improved social cognition in ASD patients (34). Furthermore, oxytocin injection into the VTA of rats caused increased DA

and DOPAC levels in medial PFC, while levels of 5-HT and 5-HIAA remained unaffected (75, 76). Therefore, oxytocin production by the PVN can also regulate dopaminergic neurotransmission in the PFC (77), which could be of importance in our study, but requires further investigation.

In conclusion, this study provides evidence that a food allergic reaction reduces social behaviour, increased repetitive behaviour and impaired spontaneous alternation in mice, accompanied by neuronal and dopaminergic changes in the brain and serotonergic changes in the intestine. We hypothesize that an allergic response regulates complex, but critical, intestinal neuroimmune interactions involving 5-HT and consequently affecting brain circuits involved in social behaviour. Together with a genetic predisposition and multiple environmental factors, these effects of allergic immune activation may exacerbate behavioural abnormalities in patients with ASD. Therefore, the intestinal tract could be a potential target in the treatment of patients with ASD and comorbid food allergic symptoms.

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SUPPLEMENTARY INFORMATION

DSS-induced colitis model

Colonic inflammation was induced in male C57BL/6 mice (8-12 weeks old, Charles River Laboratories) by administration 2 % (w/v) dextran sodium sulfate (DSS, MW = 36,000-50,000, MP Biomedicals, Aurora, OH, USA) in tap water *ad libitum* for 5 consecutive days followed by 2 days of normal drinking water. The DSS solution was refreshed every other day. Control mice received drinking water without DSS. The presence of blood in stool, stool consistency and body weight were recorded daily for each animal. The morning after two days of drinking water, mice were exposed to a social interaction test (DSS-induced colitis model, $n = 8$ per group).

Social interaction is not changed in DSS-induced colitis mice

To investigate whether deficits in social interaction are a general result of intestinal inflammation, social interaction was also measured in a murine model for DSS-induced colitis. At the day of the social interaction test, all DSS-treated mice, but no control mice, suffered from clinic-pathological signs of colitis such as blood in stool, diarrhea and loss of bodyweight, collectively resulting in a significant increase in disease activity score (median [IQR]: 0.0[0.0-0.0] and 3.0 [3.0-3.8] for control and DSS respectively, $P < 0.001$). In the social interaction test, DSS-induced colitis mice spent as much time in the interaction zone as control mice, regardless of the presence or absence of an unfamiliar social target (**Fig. S1A**). Also latency until first occurrence in the interaction zone (**Fig. S1B**) was not affected by DSS treatment. Locomotor activity of DSS mice, however, was significantly reduced both when a target mouse was absent ($P < 0.05$) and present ($P < 0.05$) compared to their healthy controls (**Fig. S1C**).

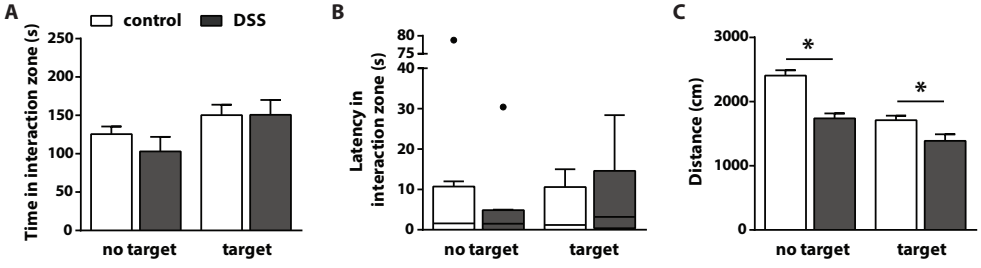

Fig. S1. Social behaviour of DSS-induced colitis mice compared to control mice. (**A**) DSS-induced colitis mice spent as much time in the interaction zone as control mice and (**B**) latency of first occurrence in the interaction zone was not significantly changed in DSS mice compared to control mice. (**C**) Locomotor activity, measured by total distance moved, was significantly reduced in DSS-induced colitis mice compared to controls. A, C: Student's *t*-test was conducted and data are presented as mean ± S.E.M. B: Mann-Whitney test was conducted and data are presented as box-and-whisker Tukey plots. * $P < 0.05$, $n = 8$ per group.

CHAPTER EIGHT

Dietary long chain n-3 polyunsaturated fatty acids prevent impaired social behaviour and normalize brain dopamine levels in food allergic mice

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ABSTRACT

Allergy is suggested to exacerbate impaired behaviour in children with neurodevelopmental disorders. We have previously shown that food allergy impaired social behaviour in mice. Dietary fatty acid composition may affect both the immune and nervous system. The aim of this study was to assess the effect of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) on food allergy-induced impaired social behaviour and associated deficits in prefrontal dopamine (DA) in mice. Mice were fed either control or n-3 LCPUFA-enriched diet before and during sensitization with whey. Social behaviour, acute allergic skin response and serum immunoglobulins were assessed. Monoamine levels were measured in brain and intestine and fatty acid content in brain. N-3 LCPUFA prevented impaired social behaviour of allergic mice. Moreover, n-3 LCPUFA supplementation increased docosahexaenoic acid (DHA) incorporation into the brain and restored reduced levels of prefrontal DA and its metabolites 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid in allergic mice. In addition to these brain effects, n-3 LCPUFA supplementation reduced the allergic skin response and restored decreased intestinal levels of serotonin metabolite 5-hydroxyindoleacetic acid in allergic mice. N-3 LCPUFA may have beneficial effects on food allergy-induced deficits in social behaviour, either indirectly by reducing the allergic response and restoring intestinal 5-HT signalling, or directly by DHA incorporation into neuronal membranes, affecting the DA system. Therefore, it is of interest to further investigate the relevance of food allergy-enhanced impairments in social behaviour in humans and the potential benefits of dietary n-3 LCPUFA supplementation.

INTRODUCTION

Food allergy affects about 6 % of young children, causing symptoms that include gastrointestinal and pulmonary distress and atopic dermatitis (1). Food allergy is provoked by abrogation of immune tolerance to harmless food antigens, resulting in Th2 polarization of the immune response. The enteric nervous system mediates intestinal immune responses, including food allergic reactions. Inflammatory signalling molecules such as cytokines, neuropeptides, and serotonin (5-hydroxytryptamine; 5-HT) can directly and indirectly activate afferent nerves, which signal to the brain to activate the HPA-axis or the cholinergic anti-inflammatory pathway (2, 3). Allergy-induced activation of the nervous system has recently been reviewed comprehensively (4). Expression of the IgE receptor on vagal afferents is enhanced in food allergic mice and partial removal of the vagus nerve suppressed Th2-mediated inflammation in the intestines (5). Vagal afferents mediate visceral nociception during food allergy via 5-HT receptor signalling (6). 5-HT in the intestines is produced by enterochromaffin cells, enteric neuronal cells and various immune cells, most predominantly mast cells. Both enterochromaffin cells (7) and mast cells (8) release 5-HT in close proximity to afferent nerves, causing receptor binding, neuronal depolarization and signalling to brain regions including the nucleus tractus solitarius (NTS) and hypothalamic paraventricular nucleus (PVN) (9).

Interestingly, these brain regions are important in emotional and social behaviour and increasing evidence shows that intestinal allergic reactions may affect behavioural responses (10). Indeed, sensitization to ovalbumin in mice resulted in neuronal activation of the PVN and central nucleus of the amygdala and increased anxiety was observed (11). Furthermore, food allergy increased neuronal activation in the NTS and PVN in rats, which was diminished after blockade of 5-HT_3 receptors and vagotomy (12), suggesting that food allergy-induced signalling to the brain is mediated by 5-HT binding to receptors on vagal afferents. In humans, food allergy has been suggested to be one of the intestinal triggers that contribute to the expression of various psychological and psychiatric traits, including anxiety, depression (13), migraine (14), schizophrenia (15), attention-deficit hyperactivity disorder (ADHD) (16) and autism spectrum disorder (ASD) (17-19). Recently, it was demonstrated that food allergy in the first year of life was associated with abnormal neurodevelopmental outcomes related to social behaviour (20). Moreover, intestinal problems are often reported in children with ASD (21, 22) and milk intake was found to be a predictor of constipation in these patients (23). A (gluten and) milk protein free diet is suggested to improve autistic behaviour (24-26) and to restore the increased intestinal permeability observed in children suffering from ASD (27).

Long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) may have a role in the prevention of allergic diseases (28). In addition to its potential immunomodulatory effects, n-3 LCPUFA have been suggested to modulate neuronal function as well. LCPUFA, predominantly docosahexaenoic acid (DHA, C22:6 n-3), arachidonic acid (AA, C20:4 n-6) and docosatetraenoic acid (DTA; 22:4 n-6), are important components of the neuronal cell membrane (29) and are essential throughout life for maintaining normal brain function (30, 31). Numerous observational studies have shown a link between peripheral n-3 and n-6 LCPUFA imbalances and neurodevelopmental disorders. For instance, ADHD (32), schizophrenia (33), and ASD (34) have been associated with a relative lack of n-3 LCPUFA in peripheral blood cells or plasma. Some clinical trials have been conducted on the beneficial effect of dietary n-3 LCPUFA supplementation on behaviour in various neurodevelopmental disorders, including ASD (35), but trials with larger sample size are required and are currently in progress (29).

Recently, we have shown that an allergic reaction to orally ingested cow's milk protein whey reduced social interaction in mice (36). Moreover, we showed that these behavioural abnormalities were associated with reduced dopaminergic activity in the prefrontal cortex (PFC). In this study, it was assessed whether dietary supplementation of fish oil, high in n-3 LCPUFA DHA, can prevent food allergy-induced abnormalities in social behaviour and in prefrontal dopamine (DA) and metabolite levels in mice. Moreover, we investigated the effect of n-3 LCPUFA supplementation on allergic sensitization and 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels in ileum of food allergic mice.

MATERIALS AND METHODS

Table 1. Diet composition of n-3 LCPUFA-enriched diet

A. Diet composition of chow, based on AIN-93G

	control diet	**n-3 LCPUFA enriched diet**
	(g/kg diet)	(g/kg diet)
Carbohydrates		
Cornstarch	367.5	367.5
Dextrinized cornstarch	132.0	132.0
Sucrose	100.0	100.0
Cellulose	50.0	50.0
Protein		
Soya	200.0	200.0
Methionine	3.0	3.0
Fat		
Soybean oil	100.0	40.0
Tuna oil	0	60.0
Others		
Mineral mix AIN-93G	35.0	35.0
Vitamine mix AIN-93VX	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

B. Fatty acid composition of lipid source

	Soybean oil (%)	**Tuna oil** (%)
Fatty acid		
Saturated	15.1	28.9
Monounsaturated	24.9	22.8
Polyunsaturated	59.1	44.5
n-6	53.1	5.5
C18:2 LA	53.1	1.3
C20:4 AA		1.8
C22:5		1.6
n-3	5.6	38.5
C18:3 ALA	5.6	0.5
C20:5 EPA		7.0
C22:5 DPA		1.4
C22:6 DHA		27.8
Minor components	0.9	3.8

LA: linoleic acid, AA: arachidonic acid, ALA: a-linolenic acid, EPA: eicosapentaenoic acid, DPA: docosapentaenoic acid, DHA: docosahexaenoic acid

Diets

Semi-purified cow's milk protein-free AIN-93G-based diets were composed of either 10 % soybean oil (control diet) or 4 % soybean oil combined with 6 % tuna oil (n-3 LCPUFA-enriched diet) and prepared at Research Diet Services (Wijk bij Duurstede, The Netherlands). The fat percentage of AIN-93G was enhanced from 7 % to 10 % at the expense of cornstarch (**Table 1A**), as described before (37). The ratio n-3: n-6 LCPUFA was 1:9.5 for the soybean oil control diet and 1:1 for the n-3 LCPUFA-enriched fish oil diet (**Table 1B**). Tuna oil was a kind gift from Bioriginal (Den Bommel, The Netherlands). Diets were stored at -20 °C prior to use to prevent fatty acid oxidation.

Animal experiments

Three-week-old, specific pathogen free, male C3H/HeOuJ mice, purchased from Charles River Laboratories (L'Arbresle Cedex, France) were housed at the animal facility of the Utrecht University on a 12 h light/dark cycle with access to food and water *ad libitum*. Mice were fed either the control or n-3 LCPUFA-enriched fish oil diet, starting two weeks prior to first sensitization and continued during the entire experiment. Mice were sensitized intragastrically (i.g.) with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS containing 10 µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks as previously described (38). One week after the last sensitization, sham and whey-sensitized mice were challenged i.g. with 50 mg whey/0.5 mL PBS and a social interaction test was conducted the next morning. In the first experiment, (n = 11 per group), the allergic skin response was measured 4 days after the social interaction test (**Fig. 1A**). Mice received a second oral whey challenge (50 mg whey/ 0.5 mL PBS) and were decapitated the next morning to measure levels of fatty acids in the brain and immunoglobulins in serum. In the second experiment, mice (n = 8 per group) were sacrificed after the social interaction test to measure monoamines in brain and intestine (**Fig. 1B**). All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2012.I.04.053).

Acute allergic skin response

Ear thickness (n = 11 per group) was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands) prior to and 1 h after intradermal (i.d.) whey challenge (10 µg/20 µL PBS) in the ear. Isoflurane was used for inhalational anaesthesia during measurements. Ear swelling was calculated by subtracting the mean basal thickness of duplicates per ear from the mean thickness measured after i.d. challenge, to express ear swelling as delta µm. Mean of left and right delta ear swelling were calculated for each mouse.

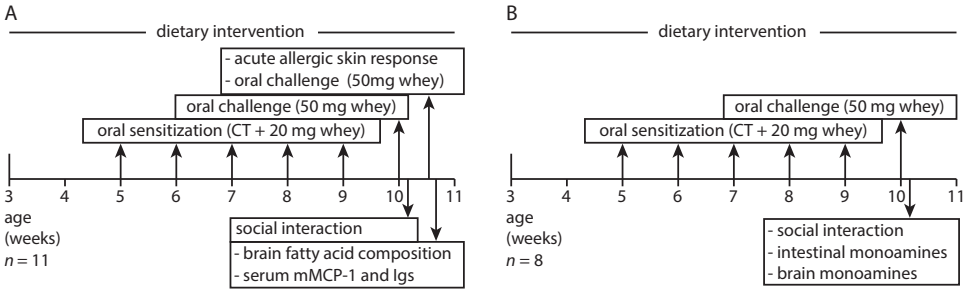

Fig. 1. Schematic overview of the two experiments. (**A**) In the first experiment, ($n = 11$ mice per group) the acute allergic skin response was measured four days after the social interaction test. Mice underwent a second oral challenge and the next morning, serum was collected for measuring levels of immunoglobulins (Igs) and mouse mast cell protease-1 (mMCP-1) and brain was collected to determine fatty acid composition. (**B**) In the second experiment, mice ($n = 8$ per group) were sacrificed after the social interaction test to measure monoamines.

Social interaction test

The behavioural assessment was adapted from a previous description (36, 39). The morning after oral challenge mice were exposed to a social interaction test ($n = 11$ per group). Mice were placed in a 45 x 45 cm open field with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (**Fig. 4A**). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands) an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved were measured. Data from the first experiment was presented, but comparable results were obtained from the second experiment (data not shown).

Measurements of whey-specific immunoglobulins and mouse mast cell protease-1

Blood ($n = 11$ per group) was collected 16 h after oral challenge, centrifuged for 15 min at 14,000 rpm and serum was stored at -70 °C. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 µg/mL whey in carbonate/ bicarbonate buffer (0.05 M, pH = 9.6; Sigma-Aldrich, Zwijndrecht, The Netherlands) overnight at 4 °C. Plates were blocked in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05 % Tween-20 and 0.5 % BSA in PBS) and serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 µg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h and subsequently with streptavidin-horse radish peroxidase (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h. Plates were developed using *o*-phenylendiamine (Sigma-Aldrich) and reaction was stopped after

15 min with 4 M H_2SO_4. Absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU), composed using a titration curve of pooled sera from whey-alum i.p. immunized mice serving as an internal standard. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (BD Biosciences) according to the manufacturer's protocol.

HPLC for analysis of monoamines and metabolites in brain and intestines

After decapitation, brains (n = 8 per group) were rapidly removed, frozen in isopentane (Sigma-Aldrich) and brain and 1 cm of distal ileum were stored at -70 °C until further analysis. PFC was isolated with 500 µm coronal sections using a cryostat (Model 700, Laméris Instruments, Utrecht, The Netherlands). Brain and intestinal tissues were sonicated in 50-200 µL ice-cold solution containing 5 µM clorgyline and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL tissue homogenate, 12.5 µL 2 M $HClO_4$ was added. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000g (4 °C). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM. Settings of HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Lelystad, The Netherlands) were described elsewhere (36).

Fatty acid composition brain

Brain was removed (n = 11 per group) and stored at -70 °C until analysis. Whole brains were weighted and homogenized in ice cold PBS (25 mg/mL). Brain lipids were extracted as described by Bligh and Dyer (40) and the membrane fatty acid composition was assessed using gas chromatography as previously described (41).

Statistical analysis

Experimental results are expressed as mean ± S.E.M, or Box-and-Whisker Tukey plot when data were not normally distributed. Differences between groups were statistically determined with a two-way ANOVA followed by a Bonferroni's multiple comparisons test. For serum immunoglobulin levels and intestinal 5-HT turnover, log transferred data were used to obtain normality for two-way ANOVA. Latency until first occurrence in the interaction was statistically analysed with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism, version 6.02.

RESULTS

N-3 LCPUFA supplementation reduces the acute allergic skin response in whey-sensitized food allergic mice

One hour after dermal challenge, ear thickness was measured to assess the effect of n-3 LCPUFA supplementation on the acute allergic skin response. The delta ear swelling in whey-sensitized allergic mice was increased compared to sham-sensitized control mice ($P < 0.0001$, **Fig. 2A**). Whey-sensitized mice fed the n-3 LCPUFA-enriched diet showed a decreased allergic skin response when compared to whey-sensitized allergic mice fed the control diet ($P < 0.001$). Mucosal mast cell degranulation in the intestine, determined by the concentration of mMCP-1 in the serum, was increased in allergic mice fed the control ($P < 0.05$, **Fig. 2B**) and the n-3 LCPUFA-enriched diet ($P < 0.01$). Furthermore, serum levels of whey-specific IgE (**Fig. 2C**), IgG1 (**Fig. 2D**, and IgG2a (**Fig. 2E**) were increased in allergic mice fed the control diet ($P < 0.0001$ for IgE and $P < 0.001$ for IgG1 and IgG2a). and the n-3 LCPUFA-enriched diet ($P < 0.01$ for IgE and IgG1 and $P < 0.05$ for IgG2a).

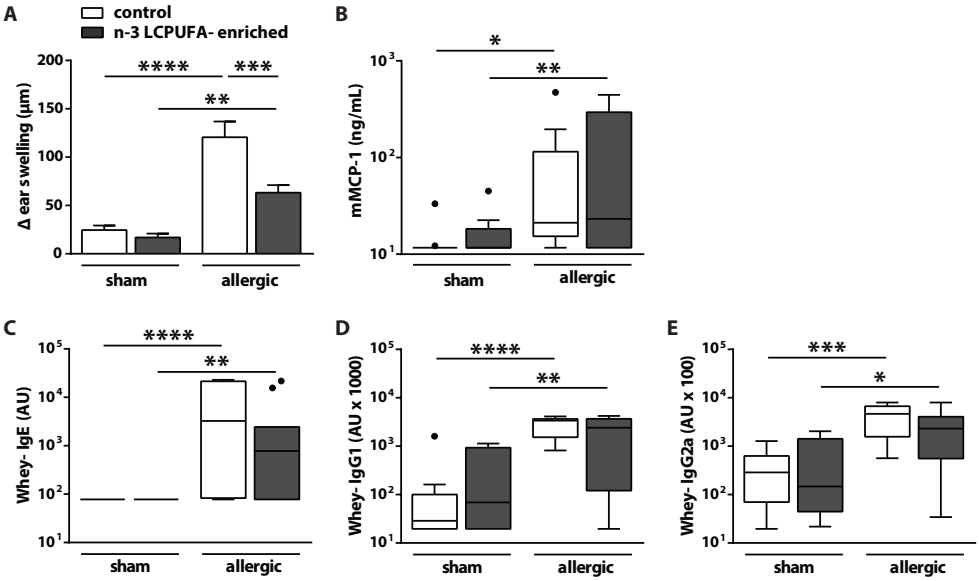

Fig. 2. The effect of n-3 LCPUFA supplementation on the allergic skin and humoral response to whey challenge. (**A**) The acute allergic skin response (delta ear swelling) after intradermal challenge (sensitization: $P < 0.0001$, diet: $P < 0.001$, interaction: $P < 0.05$). (**B**) Mouse mast cell protease-1 (mMCP-1) levels (sensitization: $P < 0.001$, diet: ns, interaction: ns). Levels of whey-specific immunoglobulins (**C**) IgE, (**D**) IgG1 and (**E**) IgG2a (sensitization: $P < 0.0001$, diet: ns interaction: ns). Two-way ANOVA followed by Bonferroni's multiple comparisons test. Data are presented as mean ± S.E.M. for ear swelling and as Box-and-Whisker Tukey plots on a log scale for mMCP-1 and immunoglobulins. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant, $n = 11$ per group.

N-3 LCPUFA supplementation prevents reduced breakdown of 5-HT in the intestine of food allergic mice

As it was previously shown that intestinal levels of 5-HT were increased and levels of 5-HIAA were decreased in food allergic mice (36), the effect of n-3 LCPUFA supplementation on the serotonergic response was determined in distal ileum homogenates. The increase in intestinal 5-HT levels in food allergic mice was not significantly different from control mice (**Fig. 3A**). However, levels of 5-HIAA were decreased in food allergic mice compared to control mice when fed a control diet ($P < 0.05$, **Fig. 3B**) and the n-3 LCPUFA-enriched diet was able to prevent this reduction in 5-HIAA levels in ileum of allergic mice. Ratio of 5-HIAA/5-HT was used as an index of 5-HT turnover. The turnover was reduced in food allergic mice compared to control mice when fed the control diet ($P < 0.05$, **Fig. 3C**), but not when fed the n-3 LCPUFA-enriched diet.

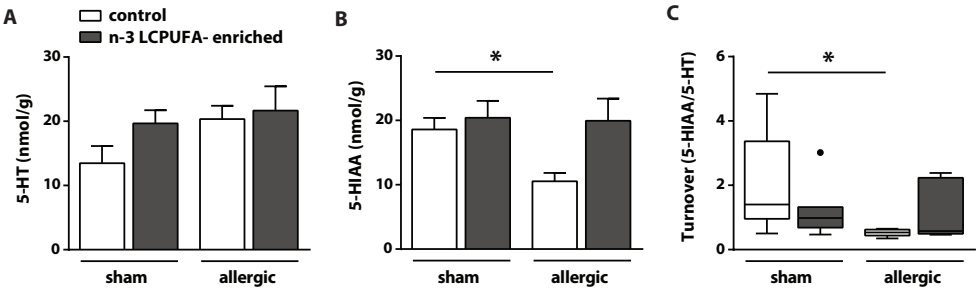

Fig. 3. Levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in ileum homogenates of whey-sensitized allergic and sham-sensitized control mice, fed the control or n-3 LCPUFA-enriched diet. (**A**) Levels of intestinal 5-HT (sensitization: ns, diet: ns, interaction: ns). (**B**) Levels of intestinal 5-HIAA (sensitization: $P < 0.05$, diet: $P < 0.05$, interaction: ns). (**C**) 5-HT turnover (sensitization: ns, diet: $P < 0.05$, interaction: $P < 0.05$). Two-way ANOVA followed by Bonferroni's multiple comparisons test. Data are presented as mean ± S.E.M. for concentrations of 5-HT and 5-HIAA as Box-and-Whisker Tukey plots for turnover rate. * $P < 0.05$, ** $P < 0.01$, ns: not significant, $n = 8$ per group.

Social interaction is reduced in food allergic mice and restored in food allergic mice fed the n-3 LCPUFA-enriched diet

The morning after oral challenge, mice were exposed to a social interaction test (**Fig. 4A**). Social interaction was determined by the amount of time that an experimental mouse spent in the interaction zone near an age- and gender-matched, unfamiliar mouse. Whey-sensitized allergic mice fed the control diet spent less time in the interaction zone compared to sham-sensitized control mice ($P < 0.05$, **Fig. 4B**), and the n-3 LCPUFA-enriched diet was able to prevent reduced social interaction. Furthermore, latency of first approach to the social target was increased in allergic mice compared to control mice fed the control diet ($P < 0.05$, **Fig. 4C**). The n-3 LCPUFA-enriched diet decreased latency of first approach in allergic mice compared to the control diet ($P < 0.01$). Of note, locomotor activity during habituation in the open field was not different between groups (data not shown).

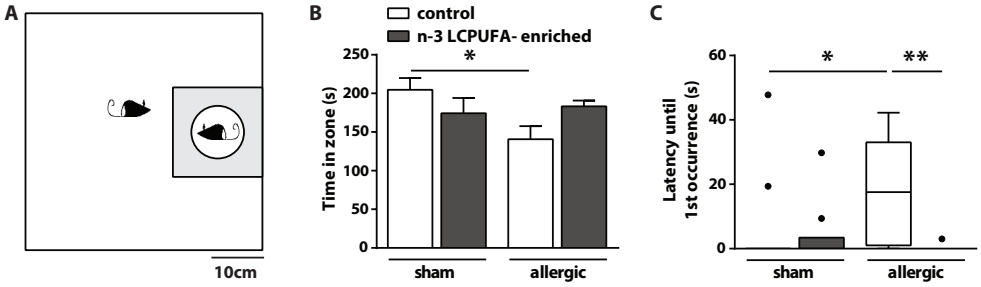

Fig. 4. Social interaction of whey-sensitized allergic and sham-sensitized control mice fed the control or n-3 LCPUFA-enriched diet. (**A**) Schematic representation of the social interaction test, illustrating the interaction zone (grey rectangle) and the cage (white circle) in which an target mouse was placed. (**B**) Time spent in the interaction zone (sensitization: ns, diet: ns interaction: $P < 0.05$). (**C**) Latency of first occurrence in the interaction zone (KW: $P < 0.01$). One allergic mouse on control diet was excluded as significant outlier (209 s, Grubb's test). (B) Two-way ANOVA followed by Bonferroni's multiple comparisons test, mean ± S.E.M. (C) Kruskal-Wallis (KW) test followed by Dunn's multiple comparisons test, Box-and-Whisker Tukey plots. * $P < 0.05$, ** $P < 0.01$, ns: not significant, $n = 11$ per group.

N-3 LCPUFA supplementation increases DHA content at the expense of AA and DTA

Dietary supplementation of n-3 LCPUFA, increased DHA content in whole brain homogenates compared to consumption of the control diet, both in allergic and in control mice ($P < 0.0001$, **Fig. 5A**). Incorporation of n-3 LCPUFA DHA in brain membranes occurred mainly at the expense of n-6 LCPUFAs AA and DTA. Decreased levels of AA ($P < 0.0001$, **Fig. 5B**) and DTA ($P < 0.0001$, **Fig. 5C**) were observed when mice were fed the n-3 LCPUFA-enriched diet compared to control diet. No difference was observed between fatty acid content in brain of whey-sensitized allergic mice compared to sham-sensitized control mice.

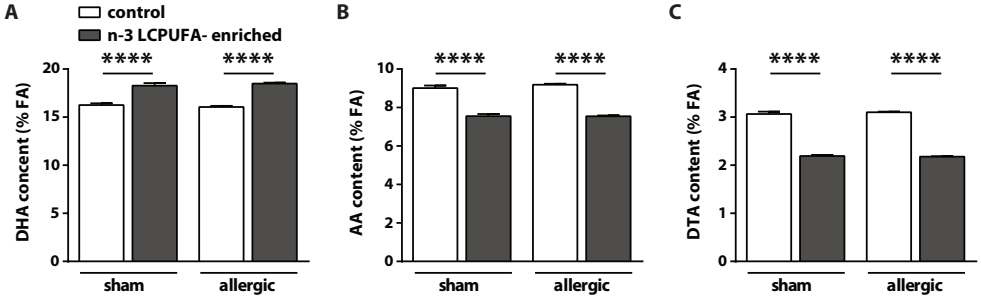

Fig. 5. The effect of n-3 LCPUFA supplementation on n-3 and n-6 LCPUFA content in brain homogenates of whey-sensitized allergic and sham-sensitized control mice. (**A**) N-3 LCPUFA docosahexaenoic acid (DHA) content (sensitization: ns, diet: $P < 0.0001$, interaction: ns). (**B**) N-6 LCPUFA arachidonic acid (AA) (sensitization: ns, diet: $P < 0.0001$, interaction: ns). (**C**) N-6 LCPUFA docosatetraenoic acid (DTA) (sensitization: ns, diet: $P < 0.0001$, interaction: ns). Two-way ANOVA followed by Bonferroni's multiple comparisons test was conducted and data are presented as mean ± S.E.M. **** $P < 0.0001$, ns: not significant, $n = 11$ per group.

N-3 LCPUFA supplementation prevents decreased levels of dopamine metabolites in PFC of food allergic mice

When fed the control diet, reduced levels of DA were observed in allergic mice compared to control mice ($P < 0.05$, **Fig. 6A**). Decreased levels of DA were not present in allergic mice when they were fed the n-3 LCPUFA-enriched diet. Furthermore, levels of DA metabolites DOPAC (**Fig. 6B**), 3-MT (**Fig. 6C**) and HVA (**Fig. 6D**) were all reduced in food allergic mice fed the control diet compared to control mice ($P < 0.05$). The n-3 LCPUFA-enriched diet was able to prevent this reduction in metabolite levels in the PFC of allergic mice. The turnover rate of DA, assessed by calculating the ratio between metabolites and DA ((DOPAC+3-MT+HVA)/DA), was not altered between groups (data not shown). Moreover, no differences were observed between groups regarding 5-HT and 5-HIAA levels in the PFC (data not shown).

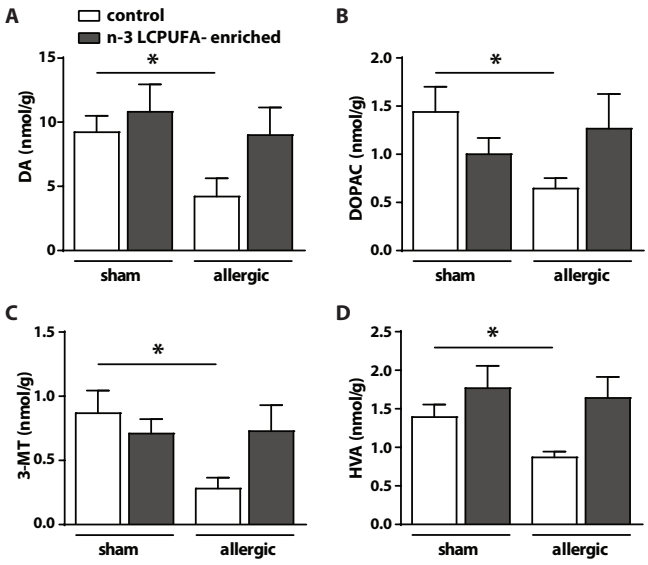

Fig. 6. Dopamine (DA) and metabolite levels in prefrontal cortex (PFC) of whey-sensitized allergic mice and sham-sensitized control mice and the effect of n-3 LCPUFA supplementation. (**A**) Levels of DA (sensitization: $P < 0.05$, diet: ns, interaction: ns). Levels of (**B**) 3,4-dihydroxyphenylacetic acid (DOPAC), (**C**) 3-methoxytyramine (3-MT), and (**D**) homovanillic acid (HVA) (all three metabolites: sensitization: ns, diet: ns, interaction: $P < 0.05$). Two-way ANOVA followed by Bonferroni's multiple comparisons test was conducted and data are presented as mean ± S.E.M. * $P < 0.05$, ns: not significant, $n = 8$ per group.

DISCUSSION

The present study demonstrates that dietary supplementation of n-3 LCPUFA prevents food allergy-induced abnormalities in social behaviour and associated dampening of the dopaminergic system in the PFC of whey-sensitized mice. We suggest that this effect of n-3 LCPUFA on social behaviour may be mediated by a reduced allergic response, decreased intestinal 5-HT signalling to afferent neuronal fibres or directly by incorporation of DHA into neuronal membranes in the brain. Locomotor activity in the open field was not different between groups, suggesting that impaired social behaviour was not affected by altered mobility and food allergic mice did not show any overt signs of sickness.

It was previously shown that n-3 LCPUFA supplementation prevented allergic sensitization and reduced the allergic skin response in female C3H/HeOuJ mice (37, 42). In contrast, we used male mice in the present experiment, because a male preponderance is observed in ASD patients (43). The n-3 LCPUFA-enriched diet was able to reduce the allergic skin response in whey-sensitized male mice. However, n-3 LCPUFA supplementation did not completely abolish the allergic skin response and it lowered neither mucosal mast cell degranulation nor production of whey-specific immunoglobulins, suggesting that the effects of n-3 LCPUFA observed at the behavioural level were not mediated by these humoral factors. Tissue content of DHA is known to be higher in females than males and is dependent on sex hormones (44), which may explain the observed gender differences.

A pro-inflammatory environment in the intestine decreases serotonin transporter activity on epithelial cells, both *in vitro* (45-47) and *in vivo* (48-50), resulting in less 5-HT reuptake and metabolism, consequently reducing 5-HIAA levels. Depressed levels of 5-HIAA were observed in intestinal biopsies of patients with coeliac disease (51) and IBS (52). In line with these observations, we showed that 5-HIAA levels were reduced in the intestine of allergic mice. The reduction in 5-HIAA levels was prevented when allergic mice were fed the n-3 LCPUFA-enriched diet. This indicates that n-3 LCPUFA supplementation restored intestinal 5-HT metabolism, while mast cell degranulation remained unaffected. Food allergy-induced signalling to the brain is suggested to be mediated by 5-HT binding to receptors on vagal afferents in the intestine (9, 12). Therefore, the beneficial effects of n-3 LCPUFA supplementation on food allergy-induced behavioural deficits may be the result of restored 5-HT metabolism. To our knowledge, the effect of LCPUFAs on intestinal 5-HT has not been investigated and could provide an interesting avenue for future research on the treatment of inflammatory and allergic diseases of the intestinal tract and activation of the gut-brain axis.

We speculate that the improvements in social behaviour may also be mediated by increased incorporation of DHA into the brain. Supplementation with N-3 LCPUFA enhanced n-3 DHA content in the brain, mainly at the expense of n-6 AA and DTA.

LCPUFA, in particular AA and DHA (53), are incorporated in the neuronal cell membrane to maintain normal brain function (29-31). It has been suggested that the ratio of n-3: n-6 PUFA in the brain regulates neuronal processes. Brain fatty acid content is dependent on nutritional intake and it was shown that suboptimal n-3 LCPUFA intake during gestation and postnatal development caused neurodevelopmental deficits in preclinical (54, 55) and clinical studies (56-58). Therapeutic effects of n-3 LCPUFA have been postulated in several neurodevelopmental (59, 60) and neurodegenerative disorders (61). Regarding ASD, conflicting results exist on the efficacy of n-3 LCPUFA supplementation on behaviour and large well-controlled randomized trials are required (35).

LCPUFAs in the brain act on physiological functions via several mechanisms. LCPUFAs are incorporated in membrane-based phospholipids of neural tissue modifying membrane integrity and fluidity (62, 63). Functioning of transmembrane proteins, such as receptors and transporters, is affected by membrane fluidity (29, 64). Monoaminergic neurotransmission, in particular mesocortical dopamine, was reported to alter upon changes in nutritional composition of fatty acids (65). Chronic n-3 LCPUFA deficiency in rats reduced levels of DA (65), the number of vesicular monoamine transporter-2 binding sites (66), and the number of storage vesicles in DA terminals in the PFC (67). This suggests that n-3 LCPUFA depletion reduces the prefrontal DA neurotransmission. On the other hand, a diet high in n-3 LCPUFA increased prefrontal DA levels of rats compared to a diet high in n-6 LCPUFA (68). This is in line with our observation that supplementation of n-3 LCPUFA restored decreased DA and metabolite levels in PFC of whey-sensitized allergic mice. As the dopaminergic system in the PFC is implicated in the regulation of social behaviour (69), n-3 LCPUFA supplementation may beneficially affect social behaviour in allergic mice directly via its incorporation into neuronal membranes, affecting dopaminergic neurotransmission in the PFC. Because the mesocortical DA system is thought to be involved in aberrant social behaviour (69) and in the pathophysiology of ASD (70), dietary n-3 LCPUFA supplementation could be of relevance in the management of abnormal social behaviour of patients with ASD and co-morbid food allergic symptoms.

In summary, we demonstrate that dietary fish oil, high in n-3 DHA, prevented food allergy-induced deficits in social behaviour of mice and restored dopamine and metabolite levels in the PFC of whey-sensitized allergic mice. Together, we suggest that n-3 LCPUFA supplementation may have beneficial effects on social behaviour of food allergic mice, either indirectly by reducing the allergic response and decreasing intestinal 5-HT signalling, or directly by incorporation of DHA into neuronal membranes in the brain improving prefrontal dopaminergic neurotransmission.

ACKNOWLEDGEMENTS

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CHAPTER NINE

A diet containing specific anti-inflammatory and neuroprotective ingredients prevents impaired behaviour, but not the allergic response, in food allergic mice

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ABSTRACT

Food allergy is suggested to exacerbate behavioural abnormalities in children with neurodevelopmental disorders, including autism spectrum disorders. We have previously shown that food allergy impaired social behaviour and induced repetitive behaviour in mice. The aim of this study was to assess the effect of a multi-nutrient supplementation diet containing specific anti-inflammatory and neuroprotective ingredients on impaired social and repetitive behaviour and allergic sensitization in food allergic mice. Mice were fed either a control or active diet before and during sensitization with whey. Social behaviour was assessed as well as serum immunoglobulin levels, mast cell activation and intestinal serotonin (5-HT) and metabolite levels. The active diet restored impaired social and repetitive behaviour in food allergic mice without affecting serum levels of immunoglobulins and mouse mast cell protease-1 as well as intestinal turnover 5-HT. Furthermore, the active diet increased 5-HT levels in the intestines of control mice. As improvement in behaviour was not the result of reduced allergic sensitization, this study suggests that the diet containing specific anti-inflammatory and neuroprotective ingredients may exert its beneficial effects directly on the brain rather than via the immune system.

INTRODUCTION

Food allergy affects about 6 % of young children and can cause symptoms that include gastrointestinal and pulmonary distress and atopic dermatitis (1). Food allergy is induced by T helper 2 (Th2) polarization of the immune response and is characterized by production of allergen-specific immunoglobulins during sensitization, and intestinal mast cell degranulation upon a second exposure to the allergen, leading to allergic symptoms (2). The enteric nervous system is involved in the regulation of intestinal immune responses. Mechanisms by which food allergy can activate the nervous system have recently been reviewed (3). Inflammatory signalling molecules such as cytokines, neuropeptides, and serotonin (5-hydroxytryptamine; 5-HT) can directly and indirectly activate afferent nerves, which signal to the brain to activate the cholinergic anti-inflammatory pathway and the hypothalamic-pituitary-adrenal (HPA)-axis (4, 5). Moreover, food allergic reactions were shown to increase the expression of IgE receptors on vagal afferents and partial removal of the vagus nerve suppressed Th2-mediated inflammation in the intestines (6). Vagal afferents mediate visceral nociception during food allergy via 5-HT receptor signalling (7). Intestinal 5-HT is predominantly derived from enterochromaffin cells, but also enteric neuronal cells and various immune cells, primarily mast cells, secrete 5-HTHT. Both enterochromaffin cells (8) and mast cells (9) release 5-HT in close proximity to afferent neurons, causing neuronal depolarization and signalling to brain regions that include the nucleus tractus solitarius (NTS) and hypothalamic paraventricular nucleus (PVN) (10). In rats it was shown that food allergy-induced neuronal activation of the NTS and PVN was diminished after blockade of 5-HT_3 receptors and vagotomy (11), suggesting that allergic signalling to the brain is mediated by 5-HT binding to receptors on vagal afferents.

Interestingly, these brain regions are important in emotional and social behaviour and increasing evidence shows that intestinal allergic reactions may also affect behavioural responses (12). We previously showed that food allergy in mice reduced social behaviour and increased repetitive behaviour (13). This was accompanied by increased neuronal activation in the orbital prefrontal cortex (PFC) and reduced activation in the PVN. Dopamine and metabolite levels were decreased in PFC of food allergic mice. Furthermore, food allergy also increased 5-HT levels and reduced 5-HT turnover in the intestines.

In humans, allergic reactions have been suggested to exacerbate impaired behaviour in children with neurodevelopmental disorders (14, 15). It was demonstrated that food allergy in the first year of life was associated with enhanced internalizing behaviour and a trend towards low social emotional scores in children at 18 months of age (16). Intestinal problems are often reported in children with autism spectrum disorder (ASD) (17, 18) and milk intake was found to be a predictor of constipation in these patients (19). A (gluten and) milk protein free diet is suggested to restore enhanced intestinal permeability observed in children suffering from ASD (20) and to improve autistic behaviour (21-23).

Previously, we showed that a nutritional intervention with a diet containing specific anti-inflammatory and neuroprotective ingredients (24-34) was able to improve social behaviour in a mouse model for ASD, using *in utero* exposure to valproic acid. Furthermore, this diet was able to restore deficits in 5-HT metabolism in the intestinal tract as well as in the brain of mice exposed to VPA *in utero*. In the present study, we assessed the effect of the active diet on food allergy-induced impairments in social and repetitive behaviour and on changes in 5-HT metabolism in the intestinal tract.

MATERIALS AND METHODS

Table 1. Diet composition

active compared to control (per kg diet)	**supplier**
carbohydrates (ref 33)	
dextrinized cornstarch and sucrose substituted by:	
41.5 wt% maltodextrin (DE6)	Roquette (Lestrem, France)
15.0 wt% free galactose	Inalco (Milan, Italy)
42.5 wt% isomaltulose	Beneo-Palatinit (Mannheim, Germany)
1 wt% fructose	Brenntag (Dordrecht, The Netherlands)
fibres (ref 24-27)	
2.8% cellulose substituted by:	
2% rice fiber RemyLiVe200	Beneo Orafti (Oreye, Belgium)
0.72% GOS*	FrieslandCampina (Amersfoort, The Netherlands)
0.08% Beneo Raftiline HP FOS^	Beneo (Leuven, Belgium)
protein (ref 29)	
protein-free diets amino acids from soy protein substituted by: amino acids from soy protein and α-lac enanced whey protein in ratio 1:1 addition of: 2.3 g tryptophan	
lipids (ref 28, 30- 32)	
to obtain 0.53% DHA$^{<}$ and 0.92% EPA$^{>}$ part of lipid fraction substituted by:	
27.5 g Nissui anchovy oil	Nippon Suisan Kaisha (Tokyo, Japan)
6.5 g Biopure DHA IF tuna oil	Bioriginal (Den Bommel, The Netherlands)
7.6 g soy lecithin Emulpur	Cargill (Mechelen, Belgium)
vitamins	
extra vitamins (reaching 200 % value): vitamin A, B6, B12, D2, folic acid	

* GOS: galacto-oligosaccharides; ^ FOS: fructo-oligosaccharides
$^{<}$ DHA: docosahexaenoic acid, $^{>}$ EPA: eicosapentaenoic acid

Diets

The iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on standard animal food for laboratory rodents AIN93-G (35). Composition of active and control diet are listed in **Table 1**. The active diet consisted of low-glycemic index carbohydrates, dietary fibres, high tryptophan content and a lipid profile that predominantly differed in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content. Diets were stored at -20 °C prior to use to prevent fatty acid oxidation.

Animal experiment

Three-week-old, specific pathogen free, male C3H/HeOuJ mice (n = 10), purchased from Charles River Laboratories (L'Arbresle Cedex, France) were housed at the animal facility of the Utrecht University on a 12 h light/dark cycle with access to food and water *ad libitum*. Mice were fed either the control or active diet, starting two weeks prior to first sensitization and continued during the entire experiment (**Fig. 1**). Mice were sensitized intragastrically (i.g.) with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS, containing 10 µg cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks as previously described (36). One day after the last sensitization, grooming behaviour was assessed. One week after the last sensitization, sham and whey-sensitized mice were challenged i.g. with 50 mg whey/0.5 mL PBS and a social interaction test was conducted the next morning. Mice were sacrificed after the social interaction test to collect serum and tissue from the distal ileum. All animal procedures were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2012.I.04.053).

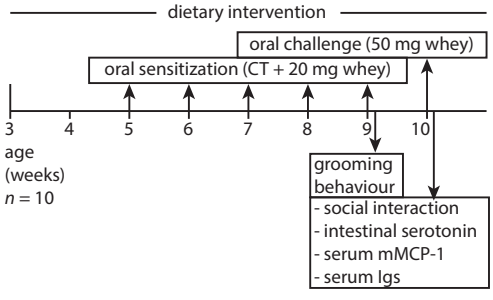

Fig. 1. Schematic representation of experimental set-up.

Social interaction test

The behavioural assessment was adapted from a previous description (13, 37). Mice were placed in a 45 x 45 cm open field with a small perforated Plexiglas cage (10 cm diameter) located against one wall allowing visual, olfactory and minimal tactile interaction (**Fig. 2A**). Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands) an interaction zone around the cage was digitally determined. Time spent in the interaction zone, latency until first occurrence in the interaction zone and total distance moved were measured.

Self-grooming

The morning after the last sensitization, mice were scored for spontaneous self-grooming behaviours as described earlier (13, 38). Each mouse was placed individually in an empty home cage (35 cm x 20 cm) without bedding and video recordings were used for behavioural scorings of frequency and cumulative time spent grooming all body regions. Open field was cleaned with water followed by 70 % ethanol after each test. After a 5 min habituation period in the cage, each mouse was scored blindly for 5 min by two independent researchers.

Measurements of whey-specific immunoglobulins and mouse mast cell protease-1

Blood was collected 16 h after oral challenge, centrifuged for 15 min at 14,000 rpm and serum was stored at -70 °C. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 µg/mL whey in carbonate/ bicarbonate buffer (0.05 M, pH = 9.6; Sigma-Aldrich, Zwijndrecht, The Netherlands) overnight at 4 °C. Plates were blocked in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05 % Tween-20 and 0.5 % BSA in PBS) and serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 µg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h and subsequently with streptavidin-HRP (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h. Plates were developed using o-phenylendiamine (Sigma-Aldrich) and reaction was stopped after 15 min with 4 M H_2SO_4. Absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU) and composed using a titration curve of pooled sera from whey-alum i.p. immunized mice serving as an internal standard. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (BD Biosciences) according to the manufacturer's protocol.

HPLC for analysis of 5-HT and 5-HIAA in intestines

5-HT and its metabolite 5-HIAA were measured in tissue from distal ileum by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Lelystad, The Netherlands) as previously described (13). Intestinal tissues were sonicated in 200 - 600 µL ice-cold solution containing 5 µM clorgyline, 5 µg/mL glutathione and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL tissue homogenate, 12.5 µL, 2 M $HClO_4$ was added and subsequently 10 µL 2.5 M potassium acetate. After 15 min in ice water, the homogenates were centrifuged for 10 min at 15,000g (4 °C). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol (pH = 3.4). Separation was performed at 45 °C using a flow rate of 0.7 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM.

Statistical analysis

Experimental results are expressed as mean ± S.E.M, or Box-and-Whisker Tukey plot when data were not normally distributed. Differences between groups were statistically determined using two-way ANOVAs followed by a Bonferroni's multiple comparisons test. For serum immunoglobulin and mMCP-1 levels and intestinal 5-HT turnover, log transferred data were used to obtain normality. Latency until first occurrence in the interaction was statistically analysed with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Results were considered statistically significant when $P < 0.05$. Analyses were conducted using GraphPad Prism, version 6.02.

RESULTS

The active diet prevents reduced social behaviour of food allergic mice

A social interaction test was conducted the morning after oral challenge. Social interaction was determined by the amount of time that an experimental mouse spent near an age- and gender-matched unfamiliar mouse (**Fig. 2A**). Whey-sensitized allergic mice fed the control diet spent significantly less time in the interaction zone compared to sham-sensitized mice after oral challenge with whey ($P < 0.05$, **Fig. 2B**). Nutritional intervention with the active diet restored social behaviour of allergic mice back to levels of sham-sensitized mice. Compared to allergic mice fed the control diet, allergic mice fed the active diet spent significantly more time in the interaction zone ($P < 0.01$). Latency of first approach to the social target was increased in allergic mice compared to sham-sensitized mice when fed the control diet ($P < 0.05$, **Fig. 2C**). This increase in latency was not observed in allergic mice when fed the active diet. Mobility during habituation in the open field was not different between groups (data not shown).

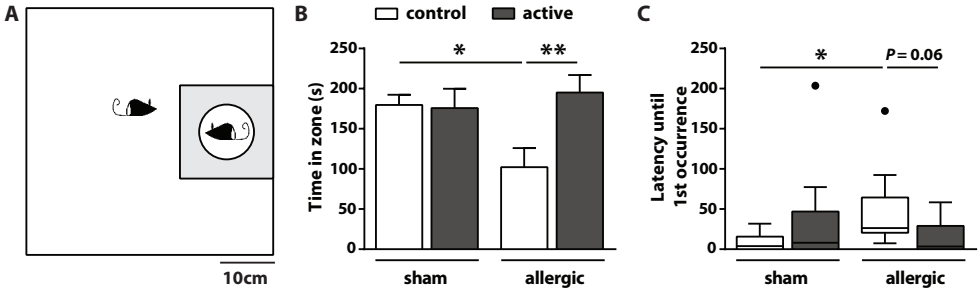

Fig. 2. Social behaviour of whey and sham-sensitized mice fed the control or active diet. (**A**) Schematic representation of the social behaviour test. (**B**) Allergic mice showed reduced social interaction compared to control mice and the active diet restored social interaction in allergic mice (sensitization: ns, diet: $P < 0.05$ interaction: $P < 0.05$). (**C**) Latency of first occurrence in the interaction zone was significantly increased in allergic mice fed the control diet, but not when fed the active diet ($P < 0.05$) . Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. For latency, Kruskal-Wallis test was conducted followed by Dunn's multiple comparisons test and data are presented as box-and-whisker Tukey plot. * $P < 0.05$, ** $P < 0.01$, ns: not significant.

The active diet normalized repetitive behaviour of food allergic mice

Duration and frequency of self-grooming was scored as a measure of repetitive behaviour. Food allergic mice fed the control diet spent more time self-grooming than sham-sensitized controls ($P < 0.01$, **Fig. 3A**). This increase in grooming duration was abolished when allergic mice were fed the active diet ($P < 0.01$). Frequency of self-grooming was also increased in allergic mice compared to sham-sensitized mice fed the control diet ($P < 0.05$, **Fig. 3B**), but frequency was not increased when allergic mice were fed the active diet.

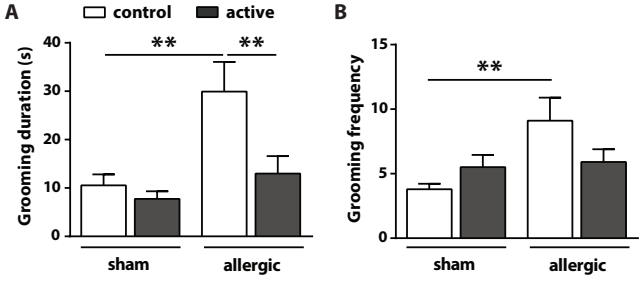

Fig. 3. Grooming behaviour of whey and sham-sensitized mice fed the control or active diet. (**A**) Cumulative time spent grooming (sensitization: $P < 0.01$, diet: $P < 0.05$ interaction: $P = 0.07$) and (**B**) frequency of grooming (sensitization: $P < 0.05$, diet: ns, interaction: $P < 0.05$) during a 5 min session were significantly increased in food allergic mice compared to control mice and this effect was ameliorated when allergic mice were fed the active diet. Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. ** $P < 0.01$, ns: not significant.

Allergic sensitization and serum mouse mast cell protease-1 levels are not affected by the active diet

Sensitization with whey protein induced high serum levels of whey-specific IgE ($P < 0.01$, **Fig. 4A**), irrespective of the dietary intervention. Whey-specific IgG1 (**Fig. 4B**) and IgG2a (**Fig. 4C**) levels were also increased in allergic mice compared to control mice, when fed the control ($P < 0.001$ and $P < 0.01$, respectively) and the active diet (both $P < 0.001$). To assess mucosal mast cell degranulation, concentration of mMCP-1 was measured in serum. Increased mMCP-1 levels were observed in allergic mice compared to sham-sensitized mice fed the control diet ($P < 0.01$, **Fig. 4D**) and the active diet ($P < 0.01$). Hence, nutritional intervention with the active diet did not alter serum mMCP-1 or immunoglobulin levels in allergic mice.

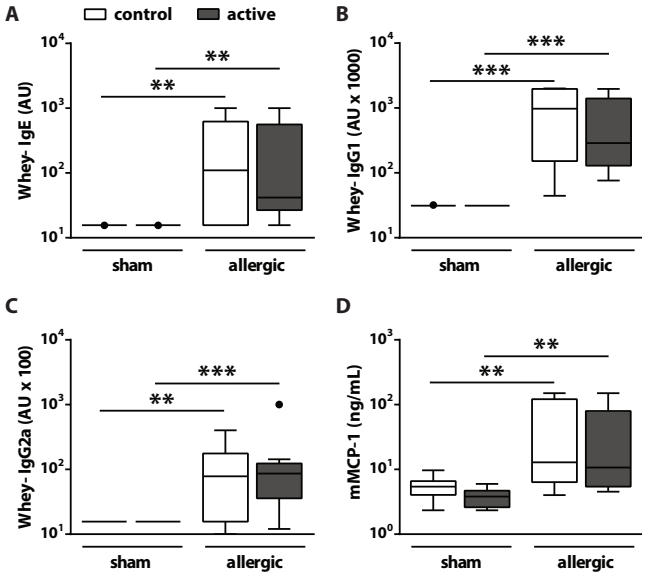

Fig. 4. The effect of the active diet on the humoral response and serum mouse mast cell protease-1 (mMCP-1) levels in whey and sham-sensitized mice fed the control or active diet. Serum levels of whey-specific (**A**) IgE, (**B**) IgG1 and (**C**) IgG2a as well as (**D**) mMCP-1 were increased in allergic mice compared to control mice and this was not affected to by the active diet (sensitization: $P < 0.001$, diet: ns, interaction: ns). Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as Box-and-Whisker Tukey plots. ** $P < 0.01$, *** $P < 0.001$, ns: not significant.

The active diet does not restore altered 5-HT turnover in ileum of food allergic mice

In accordance with previous observations (13), 5-HT levels tended to be increased in the intestines of food allergic mice compared to control mice ($P = 0.06$, **Fig. 5A**). The active diet increased levels of intestinal 5-HT in sham-sensitized mice ($P < 0.05$). Concentration of 5-HT was not different in allergic mice fed the active diet, when compared to allergic mice fed the control diet or to sham-sensitized mice fed the active diet. Although 5-HIAA levels appeared to be lower in allergic mice compared to control mice, no significant differences were found between groups (**Fig. 5B**). However, turnover of 5-HT, estimated by the ratio of 5-HIAA to 5-HT, was significantly reduced in allergic mice compared to sham-sensitized mice when fed the control diet ($P < 0.05$, **Fig. 5C**) and the active diet did not alter 5-HT turnover in either sham-sensitized or allergic mice.

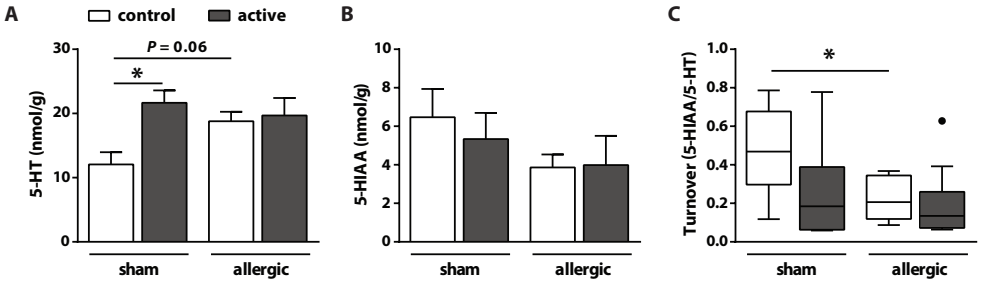

Fig. 5. Levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in ileum of whey or sham-sensitized mice fed the control or active diet. (**A**) When compared to control mice fed the control diet, 5-HT levels tended to be increased in allergic mice fed the control diet and were significantly increased in control mice fed the active diet (sensitization: ns, diet: $P < 0.05$, interaction: $P < 0.05$). (**B**) Levels of 5-HIAA were not significantly different (sensitization: ns, diet: ns, interaction: ns). (**C**) 5-HT turnover (5-HIAA/5-HT) was reduced in allergic mice compared to control mice when fed the control diet ($P < 0.05$, diet: ns, interaction: ns). Two-way ANOVAs were conducted followed by Bonferroni's multiple comparisons test and data are presented as mean ± S.E.M. for 5-HT and 5-HIAA levels and as Box-and-Whisker Tukey plots for turnover. * $P < 0.05$, ns: not significant.

DISCUSSION

In this study, we showed that nutritional intervention with a diet containing specific anti-inflammatory and neuroprotective ingredients prevented food allergy-induced impairments in social and repetitive behaviour in mice. The effects on behaviour did not appear to result from the prevention of allergic sensitization, as immunoglobulin levels were induced to the same extent in allergic mice fed the control and the active diet. Moreover, mast cell degranulation after oral challenge was also maintained in allergic mice fed the active diet, indicating that the intestinal allergic response to whey was not lower when mice were fed the active diet. We previously observed that dietary supplementation with n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) did not affect mMCP-1 and immunoglobulin levels, but did reduce the acute allergic skin response (39). Because we did not measure the allergic skin response in the present study, we cannot rule out an effect of the active diet on the skin response.

A pro-inflammatory environment in the intestine has been shown to decrease 5-HT transporter activity on epithelial cells, both in vitro (40-42) and in vivo (43-45), resulting in less 5-HT reuptake and metabolism, consequently reducing 5-HIAA levels and 5-HT turnover. Moreover, decreased levels of 5-HIAA were also found in intestinal biopsies of patients with coeliac disease (46) and IBS (47). In line with these observations, 5-HT turnover rate was reduced in allergic mice in the present study. The active diet did not restore 5-HT turnover in the intestines of allergic mice. The active diet increased 5-HT levels in control mice, which may result from enriched tryptophan in the active diet. Food allergy-induced signalling to the brain is suggested to be mediated via 5-HT binding to receptors on vagal afferents in the intestine (10, 11). The observation that the active diet restored behaviour, but not 5-HT turnover, does not support the hypothesis that food allergy-induced behavioural changes are mediated by 5-HT signalling. However, food allergy-induced 5-HT signalling cannot be excluded, as the active diet may affect the 5-HT system beyond 5-HT metabolism (e.g. via changes in extracellular 5-HT levels or 5-HT receptor activity) or it may affect neuronal signalling in the brain.

As neither the allergic response nor intestinal 5-HT metabolism was altered by the active diet, it is compelling to suggest that the active diet may exert its beneficial effects directly on the brain rather than via the immune system. Several components of the active diet may influence neuronal signalling, plasticity, and survival. Most frequently described are the effects of dietary n-3 LCPUFA on brain functioning. The n-3 LCPUFA EPA and DHA are one of the ingredients of the active diet used in this study. Dietary n-3 LCPUFA supplementation enhances incorporation of n-3 LCPUFA in the brain, at the expense of n-6 LCPUFA (39). N-3 LCPUFA are incorporated in membrane-based phospholipids of neural tissue modifying membrane integrity and fluidity (48, 49). Functioning of transmembrane proteins, such as receptors and transporter proteins, is affected by membrane fluidity

(50, 51). As a consequence, monoaminergic neurotransmission alters upon changes in nutritional composition of fatty acids (52). We have previously shown that dietary supplementation with n-3 LCPUFA restored decreased prefrontal DA and metabolite levels and normalized social behaviour in allergic mice (39). Moreover, the active diet was able to restore prefrontal serotonin levels in a mouse model of ASD (53). Also vitamin supplementation during pregnancy may reduce the risk of ASD in the newborn (55), as folate and vitamin B12 are required for methylation of DNA, proteins, phospholipids and neurotransmitters (54). Nevertheless, clinical evidence for a positive effect of nutritional supplementation with either n-3 LCPUFA or vitamins on brain and behaviour remains too limited to draw firm conclusions on the neuroprotective benefits of these components in humans (56, 57). In addition to LCPUFA and vitamin supplementation, administration of galacto-oligosaccharides (GOS) and fructo-oligosacchardes (FOS) to rats was recently shown to increase levels of brain-derived neurotrophic factor (BDNF) in the dentate gyrus of the hippocampus (34). As BDNF signalling is critical for neuronal protection, survival and plasticity (58), this study may evoke further investigation of GOS and FOS supplementation on brain development and neuroprotection.

LCPUFA and dietary fibres are thought to modulate gut microbiota composition as well as intestinal physiology and the immune system (26, 59). For example, a dietary intervention with GOS/FOS started during gestation significantly increased colon length (60) and enhanced tolerance-related immunoglobulins as well as gut barrier functions (61) in healthy murine pups. Furthermore, the rice fibre used in the multi-nutrient diet was previously shown to reduce inflammation and restore 5-HT levels in a mouse model for colitis (25). Tryptophan is the precursor of 5-HT and is suggested to modulate immune responses via its antioxidant properties and its catabolites derived from the kynurenine pathway that are able to suppress T cell responses and contribute to tolerance induction (62, 63). Supplementation with tryptophan reduced DSS-induced colitis in piglets (64). Overall, this implies that multiple components in the active multi-nutrient diet may improve intestinal homeostasis. As the active diet is a multi-nutrient supplementation diet, it is likely to target multiple pathways. Identifying the individual effects of the active components would require further extensive research subdividing components and multiple combinations to identify synergistic effects.

In summary, this study describes the beneficial effects of a multi-nutrient diet, containing specific anti-inflammatory and neuroprotective ingredients, on food allergy-induced impairments in social and repetitive behaviour. The improvement in behaviour was not the result of a reduced allergic response. Therefore, the effects observed on normalization of impaired social behaviour may be mediated by direct effects on the brain rather than via the immune system.

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CHAPTER TEN

The interplay between prenatal exposure to valproic acid and food allergy in the behavioural and allergic response of mice and the effects of a specific multi-nutrient diet -A preliminary study-

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ABSTRACT

Autism spectrum disorder (ASD) is a cluster of neurodevelopmental disorders suggested to be caused by genetic predisposition in combination with environmental factors. Gastrointestinal and immune dysfunction in patients with ASD are repeatedly reported and suggested to contribute to disease progression. In the present study, we investigated the interplay between impaired neurodevelopment, induced by *in utero* valproic acid (VPA) exposure, and food allergy in mice as well as the effects of a multi-nutrient diet, containing specific anti-inflammatory and neuroprotective ingredients. Pregnant BALB/c females, fed either the control or the active diet, were treated subcutaneously with 500 mg/kg VPA or PBS on gestational day 11. Male offspring were sensitized with whey protein for 5 weeks. The morning after challenge with whey, social and anxiety-like behaviour were assessed and serum was collected. In this pilot study we demonstrate that social and anxiety-like behaviour in VPA exposed mice was not further impaired when a food allergic reaction was induced. However, mice exposed to VPA *in utero* have a distinct immunological response to allergic sensitization, characterized by reduced serum levels of antigen-specific Th1-type IgG2a. Moreover, the specific multi-nutrient diet prevented diminished IgG2a levels in VPA-exposed allergic mice. This finding adds to the hypothesis that immune dysfunction is associated with ASD and this model may open a new window for opportunities to investigate the underlying mechanism of immune dysfunction in neurodevelopmental disorders.

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous cluster of severe neurodevelopmental disorders, characterized by impairments in social interaction and communication and the presence of stereotyped behaviours (1). Although the aetiology of ASD is unknown, it is thought that ASD is a multifactorial disorder with a strong genetic component (2, 3). A variety of environmental factors is suggested to contribute to ASD development. For example, prenatal exposure to teratogens such as valproic acid (VPA) was shown to be a significant risk factor for ASD (4). VPA is clinically used as an antiepileptic drug and mood stabilizer, but administration during pregnancy can cause adverse effects to the foetus, including congenital malformation, reduced cognitive function, and neurodevelopmental disorders such as ASD (4-6). Proposed mechanisms for VPA-induced symptoms of ASD include attenuation of folic acid metabolism, inhibition of histone deacetylases, and increased oxidative stress (5). Mice exposed to VPA *in utero* exhibit developmental and behavioural deficits comparable to those observed in ASD patients, including deficits in social behaviour (7, 8), stereotyped behaviour (9), anxiety, and impairments in cognition (8). Behavioural impairments are more prominent in male offspring compared to female offspring (8, 10), which reflects the human situation in which a marked male preponderance is observed in ASD patients (11, 12).

In addition to behavioural deficits, we have recently shown that *in utero* exposure to VPA in mice induced an inflammatory intestinal phenotype and an altered microbiota composition, as well as reduced serotonin (5-HT) levels in both brain and intestine (13). The presence of gastrointestinal problems in ASD patients is repeatedly reported in literature and includes chronic constipation, diarrhoea and abdominal pain (14). These symptoms have been attributed to changes in gut microflora (15, 16), increased intestinal permeability (17), intestinal inflammation (18), and food allergy (19). Associations have been observed between allergic diseases and impaired neurodevelopment in patients with ASD and attention-deficit hyperactivity disorder (ADHD) (19, 20). Psychological factors and psychiatric traits have been associated with triggering the onset or exacerbation of atopic disorders (21, 22) and vice versa, development of atopic disorders was suggested to contribute to impaired behaviour in children with ASD and ADHD (19, 20). Supporting the hypothesis that food allergy may affect mental disorders of psychosocial relevance, we have recently shown reduced social behavioural and neurochemical changes in a mouse model of cows milk allergy (23). In addition, enhanced internalizing behaviour and a trend towards low social emotional scores were observed in food allergic children at 18 months of age (24). In children with ASD, milk intake was found to be a predictor of constipation (25) and a gluten and milk protein free diet restored the increased intestinal permeability (17). Moreover, gluten and milk free diets are suggested to improve autistic behaviours in these children (26-28).

In the present study, we investigated the interplay between impaired neurodevelopment, induced by *in utero* VPA exposure, and food allergy in mice. We hypothesized that food allergy augmented VPA-induced impairment in social behaviour and that impaired neurodevelopment, leading to ASD-like behaviour, induced a higher allergic response to whey. Finally, we investigated the effects of a multi-targeted nutritional intervention containing specific anti-inflammatory and neuroprotective ingredients (29-39) on behaviour and the allergic response in this model.

MATERIALS AND METHODS

Diets

The iso-caloric diets were produced by Research Diet Services (RDS, Wijk bij Duurstede, The Netherlands) and were based on standard animal food for laboratory rodents AIN93-G (40). Composition of active and control diet are listed in **Table 1**. The active diet consisted of low-glycemic index carbohydrates, dietary fibres, high tryptophan content and a lipid profile that only differed in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Diets were stored at -20 °C prior to use to prevent fatty acid oxidation.

Animal experiment

Specific pathogen-free BALB/c breeding pairs from Charles River laboratories (Maastricht, The Netherlands) were housed under a 12 h light/dark cycle with free access to food and water. Starting two weeks before mating, females were fed either the control or the active diet and diets were fed to mothers and offspring throughout the experiment (**Fig. 1**). All females were mated until a vaginal plug was detected, indicated as gestational day 0 (G0). On G11, pregnant females were treated subcutaneously with 500 mg/kg VPA (Sigma-Aldrich, Zwijndrecht, The Netherlands; 100 mg/mL) or PBS. Litters were culled to 4 mice per litter and offspring were housed with their mother until weaning on postnatal day 21 (P21). Two weeks after weaning, mice were sensitized intragastrically (i.g.) with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS containing 10 µg cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized mice received CT alone. This resulted in the following groups: PBS-exposed mice fed the control diet: sham $n = 7$, whey $n = 6$; VPA-exposed mice fed the control diet: sham $n = 2$, whey $n = 4$, PBS-exposed mice fed the active diet: sham $n = 11$, whey $n = 12$; VPA-exposed mice fed the active diet: sham $n = 5$, whey $n = 6$. Mice were sensitized once a week for 5 consecutive weeks as previously described (41). One week after the last sensitization, sham and whey-sensitized mice were challenged i.g. with 50 mg whey/ 0.5 mL PBS and a social interaction test was conducted the next morning. Mice were sacrificed after the social interaction test to collect serum. All animal procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University (Utrecht, The Netherlands) (DEC2013.I.01.013).

Table 1. Diet composition

active compared to control (per kg diet)	supplier
carbohydrates (ref 38)	
dextrinized cornstarch and sucrose substituted by:	
41.5 wt% maltodextrin (DE6)	Roquette (Lestrem, France)
15.0 wt% free galactose	Inalco (Milan, Italy)
42.5 wt% isomaltulose	Beneo-Palatinit (Mannheim, Germany)
1 wt% fructose	Brenntag (Dordrecht, The Netherlands)
fibres (ref 29-32, 39)	
2.8% cellulose substituted by:	
2% rice fiber RemyLiVe200	Beneo Orafti (Oreye, Belgium)
0.72% GOS*	FrieslandCampina (Amersfoort, The Netherlands)
0.08% Beneo Raftiline HP FOS^	Beneo (Leuven, Belgium)
protein (ref 34)	
protein-free diets amino acids from soy protein substituted by: amino acids from soy protein and α-lac enanced whey protein in ratio 1:1 addition of: 2.3 g tryptophan	
lipids (ref 33,35-37)	
to obtain 0.53% DHA< and 0.92% EPA> part of lidpid fraction substituted by:	
27.5 g Nissui anchovy oil	Nippon Suisan Kaisha (Tokyo, Japan)
6.5 g Biopure DHA IF tuna oil	Bioriginal (Den Bommel, The Netherlands)
7.6 g soy lecithin Emulpur	Cargill (Mechelen, Belgium)
vitamins	
extra vitamins (reaching 200 % value): vitamin A, B6, B12, D2, folic acid	

* GOS: galacto-oligosaccharides; ^ FOS: fructo-oligosaccharides
< DHA: docosahexaenoic acid, > EPA: eicosapentaenoic acid

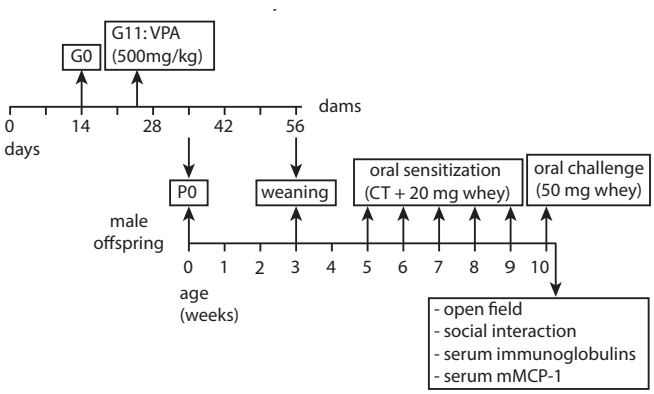

Fig. 1. Schematic overview of the experimental design.

Social interaction test

The behavioural assessment was adapted from a previous description (13, 42). Mice were placed in a 45 x 45 cm open field with a small perforated two Plexiglas cages (10 cm diameter) located against opposite walls allowing visual, olfactory and minimal tactile interaction. Mice were habituated to the open field for 5 min and an age- and gender-matched unfamiliar target mouse was introduced in one of the cages for an additional 5 min. Open fields were cleaned with water followed by 70 % ethanol after each test. By using video tracking software (EthoVision 3.1.16, Noldus, Wageningen, The Netherlands) an interaction zone around the cage was digitally determined. Time spent in the interaction zone, frequency of entries and latency until first occurrence in the interaction zone, as well as total distance moved were measured.

Open field test

Rodents naturally tend to explore the environment while avoiding open spaces. Mice that express anxiety-like behaviour tend to spend less time in the centre of the open field (43). The open field apparatus consisted of a 45 x 45 cm arena with a solid floor. After the mouse was placed in the centre of the arena, it was allowed to explore the environment for 5 min. By using Ethovision software, an inner zone was digitally determined at 10 cm from the walls. Post-acquisition analysis allowed measurement of latency of first occurrence, frequency of entries, and time spent in the inner zone, as well as total distance moved.

Measurements of whey-specific immunoglobulins and mouse mast cell protease-1

Blood was collected 16 h after oral challenge, centrifuged for 15 min at 14,000 rpm and serum was stored at -70 °C. Serum concentrations of whey-specific IgE, IgG1 and IgG2a were measured by means of ELISA. Microlon plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 20 µg/mL whey in carbonate/ bicarbonate buffer (0.05 M, pH = 9.6; Sigma-Aldrich) overnight at 4 °C. Plates were blocked in ELISA buffer (50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05 % Tween-20 and 0.5 % BSA in PBS) and serum samples were incubated for 2 h. Plates were incubated with biotinylated rat anti-mouse IgE, IgG1 and IgG2a (1 µg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 2 h and subsequently with streptavidin-HRP (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands) for 1 h. Plates were developed using o-phenylendiamine (Sigma-Aldrich) and reaction was stopped after 15 min with 4 M H_2SO_4. Absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Results were expressed as arbitrary units (AU), composed using a titration curve of pooled sera from whey and CT i.p. immunized mice serving as an internal standard. Concentration of mouse mast cell protease-1 (mMCP-1) in serum was determined using commercially available ELISA kits (BD Biosciences) according to the manufacturer's protocol.

Statistical analysis

Experimental results are expressed as mean ± S.E.M for behaviour as well as mMCP-1 results and scatter-dot plots for immunoglobulin levels. Two- and three-way interactions between groups were statistically determined using three-way ANOVAs. Two-way ANOVA post-hoc analysis was conducted by using Bonferroni's multiple comparisons tests. For serum immunoglobulin and mMCP-1 levels, log transferred data were used to obtain normality. Results were considered statistically significant when $P < 0.05$. Analyses were conducted using SPSS version 20 and GraphPad Prism, version 6.02.

RESULTS

The effect of *in utero* VPA exposure on the allergic response and mast cell activation

To investigate the effect of prenatal VPA exposure on food allergy, male mice fed the control or active diet were exposed to either VPA or PBS *in utero* and subsequently sham or whey-sensitized shortly after weaning. Sensitization with whey induced high levels of allergen-specific Th2-type IgE in mice compared to their respective sham-sensitized control groups, irrespective of VPA exposure and diet ($P < 0.001$ for all whey-sensitized groups, **Fig. 2A**). Exposure to VPA and the active diet did not significantly alter IgE levels in allergic mice. Compared to their respective sham-sensitized control groups, whey-specific Th2-type IgG1 levels were also significantly increased in serum of all allergic groups ($P < 0.001$ for all whey-sensitized groups, **Fig. 2B**). Neither VPA exposure nor dietary intervention significantly altered serum IgG1 levels.

Levels of Th1-type IgG2a were significantly increased in PBS-exposed allergic mice compared to their sham-sensitized control mice, irrespective of dietary intervention ($P < 0.001$ for both whey-sensitized PBS groups **Fig. 2C**). However, whey-specific IgG2a levels were not induced in VPA-exposed allergic mice fed the control diet and levels were significantly lower compared to PBS-exposed allergic mice fed the control diet ($P < 0.01$). When fed the active diet, serum IgG2a levels were significantly increased in VPA-exposed allergic mice ($P < 0.05$).

Levels of mMCP-1 were measured in serum as a marker of mucosal mast cell degranulation in the intestines. Levels of mMCP-1 were significantly increased in allergic mice compared to their sham-sensitized control mice, irrespective of dietary intervention and VPA exposure ($P < 0.01$ for whey-sensitized PBS-exposed mice and $P < 0.001$ for the other 3 whey-sensitized groups, **Fig. 2D**).

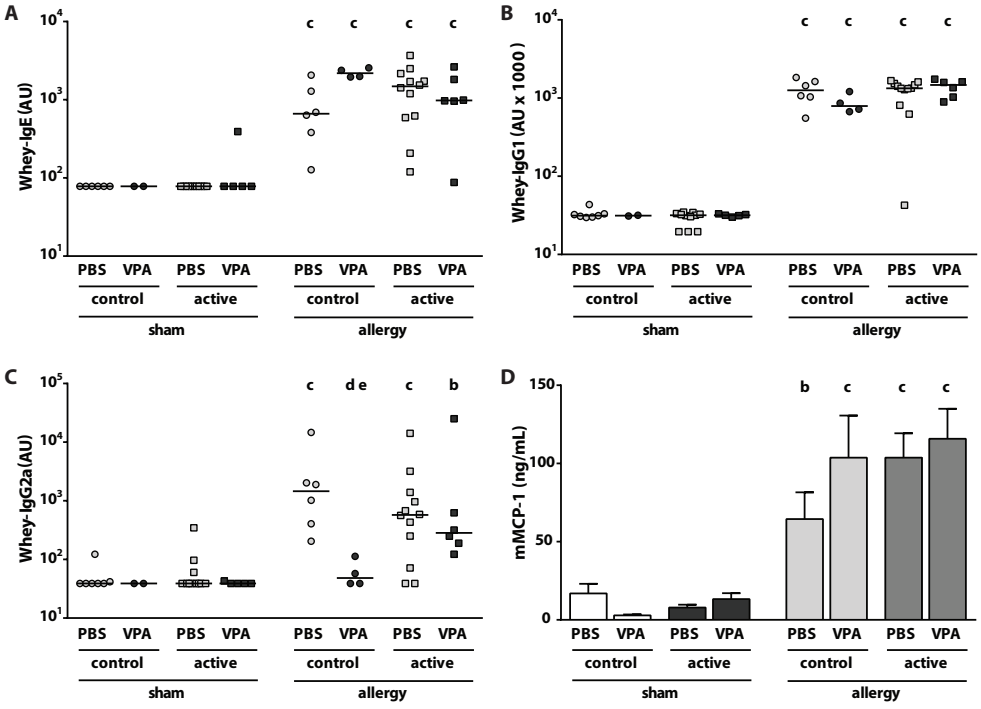

Fig. 2. Serum levels of whey-specific (**A)** IgE, (**B**) IgG1, (**C**) IgG2a and (**D**) mouse mast cell protease-1 (mMCP-1) in mice exposed to PBS or VPA *in utero*, fed either the control or active diet and sham or whey-sensitized. Three-way ANOVA analyses revealed a significant effect of food allergy ($P < 0.001$) on levels of IgE, IgG1, IgG2a and mMCP-1. Significant interactions were observed between VPA exposure * diet ($P < 0.05$) and VPA exposure * diet * allergy ($P < 0.05$) on IgG2a levels. b = $P < 0.01$; c = $P < 0.001$, compared to its respective sham-sensitized control group; d = $P < 0.01$, compared to PBS-exposed allergic mice fed the control diet; e = $P < 0.05$, compared to VPA-exposed allergic mice fed the active diet. Data are presented as scatter dot plot on log scale for immunoglobulin levels and as mean ± S.E.M. for mMCP-1 levels.

The effect of food allergy and *in utero* VPA exposure on social and anxiety-like behaviour

Social behaviour was assessed as previously described (13), by measuring the amount of time spent near an unfamiliar gender- and age-matched target mouse. Both for latency of first approach ($P < 0.01$, **Fig. 3C**) and frequency of entrance in the interaction zone ($P < 0.05$, **Fig. 3D**), significant two-way interactions were observed between VPA exposure and diet in a three-way ANOVA. Latency was significantly increased in mice exposed to VPA *in utero* compared to PBS-exposed mice fed the control diet ($P < 0.001$). The active diet was able to significantly reduce latency in VPA-exposed mice ($P < 0.01$). Food allergy did not further reduce social behaviour in mice exposed to VPA *in utero*. Distance moved was significantly affected by VPA exposure ($P < 0.001$) in a three-way ANOVA. This observation was most pronounced in VPA-exposed food allergic mice that were fed the control diet ($P < 0.05$). Pre- and postnatal intervention with the active diet did not affect mobility.

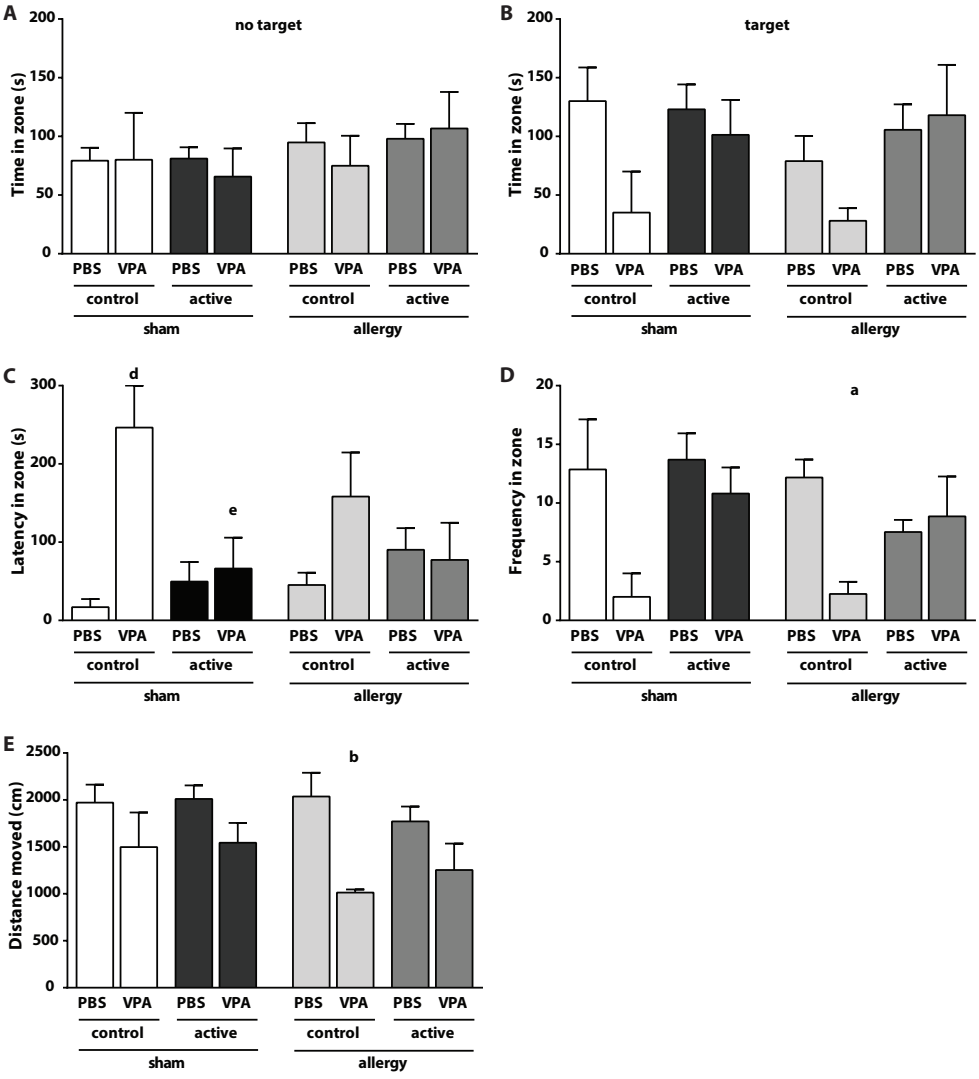

Fig. 3. Social behaviour of mice exposed to PBS or VPA *in utero*, fed either the control or active diet and sham or whey-sensitized. (**A**) While time spent in the interaction zone was not different in absence of a target mouse, (**B**) a trend towards an effect of diet ($P < 0.06$) and VPA exposure ($P < 0.09$) was observed on time spent in the interaction zone in presence of a target. (**C**) Latency of first entrance was significantly affected by VPA exposure ($P < 0.001$), almost by diet ($P < 0.07$) and a significant VPA exposure x diet interaction was observed ($P < 0.01$). (**D**) A significant effect of VPA exposure ($P < 0.01$) and a two-way interaction was observed between VPA exposure x diet ($P < 0.05$) for frequency of entrance. (**E**) Distance moved was significantly affected by VPA exposure ($P < 0.001$). a = $P < 0.10$ (trend), b = $P < 0.05$, and d = $P < 0.001$, compared to its respective PBS-exposed control group; e = $P < 0.01$, compared to VPA-exposed sham-sensitized mice fed the control diet. Data are presented as mean ± S.E.M. and three-way ANOVAs were conducted.

Anxiety-like behaviour was studied in the open field by measuring the amount of time spent in the inner zone of the field. Three way ANOVAs revealed a significant effect of exposure to VPA *in utero* on time spent in the inner zone ($P < 0.01$, **Fig. 4A**), latency of first occurrence ($P < 0.05$, **Fig. 4B**), frequency of entrance ($P < 0.001$, **Fig. 4C**), and mobility ($P < 0.001$, **Fig. 4D**). Post-hoc analysis resulted in a significant increase in latency ($P < 0.05$) and decrease in mobility ($P < 0.05$) in allergic VPA-exposed mice compared to PBS-exposed allergic mice fed the active diet. Moreover, frequency was significantly increased in VPA-exposed mice fed the active diet ($P < 0.05$) and allergic VPA-exposed mice fed the control diet ($P < 0.05$), compared to their respective PBS-exposed controls. Neither diet nor food allergy significantly affected any of the measurements in the open field test.

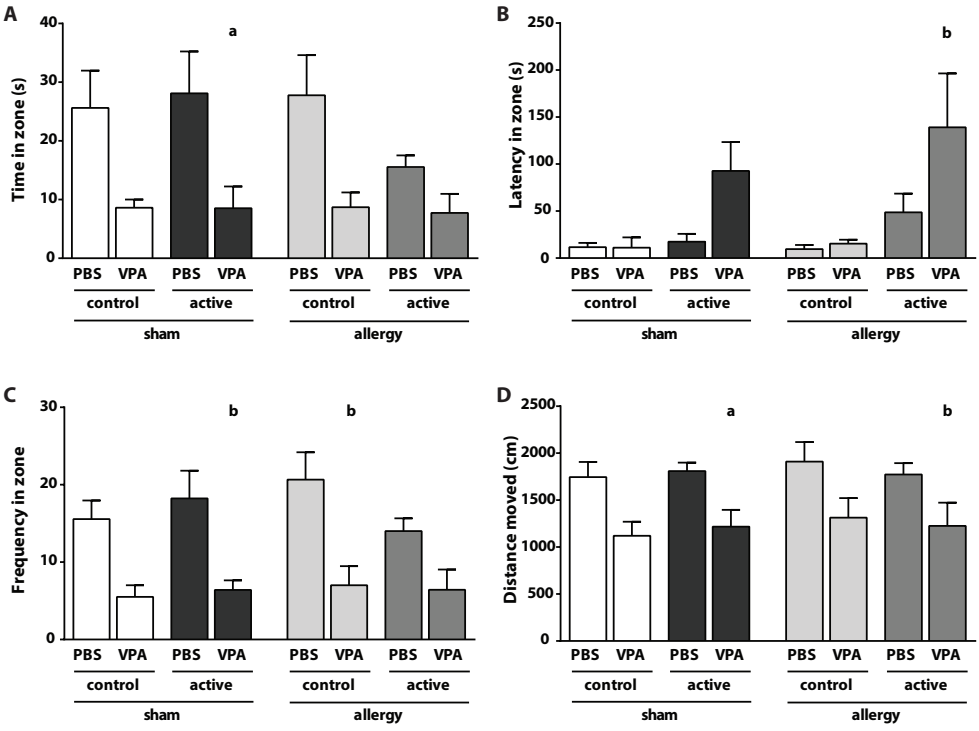

Fig. 4. Anxiety-like behaviour of mice exposed to PBS or VPA *in utero*, fed either the control or active diet and sham or whey-sensitized. Three-way ANOVAs revealed a significant effect of VPA exposure on (**A**) time spent in the inner zone ($P < 0.01$), (**B**) latency of first occurrence ($P < 0.05$), (**C**) frequency of entrance ($P < 0.001$) and (**D**) mobility ($P < 0.001$). An additional effect of diet was observed in latency of first occurrence in the inner zone ($P < 0.01$). a = $P < 0.10$ (trend); b = $P < 0.05$ compared to its respective PBS-exposed control group. Data are presented as mean ± S.E.M.

DISCUSSION

To investigate the interplay between prenatal VPA exposure and food allergy, male mice fed the control or active diet were exposed to either VPA or PBS *in utero* and subsequently sham or whey-sensitized during postnatal development. The experiment resulted in a low number of offspring in some of the control fed groups ($n = 2$ and $n = 4$ in sham-sensitized and whey-sensitized VPA-exposed groups, respectively). Therefore, results should be interpreted with care and this experiment should be regarded as a pilot experiment.

VPA exposure reduced social behaviour and food allergy did not further decrease levels of social behaviour in mice exposed to VPA *in utero*. However, exposure to VPA *in utero* reduced time, latency and frequency in the interaction zone to such low levels, that a floor-effect may have hindered the observation of further reductions in social behaviour.

In agreement with previous observations (44), pre- and postnatal dietary intervention with the active diet restored impaired social behaviour of VPA-exposed mice. However, pre- and postnatal nutritional intervention with the active diet was not able to ameliorate anxiety-like behaviour in mice exposed to VPA *in utero* and also mobility was not enhanced by the active diet in these mice. These results indicate that the active diet is able to beneficially and selectively affect social behaviour in mice exposed to VPA *in utero* with and without food allergy.

Allergic sensitization, assessed by increased levels of serum Th2-type IgE and IgG1, as well as the allergic response, assessed by mMCP-1 levels, were increased in all groups of whey-sensitized mice. On the contrary, increased serum levels of Th1-type IgG2a in allergic mice were not observed in mice that had been exposed to VPA *in utero*. Immunoglobulin class switching to IgG2a in B cells of mice is induced by Th-1 type cytokine IFN-γ (45). In rats prenatally exposed to VPA it was observed that splenocytes showed a decreased proliferative response to concanavalin A (ConA) and a reduced IFN-γ/IL-10 ratio (46). This indicates that the Th1 immune response in animals exposed to VPA *in utero* indeed may be dampened, making the individual more vulnerable to infections. Moreover, skewing of the immune response away from Th1 and towards Th2, can lead to reduced tolerance towards harmless antigens, triggering or exacerbating allergic sensitization and the subsequent allergic response after challenge. Although not significantly different, serum levels of mMCP-1 and IgE tended to be higher in VPA-exposed mice compared to PBS-exposed mice.

Imbalance of the ratio of Th1 to Th2 has been implicated in patients with ASD. Both increased and decreased Th1/Th2 ratios have been identified repeatedly (14, and ASD patients do not seem to have a consistent polarization of the immune system towards either Th1 or Th2. To our knowledge, ratios of Th1/Th2 have not been investigated in a specific subpopulation of ASD patients that were prenatally exposed to teratogens such as VPA. Transcription factor GATA-3 is crucial for T cell differentiation into the Th2 lineage and is therefore one of the important transcription factors in the regulation of the Th1:Th2 balance (47). Interestingly, it was shown that neuron-like cells incubated *in vitro* with VPA expressed increased protein levels of transcription factor GATA-3 (48). At day 11 of gestation, diffuse expression of GATA-3 is observed throughout the murine brain, which is crucial for brain development (49). GATA-3 is particularly important in the formation of serotonergic neurons in the raphe nuclei, in a gradient of increasing requirement from rostral to caudal (50). The raphe nuclei release 5-HT in many different regions in the brain, including amygdala and PFC (51). Absence of GATA-3 caused disrupted organization and morphology as well as reduced numbers of serotonergic neurons in caudal raphe nuclei (50, 52). In line with the hypothesis that VPA increases GATA-3 expression, numbers of serotonergic neurons were increased in the caudal nuclei of rats exposed to VPA *in utero* (53). Another study observed a shift of distribution of 5-HT neurons from dorsal to caudal raphe nuclei in VPA-exposed rats (54). Therefore, enhanced expression of transcription factor GATA-3 in response to teratogens such as VPA may impair development of both the nervous system and the immune system. However, this would require further investigation and it would be interesting to determine GATA-3 expression in brains and peripheral organs of food allergic mice exposed to VPA *in utero*.

In conclusion, this pilot study demonstrates that mice exposed to VPA *in utero* do not induce high serum levels of antigen-specific Th1-type IgG2a upon allergic sensitization to whey protein. Moreover, a multi-nutrient diet containing specific anti-inflammatory and neuroprotective ingredients prevented diminished IgG2a levels in VPA-exposed allergic mice. These findings add to the hypothesis that immune dysfunction is common in patients with ASD and this model may open a new window for opportunities to investigate the underlying mechanism behind immune dysfunction in neurodevelopmental disorders.

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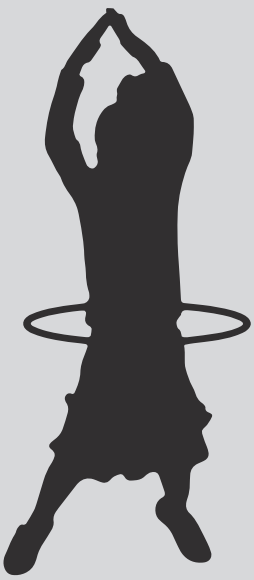
CHAPTER ELEVEN



Summarizing discussion

This thesis describes preclinical findings on intestinal disturbances in a mouse model for autism spectrum disorder (ASD) and, vice versa, reports the behavioural and neurochemical consequences of intestinal allergic immune activation in mice. Moreover, several opportunities for nutritional intervention in the prevention or early treatment of intestinal and behavioural dysfunction in these models were investigated. Here, we summarize the major findings of this thesis and hypothesize on its potential translational impact for the prevention and/or treatment of certain neurodevelopmental disorders.

INTESTINAL DISTURBANCES IN A MOUSE MODEL FOR ASD

The occurrence of gastrointestinal problems in patients with ASD is repeatedly reported and includes chronic constipation, diarrhoea and abdominal pain (reviewed in **chapter 2**). These symptoms have been attributed to changes in the gut microbiome (1, 2), increased intestinal permeability (3), intestinal inflammation (4), and food allergy (5). Although evidence is emerging, there is still considerable debate regarding the co-morbidity of gastrointestinal disturbances and ASD. **Chapter 3** demonstrates that intestinal inflammation is present in a well-established mouse model for ASD, using *in utero* exposure to valproic acid (VPA). Prenatal VPA exposure induced epithelial loss in the distal part of the ileum, which was accompanied by increased neutrophil infiltration and diarrhoea-like stools. Interestingly, both behavioural abnormalities and intestinal disturbances were observed exclusively in male VPA-exposed offspring. This is in line with the sex bias in ASD patients, where males are three times more likely to develop ASD (6). The cause of this bias is unknown, but has been attributed to sex-specific brain development and hormones, as well as to (epi-) genetic effects on the X-chomosome (7, 8). Further investigation of the male-specific deficits observed upon *in utero* exposure to VPA in mice may not only provide insight into potential causes of the male preponderance in ASD, but also provide a better understanding of the general underlying pathophysiology.

No consensus has yet been reached on the prevalence of patients with ASD suffering from co-morbid gastrointestinal problems. The prevalence has been reported to be between 10 - 90 % (**chapter 2**) and it is therefore unreasonable to further speculate on the amount of ASD patients suffering from intestinal discomfort. Diagnosis of ASD is based on behavioural observations and no biomarkers have been identified yet. In my opinion, it is plausible that multiple aetiologies and multiple developmental or neurochemical deficiencies lead to the same clinical observation of ASD. The male-specific intestinal disturbance observed upon VPA exposure may indicate that intestinal abnormalities are also sex-dependent. To gain more insight into the subtypes of ASD that are associated with gastrointestinal dysfunction, it would be of interest to investigate intestinal morphology in other models for ASD, for example in mice with targeted mutations in genes associated with ASD or in models using maternal immune over-activation.

Intestinal microbiome

In addition to an inflammatory status of the intestinal tract, distinct microbiome composition and activity were observed in mice after *in utero* exposure to VPA (**chapter 4**). VPA exposure increased composition of bacteria within the phylum of Firmicutes, mainly at the expense of bacteria within the phylum of Bacteroidetes. This is in line with clinical studies, in which a compositional dysbiosis was found in patients with ASD marked by an increased ratio of Firmicutes to Bacteriodetes (9). Furthermore, based on microbiome composition in our study, clustering of samples was observed not only by treatment and but also by gender. Comparing male and female offspring of VPA-exposed dams revealed a different microbiome profile. Genera of *Alistipes*, *Enterorhabdus*, *Mollicutes* and *Erysipelotrichalis* were more prominent in male VPA-exposed offspring and a male-specific increase in caecal levels of short chain fatty acid butyrate was observed after VPA exposure. Butyrate is a product of bacterial dietary fibre fermentation and levels are found to be increased in faeces of patients with ASD (10).

Altered microbiome composition may be a consequence of intestinal inflammation. This is supported by the observation that abundance of some microbial bacteria correlated to levels of neurotrophil infiltration (**chapter 4**). Closely related to the findings in **chapter 3** and **4**, a recent publication in Cell showed increased intestinal permeability and altered microbiome composition in a mouse model for ASD using maternal immune activation (11). In this publication, intestinal permeability and microbiome composition, as well as a subset of behaviours, were restored upon treatment with *Bacteriodes fragillis*. This implies that changes in composition of the intestinal microbiome may contribute to impaired behaviour in offspring exposed to an immune challenge *in utero*. Therefore, the altered microbiome composition in our study may also have contributed to the behavioural changes observed in mice exposed to VPA *in utero*. Investigating the behavioural effects of microbiome modulation using pre-, pro- or antibiotics in VPA-exposed offspring would provide further insights in this regard.

Involvement of serotonin

In **chapter 3** it is demonstrated that serotonin (5-HT) levels were reduced in the distal ileum as well as in the prefrontal cortex (PFC) and the amygdala of mice exposed to VPA *in utero*. Reduced 5-HT levels in the distal ileum were accompanied by reduced numbers of enterochromaffin (EC) cells in the epithelial lining. Levels of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) were increased in the brain, but not in the distal ileum. This indicates that reduced ileal 5-HT levels were not caused by increased 5-HT metabolism in the distal ileum. Approximately 90 % of intestinal 5-HT is produced by EC cells (12). Since depletion of neuronal 5-HT does not significantly reduce intestinal 5-HT tissue levels (13), reduced 5-HT levels are most likely caused by changes in EC cells. Numbers of EC cells have been shown to depend on gut microbiota (14) and the intestinal immune system (15). EC cells differentiate from pluripotent stem cells in the epithelial lining of the crypt (16). Although neuronal 5-HT promotes proliferation and turnover of the intestinal epithelium (17, 18), the effects of neuronal 5-HT on EC cell differentiation have not been examined.

Prenatal exposure to VPA was shown to impair the distribution of serotonergic neurons in the brain of rat offspring (19-21) and differentiation of serotonergic neurons *in vitro* (21). Serotonergic neurons are one of the first to arise in the developing enteric nervous system and regulate the development of subsequent enteric neuronal subtypes (22). In addition to its function as a neurotransmitter (23), enteric neuronal 5-HT is important in intestinal motility (24) and epithelial homeostasis (17, 18, 25). VPA may affect both central and enteric serotonergic neurons, thereby disturbing central serotonergic neurotransmission and intestinal homeostasis. Examining the effect of *in utero* VPA exposure on morphology of the enteric serotonergic neurons may provide insight into the pathophysiology of co-morbid signs of gastrointestinal deficiencies in patients with ASD.

In **chapter 5** we have investigated the serotonergic system throughout the intestinal tract in the VPA-induced murine model of ASD and observed that 5-HT levels were reduced in jejunum, ileum and colon. As mentioned in **chapter 3**, the decrease in 5-HT levels in the distal ileum was not caused by increased turnover but rather by decreased EC cell numbers. However, in the other regions of the intestines, increased 5-HIAA levels were observed. Therefore, the decrease in 5-HT levels may be caused by either or both reduced EC cell numbers and increased turnover of 5-HT. Intestinal inflammation was observed specifically in the distal ileum of VPA-exposed mice. As an inflammatory state generally reduces turnover of 5-HT, this may explain why 5-HIAA levels were not increased in the distal part of the ileum.

Once released, 5-HT is either transported to the portal system and taken up by platelets or transported into surrounding epithelial cells by means of the 5-HT reuptake transporter (SERT) and subsequently metabolized by monoamine oxidase (MAO) to 5-HIAA. Increased activity of SERT was reported in the amygdala of rats exposed to VPA on gestational day G12.5 (26), but not on G9 (27). Furthermore, VPA increased MAO catalytic activity and mRNA expression in neuronal cells *in vitro* (28). Therefore, VPA may also affect 5-HT reuptake or metabolism, for example by epigenetic modification of gene expression, given the reported ability of VPA to inhibit histone deacetylase (29). This would not only affect serotonergic neurons, but all cells containing 5-HT, including enterochromaffin cells, immune cells, and platelets, hence disturbing the immune system and intestinal homeostasis. Studying the effects of selective serotonin reuptake inhibitors (SSRIs) on VPA-induced deficits in behaviour as well as in central and enteric serotonin systems would provide answers on the involvement of SERT in serotonergic abnormalities in this model.

In patients with ASD, abnormalities of the serotonergic system are frequently reported. An extensive number of studies consistently observed hyperserotonemia (elevated platelet 5-HT content) in approximately 30 % of individuals with ASD (30). Possible explanations for these elevated levels are age- and brain region-dependent changes in 5-HT synthesis (31, 32) as well as altered 5-HT receptor binding (33) and increased SERT activity (34). Short-term dietary depletion of tryptophan was shown to exacerbate repetitive behaviour and to increase anxiety in ASD patients (35). Conversely, limited evidence suggests that SSRIs may be effective in improving behaviour in some but not all individuals with ASD (36). In **chapter 5**, we have observed that VPA-induced serotonergic deficiencies in the brain and intestine were highly correlated. Therefore, it may be of relevance to investigate whether there is a subtype of ASD patients with hyperserotonemia that suffer from intestinal problems mediated by 5-HT. By implementing personalized medicine approaches, this subtype of ASD patients may respond well to serotonin system-targeted treatments.

BEHAVIOURAL AND NEUROCHEMICAL CONSEQUENCES OF FOOD ALLERGY

Intestinal allergic immune activation is suggested to exacerbate psychological traits in patients with neurodevelopmental disorders (as reviewed in **chapter 6**). Supporting the hypothesis that food allergy can affect mental disorders of psychosocial relevance, diagnosis of food allergy in the first year of life was associated with enhanced internalizing behaviour in children (37). Gluten and milk protein free diets are suggested to improve autistic behaviour and to restore the increased intestinal permeability in ASD children. However, fundamental evidence remains elusive. In **chapter 7**, we demonstrate that an IgE-mediated allergic immune response in the intestinal tract of mice induced shortly after weaning is associated with disturbed social behaviour. Although these mice did not have any signs of sickness (weight loss, pilo-erection, or reduced mobility), it may be argued that reduced social interaction was a general result of 'feeling unwell'. We therefore conducted the same social behaviour test in a mouse model for colitis and observed that, although DSS-induced colitis mice do express sickness behaviour and move less in the open field, their social interaction was not affected (supplementary data of **chapter 7**). In addition to the consideration that reduced social behaviour of food allergic mice is not likely the result of sickness behaviour, it implies that reduced social behaviour is specific to an allergic immune response in the intestines.

Food allergy in mice also increased self-grooming behaviour, indicative for repetitive behaviour (**chapter 7**). Because LPS-induced sickness behaviour is accompanied by reduced self-grooming (38), this is another indication that food allergy-induced changes in behaviour are not a general result of sickness behaviour. Furthermore, food allergy reduced spontaneous alternation in the T maze test. Although this test is mainly used to investigate spatial memory, in my opinion this test also implies a diminished willingness to explore novel environmental stimuli (39, 40) and increased repetitive behaviour (41, 42). Food allergy, however, was shown not to affect anxiety-like behaviour in mice (**chapter 10** and unpublished data). In summary, **chapter 7** demonstrates that a food allergic reaction in the intestines of mice induced impaired social and repetitive behaviour, relevant to some core symptoms of ASD, without affecting levels of anxiety.

Food allergy-induced neurochemical changes

Pronounced changes in the mesocorticolimbic dopamine (DA) system were observed in food allergic mice (**chapter 7**). Levels of DA and its metabolites 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were decreased in the PFC and increased in the amygdala of allergic mice. While multiple neural systems undoubtedly underlie social-emotional behaviours, the mesocorticolimbic DA system seems a crucial pathway involved (43). This mesocorticolimbic DA system originates in the ventral tegmental area (VTA) of the midbrain and projects to various forebrain regions including PFC, amygdala, and nucleus accumbens. Social interaction in rodents

is accompanied by increased DA levels in the PFC (44, 45) and depletion of DA in the PFC markedly reduced social interactions (46). Furthermore, activation of the amygdala during emotional processing was inhibited by DA (47) and reduced amygdala functioning was suggested in ASD (48). Evidence for involvement of the mesocorticolimbic DA system in the pathophysiology of ASD is limited, although children with ASD displayed reduced levels of DA in the medial PFC (49). Dampening of the DA system in the PFC was observed also in a fragile X mouse model for ASD (50). Moreover, abnormal vagal functioning was shown to inhibit the DA system in various brain regions including PFC (51), indicating that vagal input can affect the mesocorticolimbic DA system.

In addition to an altered dopaminergic system, marked neuronal activation was observed in the lateral and ventral regions of the orbital PFC (oPFC) of food allergic mice after exposure to a social target (**chapter 7**). The oPFC is involved in cognitive processing of decision making in response to emotional stimuli that can have a rewarding or punishing value. Therefore, it is also important in guiding social-emotional behaviour (52, 53). Patients with orbitofrontal lesions have impaired abilities to recognize and interpret emotional expressions and to respond in a socially proper manner (54-57). In line with our observations, patients with ASD showed increased oPFC activation in response to tasks involving facial recognition (58), motor function (59), and attention (60). Overall, food allergy-induced changes in dopaminergic activity and neuronal activation in the PFC of mice may underlie their impaired social and repetitive behaviour and if these neurochemical responses to food allergy also occur in humans, they may exacerbate behavioural deficits in patients with ASD.

Involvement of serotonin

A food allergic reaction increased levels of 5-HT in the ileum of mice (**chapter 7**). This increase in 5-HT levels was accompanied by an increased number of intestinal EC cells and decreased levels of 5-HIAA in the ileum. Reduced levels of 5-HIAA may be explained by reduced levels of extracellular 5-HT or reduced SERT activity. A pro-inflammatory environment in the intestine was shown to decrease SERT activity, both *in vitro* (61, 62) and *in vivo* (63, 64). Increased levels of intestinal 5-HT, EC cell hyperplasia and reduced levels of 5-HIAA were observed also in intestinal biopsies of patients with coeliac disease (63) and IBD (64). These observations, however, are not always present in the inflamed bowel, as also reduced 5-HT levels and EC cell numbers have been observed in IBD (65).

Both enterochromaffin cells (66) and mast cells (67) release 5-HT in close proximity to afferent neurons. Vagal afferents respond to secreted 5-HT via 5-HT_3 receptors by signalling to the nucleus tractus solitarius (NTS) and hypothalamic paraventricular nucleus (PVN) (68). Food allergy-induced visceral nociception is mediated via 5-HT receptor signalling on vagal afferents (69). Moreover, food allergy-induced neuronal activation in rat brains was shown to be reduced after blockade of 5-HT_3 receptors and vagotomy (70). This

suggests that food allergy signals to the brain via increased release of intestinal 5-HT, binding to 5-HT_3 receptors on vagal afferents. However, DSS-induced colitis mice did not show impaired social behaviour, while increased 5-HT release has been reported in these mice (71). Moreover, in **chapter 9** we demonstrate that preventing social and repetitive behaviour in food allergic mice did not prevent changes in 5-HT and metabolite levels in ileum of food allergic mice. Therefore, immune signalling to brain regions relevant for social behaviour may be mediated by a combination of various allergy-induced signalling molecules, including 5-HT. For example, IgE can also bind to the functional high-affinity FcεRI on enteric neurons (72), resulting in an antigen-specific neuronal stimulation (73). Investigating behavioural responses to food allergy in vagotomized mice will confirm whether or not the vagus nerve is involved the behavioural changes. Alternatively, spinal afferent neurons can convey information from the gut to the brain and signalling at the blood-brain barrier has also been demonstrated upon peripheral immune activation (as reviewed in **chapter 2**).

Integrating food allergy in a model for disturbed neurodevelopment

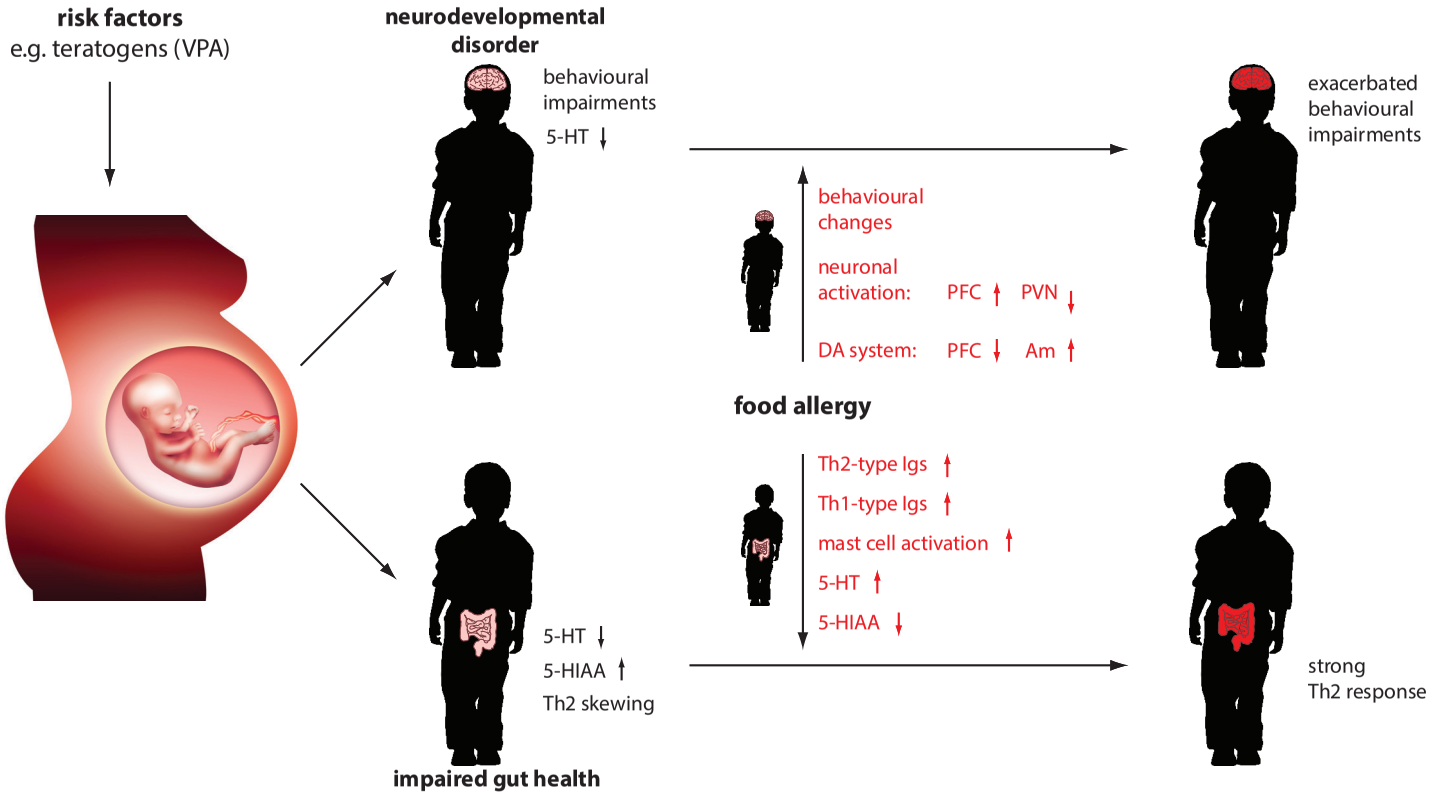
It is hypothesized that patients with neurodevelopmental disorders are more likely to develop allergic diseases and that the allergic response may exacerbate the behavioural deficits. To investigate this hypothesis in a preclinical design, mice exposed to VPA *in utero* were sensitized to cow's milk protein shortly after weaning (**chapter 10**). Food allergy did not exacerbate social behaviour in mice exposed to VPA *in utero*. However, it is important to note that exposure to VPA *in utero* reduced the time spent in the interaction zone to such low levels, that there may have been no window left to observe further reductions in social behaviour. Other social behaviour tests, such as the 3 chamber apparatus (74), may serve as a more sensitive tool to investigate the effect of food allergy on behaviour in VPA-exposed mice.

Conversely, *in utero* exposure to VPA did alter allergic sensitization to whey protein. Allergic sensitization to whey protein typically induces high serum levels of whey-specific Th2-types IgE and IgG1 and Th1-type IgG2a in mice. However, sensitization to whey did not lead to increased IgG2a levels when mice were exposed to VPA *in utero*. Immunoglobulin class switching to IgG2a in murine B cells is induced by Th1-type cytokine IFN-γ (75). In accordance with our observation, a reduced IFN-γ response was observed in splenocytes of rats exposed to VPA *in utero* (76). This indicates that the Th1-mediated immune response in animals exposed to VPA *in utero* indeed may be dampened, making the individual more vulnerable to infections. Moreover, skewing of the immune response away from Th1 and towards Th2, can lead to reduced tolerance towards harmless antigens, triggering or exacerbating allergic sensitization. Polarization of the immune response away from Th1 may affect other T cell subsets, such as regulatory T cells (Treg) or Th17 and Th9 cells, as well, but this would require further investigation.

Transcription factor GATA-3 is crucial for T cell differentiation into the Th2 lineage and is therefore one of the important transcription factors in the regulation of the Th1/Th2 balance (77). Interestingly, it was shown that neuron-like cells incubated *in vitro* with VPA expressed increased protein levels of transcription factor GATA-3 (78). At day 11 of gestation, diffuse expression of GATA-3 is observed throughout the murine brain, which is crucial for brain development (79). GATA-3 is particularly important in the formation of serotonergic neurons in the raphe nuclei, in a gradient of increasing requirement from rostral to caudal (80). Absence of GATA-3 caused disrupted organization and morphology as well as reduced numbers of serotonergic neurons in these nuclei (80, 81). In line with the hypothesis that VPA increases GATA-3 expression, distribution of 5-HT neurons was shifted from dorsal to caudal raphe nuclei in rats exposed to VPA *in utero* (21, 26). The raphe nuclei release 5-HT in many different regions in the brain, including amygdala and PFC (82) and changes during development, therefore, can have long lasting effects throughout the brain. In postmortem brains of patients with ASD, increased presence of serotonergic axons in 3 major 5-HT pathways was observed as compared to age-matched controls (83). In summary, epigenetic alteration of GATA-3 expression in response to teratogens such as VPA may impair development of both the nervous system and the immune system. Analyzing GATA-3 expression in the developing and postnatal brain of mice exposed to VPA *in utero* would provide knowledge on the involvement of GATA-3 in VPA-induced neurodevelopmental deficiencies. Moreover, if VPA also increases GATA-3 expression in T cells, it may underlie the pathophysiology of immune disturbance induced by VPA in mice. Hence, impaired GATA-3 expression may explain the association between immune disturbances and impaired neurodevelopment in humans.

Imbalances in Th1 and Th2 cytokines have repeatedly been observed in patients with ASD (as reviewed in **chapter 2**). Although the majority of papers reported a Th2-driven immune response, increases in Th1:Th2 ratios have also been identified in ASD patients. Furthermore, increased levels of Th17 cytokines (84) and diminished numbers of Tregs (85) were found in these patients. Hence, no consistent polarization of the immune system is observed, which may result from the heterogeneity between subjects with ASD. To our knowledge, T cell polarization has not been investigated in specific subpopulations of ASD patients, for example those exposed to environmental risk factors *in utero*. Expression levels of GATA-3 have never been investigated in patients with ASD and may provide additional information about immune system polarization in ASD individuals.

Overall, we hypothesize that maternal risk factors for impaired neurodevelopment reduce gut health and alter allergic immune responses, which may predispose the individual for the development of allergic diseases. Moreover, a food allergic response alters behavioural and neurochemical processes relevant to ASD, which may exacerbate behavioural deficits in patients with ASD (**Summarizing Fig. 1**).



Summarizing Fig. 1. Proposed mechanism of the gut-brain axis in neurodevelopmental disorders. Maternal risk factors alter the development of the foetus *in utero*, affecting not only the brain, but also the intestinal tract. Hence, neurodevelopmental disorders in the newborn are accompanied by impaired gut health and thus intestinal problems. Moreover, the immune system is skewed away from Th1, predisposing an individual to develop Th2-mediated allergic immune responses. A food allergic reaction in the intestines induces behavioural and neurochemical changes in the brain that are relevant to ASD. Although not observed in our studies, food allergy may therefore exacerbate behavioural impairments in individuals with neurodevelopmental disorders, such as ASD.

NUTRITION, A GUT FEELING

Brain development is a vulnerable process because of its rapid trajectory. Many nutrients are essential components of the brain and therefore affect neuroanatomy during foetal and early life. Nutrients affect not only neuroanatomy, but also neurochemistry and neurophysiology. Neurochemical alterations include changes in neurotransmitter synthesis, receptor synthesis, and neurotransmitter reuptake mechanisms (86). Various nutritional components can regulate these processes via epigenetic mechanisms that alter gene activity without changing the genetic code of the DNA. The effects of nutrition on epigenetic processes such as DNA methylation and histone acetylation have been extensively reviewed (87, 88).

First evidence for the importance of nutrition in brain development derives from a series of studies conducted in individuals born during the Dutch Hunger Winter, between 1944 and 1945. Several studies reported an association between maternal exposure to famine and two main neurodevelopmental findings: neural tube defects and schizophrenia (89). Since then, deficits in nutrient supply, both during pre- and postnatal development, have been shown to affect neurodevelopment and subsequently behaviour (86). Resulting from these studies, there is a current scientific interest in the use of dietary supplementation in the prevention or early treatment of neurodevelopmental disorders.

In **chapters 5** and **9** the effects of a multi-targeted nutritional intervention on behaviour are investigated in mouse models for autism and food allergy, respectively. The diet was composed of anti-inflammatory and neuroprotective ingredients, aimed to beneficially affect introvert behaviour. Results obtained from food allergic mice indicated that the specific multi-nutrient diet did not affect allergic sensitization or the allergic response (**chapter 9**). Nevertheless, the multi-nutrient diet normalized behavioural impairments in both individual models (**chapter 5** and **9**). In addition to beneficial effects on social behaviour of mice exposed to VPA *in utero*, serotonergic deficiencies were restored both in brains and intestines. In **chapter 8** it is demonstrated that nutritional supplementation with n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) prevented food allergy-induced reductions in social behaviour as well as impairment in dopaminergic activity in the PFC and serotonergic activity in the intestines.

Components of the specific multi-nutrient diet, such as n-3 LCPUFA (eicosapentaenoic acid; EPA and docosahexaenoic acid; DHA), are suggested to beneficially affect neurodevelopmental outcomes in the newborn (90, 91). LCPUFA in the brain act on physiological functions via several mechanisms. LCPUFA are incorporated in membrane-based phospholipids of neural tissue modifying membrane integrity and fluidity (92, 93). Functioning of transmembrane proteins, such as receptors and transporter proteins, is affected by membrane fluidity (94, 95). As a consequence, monoaminergic

neurotransmission alters upon changes in nutritional composition of fatty acids (96). For example, a diet high in n-3 LCPUFA was shown to significantly increase prefrontal DA levels in rats compared to a diet high in n-6 LCPUFA (97). This is in line with our observation that supplementation of n-3 LCPUFA restored decreased DA and metabolite levels in PFC of food allergic mice (**chapter 9**).

Maternal intake of vitamins was also associated with neurodevelopmental outcomes and ASD (98-100). Folate and vitamin B12 are required for methylation of DNA, proteins, phospholipids and neurotransmitters (101). Animal studies demonstrate that supplementing the maternal diet with methyl donors alters epigenetic regulation of gene expression in their offspring (102). Moreover, it has been suggested that vitamin supplementation during pregnancy may reduce the risk of ASD in the newborn (98). Nevertheless, recent meta-analysis failed to identify conclusive evidence for a long-term benefit of maternal LCPUFA or folic acid supplementation on neurodevelopment (103, 104).

In addition to LCPUFA and vitamin supplementation, non-digestible oligosaccharides were part of the multi nutrient diet: galacto-oligosaccharides (GOS), fructo-oligosacchardes (FOS) and rice fibres. Administration of GOS and FOS to rats was recently shown to increase levels of brain-derived neurotrophic factor (BDNF) in the dentate gyrus of the hippocampus (105). As BDNF signalling is critical for neuronal protection, survival and plasticity (106), this study may evoke further investigation of GOS and FOS supplementation on brain development and neuroprotection.

LCPUFA and dietary fibers are thought to modulate gut microbiome composition as well as the intestinal immune system (107, 108). Already during the perinatal period, supplementation with GOS/FOS was able to increase colon length (109) and enhance tolerance-related immunoglobulin levels as well as gut barrier functions (110) in healthy mice. Moreover, the rice fibre used in the multi-nutrient diet was previously shown to reduce inflammation and restore 5-HT levels in a mouse model for colitis (111). Therefore, LCPUFA and dietary fibres in the active multi-nutrient diet may improve intestinal (immune) homeostasis.

In summary, the results of these studies imply that the multi-nutrient diet may benefit in the prevention or early treatment of impaired social behaviour in mice, induced by prenatal exposure to VPA or postnatal allergic sensitization. This diet is likely to target multiple pathways in which VPA or food allergy may disturb neurodevelopment and neurochemical processes. Identifying the individual effects of the active components would require further extensive research subdividing components and investigating multiple combinations to identify synergistic effects. Although the effect of the multi-nutrient diet look promising in the studies described in this thesis, it is too soon to speculate on the potential effects of this diet in the treatment of patients with neurodevelopmental disorders. For example, timing of dietary intervention, more specifically during gestation, lactation, or later in life, would examine the opportunity for this diet to treat behavioural deficits. Moreover, it may provide important information on the mechanism by which this multi-nutrient diet may beneficially affect behaviour.

OVERALL CONCLUSION

This thesis provides evidence for the occurrence of intestinal inflammation upon impaired neurodevelopment induced by exposure to VPA *in utero* and postulates serotonergic dysfunction as a potential common underlying cause. These data support the findings of intestinal dysfunction in patients with ASD and it would be of interest to further investigate the function of the serotonergic system in this subset of ASD patients. A nutritional intervention containing specific anti-inflammatory and neuroprotective ingredients may be effective in the prevention or early treatment of disturbed social behaviour induced by *in utero* exposure to VPA. Hence, this diet may be effective as supplementation during pregnancy or early in life, when a child is at risk of developing neurodevelopmental disorders, for example when mothers use teratogens such as VPA.

Furthermore, this thesis demonstrates that food allergy reduces social and repetitive behaviour as well as it alters the mesocorticolimbic dopamine system and neuronal activation in the PFC and the PVN. A nutritional invention containing specific anti-inflammatory and neuroprotective ingredients may be effective in the management of neurdevelopmental disorders with comorbid symptoms of food allergy. Moreover, this thesis shows that *in utero* exposure to VPA alters the allergic response to whey sensitization, characterized by an increased polarization of the immune response towards Th2. Therefore, individuals exposed to neurodevelopmental risk factors *in utero* may also be predisposed to develop allergic diseases. Together, the findings in this thesis create more insight into the pathophysiological relevance of intestinal and allergic complications in neurodevelopmental disorders and demonstrate that nutritional intervention may be beneficial in the prevention or management of these disorders.

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APPENDICES

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NEDERLANDSE SAMENVATTING

De ontwikkeling van het brein is een langdurig proces dat vroeg in het leven begint en aanhoudt tot ten minste adolescentie. Breinontwikkeling in de mens begint in de derde week van de zwangerschap met de differentiatie van neurale voorlopercellen en vervolgens de formatie van de neurale buis. Het brein van de muis ontwikkelt zich op een overeenkomstige, maar snellere wijze; de neurale buis in de muis wordt gevormd op dag 8.5 van de zwangerschap. De ontwikkeling van het brein wordt gereguleerd door complexe processen die afhankelijk zijn van tijdelijke en lokale expressie van talrijke genen. Mutaties in deze genen kunnen leiden tot neuronale ontwikkelingsstoornissen, bijvoorbeeld autisme spectrum stoornis. Autisme wordt gekenmerkt door afwijkingen in sociale interactie en communicatie en de aanwezigheid van stereotiepe gedragingen. Genetische aanleg is een belangrijke factor in de ontwikkeling van autisme, zoals wordt aangetoond door het hoge risico op autisme in families met een aangedaan familielid en door de gemeenschappelijke genetische varianten die zijn waargenomen bij ongeveer 20 % van de patiënten met autisme.

Dit betekent echter, dat voor de meerderheid van de patiënten met autisme de oorzaak van hun aandoening onbekend is, wat impliceert dat naast genetische aanleg, ook omgevingsfactoren een belangrijke rol spelen. Blootstelling aan schadelijke omgevingsfactoren tijdens neuronale ontwikkeling voor en/of na de geboorte (pre- en postnataal) kunnen permanente effecten hebben op het functioneren van de hersenen en dus op het gedrag op latere leeftijd. Voorbeelden van omgevingsfactoren tijdens de zwangerschap die het risico op neuronale ontwikkelingsstoornissen verhogen zijn overactivatie van het immuunsysteem, stress, inname van bepaalde geneesmiddelen en ondervoeding. Deze zogenoemde maternale risicofactoren kunnen nakomelingen ook meer vatbaar maken voor het ontwikkelen van immuunziekten zoals allergie en ontstekingsziekten. In de laatste jaren worden neurologische stoornissen steeds vaker in verband gebracht met een afwijkend immuunsysteem, voornamelijk in darmen (zoals beschreven in **hoofdstuk 2**). Het doel van dit proefschrift was meer inzicht te krijgen in het belang van darmproblemen tijdens een verstoorde neuronale ontwikkeling en in de effecten van (allergische) immuunactivatie in de darm op brein en gedrag relevant aan autisme. Breinontwikkeling en de interactie tussen brein en immuunsysteem zijn complexe processen die niet door middel van celkweek experimenten te onderzoeken zijn. Daarom is in dit proefschrift gebruik gemaakt van twee muizenmodellen; een model voor verstoorde neuronale ontwikkeling en een model voor voedselallergie.

Darmproblemen in neuronale ontwikkelingsstoornissen

De aanwezigheid van darmproblemen in patiënten met autisme is regelmatig gepubliceerd en omvat voornamelijk chronische constipatie, diarree en buikpijn. Deze symptomen worden toegeschreven aan veranderingen in o.a. de samenstelling van de darmbacteriën (microbioom), verhoogde darm doorlaatbaarheid, darmontsteking en voedselallergie. Ondanks deze bevindingen geven diverse studies sterk uiteenlopende resultaten wat betreft de gerapporteerde prevalentie van darmproblemen in patiënten met autisme. De reden voor deze grote variatie zou verklaard kunnen worden door de diverse patiëntenpopulatie die autisme spectrum stoornis omvat. Diagnose van autisme is namelijk volledig gebaseerd op gedragsobservaties. Het is daarom aannemelijk dat verschillende oorzaken leiden tot uiting van eenzelfde type van gedragsafwijkingen. Het is mogelijk dat darmproblemen aanwezig zijn in een subpopulatie van autistische patiënten met een overeenkomstige ziekteoorzaak.

In het eerste deel van dit proefschrift is onderzocht of (en wat voor soort) veranderingen er aanwezig zijn in de darm ten gevolge van verstoorde neuronale ontwikkeling. Hiervoor is gebruik gemaakt van een muizenmodel voor autisme, waarbij zwangere muizen eenmalig blootgesteld worden aan valproïnezuur, een anti-epilepticum. Dit model is humaan relevant, omdat zwangere vrouwen die valproïnezuur gebruiken een verhoogde kans hebben op een kind met autisme. De muizen uiten afwijkingen in sociaal, communicatief en repetitief gedrag, wat gepaard gaat met een scala aan neuronale afwijkingen, zoals veranderingen in de neurotransmitter serotonine. In **hoofdstuk 3** wordt aangetoond dat deze muizen, naast afwijkend gedrag, ook een ontsteking hebben in de dunne darm. Bovendien is er diarree geconstateerd en is de samenstelling van het darm microbioom veranderd wanneer muizen tijdens de zwangerschap blootgesteld zijn aan valproïnezuur (**hoofdstuk 4**). Bacteriën in de Firmicutes stam zijn in grotere mate aanwezig, wat vooral ten koste gaat van bacteriën in de Bacteroidetes stam. Tevens is een verhoogde concentratie van het korte keten vetzuur butyraat waargenomen in de blinde darm inhoud na blootstelling aan valproïnezuur tijdens de zwangerschap. Butyraat is een product van bacteriële fermentatie van vezels en is schadelijke voor het centrale zenuwstelsel wanneer deze stof via de bloedbaan in het brein terecht komt. Vergelijkbare veranderingen in het microbioom en butyraat concentraties zijn gevonden in kinderen met autisme.

In **hoofdstuk 3** en **5** wordt beschreven dat serotonine concentraties in de nakomelingen van valproïnezuur behandelde muizen niet alleen verlaagd zijn in breingebieden relevant voor autisme (prefrontale cortex en amygdala), maar ook in de darmen (over de gehele dunne darm en dikke darm). Concentraties van het afbraakproduct van serotonine zijn tevens verhoogd in het brein en in de darm na prenatale blootstelling aan valproïnezuur.

Omdat de ratio's van deze concentraties (serotonine turnover) in beide organen statistisch sterk met elkaar gecorreleerd zijn, kan men stellen dat een verstoorde ontwikkeling of functie van het serotonerge systeem mogelijk een gezamenlijke oorzaak is voor de afwijkingen in het brein en de darm.

Zowel de afwijkingen op gedrag als in de darm zijn alleen waargenomen in mannelijke nakomelingen van valproïnezuur behandelde muizen. Dit is in lijn met de situatie in de mens, waar autisme drie maal zo vaak voorkomt in mannen dan in vrouwen. Nader onderzoek naar de discrepantie tussen de gevolgen van prenatale blootstelling aan valproïnezuur in mannelijke en vrouwelijke nakomelingen geeft niet alleen inzicht in de oorzaak voor de mannelijke dominantie in autisme, maar kan ook kennis geven over de algemene onderliggende ziekteprocessen die leiden tot autisme.

Voor een gezonde ontwikkeling en werking van het brein zijn specifieke voedingsstoffen noodzakelijk. In **hoofdstuk 5** is onderzocht of valproïnezuur-geïnduceerde afwijkingen in gedrag en het serotonerge systeem te veranderen zijn met behulp van specifieke voedingsconcepten. Hiervoor zijn muizen voor en na de geboorte blootgesteld aan een dieet dat rijk is aan voedingscomponenten die bevorderlijk zijn voor zowel het immuunsysteem als het zenuwstelsel. Wanneer muizen worden blootgesteld aan dit dieet, ontwikkelen ze geen verlaagd sociaal gedrag na prenatale blootstelling aan valproïnezuur. Tevens voorkomt consumptie van het dieet de afwijkingen in serotonine concentraties in het brein en de darm na blootstelling aan valproïnezuur tijdens de zwangerschap. Mogelijk zou inname van deze voedingscomponenten tijdens de zwangerschap of vroeg in het leven het risico op neuronale ontwikkelingsstoornissen kunnen verlagen.

Effecten van voedselallergie op brein en gedrag

Omgevingsfactoren kunnen naast prenataal, ook postnataal een blijvende invloed hebben op de hersenontwikkeling. Er wordt verondersteld dat overactivatie van het immuunsysteem bijdraagt aan de uiting en ernst van afwijkend gedrag in verscheidene neurologische aandoeningen, waaronder autisme. Met name voedselallergie wordt geassocieerd met autisme (zoals beschreven in **hoofdstuk 6**). Deze associatie wordt ondersteund door de observatie dat de diagnose van voedselallergie in het eerste levensjaar samengaat met een verhoogde kans op introvert gedrag en lage sociale emotionele scores bij kinderen van 18 maanden oud. Een aantal studies heeft aangetoond dat glutenvrije en koemelkvrije diëten kunnen bijdragen aan het verbeteren van autistisch gedrag en het herstellen van de darmfunctie in deze kinderen. Daarnaast zijn verhoogde concentraties van allergie-geassocieerde eiwitten (bepaalde antistoffen en cytokinen) herhaaldelijk waargenomen in het bloed van patiënten met autisme. Fundamenteel bewijs dat voedselallergie de hersenen kan beïnvloeden is tot nu toe niet geleverd. In het tweede deel van dit proefschrift is deze hypothese getest.

In **hoofdstuk 7** is aangetoond dat een allergische respons tegen koemelkeiwit in de darmen van jonge muizen geassocieerd is met veranderingen in gedrag (verlaagd sociaal en verhoogd repetitief gedrag). Deze veranderingen in het gedrag van allergische muizen gaan gepaard met uitgesproken veranderingen in het dopaminerge systeem in het brein. De resultaten van **hoofdstuk 7** tonen aan dat concentraties van de neurotransmitter dopamine en zijn afbraakproducten zijn verlaagd in de prefrontale cortex en verhoogd in de amygdala van allergische muizen. Het dopaminerge systeem in deze gebieden is belangrijk voor sociaal en emotioneel gedrag en verlaagde dopamine concentraties zijn waargenomen in de prefrontale cortex van kinderen met autisme. Naast veranderingen in het dopaminerge systeem is een verhoogde neuronale activatie waargenomen in de orbitale prefrontale cortex van allergische muizen. Deze regio is betrokken bij het reageren op sociale prikkels en een verhoogde activatie is tevens waargenomen in patiënten met autisme in reactie op gezichtsherkenning. Dit impliceert dat de neurochemische veranderingen in het dopaminerge systeem en de orbitale prefrontale cortex ten grondslag kunnen liggen aan de veranderingen in gedrag van muizen met een voedselallergie.

Een allergische reactie in de darmen van muizen verhoogt bovendien de concentratie van serotonine in de dunne darm (**hoofdstuk 7**). Deze toename in serotonine niveaus gaat gepaard met een verhoging van het aantal serotonine-producerende darmcellen (enterochromaffiene cellen) en verlaagde concentraties van het serotonine afbraakproduct. Zowel enterochromaffiene cellen als immuuncellen scheiden serotonine uit in de nabijheid van neuronen die naar de hersenen leiden (afferente neuronen). Serotonine kan binden aan deze neuronen, wat zorgt voor signalering naar verschillende delen van de hersenen. Het is daarom mogelijk dat de serotonine die wordt vrijgezet tijdens een allergische reactie een signaal naar de hersenen in gang zet wat zorgt voor neurochemische veranderingen in het brein en vervolgens een effect heeft op gedrag.

Een aantal studies toont aan dat patiënten met autisme vaker lijden aan allergische aandoeningen. Daarom hebben we onderzocht of muizen met een afwijkende neuronale ontwikkeling (veroorzaakt door valproïnezuur), anders reageren op het ontstaan van allergie (**hoofdstuk 10**). In dit hoofdstuk wordt aangetoond dat muizen met een verstoorde neuronale ontwikkeling een afwijkende immuunreactie hebben tegen koemelkeiwit. Dit wordt gekenmerkt door een verlaging van een antistof die geassocieerd wordt met remming van de allergische response. Hoewel verder onderzoek naar de allergische respons in valproïnezuur behandelde dieren essentieel is voor dit vraagstuk, impliceert deze studie dat individuen die tijdens de zwangerschap blootgesteld worden aan risicofactoren voor neuronale ontwikkelingsstoornissen, mogelijk ook meer vatbaar zijn voor het ontwikkelen van allergie.

In **hoofdstuk 8** en **9** is onderzocht of voeding een effect heeft op de voedselallergie-geïnduceerde afwijkingen in gedrag. Hiervoor zijn muizen blootgesteld aan een dieet voor en tijdens sensibilisatie met het koemelkeiwit. Er zijn twee type diëten getest: 1) een dieet waarin omega-6 meervoudig onverzadigde vetzuren vervangen zijn door omega-3 meervoudig onverzadigde vetzuren (**hoofdstuk 8**), en 2) een dieet dat rijk is aan voedingscomponenten die bevorderlijk zijn voor zowel het immuunsysteem als het zenuwstelsel (**hoofdstuk 9**). Beide diëten zijn in staat verlaagd sociaal gedrag in allergische muizen te voorkomen. Toevoeging van omega-3 meervoudig onverzadigde vetzuren aan het dieet voorkomt tevens de afwijkingen in het dopamine systeem in het brein, en het serotonine systeem in de darm (**hoofdstuk 8**). Het dieet dat gebruikt is in **hoofdstuk 5** en **9** is in staat afwijkend sociaal gedrag te normaliseren in zowel het model voor autisme als het model voor voedselallergie. Aangezien dit dieet bestaat uit meerdere componenten is het van belang te achterhalen welke van de componenten verantwoordelijk zijn voor de immunologische en/of neurologische effecten. Niettemin impliceren de bevindingen uit dit proefschrift dat voedingsinterventies bij kunnen dragen aan het verminderen van allergie-geïnduceerde gedragsafwijkingen. Derhalve is het interessant te onderzoeken of patiënten met autisme en voedselallergie baat hebben bij dergelijke diëten.

Samenvattend laat dit proefschrift zien dat een risicofactor voor neuronale ontwikkelingsstoornissen (prenatale blootstelling aan valproïnezuur) tevens leidt tot verstoringen in de darmen. Dit wordt mogelijk veroorzaakt door een verstoord serotonine systeem. Daarnaast toont dit proefschrift aan dat een allergische reactie in de darmen gepaard gaat met neurochemische en gedragsveranderingen die relevant zijn voor autisme. Deze bevindingen ondersteunen de frequent beschreven darmproblemen bij patiënten met autisme en laten zien dat voedingsinterventies tijdens de zwangerschap of vroeg in het leven mogelijk bij kunnen dragen aan de preventie of behandeling van een verstoorde neuronale ontwikkeling. De bevindingen in dit proefschrift suggereren dat risicofactoren voor neuronale ontwikkelingsstoornissen, tevens risicofactoren kunnen zijn voor de ontwikkeling van darmproblemen, al dan niet geassocieerd met voedselallergie. De allergische reactie die vervolgens op kan treden, leidt tot neurochemische veranderingen in het brein die de afwijkingen in gedrag mogelijk verergeren (**hoofdstuk 11**, **Summarizing Fig. 1**). Dieetinterventies met componenten die bevorderlijk zijn voor zowel het immuunsysteem als het zenuwstelsel kunnen in de toekomst mogelijk toegepast worden voor behandeling en/of voorkomen van neuronale ontwikkelingsstoornissen, zoals autisme. Vervolgstudies (klinisch en preklinisch) zullen dit moeten bevestigen en onderbouwen.

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Simon, elke dag geniet ik van de manier waarop jij mij de mooie wereld laat zien. Samen met jou voel ik me vrij.

CURRICULUM VITAE

Caroline de Theije was born on November 20, 1985 in Hulst, The Netherlands. After graduating from high school (Reynaert College, Hulst) in 2004, she studied Biology at the Radboud University in Nijmegen. Caroline received her Bachelor's degree in 2007 and continued with the Master Medical Biology. She did an internship at the department of Blood transfusion and Transplantation Immunology of the Radboud University Nijmegen Medical Center, under supervision of dr. Hans Koenen. During her stay, Caroline investigated the effects of retinoic acid on T cell differentiation. Her second internship was conducted at Danone Research Wageningen under the supervision of dr. Klaske van Norren, investigating the effects of dietary components on HIV replication via the activation of nuclear factor κB. In 2009, Caroline received her Master's degree and started as a PhD candidate at the Utrecht University under the supervision of dr. Aletta Kraneveld and prof. dr. Johan Garssen from the division of Pharmacology. During her PhD, Caroline was trained in pharmacology in the Utrecht Institute for Pharmaceutical Sciences (UIPS) PhD program. She received the Young Investigators Award at ESPGHAN 2011, the oral abstract prize at BCPT 2013, and a travel grant from NSFW in 2010 for a work visit to the Brain-Body Institute (McMaster University, Hamilton, Canada). Caroline's PhD project resulted in the completion of this thesis. In January 2014, Caroline started as a post-doctoral fellow at the division of Pharmacology (Utrecht University) in a project that is part of the NutriBrain consortium of the Utrecht Life Sciences network. She will continue her work on the importance of early life nutrition for brain and immune system development.

LIST OF PUBLICATIONS

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# Authors contributed equally to the manuscript

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