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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 comorbid 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 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.

1. Introduction

Autism spectrum disorder (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 (American-Psychiatric-Association, 2000). Although the aetiology of ASD is unknown, it is thought that ASD is a multifactorial disorder with a strong genetic component (Bailey et al., 1995; Folstein and Rosen-Sheidley, 2001). 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 (Dufour-Rainfray et al., 2011). Indeed, maternal use of the anticonvulsant valproic acid (VPA) is associated with the development of ASD in the offspring (Christensen et al., 2013; Moore et al., 2000; Rasalam et al., 2005). 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 (Ornoy, 2009). 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 (Kataoka et al., 2013; Roullet et al., 2010), stereotyped behaviour (Wagner et al., 2006), anxiety and impairments in cognition (Kataoka et al., 2013). Furthermore, observations were more prominent in male offspring compared to female offspring (Kataoka et al., 2013; Kim et al., 2013), which reflects the human situation where a marked male preponderance is observed in ASD patients (Fombonne, 2005; Lord et al., 1982).

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 (Fatemi et al., 2008; Laurence and Fatemi, 2005; Morgan et al., 2010; Tetreault et al., 2012; Vargas et al., 2005), indicative of neuroinflammation. Enhanced activation of neuroglia was also observed in various murine models of autism (Derecki et al., 2012; Ratnayake et al., 2012; Yuskaitis et al., 2010). In addition, enhanced levels of a wide range of cytokines and chemokines were found in the brain (Li et al., 2009) and in

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the cerebrospinal fluid (Vargas et al., 2005) 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 (Jyonouchi et al., 2005; Molloy et al., 2006), dysfunctional natural killer (NK) cells (Enstrom et al., 2009; Vojdani et al., 2008) and altered serum levels of immunoglobulins (Croonenberghs et al., 2002; Heuer et al., 2008; Lucarelli et al., 1995), cytokines (Ashwood et al., 2011a; Manzardo et al., 2012; Singh, 1996) and chemokines (Ashwood et al., 2011b).

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 (de Theije et al., 2011). These symptoms have been attributed to changes in gut microflora (Desbonnet et al., 2013; Louis, 2012), increased intestinal permeability (de Magistris et al., 2010) and intestinal inflammation (Ashwood et al., 2003). 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 9% to 91%, an immense range probably due to varying interpretations of gastrointestinal problems and inability of ASD patients to express their discomfort (Coury et al., 2012). 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 (Adams et al., 2011), a considerable number of ASD patients is on a specific diet to improve gastrointestinal function and behaviour (Levy and Hyman, 2003). 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.

2. Materials and methods

2.1. 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 free 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 (Ybot-Gonzalez et al., 2002), pregnant females were treated subcutaneously with 600 mg/kg valproic acid (Sigma, Zwijndrecht, the Netherlands, VPA: 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 $n = 7$). 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).

2.2. Social behaviour test

The behavioural assessment used was adapted from a previous description (Liu et al., 2012; Tabuchi et al., 2007). Mice were placed in a 45 × 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 field 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).

2.3. 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 (Breece et al., 2013; Bringas et al., 2013; Morgan et al., 2010), 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. Proinflammatory 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 (Bio Rad, Veenendaal, The Netherlands). Quantative real-time PCR analysis was performed on a CFX96 real-time PCR detection system (Bio Rad, Veenendaal, The Netherlands) using iQ SYBR green supermix (Bio Rad, Veenendaal, The Netherlands) and qPCR primers (Qiagen, Germantown, MD, USA). mRNA expression levels were calculated with CFX Manager software (version 1.6) and corrected for the expression of the housekeeping gene *Rps13*.

2.4. 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 using an Alexys 100 LC-EC system (Antec, Lelystad, the Netherlands) as previously described (Olivier et al., 2008). 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 HClO4 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×*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 × 4.6 mm i.d.) packed with Hypersil BDS C18, 5-µm particle size (Alltech