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Altered expression of circadian rhythm and extracellular matrix genes in the medial prefrontal cortex of a valproic acid rat model of autism

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ABSTRACT

Autism spectrum disorders (ASD) are a highly heterogeneous group of neurodevelopmental disorders caused by complex interplay between various genes and environmental factors during embryonic development. Changes at the molecular, cellular and neuroanatomical levels are especially evident in the medial prefrontal cortex (mPFC) of ASD patients and are particularly contributing to social impairments. In the present study we tested the hypothesis that altered neuronal development and plasticity, as seen in the mPFC of ASD individuals, may result from aberrant expression of functionally connected genes. Towards this end, we combined transcriptome sequencing and computational gene ontology analysis to identify the molecular networks impaired in the mPFC of a valproic acid (VPA) rat model of autism. This investigation identified two subsets of genes differentially expressed in the mPFC of VPA rats: one group of genes being functionally involved in the regulation of the circadian rhythm, while the second group encompasses a set of differentially expressed collagen genes acting within the extracellular matrix. Ultimately, our integrated transcriptome analysis identified a distinct subset of altered gene networks in the mPFC of VPA rats, contributing to our understanding of autism at the molecular level, thus providing novel insight into the genetic alterations associated with this neurodevelopmental disorder.

1. Introduction

Autism spectrum disorders (ASD) constitute a heterogeneous group of neurodevelopmental disorders characterized by three core symptoms: (1) social interaction impairment, (2) communication deficits, and (3) stereotypical, repetitive and restricted behaviors. Cognitive deficits associated with ASD, as assessed by intelligence quotient tests, can range from intellectual disability to the superior range (Kim, 2015). In the past few years, there have been impressive advances in understanding the pathogenesis of these disorders. Although there is no clear and consistent pathology in autism, accumulating data from brain imaging and post-mortem studies, as well as genetic investigation indicate that ASD are associated with impaired connectivity at the molecular, synaptic and neuronal systems level (Gilman et al., 2011; Boersma et al., 2013; Nair et al., 2013; Parikshak et al., 2013).

The medial prefrontal cortex (mPFC) and its connected brain regions are affected in ASD at the neuronal development and synaptic functionality levels, contributing to the role suggested for the mPFC in the development of the disorder (Mychasiuk et al., 2012; Sui and Chen, 2012; Bringas et al., 2013; Martin and Manzoni, 2014). In line with these observations, damage limited to the mPFC may cause personality changes, leading to phenotypes as observed in ASD, such as a lack of social interaction (Umeda et al., 2010). The mPFC exerts its functions through connections with the brainstem, the thalamus and the limbic system (Bringas et al., 2013; Martinez-Sanchis, 2014). This network is activated in rest and implicated in self-referential thinking, monitoring the environment, and in sensory processing tasks (Martinez-Sanchis, 2014). Although this default network seems to be intact in ASD patients, its activation is disturbed and the level of atypical activity is positively correlated with social impairment (Buckner et al., 2008; Martinez-Sanchis, 2014). Moreover, abnormal structural network integration between the mPFC and the parietal regions encompassing posterior cingulate cortex and the precuneus (PCC/PCU) has been observed in ASD patients (Valk et al., 2015). At the cellular level, disturbances in cellular functioning occur in different cell types, including neuronal progenitors, GABAergic parvalbumin-positive inter-

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neurons, glutaminergic excitatory neurons and microglia (Schubert et al., 2014).

Although it is widely accepted that the mPFC plays a critical role in the etiology of ASD, few studies have assessed gene expression levels in the PFC of autistic patients. Voineagu et al. performed microarray studies on post-mortem cortical tissue and found differential expression of 444 genes in the temporal and frontal cortices of ASD patients (Voineagu et al., 2011). The downregulated genes were enriched for gene ontology (GO) categories related to synaptic function, whereas the upregulated genes were implicated in immune and inflammatory responses. Other studies assessed differential expression of selected neuronal genes in ASD mPFC, including the genes encoding the mitochondrial calcium-binding solute carrier *Slc25a12* (Lepagnol-Bestel et al., 2008) and microtubule affinity-regulating kinase 1 (*Mark1*) (Maussion et al., 2008). Moreover, increased expression levels of astrocyte- and microglial-specific markers were detected in the PFC of autistic patients (Edmonson et al., 2014).

ASD can be triggered by a combination of genetic but also environmental factors in prenatal life (Muller, 2007). One of the environmental factors raising the risk on development of ASD in an unborn child, when used in the first trimester of a pregnancy is the anti-epileptic and mood-stabilizing drug valproic acid (VPA) (Rasalam et al., 2005; Markram et al., 2007). In rodents, a single intra-peritoneal injection of VPA to the pregnant dam at the time of embryonic neural tube closure increases the risk for ASD-like features in the offspring. Similar to human ASD patients, affected offspring exhibit brain stem injuries, diminished cerebellar Purkinje cell number (Ingram et al., 2000), social interaction deficits, enhanced anxiety, developmental delays, lowered pain sensitivity, impaired information processing and attention, and hyperactivity with lowered exploratory path finding (Schneider et al., 2006; Markram et al., 2008). The VPA animal model is thus considered one of the best-validated models for ASD and is frequently used to study the alterations in brain development at the level of morphology, gene expression, and neuronal functioning (Snow et al., 2008).

To study differential gene expression at the level of mRNA in ASD mPFC, we took advantage of a VPA rat model of autism (Olde Loohuis et al., 2015). We employed next generation sequencing to examine the transcriptome of the mPFC after prenatal VPA-exposure in rats. The outcome of our study raises the possibility that sleeping problems previously observed in the VPA animal model may partially be due to altered expression of a set of circadian rhythm genes, while differential expression of collagen-encoding genes may underlie disturbances in extracellular matrix signaling, previously associated with altered cortical development in autism.

2. Materials and methods

2.1. VPA animal model

Wistar rats (Harlan laboratories, USA) were housed individually on a 12-h light cycle in a temperature-controlled (21 ± 1 °C) environment with access to food and water ad libitum. Estrous was measured in female rats using an impedance measurement apparatus. When in estrous, the female was housed with a male rat overnight. If a vaginal plug was found the next day, female rat was deemed to be pregnant. This day was noted as day 0.5 of gestation. 12 days later the valproic acid (VPA) injection was performed. VPA sodium salt (Sigma Aldrich, Germany) was dissolved in 0.9% saline to a concentration of 150 mg/ml (pH = 8.3). The dosing volume was 3.3 ml/kg, and the dosage was adjusted according to the body weight of each rat on the day of injection. At estrous day 12.5 pregnant females received a single intraperitoneal injection (ip) of 495 mg/kg VPA sodium salt; control pregnant rats were injected with the same volume of physiological saline. The day of birth was designated postnatal day 1 (P1). Dams were allowed to raise their own young until weaning on P23. From P23 onwards animals were housed in pairs. No apparent physical malformations were detected in the VPA animals, making the exclusion of animals for the study unnecessary. Once born, pups were subjected to a set of tests to evaluate their development and (weight gain, eye opening and motor coordination) and behavior (social play, elevated plus maze, open field and prepulse inhibition). Development of the VPA-exposed pups was impaired as was shown by delayed eye-opening and motor performance and coordination. In addition, social play behavior, contact-seeking and sensorimotor-gating were impaired in rats prenatally exposed to VPA. These finding led us to conclude that this animal model exhibits “autism-like” behavior. A detailed characterization of the animals used in this study is provided in Olde Loohuis et al. (2015). All animal experiments were approved by the Committee for Animal Experiments of the Radboud University, Nijmegen, The Netherlands.

2.2. mPFC collection

Five male rats from the saline and the VPA-exposed groups were selected, each selected from four different litters per group to minimize the possibility of litter-specific data outcome biases. Animals were euthanized at P90 by decapitation (n = 5 for saline and VPA), and brains were rapidly frozen in liquid nitrogen, and stored at − 80 °C until further tissue processing. Brains were processed into 200 μm slices at − 15 °C using a microtome, and 1.2 mm mPFC punches were taken. These punches were stored at − 80 °C in RNAlater (Ambion) until RNA isolation.

2.3. RNA isolation

Total RNA was isolated from the mPFC punches of the VPA- and saline-treated rats using the RNeasy Mini kit (Qiagen). RNA integrity was assessed using the *Agilent* Tapestation analysis, which revealed RNA quality above RIN 8.0 for all samples.

2.4. RNA sequencing

Total RNA isolated as described above. For each of these ten RNA samples, 250 ng was stored for qRT-PCR analyses. RNA samples from each condition were pooled and further processed by the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA). The RNA sequencing was performed in three separate flow cells, each generating a total of 45 to 50 million 50 bp reads per sample. RNA sequencing data analysis was carried out with the GeneSifter gene expression analysis suite (Geospiza, Seattle, WA, USA). Expression data were normalized against the total number of mapped reads. The reads were assembled into genes. Differential gene expression between pooled samples of saline and VPA-exposed animals was determined using the likelihood ratio test, and the Benjamini and Hochberg correction for multiple testing was applied. Thresholds were set at an RPKM of 30 and a fold change of 1.2. Genes were assigned as differentially expressed at an adjusted P-value $P < 0.05$. To examine the validity of the calculated thresholds and the data obtained by GeneSifter, and, *qRT-PCR* was employed to quantitate the levels of a wide range of transcripts from the individual samples. Using this approach we assessed whether the individual gene expression levels were above or below the fold-change threshold, or above or below the RPKM threshold, in order to verify that the right cut-offs was set. This analysis confirmed that the appropriate thresholds were chosen.

2.5. Gene ontology (GO) analysis

Significantly differentially expressed genes in VPA-exposed animals compared to saline-exposed controls were analyzed using a combination of two independent GO analysis software programs, DAVID (Huang da et al., 2009b, 2009a) and Ingenuity Pathway Analysis® (IPA), to