Published in final edited form as:

Prog Neuropsychopharmacol Biol Psychiatry. 2017 July 03; 77: 128–132. doi:10.1016/j.pnpbp.2017.04.009.

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. Toward 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.

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Keywords

Transcriptome sequencing; gene ontology analysis; animal model of autism; Period circadian clock; collagen

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 postmortem 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 interneurons, 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 findings 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, a) and Ingenuity Pathway Analysis® (IPA), to obtain insights into the molecular pathway effects of VPA. The threshold for significance was set at $P < 0.01$. The outcomes of both software analyses were overlaid to arrive at a convergent analysis.

2.6. cDNA synthesis

For cDNA synthesis, the individual samples from the 5 saline-exposed and the 5-VPA exposed rats were used. 250 ng of total RNA was DNase-treated and reverse-transcribed using the revertAid™ H Minus First Strand cDNA Synthesis kit K1612 (Fermentas Inc., USA), according to the manufacturer's protocol. Random hexamer primers were used for reverse transcription.

2.7. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using Sensifast SYBR Green (Bioline) according to manufacturer's protocol. Polymerase activation was achieved after 2 min at 95°C, followed by 40 cycles of denaturing at 95°C for 5 seconds, annealing at 60°C for 10 seconds and elongation at 72°C for 15 seconds. mRNA expression was normalized to the housekeeping genes U6 and β -actin using the GeNorm method (Vandesompele et al., 2002). The sequence Gene-specific primers employed in the analysis are presented in the supplementary information.

2.8. Statistical analysis

Statistical analyses were performed using Prism software (Graphpad). Two group comparisons were evaluated using non-paired two-tailed t-test. The level of significance was set at 0.05. Data are plotted as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Transcriptomics analysis in the mPFC of the VPA rat model of autism

To examine the consequences of prenatal VPA exposure on mRNA expression in the mPFC, RNA sequencing was performed. For differential expression analysis, the threshold for the P-value after correction for multiple testing (adjusted P-value) was set at 0.05. In combination with a threshold of 1.2-fold change in mRNA expression. Using these criteria, 732 genes showed altered expression levels, 305 of which were downregulated, while 426 genes were upregulated (Fig. 1A). With an expression level threshold of RPKM ≥ 30.0 in at least one condition (either saline- or VPA-exposed), a set of 74 differentially expressed genes was identified, of which 34 genes were downregulated and 40 genes exhibited increased expression levels (Fig. 1B). Details on the differentially expressed genes are summarized in Supplementary Table I.

3.2. Pathway analysis

To identify the most biologically relevant processes in which the 74 differentially expressed genes were most likely functional, we employed two independent gene ontology (GO) analysis software programs, the Ingenuity Pathway Analysis (IPA) and DAVID (<http://david.abcc.ncifcrf.gov/summary.jspv>). Pathway analysis using IPA identified a total of four highly enriched canonical pathways, whereas DAVID analysis revealed three significantly enriched pathways (Figure 2A, B). Both analyses identified abnormal functioning of circadian rhythm signaling as the top pathway. The three affected genes in this signaling pathway were: 1. Basic Helix-Loop-Helix Family, 2. Member E40 (*Bhlhe40*), and 3. Period circadian clock 1 (*Per1*) and period circadian clock 2 (*Per2*), all of which were downregulated in their expression levels as a consequence of prenatal VPA exposure. Moreover, three collagen encoding genes were represented in the top pathways of both IPA and DAVID all of which exhibited an increased expression level: Collagen, Type I, Alpha 1 (*Col1a1*) Collagen, Type I, Alpha 2 (*Col1a2*) and Collagen, Type III, Alpha 1 (*Col3a1*) (Fig. 2C).

3.3. Validation of RNA sequencing results

Quantitative qRT-PCR analysis was used to validate the outcome of the RNA sequencing by determining the expression levels of eight genes incorporated within the aforementioned pathways. Expression levels of three significantly altered circadian rhythm genes (*Bhlhe40*, *Per1* and *Per2*) were assessed. Whereas decreases in mRNA levels could be confirmed for *Per1* (P=0.0028) and *Per2* (P=0.0008), *Bhlhe40* showed only a tendency towards downregulation (P=0.0854). In addition, the expression levels of three circadian rhythm genes that had a fold-change below 1.2 value was measured, which was taken as a threshold for significance. Expression levels of these three circadian rhythm genes (*Cry2*, *Grin2c* and *Crebbp*) were not significantly changed when measured using qPCR, confirming the fold-change threshold should not be lower than 1.2. Lastly, Potassium Channel, Inwardly Rectifying Subfamily J, Member 10 (*Kcnj10*) and member 16 (*Kcnj16*), were measured to confirm the RPKM threshold that we set at 30. As expected, *Kcnj10* (RPKM saline 17.9 and RPKM VPA 23.4) and *Kcnj16* (RPKM saline 5.0 and RPKM VPA 6.7) mRNA levels were not significantly altered (Suppl. Fig. 1; Suppl. Table II).

4. Discussion

Abnormalities in the mPFC are associated with socio-cognitive dysfunction in several groups of ASD patients (Umeda et al., 2010; Etkin et al., 2013; Schubert et al., 2014). Our studies aimed at assessing the effects of prenatal VPA-exposure on gene expression in the mPFC. The VPA animal model of autism and has become one of the best-validated animal models for autism. Prenatal exposure to VPA at the time of embryonic neural tube closure causes the offspring to display characteristics similar to human ASD patients regarding: behavioral performance, neuroanatomical characteristics, cellular functioning and gene expression (Roulet et al., 2013). Transcriptome analysis of VPA mPFC identified 74 differentially expressed genes, and subsequent GO analysis revealed functional alterations in the circadian rhythm pathway. Moreover, collagens appeared as a main class of affected genes encoding proteins of extracellular matrix.

4.1. Circadian rhythm disturbances in ASD

Problems in sleep, memory and timing are characteristics of ASD, and each of these aspects is commonly regulated by a clock gene (Nicholas et al., 2007). Previously, multiple studies have identified high rates of sleep problems in children suffering from ASD (Richdale and Prior, 1995; Patzold et al., 1998; Schreck et al., 2004; Wiggs and Stores, 2004). Studies measuring blood levels of melatonin (Nir et al., 1995) and melatonin metabolites in urine of ASD patients mirrored the sleeping disturbances observed in autism patients at the metabolic level (Tordjman et al., 2005). More recently, gene expression studies on lymphoblastoid cell lines derived from autism patients revealed that severely affected patients show dysregulation of genes of the circadian rhythm pathway (Hu et al., 2009). Moreover, single-nucleotide polymorphisms (SNPs) in the circadian rhythm genes *PER1* and *NPAS2* have been identified in ASD patients, leading the authors to conclude that clock genes may be involved in the etiology of this disorder (Nicholas et al., 2007).

In our VPA animal model, we show decreased expression levels of *Per1* and *Per2*, while *Bhlhe40* showed a trend towards downregulation. These finding raises the possibility that altered expression of the circadian rhythm genes is associated in the etiology of previously observed phenotype. Interestingly, a recent study on sleeping patterns in rats prenatally exposed to VPA showed that the animals spent more time in wake and less time in non-rapid eye movement sleep (Cusmano and Mong, 2014). In addition, animals exhibited increased high-frequency activity during wake and rapid eye movement sleep, while theta-power was reduced in all vigilance states. Moreover, the connection between VPA and *Per2* has been studied in cell cultures derived from the suprachiasmatic nuclei (SCN) together with skin fibroblasts from transgenic mice expressing *Per2*-luciferase. VPA was shown to significantly phase-advance or phase-delay the *Per2*-luciferase rhythm depending on the time point of application (Johansson et al., 2011). However, it should be noted that previous studies suggested that *Per* mRNAs levels oscillate throughout the day (Keene, 2007). We would like to note that all animals were euthanized concomitantly and their brains dissected within a very short time (60 minutes) to avoid large gene expression variances in the animals examined. Our study suggested that VPA decreases *Per2* expression in the mPFC when administered systemically during prenatal life, providing a possible molecular mechanism for the adverse effect on the VPA animals' sleeping behavior previously reported by others.

4.2. Collagens and extracellular matrix (ECM) functioning in ASD

In addition to the clock genes, expression levels of three collagens, including *Col3a1*, were increased in the mPFC of rats prenatally exposed to VPA. COL3A1 is important in the development of the cortical areas of the brain by regulating neuronal migration (Luo et al., 2011; Jeong et al., 2012) through activation of the RhoA pathway (Luo et al., 2011). Disturbed neuronal migration may lead to defective layering of the cortical areas. Interestingly, in some children with ASD, cortical enlargement was observed (Hazlett et al., 2011), as well as increased cortical thickness at later ages (Valk et al., 2015). Type I collagen is involved in signal transduction and ECM remodeling (Sweeney et al., 2008). The ECM is tightly connected to the intracellular environment, playing a part in intracellular signaling. Therefore, disturbances in the expression of ECM components could lead to altered signaling disturbing proper cellular functioning, and, in the case of neurons, in abnormal

outgrowth and synaptic functioning. In the VPA animal model, neurons of the mPFC displayed altered arborization (Mychasiuk et al., 2012; Bringas et al., 2013), decreased spinogenesis (Bringas et al., 2013) and altered synaptic plasticity (Sui and Chen, 2012; Martin and Manzoni, 2014). In addition, in ASD patients, defects in spinogenesis have been observed (Schubert et al., 2014).

5. Conclusions

This study identified a set of significantly altered genes in the mPFC of a VPA rat model of autism. These genes converged on pathways affecting circadian rhythm and synaptic connectivity, two critical gene networks previously shown to be impaired in individual with autism. This animal model of autism thus provides a valid resource to examine altered circadian rhythm and mPFC synaptic connectivity in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research of the authors is supported by grants from the “FP7-Marie Curie International Reintegration Grant” [to A.A. grant number 276868]. This work was also supported by the Division of Intramural Research Programs of the National Institute of Mental Health (MH002768).

References

- Amaral DG, Schumann CM, Nordahl CW. Neuroanatomy of autism. *Trends in neurosciences*. 2008; 31:137–145. [PubMed: 18258309]
- Boersma M, Kemner C, de Reus MA, Collin G, Snijders TM, Hofman D, Buitelaar JK, Stam CJ, van den Heuvel MP. Disrupted functional brain networks in autistic toddlers. *Brain connectivity*. 2013; 3:41–49. [PubMed: 23259692]
- Bringas ME, Carvajal-Flores FN, Lopez-Ramirez TA, Atzori M, Flores G. Rearrangement of the dendritic morphology in limbic regions and altered exploratory behavior in a rat model of autism spectrum disorder. *Neuroscience*. 2013; 241:170–187. [PubMed: 23535253]
- Buckner RL, Andrews-Hanna JR, Schacter DL. The brain’s default network: anatomy, function, and relevance to disease. *Annals of the New York Academy of Sciences*. 2008; 1124:1–38. [PubMed: 18400922]
- Cusmano DM, Mong JA. In utero exposure to valproic acid changes sleep in juvenile rats: a model for sleep disturbances in autism. *Sleep*. 2014; 37:1489–1499. [PubMed: 25142574]
- Edmonson C, Ziats MN, Rennert OM. Altered glial marker expression in autistic post-mortem prefrontal cortex and cerebellum. *Molecular autism*. 2014; 5:3. [PubMed: 24410870]
- Etkin A, Gyurak A, O’Hara R. A neurobiological approach to the cognitive deficits of psychiatric disorders. *Dialogues in clinical neuroscience*. 2013; 15:419–429. [PubMed: 24459409]
- Gilman SR, Iossifov I, Levy D, Ronemus M, Wigler M, Vitkup D. Rare de novo variants associated with autism implicate a large functional network of genes involved in formation and function of synapses. *Neuron*. 2011; 70:898–907. [PubMed: 21658583]
- Hazlett HC, Poe MD, Gerig G, Styner M, Chappell C, Smith RG, Vachet C, Piven J. Early brain overgrowth in autism associated with an increase in cortical surface area before age 2 years. *Archives of general psychiatry*. 2011; 68:467–476. [PubMed: 21536976]
- Hu VW, Sarachana T, Kim KS, Nguyen A, Kulkarni S, Steinberg ME, Luu T, Lai Y, Lee NH. Gene expression profiling differentiates autism case-controls and phenotypic variants of autism spectrum

- disorders: evidence for circadian rhythm dysfunction in severe autism. *Autism research: official journal of the International Society for Autism Research*. 2009; 2:78–97. [PubMed: 19418574]
- Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*. 2009a; 37:1–13. [PubMed: 19033363]
- Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*. 2009b; 4:44–57. [PubMed: 19131956]
- Ingram JL, Peckham SM, Tisdale B, Rodier PM. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicol Teratol*. 2000; 22:319–324. [PubMed: 10840175]
- Jeong SJ, Li S, Luo R, Strokes N, Piao X. Loss of Col3a1, the gene for Ehlers-Danlos syndrome type IV, results in neocortical dyslamination. *PloS one*. 2012; 7:e29767. [PubMed: 22235340]
- Johansson AS, Brask J, Owe-Larsson B, Hetta J, Lundkvist GB. Valproic acid phase shifts the rhythmic expression of Period2::Luciferase. *Journal of biological rhythms*. 2011; 26:541–551. [PubMed: 22215612]
- Keene JD. Biological clocks and the coordination theory of RNA operons and regulons. *Cold Spring Harb Symp Quant Biol*. 2007; 72:157–165.
- Kim SK. Recent update of autism spectrum disorders. *Korean journal of pediatrics*. 2015; 58:8–14. [PubMed: 25729393]
- Lepagnol-Bestel AM, Maussion G, Boda B, Cardona A, Iwayama Y, Delezoide AL, Moalic JM, Muller D, Dean B, Yoshikawa T, Gorwood P, Buxbaum JD, Ramoz N, Simonneau M. SLC25A12 expression is associated with neurite outgrowth and is upregulated in the prefrontal cortex of autistic subjects. *Molecular psychiatry*. 2008; 13:385–397. [PubMed: 18180767]
- Luo R, Jeong SJ, Jin Z, Strokes N, Li S, Piao X. G protein-coupled receptor 56 and collagen III, a receptor-ligand pair, regulates cortical development and lamination. *Proc Natl Acad Sci U S A*. 2011; 108:12925–12930. [PubMed: 21768377]
- Markram H, Rinaldi T, Markram K. The intense world syndrome--an alternative hypothesis for autism. *Front Neurosci*. 2007; 1:77–96. [PubMed: 18982120]
- Markram K, Rinaldi T, La MD, Sandi C, Markram H. Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology*. 2008; 33:901–912. [PubMed: 17507914]
- Martin HG, Manzoni OJ. Late onset deficits in synaptic plasticity in the valproic acid rat model of autism. *Frontiers in cellular neuroscience*. 2014; 8:23. [PubMed: 24550781]
- Martinez-Sanchis S. Neurobiological foundations of multisensory integration in people with autism spectrum disorders: the role of the medial prefrontal cortex. *Frontiers in human neuroscience*. 2014; 8:970. [PubMed: 25538597]
- Maussion G, Carayol J, Lepagnol-Bestel AM, Tores F, Loe-Mie Y, Milbreta U, Rousseau F, Fontaine K, Renaud J, Moalic JM, Philippi A, Chedotal A, Gorwood P, Ramoz N, Hager J, Simonneau M. Convergent evidence identifying MAP/microtubule affinity-regulating kinase 1 (MARK1) as a susceptibility gene for autism. *Human molecular genetics*. 2008; 17:2541–2551. [PubMed: 18492799]
- Muller RA. The study of autism as a distributed disorder. *Mental retardation and developmental disabilities research reviews*. 2007; 13:85–95. [PubMed: 17326118]
- Mychasiuk R, Richards S, Nakahashi A, Kolb B, Gibb R. Effects of rat prenatal exposure to valproic acid on behaviour and neuro-anatomy. *Developmental neuroscience*. 2012; 34:268–276. [PubMed: 22890088]
- Nair A, Treiber JM, Shukla DK, Shih P, Muller RA. Impaired thalamocortical connectivity in autism spectrum disorder: a study of functional and anatomical connectivity. *Brain: a journal of neurology*. 2013; 136:1942–1955. [PubMed: 23739917]
- Nicholas B, Rudrasingham V, Nash S, Kirov G, Owen MJ, Wimpory DC. Association of Per1 and Npas2 with autistic disorder: support for the clock genes/social timing hypothesis. *Molecular psychiatry*. 2007; 12:581–592. [PubMed: 17264841]

- Nir I, Meir D, Zilber N, Knobler H, Hadjez J, Lerner Y. Brief report: circadian melatonin, thyroid-stimulating hormone, prolactin, and cortisol levels in serum of young adults with autism. *Journal of autism and developmental disorders*. 1995; 25:641–654. [PubMed: 8720032]
- Olde Loohuis NF, Kole K, Glennon JC, Karel P, Van der Borg G, Van Gemert Y, Van den Bosch D, Meinhardt J, Kos A, Shahabipour F, Tiesinga P, Van Bokhoven H, Martens GJ, Kaplan BB, Homberg JR, Aschrafi A. Elevated microRNA-181c and microRNA-30d levels in the enlarged amygdala of the valproic acid rat model of autism. *Neurobiology of disease*. 2015; 80:42–53. [PubMed: 25986729]
- Parikshak NN, Luo R, Zhang A, Won H, Lowe JK, Chandran V, Horvath S, Geschwind DH. Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. *Cell*. 2013; 155:1008–1021. [PubMed: 24267887]
- Patzold LM, Richdale AL, Tonge BJ. An investigation into sleep characteristics of children with autism and Asperger's Disorder. *Journal of paediatrics and child health*. 1998; 34:528–533. [PubMed: 9928644]
- Rasalam AD, Hailey H, Williams JH, Moore SJ, Turnpenny PD, Lloyd DJ, Dean JC. Characteristics of fetal anticonvulsant syndrome associated autistic disorder. *Developmental medicine and child neurology*. 2005; 47:551–555. [PubMed: 16108456]
- Richdale AL, Prior MR. The sleep/wake rhythm in children with autism. *European child & adolescent psychiatry*. 1995; 4:175–186. [PubMed: 8846206]
- Roullet FI, Lai JK, Foster JA. In utero exposure to valproic acid and autism--a current review of clinical and animal studies. *Neurotoxicology and teratology*. 2013; 36:47–56. [PubMed: 23395807]
- Schneider T, Turczak J, Przewlocki R. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. *Neuropsychopharmacology*. 2006; 31:36–46. [PubMed: 15920505]
- Schreck KA, Mulick JA, Smith AF. Sleep problems as possible predictors of intensified symptoms of autism. *Research in developmental disabilities*. 2004; 25:57–66. [PubMed: 14733976]
- Schubert D, Martens GJ, Kolk SM. Molecular underpinnings of prefrontal cortex development in rodents provide insights into the etiology of neurodevelopmental disorders. *Molecular psychiatry*. 2014
- Snow WM, Hartle K, Ivanco TL. Altered morphology of motor cortex neurons in the VPA rat model of autism. *DevPsychobiol*. 2008; 50:633–639.
- Sui L, Chen M. Prenatal exposure to valproic acid enhances synaptic plasticity in the medial prefrontal cortex and fear memories. *Brain research bulletin*. 2012; 87:556–563. [PubMed: 22326482]
- Sweeney SM, Orgel JP, Fertala A, McAuliffe JD, Turner KR, Di Lullo GA, Chen S, Antipova O, Perumal S, Ala-Kokko L, Forlino A, Cabral WA, Barnes AM, Marini JC, San Antonio JD. Candidate cell and matrix interaction domains on the collagen fibril, the predominant protein of vertebrates. *The Journal of biological chemistry*. 2008; 283:21187–21197. [PubMed: 18487200]
- Tordjman S, Anderson GM, Pichard N, Charbuy H, Touitou Y. Nocturnal excretion of 6-sulphatoxymelatonin in children and adolescents with autistic disorder. *Biological psychiatry*. 2005; 57:134–138. [PubMed: 15652871]
- Umeda S, Mimura M, Kato M. Acquired personality traits of autism following damage to the medial prefrontal cortex. *Social neuroscience*. 2010; 5:19–29. [PubMed: 19637100]
- Valk SL, Di Martino A, Milham MP, Bernhardt BC. Multicenter mapping of structural network alterations in autism. *Human brain mapping*. 2015; 36:2364–2373. [PubMed: 25727858]
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*. 2002; 3:RESEARCH0034. [PubMed: 12184808]
- Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S, Mill J, Cantor RM, Blencowe BJ, Geschwind DH. Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature*. 2011; 474:380–384. [PubMed: 21614001]
- Wiggs L, Stores G. Sleep patterns and sleep disorders in children with autistic spectrum disorders: insights using parent report and actigraphy. *Developmental medicine and child neurology*. 2004; 46:372–380. [PubMed: 15174528]

Highlights

- RNA sequencing and gene ontology analysis identified the molecular networks impaired in the mPFC of a VPA rat model of autism.
- Expression of a subset of circadian rhythm genes are altered by prenatal VPA exposure.
- Collagen mRNA levels are differentially expressed in the mPFC of the VPA animal model as compared to control animals.

FIG. 1. Volcano plots representing the RNA sequencing data

A) Volcano plot presenting the RNA sequencing data as $-\log_{10}(\text{adj } P)$ plotted against the $\log_2(\text{fold change})$. The $-\log_{10}(\text{adj } P)$ and $\log_2(\text{fold change})$ analysis thresholds are indicated by dotted lines. Green dots represent the genes used for further analysis.

B) Volcano plot on the genes with an adjusted P-value ≥ 0.05 , plotted as $-\log_{10}(\text{RPKM})$ against $\log_2(\text{fold change})$. Green dots represent the genes used for GO analysis.

FIG. 2. Circadian rhythm genes are dysregulated in the mPFC due to prenatal VPA exposure

A) IPA-based GO analysis on the 74 VPA-affected genes resulted in a top four significantly enriched pathways. Values within the grey bars indicate the P-values assigned by the GO analysis software.

B) DAVID-based GO analysis indicates three significantly affected pathways in the VPA animal model, being the circadian rhythm pathway, ECM receptor interaction and focal adhesion. The value within the bar indicates the P-values assigned by the GO analysis software.

C) *Per1*, *Per2* and *Bhlhe40* are part of the circadian rhythm pathway indicated significantly altered by both GO software analysis (Dark blue). *Col1a1*, *Col1a2* and *Col3a1* were present in the top pathways of both IPA and DAVID (blue). *Itga5* was unique in the top ECM receptor interaction pathway of DAVID (light blue) while *Col27a1*, *Igf2*, *Lhx2* and *Lyz* were unique in the top pathways of IPA (purple).