Neurotoxicology and Teratology 71 (2019) 64–74

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Full length article

S-adenosyl methionine prevents ASD like behaviors triggered by early postnatal valproic acid exposure in very young mice

Asher Ornoy[a,*], Liza Weinstein-Fudim[a,1], Matanel Tfilin[b], Zivanit Ergaz[a], Joseph Yanai[a], Moshe Szyf[c], Gadi Turgeman[b]

[a] Department of Medical Neurobiology, Hebrew University Hadassah Medical School, Israel
[b] Department of Molecular Biology and Pre-Medical Studies, Ariel University, Ariel, Israel
[c] Department of Pharmacology and therapeutics, McGill University Medical School, Montreal, Quebec, Canada

ARTICLE INFO

Keywords:
ASD
Mice
VPA
Postnatal injection
SAM
Epigenetics

ABSTRACT

Introduction: A common animal model of ASD is the one induced by valproic acid (VPA), inducing epigenetic changes and oxidative stress. We studied the possible preventive effect of the methyl donor for epigenetic enzymatic reactions, S-adenosine methionine (SAM), on ASD like behavioral changes and on redox potential in the brain and liver in this model.

Methods: ICR albino mice were injected on postnatal day 4 with one dose of 300 mg/kg of VPA, with normal saline (controls) or with VPA and SAM that was given orally for 3 days at the dose of 30 mg/kg body weight. From day 50, we carried out neurobehavioral tests and assessment of the antioxidant status of the prefrontal cerebral cortex, liver assessing SOD and CAT activity, lipid peroxidation and the expression of antioxidant genes.

Results: Mice injected with VPA exhibited neurobehavioral deficits typical of ASD that were more prominent in males. Changes in the activity of SOD and CAT increased lipid peroxidation and changes in the expression of antioxidant genes were observed in the prefrontal cortex of VPA treated mice, more prominent in females, while ASD like behavior was more prominent in males. There were no changes in the redox potential of the liver. The co-administration of VPA and SAM alleviated most ASD like neurobehavioral symptoms and normalized the redox potential in the prefrontal cortex.

Conclusions: Early postnatal VPA administration induces ASD like behavior that is more severe in males, while the redox status changes are more severe in females; SAM corrects both. VPA-induced ASD seems to result from epigenetic changes, while the redox status changes may be secondary.

1. Introduction

As of today, there are no biological markers that predict a diagnosis of ASD, and the etiology is largely unknown. Both genetics and *prenatal or* early postnatal environmental exposures, or potential interactions of the two, are known to cause ASD (DSM 5, 2013; Developmental Disabilities Monitoring Network Surveillance Year Principal et al., 2014; Ornoy et al., 2015; Rodier et al., 1996). Typical neurobehavioral symptoms of ASD can be detected in animals, such as mice, rats and various genetically and environmentally-induced animal models of ASD have been described (Ornoy et al., 2016). Their advantage is the possibility to perform different behavioral, morphological and molecular studies in the search of understanding the etiology of ASD. Although most models of ASD are genetic, there are several animal models where ASD-like symptoms were produced by exposure of the pregnant dams to a teratogenic agent during different stages of gestation, especially valproic acid (VPA), and a few following early postnatal insults (Ornoy, 2009; Ornoy et al., 2015; Rodier et al., 1996; Wagner et al., 2006).

Christianson et al. (1994) first reported in 1994 that VPA during pregnancy leads to significant increased rate of ASD in the offspring; which was further corroborated by others (Williams et al., 2001). In a large Danish population-based study (Christensen et al., 2013), the rate of ASD among children exposed in utero to VPA was about 4%. The association between VPA use in pregnancy and ASD prompted investigators to produce experimental ASD models in rodents by VPA administered in high doses during different stages of pregnancy (Ornoy, 2009; Rodier et al., 1996).

Various investigators have produced in mice and rats VPA–induced behavioral changes reminiscent of ASD-like behaviors (Bambini-Junior et al., 2011; Ingram et al., 2000; Kolozsi et al., 2009; Ornoy, 2009;

* Corresponding author.
E-mail address: ornoy@cc.huji.ac.il (A. Ornoy).
[1] This study serves as part of the requirements for PhD degree of Liza Weinstein - Fudim of the Hebrew University.

https://doi.org/10.1016/j.ntt.2018.01.005
Received 12 July 2017; Received in revised form 21 November 2017; Accepted 11 January 2018
Available online 16 January 2018
0892-0362/

Rodier et al., 1997, 1996; Wagner et al., 2006). The exposed offspring exhibited developmental delay, lifelong deficits in motor performance and social behavior, anxiety-like behavior and alterations in postnatal growth and development (Bambini-Junior et al., 2011; Ingram et al., 2000; Kolozsi et al., 2009; Rodier et al., 1997; Wagner et al., 2006). As in humans, anatomical alterations such as reduced number of cerebellar Purkinje cells, abnormal neocortical neuronal organization, enhanced synaptic plasticity of the prefrontal cortex and damage to cranial nerve nuclei have been described (Bambini-Junior et al., 2011; Ingram et al., 2000; Kolozsi et al., 2009; Rodier et al., 1997). Wagner et al. (2006) induced ASD-like behaviors in mice by injecting VPA, during both, pregnancy and on postnatal day 14. The advantage of this rodent's postnatal model, that is equivalent to the developmental stages of the third trimester of human fetal brain (Maciag et al., 2006), is the lack of possible interference of maternal metabolism and the possibility to track the sequence of the behavioral changes induced by VPA.

ASD is more prevalent in males than in females (Ornoy et al., 2016). Indeed, in humans, different clinical presentation, especially in socialization, were found between genders (Beggiato et al., 2016; Goddard et al., 2014; Head et al., 2014; Sedgewick et al., 2016). However, the same diagnostic criteria were used for males and females. There seems to be few animal models that studied the gender differences. In the VPA induced ASD rat model, Kim et al. (2013) found a higher susceptibility of male rats to social interaction impairment over females. Schneider et al. (2008) also observed more behavioral and other changes in VPA exposed male rats compared to females. There seems to be no studies describing gender differences in a murine model for ASD like behavior.

Several mechanisms that may explain the teratogenic effects of VPA in different animal species have been proposed as following: 1. VPA being a potent histone deacetylase inhibitor affects the epigenome by increasing DNA demethylation (Detich et al., 2003a; Dong et al., 2007). 2. VPA induces increased oxidative stress that may cause oxidative damage to lipids, DNA and proteins (Detich et al., 2003a; Moldrich et al., 2013; Ornoy, 2009; Phiel et al., 2001). Indeed, treatment of VPA exposed dams with high doses of folic acid, vitamin E (antioxidants) and the methyl donor methionine were demonstrated to ameliorate or prevent the VPA-induced teratogenicity (Ehlers et al., 1996; Ornoy, 2009).

Proof for possible epigenetic changes in ASD came from a variety of studies demonstrating dysfunction of various genetic pathways, especially associated with inflammation, immunity (Loke et al., 2015), and transcription factors such as ADNP and ASH1L. Genome-wide studies of gene expression and DNA methylation in ASD have directly shown changes in DNA methylation patterns. For example, the description by Yip et al. (2008) of changes in the expression of glutamate decarboxylase 1 (GAD1) in Purkinje cells of the cerebellum in ASD patients and increased methylation of the promoter of GAD1 demonstrating increased gene silencing. Reduced MECP2 expression was found in the frontal cortex of patients with Rett syndrome that manifest ASD like symptoms and in many patients with ASD (Loke et al., 2015; Nagarajan et al., 2008). Studies analyzing DNA methylation in the frontal cortex or cerebellum have observed changes in the methylation patterns of many genes, demonstrating the importance of epigenetic changes in ASD (Tordjman et al., 2014). Kim et al. (2016) showed downregulation of several genes in the brain of VPA-induced ASD rats, including the methylated DNA binding protein, MECP2 gene. Similarly, Aizawa and Yamamoto found altered DNA methylation of the *p21* gene which was correlated with expression changes in the hippocampus of adult VPA treated mice (Aizawa and Yamamuro, 2015). Tremolizzo et al. (2005) found decreased methylation in CG sites in the promoter of *Reln* and *Gad67* genes.

DNA methylation inhibitors and enhancers as potential therapeutic agents (Szyf, 2015). Indeed, some inhibitors of DNA methylation including 5-azacytidine, zebularine and decitabine were approved for cancer treatment (Szyf, 2015); methylation enhancers were considered for use in cancer treatment and possibly for several mental and psychiatric disorders.

A potent enhancer of DNA methylation is S-adenosine methionine (SAM) (Szyf, 2015). SAM is an endogenous physiological co-factor involved in methyl group transfers, trans-sulfuration, aminopropylation and many other metabolic processes occurring in all organs. A large number of methyl transferase reaction utilise SAM as a methyl donor including nucleic acids, proteins, lipids and secondary metabolites. SAM is bound by the SAM riboswitch which regulates genes involved in methionine or cysteine biosynthesis. It is a widely used nutritional supplement without distinct side effects. In a recent phase 1 clinical trial, the addition of SAM to antidepressant drugs promoted a favorable response (Mischoulon et al., 2014). SAM treatment was shown to affect cocaine craving in rat models (Massart et al., 2015) and to prevent cognitive decline in a trangenic AD mouse model (Do Carmo et al., 2016). In both examples, SAM altered DNA methylation in condition-relevant genes. SAM was also shown to have antioxidant effects such as it decreased lipid peroxidation in rat brains and other tissues (De La Cruz et al., 2000; Michael Brown et al., 2012; Cavallaro et al., 2010); affected antioxidant enzyme activity, increased total glutathione levels (Lozano-Sepulveda et al., 2016) and inhibited Fe oxidation (Caro and Cederbaum, 2004).

Our aim was to test the hypothesis that early methyl supplementation reduces/prevents ASD symptoms triggered by postnatal valproic acid exposure. We established a mouse model of ASD like behavior by treating mouse pups on postnatal day 4 with VPA, examining sex differences in development of ASD symptoms and evaluating whether concomitant treatment with the methyl donor-SAM affects the development of ASD symptoms and prefrontal cerebral cortex redox potential.

2. Materials and methods

2.1. Animals

Male and female (four days old) outbred ICR albino pups, were injected subcutaneously on postnatal day 4, either 300 mg/kg body weight of VPA dissolved in normal saline or normal saline (NS). The dose chosen was the minimal dose that was reported to produce ASD in mice offspring. This time is developmentally equivalent to months 7–8 of human pregnancy. Each of these two groups (VPA and NS treated) were further subdivided into two groups - one receiving daily by intraoral gavage, from day 5 for 3 days, 30 mg/kg body weight of SAM dissolved in NS, and the other receiving NS. We postponed the SAM administration by 24 h to enable the recovery of the pups from the VPA injection and to achieve clearance of the drug. Pups were weighed twice weekly, and on day 50 they were exposed for 10 days to a variety of neurobehavioral tests. Each treatment or control group consisted of 12–16 males and a similar number of female mice.

The experiments were conducted in two parts. About half of the pups were treated in the first part and divided into the 4 groups. After several months, we similarly treated the second group of pups; also divided into the 4 groups. As the results were similar, we reported the combined data of both experiments.

On day 60, the animals were euthanized, brains (prefrontal cerebral cortex) and livers were removed and weighed; the redox status was examined as detailed below. All pups were handled similarly in the animal quarters under optimal temperature and light. The University of Ariel Ethics committee for experiments on animals received approval for the study.

2.2. Neurobehavioral assessment

During days 50–60, mice were exposed to a comprehensive set of behavioral assays considered to be of high relevance and sensitivity for the assessment of symptoms associated with ASD (Silverman et al., 2010). The following tests were used during the light cycle:

1. Water T-maze: assessing reversal learning, cognitive rigidity and repetitive behavior. A four days trial was applied. In the first 2 days (10 trials/day), mice were learning the location of the platform in one of the arms. Latency duration to finding the platform confirms and validates the learning process. On the third day, platform place is switched and re-learning its location is assessed by measuring latency duration to finding the platform in two consecutive days (10 trials/day) and by counting the number of correct turning across the trials (Moy et al., 2007; Segal-Gavish et al., 2015).
2. Open Field test: assessing general locomotor activity and exploration behavior. During a 10 minute assay, total distance travelled and time spent in the center of the arena (duration) were measured. In addition, frequency of self-grooming was measured (Moy et al., 2007; Segal-Gavish et al., 2015).
3. Three chamber social interaction tests: assessing social behavior and preference for social novelty, typically impaired in mice presenting ASD-like phenotype. Social behavior is assessed as the time spent with a social stimulus versus non-social stimulus. Whereas, social preference is evaluated as a ratio between the times spent with a novel social stimulus versus a familial social stimulus (Crawley, 2004; Moy et al., 2007; Segal-Gavish et al., 2015).
4. Elevated Plus Maze: assessing the level of anxiety. Percent open arms were calculated as the ratio between the time spent in open arms and the total time spent in both open and closed arms for 5 min trial (Moy et al., 2007). The Number of head dips beyond the open arms was also counted (Walf and Frye, 2007).

2.3. Autism composite score

In order to assign for each animal an integrated value indicating for its ASD-like phenotypic severity, an integrated Autism composite score was calculated for each mouse by combining Z-scores from three behavioral parameters: Self-grooming frequency, T-maze (% of incorrect turns) and social novelty preference (preference to familiar stimulus). Z-scores were obtained by Z-standardization of the measured results for each parameter, in a manner in which higher scores will reflect a more severe autistic trait as previously described (El-Kordi et al., 2013; Segal-Gavish et al., 2015).

2.4. Antioxidant gene expression studies

Total RNA was extracted from the right prefrontal cortex of the mice using the RNA/DNA/protein purification plus kit (47700; Norgen) according to the manufacturer's protocol. RNA was quantified absorbance at 260 nm. A Perfecta DNase 1 kit (Quanta bio) was used to eliminate possible DNA product. Complementary DNAs (cDNA) were transcribed from 1-microgram RNA samples using the qScript cDNA Synthesis Kit (Quanta-bio) and then served as template for real-time PCR analyses to quantify expression of *antioxidant genes* using StleepOnePlus Real-Time PCR System (Life Technologies). *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) served as a reference gene to normalize for differences in total mRNA between the samples. Primers are listed in Table 1. Amplification was performed using PerfeCTa SYBR Green FastMix (Quanta-bio) using the following conditions: 30 s denaturation at 95 °C, followed by 30 s annealing at 60 °C for a total of 40 cycles and normalized quantification of the target genes was calculated with the $2-\Delta\Delta Ct$ method. PCR product specificity was confirmed using melting curve analysis. RNA samples that were not reverse-transcribed were used as negative controls.

Table 1
Primers used for qPCR.

SOD1 forward	TTGGCCGTACAATGGTGGT
SOD1 reverse	ACTGCGCAATCCCAATCACTC
SOD2 forward	GTGGTGGAGAACCCAAAGGA
SOD2 reverse	ACCTTGGACTCCCACAGACA
CAT forward	CCCGAGTCTCTCCATCAGGT
CAT reverse	CTGCCTCTCCATCTGCATTAACC
GPX1 forward	AATCAGTTCGGACACCAGGA
GPX1 reverse	CACTTCGCACTTCTCAAACAATG
GAPDH forward	GGGGCTCTCTGCTCCTCCCTGT
GAPDH reverse	TGACCCTTTTGGCCCCACCCT

2.5. Assessment of redox status in the cerebral cortex and liver

The biochemical analyses were carried out from homogenates of the left prefrontal cerebral cortex and liver.

2.5.1. Protein content

Measured in the crude homogenate of the brain and liver according to Bradford, using bovine serum albumin as a standard (Bradford, 1976).

2.5.2. Oxidative stress and antioxidant capacity

1. Lipid peroxidation: assayed by measuring thiobarbituric acid reactive substances (TBARS) according to Mihara and Uchiyama (1978). Sixty microliters of TCA (60%) were added to 300 µl of brain and liver homogenate in 1.5 ml microtubes. Following vigorous mixing, the tubes were centrifuged for 10 min at 14,000*g*. Supernatant samples (200 µl) were placed into 96-well plates, and 80 µl of thiobarbituric acid (1.3%, dissolved in NaOH 0.3%) was added to the samples in the wells. The plate was wrapped with Saran (Ziploc, Indianapolis, IN), incubated for 20 min at 90 °C bath, and then cooled on ice. The samples were spectrally analyzed in the plate at 532 nm (Mihara and Uchiyama, 1978). Data is expressed by micromole Malone dialdehyde (MDA) per milligram protein.
2. Superoxide dismutase (SOD) total activity: determined as described by McCord and Fridovich (McBride et al., 2010). Xanthine oxidase 0.215 U/l (Sigma) was added to a solution containing hypoxanthine (1.2 mM) that produces ROS. Cytochrome C, 48 µM (Sigma) was used as a detector. $O_2\cdot^-$ reduces the cytochrome C, and the reduced cytochrome C is measured spectrophotometrically (Uvicon 933, Kontron, Switzerland) at 550 nm. SOD present in the samples decreases the amount of $O_2\cdot^-$, and hence decreasing the amount of reduced cytochrome C detected by the spectrophotometer. By this method the activity of both Cu/Zn SOD and Mn SOD are assessed. Data is expressed by percent of inhibition of cytochrome C reduction. The higher the inhibition, the higher is SOD activity.
3. Catalase-like activity: The method was described by Thurman et al. (1972) Aliquots of 15–20 µl of the homogenate were added to the reaction mixture containing H_2O_2 (0.15 mM). Following 10 min of incubation at room temperature, the reaction was stopped by adding 200 µl trichloroacetic acid 30% and the residual H_2O_2 was determined according to the Thurman procedure. In brief, it measures the red complex that is formed by H_2O_2, ferrous ammonium sulfate (Sigma) and 25% thiocyanate (Sigma). The concentration of the complex, which is directly related to the concentration of H_2O_2 in the tested solution, is read by a spectrophotometer at 480 nm. By this method, the activity of catalase and glutathione like enzymes are assessed together. Results of enzyme activities are expressed as U/mg protein.

2.6. Statistical analysis

Results are presented as mean ± standard error in the figures or standard deviation in the table. Statistical significance was calculated using analysis of variance (ANOVA), followed by Tukey's post–hoc analysis of pairwise comparisons. In the analysis of latencies in the water T-maze, differences between the latency curves were analyzed with ANOVA repeated measures followed as well by Tukey's post-hoc test. Pearson correlation test was applied for correlating related

variables. Graphs and statistical analysis was performed using Prizm Graph pad 5 software.

3. Results

3.1. General

There was about 10% mortality, mainly in the first day following VPA injection. Apart from VPA toxicity, we assume that temporary malnutrition contributed to the mortality since, during the first several days, VPA treated neonates were less active than those injected with saline. Indeed, the VPA injected pups had reduced weight gain during the first week post treatment; however, this was normalized thereafter. At the end of the experiment, weight ranged between 29.6 ± 14 to 32.9 ± 0.4 g in females and 39.1 ± 1.3 to 41.2 ± 0.7 g in males, with no differences between the groups. No additional mortality was noticed during the administration of SAM.

3.2. Redox potential studies in the prefrontal cortex

Increased lipid peroxidation (MDA) was found in the brain of the VPA injected female mice. The administration of SAM reduced MDA levels to those of control animals (Fig. 1). There was no change in the activity of total SOD between the different groups except in the VPA + SAM treated females that had increased SOD activity. The activity of CAT was significantly increased in the VPA injected female mice, and normalized with the administration of SAM.

3.2.1. Males

In males, VPA increased SOD activity that was not reduced by the addition of SAM. SAM increased CAT activity compared to all other groups but VPA or VPA plus SAM induced no changes in CAT activity (Fig. 1). There was no change in MDA levels.

3.3. Antioxidant gene expression in the prefrontal cortex

3.3.1. Females

VPA administration increased the expression of CAT that was not reduced by the addition of SAM. SAM increased the expression of SOD1 in the controls, but there was no change by VPA, or VPA plus SAM. VPA, VPA plus SAM or SAM plus saline increased the expression of SOD2 (Fig. 2).

3.3.2. Males

VPA treatment increased the expression of CAT that was normalized by the co-administration of SAM (Fig. 2). SAM alone had no effect on CAT gene expression. There were no changes in the expression of SOD1 or Glutathione peroxidase. VPA increased the expression of SOD2 that was not reduced by SAM; the addition of SAM to the control pups also increased the expression of SOD2. There was no change in the expression of GSHPx in males or females.

No changes were observed in MDA levels and in SOD and CAT activity in the liver among VPA, VPA and SAM or control female mice, suggesting that there was no oxidative stress in the mice at 60 days of age (Table 2).

3.4. Neurobehavioral studies

1. Water T-maze: there were no significant differences in learning curve in male mice (Fig. 3A). In females, the learning curve for detecting the new location of the platform was significantly less effective and there were significantly greater latencies in trials in the VPA-exposed mice compared to saline. This higher latency was normalized by the co-addition of SAM (Fig. 3B). VPA also decreased the percent of correct entrances in males but not in females. This was normalized by the addition of SAM (Fig. 4a,b). While the learning curve was affected by VPA in females, the abnormal turning was evident in males, implying that tasks were altered differently in each sex.
2. Open field: Total distance walked did not differ between groups in both sexes (data not shown) implying no effect of VPA or SAM on general locomotion. VPA increased grooming frequency only in males that normalized upon co-administration of SAM (Fig. 5). VPA significantly decreased accumulating duration in the center of the arena only in females and normalized by co-administration of SAM (Fig. 6).
3. Elevated plus maze: No differences were observed between the groups in entries and durations in open versus closed arms (data not shown). Greater anxiety demonstrated by significantly lower frequency of head dipping over the edges of the open arm compared with controls was demonstrated only among females; co-administration of SAM was associated with an increase in head dipping in both genders (Fig. 7).
4. Social interaction: Males, but not females, exposed to VPA showed significantly lower preference for social novelty and preference to familiar social stimuli that was normalized by the co-administration of SAM (Fig. 8).

Autism composite score was significantly higher in VPA exposed male mice and was reduced following SAM treatment. Same trend was observed in females; while without statistical significance due to average lower score in the VPA exposed female mice (Fig. 9).

4. Discussion

We have shown that VPA administration to 4-day-old mice induces autistic like behavior, which is still manifested even at the age of 60 days. In addition, there were some changes in the redox potential of the prefrontal cortex in the treated animals, but not in the liver. This implies that the brain is particularly sensitive during this period of development to the effects of VPA. There were noted sex-differences in the behavioral changes and in the redox potential in response to VPA. Notwithstanding these sex differences, both females and males exhibited typical autistic-like behavior. The addition of SAM generally improved the behavior of the VPA treated animals of both sexes, becoming similar to that of control animals. While VPA increased the expression of *catalase* and *SOD2* genes in both sexes, SAM normalized CAT gene expression only in males. Lipid peroxidation and CAT activity were increased by VPA in females; it was normalized by the addition of SAM, but SAM increased SOD activity in the brain of VPA treated female mice. In males, VPA did not increase lipid peroxidation or SOD activity but decreased CAT activity. The fact that SAM ameliorated behavioral effects but had a mixed effect on the redox status suggests that these changes in redox state are not critical for the autistic-like behavioral phenotype.

4.1. Differences between males and females

Sex differences in the clinical presentation of ASD are infrequently described in humans. Head et al. (2014) found among 25 autistic girls at 10–16 years of age, that they scored better on a “friendship questionnaire” than 25 autistic boys of similar age. Sedgewick et al. (2016) found that autistic adolescent males, but not females, have significant reduced motivation for friendship compared to non-autistic adolescents. These differences, which generally show weaker deviations in sociability of autistic girls compared to boys, might cause an underdiagnosis of autistic girls (Head et al., 2014).

Most studies on autistic like behavior induced by VPA did not assess the effects of sex. Schneider et al. (2008) found that among the offspring of VPA treated rats, males exhibited lower sensitivity to pain, increased anxiety, increased repetitive stereotypic like behavior and decreased social behavior; while treated females only showed increased

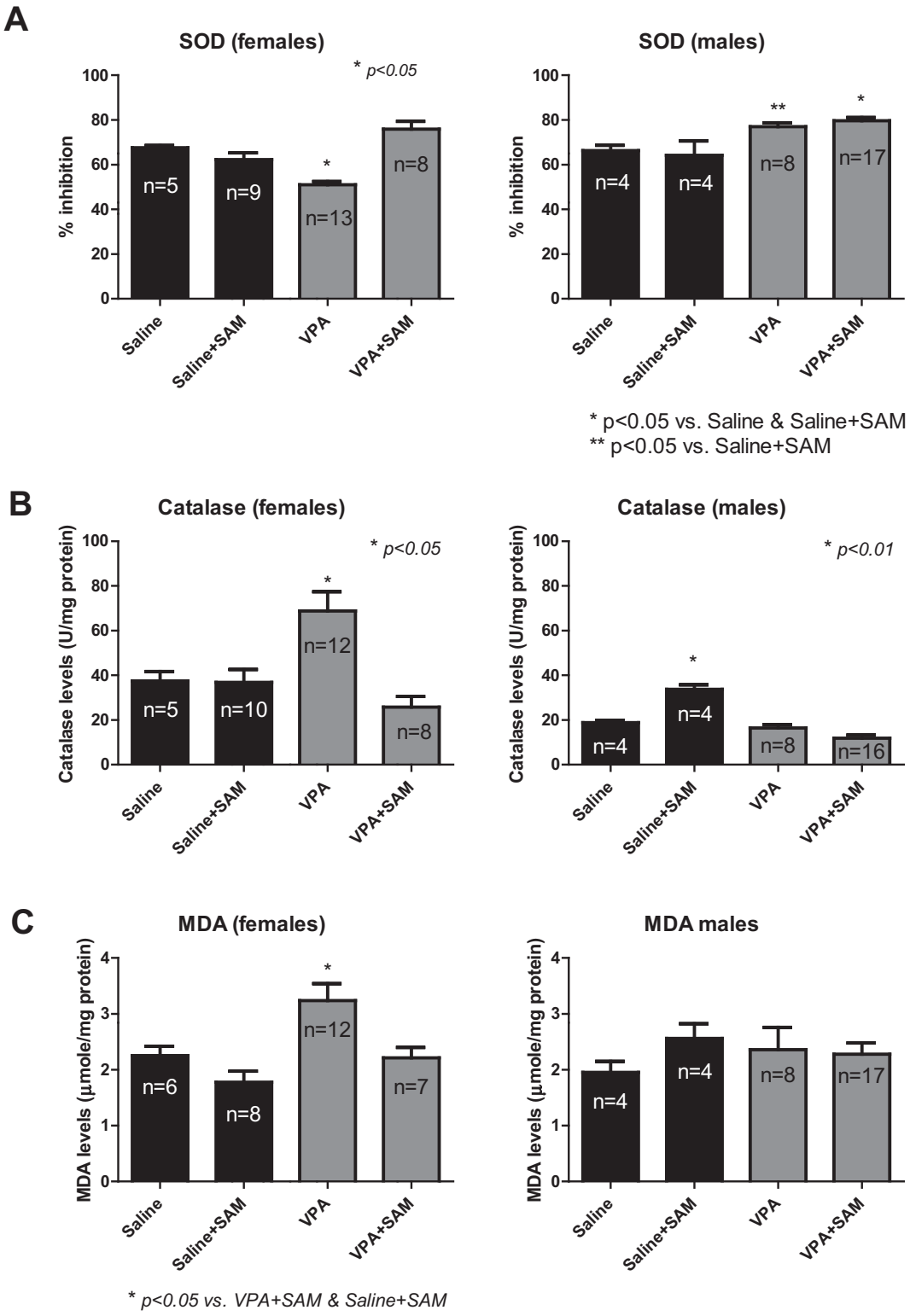

Fig. 1. Redox potential in the prefrontal cortex of male and female mice. The activity of the antioxidant enzymes SOD and Catalase as well as the levels of lipid peroxidation assessed redox potential. Percent inhibition of cytochrome C reduction is presented in A for SOD activity in male and female mice. Catalase activity is presented in B. The levels of peroxidized lipids (MDA) are presented in C. Data is presented as mean ± standard error. *Marks statistical significance ($p < .05$, *ANOVA*) versus all other groups unless stated differently in the graphs.

repetitive movements. They also found sex differences in immune response and in blood cortisol level, which were more abnormal in males. Others (Kim et al., 2013) also found in the offspring of VPA treated rats that the impairment in social interaction in male offspring was more severe than in females.

Our study showed that in spite of the fact that both genders exhibited autistic-like behaviors, they differed in several neurological functions. Whereas, following exposure to VPA, females were more prone to present anxiety-related traits as observed in the open field (center duration) and Elevated plus maze (head dipping); males

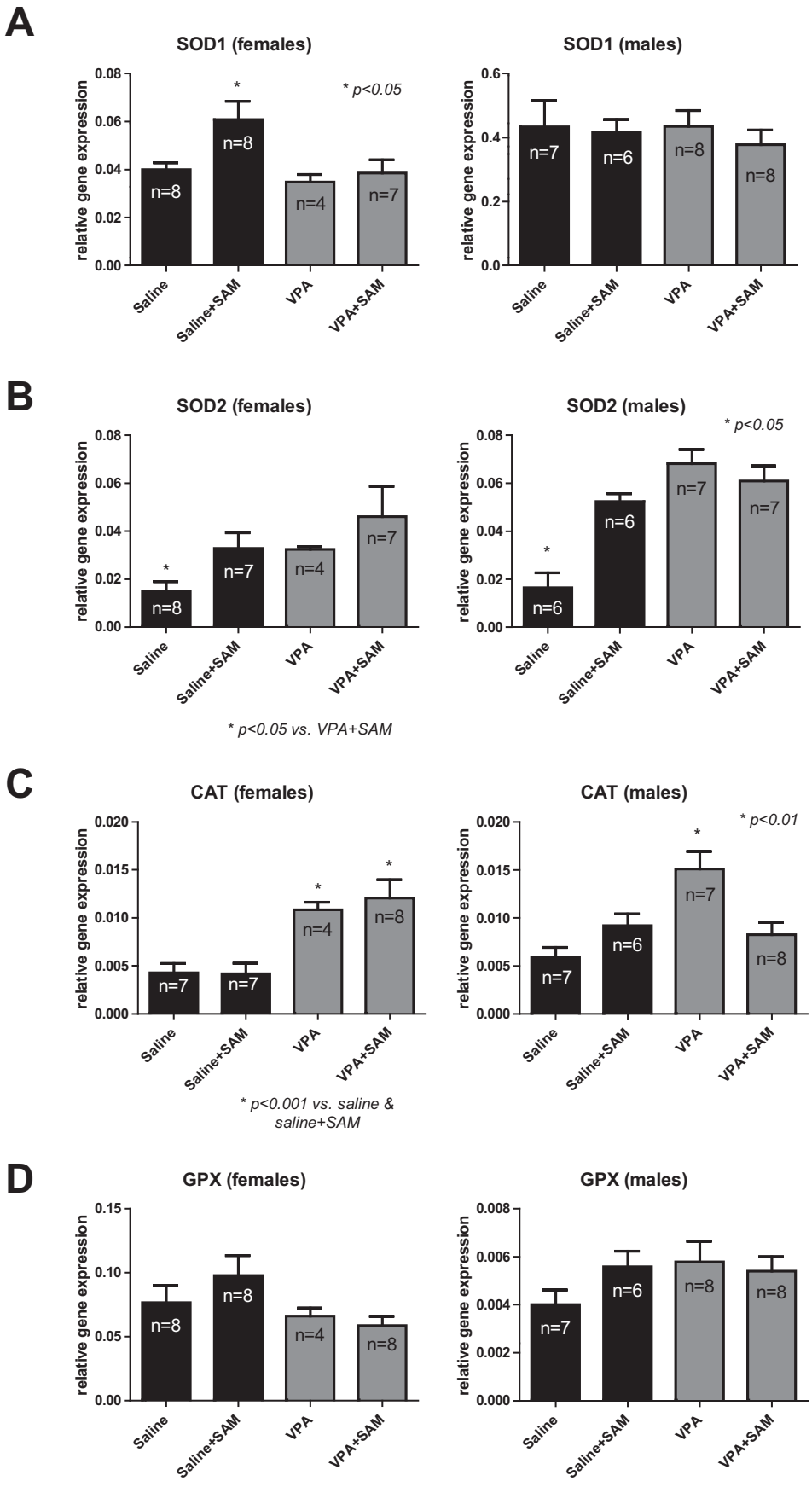

Fig. 2. Antioxidant gene expression. The expression of genes encoding for antioxidant enzymes in the prefrontal cortex of male and female mice were determined by real time RT-PCR normalized to the housekeeping gene GAPDH. Relative gene expression levels were assessed for SOD1 (A), SOD2 (B), Catalase (C) and glutathione peroxidase (D) in male and female mice of the different groups. Data is presented as mean ± standard error. *Marks statistical significance ($p < .05$, *ANOVA*) versus all other groups unless stated differently in the graphs.

Table 2
a, b. SOD, CAT activity and lipid peroxidation in the liver. There are no differences among the groups in females (panel a) or in males (panel b).

a: Females			
	SOD inhibition rate %	MDA (micromole/mg protein)	Catalase (U/mg protein)
Control (10)	82.52 ± 12.69	6.67 ± 1.89	1858.74 ± 829.54
VPA (11)	83.59 ± 9.48	5.17 ± 0.93	1494.16 ± 597.50
P(T ≤ t) two-tail	0.57	0.26	0.25
VPA + SAM (8)	85.68 ± 2.93	6.71 ± 1.79	1690.86 ± 340.45
P(T ≤ t) two-tail	0.47	0.24	0.36

b: Males			
	SOD inhibition rate %	MDA (micromole/mg protein)	Catalase (U/mg protein)
Control (7)	89.09 ± 1.36	5.75 ± 1.30	1742.12 ± 223.58
VPA (7)	87.93 ± 1.63	4.81 ± 1.19	1627.18 ± 438.03
P(T ≤ t) two-tail	0.18	0.18	0.55
VPA + SAM (8)	88.48 ± 1.89	5.65 ± 0.89	1971.16 ± 228.25
P(T ≤ t) two-tail	0.56	0.16	0.09

displayed greater impairment in social novelty preference, grooming frequency and cognitive rigidity (T-maze) implying that the autistic-like behavior was stronger in males. The differences were also reflected in the higher autism composite score induced by VPA in males.

4.2. Oxidative stress in ASD

Studies have shown that the brains of ASD patients are under increased oxidative stress, (low GSH/GSSG), often as a result of some mitochondrial dysfunction (Frye et al., 2013; Ornoy et al., 2015; Rose et al., 2012; Rossignol and Frye, 2014; Yui et al., 2015) and about one third of children with ASD showed definite signs of mitochondrial dysfunction and oxidative stress (Frye et al., 2013). Impaired glutathione synthesis and dysregulation of the mTOR signaling pathway was also described (Yui et al., 2015). Decreased antioxidant capacity causing oxidative stress and abnormal redox status seem to be important contributors to ASD.

Studies have also shown increased oxidative stress in animals that exhibit ASD-like phenotype, including animals exposed to VPA. Antioxidants ameliorated the ASD-like behaviors and reduced the degree of oxidative stress (Yui et al., 2015).

In our study, the redox status was more affected in females than in males as observed by the significant increase in MDA in the brain of females only. This might imply that the neurobehavioral changes are not related primarily to the increased oxidative stress, and oxidative stress is a secondary phenomenon resulting from other changes induced in the brain by VPA, most probably in the epigenome.

4.3. Preventing ASD by SAM treatment

Several researchers attempted to ameliorate the VPA – induced ASD-like symptoms in animal models by postnatal treatments. They generally used brain protective agents. For example, the antioxidant Astaxanthin was administered during days 25–56 and decreased the brain oxidative stress, improving animal's behavior (Al-Amin et al., 2015). Sub-chronic treatment with Donepezil, a drug used for the improvement of cognition in patients with Alzheimer disease, reduced repetitive behavior and hyperactivity and improved sociability in prenatally VPA treated mice. Administration of piperine that was given daily up to day 40 in VPA treated mice ameliorated the ASD-like symptoms (Pragnya et al., 2014). Piperine reduced the extent of oxidative stress in the brain and improved behavior. These models apparently induced local and temporary improvement in brain function. We used a different strategy in our study trying to reverse the underlying long-term abnormal gene programming that drives ASD in the first place.

In our model, we showed that a single acute early postnatal administration of VPA was sufficient to trigger persistent ASD-like symptoms in the animals that were detectable 60 days later. The fact that differences in expression of oxidative enzymes are detected in the brain long after a short transient exposure to the drug suggests that VPA caused stable changes in programming of gene expression by epigenetic processes. VPA is a histone deacetylase inhibitor (Phiel et al., 2001) and triggers as well DNA demethylation (Detich et al., 2003a; Milutinovic et al., 2007), which is consistent with a long-term epigenetic mechanism underlying ASD-like behaviors triggered by VPA. Moreover, we presume that the observed changes in the redox potential of the brain, but not of the liver, result from the specific changes in the expression of antioxidant and other genes induced by VPA. However, future experiments are required to map the particular epigenetic changes triggered by VPA that are involved in the autistic phenotype.

VPA is a general histone deacetylase inhibitor and could potentially affect all genes and all tissues. Nevertheless, we see in our study particular behavioral effects as well as tissue specificity in the response of the redox enzymatic machinery to VPA with noted effects in the brain but not in the liver. The brain is particularly sensitive to epigenetic modulation. A classic example is RETT syndrome which is caused by mutations in MeCP2, a methylated DNA binding protein which has a general role in controlling expression of methylated genes (Meehan

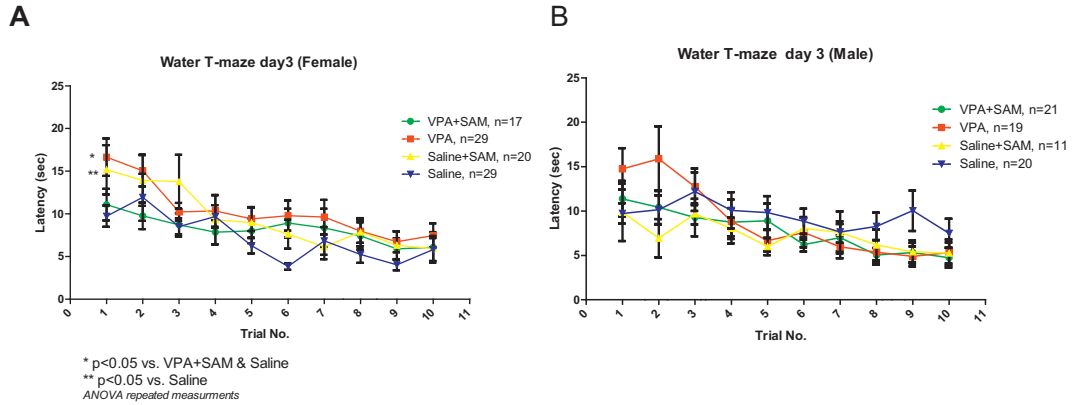

Fig. 3. T-maze test (latency). Latencies to reach the reversed positioned platform in the 3rd day of the assay were measured in 10 consecutive trials. Differences between the groups were noted in females (A), but not in males (B). Data is presented as mean ± standard error. Statistical significance ($p < .05$, *ANOVA repeated measures*) is between VPA group and VPA + SAM or Saline groups.

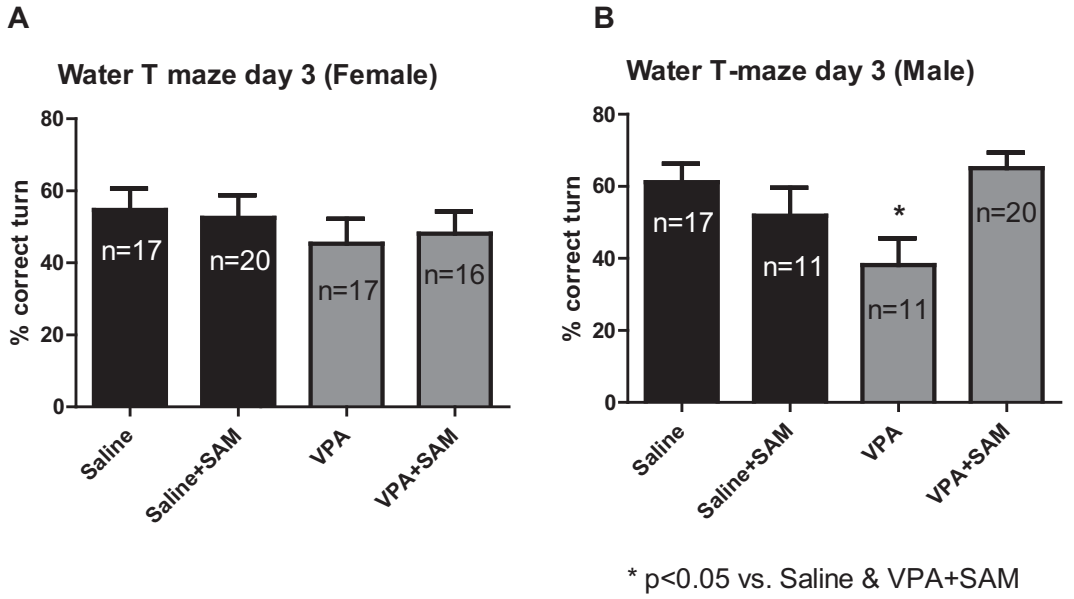

Fig. 4. T-maze test. The percent of correct turns across the 10 trials in the 3rd day of the T-maze assay was calculated for each group. Differences were noted in males (B) but not in females (A). Data is presented as mean ± standard error. *Marks statistical significance ($p < .05$, *ANOVA*) between VPA group and VPA + SAM or Saline groups.

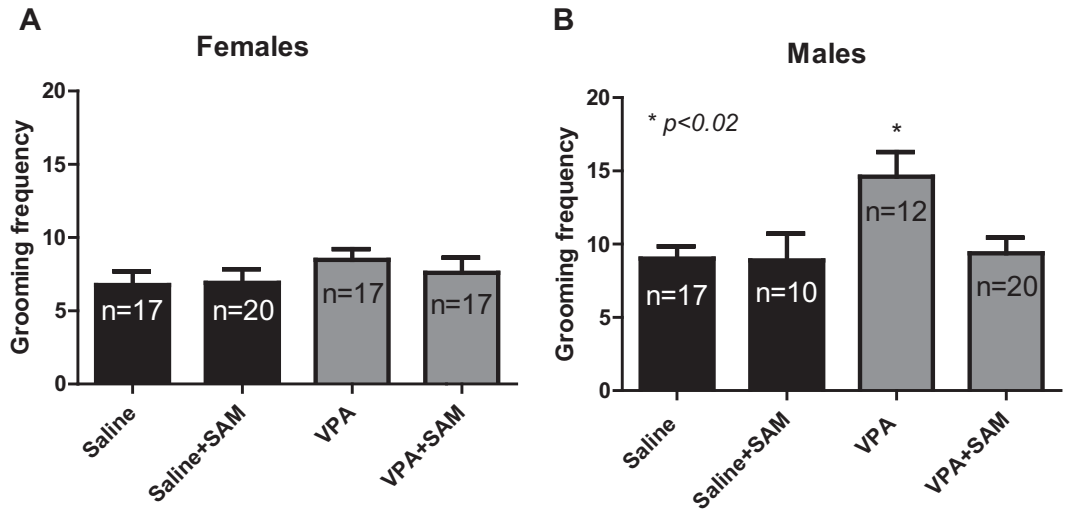

Fig. 5. Open field test (self-grooming). Grooming frequency was measured during the 5 min trial of the open field test. Increased grooming frequency in the VPA group was observed in males (B) but not in females (A). Data is presented as mean ± standard error. *Marks statistical significance ($p < .02$, *ANOVA*) versus all other groups.

et al., 1992) but nevertheless the notable phenotype is neurodevelopmental (Amir et al., 1999). It is also expected that effects of epigenetic modulators will be particularly notable at a time when developmental epigenetic reprogramming is ongoing. Inhibition of epigenetic enzymes such as HDACs should have a stronger impact when these enzymes are involved in setting up gene expression programs. VPA was administered at a time when the mouse brain is still undergoing developmental changes which parallel the developmental stage of the third trimester of human fetal brain (Maciag et al., 2006).

Taking together, the emerging evidence for an epigenetic mechanism in ASD (Ciernia and LaSalle, 2016) and the fact that VPA triggers DNA demethylation (Detich et al., 2003a) as well as extensive epigenetic reprogramming, we reasoned that we could counteract this effect by using a methyl donor. SAM that was shown previously to prevent active demethylation triggered by HDAC inhibitors (Detich et al., 2003b) and to reverse demethylation of prometastatic genes in cancer models (Pakneshan et al., 2004; Parashar et al., 2015; Shukeir et al., 2006). There is evidence that SAM can reverse demethylation of genes in the brain as well (Do Carmo et al., 2016; Massart et al., 2015). SAM is highly attractive agent for treating ASD since it is an approved nutritional supplement that has been used by humans for a long time without notable toxic side effects. Our results suggest that co-administration of SAM with VPA protected the animals against the development of ASD-like behavioral symptoms. Future experiments are required to map the epigenetic changes that are triggered by VPA and are inhibited by SAM. Such genes might be excellent candidates for playing a causal role in development of ASD phenotypes.

SAM has also antioxidant activity, apparently through its effects on antioxidant enzymes and on glutathione (Michael Brown et al., 2012; Caro and Cederbaum, 2004; De La Cruz et al., 2000). This may possibly be an alternative mechanism by which SAM ameliorated the VPA –induced ASD-like symptoms. However, we found no changes in the antioxidant enzyme activity or lipid peroxidation by SAM, and the CAT gene expression that was increased in males, was carried out only in 4 animals. Hence, although this possibility cannot be completely ruled out, it seems clear that VPA indeed induced epigenetic changes, as evidenced by the changes in the expression of antioxidant genes, and SAM prevented this. Further studies need to be carried out to examine

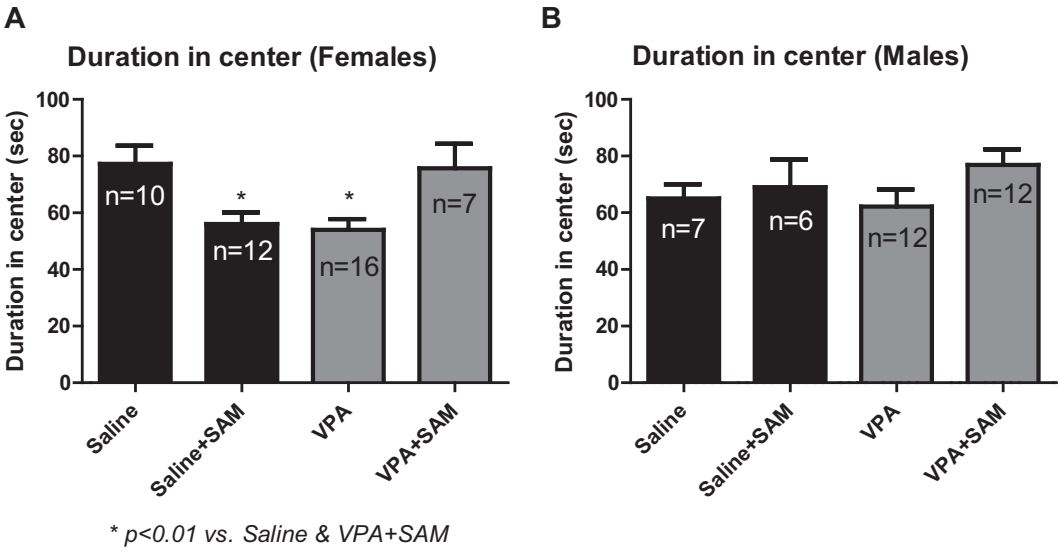

Fig. 6. Open field test (time in center): During the 5 min trial of the open field assay, total duration in the arena center was measured for each mouse. Duration in the center was reduced in VPA group in females (A) and not in males (B). Data is presented as mean ± standard error. *Marks statistical significance ($p < .01$, *ANOVA*) between VPA group and VPA + SAM or Saline groups.

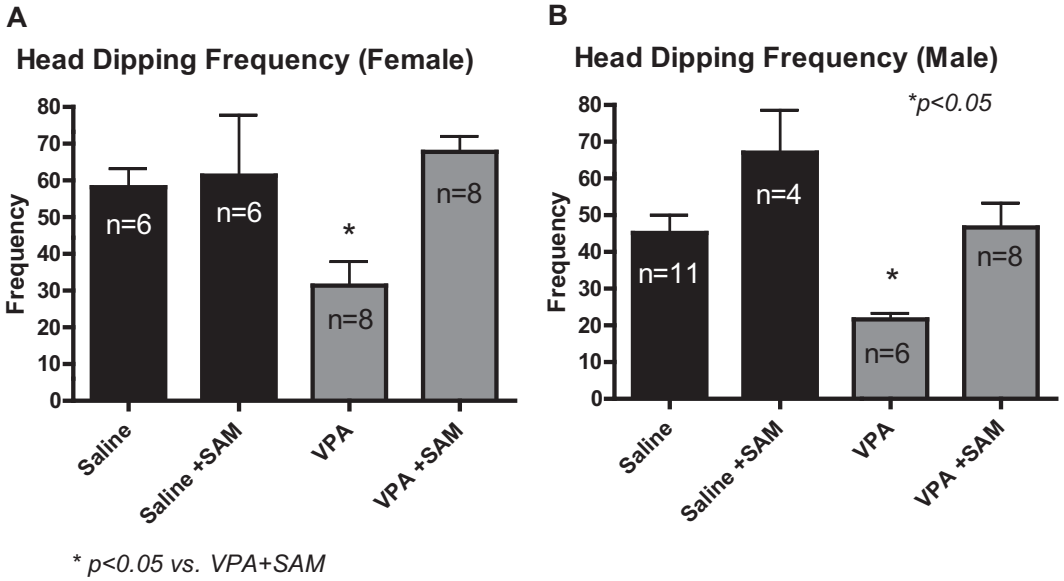

Fig. 7. Elevated plus maze (head dipping). Head-dipping frequency over the open arm edges was measured for each mouse in the elevated plus maze assay. Reduced head-dipping frequency was observed in VPA group both in females (A) and in males (B). Data is presented as mean ± standard error. *Marks statistical significance ($p < .05$, *ANOVA*) versus all other groups unless stated differently in the graphs.

other genes whose expressions might also be changed by VPA.

In addition to its inhibition of histone deacetylase, VPA was also found to inhibit GSK2β (Hall et al., 2002); thus, enhancing Wnt signaling. This may directly interfere with the differentiation of cortical neurons, inducing autistic-like behavior. The fact that VPA induced ASD-like behavior after postnatal administration at relatively late cortical brain development, points more to the histone deacetylase inhibition as the underlying mechanism, as these late developments are no more related to Wnt signaling.

Our data points to possible translational opportunities and the possibility that methyl supplementation during pregnancy might reduce the risk for ASD in offspring as a possible intervention. In our study, SAM was provided at a time that parallels the third trimester in humans and translating this to humans might suggest dietary methyl supplementation using folic acid and vitamin B12 or perhaps even SAM administration during the third trimester in high risk pregnancies. We should however, remember that SAM was effective in VPA induced ASD-like symptoms.

Our study design aimed to test whether SAM could prevent emergence of ASD-like symptoms in a postnatally VPA induced ASD in a mouse model. An attractive possibility is that SAM might also relieve ASD symptoms after they had been established, as we started SAM 24 h after the administration of VPA. If indeed this is possible, then SAM, a nutritional supplement, could become a candidate for treating VPA related ASD in treated women. Future studies are required to test this possibility.

This study has the limitation of a specific, genome – wide analysis of DNA methylation studies, were not yet carried out in our model. Hence, the explanations as to the possible epigenetic mechanisms underlying our finding are, to some extent, speculative. We indeed plan to carry out such studies by Methylated DNA immunoprecipitation (MeDIP) arrays.

In summary, our study provides further support for an epigenetic

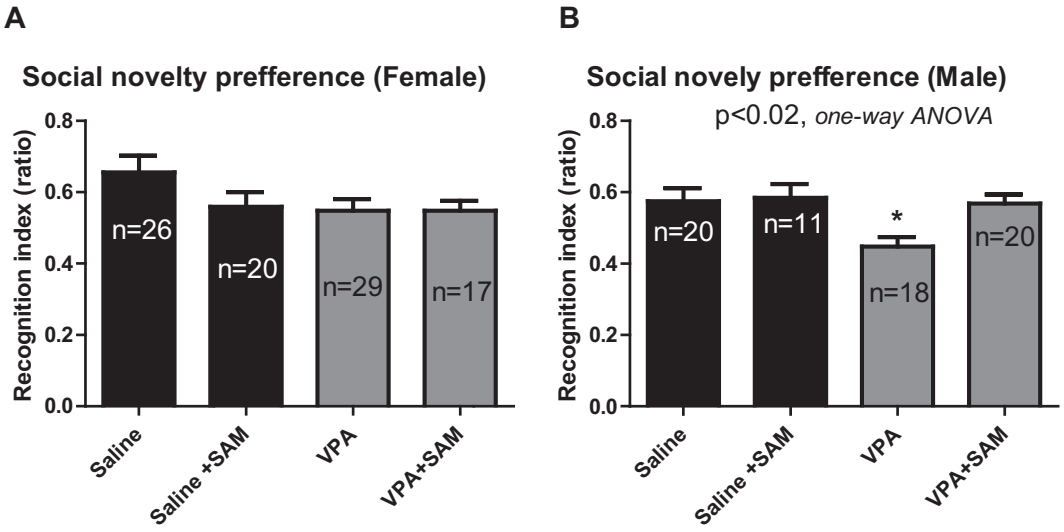

Fig. 8. Social novelty preference. In the three-chamber social interaction assay, social novelty preference was estimated using recognition index, calculated as the ratio of the interaction time with novel stimulus and the total interaction time of both novel and old social stimuli. Reduced social novelty preference in the VPA group was noted only in males (B) and not in females (A). Data is presented as mean ± standard error. *Marks statistical significance ($p < .02$, *ANOVA*) versus all other groups.

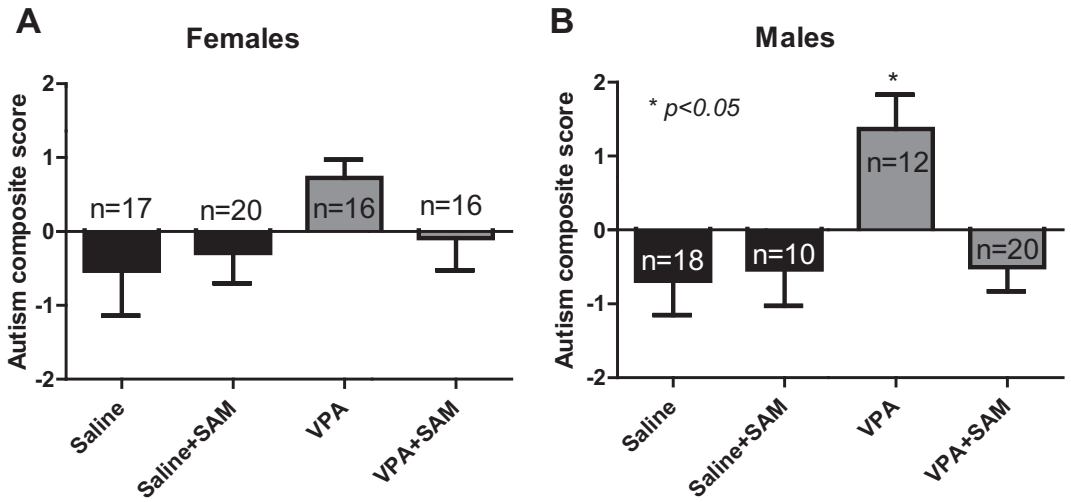

Fig. 9. Autism composite score. A composite ASD score combining the social novelty preference, T-maze test and self-grooming assay was calculated for each mouse. An elevated score in the VPA group with correction in the SAM treatment group was noted both in females (A) and in males (B). However, differences were significant in males only. Data is presented as mean ± standard error. *Marks statistical significance ($p < .05$, *ANOVA*) versus all other groups.

mechanism driving ASD and points to the attractive possibility of using SAM to prevent, or possibly even reverse, VPA-related ASD. Future experiments are required to test this possibility.

Transparency documents

The Transparency documents associated with this article can be found, in online version.

References

Aizawa, S., Yamamuro, Y., 2015. Valproate administration to mice increases hippocampal p21 expression by altering genomic DNA methylation. Neuroreport 26, 915–920. http://dx.doi.org/10.1097/WNR.0000000000000448.

Al-Amin, M.M., Rahman, M.M., Khan, F.R., Zaman, F., Mahmud Reza, H., 2015. Astaxanthin improves behavioral disorder and oxidative stress in prenatal valproic acid-induced mice model of autism. Behav. Brain Res. 286, 112–121. http://dx.doi.org/10.1016/j.bbr.2015.02.041.

Amir, R.E., Van den Veyver, I.B., Wan, M., Tran, C.Q., Francke, U., Zoghbi, H.Y., 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2 [see comments]. Nat. Genet. 23, 185–188.

Bambini-Junior, V., Rodrigues, L., Behr, G.A., Moreira, J.C., Riesgo, R., Gottfried, C., 2011. Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. Brain Res. 1408, 8–16. http://dx.doi.org/10.1016/j.brainres.2011.06.015.

Beggiato, A., et al., 2016. Gender differences in autism spectrum disorders: divergence among specific core symptoms. Autism Res. http://dx.doi.org/10.1002/aur.1715.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254 (doi:S0003269776699996 [pii]).

Caro, A.A., Cederbaum, A.I., 2004. Antioxidant properties of S-adenosyl-L-methionine in Fe(2+)-initiated oxidations. Free Radic. Biol. Med. 36, 1303–1316. http://dx.doi.org/10.1016/j.freeradbiomed.2004.02.015.

Cavallaro, R.A., Fuso, A., Nicolia, V., Scarpa, S., 2010. S-adenosylmethionine prevents oxidative stress and modulates glutathione metabolism in TgCRND8 mice fed a B-vitamin deficient diet. J. Alzheimers Dis. 20, 997–1002. http://dx.doi.org/10.3233/JAD-2010-091666.

Christensen, J., et al., 2013. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. JAMA 309, 1696–1703. http://dx.doi.org/10.1001/jama.2013.2270.

Christianson, A.L., Chesler, N., Kromberg, J.G., 1994. Fetal valproate syndrome: clinical and neuro-developmental features in two sibling pairs. Dev. Med. Child Neurol. 36, 361–369.

Ciernia, A.V., LaSalle, J., 2016. The landscape of DNA methylation amid a perfect storm of autism aetiologies. Nat. Rev. Neurosci. http://dx.doi.org/10.1038/nrn.2016.41.

Crawley, J.N., 2004. Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment. Retard. Dev. Disabil. Res. Rev. 10, 248–258. http://dx.doi.org/10.1002/mrdd.

20039.
De La Cruz, J.P., Pavia, J., Gonzalez-Correa, J.A., Ortiz, P., Sanchez de la Cuesta, F., 2000. Effects of chronic administration of S-adenosyl-L-methionine on brain oxidative stress in rats. Naunyn Schmiedeberg's Arch. Pharmacol. 361, 47–52.
Detich, N., Bovenzi, V., Szyf, M., 2003a. Valproate induces replication-independent active DNA demethylation. J. Biol. Chem. 278, 27586–27592. http://dx.doi.org/10.1074/jbc.M303740200.
Detich, N., Hamm, S., Just, G., Knox, J.D., Szyf, M., 2003b. The methyl donor S-adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-adenosylmethionine. J. Biol. Chem. 278, 20812–20820.
Developmental Disabilities Monitoring Network Surveillance Year Principal, I., C. Centers for Disease, Prevention, 2014. Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2010. In: Morbidity and Mortality Weekly Report Surveillance Summaries. 63. pp. 1–21.
Association, A.P. (Ed.), 2013. Diagnostic and Statistical Manual of Mental Disorders, Fifth edition. .
Do Carmo, S., et al., 2016. Rescue of early bace-1 and global DNA demethylation by S-adenosylmethionine reduces amyloid pathology and improves cognition in an Alzheimer's model. Sci. Rep. 6, 34051. http://dx.doi.org/10.1038/srep34051.
Dong, E., Guidotti, A., Grayson, D.R., Costa, E., 2007. Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc. Natl. Acad. Sci. U. S. A. 104, 4676–4681. http://dx.doi.org/10.1073/pnas.0700529104.
Ehlers, K., Elmazar, M.M., Nau, H., 1996. Methionine reduces the valproic acid-induced spina bifida rate in mice without altering valproic acid kinetics. J. Nutr. 126, 67–75.
El-Kordi, A., et al., 2013. Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. Behav. Brain Res. 251, 41–49. http://dx.doi.org/10.1016/j.bbr.2012.11.016.
Frye, R.E., et al., 2013. Redox metabolism abnormalities in autistic children associated with mitochondrial disease. Transl. Psychiatry 3, e273. http://dx.doi.org/10.1038/tp.2013.51.
Goddard, L., Dritschel, B., Howlin, P., 2014. A preliminary study of gender differences in autobiographical memory in children with an autism spectrum disorder. J. Autism Dev. Disord. 44, 2087–2095. http://dx.doi.org/10.1007/s10803-014-2109-7.
Hall, A.C., Brennan, A., Goold, R.G., Cleverley, K., Lucas, F.R., Gordon-Weeks, P.R., Salinas, P.C., 2002. Valproate regulates GSK-3-mediated axonal remodelling and synapsin I clustering in developing neurons. Mol. Cell. Neurosci. 20, 257–270.
Head, A.M., McGillivray, J.A., Stokes, M.A., 2014. Gender differences in emotionality and sociability in children with autism spectrum disorders. Mol. Autism 5, 19. http://dx.doi.org/10.1186/2040-2392-5-19.
Ingram, J.L., Peckham, S.M., Tisdale, B., Rodier, P.M., 2000. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. Neurotoxicol. Teratol. 22, 319–324.
Kim, K.C., et al., 2013. Male-specific alteration in excitatory post-synaptic development and social interaction in pre-natal valproic acid exposure model of autism spectrum disorder. J. Neurochem. 124, 832–843. http://dx.doi.org/10.1111/jnc.12147.
Kim, K.C., et al., 2016. MeCP2 modulates sex differences in the postsynaptic development of the valproate animal model of autism. Mol. Neurobiol. 53, 40–56. http://dx.doi.org/10.1007/s12035-014-8987-z.
Kolozsi, E., Mackenzie, R.N., Roullet, F.I., deCatanzaro, D., Foster, J.A., 2009. Prenatal exposure to valproic acid leads to reduced expression of synaptic adhesion molecule neuroligin 3 in mice. Neuroscience 163, 1201–1210. http://dx.doi.org/10.1016/j.neuroscience.2009.07.021.
Loke, Y.J., Hannan, A.J., Craig, J.M., 2015. The role of epigenetic change in autism spectrum disorders. Front. Neurol. 6, 107. http://dx.doi.org/10.3389/fneur.2015.00107.
Lozano-Sepulveda, S.A., et al., 2016. S-adenosyl-L-methionine modifies antioxidant-enzymes, glutathione-biosynthesis and methionine adenosyltransferases-1/2 in hepatitis C virus-expressing cells. World J. Gastroenterol. 22, 3746–3757. http://dx.doi.org/10.3748/wjg.v22.i14.3746.
Maciag, D., Simpson, K.L., Coppinger, D., Lu, Y., Wang, Y., Lin, R.C., Paul, I.A., 2006. Neonatal antidepressant exposure has lasting effects on behavior and serotonin circuitry. Neuropsychopharmacology 31 (1), 47–57.
Massart, R., et al., 2015. Role of DNA methylation in the nucleus accumbens in incubation of cocaine craving. J. Neurosci. 35, 8042–8058. http://dx.doi.org/10.1523/JNEUROSCI.3053-14.2015.
McBride, K.L., et al., 2010. Confirmation study of PTEN mutations among individuals with autism or developmental delays/mental retardation and macrocephaly. Autism Res. 3, 137–141. http://dx.doi.org/10.1002/aur.132.
Meehan, R.R., Lewis, J.D., Bird, A.P., 1992. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Res. 20, 5085–5092.
Michael Brown, J., Ball, J.G., Wright, M.S., Van Meter, S., Valentovic, M.A., 2012. Novel protective mechanisms for S-adenosyl-L-methionine against acetaminophen hepatotoxicity: improvement of key antioxidant enzymatic function. Toxicol. Lett. 212, 320–328. http://dx.doi.org/10.1016/j.toxlet.2012.05.018.
Mihara, M., Uchiyama, M., 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–278 (doi:0003-2697(78)90342-1 [pii]).
Milutinovic, S., D'Alessio, A.C., Detich, N., Szyf, M., 2007. Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. Carcinogenesis 28, 560–571.
Mischoulon, D., et al., 2014. A double-blind, randomized, placebo-controlled clinical trial of S-adenosyl-L-methionine (SAMe) versus escitalopram in major depressive disorder. J. Clin. Psychiatry 75, 370–376. http://dx.doi.org/10.4088/JCP.13m08591.
Moldrich, R.X., et al., 2013. Inhibition of histone deacetylase in utero causes sociability deficits in postnatal mice. Behav. Brain Res. 257, 253–264. http://dx.doi.org/10.1016/j.bbr.2013.09.049.
Moy, S.S., et al., 2007. Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behav. Brain Res. 176, 4–20. http://dx.doi.org/10.1016/j.bbr.2006.07.030.
Nagarajan, R.P., et al., 2008. MECP2 promoter methylation and X chromosome inactivation in autism. Autism Res. 1, 169–178. http://dx.doi.org/10.1002/aur.24.
Ornoy, A., 2009. Valproic acid in pregnancy: how much are we endangering the embryo and fetus? Reprod. Toxicol. 28, 1–10. http://dx.doi.org/10.1016/j.reprotox.2009.02.014.
Ornoy, A., Weinstein-Fudim, L., Ergaz, Z., 2015. Prenatal factors associated with autism spectrum disorder (ASD). Reprod. Toxicol. 56, 155–169. http://dx.doi.org/10.1016/j.reprotox.2015.05.007.
Ornoy, A., Weinstein-Fudim, L., Ergaz, Z., 2016. Genetic syndromes, maternal diseases and antenatal factors associated with autism spectrum disorders (ASD). Front. Neurosci. 10, 316. http://dx.doi.org/10.3389/fnins.2016.00316.
Pakneshan, P., Szyf, M., Farias-Eisner, R., Rabbani, S.A., 2004. Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. J. Biol. Chem. 279, 31735–31744.
Parashar, S., et al., 2015. S-adenosylmethionine blocks osteosarcoma cells proliferation and invasion in vitro and tumor metastasis in vivo: therapeutic and diagnostic clinical applications. Cancer Med. http://dx.doi.org/10.1002/cam4.386.
Phiel, C.J., Zhang, F., Huang, E.Y., Guenther, M.G., Lazar, M.A., Klein, P.S., 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J. Biol. Chem. 276, 36734–36741. http://dx.doi.org/10.1074/jbc.M101287200.
Pragnya, B., Kameshwari, J.S., Veeresh, B., 2014. Ameliorating effect of piperine on behavioral abnormalities and oxidative markers in sodium valproate induced autism in BALB/C mice. Behav. Brain Res. 270, 86–94. http://dx.doi.org/10.1016/j.bbr.2014.04.045.
Rodier, P.M., Ingram, J.L., Tisdale, B., Nelson, S., Romano, J., 1996. Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. J. Comp. Neurol. 370, 247–261. http://dx.doi.org/10.1002/(SICI)1096-9861(19960624)370:2<247::AID-CNE8>3.0.CO;2-2.
Rodier, P.M., Ingram, J.L., Tisdale, B., Croog, V.J., 1997. Linking etiologies in humans and animal models: studies of autism. Reprod. Toxicol. 11, 417–422.
Rose, S., et al., 2012. Evidence of oxidative damage and inflammation associated with low glutathione redox status in the autism brain. Transl. Psychiatry 2, e134. http://dx.doi.org/10.1038/tp.2012.61.
Rossignol, D.A., Frye, R.E., 2014. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. Front. Physiol. 5, 150. http://dx.doi.org/10.3389/fphys.2014.00150.
Schneider, T., et al., 2008. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33, 728–740. http://dx.doi.org/10.1016/j.psyneuen.2008.02.011.
Sedgewick, F., Hill, V., Yates, R., Pickering, L., Pellicano, E., 2016. Gender differences in the social motivation and friendship experiences of autistic and non-autistic adolescents. J. Autism Dev. Disord. 46, 1297–1306. http://dx.doi.org/10.1007/s10803-015-2669-1.
Segal-Gavish, H., et al., 2015. Mesenchymal stem cell transplantation promotes neurogenesis and ameliorates autism related behaviors in BTBR mice. Autism Res. http://dx.doi.org/10.1002/aur.1530.
Shukeir, N., Pakneshan, P., Chen, G., Szyf, M., Rabbani, S.A., 2006. Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis in vitro and in vivo. Cancer Res. 66, 9202–9210.
Silverman, J.L., Yang, M., Lord, C., Crawley, J.N., 2010. Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. 11, 490–502. http://dx.doi.org/10.1038/nrn2851.
Szyf, M., 2015. Prospects for the development of epigenetic drugs for CNS conditions. Nat. Rev. Drug Discov. 14, 461–474. http://dx.doi.org/10.1038/nrd4580.
Thurman, R.G., Ley, H.G., Scholz, R., 1972. Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. Eur. J. Biochem. 25, 420–430.
Tordjman, S., et al., 2014. Gene × environment interactions in autism spectrum disorders: role of epigenetic mechanisms. Front. Psych. 5, 53. http://dx.doi.org/10.3389/fpsyt.2014.00053.
Tremolizzo, L., et al., 2005. Valproate corrects the schizophrenia-like epigenetic behavioral modifications induced by methionine in mice. Biol. Psychiatry 57, 500–509. http://dx.doi.org/10.1016/j.biopsych.2004.11.046.
Wagner, G.C., Reuhl, K.R., Cheh, M., McRae, P., Halladay, A.K., 2006. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. J. Autism Dev. Disord. 36, 779–793. http://dx.doi.org/10.1007/s10803-006-0117-y.
Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2, 322–328. http://dx.doi.org/10.1038/nprot.2007.44.
Williams, G., King, J., Cunningham, M., Stephan, M., Kerr, B., Hersh, J.H., 2001. Fetal valproate syndrome and autism: additional evidence of an association. Dev. Med. Child Neurol. 43, 202–206.
Yip, J., Soghomonian, J.J., Blatt, G.J., 2008. Increased GAD67 mRNA expression in cerebellar interneurons in autism: implications for Purkinje cell dysfunction. J. Neurosci. Res. 86, 525–530. http://dx.doi.org/10.1002/jnr.21520.
Yui, K., Sato, A., Imataka, G., 2015. Mitochondrial dysfunction and its relationship with mTOR signaling and oxidative damage in autism spectrum disorders. Mini-Rev. Med. Chem. 15, 373–389.