Prenatal Exposure to Valproate in Animals and Autism

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Introduction

Scientific research clearly shows that environmental factors play a primary role in the development of *Autism Spectrum Disorders (ASD)* (Fombonne 2009). Prenatal exposure to several factors such as infections, alcohol, thalidomide and valproic acid (VPA) may predispose that child to developing *ASD* features (Dufour-Rainfray 2011). The following overview emphasizes the main findings obtained using an animal model induced by prenatal exposure to VPA, and highlights the next challenges in ASD research.

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Valproic Acid

Valproic acid (Fig. 1) is a simple eight-carbon branched-chain fatty acid, which is dissociated in a physiological environment (pH 7.4) (Fig. 2) and is used medicinally as an anticonvulsant and mood-stabilizing agent for the treatments of epilepsy and bipolar disorder (Bowden and Singh 2005). Valproic acid was first synthesized by B.S. Burton in 1882, who was studying organic solvents (Burton 1882).

In 1963, VPA was found to be an efficient seizures inhibitor (Meunier et al. 1963) and in 1966, it was described as effective for treatment of bipolar disorder (Lambert et al. 1966). Depending on the severity of the patient's condition, the daily dose for VPA ranges between 200 mg and 3,000 mg (Perucca 2002). After intravenous administration, VPA rapidly crosses the blood brain barrier reaching a plateau value after 10–15 min (Lücke et al. 1994).

Research has demonstrated that VPA increasesbrain concentrations of gamma-aminobutyric acid *(GABA)* by decreasing degradation, and increasing synthesis, thereby stimulating its inhibitory activity in some specific brain regions. Furthermore, the effect of VPA on neuronal excitation mediated by the N-methyl-D-aspartate *(NMDA)* subtype of glutamate receptors might be important for its anticonvulsant effects (Johannessen 2000). In addition, VPA has physiological activity as an inhibitor of histone deacetylase *(HDAC)* (Phiel et al. 2001). *Histone proteins* are rich in positive charges that bind to the negative charges of DNA, keeping it wrapped and thus decreasing *gene transcription* (Williamson and Pinto 2012). Acetylation of the lysine residues at the N-terminus of histone proteins removes the positive charges, thereby reducing the affinity between histones and DNA. This allows *RNA polymerase* and transcription factors to access the promoter region. Once HDAC is active, it decreases the transcription of certain genes. As VPA diminishes HDAC activity, it promotes altered gene transcription (Fig. 3), followed by distinct protein expression through the autism spectrum.

Developing the Concept of an Animal Model of Autism

In 1984, fetal *valproate* syndrome (FVS) was proposed after evaluating seven children exposed to VPA *in utero* who presented craniofacial anomalies, such as midface hypoplasia and epicanthal folds (DiLiberti et al. 1984). Exposure to VPA during the first *trimester* of pregnancy was found to be associated with significantly increased risks of several congenital malformations, such as spina bifida, *atrial septal defect*, *cleft palate* and *polydactyly* (Jentink et al. 2010). Demonstration of a link between autism and VPA began with *case reports* of patients with FVS demonstrating autism-like behavior (Table 1). One of the case reports described a 5 year old boy who presented with several anatomical alterations, such as a small midface, mild *micrognathia*, and clinodactyly. His speech development was delayed and his language consisted of a few single words. He exhibited *echolalia*, used *gestures* to communicate, did not interact with other children, and preferred to

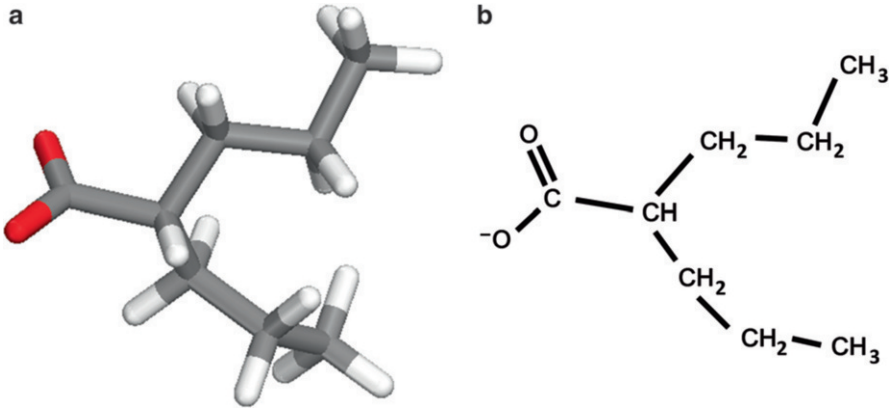

Fig. 1 Valproic acid structure. (**a**) Structural representation of VPA. Oxygen atoms are represented by *red sticks*. Carbon and carbon-carbon bonds are represented by *grey sticks*. Hydrogens are represented by *white sticks*. (**b**) Chemical and structural representation of VPA

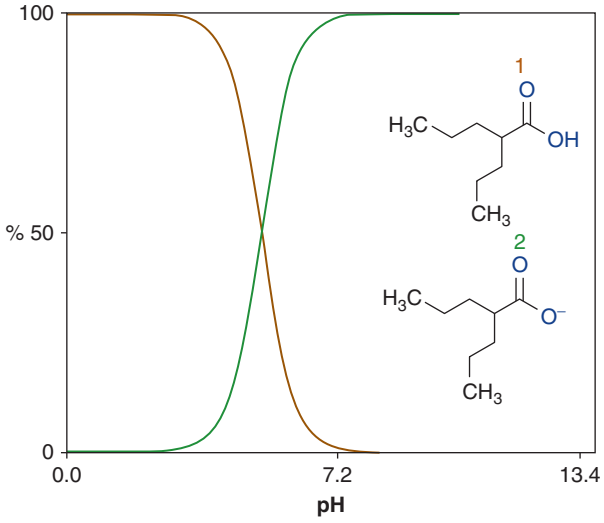

Fig. 2 Valproic acid response to pH variation. Different concentration of VPA, dissociated or not, depending on the pH

play alone. The boy's mother began taking VPA 2–3 years before pregnancy, and kept using it (500 mg – four times per day) until the end of the fifth month of pregnancy (Williams and Hersh 1997). After observing the consequences of prenatal exposure to VPA, a relationship was proposed between exposure and the development of autism (Table 2).

In this context, examination of the triggers of autism has been used to elucidate the role of VPA in the etiology of autism. Therefore, an animal model of autism

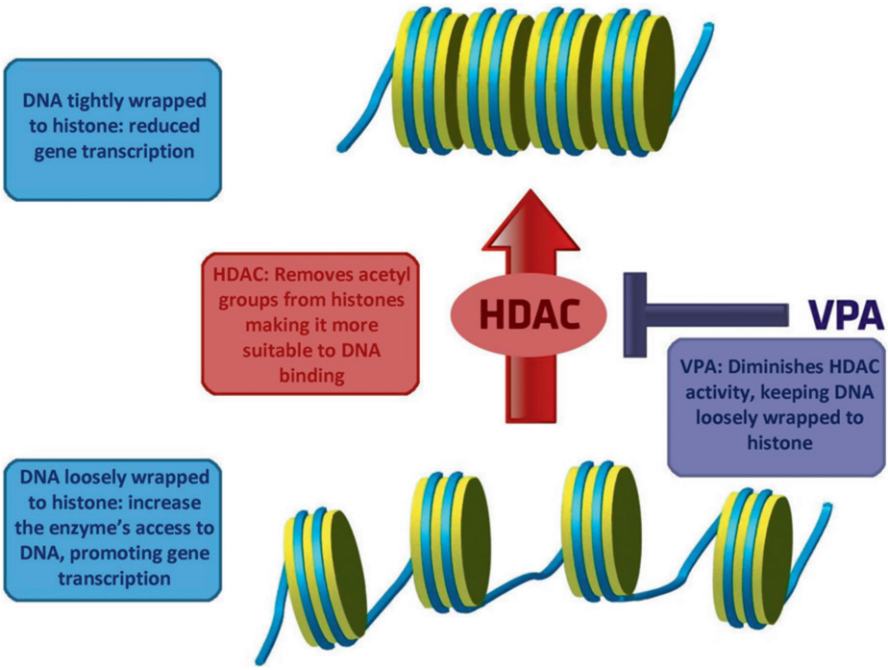

Fig. 3 ***Histone deacetylase*** **inhibition by VPA**. Valproic acid acting as an HDAC inhibitor, leading to enhanced gene transcription. *HDAC* histone deacetylase

Table 1 First evidence of an association between prenatal exposure to VPA and autism

Valproic acid (VPA)	Valproic acid was serendipitously discovered by Burton in 1882 while he studied organic solvents. Until today it is largely used to prevent seizures and to treat bipolar disorder
Fetal valproate syndrome	After reports of malformations in children prenatally exposed to VPA, a Fetal Valproate Syndrome was proposed in 1984 by DiLiberti and colleagues. Several *autistic* characteristics were found in patients with FVS
Autistic patients prenatally exposed to xenobiotics	In 1994, Strömland observed that a number of autistic patients in Sweden were prenatally exposed to xenobiotics between gestational days 20–24. These days matches with the neural tube closure
Rats prenatally exposed to VPA	In 1996 Rodier et al. observed morphological alterations in rats prenatally exposed to VPA that were similar to those observed in autistic patients. The first work reporting behavioral alterations in VPA model was performed by Schneider and Przewlocki in 2005
Animal model of autism induced by prenatal exposure to VPA	The VPA animal model is becoming a well explored instrument in autism research and has been showing several behavioral, neural and biochemical alterations corresponding to those found on autistic patients

Table 2 Key facts. Prenatal exposure to VPA and autism

Study	Year	Children's outcomes
Winter	1987	Low response to stimulation, infrequent smile, no attempt to communicate
Ardinger et al.	1988	Neurologic abnormalities
Laegreid et al.	1993	Mental retardation, communication disability
Thisted and Ebbesen	1993	*Irritability*, jitteriness, seizures
Christianson et al.	1994	Difficult behavior, developmental delays, speech disabilities
Williams and Hersh	1997	Autism
Moore et al.	2000	Autism, Asperger's Syndrome, poor social interaction, poor *communication skills*
Bescoby-Chambers et al.	2001	Autism, Asperger's Syndrome
Kozma	2001	Mental retardation, speech disabilities
Williams et al.	2001	Autism
Adab et al.	2001	Additional educational needs
Adab et al.	2004	Low *verbal IQ*

induced by prenatal exposure to VPA became an alternative tool of research. Although the model does not exactly replicate a human disorder, there are several behavioral and anatomical similarities, which are useful to improve the understanding of *autism spectrum disorder*. In this context, translational studies of neuropsychiatric conditions employing animal models are reliable and allow a wide range of research possibilities such as the search for an etiology, *molecular targets*, and biomarkers.

It should be emphasized that a trustworthy animal model would be based on: (1) Construct validity, which means to reproduce a circumstance that would lead to a certain state, for example, inducing a genetic disease by manipulating a specific gene; (2) *Face validity*, is to induce similar patterns found in the studied condition, for example, observing the same behavioral alterations found in a particular impairment; and (3) *Predictive validity*, which is how well a test answers a future performance, for example, if an animal model reacts similarly to a human when exposed to certain treatment (Crawley 2008). Considering this, the creation of an animal model to study autism is particularly important and provides a challenge in *ASD* research. These difficulties led, for example, the National Institute of Health to *sponsor* in 1998 the conference Building Animal Models for Autism through Translational Neuroscience Research.

Research suggests that prenatal exposure to VPA is directly related to autism, in this context, exposing rats prenatally to the same chemical agent (construct validity) should give an indication about *what is happening* and *how it happens* in the autism's pathophysiology. Autism is diagnosed exclusively by behavioral alterations in three main areas: sociability, communication and behavioral stereotypes and narrow range of interests. Therefore, a consistent animal model should

demonstrate similar behavioral abnormalities (face validity), which might indicate common neural alterations. Animal models using VPA as an environmental factor generally use prenatal exposure, but postnatal exposure to VPA was also used to induce autism-like behavior (Yochum et al. 2008).

In rats, postnatal day (PND) 14, corresponds to the third *trimester* of human development when cerebellar and hippocampal granule cells migrate and differentiate, therefore it is considered a *critical period* in *neural development* (Rice and Barone 2000). Pre and postnatal exposure to VPA were used in a same experiment, and the animals of this model displayed autistic-like behaviors. One of the major findings of this article is retardation in the maturation of negative *geotaxis* and water maze performance (Wagner et al. 2006). In a Swedish group of patients with thalidomide embryopathy, researchers found several patients with autism who were exposed *in utero* to thalidomide between gestational days (GD) 20 and 24 (Strömland et al. 1994). During this time period in humans the neural tube closes, the corresponding gestational period in rats is day 11.5 (Altman and Bayer 1980), these evidences contributed to the formulation of a reliable animal model using prenatal exposure to VPA.

Examination of the effects of various VPA dosages is extremely important to confidently develop an effective ASD animal model. In a 1988 study, pregnant rats were exposed to VPA doses from 200 mg/kg to 800 mg/kg (5–20× the therapeutic dose), from GD 8–17. The offspring showed dose-dependent effects, with the highest dose causing 100 % maternal lethality. A VPA dose of 600 mg/kg, a very often used dosage to induce autism-like behavior, has a *half life* of 2.3 h and reaches 900 μg/mL in maternal plasma in less than 1 h (Binkerd et al. 1988).

Recently, Kim and colleagues examined the critical period necessary to induce autism-like behavior in rats using prenatal VPA exposure. Pregnant rats received injections of 400 mg/kg VPA at GDs 7, 9.5, 12 and 15. Rats' sociability was measured at 4 weeks of age. The results of the sociability experiment showed that rats exposed to VPA at GD 12 were less sociable than both the control group and animals exposed at GDs 7, 9.5, and 15 (Kim et al. 2011). Although researchers have exposed rodents to VPA as early in development as GD 7 and as late as GD 15, the most utilized exposure time in VPA experiments is day is GD 12.5, a time not evaluated by Kim. Several works used different induction's day, dosage and procedure to injection VPA in pregnant rats. Table 3 summarizes methods employed to induce the VPA animal model.

First Steps in the Implementation of the VPA Model

Prenatal exposure to VPA in rodents is causative of several behavioral and morphological abnormalities similar to those displayed by individuals with autism. For example, abnormalities in the monoamine system, neural hyper-excitability, decreased nociception, deficit in weight gain, anxious-like behavior, deficits in sociability and aberrant *exploratory behavior* are all noted in the animal model (Tsujino et al. 2007; Schneider et al. 2006). These findings indicate that the VPA

Table 3 General methods to VPA induction

Studies	Induction Day	Rodent	Dosage (mg/kg)	Procedure
Bambini-Junior et al. 2011	GD12.5	Rats	600	Injection (i.p.)
Dendrinos et al. 2011	GD12.5	Rats	400–600	Injection (i.p.)
Downing et al. 2010	GD9	Mice	200–800	Injection (i.p.)
Dufour-Rainfray et al. 2010	GD9	Rats	600	Injection (i.p.)
Felix-Ortiz and Febo 2012	GD12.5	Rats	600	Injection (i.p.)
Foley et al. 2012	GD12.5	Rats	600	Injection (i.p.)
Fucic et al. 2010	GD12 to GD14	Mice	100	Injection (i.p.)
Gandal et al. 2010	GD13	Mice	600	Injection (subcutaneous)
Go et al.2011	GD12	Rats	400	Injection
Hara et al. 2012	GD12.5	Mice	500	Injection
Ingram et al. 2000	GD12.5	Rats	600	Injection (i.p.)
Kataoka et al. 2011	GD9, GD12.5 or GD14.5	Mice	500	Injection
Kim et al. 2011	GD7, GD9.5, GD12 or GD15	Rats	400	Injection
Kolozsi et al. 2009	GD11	Mice	800	Oral (mixed with *peanut* butter)
Kuwagata et al. 2009	GD9 or GD11	Rats	800	Oral (gavage)
Lukose et al. 2011	GD12.5	Rats	600	Injection
Mehta et al. 2011	GD13	Mice	600	Injection (subcutaneous)
Miyazaki et al. 2005	GD9	Rats	800	Oral (infant feeding tube)
Narita et al. 2002	GD9	Rats	800	Oral (infant feeding tube)
Rinaldi et al. 2007	GD11.5	Rats	500	Injection (i.p.)
Rodier et al. 1996	GD11.5, GD12 or GD12.5	Rats	350	Injection (i.p.)
Roullet et al. 2010	GD11	Mice	800	Oral (mixed with peanut butter)
Schneider et al. 2006	GD12.5	Rats	600	Injection (i.p.)
Tsujino et al. 2007	GD9	Rats	800	Oral (infant feeding tube)

model of autism provides a close parallel between an experimentally created model to study autism and *ASD*, making the rodent model a well established tool in autism research.

In 1996, Rodier and colleagues performed the first study aiming to induce *autistic* morphological characteristics in rat embryos exposed to VPA during gestation. No test was performed to confirm autism-like behaviors in the animals, however it was stated that with such severe morphological brain alterations, behaviors would be aberrant. Dams received 350 mg/kg of VPA at GDs 11.5, 12, or 12.5. The VPA exposure decreased the number of motor neurons counted in

matched sections in the earliest-forming motor nuclei (V, XII). These effects were more evident at GD 12.5 (Rodier et al. 1996). By determining the time period when exposure to VPA would cause the greatest effect, researchers would be able to target exposure to a specific time period and create a reproducible model.

Exposure to VPA during pregnancy can cause malformations, including neuroanatomical (Thisted and Ebbesen 1993). Ingram and colleagues described similar changes in the brains of rats exposed to VPA during gestation. Treated rat dams received a single injection of 600 mg/kg VPA at GD 12.5. Pups presented 11.2 % reduction in brain weight when compared to control animals. Examination of the cerebellar vermis revealed that the animals exposed to VPA had a greater reduction of cell number in the posterior lobe than in the anterior lobe. However, a difference in cell density was not observed (Ingram et al. 2000). The *Purkinje cells* form primarily around GD 15, so it is probable that the proliferating cells did not have any contact with VPA itself. The reduction in number of Purkinje cells is one of the most robust findings in both humans with autism and the VPA model, which lends evidence for the efficacy of the model.

Although there were many experiments comparing the anatomical abnormalities of the VPA model to the brains of humans with autism, the first experiment using VPA exposure to verify autism-like behavior was performed in 2005. The elegant paper written by Schneider and Przewłocki produced a series of evidence showing behavior was altered in rats exposed to VPA during gestation. Some of the characteristics exhibited by VPA animals, when compared to controls were: stereotypic-like hyperactivity combined with lower exploratory activity, decreased number of social behaviors and increased latency to social contact (Schneider and Przewłocki 2005).

A Consistent Model

Similar to humans with autism, the VPA animal model exhibits impairments in social behavior, for example, impairments in initiating social interaction. Rodents exposed to VPA demonstrate the same interest exploring an object or an animal, while a control rat normally chooses to spend time socializing (Dufour-Rainfray et al. 2010; Bambini-Junior et al. 2011). The VPA animal model also exhibits additional behavior that is comparable to humans with autism for example, circadian rhythm is often altered in people with autism and this feature is also noted in the animal model (Tsujino et al. 2007). Also, the VPA model shows increased latency to change strategy in the reverse Y-maze task (which could be an indicative of behavioral rigidity) (Bambini-Junior et al. 2011), and repetitive-like behavior (Mehta et al. 2011). Therefore, data is revealing the efficiency of the prenatal exposure to VPA in rodents to generate autism-like behaviors.

A dysregulation in the serotonergic system is considered to be a possible mechanism in the pathophysiology of autism. Serotonin is part of a neurotransmitter family called *monoamines* and among other functions; it is involved in emotional

and mood control. High levels of serotonin have been found in the blood of patients with autism (Anderson et al. 1990). Several alterations in the monoamine system have been found in the VPA model, including increased monoamine levels in brain and blood, and abnormal serotoninergic neurons have been noted (Narita et al. 2002; Tsujino et al. 2007; Miyazaki et al. 2005). Alternatively, a decrease of 46 % in the levels of serotonin in the hippocampus of the animals exposed prenatally to VPA, and unaltered levels in the cerebellum and cortex were also described. The *serotonin transporter* expression was the same in control and VPA exposed animals. Decreased serotonin levels in the hippocampus could reflect a sociability alteration, as it is a *brain structure* that functions in social behaviors (Dufour-Rainfray et al. 2010). The role of the serotonergic system should be more explored in the future.

Brain-derived neurotrophic factor *(BDNF)* is another molecule altered in the blood of patients with autism. BDNF is involved in *neuronal plasticity* and regulation (Nelson et al. 2001). Reduced cortical BDNF *mRNA* expression was detected in mice exposed to VPA and was accompanied by behavioral changes, such as olfactory discrimination deficit (Roullet et al. 2010). A possible role for BDNF in the development of autism-like behavior should be well studied, but is likely that reduced BDNF expression contribute to altered synaptic development.

Alterations in synaptic function have been reported in patients with autism (Laumonnier et al. 2004). This abnormality has also been noted in the VPA model. Significantly lower Neuroligin 3 mRNA expression in VPA mice compared to control animals in the cornu ammonis (CA1) and *dentate gyrus* regions of the hippocampus and somatosensory cortex have been found. *Neuroligins* are postsynaptic cell-adhesion molecules involved in synaptic maturation (Kolozsi et al. 2009). Behavioral alterations found in knockout mice to Neuroligin 3 are similar to those found in VPA rats, such as deficits in response to social *novelty*.

The consequences of prenatal exposure to VPA have been thoroughly studied in the brain by examining behavioral, chemical, and morphological factors. However, information about the effects VPA has on other regions of the body is lacking. In one study, hepatic parameters were evaluated in order to check if metabolic dysfunction could generate some of the behavioral and neurological alterations; however no sign of liver damage was detected (Bambini-Junior et al. 2011). To gain a better understanding of the systemic alterations that might be involved in the pathophysiology of autism, the VPA model should be examined further.

Approaches to Ameliorate Characteristics of Autism

The evaluation of prospective strategies to ameliorate the symptoms of autism is one of the several possibilities enabled by the VPA animal model, making this approach an essential tool in autism research. A variety of therapeutic interventions have been proposed in animal models of autism to *attenuate*, behavioral, neurochemical and anatomical impairments.

One of these interventions is environmental enrichment, which is a well documented influence on *brain plasticity*. Since the 1960s, many experiments have shown that environmental enrichment is a means to enhance cognition. Rats housed in cages equipped with toys, such as ladders and swinging blocks, showed neurochemical changes when compared to controls. These animals had lower cortical activity of the enzyme *cholinesterase*, allowing an increase in the turnover of acetylcholine, a neurotransmitter related with synaptic plasticity (Krech et al. 1960).

In rats prenatally exposed to VPA, environmental enrichment improves behavioral alterations most notably, anxiety (Schneider et al. 2006). Anxiety was measured by verifying the time animals spent in open arms and open arm entries in an elevated plus-maze. Spending time in the open arms is indicative of low anxiety levels, since rodents prefer closed environments. VPA exposed animals subjected to this enrichment spent more time in open arms than the animals that were not placed in the enriched environment. In fact, the VPA animals that did not have access to the enriched environment presented a decrease in time spent in the open arms. It is hypothesized that reduced anxiety would improve exploratory activity and social behavior, as anxiety in animals generally leads to impairments in exploration and social interaction.

The pathophysiology of autism includes several likely mechanisms. It is speculated that oxidative stress, a consequence of toxic environmental insults, injures neural cells in predisposed individuals leading to the characteristic alterations of autism (Frustaci et al. 2012). *Antioxidant capacity* has been shown to be decreased in children with autism. A study examining 80 children with autism showed lower levels of cysteine, glutathione and the ratio of reduced to oxidized glutathione, when compared to 73 controls (James et al. 2006). These are important indicatives of oxidative stress presence in autism's pathophysiology.

Flavonoids, a group of polyphenolic compounds, are known for their antioxidant (Lu and Chen 2008) and anti-inflammatory properties (Meki et al. 2009). The leaves of *green tea* (*Camellia sinensis*) are source of many different antioxidants, including the flavonoid catechin. Mice exposed to VPA (400 mg/kg) on PND 14 and treated with daily doses (300 mg/kg) of green tea extract from PND 13 to PND 40 were compared to animals only treated with VPA and also to control animals. Animals that received the extract displayed improved behavioral characteristics when compared to VPA animals, and spatial *learning and memory* were similar to controls. Animals treated with the extract showed increased exploratory activity on open field and enhanced memory and spatial learning. Upon microscopic examination of the brains of animals exposed to VPA, a damaged Purkinje cell layer was noted, while the animals that received the extract did not present this *histopathological* alteration. Malondialdehyde, a marker of oxidative stress, was also measured. VPA animals were found to have higher plasma levels of malondialdehyde than treated animals and control animals (Banji et al. 2011).

Excitatory activity in the brain is mainly regulated by glutamate. Patients with autism and related disorders display impaired glutamatergic signaling, due a dysfunction of the *metabotropic glutamate receptor* 5 (mGluR5) (Carlson 2012). Exposure to *teratogens*, such as VPA, alters neural circuitry leading to exacerbated excitation and lowered inhibition signals in the brain (Rinaldi et al. 2007). It is

hypothesized that a misbalance in excitatory and inhibitory signaling during certain periods of development could be part of autism pathophysiology (Rubenstein and Merzenich 2003).

Mice exposed to VPA and treated with 2-methyl-6-phenylethyl-pyrididine *(MPEP)* were evaluated for spontaneous grooming time as a measure of *repetitive behavior*. Animals exposed to VPA spent more time grooming than controls. The VPA exposed animals that received treatment with MPEP, an antagonist of mGluR5, showed a significant reduction in time spent grooming compared to the other animals (Gandal et al. 2010), indicating that repetitive behaviors were reduced. To evaluate anxiety-like and repetitive-like behaviors in the animals, marble burying activity was measured. Marble burying activity was measured in order to evaluate anxiety-like and repetitive-like behaviors in the animals. VPA animals treated with MPEP buried significantly less marbles, indicating decreased anxiety and repetitive behaviors (Mehta et al. 2011).

Conclusions and Perspectives

From the initial model established by Rodier et al., which described morphological alterations similar to what is found in autism, to the behavioral alterations studied by Schneider and Przewłocki; and the several evaluations of treatments possibilities, many information has emerged from the animal model of autism induced by prenatal exposure to VPA. There are, though, several challenges in using the VPA animal model. As noted in this chapter, it is a valuable tool, however, like every animal model it is not a perfect replication of a human condition.

It was proposed that *autistic behaviors*, like decreased social interaction, anxious-like behavior and decreased learning, caused by prenatal exposure to VPA, are consequences of the inhibition of classes I and/or II *HDACs* at a certain embryonic stage (Kataoka et al. 2011), as seen in Fig. 3. However, the exactly machinery involved triggering autism-like behavior is not clear yet. The elucidation of *how this is happening* could bring forth very interesting data about the autism etiology and pathophysiology.

From the translational perspective, the VPA animal model can, as an example, link etiological triggers with treatment possibilities for autism. It should be stated that results coming from animal models should be handled carefully, once those observations come from *basic research* and in most cases, are not directly applied to human beings.

Key Terms

Valproic Acid. A drug used to prevent seizures and treat bipolar disorder. At physiological pH it is mostly found as sodium *valproate*.

Prenatal Exposure. When an organism is exposed to any kind of factor while it is still *in utero*.

Teratogen. An agent (chemical, physical, or biological) causative of embryonic developmental impairments.

Animal Models. When animals are used to study a certain condition. They are tools frequently utilized in basic research to gain a better understanding of the pathophysiology of different disorders.

Behavioral Analysis. Behaviors are how an organism responds to and interacts with its environment. There are some human features that are difficult to observe (or even absent) in animals. Therefore, certain tasks are employed to verify animal reactions and then, associate them with human behaviors.

Summary Points

- This chapter focuses on the effects of prenatal exposure to the teratogen, VPA, exposure to which could lead to the development of autism.
- Valproic acid is a simple eight-carbon branched-chain fatty acid.
- Valproic acid is a drug used to prevent seizures and as treatment for bipolar disorder.
- It was observed that women who used VPA during the first *trimester* of pregnancy had an increased chance to have a child with autism.
- An animal model to study autism induced by prenatal exposure to VPA, was proposed in 1996.
- Since 1996, several experiments showed neural and behavioral similar abnormalities in the VPA model of autism and those in human autism.
- The animal model induced by prenatal exposure to VPA demonstrates potential to be a valuable tool to study and better understand the pathophysiology of autism.

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