Perinatal Influences of Valproate on Brain and Behaviour: An Animal Model for Autism

Peter Ranger and Bart A. Ellenbroek

Abstract Valproic acid or valproate (VPA) is an anti-convulsant and mood stabiliser effective in treating epilepsy and bipolar disorders. Although in adults VPA is well tolerated and safe, there is convincing evidence that it has teratogenic properties, ranging from mild neurodevelopmental changes to severe congenital malformations. In particular, studies involving humans and other animals have shown that prenatal exposure to VPA can induce developmental abnormalities reminiscent of autism spectrum disorder (ASD). In this chapter, we discuss the connection between VPA and ASD, evaluate the VPA animal model of ASD, and describe the possible molecular mechanisms underlying VPA's teratogenic properties.

Keywords Autism · ASD · Valproate · VPA · Valproic Acid · HDAC-I · ROS · Oxidative stress · Animal models · Behaviour · Teratogen

Contents

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1 Introduction

The thalidomide disaster in the 1950s and 1960s signalled an unprecedented increase in the awareness that the effects of drugs which are relatively safe in adulthood can have severe consequences for the developing foetus. Originally marketed as a sedative, thalidomide was widely used to treat pregnancy-related nausea until it was discovered to lead to pronounced limb reductions, congenital heart disease and other developmental malformations (Kim and Scialli 2011). As exceptional as the thalidomide case was, it is now well recognised that many drugs can induce developmental alterations when given during pregnancy. These alterations can range from severe malformations to much more subtle changes in behaviour and personality. In this chapter, we focused on valproic acid (VPA), a drug that, like thalidomide, is considered relatively safe in adulthood.

2 Valproic Acid (VPA)

VPA is an anti-convulsant and mood stabiliser used predominantly to treat epilepsy, bipolar disorder, and migraine (Lloyd 2013; Mulleners et al. 2014; Trinka et al. 2014). However, its therapeutic potential in Alzheimer's disease, cancer, and HIV has also been explored (Lehrman et al. 2005; Qing et al. 2008; Hu et al. 2011; Avallone et al. 2014; Brodie and Brandes 2014; Grishina et al. 2015). In addition to its prophylactic properties, VPA is a known teratogen (Wyszynski et al. 2005; Koren et al. 2006; Morrow et al. 2006; Diav-Citrin et al. 2008; Meador et al. 2008). A systematic review of the literature concluded that taking VPA during pregnancy was associated with a 3.77-fold increased risk of major congenital malformations in offspring, relative to healthy women, a 2.59-fold increased risk relative to women treated with other anti-epileptic medication, and a 3.16-fold increased risk relative to those with untreated epilepsy (Koren et al. 2006). Together, the literature investigating VPA exposure and congenital malformations indicates an approximate threefold increase in major malformations in children exposed prenatally to VPA (Ornoy 2009).

The potential teratogenic effects of VPA provide a dilemma for pregnant women required to take VPA in their course of treatment (Hill et al. 2010; Tomson and Battino 2012). Although approximately 94 % of children born to mothers medicated with VPA are completely normal (Morrow et al. 2006), the decision to

medicate with VPA must involve a calculation of the dangers imposed to a foetus by the drug against the dangers imposed to the mother and foetus by the disease (Meador et al. 2008; Hill et al. 2010). Conditions such as epilepsy provide real risks to pregnant mothers and their offspring due to the possibility of severe seizures (Tomson and Battino 2012), although the risk of congenital malformations resulting from maternal epilepsy alone is strongly disputed (Kaaja et al. 2003; Fried et al. 2004).

A teratogen is predominantly defined as an agent that causes malformation to, and/or disrupts the development of, the embryo or foetus. What constitutes a teratogenic *outcome* is somewhat less clear. Typically, discussion of outcomes surrounds congenital malformations such as neural tube defects present from birth. However, in addition to the increased likelihood of congenital malformations, children exposed prenatally to VPA are also significantly more likely to suffer from more subtle neurodevelopmental deficits (Williams et al. 2001; Wyszynski et al. 2005; Diav-Citrin et al. 2008; Ornoy 2009; Christensen et al. 2013). There is especially strong evidence suggesting that prenatal exposure to VPA significantly increases the likelihood of children developing autism spectrum disorders (ASD) (Christianson et al. 1994; Moore et al. 2000; Rasalam et al. 2005; Christensen et al. 2013; Roullet et al. 2013; Smith and Brown 2014). Most recently, data from a Danish population-based study concluded that prenatal exposure to VPA was associated with an absolute risk factor of 4.42 % for ASD (Christensen et al. 2013). Consequently, prenatal exposure to VPA has been thoroughly assessed as an animal model for this disorder and has been used in cell-cultures, tadpoles, zebrafish, and rodents (Miyazaki et al. 2005; Schneider and Przewlocki 2005; Bauman et al. 2010; Kim et al. 2011; Patterson 2011; Jacob et al. 2014; James et al. 2015). The focus of this chapter is primarily on the link between VPA and ASD. However, as we will discuss later on, there is convincing evidence to suggest that the congenital malformations and the ASD-like pathology depend, to a large degree, on similar mechanistic processes including alterations in epigenetic processes and oxidative stress (Dufour-Rainfray et al. 2011).

3 ASD

Before discussing the relationship between VPA and ASD, it is relevant to briefly describe the disorder. ASD is a heterogeneous neurodevelopmental disorder characterised by deficits in social interaction and communication, as well as an increase in restricted or repetitive behaviour (Fig. 1) (Levy et al. 2009b). Repetitiveness is seen not only in motor patterns, but also in cognition and thought. Although these three classes of symptoms form the core of ASD, it is important to realise that most ASD patients also show a plethora of other symptoms. These auxiliary symptoms can be psychiatric (including depression and anxiety), behavioural (including aggression), intellectual (lower IQ in many cases), and neurological (including epilepsy). These clinical symptoms manifest themselves at a very early age (often

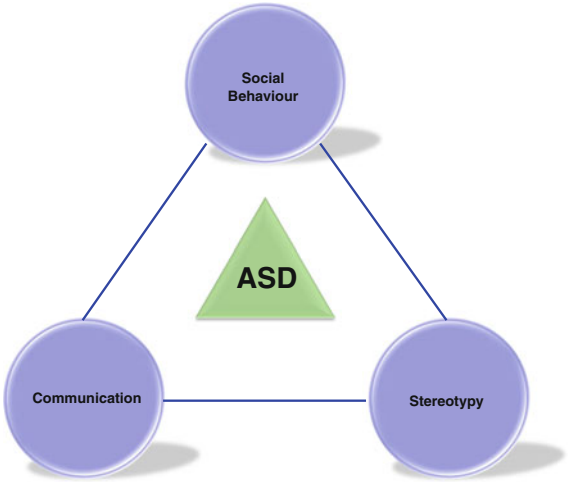

Fig. 1 The three core symptom domains of ASD: social behaviour, communication, and stereotypy

before the age of 3) and usually persist into adulthood (Kinney et al. 2008b; Levy et al. 2009a). One of the most interesting and troublesome aspects of ASD is that over the last fifty years, the incidence of ASD has increased significantly. Indeed, a study in the USA found that the incidence increased from 0.67 % in 2000 to 1.5 % in 2010, with males being 4–5 times more likely affected than females (Wingate et al. 2014). Although there are several different theories why the incidence may be rising (changes in diagnostic criteria, in awareness, or an actual increase in cases), one of the important consequences is that costs relating to the treatment and care of ASD patients are also dramatically rising. A recent study from Great Britain has estimated the total costs of children with ASD at £ 2.7 billion, and for adults at over £ 25 billion per year (Knapp et al. 2009).

In addition to the increased costs, treatment options for patients with ASD are fairly limited (Vorstman et al. 2014), especially in the area of pharmacological interventions. Currently, there are only two FDA-approved drugs for ASD: the second generation antipsychotics risperidone and aripiprazole, both specifically for the treatment of irritability and stereotyped behaviour. Furthermore, drugs such as antidepressants (especially selective serotonin reuptake inhibitors), anxiolytics, and even psychostimulants such as methylphenidate are being used to treat some of the auxiliary symptoms of ASD, such as aggression, anxiety, and irritability (Levy et al. 2009a; Hsia et al. 2014; Murray et al. 2014; Vorstman et al. 2014). However, especially in relation to the social and communication domains, effective therapy is sadly lacking.

The precise causes of ASD are not well understood. However, what *is* known is that ASD has both genetic and environmental determinants, and that its aetiology is likely multifactorial (Tordjman et al. 2014). In fact, ASD is probably the most genetic of all major psychiatric disorders with heritability estimates of around 56-95 % and a substantial number of different risk genes have been identified in patients (Betancur 2011; Chaste et al. 2015; Colvert et al. 2015b). In addition, given

that the concordance rate for monozygotic twins consistently falls short of 100 % (Ronald and Hoekstra 2011), non-genetic environmental factors must be involved in the aetiology of ASD as well. In line with this, several environmental factors have been found to significantly enhance the risk of ASD, including prenatal exposure to infectious agents (Atladottir et al. 2010), prenatal stress such as experiencing hurricanes (Kinney et al. 2008a), and, as previously mentioned, VPA.

4 Animal Models

The major reason for using animal models of ASD, and indeed for any disorder, is that they allow the testing of specific hypotheses and the identification of novel therapies (Bauman et al. 2010). An animal model's validity is usually judged on three distinct criteria:

First, *construct validity*: the extent to which the model replicates the theoretical underpinnings of the disorder, i.e. is the model constructed by the same factors that are known (or at least hypothesised) to cause the disorder in humans? Second, *face validity*: the extent to which the signs and symptoms of the model mimic those seen in humans with the disorder. Third, *predictive validity*: the extent to which the administration of pharmacological treatments that are effective in humans is also effective in reversing the signs and symptoms in the animal model (Ellenbroek and Cools 2000; van der Staay et al. 2009; Bauman et al. 2010; Nestler and Hyman 2010).

Thus, the ideal animal model for ASD would be generated by mimicking both genetic and environmental risk factors (construct validity), would produce the three major behavioural hallmarks of ASD, some of the co-morbid symptoms, and biochemical changes found in patients (face validity), and be responsive to medication that also works in patients (predictive validity). Creating the *perfect* animal model of any human disease, especially in the field of brain disorders, has been notoriously difficult, so a "*best-we-can-do*" approach is traditionally taken. The major barriers in creating animal models of ASD are the complex and unclear aetiology of the disorder, the heterogeneity of the symptomatology, and the lack of effective treatments for humans, making it difficult to achieve suitable construct, face, and predictive validity, respectively.

5 Animal Models for ASD

Before analysing the VPA animal model of ASD in more detail, we will briefly describe several other animal models for ASD, based on either genetic or environmental factors. As such models have been extensively described in recent reviews (Oddi et al. 2013; Ellegood and Crawley 2015; Servadio et al. 2015), we will only briefly mention the most relevant ones.

The maternal immune activation (MIA) model of ASD is one of the most prominent environmentally based models in the literature. There is good evidence indicating that maternal infection leads to an increase in ASD in offspring, and that this likely acts via the maternal immune response (Shi et al. 2003; Atladóttir et al. 2010; Bauman et al. 2014). Animal models using substances that elicit a maternal immune response, such as lipopolysaccharide (LPS, mimicking a bacterial infection) and polyriboinosinic-polyribocytidilic acid (Poly I:C, mimicking a viral infection), have demonstrated the ability to generate ASD-like symptomatology in animals, including all three of the major behavioural hallmarks of ASD and additional neuroanatomical and immunological changes observed in patients with ASD (Shi et al. 2009; Bauman et al. 2014; Ohkawara et al. 2015).

Dysregulation of serotonin (5-HT) is a consistent finding with ASD patients (Devlin et al. 2005). In fact, 5-HT has been suggested to be the neurochemical with the most consistently proven involvement in ASD (Lam et al. 2006). Tryptophan hydroxylase 2 knockout (TPH2-KO) mice, lacking any brain 5-HT, display all three of the major behavioural hallmarks of ASD; moreover, they exhibit key developmental delays related to central nervous system (CNS) functioning. These TPH2-KO mice have been suggested as a promising genetic model of ASD (Kane et al. 2012). Intriguingly, animals lacking the serotonin transporter, and therefore displaying significantly increased (extracellular) serotonin levels have also been proposed as animal models for ASD (Kinast et al. 2013).

The BTBR +tf/J inbred mouse strain exhibits behavioural symptom relevant to all three of the major hallmarks of ASD (Bolivar et al. 2007; McFarlane et al. 2008; Wöhr et al. 2011) and is used as a model of ASD. However, one key problem with the BTNR +tf/J mouse model of ASD is that it is unclear which other genetic mouse strains should be used as a control group for comparison (Patterson 2011).

Accumulating evidence suggests disruption of synaptic pathways plays a key role in patients with ASD (Betancur et al. 2009). Neuroligins (NLGN) are cell adhesion molecules that facilitate synapse formation (Chih et al. 2005). Interestingly, mutations in genes encoding NLGN3 and NLGN4 have been associated with ASD (Jamain et al. 2003). Modelling of these mutations in animals has produced multiple behavioural phenotypes relevant to ASD. Specifically, NLGN4 (the murine orthologue of NLGN4) knockout mice demonstrate selective deficits in two out of the three major behavioural hallmarks of ASD: social interaction and communication (Jamain et al. 2008). The data suggest that NLGN models of ASD show good construct validity, although other models have demonstrated superior face validity.

In addition to these models, many other genetic models have been developed (Ellegood et al. 2015), many of them based on single gene mutations with a link to ASD. Although these models therefore have some construct validity, it is important to realise that only very few cases ASD can be explained by a single genetic deficit (such as fragile X syndrome). Therefore, forward genetic models (i.e. those starting with the symptoms of ASD) may be more relevant. In addition, future research should be aimed at combining genetic and environmental factors in a single animal model, as ASD is caused by both genetic and environmental factors, and thus

models which include both will have stronger construct validity. In fact, as was found in several humans studies as well, the true effects of both genetic and environmental factors may be uncovered when investigating their interactive properties that may never have been discovered when looking at the specific genetic or environmental factor in isolation. In other words, ignoring investigations of gene x environment interactions may lead to a failure to identify effects in both areas.

6 VPA as an Animal Model of ASD

Given the link between maternal treatment with VPA and ASD in humans (see above), prenatal exposure to VPA has been proposed as an animal model of ASD (Rodier et al. 1997; Schneider and Przewlocki 2005). The typical method of creating this model is to inject pregnant rats with a single dose of VPA around the time of the foetal neural tube closure, approximately gestational day 12 (Kim et al. 2011). However, the exact dose, method of injection, day of exposure, and whether the exposure is acute or chronic can vary from study to study; often leading to varying outcomes (Cohen et al. 2013; Štefánik et al. 2015). In fact, it has been proposed that the dose of VPA determines the mechanism of influence and thus the outcome (Johannessen and Johannessen 2003). The VPA animal model of ASD will now be discussed with regard to the previously mentioned validity criteria.

6.1 Construct Validity

Although the VPA model is created using a well-established environmental risk factor for ASD, it does not take into account the large genetic component of ASD (Colvert et al. 2015a). Thus, from a construct validity point of view, the model is clearly limited as it only incorporates an environmental factor involved in ASD and ignores the genetic contribution to the disorder. Moreover, VPA is likely only *one* of the environmental determinants that interact with genotype in the development of ASD (Bauman et al. 2010). Finally, as timing is a crucial element in the outcome of early environmental challenges (see also below), it is important to realise that in most studies only a single injection of VPA is administered, whereas in humans VPA is typically taken throughout pregnancy.

6.2 Face Validity

The VPA model achieves very good face validity. Numerous behavioural and biochemical outcomes associated with ASD in humans are produced by this model; importantly, these outcomes are observed in a variety of species, including

"outbred" genetically heterogeneous rat and mouse strains with stronger translational validity to the genetically diverse human population. It is quite remarkable given VPA's broad spectrum of action and the complexity of ASD that the pattern of outcomes associated with this model overlaps the pattern of deficits observed in ASD so well.

Specifically, prenatal exposure to VPA can produce the following behavioural abnormalities that are associated with ASD in humans: lower sociability, deficits in communication, increased repetitive behaviour/stereotypies, pre-pulse-inhibition deficits, lowered sensitivity to pain, increased anxiety, and hyperlocomotor activity (Schneider and Przewlocki 2005; Schneider et al. 2008; Dufour-Rainfray et al. 2010; Gandal et al. 2010; Mehta et al. 2011; Choi et al. 2014; James et al. 2015). In addition, prenatal exposure to VPA produces the following biochemical, anatomical, or neuronal deficits, many of which are associated with ASD in humans: a reduction in Purkinje cells, cerebellar, and gastrointestinal abnormalities (Rodier et al. 1997; Ingram et al. 2000; Kim et al. 2013a), deficits in the Akt/mTOR pathway (Nicolini et al. 2015), increased cortical thickness and number of neurons in the neocortex (Sabers et al. 2015), an increase in the basolateral nucleus of the amygdala (Loohuis et al. 2015), a reduction in spine density in the hippocampus (Takuma et al. 2014), decreased cortical brain-derived neurotrophic factor (BDNF) mRNA (Roullet et al. 2010) abnormal serotonergic differentiation, migration, and maturation (Miyazaki et al. 2005), hyperserotonemia (Narita et al. 2002), both increased and decreased hippocampal serotonin levels (Narita et al. 2002; Dufour-Rainfray et al. 2010), and the failure of serotonin expression (Jacob et al. 2014) (see Table 1).

The VPA model has even been able to replicate the gender imbalance found in ASD. VPA exposure in animals has a more detrimental impact on behaviour, morphology, and the immune system in males than it does in females (Schneider et al. 2008; Kataoka et al. 2013a; Kim et al. 2013b; Mowery et al. 2015). The reasons for this differential impact of VPA are uncertain (Schneider et al. 2008; Mowery et al. 2015). However, the preponderance of evidence suggests the likely answer is that natural differences between the sexes exacerbate or protect against the teratogenic impact of VPA. In other words, female-specific biochemical patterns during critical developmental periods may protect against VPA (Schneider et al. 2008). Indeed, female oestrogen has been described as protective against harmful toxins implicated in the onset of ASD (Geier et al. 2010).

In short, the VPA animal model's strength rests upon its very high degree of face validity. The model is able to produce the varied symptomatology of ASD, and its unique gender expression profile.

6.3 Predictive Validity

The ability to achieve pharmacological predictive validity in ASD animal models is currently impossible, due to the lack of effective treatments of the core symptoms in

Table 1 ASD-like behavioural and neurochemical alterations induced by prenatal VPA administration in animals

Reference	Effect	Species
Choi et al. (2014)	Hyperlocomotor activity	Rat (Sprague-Dawley)
Gandal et al. (2010)	↓ Social interaction ↓ USV ↑ Rep. behav.	Mice
Mehta et al. (2011)	↑ Anxiety ↑ Rep. behav.	Mice
Schneider and Przewlocki (2005)	↓ Sensitivity to pain ↓ p.p.i ↓ Social behav. ↑ Stereotypies	Rat (Wistar)
Schneider et al. (2008)	↓ Sensitivity to pain ↑ Rep. behav. ↑ Anxiety ↓ Social behav.	Rat (Wistar)
James et al. (2015)	Abnormal social behaviour	Xenopus laevis Tadpoles
Ingram et al. (2000)	↓ Purkinje cells	Rat (Long Evan)
Kim et al. (2013a)	Gastrointestinal abnormalities	Rat (Sprague-Dawley)
Rodier et al. (1997)	Cerebellar abnormalities	Rat
Nicolini et al. (2015)	Deficits in the Akt/mTOR pathway	Rat (Wistar Han)
Sabers et al. (2015)	↑ Cortical thickness ↑ Neurons neocortex	Rat (Wistar)
Loohuis et al. (2015)	↑ In the basolateral nucleus of the amygdala	Rat (Wistar)
Takuma et al. (2014)	↓ Spine density in hippocampus	Mice
Roullet et al. (2010)	↓ BDNF mRNA	Mice
Miyazaki et al. (2005)	Abnormal 5HT differentiation, migration and maturation	Rat (Wistar)
Narita et al. (2002)	Hyperserotonemia ↑ 5HT in hippocampus	Rat (Sprague-Dawley)
Dufour-Rainfray et al. (2010)	↓ 5HT in hippocampus	Rat (Wistar)
Jacob et al. (2014)	Failure of 5HT expression	Zebrafish

humans. However, VPA animal models have offered predictions in the other direction, i.e. predictions as to what might benefit humans based upon their impact in the VPA model. Several research groups have been able to show attenuation of the deficits produced by the VPA model, all via differing methods.

Specifically, the acetylcholinesterase inhibitor (AChEI) donepezil ameliorated social deficits, repetitive behaviour, and hyperactivity in mice prenatally exposed to VPA (Kim et al. 2014). Ciproxifan (CPX), an H3R antagonist, improved social

behavioural deficits and repetitive behaviours in mice (Baronio et al. 2015). Moreover, treatment with atomoxetine (ATX), a norepinephrine reuptake inhibitor, reversed the hyperactivity induced by prenatal VPA exposure (Choi et al. 2014). In addition, post-natal environmental enrichment has also been shown to reverse a wide array of the expected outcomes of the VPA rat model, including social behavioural deficits, repetitive behaviour, and anxiety (Schneider et al. 2006).

Finally, antioxidants have been investigated for their attenuating properties. Astaxanthin was able to ameliorate VPA-induced deficits in social behaviour, and lowered sensitivity to pain, as well as significantly reducing oxidative stress in the liver and brain (Al-Amin et al. 2015). In addition, green tea extract attenuated some of the effects in a rodent VPA model, including cognitive and motor deficits, hyperlocomotion, and anxiety (Banji et al. 2011).

Taken together, these findings suggest that the cholinergic system, the histaminergic system, and the norepinephrine transporter may all play important roles in attenuating the deficits observed in ASD and, in turn, offer promising pharmacological targets in the drug discovery process. Further, environmental enrichment and specific antioxidants may be effective in helping patients with ASD. However, with regard to *environmental enrichment*, the translational value for humans is currently unclear. The identification of new targets for drug treatment is one of the key aims of animal models of disease (van der Staay et al. 2009), and in this respect the VPA model of ASD seems to offer great potential. However, clearly clinical trials with these compounds will need to be performed to test the validity of these predictions.

In summary, the prenatal exposure to VPA model of ASD is as good a model as there currently is for ASD. The strength of the model rests on its high degree of face validity across a range of different species and its ability to work in heterogeneous "outbred" rat strains that exhibit a genetic heterogeneity more representative of the human population. Although the VPA model has limited construct validity, given the complexity and heterogeneity of the disorder this is not surprising. In addition, despite the lack of treatments for ASD currently preventing the examination of predictive validity in animal models of this disorder, the VPA model has identified several distinct new targets/pathways for potential therapeutic intervention worthy of future research.

7 Mechanisms of Action of VPA

We have seen that prenatal exposure to VPA is a well-established animal model for ASD; however, the molecular mechanism(s) underlying these effects are far from clear (Jeong et al. 2003; Fujiki et al. 2013; Fathe et al. 2014; Bollino et al. 2015). The literature on the mechanisms of VPA is filled with entirely different explanations, conflicting results, failed replications, and lingering unproven hypotheses. However, from the complexity, at least three points can be gleaned: VPA has a broad spectrum of action, it works via multiple different mechanisms, and it

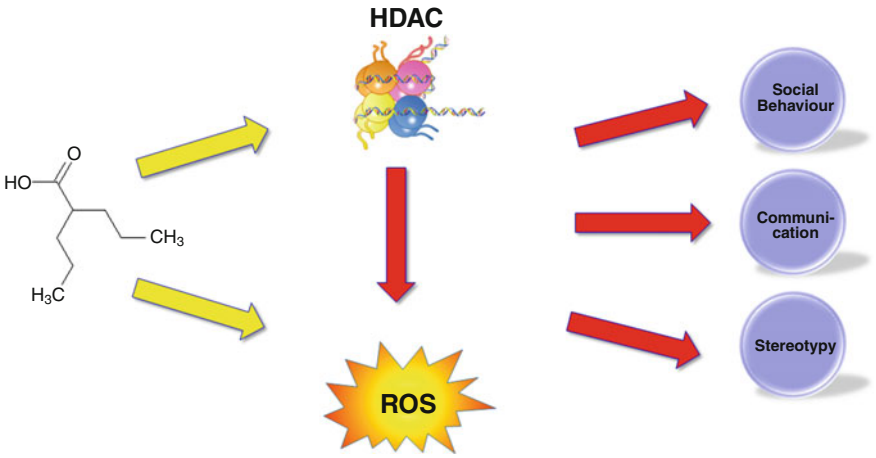

Fig. 2 Although the precise mechanism(s) by which VPA affects (neuro) development are as yet unknown, inhibition of histone deacylation (*HDAC*) and increase in reactive oxygen species (*ROS*) are two processes likely to contribute. As discussed in the text, there is evidence supporting an interaction between these two processes, with alterations in HDAC leading to changes in ROS

involves multiple different neurotransmitters, proteins, and enzymes (Johannessen and Johannessen 2003).

Although a variety of mechanisms for VPA-induced teratogenesis have been proposed, including VPA interference of folate metabolism (Wegner and Nau 1992) and inhibition of folate receptors (Fathe et al. 2014), here we focus on the two major mechanisms for which there is compelling evidence (see Fig. 2). A discussion of the mechanisms underlying VPA's therapeutic properties is beyond the scope of this chapter.

8 Histone Deacetylase Inhibition (HDAC-I)

One of the most convincing mechanisms through which VPA could exert its teratogenic activity is through inhibition of histone deacetylase (Phiel et al. 2001; Menegola et al. 2005; Eikel et al. 2006; Tung and Winn 2010; Fujiki et al. 2013; Lloyd 2013). Deoxyribonucleic acid (DNA) molecules are surprisingly long and in order to fit within the small confines of the cell nucleus, it has to be dramatically compressed. This compression is accomplished by tightly wrapping the DNA molecule around proteins known as histones, to form nucleosomes: the repeating units of chromatin (Kornberg 1977; Li and Reinberg 2011). However, this high degree of compression makes it more difficult for gene transcription to occur, as this involves large proteins (such as transcription factors) binding to DNA before RNA polymerase can bind and initiate gene transcription. Thus, gene expression is

regulated, among other factors, by how tightly DNA is wrapped around the core histones. Several different modifications of the core histones are known to influence this wrapping, including histone methylation, histone phosphorylation, and histone acetylation. This latter process is regulated by two classes of enzymes: Histone acetyltransferases (HATs), which attach acetyl groups to lysine residues on histones, and histone deacetylases (HDACs) which subsequently remove these acetyl groups. As acetylation reduces the positive charge of lysine residues, the chromatin becomes less tight (as DNA is negatively charged) and therefore gene transcription is promoted. Conversely, by removing the acetyl groups, HDACs lead to a more compact DNA-histone package and, in turn, reduced gene transcription (Lloyd 2013; Ivanov et al. 2014).

Four different classes of HDACs are known in mammals: the Zn-dependent HDAC-I, II, and IV, and the NAD^+-dependent HADC-III (also known as sirtuins). VPA has been shown to non-selectively inhibit most HDAC, (Phiel et al. 2001; Menegola et al. 2005; Fujiki et al. 2013), thus preventing HDACs from removing acetyl groups and consequently resulting in hyperacetylation and increased *gene activation*. By disturbing the natural HAT and HDAC dynamic processes, VPA has the ability to impact many different genes at the same time (Lloyd 2013). These HDAC-inhibitory properties are thought to underlie the teratogenic influence of VPA (but also its potential therapeutic effects in cancer and cognitive deficits). Indeed, like VPA, other HDAC-Is such as TSA (trichostatin A) and sodium butyrate have teratogenic effects. Interestingly, whereas VPA analogues that retain HDAC-inhibitory activity also induce teratogenicity, analogues that lack this effect do not (Gurvich et al. 2005).

Histone modifications that can alter gene expression come under the umbrella term of epigenetics. Epigenetics is defined as the study of changes of function to the genome that modifies the expression of genes but does not change the nucleotide sequence (Ivanov et al. 2014; Tordjman et al. 2014). It is now believed that specific environmental factors can induce changes in gene expression via epigenetic mechanisms. These changes in gene expression are functionally expressed by the affected organism and can be responsible for a variety of phenotypes, both positive and negative. Importantly, histone modifications can be long-lasting, thus affecting gene transcription for prolonged periods of time. Epigenetics plays an important role in the broader explanatory model of gene x environment interactions, and therefore, VPA can be viewed as an environmental agent that has the capability to interact with specific genes that lead to an ASD phenotype.

The main question that follows from VPA's influence on HDAC is: why does HDAC-I-induced *gene activation* lead to teratogenic effects? At present, it is very difficult to answer this question for a number of reasons. First, depending on the type of histone that is acetylated (there are four basic histones (H2A, H2B, H3, and H4) that make up the nucleosome, plus H1 that connects nucleosomes together), histone acetylation can induce different effects. Second, the histone acetylation can occur in many different genes, thus leading to an increased transcription of many genes. Third, epigenetic changes, such as histone acetylation, may be very local, i.e. differ between different brain regions or within a single brain region even between

different types of cells. Finally, HDACs are known to interact with other epigenetic alterations (such as histone methylation and DNA methylation). Altogether, the puzzle of which genes are activated by VPA and when and where has not been solved. However, the types of genes that were activated can be inferred from the behavioural or biochemical outcomes. In other words, if we observe a teratogenic outcome, we can infer that the types of genes that lead to this outcome were the ones activated in this instance. The field of toxicogenomics has the potential to make significant strides in our understanding of VPA-responsive genes.

9 Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) may also play an important role in the mechanism of VPA-induced teratogenesis (Tung and Winn 2011). ROS are highly reactive molecules that, in excess, have the capacity to damage many elements of a cell (Andersen 2004; Wells et al. 2009). There are a variety of endogenous mechanisms by which ROS are generated, including mitochondrial respiration and the immune response system (Wells et al. 2009; Lloyd 2013), but ROS can also be enhanced exogenously by xenobiotics, including VPA (Na et al. 2003; Defoort et al. 2006; Kawai and Arinze 2006; Wells et al. 2009; Tung and Winn 2011). Although generation of ROS is both normal and beneficial, excessive generation of ROS can have major detrimental effects through either disruption of signal transduction and/or damage to lipids, DNA, RNA, proteins, and carbohydrates (Wells et al. 2009). A variety of defence mechanisms exist that help fight against the excess generation of ROS and regulate this environment, including antioxidant enzymes and compounds, and direct and indirect repair systems (Sies 1997; Davies 2000). When the generation of ROS overwhelms the multitiered defence mechanisms, a state of oxidative stress ensues and deleterious effects to the host can occur. The balancing act of ROS generation versus the host's defence mechanisms to keep a regulated and healthy ROS environment reflects what is referred to as the *oxygen paradox*—the concept that although aerobic life requires oxygen to survive, oxygen is also intrinsically dangerous to its existence (Davies 1995).

Importantly, the embryo and foetus have lower antioxidant enzyme levels, and in turn, a lowered defence system against excess generation of ROS (Winn and Wells 1999; Wells et al. 2009). This lowered defence system theoretically leaves the embryo and foetus with a higher susceptibility to the effects of ROS-generating mechanisms or xenobiotics, such as VPA (Zaken et al. 2000). Numerous reports have demonstrated that exposure to VPA increases the production of ROS and has negative consequences for cell survival and development (Na et al. 2003; Defoort et al. 2006; Tung and Winn 2011). One mechanism whereby this may be achieved is through enzymatic bioactivation (Winn and Wells 1997). Xenobiotics can be bioactivated by certain enzymes that *are* highly prevalent in the embryo, such as prostaglandin H synthase (PHS) and lipoxygenase (LPO) and converted to free radical reactive intermediates that commence ROS generation (Wells et al. 1997).

If the excess ROS generation overwhelms the host's defence mechanisms and oxidative stress results, then adverse developmental effects may be produced (Wells et al. 2010). Put simply, the teratogenic effect of VPA could result from a combination of an undeveloped defence mechanism and VPA's ability to initiate ROS production.

Furthermore, VPA has the ability to interfere with the defence mechanisms themselves. Superoxide dismutase (SOD) and glutathione (GSH) are two important antioxidants involved in the defence against ROS, and a downregulation of both SOD and GSH have been observed following VPA exposure (Zhang et al. 2010; Hsieh et al. 2012). GSSG (glutathione in its oxidised form) and its ratio with GSH can be used as a measure of oxidative stress, with increases in GSSG: GSH ratio indicative of increased oxidative stress. Dose-dependent increases in embryonic GSSG: GSH ratio have been observed following VPA exposure at doses ≥100 μg/ml (Zhang et al. 2010). Together, these data suggest VPA's ability to alter antioxidant homoeostasis in the embryo may play an important role in VPA's teratogenic influence.

ROS can directly induce DNA double-strand breaks (Winn 2003). Homologous recombination (HR) is a DNA repair mechanism that can repair DNA double-strand breaks (Haber 1999). However, HR is not an entirely error-free procedure and has the potential to contribute to detrimental genetic changes. Thus, increased levels of HR theoretically would increase the odds of important genes in the developmental process being disrupted at critical time points, possibly resulting in teratogenesis (Defoort et al. 2006). Interestingly, VPA has been demonstrated to cause oxidative stress and, in turn, increase HR levels in vitro. Furthermore, the antioxidative enzyme *catalase*, one of the cellular defence mechanisms against oxidative stress, completely blocked the increased HR following VPA treatment (Defoort et al. 2006). These data suggest HR could be an underlying mechanism of VPA-induced teratogenesis and that oxidative stress plays an important role (Defoort et al. 2006).

Finally, the role of oxidative stress and ROS in the mechanism of VPA-induced teratogenesis is further supported by data demonstrating the attenuating effects of certain antioxidants in prenatal VPA animal models. For instance, green tea extract was found to exhibit neuroprotective effects, possibly due to its antioxidant properties (Banji et al. 2011). Likewise, the antioxidant Vitamin E attenuated the VPA-induced teratogenic effects in mice (Al Deeb et al. 2000). Embryonic models have suggested the main mechanisms of Vitamin E in attenuating VPA-induced teratogenicity are through the inhibition of ROS and the restoration of GSH (Hsieh et al. 2014). In addition, the neuroprotective antioxidant Astaxanthin (Liu and Osawa 2009) was seen to improve ASD-related behavioural outcomes in mice; an effect also attributed to its antioxidant properties (Al-Amin et al. 2015).

Overall these data provide convincing evidence for a role of HDAC inhibition and ROS production in VPA's effects on the unborn foetus. However, it should be realised that these processes are not necessarily independent (Fig. 2). As HDAC inhibition is likely to affect the expression of a multitude of genes, it is at least conceivable that the alterations in ROS production and the subsequent oxidative

stress may be secondary to the inhibition of HDAC. In line with this, studies in the cancer field have clearly shown that HDAC inhibitors can increase ROS production and programmed cell death (Carew et al. 2008).

10 Experimental Models and Mitigating Factors

The broad spectrum of action and complex nature of VPA make investigation of the underlying mechanisms and linking them to the behavioural and biochemical changes very difficult. The heterogeneity in the data on the outcomes and mechanisms of VPA is substantial. It is therefore vitally important to study the factors contributing to the variability of the effects of VPA (Bielecka and Obuchowicz 2008; Roullet et al. 2013).

Cell, animal, and human research on VPA have demonstrated several key points:

First, response to VPA differs as a function of the developmental age, brain region, and gender investigated (Bittigau et al. 2002; Kataoka et al. 2013a). For instance, the apoptotic effects of VPA in 14 different brain regions were studied in rat pups exposed to VPA at various developmental stages. Results revealed the response to VPA differed as a function of both developmental age and brain region (Bittigau et al. 2002). Regional specific neuronal cell loss has also been observed in mouse models of VPA (Kataoka et al. 2013a). In addition, mice treated with VPA at gestational day (GD)12.5 led to social interaction deficits in male, but not female mice, highlighting the importance of gender in VPA exposure (Kataoka et al. 2013a).

The second key point is that response to VPA is both differentiation stage and cell-type dependent (Wang et al. 2011; Fujiki et al. 2013). For instance, VPA was found to have a proapoptotic effect on embryonic stem cell-derived neural progenitor cells of glutamatergic neurons, but this effect was not observed in their neuronal progeny (Fujiki et al. 2013). Moreover, a neuron–astrocyte culture mix treated with VPA induced apoptotic effects that were not observed in a simple neuron-enriched culture, implicating the importance of cell-type in VPA-induced neurodegeneration (Wang et al. 2011).

The third key point is that even seemingly small experimental changes can lead to not just different but opposing findings. A clear example of this comes from two different research groups investigating VPA exposure at GD9 and measuring hippocampal serotonin at post-natal day (PND)50 (Narita et al. 2002; Dufour-Rainfray et al. 2010). Whereas one paper found an *increase* in serotonin in Sprague-Dawley rats following 800 mg/kg VPA (Narita et al. 2002), the other found a 46 % *decrease* in serotonin in Wistar rats following 600 mg/kg VPA (Dufour-Rainfray et al. 2010). The discrepant findings were hypothesised to be a result of the differences in the experimental procedure (Dufour-Rainfray et al. 2010).

The final key point is that VPA-induced outcomes are highly dependent on dosage and timing, or the window of exposure, to the drug (Jeong et al. 2003; Johannessen and Johannessen 2003; Takuma et al. 2014). The *amount* of VPA

administered has been repeatedly shown to affect the outcome of the drug in humans, with higher doses associated with higher rates of teratogenicity (Koren et al. 2006; Diav-Citrin et al. 2008; Meador et al. 2008). The timing of VPA administration also has significant implications for the response to the drug. For instance, mice treated with VPA at GD12.5, but not GD9 and GD14.5, exhibited ASD-like symptomatology, including deficits in social interaction (Kataoka et al. 2013b). Another, particularly striking, example of the role of timing in VPA-induced-outcome was seen in a paper exposing mice prenatally to VPA and then treating these same mice with VPA post-natally. VPA-exposed mice had deficits in novel object recognition, and decreased spine density in the hippocampus. Remarkably, post-natal chronic treatment of VPA attenuated both deficits (Takuma et al. 2014). In other words, the very drug that created the deficits prenatally attenuated the deficits when given post-natally. On a related note, studies with ASD patients have found that although prenatal exposure to VPA can increase the likelihood of ASD, treatment with Divalproex (a derivative of VPA) was actually seen to benefit patients—helping with repetitive behaviours and irritability (Hollander et al. 2006, 2010). One reason for the strong effect of timing may relate to VPA's HDAC-I and ROS-inducing properties, and the fact that different genes may be activated at different developmental periods. The effect of timing may be exactly why VPA is used prophylactically in adulthood for several conditions, but still causes damage when exposure occurs prenatally.

Taken together, the data suggests strongly that VPA has a particularly high sensitivity for experimental variables and therefore even slightly different experimental models can produce very different results. It is clear that the timing, dosage, cell-type, differentiation stage, strain-type, gender, and brain region studied can all have a meaningful impact on the outcome of research using VPA. These mitigating factors likely explain some of the diversities in the VPA literature.

11 Concluding Remarks

Prenatal exposure to VPA is as good an animal model as there is for ASD. The VPA model has limitations at several levels in the traditional concept of animal model validity, most prominently with regard to construct and predictive validity. However, these limitations say more about the state of knowledge on ASD, the heterogeneity of ASD itself, and the traditional criteria of validity, than the VPA model in question.

The mechanisms under which VPA exerts its teratogenic influence are not well understood. At this point, the preponderance of evidence suggests that VPA's HDAC-inhibitory properties are likely the major source of its influence. However, given VPA's enormously broad spectrum of action, it is probable that multiple mechanisms contribute, but to varying degrees. In addition, the genetic make-up of mother and foetus combined with other environmental factors will be critical in determining the susceptibility to VPA-induced teratogenesis.

Future research would benefit from combining this model with various genetic animal models, in order to investigate gene x environment interactions and improve its construct validity and explanatory power. In addition, more research investigating chronic prenatal VPA exposure, as opposed to a single exposure, would be of great value. The major reason for this would be because it would more accurately represent the human condition of VPA administration; this is especially important considering the enormous role that timing plays in response to VPA. More toxicogenomic research aimed at identifying VPA-responsive genes, when and for how long these genes are responsive, and then mapping these genes onto biochemical and behavioural outcomes would be enormously beneficial. Research of this nature would allow a clearer understanding of the molecular mechanisms of VPA-induced teratogenesis and may also reveal biomarkers that indicate genetic susceptibility to such teratogenic effects. The identification of biomarkers should be a high priority for research, as they can be used as targets and exploited for therapeutic action.

The ability of VPA to act as a model for ASD demonstrates how even a single exposure to a neuroteratogen at developmentally critical time points can lead to permanent biochemical and behavioural outcomes in offspring. The high sensitivity to experimental variables is indicative of the complexity of VPA, the developmental process, and the interaction between them.

It is perhaps fitting that one of the more complex and poorly understood disorders in neuroscience, ASD, is linked to one of the most complex and poorly understood environmental agents: VPA. That their respective complexities overlap in such a manner is nothing short of remarkable.

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