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Original Article

Epigenetic studies in insects and the valproic acid perspective

Estudos epigenéticos em insetos e a perspectiva do ácido valpróico

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Abstract

Valproic acid in association with sodium valproate (VPA) is an important anticonvulsant drug used for decades to treat neurological disorders. VPA also acts as an epigenetic modulator by inhibiting histone deacetylases, permitting histone acetylation, affecting the DNA and histone methylation status and gene expression, and inducing chromatin remodeling. Insects represent an important animal model for studies in several areas of science. Their high phenotypic plasticity makes them alternative models for epigenetic studies. This brief review emphasizes recent reports on insect epigenetics and the contribution of studies on the VPA action in insects, including effects on epigenetic markers, extending the pharmacological understanding of the potential of this drug, and demonstrating the usefulness of insects as an alternative animal model to drug studies.

Keywords: sodium valproate, DNA methylation, histone modification, non-coding RNAs, chromatin.

Resumo

O ácido valproico associado ao valproato de sódio (VPA) é um importante agente anticonvulsivo usado há décadas no tratamento de distúrbios neurológicos. Também atua como um modulador epigenético ao inibir as desacetilases de histonas, permitindo a acetilaçao de histonas, afetando o estado de metilação do DNA e das histonas e a expressão gênica, e induzindo a remodelação da cromatina. Os insetos representam um importante modelo animal para estudos em diversas áreas da ciência e dada a sua alta plasticidade fenotípica os torna modelos alternativos para estudos epigenéticos e de resposta a agentes moduladores epigenéticos. Esta breve revisão enfatiza relatos recentes sobre epigenética em insetos e a contribuição de estudos sobre a ação do VPA nesse grupo animal, incluindo efeitos sobre marcadores epigenéticos, estendendo a compreensão farmacológica do potencial desta droga e demonstrando a utilidade dos insetos como um modelo animal alternativo para estudos de drogas.

Palavras-chave: valproato de sódio, metilação do DNA, modificação de histonas, RNAs não-codificantes, cromatina.

1. Epigenetics: General Concepts

Epigenetics is specifically concerned with reversible changes in gene expression or silencing that do not involve changes in DNA base sequences and that furnish a cell memory that goes beyond the potential of the DNA genetic code and that can be heritable through mitosis or meiosis, passing to the next generation (Wu and Morris, 2001). Gene expression can thus be altered without modification of the genetic code.

The mechanisms of epigenetic changes include 1) DNA methylation, which results from the incorporation of a methyl group to the cytosine carbon 5, mostly in cytosine followed by guanine (CpG) and leading to the formation of 5-methylcytosine (5mC), 2) histone post-translational modifications, including acetylation and methylation, and histone variants, and 3) mechanisms mediated by non-coding RNAs. All these epigenetic mechanisms with respective effects on cells and organisms have been widely described and the subject of several reviews, with predominant application to vertebrate models. In insects, epigenetic studies have been restricted to a few model groups and species and even in these cases, a few reports have been addressed to disease vectors (Sharakhov and Sharakhova, 2015), possibly because non-efficient functional validation tools are sufficiently available for these non-model organisms (Richard et al., 2021). It is always worth mentioning that the pioneering studies and concepts of Waddington, who coined the term Epigenetics in 1942 (Waddington, 2012), refer to the environmental influences on the development of phenotype in *Drosophila melanogaster* (Tronick and Hunter, 2016).

Epigenetic mechanisms regulate the development of extreme phenotypic divergences and environmental adaptations, permitting dynamic changes of insect phenotype, conservation of insect forms and functions, and interconnections with other forms of biological regulation (Villagra and Frías-Lasserre, 2020). Regarding social insects, including ants, bees, wasps, and termites, epigenetics has revealed an important role in eusocial

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societies, participating in caste determination and phenotypic plasticity, and leading to the evolutionary success of these groups (Bonasio, 2014; Yan et al., 2015; Sieber et al., 2021).

In this brief review, we summarize some relevant aspects of the epigenetic mechanisms reported for insects and the emerging role of drugs like valproic acid, that while perturbing the epigenome in human and animal cells, may contribute to a better understanding of the diversity of the epigenetic metabolic pathways followed by these drugs when using insects as complimentary research models.

2. Epigenetic Markers in Insects

2.1. DNA methylation

DNA methylation is generally associated with the repression of gene activity and depends on the action of enzymes of the DNA methyltransferase (DNMT) family. Although it does not cause changes in the DNA sequence, it significantly affects the interaction of DNA with proteins (Kozeretska et al., 2017). The analysis of the DNA methylation and demethylation processes in insects indicates that these epigenetic events not only allow them to control and tolerate stress insults but also furnish the ability to change their structures and behavior, and affecting their offspring (Villagra and Frias-Lasserre, 2020).

Even though DNA methylation is a widely heritable form of epigenetic information especially residing in the CpG context in several organisms, it is absent or reduced from the genomes of many insect species (Suzuki and Bird, 2008; Bewick et al., 2017; Provataris et al., 2018; Glastad et al., 2019). In dipterans, including *Drosophila melanogaster,* there is little presence of DNA methylation (Lyko et al., 2000; Provataris et al., 2018). In the beetle *Tribolium castaneum*, the levels of DNA methylation are also close to zero (Schulz et al., 2018). In the hemipteran species *Aphis nerii* and *Triatoma infestans*, although cytosine methylation is revealed in euchromatin, it is not identified in heterochromatin (Alvarenga et al., 2011; Mandrioli et al., 2011).

In highly eusocial bees, the bumblebee *Bombus terrestris*, ants, and non-social wasps, where DNA methylation was confirmed, shared methylation sites associated with key biological processes (development, caste differentiation, lifespan, sex determination, cognition and memory, among others) have been suggested by several authors (Villagra and Frias-Lasserre, 2020 – review, Araujo and Arias, 2021), although DNA methylation as a causal role in the divergent development of queen and worker castes has been questioned for honeybees (Oldroyd and Yagound, 2021). In the larval genome of *Apis mellifera* around 49 million cytosines are present, of which only 90,000 cytosines are methylated; genomic analysis showed 38 differentially methylated genes between queen larvae and worker larvae associated with differentiation of specific organs, including those involved in reproduction, morphology, and vision differentiation (Wang et al., 2020). In addition, involvement of non-CG methylation in bee eusociality has recently been proposed (Araujo and Arias, 2021).

Planococcus citri, a citrus mealybug that exhibits a sex-specific genomic imprinting with the elimination of the entire paternal genome during spermatogenesis, has been analyzed with reference to its DNA methylation profile (Bain et al., 2021). Genome-wide-analysis of sex-specific gene expression and DNA methylome performed in adult specimens revealed that while males exhibit overall higher levels of DNA methylation manifested at more uniform low sites across the genome, females display more targeted higher levels of DNA methylation (Bain et al., 2021). These findings were suggested to be associated with chromosomal differences caused by the paternal genome elimination and possibly related to ploidy compensation.

In contrast to vertebrates in which most DNA methylation occurs in both intragenic and intergenic regions, in insects DNA methylation occurs within gene bodies affecting exon sequences in holometabolous species, or occurs largely in the genome in hemimetabolous species (Glastad et al., 2019). The functional significance of this event is yet not clear, especially considering that the DNA methylation events have a general effect on transcription factor binding and recruitment which in turn requires patterns of transcript splicing (Glastad et al., 2019).

2.2. Histone modifications

Histones are basic proteins that make complexes with the DNA forming chromatin in eukaryotes; they not only play a structural role by packaging the DNA but also represent regulators of the DNA function. Post-translational modifications of histones, which include acetylation, methylation, phosphorylation, and ubiquitination, among others, of several of their different amino acid residues, and that are mainly focused on histones H3 and H4, are involved in the regulation of cell differentiation and disease (Lennartsson and Ekwall, 2009). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the enzymes responsible for inserting and removing the acetyl groups, in and from, respectively, the amino acids of histones. Histone acetylation mostly affecting lysine (K) residues reduces the electrostatic binding of histones to DNA, facilitating the accessibility of binding factors to DNA and permitting its transcription. Histone methylation, on the other hand, may be associated with actively expressed or repressed genes depending on the metabolized residues, the number of inserted -CH_3 groups to these residues, and wherein the genome such inserted groups are associated. For example, H3K27me is correlated with active genes whereas H3K27me3 is associated with silent genes; H3K9me3 correlates with repressed genes when present at the gene promoter but correlates with gene expression when enriched within the body of a gene (Black and Whetstine, 2011). Non-histone proteins, for instance, the heterochromatin protein HP1, may associate with histone modifications, participating in chromatin structure and remodeling (Felisbino et al., 2014).

It appears that histone modifications or an alternative sequence variant of a histone may be more involved in the mediation of phenotypic plasticity in insects than DNA methylation (Glastad et al., 2019). However, except for flies of the genera *Drosophila* and *Anopheles*, a few studies

are still devoted to exploring post-translational histone modifications in association with transcription control and the regulation of chromatin structure and function (Swaminathan et al., 2012; Neafsey et al., 2015; Sharakhov and Sharakhova, 2015). In *Drosophila*, different members of HDACs are related to specific functions and processes such as aging, segmentation, suppression of apoptosis, and circadian rhythms, and the histone demethylase KDM5 is responsible for regulating social behavior through immunological maintenance of the intestinal microbiota, and development control (Chen et al., 2019; Drelon et al., 2019).

Epigenetic research to evaluate interferences of pathogenic bacteria on the regulation of HDACs and HATs in insects demonstrated that histone acetylation/deacetylation intervenes in transcriptional reprogramming during metamorphosis and in response to injuries and infections, which can modulate directly responses on immunity and development (Mukherjee et al., 2015, 2017). Histone acetylation can also play an important role in chromosomal imprinting in the germline of sciarid flies; in male meiosis, the maternal chromosomes are highly acetylated for histones H3 and H4, whereas the entire set of the paternal chromosomes that will undergo elimination appears under-acetylated (Goday and Ruiz, 2002; Matsuura, 2020). *Locusta migratoria* represents one of the richest species in histone-modifying enzymes, that are highly expressed in male eggs and testes, and that can play important roles in embryogenesis and spermatogenesis (Guo et al., 2016; Lo et al., 2018).

Twelve HDACs have been identified in the coleopteran *Tribolium castaneum*. HDAC1, particularly, plays a role in the regulation of cellular processes necessary for the postembryonic development of this insect, affecting the acetylation status of histones and suppressing the expression of genes involved in the action of the juvenile hormone (George et al., 2019). In the absence of juvenile hormone, multiprotein complexes suppress the expression of juvenile hormone response genes. On the other hand, suppression of the expression of the HDAC1 gene by juvenile hormone induces increase in the acetylation levels of core histones regulating the expression of juvenile hormone response genes such as *Kr-h1* (George et al., 2019). These findings demonstrate that the juvenile hormone action is affected by epigenetic modifications (George et al., 2019).

Differential identification of acetylated H3K9, H4K8 and H4K16 and mono-, di-and trimethylated H3K9 (H3K9me/me2/me3) between euchromatin and heterochromatin has been reported in the hemipteran species *Triatoma infestans* and *Panstrongylus megistus*, vectors of Chagas disease, and are consistent with the transcriptionally active and relatively inert status, respectively, of these chromatin compartments (Alvarenga et al., 2016, 2018). More details on histone acetylation, histone methylation, and histone phosphorylation in insects have been recently revised by Villagra and Frías-Lasserre (2020).

Histone variants also alter gene expression. Variants of the canonic histone H2A, for instance, affect the chromatin repeating elements, the nucleosomes, resulting in increased accessibility of the DNA. Histone variants have been detected in *Drosophila* and are considered promising candidates for investigation in other insect species (Glastad et al., 2019).

2.3. Non-coding RNAs

Regarding the epigenetic effects carried out by non-coding RNAs, small RNAs like microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs), with 20-40 nucleotides, and long non-coding RNAs (lncRNAs), with more than 200 nucleotides, have particularly been identified in the regulation of gene expression of insects, especially in *Drosophila* (Chambeyron and Seitz, 2014; Kelleher, 2016; Glastad et al., 2019; Villagra and Frías-Lasserre, 2020). miRNAs are post-transcriptional regulators of gene expression by interacting with mRNAs and affecting protein levels (Bartel, 2009; Asgari, 2018; Richard et al., 2021). A review by Asgari (2018) has emphasized that although studies of insect miRNAs are recent, they are already revealing involvement of these molecules in development, reproduction, caste determination, lifespan, insecticide resistance and immune responses to infection, among other physiological events. Caste-specific expression of miRNAs has been reported in ants, honeybees, and bumblebees (Guo et al., 2013), ranging from 257 in the silkworm *Bombyx mori*, to 883 in *Locusta migratoria* (Wang et al., 2015; Wang et al., 2019).

Recent studies have shown the use of an artificial miRNA (amiRNA) approach for gene silencing to generate insect pest-resistant tomato plants. Plant amiRNAs are produced by expression of a miRNA gene genetically modified to silence a desired target gene. amiRNAs manipulate gene functions because of their efficiency and specificity to decrease target gene expression. Plant amiRNAs are thus a potential strategy for engineering plant resistance to virus and insects (Agrawal et al., 2015; Yogindran and Rajam, 2021). *Helicoverpa armigera* specimens that were continuously fed with tomato leaves expressing amiRNA-319a-HaEcR, showed a reduction in the target gene (ecdysone receptor *EcR*) transcripts, affecting the general growth and survival of this lepidopteran pest. Furthermore, the expression of downstream genes involved in the ecdysone signaling pathway (*E74A, Et4B, BR-C7* and *HR3*) was affected. Interference in the ecdysone signaling results in impaired embryogenesis and disrupted metamorphosis. These results helped to understand the role played by ecdysone receptors as well as the effectiveness of amiRNA technology in the control of *H. armigera* (Agrawal et al., 2015; Yogindran and Rajam, 2021).

Following the same line of reasoning on pest control, a methodology based on pre-microRNA proved to be efficient in controlling herbivorous insect pests. The study was conducted with insect pre-microRNA (pre-miR) transcripts that were modified to contain microRNAs targeted to insect genes and expressed in transgenic *Nicotiana benthamiana* plants. These modified pre-miRs remained largely unprocessed in plants. Thus, the insect pest *H. armigera*, when fed on the leaves of these plants, had a significant increase in mortality, developmental abnormalities, and delays in growth rates (Bally et al., 2020).

piRNAs that were identified for the first time in *D. melanogaster*, are single-stranded noncoding RNAs that

can initiate epigenetic events within insect genomes (Glastad et al., 2019). They interact with transcribed genes inducing co-transcriptional silencing and generating heterochromatic marks where transposable elements are present, thus protecting the genome from damages the transposable elements could bring (Glastad et al., 2019).

lncRNAs are particularly suggested to be involved in caste development in Hymenoptera. In this insect group, lncRNA regulates the size of the worker ovaries in honeybees (Humann et al., 2013; Richard et al., 2021), binds to specific targets, and recruits chromatin-modifying enzymes, initiating the formation of a silent or an active chromatin site (Glastad et al., 2019).

3. Valproic Acid Effects on Epigenetic Markers

Some drugs may affect the epigenetic information by acting on the deposition or removal of epigenetic markers. One of these drugs is valproic acid which may work through different metabolic pathways, affecting several types of epigenetic markers and behaving as an epigenetic modulator.

Valproic acid is a short-chain fatty acid that in association with its sodium salt (VPA) has been widely prescribed as an anticonvulsive drug. The effects of VPA involved with the treatment of seizure disorders revealed activities as inhibition of transamination of the neurotransmitter gamma-aminobutyric acid (GABA) and blockage of the voltage-gated sodium and T-type calcium channels (Chateauvieux et al., 2010). VPA has also been demonstrated to have a binding affinity for chromatin components by acting directly on histones and DNA (Sargolzaei et al., 2017; Vidal and Mello, 2020, 2022) and altering histone H1/nucleosome ratios (Baumann et al., 2021).

In addition to these pharmacological effects, VPA has been found to inhibit cell proliferation, affecting mitotic indices and eliciting cell death pathways, especially in tumor cells. These findings allowed it to be proposed alone or synergistically combined with other drugs as a promising antitumor agent that has been evaluated under phase I and II clinical trials (Duenas-Gonzalez et al., 2008; Mohammed et al., 2011; Goyal and Rodriguez, 2013; Heers et al., 2018; Zhang et al., 2019). Although many advantageous effects have been demonstrated for the use of VPA, it is not to be neglected that some adverse effects like hepatotoxicity, teratogenesis, and expansion of leukemic stem cell population are risks that limit its use in humans (Bug et al., 2007; Lennartsson and Ekwall, 2009; Chateauvieux et al., 2010).

VPA was also demonstrated as a potent drug that affects epigenetic markers in a vast number of human and animal cells, changing gene expression and chromatin interaction with regulatory factors. Among these effects, inhibition of class I HDAC, with the activation of diverse gene promoters, and promoting chromatin remodeling was soon revealed (Göttlicher et al., 2001; Phiel et al., 2001; Eyal et al., 2004; Felisbino et al., 2011; Mello, 2021). In rats, many genes involved in epileptogenesis were demonstrated to become upregulated when hyperacetylation of histones on their promoters was induced by VPA (Fukuchi et al., 2009). A higher number of differentially expressed genes can result from the VPA action, as demonstrated, for instance, in HeLa cells (Dejligbjerg et al., 2008) and, under a hyperglycemic microenvironment, in HepG2 cells (Felisbino et al., 2021).

The methylation status of histones and DNA is also affected by the VPA action in many cell types. Methylation and demethylation of histones especially affecting lysine residues are events modulated by the VPA action that may occur simultaneously with HDAC inhibition (Ganai et al., 2015), affecting the intensification of global gene expression and depending on the metabolized residues (Lanouette et al., 2014; Mello, 2021). DNA demethylation promoted by VPA is a complex process that generally stands longer than histone acetylation (Detich et al., 2003; Lee et al., 2010; Perisic et al., 2010). It flows through a passive or an active pathway, depending on the cell type, and is especially verified to affect tumor suppressor genes (Milutinovic et al., 2007; Gu et al., 2012; Veronezi et al., 2017). The passive pathway presumes suppression of the activity of enzymes of the DNMT family that are responsible for the maintenance of the methylation status in cytosines. The active pathway of DNA demethylation involves the action of enzymes of the *ten-eleven-translocation* (TET) family that converts 5mC into C derivatives (Guo et al., 2011; Wu and Zhang, 2014; Rocha et al., 2019). There are cells in which, although acting predominantly within an active pathway, the DNA demethylation phenomenon induced by VPA may also occur within a passive pathway (Rocha et al., 2019).

4. VPA Effects in Insects

The use of mammals in scientific activities has provoked considerable discussions over the years regarding the ethical character, mainly due to the use of a high number of animals and the suffering caused in various scientific procedures (Liguori et al., 2017; Santos, 2019). Insects have been proposed as an alternative for analysis of potential epigenetic side effects, including those transferable to offspring, on account of their shorter generation intervals, high fecundity, facilities for using larger samples, and resistance to pathogens, parasites, and environmental stress, thus diminishing ethical concerns (Mukherjee et al., 2015; Bingsohn et al., 2016). Furthermore, insects present themselves as remarkable and important models for the research of epigenetic inheritance, since many insect species show high phenotypic plasticity, that is, the ability of an individual organism to respond to the environment by producing alternative phenotypes based on the same genotype (Manfredini et al., 2019). Epigenetic research on insects is an important tool for studying biodiversity, as environmental stimuli result in heritable phenotypic changes with biological variation without mutations and independent alterations in the DNA sequence to the detriment of variation in gene expression levels (Amiri, 2019).

A few experimental studies using VPA have been undertaken in insects. Among these, some are proposed as alternative models for epigenetic researches while

others are devoted to search for effects on precise epigenetic markers and action mechanisms or involve the determination of toxicity induction.

MJDtr-Q78 transgenic *Drosophila* specimens have been revealed as a strategic model for studies addressed to a VPA treatment of a human disorder known as Machado-Joseph Disease (MJD), which is a polyglutamine (polyQ) neurodegenerative disorder and that involves an imbalance in histone acetylation (Yi et al., 2013). This transgenic model exhibits neurodegenerative phenotypes that could be considered similar to characteristics of the human disease. Using this model, VPA treatment was found to alleviate neurodegeneration, suppressing apoptosis and restoring the imbalance in the acetylation levels of histones H3 and H4 (Yi et al., 2013). At a dose of 2.5 mM for 15 days VPA prevented eye depigmentation while a dose-dependent increase in climbing activity was verified for flies under gentler VPA doses (< 1.5 mM for 5 days). Life span was mildly prolonged under a 0.5 mM VPA treatment. Based on these data, VPA was suggested as a possible therapeutic drug in the MJD disease, suppressing retinal cell death via an anti-apoptosis pathway (Yi et al., 2013).

Drosophila has also been considered an attractive system to unravel potential pathway mechanisms of anti-epilepsy drugs, including VPA, in the treatment of several other neurological disorders, revealing differential expression of only a small number of genes (Singh et al., 2011). As concerned with the modulation of bipolar disorder using the *Drosophila* model, VPA has been found to induce transcriptional responses at gene ontology and pathway level with similar ontology as lithium (Herteleer et al., 2016). Using *Drosophila* culture cells and adult flies, it was demonstrated that VPA and lithium affect significantly overlapping genes similar to the effects described for mammals, showing that these drugs act on evolutionarily conserved pathways (Herteleer et al., 2016).

When screened for the VPA action, the beetle *T. castaneum* has been revealed to experience a longer-lasting effect than vertebrates. In this model, VPA induces delayed development, reduced longevity, declined fertility, and fecundity and revealed a warning system for transgenerational epigenetic side effects (Bingsohn et al., 2016).

A positive longevity effect was reported for *A. mellifera* under treatment with VPA (Rasmussen et al., 2021). Because this insect is reported to contain a functional DNA methylation system similar to humans, Rasmussen et al. (2021) investigated whether DNA global methylation changes induced by VPA occurred along with the insect lifespan. The fact that they did not detect significant effects on this level may have been due to the use of ELISA assay for such investigation, which was not effective enough to detect response changes at specific genome sites.

In Malpighian tubules of *T. infestans* injected with 0.5 mM VPA or cultivated *in vitro* in presence of 0.05 mM VPA for 4 h, a few cells showed decondensation of the heterochromatin bodies that constitute chromocenters (Alvarenga et al., 2016; Bassani et al., 2021). It is interesting that although this VPA-induced heterochromatin imaging change affected only a few cells, this event could not be associated with induction of H3K9 acetylation or DNA demethylation (Alvarenga et al., 2016; Bassani et al., 2021). Other epigenetic markers are thus possibly involved with this response, including non-histone proteins that may associate with histone demethylation, which certainly requires further studies (Bassani et al., 2021). Much longer exposition to VPA or the use of more concentrated doses of VPA leads Malpighian tubule cells to apoptosis and necrosis (Bassani et al., 2021). Regarding the response of the euchromatin of these cells to VPA treatment as assessed by H3K9ac immunofluorescence signals, it was slightly intensified under cultivation of the Malpighian tubules in the presence of 0.5 or 1.0 mM VPA for 4 h especially around the chromocenters, a nuclear area previously demonstrated to contain 18S rDNA (Imperador et al., 2020).

In terms of methodological approaches for studies on the VPA action in insects, injection rather than drug-feeding methods has been preferred to ameliorate seizure-sensitive phenotypes in several mutant genotypes of *Drosophila* (Howlett and Tanouye, 2013). In *T. castaneum* and *A. mellifera*, VPA has been furnished as a supplement on diet (Bingsohn et al., 2016; Rasmussen et al., 2021). In *T. infestans*, both injection and tissue culture revealed efficient to search for epigenetic changes following VPA treatment (Alvarenga et al., 2016; Bassani et al., 2021).

5. Conclusion

Epigenetics has gained significant attention from the scientific audience as it explains changes in gene expression without the modification in the genetic code of animal and plant species. Progress in this matter has been benefited from the use of drugs that act as epigenetic modulators. However, although Waddington's pioneering epigenetic studies initially referred to an insect (*D. melanogaster*) (Waddington, 2012), the investigation of epigenetic markers, interpretation of epigenetic mechanisms and use as alternative models for revealing responses to epigenetic modulators in another insect species is relatively recent. VPA, a drug widely prescribed for the treatment of neurological disorders as well as a promising antitumor agent and a potent epigenetic modulator may reveal as an interesting candidate for unravel new pathway mechanisms of drug effects, including those of pharmacological interest, when administered to insects. Using insects to evaluate VPA actions may allow tracking effects on complex fitness parameters and the expression of epigenetic regulatory genes, being a valuable early warning system for epigenetic risk factors that otherwise would be difficult to detect in mammals (Bingsohn et al., 2016). Many investigations that will bring new knowledge and possibly unexpected new information would certainly be developed and reported in forthcoming years.

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