Research report

Sodium valproate alters GnRH–GABA interactions during development in seizure-prone mice

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Abstract

During reproductive maturation, characteristic changes occur in the morphology of the gonadotropin releasing hormone (GnRH) cell population within the hypothalamus. In the early stages of development, GnRH neurons are bipolar cells; however, just before pubertal onset, the majority of these neurons transform into unipolar cells. Our laboratory has reported that valproic acid (VPA), an antiepileptic medication that has previously been shown to slow the velocity of pubertal development in both humans and seizure-prone mice, is capable of delaying the normal process of GnRH morphological differentiation. As VPA is primarily believed to act via a GABAergic mechanism, the present study investigated potential influences of VPA on GnRH–GABA interactions within the medial preoptic area (mPOA) across pubertal development (experiment 1), as well as in adult animals (experiment 2). The results from experiment 1 revealed the expected drug effects on GnRH cell morphology. For VPA animals, there was a greater percentage of bipolar neurons at every time period except for the 24-day sample. Additionally, VPA animals had greater numbers of bipolar and unipolar GnRH neurons with GABA associations across all ages. However, experiment 2 showed a lack of drug effects on GnRH–GABA interactions in adulthood. These results suggest that VPA may delay GnRH cell morphological maturation by altering the density of GABAergic inputs to GnRH neurons. These inputs may normally play a role in timing the activation of the GnRH pulse generator. However, any neuroendocrine effects of VPA in adulthood are most likely due to the actions of VPA at another level of the hypothalamic-pituitary-gonadal axis. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Valproic acid (VPA, 2-propylpentanoic acid) is an extremely potent and widely prescribed anticonvulsant agent. Originally synthesized by Burton in 1882, VPA was later shown to have anticonvulsant properties in 1963 [31]. VPA was licensed for use in the United States as an anticonvulsant in 1978. Since that time, the use of VPA in the clinical population has increased dramatically, making it one of the most frequently prescribed agents for the treatment of generalized seizure disorders [26]. In recent years, VPA has also become increasingly popular as a mood stabilizer in the treatment of several affective disorders, including major depression [21] and bipolar disorder [30].

Despite its widespread use, the exact mechanism of action of VPA still remains relatively unclear. However, it is generally agreed upon that VPA acts to potentiate GABA-mediated postsynaptic inhibition within the CNS via some, as of yet, unknown cellular mechanism [20]. The first major hypothesis for the cellular action of VPA was made by Godin et al. [12], who proposed that VPA produces an increase in the level of gamma-aminobutyric acid (GABA) in the CNS. Since that time, several mechanisms have been postulated to explain the GABA-elevating effects produced by VPA, including inhibition of several degradative enzymes in the GABA shunt pathway; as well
as an increase in the activity of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the synthesis of GABA [10].

MacDonald and Bergey [28] proposed a second major hypothesis for VPA’s action, suggesting that VPA may be enhancing neuronal responsiveness to GABA. These investigators demonstrated that iontophoresed VPA was capable of augmenting postsynaptic inhibition mediated by iontophoresed GABA in cultured spinal cord neurons. Subsequently, Kerwin et al. [22] reported the ability of VPA to potentiate GABA responses in cortical neurons in vivo.

A third major hypothesis, which has received much less attention, suggests that VPA may have direct membrane-stabilizing effects on neurons. However, precisely how VPA exerts these effects continues to be controversial. Using isolated frog spinal cord, Hackman et al. [13] found that low concentrations of VPA hyperpolarize dorsal roots, while higher concentrations produce depolarizations. Thus, VPA may retard rapid neuronal firing in a manner that mimics the actions of endogenous GABA [10]. In addition to the GABA hypothesis, some evidence suggests that VPA may function to decrease excitatory amino acid and norepinephrine activity within the CNS [4,7].

VPA has been shown to suppress the secretion of several pituitary hormones, including growth hormone (GH), adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) [5,18,23,27]. Additionally, reports of various symptoms associated with decreased pituitary hormone release have been described in the clinical population. In adults, VPA administration has been shown to induce unresponsiveness to GnRH challenge, transient amenorrhea, polycystic ovarian syndrome, and hyperandrogenism [19,27,29].

Furthermore, in a subset of the juvenile clinical population, chronic treatment with VPA has been shown to elicit a retardation of pubertal development and skeletal growth [6]. Specifically, Cook et al. [6] first described the case of a 12-year-old girl who experienced an arrest of normal reproductive development during chronic treatment with VPA for complex partial seizures. Shortly after this observation, VPA treatment was discontinued and normal pubertal development and body growth resumed 2 months later. Taken together, all of this clinical evidence raises the possibility that chronic VPA administration may result in adverse effects on the neuroendocrine system, including disturbances of growth, sexual development, and fertility.

Although a large body of literature has accumulated on the adverse effects of chronic VPA administration on the neuroendocrine system, its mechanism and sites of action for inhibition of the reproductive axis remain to be clarified. In our laboratory, we have reported that VPA produces endocrine-related side effects in genetically seizure-prone mice, which correspond to those commonly observed in human patients with epilepsy. In particular, administration of VPA was found to delay both gonadal and skeletal maturation, and to alter serum FSH levels in inbred mice of the DBA/2J strain [35]. This finding has created an animal model that has proven to be useful in examining the effects of VPA on the endocrine system, and in particular, attempting to determine the precise site of VPA’s action on the hypothalamic-pituitary-gonadal (HPG) axis.

Reproductive maturation is an extremely complex process that is principally regulated by both central and peripheral mechanisms that control the development of the HPG axis. Although the exact mechanisms that drive this development remain to be fully elucidated, it is generally believed that the pulsatile release of gonadotropin releasing hormone (GnRH) from GnRH neurons within the hypothalamus plays a pivotal role in timing the onset of puberty [34,38].

In rodents, the majority of GnRH cell bodies are located within the medial preoptic area (mPOA) [38]. During normal sexual development, distinct morphological changes occur within the GnRH cell population prior to the onset of puberty. It has been widely reported that early in development, GnRH neurons are bipolar cells with smooth surfaces, however, just prior to puberty, many of these neurons transform into unipolar cells with irregular spine-like surfaces. Although the total number of GnRH cells does not change, the percentage of irregular, ‘spiny’ GnRH cells increases during reproductive development [34,38].

It has been suggested that these morphological changes associated with the pubertal development process are related to an increased function of the GnRH cell population, as development of the normal diurnal pattern of LH release [37], as well as responsiveness of the hypothalamic-pituitary axis to the positive feedback [1] have been found to coincide with the increase in the number of unipolar neurons in the preoptic region.

Many neurotransmitters and peptide neuromodulators have been implicated in the regulation of GnRH function. In particular, several lines of evidence provide support for an inhibitory role of GABA in the modulation of the activity of GnRH cells. Firstly, ultrastructural data indicate that GABAergic neurons establish symmetric (inhibitory) synapses directly onto GnRH neurons within the mPOA [25]. Additionally, administration of GABA and GABA agonists directly into the mPOA has been shown to reduce GnRH gene [2], suppress the release of LH from the anterior pituitary [24,33], and block the estrogen-induced LH surge that normally occurs at the time of proestrus [15]. More recently, infusion of GABA agonists into a superfusion medium containing nuclei from the mPOA has been found to directly decrease levels of GnRH secretion [11].

Although the role of mPOA GABA in the pubertal development of rodents has received less attention, work conducted in primates suggests that GABA exerts a powerful inhibition on the GnRH neurosecretory system during the prepubertal period [32]. Taking this evidence into consideration, it is likely that a decrease in tonic
GABA inhibition may be a key factor for the activation of the GnRH pulse generator and, thus, the onset of puberty.

The previously discussed animal model established by this laboratory, which mimics the clinical neuroendocrine abnormalities frequently associated with VPA treatment, was recently utilized in a preliminary morphological study exploring the effects of VPA on the normal development of the GnRH cell population within the mPOA [36]. Interestingly, Snyder and Badura [36] found that chronic administration of VPA in DBA/2J mice delays the typical pattern of GnRH cell morphological changes occurring during reproductive maturation. As the effects of VPA on GnRH cell morphology are correlated with a slowing of gonadal development, it is likely that this delayed maturation of GnRH cells within the mPOA may lead to diminished anterior pituitary activity. Such a mechanism might explain the reproductive maturational delays that are observed in some epileptic patients.

Thus, if it is assumed that these morphological changes are functional in nature, it appears that VPA may exert at least some of its inhibitory effects on the HPG axis at the level of the hypothalamic GnRH cell population. Taking into consideration both the inhibitory role of GABA as a neurochemical regulator of GnRH cells, as well as VPA’s proposed GABAergic mechanism of action, it is possible that VPA may mimic and/or elevate the activity of the GABAergic system to slow normal morphological maturational changes occurring within the GnRH neuronal population.

The goal of the present study was to expand upon the preliminary findings of Snyder and Badura [36] by examining the putative effects of VPA on GnRH–GABA interactions within the mPOA across pubertal development, as well as in reproductively mature adult animals. As these mice typically become reproductively mature around 30–45 days of age, the addition of adult animals to this study allows for an examination of any residual effects of VPA treatment on the GnRH cell population after, under normal circumstances, reproductive maturity should have been reached.

This investigation was accomplished via a dual-labeling immunocytochemical technique for both GnRH and GABA. By using light level microscopy with immunohistochemical visualization techniques, this study allowed any significant gross morphological changes occurring in the relationship between these two cell groups as a result of VPA administration to be detected.

2. Methods

2.1. Animals and housing

Male inbred DBA/2J mice were obtained from a breeding colony at the University at Buffalo, which was originally derived from progenitors acquired from the Jackson laboratory (Bar Harbor, ME). Animals were group housed (n=4/cage) in polypropylene cages and had access to food (Agway Lab Chow 3000) and water ad libitum. All animals were maintained from birth in a colony room equipped with a controlled photoperiod (12 L:12 D, with lights off at 18:00 h). All procedures were approved by the Institutional Animal Care and Use Committee at the University at Buffalo prior to implementation.

2.2. Drug administration

Juvenile male DBA/2J mice were weaned at 14 days of age and were placed either on oral administration of VPA (17 mg/kg/day) or control vehicle solution (CON). Since VPA is highly water-soluble and is rapidly absorbed from the digestive tract [3], it was administered via the drinking water to which 1% sucrose was added to increase palatability. This dosage of VPA was calculated to correlate with therapeutically relevant serum concentrations of VPA when used as an anticonvulsant in the clinical population [3]. Where appropriate, drug dosage was increased in 2-week steps in order to account for developmental differences in body weight, as well as tolerance effects. Previous work from this laboratory has revealed that, under these conditions, individual mice consume 6–8 ml of fluid/day and there are no differences in fluid consumption between the VPA and CON groups [35].

2.3. Tissue preparation

Subsets of animals (n=5–9/age group) receiving VPA or control solution were sacrificed via anesthetic overdose with sodium pentobarbital (100 mg/kg). Animals were either sacrificed at 21, 24, 28, or 32 days of age for the developmental portion of the study (experiment 1), or following 6 weeks of drug administration for the adult (8 weeks of age) portion of the study (experiment 2). The animals were then administered 0.1 ml of heparin and perfused transcardially with 0.87% physiological saline, followed by 4% paraformaldehyde in 0.1 M NaPO₄ buffer (pH 7.3) using a perfusion pump. Brains were removed and post-fixed in the same fixative overnight. Tissue was then cryoprotected with 4% paraformaldehyde, 20% sucrose (pH 7.3) for 2 days prior to frozen-sectioning (40 μm) through the preoptic area on a sliding microtome with a freezing stage. All sections were stored in 0.1 M NaPO₄ buffer (pH 7.3) until immunocytochemical staining for GnRH and GABA.

2.4. Dual-labeling immunocytochemistry

All reactions were carried out with free-floating sections on an orbital shaker table. Sections were treated for sites of GnRH and GABA immunoreactivity in sequential order. In between each incubation step, sections were rinsed three
averaged for all statistical analyses. Every sampling age (\( P \))

Ratings from the two experimenters were combined and had more bipolar neurons with GABA associations at

study were analyzed separately. For the developmental age (\( F_{1,37} = 32.972, P<0.001 \)), but no main effect of age on the total number of bipolar neurons among groups. Follow-up analyses revealed that the VPA-treated group had more bipolar neurons than the CON group at every age except the 24-day sample (\( P<0.001; \) Fig. 1). However, for the total number of unipolar neurons, there were no significant differences between groups at any time point (Fig. 2).

There were also no significant differences at any time point for the percentage (of total) of bipolar neurons among groups (Fig. 3). However, for the percentage (of total) of unipolar neurons, there was a significant main effect of drug (\( F_{1,37} = 53.958, P<0.001 \)), but no main effect of age. Follow-up comparisons revealed that the percentage of unipolar neurons was greater for the CON group at all time points except for the 24-day period (\( P<0.001; \) Fig. 4).

There was a significant main effect of drug (\( F_{1,37} = 34.758, P<0.001 \)), but no main effect of age, on the mean number of bipolar neurons with GABA associations. Follow-up analyses revealed that the VPA-treated group had more bipolar neurons with GABA associations at every sampling age (\( P<0.001; \) Fig. 5). Furthermore, for the mean number of unipolar neurons with GABA associations, there was also a significant main effect of drug (\( F_{1,37} = 50.362, P<0.001 \)), but no main effect of age. Follow-up comparisons indicated that the mean number of unipolar neurons with GABA associations was significantly greater for the VPA-treated group at every sampling age (\( P<0.001; \) Fig. 6).

3. Results

The total number of GnRH-immunoreactive neurons did not differ significantly among drug or age groups in either experiment 1 or experiment 2. Stained neurons were divided into unipolar and bipolar groups based upon their morphology. The processes of these neurons were followed through several planes of focus in order to ensure that they extended out from the cell bodies of the neurons of interest and were not part of other GnRH neurons located in proximity to them.

3.1. Experiment 1: effects of VPA on GnRH–GABA interactions across development

There was a significant main effect of drug (\( F_{1,37} = 32.972, P<0.001 \)), but no main effect of age on the total number of bipolar neurons among groups. Follow-up analyses revealed that the VPA-treated group had more bipolar neurons than the CON group at every age except the 24-day sample (\( P<0.001; \) Fig. 1). However, for the total number of unipolar neurons, there were no significant differences between groups at any time point (Fig. 2).

There were also no significant differences at any time point for the percentage (of total) of bipolar neurons among groups (Fig. 3). However, for the percentage (of total) of unipolar neurons, there was a significant main effect of drug (\( F_{1,37} = 53.958, P<0.001 \)), but no main effect of age. Follow-up comparisons revealed that the percentage of unipolar neurons was greater for the CON group at all time points except for the 24-day period (\( P<0.001; \) Fig. 4).

There was a significant main effect of drug (\( F_{1,37} = 34.758, P<0.001 \)), but no main effect of age, on the mean number of bipolar neurons with GABA associations. Follow-up analyses revealed that the VPA-treated group had more bipolar neurons with GABA associations at every sampling age (\( P<0.001; \) Fig. 5). Furthermore, for the mean number of unipolar neurons with GABA associations, there was also a significant main effect of drug (\( F_{1,37} = 50.362, P<0.001 \)), but no main effect of age. Follow-up comparisons indicated that the mean number of unipolar neurons with GABA associations was significantly greater for the VPA-treated group at every sampling age (\( P<0.001; \) Fig. 6).

2.5. Histological evaluation

Two representative sections from each animal were subjected to histological examination with an Olympus BH-40 microscope. The sections chosen for analysis extended in 40-\( \mu \)m increments beginning rostrally at the decussation of the anterior commissure. The total number of neurons immunoreactive for GnRH was counted for each section, and the percentages (of total) of unipolar and bipolar neurons were determined for the analyses. Additionally, the total number of unipolar and bipolar neurons having GABA associations was determined. For the purpose of this study, a GnRH-immunoreactive neuron was defined as having a GABA association if at least one GABA-immunoreactive process was found to cross over its cell body and/or one of its processes within the same plane of section. All ratings were conducted by two experimenters who were blind to the treatment condition of each animal (inter-rater reliability was high, \( r=0.92 \)). Ratings from the two experimenters were combined and averaged for all statistical analyses.

2.6. Statistical analyses

The immunocytochemical data for the developmental (experiment 1) and adult portions (experiment 2) of the study were analyzed separately. For the developmental data obtained in experiment 1, a two-way (drug×age) analysis of variance (ANOVA) was utilized. For the adult data obtained in experiment 2, a one-way ANOVA for drug effects was used. Significant interactions were broken down using analysis of simple main effects, with Fisher’s LSD post-hoc tests where appropriate. All results were considered significant if \( P<0.05 \).
3.2. Experiment 2: effects of VPA on GnRH–GABA interactions in adulthood

There was no significant effect of drug on the total number of bipolar neurons between the two adult treatment groups (Table 1). Likewise, there was no significant effect of drug on the total number of unipolar neurons (Table 1). Additionally, there were no significant differences between the two treatment groups on the mean number of bipolar or unipolar neurons with GABA associations (Table 2).
4. Discussion

As expected, the total number of GnRH-immunoreactive neurons did not change across development. This finding is consistent with previously reported work on the development of the GnRH neurosecretory system during the maturational process [34,38], as well as with the morphological study previously conducted in this laboratory [36]. Additionally, the results obtained from this study add further support to the previous tenet proposed by Snyder.
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and Badura [35,36] that administration of VPA delays normal GnRH cell morphological differentiation. VPA-treated animals had significantly greater numbers of bipolar GnRH-immunoreactive neurons than CON animals at every time period except for the 24-day sampling period. Likewise, the percentage of unipolar GnRH-immunoreactive neurons was greater for the CON group at every sampling period except for 24 days of age. If these morphological changes are, in fact, functional in preparing the GnRH pulse generator for the onset of puberty, then any alteration might explain the previously reported delays in reproductive development induced by chronic VPA administration in DBA/2J mice [26,35].

In particular, VPA-treated animals were found to have more bipolar and unipolar GnRH-immunoreactive neurons with GABA associations when compared with age-matched controls at all sampling ages utilized in the present study. Thus, it appears that VPA may elicit a delay in GnRH cell morphological maturation by acting on GABAergic cells that provide direct input to GnRH neurons located within the hypothalamus. However, with a lack of evidence for changes in the density of GnRH-GABA associations throughout development, it is still unclear precisely how VPA exerts these effects.

Although the sites and mechanisms of VPA’s action remain to be fully elucidated, it is generally accepted that VPA produces an increase in the level of GABA in the brain, thereby, augmenting GABA-mediated postsynaptic inhibition. Pilot work from this laboratory indicates that VPA does increase GABA release in the mPOA of male DBA/2J mice [9], thereby, suggesting that VPA’s inhibitory actions on GnRH cell development may be mediated by enhancing extracellular GABA concentrations within the mPOA.

Several mechanisms have been postulated to explain the GABA-elevating effects produced by VPA, including inhibition of several degradative enzymes in the GABA shunt pathway, as well as an increase in the activity of GAD, the rate-limiting enzyme in the synthesis of GABA [10]. In particular, Harvey et al. [14] have shown that VPA is, in fact, capable of inhibiting GABA aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSAD). These two mitochondrial enzymes are necessary for the intracellular degradation of GABA. In this way, VPA could act to increase the amount of GABA available for release, which could subsequently lead to an increase in overall GABA release.

Given this evidence for at least one of VPA’s potential mechanisms of action, it seems unlikely that, by simply increasing GABA synthesis and release, VPA could induce such a significant increase in the overall number of GABAergic inputs to GnRH neurons as was observed in this study. However, one theory can be postulated to explain the robust nature of its effects on GnRH–GABA associations throughout development. In particular, by increasing GABA synthesis and release, VPA may have
maintained GABAergic contacts with GnRH neurons that were already present prior to the start of drug treatment.

Although a decline in GABAergic inputs was not observed across developmental age, it remains possible that this decline could normally begin at an earlier age than was examined in the present study (i.e. earlier than 21 days of age). This rapid early decline of GABAergic inputs to GnRH cells is quite possible, as most synapse elimination has been shown to occur very early in postnatal life [26]. In other words, CON animals may have had higher numbers of GnRH–GABA associations at an earlier age group than was assessed in the present study, at which time they would have been no different from VPA-treated animals on this variable. But sometime between 14 (the initiation of drug treatment) and 21 days of age, the density of these GABAergic inputs in the CON group may have begun to decline rapidly, reaching somewhat of a stabilization around 21 days of age.

It is generally believed that mPOA GABA levels are higher prior to the onset of puberty in order to maintain tonic inhibition on the GnRH cell group [8,32]. If this is presumed to be true, then a greater number of GnRH–GABA interactions early in postnatal life could result from higher levels of activity in the GABAergic cell population. This activity increase could elevate extracellular GABA levels, thereby, augmenting the degree of inhibition on GnRH neurons. Likewise, a rapid decline in GABAergic inputs with increasing developmental age might represent a normal form of ‘plasticity’ that exists within the hypothalamic-pituitary axis. In this manner, the HPG axis could prepare for the eventual activation of the GnRH pulse generator, and the consequent onset of puberty.

VPA may be capable of interfering with this ‘plasticity’ by maintaining high levels of activity in the GABAergic cell group. In accordance with the activity-dependent phenomenon of synapse elimination, these high activity levels could strengthen the connections between GABAergic and GnRH neurons. This would then result in the stabilization and maintenance of a large number of GnRH–GABA associations throughout reproductive development, thereby, explaining the results obtained from experiment 1 of the present study. By maintaining an increased number of GnRH–GABA interactions within the mPOA during development, VPA could enhance GABA-mediated inhibition on GnRH neurons, thus, delaying their differentiation, the activation of the GnRH pulse generator, and the eventual onset of puberty.

Finally, the data obtained from experiment 2 of this study indicate that, by 8 weeks of age (following 6 weeks of drug treatment), there was no longer any effect of VPA on either the number of bipolar or unipolar GnRH neurons within the mPOA. These results were as expected based on the preliminary study using this animal model, in which VPA-treated animals no longer differed from their control counterparts on measures of endocrine or gonadal status at this age [35]. In addition, there was no effect of drug on the mean number of bipolar or unipolar neurons with GABA associations. These results were also expected and provided support for the final hypothesis, which stated that any VPA-induced increase in the density of GnRH–GABA associations might no longer be present in reproductively mature adult animals. Thus, it appears that VPA’s effects on the GnRH neurosecretory system may only act to delay reproductive development in DBA/2J mice, but does not stop it completely.

Although VPA is capable of increasing the number of GnRH–GABA associations during development via the mechanism proposed above, it may be possible that this effect diminishes over time. The VPA-induced increase in GABAergic activity might decline in response to an increase in stimulatory inputs (including NE and gluta-mate) to the GnRH cell group. In particular, it has previously been mentioned that NE decreases mPOA GABA release both in vitro and in vivo [16,17]. Thus, given enough time, these stimulatory inputs might override the increased GABAergic inhibition on the GnRH cell group induced by VPA, thereby, resulting in normal GnRH cell differentiation, activation of the GnRH pulse generator, and the onset of reproductive maturity. This would explain the fact that, by 8 weeks of age, VPA-treated animals no longer differed from CON animals on either endocrine or gonadal measures.

However, in the clinical population, various neuroendocrine abnormalities are still reported in adults [17,27,29]. Although these side effects are somewhat less severe than those observed in juvenile patients, they are still common. Based on the findings from this study, it appears that the side effects observed in adult epileptic patients might be due to the actions of VPA at yet another level of the HPG axis, possibly at the level of the anterior pituitary itself.

In conclusion, this series of experiments not only provides further knowledge regarding VPA’s actions at the level of GnRH neurons within the mPOA during development, but it also suggests that VPA has yet another potential mechanism of action on the HPG axis, by which it exerts its commonly reported neuroendocrine side effects in adulthood. Future investigations should focus on attempting to elucidate this, as of yet, unknown mechanism of action.

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