Research Article

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Valproic Acid Accelerates Neural Outgrowth during Dorsal Root Ganglia Neurogenesis In Vitro

Wiens D*, Bergan H, Walter OS, McGinley A and Ahlrichs B

Department of Biology, University of Northern Iowa, Cedar Falls, Iowa, USA

***Correspondence:** Darrell Wiens, Department of Biology, University of Northern Iowa, Cedar Falls, IA 50614-0421, USA, Tel: 319 273 6880; Fax: 319 273 7125; E-mail: wiens@uni.edu

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Abstract

Background: Valproic acid (VPA) is an anti-convulsant drug used to treat seizures and a variety of neural pathologies. Studies have shown that VPA exposure in rodent embryos leads to behavioral characteristics similar to those in humans with autism spectrum disorder (ASD). Utilizing this rodent model of ASD, research has led to a recognized mechanism of action of VPA involving brain overgrowth and hyperconnectivity, likely caused by epigenetic alteration of gene expression through inhibition of histone deacetylases.

Objective: To gain further insight concerning this mechanism we modeled the development of neural connectivity at the cellular level.

Method: We cultured dorsal root ganglia (DRGs) taken from eight-day old chick embryos in a range of VPA concentrations and investigated aspects of neuronal structure and behavior. DRGs were cultured 48 hours, fixed, and immunostained to reveal the locations of neural networks with synaptic vesicles.

Results: We found a concentration-dependant relationship with a significant increase in neurite length in VPA concentrations of 1 and 2 mM, and the effect was still present though weaker at 4 and 6 mM. Trichostatin A (TSA), another histone deacetylase inhibitor, caused similar responses. To further characterize the effects, we carried out time-lapse imaging of growth cones of extending neurites. We found that VPA increased the area-changing activity of growth cones, augmenting their exploratory capabilities, along with significantly enhancing overall advancement, thus increasing the ability to extend and form synapses. The average total of stained synaptic areas surrounding each cultured DRG was significantly increased in 6 mM VPA, but not significantly at the lower concentrations compared to controls.

Conclusion: Our results show that VPA, at 1 mM and higher concentrations increases growth cone activity, and increases the number of neurites and their extension, a neurotrophic effect. It also increases synaptogenesis at 6 mM, supporting the theory of developmental neuronal overgrowth.

Keywords: Neurogenesis, Valproic acid, Dorsal root ganglia, Chick embryo

Abbreviations: ASD: Autism Spectrum Disorder; BDNF: Brain-Derived Neurotrophic Factor; DIC: Differential Interference Contrast; DRG: Dorsal Root Ganglia; HDAC: Histone Deacetylase; NMDA: N-Methyl-D-Aspartate; TSA: Trichostatin A; VPA: Valproic Acid

Introduction

The symptoms of autism spectrum disorder (ASD) can

be observed following exposure to the anti-seizure drug VPA. Autism is a neurodevelopmental disorder typified by deficiencies in social interaction and communication,

repetitive speech and behaviors, oversensitivity to changes, and dependence on strict routines and schedules. It is one of an assorted collection of disorders (ASD) with overlapping symptoms but distinctive courses, patterns and function levels [1]. The incidence of ASD has shown alarming increase since around 1990. According to data from the United States Center for Disease Control and Prevention [2] the incidence rate was one in 150 births in 2002, one in 68 births in 2010, and had climbed to one in 59 by 2014. Within this data, the CDCP reported that ASD is about four times more common among boys than among girls [2]. This represents enormous costs for parents and for social service and educational systems, and because there is no cure or diagnostic test, ASD is a rapidly growing public health issue. The alarming increase in incidence of ASD is partly explained by improved recognition and awareness, by newer diagnostic criteria changing the definition, and by screening at an earlier age in some areas, however these do not likely account for all of the increase. ASD is known to have a heritable component [3], and environmental agents or conditions *in utero* such as heavy metal exposure, maternal infection, folic acid supplementation, vitamin D metabolism defects, or epigenetic effects have all been implicated in causing it [4-7].

One environmental agent known to cause ASD-like symptoms is the widely used anti-convulsant medicine valproic acid (VPA). Chemically a short fatty acid chain with known teratogenicity, the drug transfers across the placenta and has been shown to concentrate in fetal serum up to five times its level in maternal serum [8]. VPA use is associated with an increased risk for neural tube defects, cardiac anomalies, facial clefts, and limb defects, and there is a clinically recognized set of facial malformations that accompanies these defects, first described as fetal valproate syndrome by DiLiberti et al. [9]. Along with these physical symptoms there is often neurological impairment ranging from reduced verbal intelligence to mental retardation [8]. In 2013, published reports provided evidence that associates prenatal VPA exposure to an increased risk for ASD [10,11]. A substantial body of research with VPA and animals has now accumulated that has introduced and established a VPA rodent model of ASD [12-15]. These studies have illustrated the strong similarities of the symptoms shown by rodents exposed prenatally to VPA to those in humans diagnosed with ASD, and have led to greater understanding at the cellular level. Bringas et al. [16] showed that in rats prenatally exposed to VPA exploratory behavior was altered compared to control rats, and that this was accompanied by changes

in the brain's limbic system region dendritic morphology. Their findings are consistent with a local excitatory over-connectivity hypothesis or "intense world" theory of the neurobiology of ASD that proposes hyperactivity in local neural circuits [17,18].

There is considerable evidence that VPA exerts its effects during brain development through epigenetic regulation. VPA is an inhibitor of histone deacetylases (HDACs), inhibiting both HDAC1 and HDAC2 of the CNS [19-21]. These two HDACs are required for brain development by acting as transcriptional co-repressors, silencing specific growth-inhibitory genes [22,23]. Unexpectedly, VPA inhibits HDAC1 by interfering with its active site but inhibits HDAC2 by promoting degradation of HDAC2 on proteasomes. Thus it can have effects that promote multiple aspects of neural overgrowth [24,25]. Blocking HDACs can lead to synaptogenesis, excitatory synapse maturation, increased neurite growth, and neural proliferation; and VPA is known to promote neural proliferation and increase neurite growth (lithium and VPA selectively activate promotor IV of BDNF in neurons [26]). That inhibition of HDACs by VPA can induce neural differentiation has been demonstrated in adult rat hippocampal neural progenitor cells as well, even inhibiting astrocyte and oligodendrocyte differentiation while inducing neural differentiation [27]. A principle target of VPA's epigenetic effects appears to be through the activation of the mammalian target of rapamycin (mTOR) or PI-3K/Akt/mTOR signaling pathway. Using neural stem cells Zhang et al. [28] showed that mTOR activation promoted and was also necessary for differentiation of the cells as neurons, and that this activation involved methylation of the neural regulatory factor neurogenin 1, an epigenetic action. Interestingly, reduced AKT/mTOR signaling has been implicated in idiopathic autism in humans as well as in rats exposed to VPA [29]. Furthermore, Qin et al. [30] demonstrated in the rat model of autism that VPA causes sequential activation of Wnt and mTOR signaling, and that suppression of the Wnt signaling relieved autistic-like behaviors in the rats partially by deactivating the mTOR pathway.

The epigenetic effects of VPA on these signaling pathways may involve promotion of growth and survival as well as differentiation, and all of these effects are likely mediated by regulation of specific gene expression. Evidence for upregulation of BDNF [26] and neurogenin 1 [28] are two examples. Another compelling example is the activation of promotor activity for the Oct4 gene in induced pluripotent stem cells by VPA. This activation

was shown to be highly specific for VPA, to occur via the PI-3/Akt/mTOR pathway, to target the proximal hormone response element in the promotor, and to involve nuclear receptors [31]. There is evidence for the effects of VPA and other agents that affect epigenetic regulation on many other genes affecting other signaling pathways to induce and regulate neurite formation [32,33].

Moreover, VPA is known to affect neural development in non-rodent vertebrates as well. *Xenopus laevis* tadpole brain development, behavioral characteristics, and neuronal circuit excitability and connectivity have been shown to respond to VPA. In an investigation of the excitability and level of connectivity in tadpoles exposed to VPA, an increase in neural connections resulted in a hyper-connected network of neurons throughout the brains with heightened excitability [34], supporting the “intense world” theory. In Zebrafish adult optic tectum cells, VPA has been shown to upregulate *Notch* signaling to reduce cell proliferation via the inhibition of HDAC activity [35].

The direct influences of VPA on embryonic neurons as they form neurites with growth cones, extend in length, and establish synapses, have not been elucidated. One model for neurogenesis in which neurites led by growth cones can be observed and measured as they extend and form synaptic networks is the neurotrophin-mediated development of dorsal root ganglia (DRG) in the chick embryo *in vitro* [36-41]. After they become established from neural crest cells during early development, DRG bipolar neuroblasts begin to innervate the spinal cord and establish sensory receptor dendrites from day six of development onward. Neurogenesis processes are then occurring in both DRG and spinal cord, as well as in the brain during this time. In culture, ganglion neurons advance neurites outward across the surface and these interact to establish synaptic communication and networks. To gain further insight, we treated some cultures with another inhibitor of histone deacetylases, TSA. TSA inhibits HDACs 1, 3, 4, 6, and 10 [42]. Thus it would be expected to diminish activity of HDAC 1, as does VPA, but not HDAC 2, and to diminish the additional HDACs 3, 4, 6 and 10. Therefore, unless HDAC 2 is key, TSA is predicted to exhibit similar but perhaps stronger neurogenesis than VPA, and this would confirm HDAC inhibition as the general mechanism.

The alteration by VPA of multiple signaling pathways and expressed genes via inhibition of HDACs has been well elucidated, and the elaboration of this approach

continues. In this study we focus on the cellular motility behavior and developmental tissue construction. The study of cultured DRGs described here provides some evidence for a significant, dose-dependent stimulatory effect of the histone deacetylase inhibitor VPA on neurite outgrowth, extension, growth cone activity, and on the differentiation of synaptic vesicle exhibiting networks.

Materials and Methods

Dorsal root ganglia culture

Chicken eggs (obtained from Mixdorf Farms, Waterloo, Iowa) were incubated at 38°C with approximately 80% humidity until day eight of development. DRG were excised from the lumbar-sacral region using microdissection tools (Minitool, Inc., Los Gatos, CA) and placed into Falcon Primaria 35 mm diameter culture dishes. The DRGs were washed in Earle's Balanced Salt Solution (Sigma-Aldrich) and then cultured in 2 mL of Medium 199 (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich or MP Biomedicals, Santa Ana, CA) and 1% antibiotic/antimycotic (CellnTec, Bern, Switzerland). VPA sodium salt (Sigma-Aldrich) was added from a 0.1 M stock solution in medium directly to dishes to final concentrations of 1.0, 2.0, 4.0 or 6.0 mM. In some experiments Trichostatin A (Tocris Bioscience, Bristol, UK) was added directly from the dishes from a 1.0 mM stock solution in DMSO to a concentration of 200 nM. The dishes were then incubated at 38°C with 5% CO₂ for 48 hours. Attachment was observed by 24 hours for all conditions, and those explants not attached were excluded from the experiment.

Immunocytochemical staining

Immunostaining to reveal localization of synaptic vesicles was begun after fixation at 48 hours of explant incubation. The explants were fixed in ice-cold 20% dimethyl sulfoxide in methanol for 60 minutes on ice. The explants were then washed in phosphate buffered saline (PBS) three times for two minute search. Following the washes, the explants were treated for ten minutes with peroxidase quenching solution (3% hydrogen peroxide in methanol). The cultures were next permeabilized with gentle agitation for 30 minutes in 0.1% Triton X-100 in PBS. Blocking solution (0.5% Tween-20, 2.5% bovine serum albumen, 0.5% nonfat dry milk in PBS) was then added and they were incubated for one hour with gentle agitation. Mouse monoclonal antibody SV2 (or 23.4-5 against TAG-1 in some experiments) secreted into culture supernatant (Developmental Studies of Hybridoma Bank, University of Iowa Department of Biological Sciences, Iowa City, IA) and diluted 1:50 in blocking solution, was introduced

and the dishes were incubated overnight at 4°C. The cultures were then warmed to ambient temperature with gentle agitation and washed as before three times in PBS. Biotinylated anti-mouse IgG from a Histostain SP kit (Invitrogen) was added to dishes for 20 minutes followed by washing and then streptavidin-peroxidase conjugate solution, also from the kit, for 15 minutes with gentle agitation. The chromagen mixture from the kit was next added to develop color in darkness for ten minutes. This was then removed and dishes were immediately rinsed with dH₂O to stop the reaction. The stained cultures were finally post-fixed in 3.7% paraformaldehyde solution at 4°C for eight minutes to stabilize the red color and they were rinsed and stored in dH₂O.

Microscopy and analysis

Following immunostaining the cultures were observed and images were captured using a Leica DMIRE-2 inverted microscope connected a Q-Imaging CCD camera (Surrey, BC, Canada) and *Image-Pro Premier* software (Media Cybernetics, Inc., Rockville, MD). To define a random sample of neurites extending outward from each DRG image, twelve radial lines originating from the center of the explant at 30° intervals were drawn over each image using *Image-Pro Premier* software and each neurite touching any of these twelve lines was enumerated and measured for length as previously described [40]. Statistical and graphic analyses were conducted using programming in the R language for statistical computing [43] or Microsoft Excel. When data with normal distribution were observed, significance was tested through one-way analysis of variance (ANOVA) and the Bonferroni correction was used as a *Post Hoc* test. When data were not distributed normally they were tested through the Wilcoxon rank sum test. For all tests a probability level of less than 5% was considered to indicate a significant difference.

To estimate the area of positive synaptic vesicle immunostaining around each explant, differential interference contrast (DIC) images at higher magnification were captured using the Leica DMIRE-2 inverted microscope connected to a Dell Optiplex 980 computer running *Image-Pro Premier* (Media Cybernetics) software. Multiple high-resolution .tiff images were assembled into tiled aggregates, which could be moved and magnified on screen. The synaptic stained areas were measured in square microns using the color thresholding feature in *ImageJ* software [44]. The thick three-dimensional area of the original explant was excluded from this analysis because of its high cellular density and impossibility of single plane focus. Before analysis, a border was carefully

traced in the image around the body of the explant where there was an apparent change of depth, and it was eliminated. Analysis of these areas included a correction for DRG explant size, and they were compared using the Wilcoxon rank sum test because the measurements were not normally distributed.

Time lapse analysis of neurite and growth cone activity

Time-lapse measurements of changes in growth cone area and neurite length were taken to determine the effect of VPA or trichostatin A (TSA) on the dynamic aspects of neurite outgrowth and synapse formation. Live cultures were imaged on the stage of a Leica DMIRE-2 inverted microscope on a heated stage at 38°C. DIC images of the selected region with clear growth cones were collected every minute for 30 minutes using the Q-Imaging camera and *Image-Pro Premier* software. After 30 minutes, 40 µL of 0.1 M VPA (resulting in 2.0 mM) or 0.4 µL of 1.0 mM TSA (200 nM) was added to the control dish and an additional 30 images were captured. The image files were assembled into a movie using *iMovie* software. Changes in exploratory behavior in the growth cones were observed and analyzed frame-by-frame using *ImageJ* software. The images were enhanced using the software to more clearly see the borders of the growth cones. Each growth cone perimeter was traced with the computer mouse and its area was calculated using calibrated measurements. The same approach was used for neurite length. Box plots were produced and P-values calculated using R language from the compiled data.

Results

Effect of VPA on neural differentiation

Within the first day of culture, the DRGs attached to the *Primaria* culture dish surface (if unattached they were excluded). Without delay cells migrated out from the explants to form a dense carpet, but cell density tapered at the outer edges to reveal flat migratory cells with irregular shapes (Figure 1). These flat cells did not stain with the neural/glial marker antibody 23.4-5 to TAG-1 protein [45,46], however the initial body of the attached DRG contained a majority of cells that did stain positively with the antibody to TAG-1 (Figure 1), identifying them as neural or glial. Furthermore, many cells that had migrated outward and were recumbent upon the flat and irregular cells appeared to be neurons and these did stain positively for TAG-1. Many of these cells had thin, straight processes that we identified as neurites, and usually the cell bodies were less flat and more rounded than the flat

migratory cells. In some areas there were networks of neurites interconnecting neurons and clusters of neurons (Figure 2).

Figure 1: Dorsal root ganglion developing in culture, differential interference contrast optics.

The images and all others are composites of 15-25 individual photomicrographs taken at high magnification and stitched together using Image-Pro Premier software.

- After nine hr in culture the DRG has attached and the earliest migrating cells are emerging from its edge in all directions. A short stub of nerve is seen on its right side.
- After 21 hr of culture the same DRG shows many more emigrated cells. Some are separate or associated with a few others in clusters, but many appear to be forming an epithelial pavement of cells expanding outward from the DRG's edge.
- At 33 hours the individuals, clusters, and sheet are much more expanded and the center of the DRG is undergoing some depletion of cells. Some of the emigrated cells are now elongated and

appear to be extending neurites.

- The edge of a different DRG immunostained with TAG-1 antibody after 48 hr in culture. The magnification is less than for the DRG in A, B, and C. Neural and glial cells positive for the antibody form a red-stained network of interconnecting neurites and nerves that resides upon a flat epithelium of non-staining cells.

Treatment of DRGs with VPA appreciably stimulated formation of neurites and neural networks (Figure 2). The numbers of neurites per explant as well as their lengths were clearly greater. We counted the number of neurites that were observed to cross any of the twelve radial lines drawn over each explant image from the explant's center (lines were drawn to generate a random sample) and found that exposure to 1-6 mM VPA increased the average number from sixteen to 23 or more, a significant change, with the greatest effect at 2 mM (Table 1). As we have noted previously with these cultures [38], the distribution of neurite lengths was not statistically normal because of radial density tapering. Therefore we used box plots showing medians, first and third quartiles, and maxima and minima to represent the samples (Figure 3). The effect of VPA was to stimulate neurite median length (when average lengths were calculated, they also showed the concentration-dependent stimulation). Because we placed multiple dissected DRGs (usually six) into each dish, the distances among them were several millimeters. In control dishes, cells migrating and neurites extending outward from the DRGs rarely could interact. But in VPA-treated dishes, the neurites and neural networks were so long that they occasionally interacted between DRGs (Figure 2). Average and median lengths of neurites in the random sample were also increased. Median length measured in controls and at four concentrations of VPA is displayed in figure 3. Compared to controls, the concentrations from 1-6 mM all significantly increased length, with the greatest increase seen, again, at 2 mM. We treated some DRGs with TSA to test whether the stimulation of neurite formation and length was because of epigenetic activity via inhibition of HDACs, or through some other mechanism. Its effect on number of neurites (Table 1) and on their length (Table 2) was similar to the effect of VPA.

Immunostaining the cultures with a monoclonal antibody (SV2) to synaptic vesicles revealed positive staining areas that were a peroxidase-generated red-brown color (a staining control dish with primary antibody omitted during the staining process showed only slight

discernible red-brown staining trapped within the elevated DRG explant center (Figure 4). The DRG explants bodies maintained some elevation above the culture surface because of their spherical shape, and showed a prominent level of immunostaining with heavily stained dots within, indicating many dense synaptic areas. In untreated (control) dishes there were plentiful regions of red staining peripheral to the explant, where extending neurites had made contact and had begun to create synaptic associations (Figure 2A). In dishes with explants treated with VPA, these were also plentiful and they did show somewhat more intensity and more extensive area coverage of immunostaining (Figure 2B).

Figure 2: Control and 2 mM VPA treated DRGs showing the stimulation of neurites and neural network formation.

Images are composites of high-resolution images taken with the 20X phase objective of the inverted microscope.

- Control DRG at 48 hr of culture. A peripheral layer of flat migratory cells surrounds the original DRG and numerous neurites are visible (short white arrows). These are beginning to form neural networks (nn) in some areas. Red staining with antibody SV-2 identifies synaptic vesicles and reveals synaptogenic areas (black arrows).
- VPA-treated culture (2 mM) at 48 hr with two DRGs interconnected by very long neurites (short white arrows) that have formed networks. Otherwise similar to controls, treated DRGs showed more neural networks created from much longer and more numerous neurites. However, antibody-positive areas did not appear to be more intense or more extensive.

Figure 3: Box plots showing neurite length in the absence or presence of four concentrations of VPA.

Box plots showing neurite length in the absence or presence of four concentrations of VPA. Because the lengths were not normally distributed, medians are represented with heavy lines, the first and third quartiles with bottom and top of boxes, and maxima and minima with whiskers. VPA stimulated the formation of neurites that were significantly longer than controls with the maximum effect at 2.0 mM. Significance was tested using the Wilcoxon rank sum test.

*Significantly greater than control, $P \leq 0.05$.

**Significantly greater than controls or other concentrations, $P \leq 0.05$.

Effect of VPA on synaptogenic differentiation

Following immunostaining with monoclonal antibody SV2, which binds to synaptic vesicles and their precursor molecular components, the areas in the culture dishes where differentiation of neurons had resulted in the production and assembly of synaptic vesicles were apparent. These areas showed red-brown staining as described above. Simple inspection suggested that the average number of these red areas was augmented by VPA treatment (Figure 4). However, these areas varied greatly in size and shape. In order to measure the extent of this differentiation, we used the color thresholding feature in the *ImageJ* software to measure the total amount of red-stained area around each explant where new synaptic areas became established (the thick three-dimensional area of the original explant was excluded as described in the materials and methods section). The software thresholding includes only image pixels that are color-defined, and then converts these to μm^2 . The median total synaptic area for each of ten explants is shown in figure 4D. The control was approximately 5,000 μm^2 , but the median for ten 6.0 mM VPA-treated explants

was 10,150 μm^2 . This difference was significant when tested using the Wilcoxon Rank Sum Test. Treatment at 1.0, 2.0, or 4.0 mM VPA also increased the measured synaptogenic areas, however these increases were not significant. Thus VPA did appear to have a stimulatory effect on synaptogenesis, however it was significant only at the highest concentration used in these experiments.

Figure 4: Composite differential interference contrast images of DRGs with monoclonal antibody SV2 immunostained synaptogenic areas.

Composite differential interference contrast images of DRGs with monoclonal antibody SV2 immunostained synaptogenic areas.

- DRG shows antibody-positive areas that appeared red-brown within the original explant at the center, often with a darker dotted pattern, and in the surrounding margin of spreading cell layers that were abundant with neurites and bundles of neurites.
- The same DRG explant shown in A, but with immunostaining digitally converted to bright red false color to enhance visibility. Synaptogenic networks are identical but more apparent.

- Antibody staining control DRG in which the primary antibody was omitted during the staining procedure.
- Box plot graph displaying the effect of VPA on the development of synaptogenic areas. The ImageJ color-thresholded red areas were measured in square microns after removing the original central DRG explant from the image. The total of antibody-positive areas around ten DRGs at each VPA concentration was averaged. The heavy line in each box represents the median, the bottom and top of each box represent first and third quartiles, and the bottom and top whiskers show minimum and maximum for the group of ten. VPA appeared to increase synaptogenesis somewhat, however only at 6.0 mM was the increase significant ($P \leq 0.05$, Wilcoxon rank sum test).

The effect of VPA on growth cone and neurite dynamic activity

In order to examine the effect of VPA on growth cone motile behavior, we examined untreated cultures of DRGs on the warmed (38°C) stage of an inverted microscope to locate neurites that had visible terminal growth cones. At one-minute intervals we captured images of these for thirty minutes and then added VPA to a concentration of 2.0 mM or TSA to a concentration of 200 nM. We then continued time-lapse image capture for thirty minutes. Analysis of these image sequences frame-by-frame using *ImageJ* software allowed measurements of neurite lengths and growth cone areas by tracing with the computer mouse. This data allowed us to calculate neurite length and growth cone area change with time. These results from many such analyses showed that there was a significant stimulation of growth cone area changing activity following the addition of VPA (Table 3). Prior to the addition the average (and also median) growth cone area was $5.7 \mu\text{m}^2$ and they showed an average area change of $0.78 \mu\text{m}^2$ per minute (median of $0.35 \mu\text{m}^2$ per minute). But after the addition of VPA to the dish at the 30-minute time point, the average growth cone area was $7.9 \mu\text{m}^2$ (median = $8.0 \mu\text{m}^2$) and they showed an average area change of $1.1 \mu\text{m}^2$ per minute (median = $0.5 \mu\text{m}^2$ per minute), a stimulation of dynamic activity of about 27%. TSA also stimulated dynamic activity in growth cones. The average area change per minute was increased from 0.25 to $0.82 \mu\text{m}^2$ per minute (median from 0.23 to 0.78), an increase of 70%.

Any growth cone advance that we observed in the time-lapse sequences was supported by neurites that clearly

displayed both extension and retraction during the period of image collection. These data are summarized in table 4. Although net advancement did increase somewhat after addition of VPA, the net advancement and net retraction were not changed significantly. To describe this behavior further however, we calculated neurite length changing (extension plus retraction) per minute, also during the thirty minutes before and after addition of VPA. This measure of dynamic length was increased significantly after addition of VPA (Table 4).

Table 1: The effects of VPA and TSA on number of neurites extending from cultured DRGs.

Treatment	Average Number	Median Number	Minimum and Maximum Number
None (control)	16	15	7, 29
VPA 1.0 mM	23	22.5	10, 36
VPA 2.0 mM	26 ^a	25	12, 42
VPA 4.0 mM	25 ^b	23.5	13, 35
VPA 6.0 mM	23 ^c	23	10, 30
TSA 200 nM	25	27	20, 40

^aSignificantly more than control, $p \leq 10^{-7}$.

^bSignificantly than control, $p \leq 0.002$.

^cSignificantly more than control, $p < .01$.

Table 2: The effect of TSA on neurite total length (μm).

	Average+SD	Median
Control n=13 DRGs	85+21	81
TSA-treated n=11 DRGs	117+36	100*

*Significantly longer than controls, $p \leq 0.01$.

The data for both area changing and length changing with time were skewed with much more frequency for smaller length changes. The data for these were not normally distributed then, and the Wilcoxon rank sum test was used to assess significance of differences in medians. In all thirteen experiments the area changing per minute of the growth cone was significantly increased by VPA, and overall, neurite length changing was as well. Similar experiments with TSA showed that neurite length change with time was not affected, but area changing was (data not shown). As with VPA, the increased activity was significantly greater ($p < 8.6 \times 10^{-5}$).

Table 3: The Effect of VPA on growth cone area and area change per minute (μm²).

	Average ± SD	Median
Growth Cone Area: Control	5.7 ± 1.3	5.7
Growth Cone Area: VPA-treated	7.9 ^a ± 1.6	8
Growth Cone Area Change: Control	0.78 ± 1.1	0.35
Growth Cone Area Change: VPA-treated	1.10 ^b ± 1.4	0.53

n = 390 growth cone area changes measured during 30 minutes from 13 DRGs.

^aSignificantly greater than control, $p \leq 0.003$.

^bSignificantly greater than control, $p \leq 3 \times 10^{-5}$.

Table 4: Neurite length dynamics before and after addition of VPA.

	Control	VPA
Net Advancement	20.6 μm	30.3 μm
Net Retraction	-21.5 μm	-21.4 μm
Average Length Change per minute	0.1 μm	0.3a μm
Median Length Change per minute	0.03 μm	0.27 μm

n = 390 neurite length changes measured during 30 minutes from 13 DRGs.

^aSignificantly more than control, $p \leq 0.02$.

Discussion

We have investigated six aspects of the cellular mechanism of action of the anti-seizure drug VPA on neural development in culture. Our results provide evidence that VPA stimulates the formation of more neurites with greater neurite length, having a maximum effect at 2 mM. Corroborating this neurotrophic effect, we found greater growth cone spread area, growth cone area changing and neurite length changing activity levels. We also found significant stimulation of the development of synaptogenic areas around the DRG cultures at 6.0 mM, and the data showed that there was an increase in median area at three lower concentrations, although this was not significant. In addition, we found quite similar responses evoked by alternate drug TSA, which also acts to inhibit histone deacetylases. We found that VPA concentration affected the neurite number formed per DRG and neurite length maximally at 2.0 mM, and that higher concentration had lesser effect, suggesting an

upper limit to the response. Omtzigt et al. [47] reported a concentration of VPA measured in maternal serum for 29 epileptic women taking 14.1 mg of VPA per kg of body weight daily to be 49 ± 24 mg/ml (average and standard deviation), and ranging from 2.0 to 108 mg/ml. These authors reported that the concentration of 13 metabolites of VPA were all much lower, from 0.0 to 2.7 mg/ml in serum, and that VPA in amniotic fluid was also considerably lower, ranging from 0.5 to 11.5 mg/ml. These values are in the range of 7.0 to 750 mM (0.75 mM). But they also found that when higher doses of VPA were used for women whose pregnancies were associated with neural tube defects, the serum concentrations were significantly higher. We determined VPA concentrations to use for our culture system empirically, arriving at 1-6 mM. These are higher than the concentrations found in human maternal serum reported by Omtzigt et al. [47]. However, VPA transfers across the placenta and has been shown to concentrate in fetal serum up to five times its level in maternal serum [8]. Thus our concentrations may well be similar to those that developing neurons in the fetal brain are experiencing.

As an autism-inducing agent (rodent model) VPA causes symptoms in the offspring of treated mothers that remarkably resemble the symptoms of ASD in children. Putatively, it does this epigenetically by inhibiting HDAC enzymes. These enzymes, in turn, are necessary for brain development because they assist in the repression of genes that inhibit neural growth [22,23]. By inhibiting HDACs, VPA exposure can lead to developmental brain overgrowth through stimulation of neural proliferation, neurite growth, greater synaptogenesis, excitatory synapse maturation [15,19,24], and even stimulation of higher levels of the NMDA receptor [48-50]. Collectively, this leads to developmental brain overgrowth in certain areas and certain hyper-connected neuronal networks ("intense world" theory [18,19,50]. Our results support the neurotrophic aspects of this mechanism, although our evidence of enhancement of synaptogenesis was not convincing unless the highest concentration was used. We observed that the median synaptogenic area surrounding ten DRG explants was more than twice as large as controls when treated with 6.0 mM VPA, but it was higher at 1.0 mM than at 2.0 or 4.0 mM, and the maximum values were all much higher than the median and quite variable. The minimum values were not so much lower than the medians and were not so variable. It is noteworthy that our analysis did not measure the intensity of immunostaining but rather the summed square microns of area above threshold. If intensity of

staining could be assessed from the images it might have revealed more consistency in synaptogenesis. However, it may be that the complex pathways that control the extension of neurites and the exploratory behavior of growth cones are repressed through regulation that is separable from that which controls the formation and maturation of synapses. Some HDACs and some of their regulated pathways could be selective or be differentially sensitive to VPA. Alternatively, it may be that the limitations of our system prevented detection of any significant effects on synaptogenesis. We found no evidence that synaptogenesis was inhibited in any way since treatment at all concentrations showed higher median areas than the untreated controls. Cultured DRGs have been used to study peripheral neuropathies and evaluate potential therapeutic compounds [51], for example diabetic neuropathy [52,53]. DRGs from rats have been used together with immunocytochemistry to assess the formation of nociceptive-like DRG neurons that form synapses with dorsal horn neurons. In this case synaptogenesis was studied after separately purifying the neurons and subculturing on astrocyte micro islands. However, to our knowledge, assessment of overall synaptogenesis in DRG explants in response to environmental agents has not been reported previously [54].

Logical candidates for VPA-mediated genetic upregulation in the neurons in the present study are the neurotrophins, including NGF, BDNF, NT-3 and NT-4. These are active in both CNS and PNS [55] to support survival, proliferation, synaptogenesis and neural development in general [56,57]. After binding their Trk family and p75 neurotrophin receptors, biological effects are mediated through activation of Ras, phosphatidylinositol 3-kinase, phospholipase C-g1, and the signaling pathways that these enzymes control [56,57]. BDNF, for example, acts to maintain sufficient populations of neural stem cells and neural precursor cells by promoting protective and inhibiting degradative pathways, and by stimulating their proliferation and differentiation [58,59]. BDNF may also promote their migration and ordering into layers [60] along with its control of the cytoskeleton to produce neurite outgrowth [56]. Although our data does not directly connect VPA to these neurotrophic actions, it is consistent with such a mechanism, and the presence and abundance of these neurotrophins and their receptors could be investigated using this culture system in the presence and absence of VPA and TSA. Evidence that VPA mediates neurite outgrowth in cultured neuroblastoma cells via activation of the c-Jun N-terminal kinase (JNK)

cascade that in turn phosphorylates the focal adhesion protein paxillin has also been demonstrated [61,62].

The image analysis data that we acquired using time-lapse images taken at one-minute intervals revealed a stimulatory effect of VPA on growth cone and neurite dynamic behavior within thirty minutes. This is a remarkably quick response that would seem to exceed the rate of typical developmental events. We have reported previously that chick embryo DRG growth cone and neurite behavior do show detectable inhibition caused by supplementing with folic acid within this time scale [40]. Although the mechanism of inhibition by folic acid is unknown, our evidence was consistent with a mechanism of biochemical competition. We have proposed that folic acid may compete with the neurotransmitter glutamic acid for binding to the NMDA receptor in both presynaptic [63] and postsynaptic membranes, and that this could lead to calcium ion fluxes that could affect neuronal development [41]. The hypothesis that dysregulation of activity-dependent signaling networks that are controlling synapse formation and maturation underlies the etiology of ASD has been discussed [64].

However, if VPA's stimulation of path-finding activity by growth cones occurs through inhibition of HDACs, then many subsequent steps would be required. It would lead to upregulation of neurotrophin(s), followed by activation of their ensuing signaling pathways to achieve cytoskeletal motility changes, among other things. Despite the presence of receptor sensitive mRNA translation systems in growth cones [65,66], stimulation of their responses via inhibition of HDACs would require some time. Based on measurements of muscle protein synthesis following strong resistance training, hours are required to achieve detectable elevation [67]. Thus the action of VPA on growth cone and neurite dynamic behavior may involve another mechanism. It has been reported that both VPA and lithium selectively activate promoter IV of BDNF in embryonic rat neurons in culture, and that the effect can be mediated via inhibition of glycogen synthase kinase-3 (GSK-3) as well as by inhibition of HDAC in cultured rat cortical neurons [24,26]. Because GSK-3 regulates an array of transcription factors and affects neurogenesis as well as mood stability in the adult brain [68], inhibition of it by VPA is a mechanism that could respond more quickly than inhibition via HDACs.

A limitation of this study is that the chick embryo DRG model system that we employed utilizes peripheral nervous system neurons developing in a non-human

species. It would be ideal to use human developing brain neurons in a tissue-replicating format. Yet human brain development is enormously complex and the neurons affected by VPA and involved in the etiology of ASD are unknown. Brain neurons derived from rodents, human cell lines, or even humans brain regions may not be a better model. Chick embryo DRGs in culture are a convenient, venerable and well-tested model in which basic and important aspects of neuronal behavior can be observed and measured with a level of understanding [36-41]. They model neurite extension, growth cone exploratory motility, synapse formation and other aspects of differentiation, all universal in neural development.

Conclusions

We have presented evidence that VPA stimulates the formation of more neurites with greater length, and has a maximum effect at 2 mM. Accompanying this neurotrophic effect, we found greater growth cone and neurite activity levels and a tendency for greater extension, augmenting the neurons' exploratory capabilities and thus the ability to extend and form synapses. The exploratory growth cone provides leading tractional force and determines direction by sensing via receptors and by continually adjusting its motility based on recognition of extracellular matrix binding sites and soluble factors in the environment. The activities of tubulin, actin, myosin and hundreds of their binding partners orchestrate these responses. The growth cone determines final synaptic innervation as it leads a dynamically extending neurite. This behavior constitutes the basis for neurogenesis. If the extraordinarily complex activity of this apparatus is accelerated, more contacts will be made. Differentiation to form synaptic vesicles will follow growth cone motility, and will become established where viable contacts are made. The creation of a regionally over-connected network would thus depend on growth cone behavior. Many opportunities for therapeutic intervention might be investigated by analyzing the growth cone as it responds to agents that are able to interact with it directly or indirectly. Our findings are consistent with a mechanism in which VPA acts to cause brain overgrowth in certain areas through its inhibition of HDACs (and probably also GSK-3) to increase neurotrophin expression and its consequent downstream signaling. The cellular basis of ASD may come to be understood better as research aims to clarify this mechanism further.

Declarations

Ethics approval and consent to participate

This work did not involve the use of any hatched or born vertebrate animals nor any human subjects. It therefore required neither ethics approval nor consent to participate.

Consent for publication

The authors declare their consent to the publisher for publication.

Availability of data and material

The SV2 monoclonal antibody, developed by K. M. Buckley, Harvard Medical School, was obtained from the Developmental Studies Hybridoma Bank, created by the NIHCD of the NIH and maintained at the Department of Biology at the University of Iowa, Iowa City, IA 52242.

Competing interests

The authors have no competing interests or ethical conflicts to disclose.

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Authors' contributions

H.B., O.S.W, A.M. and B.A. carried out the experiments and analyzed data. D.W. and H.B. designed the experiments. D.W. wrote the manuscript with contributions from H.B. and A.M.

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