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Effect of Valproic Acid on Morphology and Behavior of *Hydra viridissima*: Possibility of using *Hydra* for Screening Neuromodulators

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Abstract

Nervous system of *Hydra* responds to mechanical, chemical, light and temperature stimuli, despite lack of organizational complexities like ganglion or sense organs. Nerve net of *Hydra* shows remarkable similarities with vertebrates at cellular and molecular level. Chemical and electrical synapses of *Hydra* modulate array of behavioral responses exhibited by *Hydra*. We have assayed toxicity and tested effect of Valproic Acid (VPA), an anti-epileptic drug on *Hydra*. We have performed whole animal toxicity testing, cytological staining using toluidine blue and assayed feeding behavior post VPA treatment. We conclude mild toxicity and loss of cell organization pattern in tentacles of *Hydra* upon prolonged VPA exposure. Our results indicate the possibility of using invertebrates like *Hydra* for screening of chemical modulators of molecular pathways.

Keywords: Battery Cell Complex, Chloro *Hydra*, Cnidarians

1. Introduction

Hydra is a diploblastic cnidarian that displays complex behavioral responses to food and light using its preliminary nerve net. Body of *Hydra* has few small sized sensory and ganglionic neurons that are present in between ectodermal and endodermal layers of epithelial cells[1]. Nervous system of *Hydra* lacks organizational intricacies of vertebrates, however at cellular and molecular level remarkable similarities are observed. Ultra-structural, histological and biochemical evidences support presence of polarized chemical synapses in *Hydra*[2,3]. Remarkable sequence homologies are found in few neurotransmitter receptors between *Hydra* and vertebrates. Different antagonists and agonists of neurotransmitter receptors alter behavioral response in *Hydra*. This indicates functional role for neurotransmission related genes in *Hydra* genome[4-6].

The body of *Hydra* is divided into three parts. Mouth-including hypostome and tentacles, central body column and basal disc. The tentacles are involved in catching, paralyzing and bringing prey closer to mouth. Mouth ingests the prey. Central body column is a slender tube that contracts and elongates according to situation. Basal disc attaches to substratum providing support for *Hydra* against water current. *Hydra* can coordinate all its body parts and exhibit complex behavioral patterns like feeding response in presence of prey and phototaxis. *Hydra* is good experimental model as it is inexpensive to maintain. It is possible to image *Hydra* at cellular level, without sectioning due to its diploblastic nature[7,8]. *Hydra* can be used for studying toxicological properties at molecular, cellular and behavioral level[9,10]. It is not known whether *Hydra* can also be used as a model for testing neuro-pschycotic drugs.

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Amongst various neuromodulators used in mammals, Valproic Acid (VPA) has been tested for its effect on growth and regeneration in *Hydra*[11]. VPA is a widely used anti-epileptic drug that is also used to treat depression and bipolar disorder. Physicians administer VPA as prophylactic measures to treat migraine[12]. Overnight treatment with 0.1 mM VPA inhibits head regeneration in *Hydra*[13]. This report is in corroboration with developmental abnormalities and teratogenic effects of VPA treatment on mammalian systems. Whether similar concentration of VPA induces toxicity or alters behavioral responses of *Hydra* has not been studied. We report VPA induced toxicity at organismic level and cellular organization level in *Hydra viridissima*. The toxicity was statistically significant, but not lethal. We observed shortening of tentacle length and distorted organization of tentacular cell complexes in *Hydra viridissima* treated with 0.1 mM VPA. We also observed that these changes did not affect tentacle contraction during glutathione induced feeding behavior.

2. Methods

2.1 Maintenance of *Hydra*

Hydra viridissima (Green symbiotic *Hydra*) culture was kind gift from Prof. Ghaskadbi, ARI, Pune. *Hydra viridissima* were grown in glass bowls containing de-chlorinated water at a constant temperature of 22 + 1 ^{0}C with 12 hrs of day and night cycle. Light source was routine white tube light in culture room. Tap water was kept in open containers (bottle or tubs covered with muslin cloth) for 48 hrs. *Hydra* was fed daily with freshly hatched, thoroughly washed, Artemia. Approximately 10 artemia per polyp were used as feed. Alternate day feeding pattern was followed when maintaining a fairly constant number was required. *Hydra* was fed daily when increasing number of *Hydra* was required. Water was changed after two hrs of feeding[14].

2.2 Preparation of *Hydra* Feed

Artemia cysts were procured from local shop. They were maintained and allowed to hatch for 24 to 48 hrs in tap water containing (33 gm/L) sea salt with constant aeration. Hatched artemia were collected as they aggregate towards source of light. A muslin cloth was used as a strainer of artemia. Aggregated artemia washed with tap water to remove all the salt. The washed artemia were transferred from cloth to a beaker containing de-chlorinated water.

2.3 Microscopy

The polyp response and tentacle length of *Hydra* were recorded using Nikon SMZ1500 stereoscope. Feeding response of *Hydra* was quantified using Video recordings of *Hydra* behavior. The videos were recorded at 8 frames per second on Nikon SMZ1500A. Toluidine blue staining of the battery cell complex was observed using 20x and 40x objective of Zeiss Axioscope2 plus.

2.4 Toluidine Blue Staining

Hydra was fixed in 10% formalin overnight. After 3 Phosphate Buffered Saline (PBS) washes of 10 minutes each, *Hydra* were incubated with (0.05%) Toluidine blue (dissolved in 10 mM Tris-Cl) for 30 minutes. Quick washes of 50% and 60% alcohol were used to remove excess stain. Samples were Glycerol mounted and observed under microscope.

2.5 Feeding Behavior of *Hydra*

Mature polyps were transferred into a twenty-four well plate, at density of one polyp per well. 0.5 ml of fresh de-chlorinated water was added to each well. The plate was incubated at 22 $\pm$ 1 ^{0}C in culture room for 24 hrs. Plate was placed in moderately lit conditions underneath stereo microscope. *Hydra* was allowed to get accustomed to the light for 10 minutes. Each *Hydra* was sequentially and gently exposed to 3.2 uM of freshly prepared glutathione. A two minute video was recorded before adding glutathione and another 5 minute video was recorded after adding glutathione. Average tentacle spread before and after adding glutathione was calculated[15] for a total of 30 *Hydra* under each condition. Stock solutions and dilutions of 0.1 mM/0.5 mM VPA and 3.2 uM Glutathione were prepared and diluted in de-chlorinated water.

2.6 Image Analysis and Statistical Analysis

ImageJ 1.8.0_112 (scientific image analysis software) was used for length measurements and for calculating average tentacle spread. Median, standard deviation, quartiles,

averages and all statistical calculations and tests (ANOVA and T test) were performed using Microsoft excel (Office 2007). All graphs, box and whisker plots were plotted using Microsoft excel.

3. Results

3.1 VPA Induced Toxicity to Entire Body of *Hydra* Polyp

Effect of VPA on head regeneration has been demonstrated in *Hydra*. However, previous reports have not assessed toxicity of VPA on *Hydra*. *Hydra* is a well known model for assessment of ecotoxicity[16]. Potentially toxic chemical is added to de-chlorinated water or *Hydra* medium and alteration in phenotype of *Hydra* is recorded. Morphology of *Hydra* is altered in response to unfavorable environmental changes[9,10]. Altered morphology of *Hydra* can be grouped into 0 to 10 ascending categories, called *Hydra* Health Index (HHI) score[14] based on extent of damage caused by chemical under study. Representative HHI scores of 10, 8, 7 and 6 can be seen in Figure 1 A, B, C and D. We treated *Hydra viridissima* with 0.1 mM VPA for 24 hrs and 0.5 mM VPA for 3 hrs[11]. We assessed 30 *Hydra* under each condition on three separate days, using Nikon SMZ1500 stereoscope. *Hydra* showed extended tentacles and their body was highly reactive (Figure1.A). Median HHI of wild type *Hydra* was 10. Overnight exposure to 0.1 mM VPA hrs caused shortened tentacles and polyp size (Figure 1. C, D). We observed shortened tentacles in VPA treated *Hydra*. Average length of *Hydra* tentacles was reduced to half in 0.1 mM VPA treated *Hydra* (Figure 1.F). Median HHI for VPA treated *Hydra* was 7 and 8 (Figure 1. B, C, E). The HHI score of VPA treated *Hydra* was significantly low than wild type (Figure1.E). Morphological change caused in *Hydra* post VPA treatment was classified as reversible[9]. VPA dose was sub-lethal for *Hydra viridissima* at 0.1 mM (24 hrs) and 0.5 mM (3 hrs) concentrations. We went ahead with the further studies using the same dose as VPA did not appear lethal at this concentration. ANOVA followed by the student's t-test confirmed that the reduction in length and reduction in HHI, post VPA treatment was statistically significant (P<0.001) as Indicated (Figure 1).

The results indicate that VPA is causing changes in morphology of tentacles. Tentacles are involved in catching of prey. Accidental touch of swimming prey to *Hydra* tentacles induces feeding response in *Hydra*. Feeding response is a series of sequential behavioral responses including nematocysts discharge, wriggling of tentacles and opening of mouth[15,17,18]. Nematocytes are stinging capsules of *Hydra*. The organelle of Nematocytes that stings prey is called Nematocyst[19]. Wriggling tentacles physically bring prey close to mouth. Mouth rips open to ingest the prey. Chemical sensation by tentacles is essential for *Hydra* survival[17]. Tentacle ectoderm, which is essential for chemical sensation in *Hydra*, contains three types of cells: epithiliomuscular cells, nematocytes and neurons. We speculated that VPA treatment could alter tentacle morphology of *Hydra* at cellular level as well.

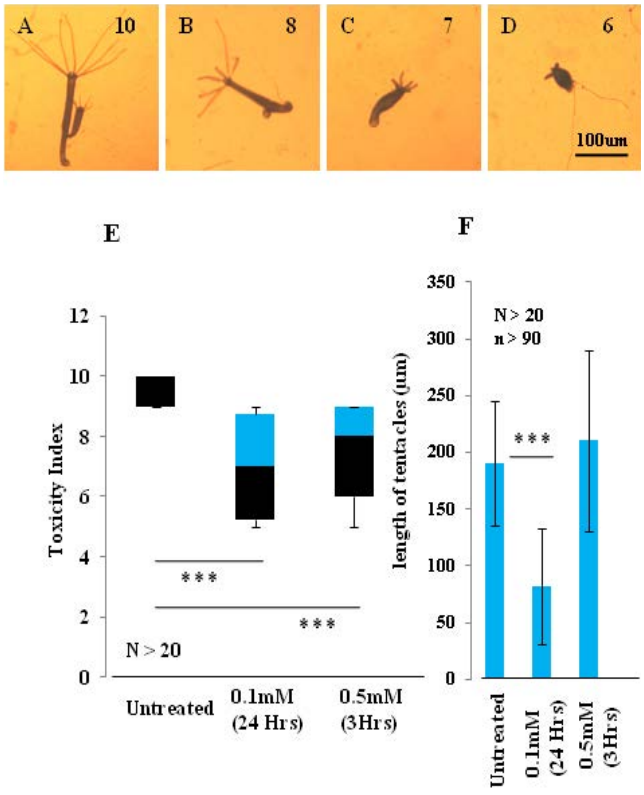


Figure 1. Long term exposure to 0.1 mM VPA or short term exposure to 0.5 mM VPA is toxic and not lethal to *Hydra viridissima*. A, B, C, D) *Hydra viridissima* of Wilby score 10, 8, 7 and 6 respectively. E) Toxicity testing of 0.1 mM VPA (24 hrs) and 0.5 mM VPA (3 hrs) treated *Hydra*. F). Average tentacle length post VPA treatment. Error bars represent standard deviation. Lengths of tentacles of 0.1 mM VPA treated *Hydra* are lower than that of untreated *Hydra*. ANNOVA was followed by unpaired t-test. ***indicates P< 0.001.

3.2 Effect of VPA on Organization of Battery Cell Complex in *Hydra*

Various cells in tentacles of *Hydra* are organized in clusters called battery cell complexes (BCC)[20,21]. Each BCC has different types of cells. At center of BBC is a stenotele bearing nematocyte, surrounded by isorhyza, desmoneme, sensory neurons and myoneme. Each cell has its own specialized function. Stenotele and desmoneme are two types of nematocysts. Stenoteles penetrate into prey and release neurotoxin. Isorhyza helps in attachment to substratum. Stimulation of desmonemal discharge of nematocyte causes contraction of tentacles and helps in the physical capture of prey. Thus, BCC is involved in perception and conduction of signals from environment, stinging prey, contraction of tentacles and capturing prey[22,23].

We performed Toluidine blue staining and BCC (Protocol with modifications from Murugadas et al., 2016) and reported that BCC organization was disrupted with VPA treatment. We observed that Toluidine blue staining of tentacles of *Hydra* shows bands of distinctly spaced battery cell complexes (Figure 2.A-A'). The pattern of banding is lost and a stenotele like structure surrounded by many other cells could not be visualized in prolonged VPA treated *Hydra*. Figure 2.B and B' show Toluidine blue staining of 0.1 mM VPA treated *Hydra*, that clearly shows disrupted organization of battery cell complexes.

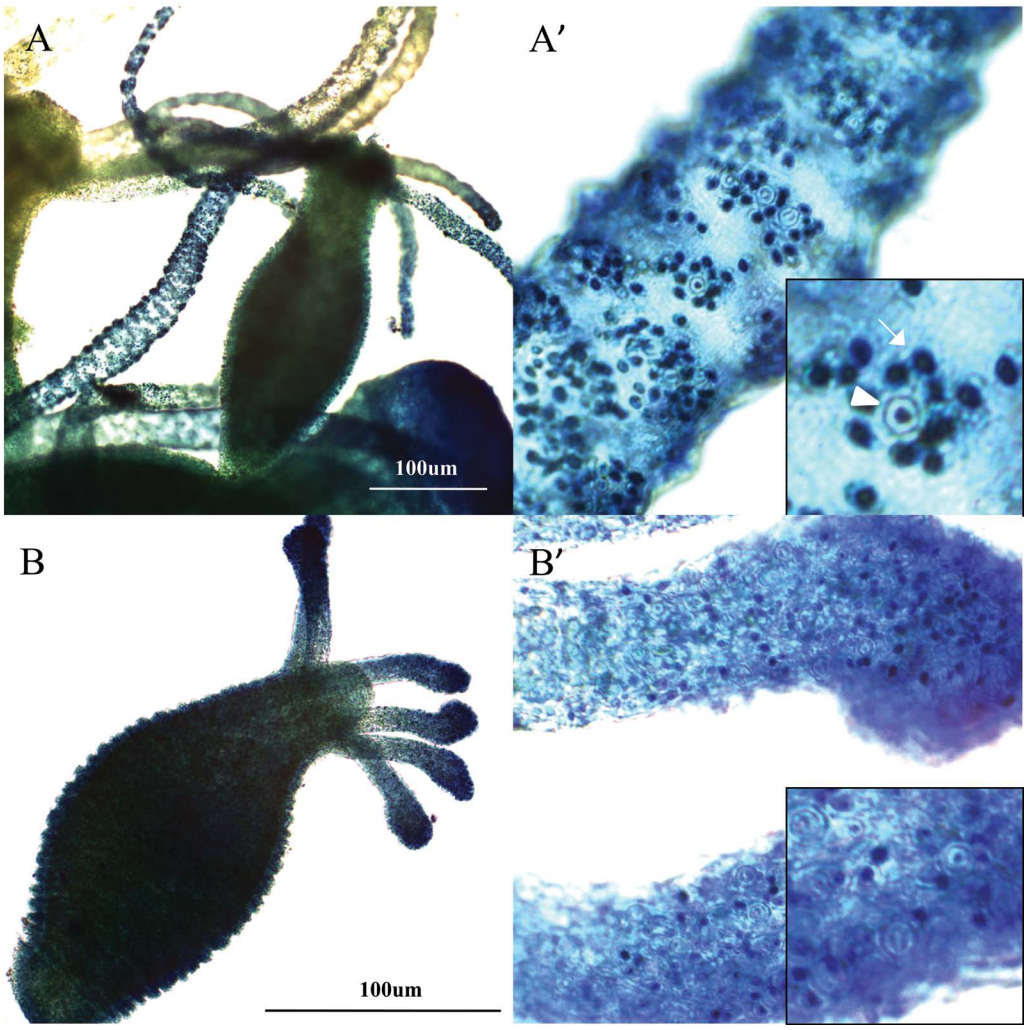


Figure 2. Organization of battery cell complex is perturbed post VPA treatment A) Concentric bands of battery cell complex stained with Toluidine blue in Wild Type. A') Magnified view of battery cell complex. Arrowhead marks cell that appears like Stenotele. Other types of cells surrounding stenotele are marked with arrow. B-B') Tentacles and battery cell complex in 0.1mM VPA (24 hrs) treated *Hydra*. N>20.

3.3 Effect of VPA on Feeding Behavior in *Hydra*

Battery cell complex plays important role in *Hydra* survival. In absence of well-organized battery cell complex, feeding response of *Hydra* could be adversely affected. Measuring extent of tentacle contractions in response to induced feeding behavior in wild type and VPA treated *Hydra* gives insights into VPA induced toxicity at behavioral level. When a prey moves in water, mechanical stimulus is generated. Accidental touch of prey to *Hydra* tentacles activates nematocytes of *Hydra*, to sting the prey. Injured prey releases glutathione (GSH). GSH generates feeding response in *Hydra* by further initiating tentacle contraction and mouth opening. The feeding behavior can be chemically induced in *Hydra* by adding freshly prepared glutathione to dechlorinated water surrounding *Hydra*[15].

We used 3.2 uM GSH for induction of feeding response in 30 *Hydra* polyps under wild type and VPA treated conditions. Extent of tentacle contraction was assessed in

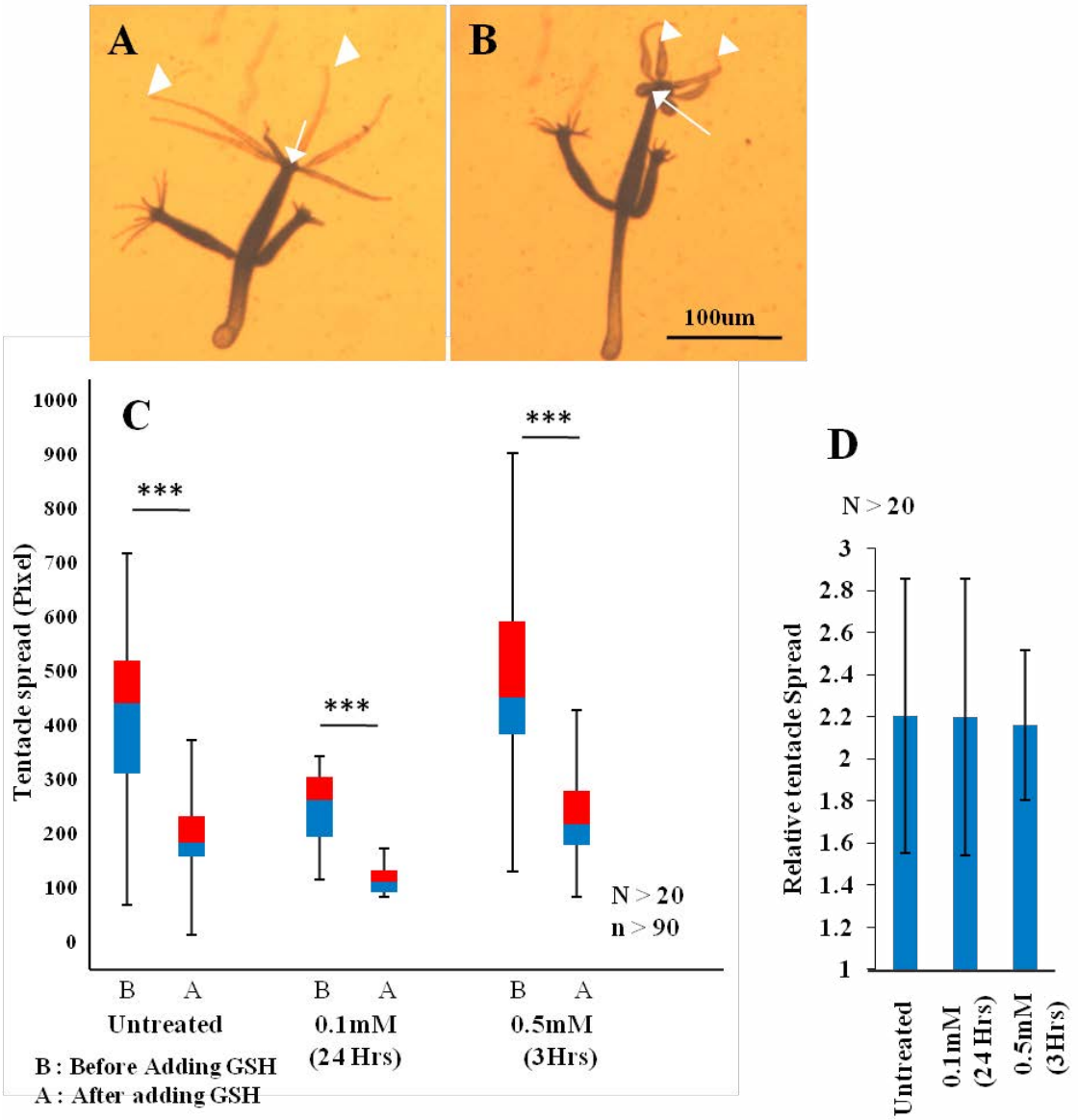


Figure 3. Extent of GSH induced tentacle contraction is not affected post VPA treatment. A, B) Tentacle spread of *Hydra viridissima*, before GSH treatment and within 180 seconds of GSH exposure. C) Feeding response of Control, 0.1 mM VPA (24 hrs) and 0.5 mM (3 hrs) treated *Hydra*. D) Relative tentacle spread of control, 0.1 mM and 0.5 mM VPA treated *Hydra viridissima*. *** indicates P<0.001.

order to quantify feeding behavior of *Hydra*, as described in methods section. We found significant decrease in tentacle spread post GSH induction in both, control and VPA treated *Hydra*. (Figure 3. A-C) This indicates that despite distortion of BCC, *Hydra* is able to elicit tentacle contraction step of feeding response. In order to comment on magnitude of tentacle contraction, we measured relative tentacle spread. Relative tentacle spread was calculated as ratio of average tentacle length after and before GSH treatment. Relative tentacle spread did not show any significant change after VPA treatment. The tentacles of VPA treated *Hydra* contracted like controls in response to GSH (Figure 3. D). Despite shortened length of tentacles, distorted organization of BCC and near absence of few types of cells in BCC, tentacle contraction of VPA treated *Hydra* is unaltered.

4. Discussion and Conclusion

Valproic Acid is an anti-convulsant drug. It is used to manage epilepsy, bipolar disorder or migraine in mammals. It is also a well known teratogen in mammals. Several studies have indicated three modes of action of VPA. VPA can act as a Histone Deacetylase (HDAC) inhibitor, Sodium ion channel blocker or/and as an activator of neurotransmitter Gama-amino Butyric Acid (GABA)[12,24,25]. HDAC inhibitors (HDACi) are known to interfere in mechanisms regulating eukaryotic cell cycle. HDAC is may affect proliferation, differentiation and could activate apoptosis in cells (Bose et al., 2013). Anti-convulsant property of VPA is attributed to its role as a sodium ion channel blockers and as an activator of GABAnergic pathways. We administered *Hydra* with a lower dose of VPA for longer duration and a higher dose with shorter duration[11].

Hydra is a diploblastic animal with remarkable regenerative ability and it shows no senescence. *Hydra* has two layers of epithelia surrounding central gastric cavity. This interstitial cavity harbors a population of interstitial stem cells. Interstitial stem cells have a cycle of 18-30 hrs[27]. Therefore, a 24 hour long VPA treatment is likely to affect cell cycle regulation of most of the interstitial stem cells of *Hydra*, via its HDACi activity. Shorter treatment may affect only a small population of interstitial stem cells. Most of the cells of *Hydra* are either continuously self renewing or they are rapidly lost from tentacle or foot region. Shorter length of tentacle and disrupted organization of BCC (Figures 1 and 2) post long term VPA treatment indicates that the cells that are lost from tentacles may not be replaced by actively dividing interstitial stem cells. Short term exposure to VPA, probably does not affect a good number of interstitial stem cells.

Tentacle of VPA treated *Hydra* contracted like untreated *Hydra*. Shortened length of tentacles, disrupted organization of BCC and near absence of few types of cells in BCC does not alter feeding behavior of VPA treated *Hydra*. Direct stimulation of desmoneme could bend *Hydra* tentacles[20,21]. Myonemes-muscle cells of BCC are innervated by nerves and excitatory input from nerves can also cause tentacles to bend. Our experiments suggest that tentacle contraction may not be a result of activation of only one type of cell present on surface of battery cell complex[21]. Tentacle bending could involve extensive use of neural circuitry downstream sensory cell of each individual battery cell complex. Certain neurotransmitter receptors, like that of GABA, Glutamate and Glycine are known to be present near tentacle and Hypostome region of *Hydra*[4,5]. Studies using various antagonists and agonists have proven that they are involved in modulation of feeding response. GABA, via its metabotropic receptor GABAB is known to be involved in desmoneme stimulation and tentacle contraction. Structure of GABAnergic receptors is conserved across *Hydra* and Verterbrates[4]. Neuro-protective effect of VPA is correlated to its function as a of Histone Deacetylase (HDAC) enzyme inhibitor and as an activator of Gamma-amino Butyric Acid (GABA) nergic pathways[24]. Molecular mechanisms of VPA action in *Hydra* require detailed refined study at molecular level. Whether VPA induces activation of GABAnergic neurons can be verified. GABAnergic inhibition of inhibitory signals, increases duration of mouth opening during induced feeding response[5]. It is possible that VPA induced activation of GABAnergic neurons could have compensated for loss of signals from desmonemes of deformed battery cell complexes. This could have caused normal feeding behavior of *Hydra* in our experiments.

We tested the effect of Valproic Acid (VPA) in an invertebrate, *Hydra viridissima* at organism, cellular and behavioral level. Working on *Hydra* offered us several advantages. *Hydra* being a diploblastic aquatic animal absorbs materials from the aquatic environment by filtration or diffusion. Material can also enter *Hydra* by direct ingestion or by ingestion of contaminated prey organisms. Thus administration of VPA to *Hydra* and monitoring its dosage was relatively easy by simply dissolving VPA in water. We could study by cell biology of tentacles

without sectioning. *Hydra* proved to be very simple and efficient in vivo test system, providing scope for analysis at the level of individual cells, at level of whole organism toxicity and also at level of individual animal behavior. Our results provide primary indication for possible utility of invertebrates like *Hydra* for screening of chemicals for possible modulations in molecular pathways. Detailed molecular, phylogenetic analysis and biochemical studies are required to validate whether molecular mechanism of action of molecules like VPA, are conserved across phyla and to what extent.

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