Negative geotaxis: An early age behavioral hallmark to VPA rat model of autism

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KEY WORDS

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ABSTRACT

Background: Negative geotaxis (NG) is an important parameter, commonly used in study of different CNS diseases and neurodevelopmental disorders. Neurobehavioural change following brain injury was easily identified by negative geotaxis.
Purpose: Although NG is evaluated in the settings of ASD, most of the studies are conducted for short duration (1–3 day) and the overall trend of acquisition of NG is not evaluated. In this context, we wanted to evaluate the trend of acquisition of negative geotaxis as a behavioural marker of autism in Valproic acid (VPA) model of ASD.
Methods: Dams in the VPA group were treated with intraperitoneal injections of VPA 600 mg/kg single dose on gestational day 12.5, while the control animals received normal saline of similar volume. Developmental parameters {body weight (PND 8, 10 & 12), body length (PND 4, 5, 6 8, 10), eye opening (PND 10, 12, 14, 15 and 16) and motor development (grid walking test on PND 20)} were monitored. Negative geotaxis test was performed at PND 6, 10, 15 and 17.
Results: The results of the present experiments demonstrate that VPA exposed rats exhibited delayed developmental parameters, aberration of the pattern of acquisition of negative geotaxis, enhanced negative geotaxis in early postnatal period (PND 6) and enhanced negative geotaxis in absence of visual clues (PND 17).
Conclusion: NG can be a valuable biomarker in early detection of autistic behavior and in absence of visual clues. The abberant negative geotaxis developmental pattern can serve as a marker to detect ASD. Thus NG can serve as an important early age biomarker of ASD. Further studies are required to validate this finding.

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Introduction

Autism is a neurodevelopmental disorder with unclear etiopathogenesis [1]. It is grossly characterized by impairment in social interaction, communication abnormalities and stereotyped behaviors [2]. DSM-IV-TR classified the spectrum of autistic disorders into three classes: Autism, Asperger's syndrome and PDD-NOS [3,4]. But the DSM-V combines all these three categories into one umbrella i.e. autism spectrum disorder (ASD). The hallmark clinical presentation are [1] deficits in social communication, and restricted, repetitive behavior [1,3,4].

Study using animal model is a very crucial tool for understanding the neurobiology of autism. The Valproic acid (VPA) model is a well established model of ASD. Prenatal VPA exposure is associated with high risk of autism in the offsprings [5]. Similarly, in rats, VPA exposure on day 12.5 (time of neural tube closure) leads to neurodevelopmental aberration with autism like clinical, molecular and behavioural alterations. This model has strong construct and clinical validity [2].

Negative geotaxis is an automatic unlearned response and directional movement against gravitational cues that help to study sensory or proprioceptive function. It is one of the early behavioral tests which is conducted to evaluate motor development (reflexes), activity and vestibular function [6–8]. In early behavioral tests, NG has their own importance as controlling vestibular functions in developmental sequence after sensory function onset [9]. This is an innate postural response that develops in the early age of pups.

Use of negative geotaxis is validated in the settings of many neurological and neurodevelopmental disorders e.g. amyloid beta induced locomotor decline [10], recurrent neonatal seizure [11], infantile spasm [12] and many others. Few studies have evaluated NG in the settings of ASD. Schneider T *et al*, 2005 did not find any difference in negative geotaxis when tested from PND 7 to 10 (VPA model, rat) [13], but in rett syndrome (MECP2 heterozygous and MECP2 null mice),

Santosh M *et al*, 2007 found that on 10th day the MECP2 heterozugous and MECP2 null mice group, more percentage of animals showed positive NG (PND 10) when compared to wild type and the trend continued till the end of the experiment [14]. Again, Schreider T *et al*, 2005 conducted the experiment for a limited period (3 consecutive days) [13]. In endotoxin-exotoxin + paracetamol rat model, on PND 17 (they evaluated NG on 3 consecutive days PND 15, 17 and 18), the autistic pups showed enhanced negative geotaxis, when compared to the control group [15]. So the role and validity of the negataxis geotaxis experiment in ASD remains blurred, as the results are contradictory and most of the studies are limited by short duration of observation (3 consecutive days) and the trend of negative geotaxis behavior delopment or aquisition is not analyzed. In this context we tried to elucidate the trend of negative geotaxis development in VPA rat model of autism with longer follow up data collection.

Methods

Animals

Wistar rats (200–300g) born and raised at the advanced small animal facility, PGIMER, Chandigarh (India) were used in the study. Food and water *ad libitum* were supplied in standard light conditions (12 hour light/dark cycle). Animal housing, caring and experimental procedures were followed as per CPCSEA guidelines. The protocol was approved by the Institutional Animal Ethics Committee, PGIMER, Chandigarh, India (vide letter no 457/2012 dated 23/07/2012).

Reproduction cycle recognition in rats

Generally, estrous cycle is divided into four phases: proestrus (12h), estrus (12h), metestrus (21h) and diestrus (57h) [16,17]. As the female rats accept male in their estrous phase, proper identification of the reporoductive cycle is of utmost importance. In our study, the exact phase of reproductive cycle was identified by vaginal smear method [18]. The vaginal smear of pro-estrus phase shows nucleated epithelial cells, estrus phase shows both nucleated epithelial cells & cornified cells, meta-estrus phase shows many leukocytes with nucleated & cornified cells and diestrous phase is characterized by predominance of leukocytes [17].

Pregnancy determination

In our experiment, female rats were identified in the estrus phase and matted overnight in a male: female ratio of 1:3. Pregnancy in female rats was identified by vaginal plug observation (Fig. 1) and it was confirmed by the presence of sperms in vaginal smear (Fig. 2) [19]. The presence of spermatozoa in vaginal smear was considered the 0.5 days of gestation (GD 0.5) [2,20,21]. Although, vaginal plug does not persist for a long time in the female; therefore sperm detection in vaginal smear considers as excellent predictor of pregnancy [22]. Females were housed in separate cages after the pregnancy confirmation with food and water *ad libitum*.

VPA model

Dams in the ASD group received a single intraperitoneal injection of sodium valproate (VPA; Sigma, USA) dissolved in saline (pH = 7.3; 250 mg/ml) in the dose of 600 mg/kg [4,19,20] on gestational day 12.5. Dams in the control group (c) received saline (pH = 7.3; 250 mg/ml) at the same time of gestation [20].

Grouping of pups

Valproate-treated (VPA) and control (C) females were allowed to raise their litter. A daily inspection for the presence of new litter was carried out twice a day. We scored postnatal day (PND) 0 for the litter when it was observed for the first time. Mother and new litters were kept together in home cage. On PND 4, the animals were tagged with their feet or the ear tip. Six male rat pups were selected from both control and VPA group and they were included in the study. Neurodevelopmental evaluation tests were started on PND 4. Weaning was done on PND 21.

Developmental monitoring

Postnatal developmental measures: body weight, body length and eye opening score data was collected for each included animal. Body weight data of each pups were collected on PNDs 8, 10 & 12. Milk band was observed on PND 6 using Ethovision software. Body length measurements were performed from nose-to-rump on PNDs 4, 5, 6, 8 and 10. Eye opening: To measure this developmental parameter, we followed a particular score for each stage of eye opening (0 = both eyes closed, 1 = one eye open, 2 = both eye open). Eye opening was measured from PND10, 12, 14, 15 and 16 [13].

Behavioral tests

Grid walking

Grid walking test helps to detect motor coordination and development in pups. Pups are allowed to walk freely on a grid for one minute and "foot fault" was counted on PND 20. Both controls (n = 6) and VPA treated (n = 6) rats were placed on a square plane one by one [23,24].

Negative geotaxis

NG is an automatic vestibular response to detect geogravitational stimuli and to measure sensorimotor competence in pups. During NG experiment, we used a plexiglass platform (12.5 × 45 cm w × h) and covered it with a rough clothe paper. The offspring of VPA (n = 6) and control group (n = 6) were tested in the negative geotaxis experiment at different PNDs (6, 10, 15 & 17). In this test, rat pups were kept on a 45° inclined plane and mean time to rotate 180° was recorded. Two independent evaluators, who were blind to study group allocation performed and evaluated the resuls of NG experiments to enhance the validity of NG experiment [24]. On PND 17, the experiment was performed both in presence and in absence of light.

Statistical analysis

Data was presented as mean ± SD. SPSS version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used for data analysis. The $p<0.05$ was taken as the level of significance. Repeated measure ANOVA was applied to analyze repeated measure data e.g. body weight, body length, eye opening and negative geotaxis. Student's *t*-test (independent) was applied to evaluate results of grid walking test and negative geotaxis data (with light and without light).

Results

Vaginal smears of different phases of mensrual cycle of rat is shown in figure 1. Sperm plug is shown in figure 2.

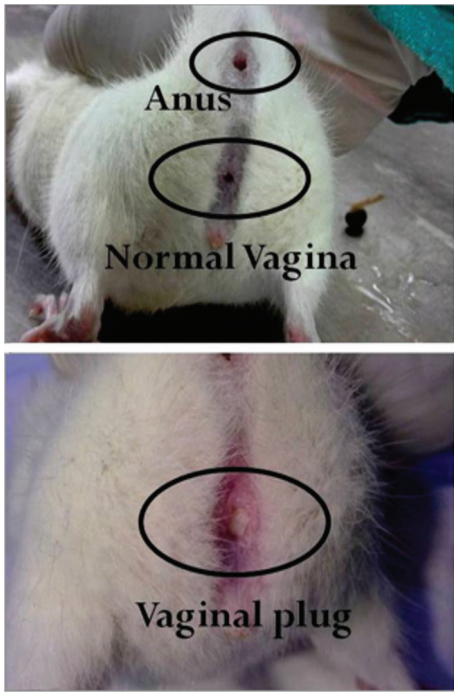

Fig. 1: Sperm plug observation as a measure of confirmation of pregnancy.

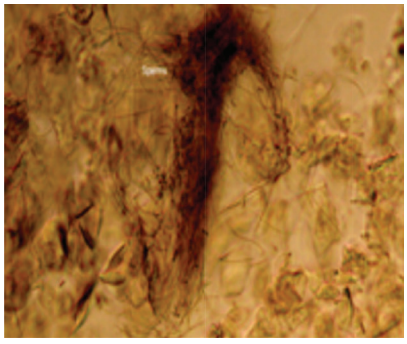

Fig. 2: Pregnancy conformation by observation of presence of sperms in vaginal smear (sperms seen as thread like structures).

Body weight

We measured body weight of rats on PND8, 10 and 12. Data is shown in figure 3. In each of the PNDs, the body weight of VPA group was significantly lower, when compared to the control group at that respective time point. Milk band observation in early PNDs (figure 4) also supported the less food intake in autistic pups (data not shown).

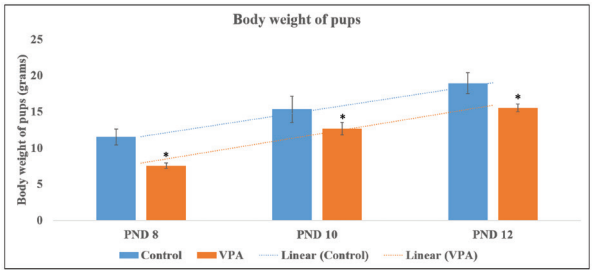

Fig. 3: Body weight on different PNDs. Overall p value is <0.05 between the two groups. * denotes $p<0.05$ when compared to control group at that respective time point.

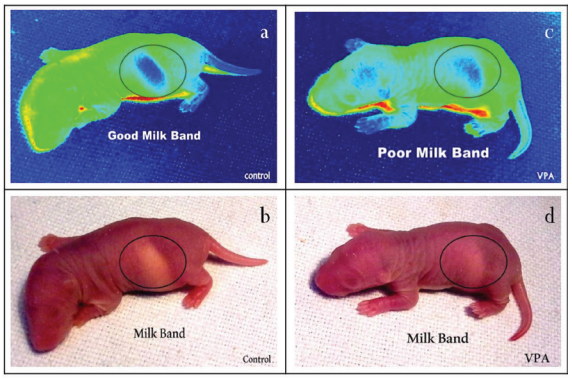

Fig. 4: Milk band in control and autistic pups(poor milk band seen in autistic pup).

Body length

Body length was measured on PND 4, 5, 6, 8 and 10. Data is shown in figure 5. Prenatal VPA exposed group showed a significantly less body length ($p<0.05$), when compared to the control group at respective time point.

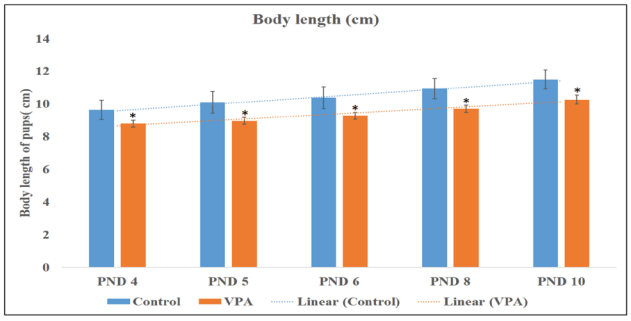

Fig. 5: Body length on different PNDs. Overall p value is <0.05 between the two groups. * denotes $p<0.05$ when compared to control group at that respective time point.

Eye opening

To examine the maturation process in the pups, we monitored eye opening status on PND 10, 12, 14, 15 and 16 in both control and VPA treated rats. Tha data is shown in Fig. 6. Repeated measure analysis showed a significant difference in the eye opening trend between the two groups, with a delayed eye opening trend being seen in the VPA group. The eye opening score between the two groups were significantly different on PND 15 with significantly lower eye opening score in the VPA group. On PND 16, the eye opening score was similar (eye opening score of 2 in all the animals) and no statistically significant difference was seen between the two groups.

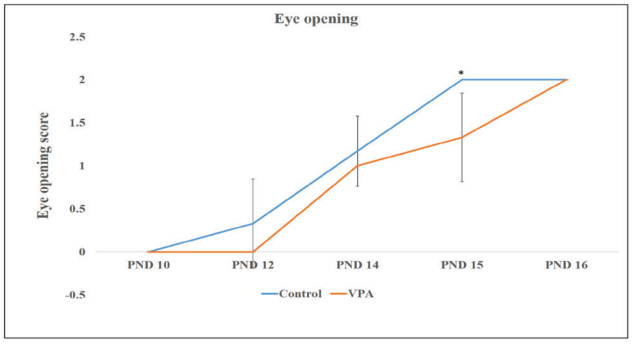

Fig. 6: Eye opening score on different PNDs. Overall p value is <0.05 between the two groups. * denotes p<0.05 when compared to control group at that respective time point.

Grid walking

The pups are placed on a an elevated levelled grid with openings was used for the grid walking test. The grid walking test is conducted on PND 20 in both control and VPA treated group. Normally animals place their paws on the wire frame precisely while they travel on the grid. In case of a paw slipping through the grid, a foot fault is recorded. Number of foot faults were carefully counted for each group. The data is shown in figure 7. The number of footfault were significantly higher in the VPA group, when compared to the control group (p<0.05).

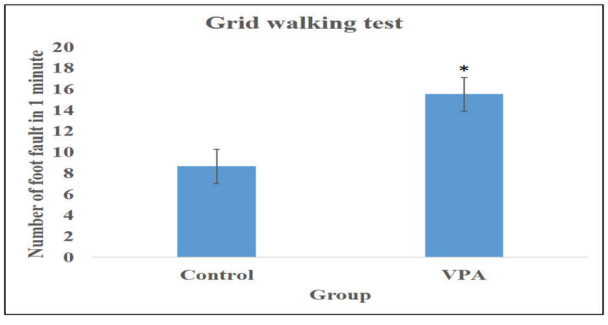

Fig. 7: Number of foot faults in Grid walking. * denotes p<0.05 when compared to control group.

Negative geotaxis

Data shown in figure 8.1. Repeated measurements of NG in different time course (PNDs 6, 10, 15, 17), revealed that the VPA treated group took less time on PND 6 (p<0.01) and 15 (p<0.05), while higher time was taken on PND 10 and 17, when compared to the control group at respective time point. Regarding trend of acquisition of negative geotaxis, the control group showed a trend of gradual decrease in time taken to rotate 180° as the PNDs advanced. On the contrary, the trend of acquisition of negative geotaxis was abberated in the VPA group. In the early phase, enhanced negative geotaxis was seen, but the trend was not maintained as PNDs advanced and somewhat an abberated trend was seen.

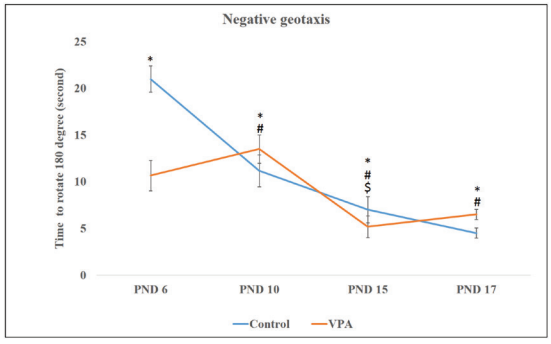

Fig. 8.1: Negative geotaxis data on different PNDs. Overall p value is <0.05 between the two groups. * denotes p<0.05 when compared to control group at that respective time point. # denotes p<0.05 when compared to PND 6 in the control group. $ denotes p<0.05 when compared to PND 6 in the VPA group.

Negative geotaxis in presence and absence of light on PND 17

Data shown in figure 8.2. On PND 17, in presence of light, the control group showed significantly less time to rotate 180° when compared to the VPA group. But in the absence of light, performance of the autistic pups were significantly better than the control group (p<0.05).

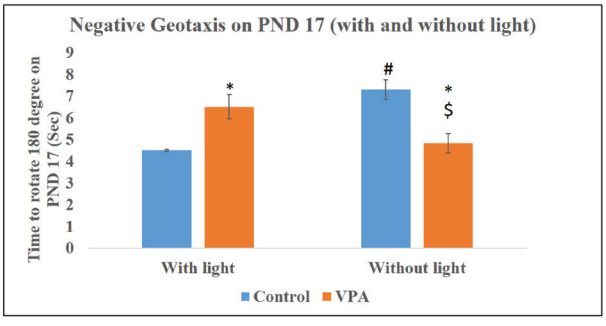

Fig. 8.2: Negative geotaxis data on PND 17 (in presence and in absence of light) * denotes p<0.05 when compared to control group at that respective time point. # denotes p<0.05 when compared to control group (with light). $ denotes p<0.05 when compared to VPA group (with light).

Discussion

VPA model is a well validated model of ASD [25,26]. The model has striking anatomical, pathological and etiological similarities with the human disease [26]. In our study, the body weight was significantly less in the VPA exposed rats. Similar observation is also made by Schneider T et al, 2006 [20]. Similarly, the mean body length of prenatal VPA treated rats were also significantly less when compared to controls. The eye opening pattern described in our results also suggests maturational delay in prenatal exposed VPA rats. This delayed eye opening time also support impaired glutamatergic synapse maturation in the superior colliculus [27,28]. So, summarizing findings of these three developmental parameters of our study, the autistic pups (VPA model) showed delayed attainment of developmental milestones when compared to the control group in terms of body weight, body length and eye opening. Autism is a neurodevelopmental disorder [28]. Delayed attainment of development milestones in autistic children is reported in many clinical studies [29,30] and this finding highlights the validity of the model.

As far as motor co-ordination and development is concerned, our study has demonstrated significantly higher number of "foot faults" in grid walking test in the autistic group, when compared to the control group. Motor delays and abnormalites can be seen in autistic individuals [31–36]. So, increased number of foot faults can be attributed to motor development delays and abnormalities associated with autism.

Geotaxis is considered as an important tool in behavioral analysis [6]. Geotaxis is characterized as negative and positive geotaxis; in which negative geotaxis is upward movement of rodents on an inclined plane and positive geotaxis is downwards movement on an inclined plane [37]. Many studies have reported negative geotaxis as an important component in evaluation of different neurological [14,38] and neurodevelopmental disorders [20,39]. Neurobehavioral dysfunction in brain injury was easily identified via negative geotaxis [40]. Furthermore, it played a significance role in evaluation of brain damage in kaolin induced hydrocephalus rat model [41]. Study of negative geotaxis performance before and after drug therapy has also been used to identify effective drug treatment in various CNS disorders [42,43]. NG was used before giving swim stress in autism animal model to study the activity of mesocortical dopaminergic system [44,45]. Thus, NG is coming up as an important behavioural tool for evaluation of CNS diseases, which can even be helpful at very early age, when other behavioural tests are not possible.

Therefore, our study aimed at evaluating the role of negative geotaxis as a early behaviour hallmark of autism. In our study, the control group showed a gradual decrease in time taken to rotate 180 degree, when placed in an inclined plane with head side downwards. The enhancement in negative geotaxis performance correlated well with eye opening and visuo-spatial development timelines. On the other hand the pattern of acquisition of negative geotaxis was abberated amongst the autistic pups.

Visuo-spatial development is one confounding variable, which can influence the result of the negative geotaxis experiment. Before visuospatial development phase, gravitational clues are the main driving factors in rotationg 180 degree by the pups. But, after opening of eye, which occurs typically between PND 10–14 days, visuo-spatial orientation can have significant role on the acquisition of negative geotaxis. So, we performed the negative geotaxis experiment both in presence and in absence of light (on PND 17) to differentiate the effect of visuospatial development and gravitational clues. In absence of light, gravitational clues will play the main role in rotating 180 degree in negative geotaxis experiment, while in presence of light, visual clues plays the dominant in negative geotaxis.

In presence of light, the autistic animals took more time in rotating 180 degree when compared to their control counterpart. Going into more detail, alteration in visuo spatial processing is noted in autism [46–48]. Weak central coherence is attributed for this abnormality as they fail to integrate local features into coherent global Gestalts and/or they show a trend of bias towards local processing [46–48]. Moreover, austistic subjects are susceptible to visual illusions [46]. The cause of underperformance of the autistic animals in presence of light may be late eye opening, neurodevelopmental abberations and weak central coherence.

In the absence of light, the VPA group took significantly lower time to rotate 180 degree when compared to the control group. Again, on PND 6, the autistic group (VPA treated) performed better, as at that time geotaxis was solely dependent on gravitational clues as eye opening was not there and on PND17 also in absence of light, the autistic group performed significantly better than the control group. We can conclude that, they can better perceieve gravitational clues. Higher performance of autistics in absence of light is already reported. In population based studies also, autistic individuals were more accurate in judging the shape of slanted circle, when visual clues were eliminated [46]. This finding highlights and adds validity to our observations.

Conclusion

To summarize, this is the first detailed evaluation of the role of negative geotaxis (both in light and without light) as a behavioural test of autism in early stage of life of rats exposed to VPA in 12.5th day of gestation. NG can serve as an important biomarker of autism in rat pups as early as PND 6, when evaluation by other neurobehavioral tests are not possible. As the eye opening starts, the abberant trend of acquisition of negative geotaxis may be an important early marker of ASD. Again, NG in absence of light also can also serve as an important predictor of the same. Additional research is required to connect this behavioral parameter with biochemical and molecular changes. We need more studies to validate this finding in different settings.

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Authorship contribution

All the authors contributed in the concept generation, design by RR, SS and PS, all the authors contributed equally towards intellectual content, experimental studies were conducted bt RR, SS and PS. Data acquisition was done by RR, SS and PS, statistical analysis was done by RR, PS and AP, manuscript was prepared by RR, SS and PS, manuscript was reviewed and editing by all.

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Conflict of interest

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