Congenital malformation and autism spectrum disorder: Insight from a rat model of autism spectrum disorder

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Abstract:
AIMS AND OBJECTIVES: The primary aim was an evaluation of the pattern of gross congenital malformations in a rat model of autism spectrum disorder (ASD) and the secondary aim was characterization of the most common gross malformation observed.

MATERIALS AND METHODS: In females, the late pro-oestrous phase was identified by vaginal smear cytology, and then, they were allowed to mate at 1:3 ratio (male:female). Pregnancy was confirmed by the presence of sperm plug in the vagina and presence of sperm in the vaginal smear. In the ASD group, ASD was induced by injecting valproic acid 600 mg/kg (i.p.) to pregnant female rats (n = 18) on day 12.5 (single injection). Only vehicle (normal saline) was given in the control group (n = 12). After delivery, pups were grossly observed for congenital malformations until the time of sacrifice (3 months) and different types of malformations and their frequency were noted and characterized.

RESULTS: In the ASD group, congenital malformation was present in 69.9% of the pups, whereas in the control group, it was 0%. Male pups were most commonly affected (90% in males vs. only 39.72% in female pups). The tail deformity was the most common malformation found affecting 61.2% pups in the ASD group. Other malformations observed were dental malformation (3.82%), genital malformation (3.28%) and paw malformation (1.1%). Hind limb paralysis was observed in one pup. The tail anomalies were characterized as per gross appearance and location of the malformation.

CONCLUSION: In this well-validated rat model of ASD, congenital malformation was quite common. It seems screening of congenital malformations should be an integral part of the management of ASD, or the case may be vice versa, i.e., in the case of a baby born with a congenital deformity, they should be screened for ASD.

Keywords: Autism spectrum disorder, behavioral impairment, developmental malformation, valproic acid

Introduction

Autism is a clinically and etiologically heterogeneous group of disorders, together referred as the “autism spectrum disorders” (ASDs). Kanner was first to describe autism in the United States in the early 1940s. However, autism became popular in the early 1980s.

In the Diagnostic and Statistical Manual of Mental Disorders I (DSM-I) (1958) and DSM-II (1968), we cannot find the word autism. This term was first introduced in DSM-III (the 1980s). In DSM-IV autism term was mentioned, but it is changed to ASD in DSM-V. ASD is a spectrum of neurodevelopmental disorder of heterogeneous etiology. The behaviors that are characteristics of autism are (1) social communication and interaction deficit and (2) restricted repetitive behaviors (RRBs), interests, and activities. Diagnosis of ASD requires the presence of both of these components.

How to cite this article: Ruhela RK, Sarma P, Soni S, Prakash A, Medhi B. Congenital malformation and autism spectrum disorder: Insight from a rat model of autism spectrum disorder. Indian J Pharmacol 2017;49:243-9.
Social communication disorder is diagnosed if RRBs is absent.\(^6\)

In the early epidemiological studies conducted, prevalence estimates from European studies were 1:2500 children (data from the late 1960s and early 1970s). In the year 2000, prevalence was 1%–2% in children age group. In the year 2010, overall ASD prevalence in the United States was 14.7/1000, i.e., 1 case per 68 children. Thus, the prevalence of autism has increased to 20–30 times compared to early studies conducted in the late 1960s.\(^3\) Among autistic individuals, the frequency of minor physical anomalies is quite high.\(^4\) In a population-based cohort study, 6% of children with autism had birth defects, and the birth defect was associated with nearly two-fold increased risk for autism overall.\(^5\) Intellectual disability in autism was found to be strongly associated with severe birth defects. However, the link between intellectual disability and the birth defect is not clear.\(^5\) Miles and Hillman proposed that different minor physical anomalies may indicate the involvement of different etiological pathways in the disease process.\(^6\)

Use of valproate during pregnancy is directly associated with high risk of autism in the offspring (absolute risk 4.42% [95% confidence interval (CI), 2.59%–7.46%] and adjusted hazard ratio, 2.9 [95% CI, 1.7–4.9]).\(^7\)

In animals (rat and mice), in utero valproic acid (VPA) exposure leads to autistic-like behaviors in the offspring characterized by a deficit in social behavior and communication and increased repetitive behaviors. Based on these findings, the VPA model is increasingly accepted as a good model for autism with face and construct validity.\(^8\) The model is anatomically, pathologically, and etiologically similar to human disease and the model also shows behavioral similarities to human disease (e.g. hyperactive repetitive/stereotypic behavior, diminished acoustic prepulse inhibition, low sensitivity to pain and high sensitivity to nonpainful stimuli and decreased locomotor behavior, decreased exploratory activity, social behavior deficit and increased latency to social behaviors).\(^9,\)\(^10\) Besides this, VPA rats also show low birth weight, delay in motor development, delay in maturation, and attenuated integration of series of reflexes. Another important feature of this model is that behavioral aberrations observed appear before puberty. This feature can distinguish the VPA rat model of autism from other animal models of other disorders of neurodevelopment, for example, rodent models of schizophrenia.\(^9,\)\(^10\)

From the literature review, it seems that there is an important link between ASD and congenital malformations. In this study, we are going to present our findings on the incidence and pattern of gross congenital morphological alterations in a rat model of ASD and characterization of the most common gross congenital malformation (tail malformation). Furthermore, we have proposed a new classification system for tail malformation found among animals prenatally exposed to VPA. The earlier classification system proposed by Saft et al. did not take care of several points, like no mention of the simple distal bend, simple proximal bend. The only importance of tail with a simple bend in the middle was emphasized. Again emphasis was given on double flexure tail, but there was no mention of more complex flexure or gross deformation of the tail.\(^11\) Earlier classification system only mentions as a short tail but fails to incorporate other details of short tail. Hence in our new classification system, we incorporated the wide spectrum of tail morphological alterations. This new morphological classification takes care of the discontinuous nature of neural tube closure as well as we can have information on severity of effect on the differentiation process.

### Materials and Methods

**Animals**

Adult Wistar male and female rats weighing about 180–250 g were obtained from the central animal house of the Institute. A total of 14 adult male and 42 female healthy Wistar rats were used for the breading. The animals were housed in standard laboratory conditions in groups of three per cage initially at 25°C ± 2°C, humidity of 60% ± 2% and 12 h light:dark cycle. Animals had free access to standard laboratory chow diet and water. The protocol was approved by the Institutional Animal Ethics Committee, and for animal housing, care, handling and other issues, CPCSEA guideline was followed.

**Grouping and data collection**

Females were identified for late pro-estrous phase by vaginal smear cytology, and then they were allowed to mate (1:3 ratio). Pregnancy was confirmed by the presence of vaginal plug. It was reconfirmed by observing sperm in vaginal smear by microscopy. The day of confirmation was taken as gestation day 0.5.

In the ASD group, pregnant female rats (n = 26) were given a single injection of VPA 600 mg/kg by I.P. route on day 12.5 to induce ASD. In the control group, pregnant female rats (n = 16) were given only vehicle (normal saline). On postnatal day 21, pups were separated from their mother. The animals were grossly observed for congenital malformations until the time of sacrifice (3 months), and different types of malformations and their frequency were noted and characterized.\(^9,\)\(^10\)

Animals were observed by two independent observers to identify any gross malformations and in the case of any
conflict, it was discussed with the third observer and the issue was resolved. Animals were observed from caudal to the cephalic region. The sequence of observation was tail, right hind limb, left hind limb, pelvis area, vagina, anus, abdomen, back, thorax, right upper limb, left upper limb and then head and neck regions (including hard and soft palate by opening the mouth with gentle pressure) and observed for any gross morphological abnormality. Any abnormality found is noted and tried to characterize in light of literature.

Characterization of the most common deformity (tail deformity) was done as per methodology of Saft et al. (2014). We have suggested an alternative morphological classification of tail malformation. Data are shown as per both the classification systems.

Statistical analysis
Data were presented as mean ± SEM. SPSS version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used for data analysis. P < 0.05 was taken as the level of significance. Student’s t-test (independent) was applied to compare number of pups delivered per dam, live male pup and female pup delivered per dam. The frequency of different malformations and different morphological patterns were presented as percentage and intergroup comparison was made by using Student’s t-test (independent).

Results
Pregnancy outcome in dams exposed prenatally to valproic acid
The results are presented in Table 1 and Figure 1. The number of live births per dam (litter size) was significantly high (P < 0.05) in the control group (8.87 ± 0.768) compared to the valproate-treated autistic group (7.04 ± 0.530). No significant difference was found with regard to live male pup delivered per dam and live female pup delivered per dam between the two groups.

Malformations
The spectrum of different types of malformations observed in our study is presented in Table 2 and Figure 2. The percentage of congenital malformations in ASD group was significantly higher in the ASD group than control group (69.9% vs. 0%) and the most common deformity observed was tail deformity (61.2%). Other deformities observed in the ASD group were dental malformation, genital malformation, paw malformation, and hind limb paralysis.

Tail deformities
The tail deformity was the most common congenital anomaly observed in the ASD group (61.2%), and there was a significant difference with the control group (0%). The tail deformity was significantly (P < 0.05) higher among male ASD pups (75.45%) than their female counterpart (39.72%).

Teeth deformity
Seven cases of teeth overgrowth (3.82%) were observed in the ASD group. No case of teeth overgrowth was seen in the control group. The overgrown teeth significantly affected the day-to-day life of the animal. Male pups were mostly affected (6.36% vs. 0%).

Genital abnormality
Six pups with the genital abnormality (3.28%) were seen in the ASD group. No case of genital overgrowth was seen in the control group.

Paw malformation
Two cases of paw malformation (1.1%) were observed in the ASD group (only male pups affected). In the control group, no case of paw malformation was seen.

Hind limb paralysis
One case of hind limb paralysis (0.55%) was encountered in the ASD group (affected pup is a male pup).

Table 1: Pregnancy outcome in dams

| Parameter                        | Control (n=16 dam) | ASD group (n=26 dam) |
|----------------------------------|--------------------|----------------------|
| Number pups delivered per dam    | 8.87±0.768         | 7.04±0.530*          |
| (litter size)                    |                    |                      |
| Live male pup delivered per dam  | 4.94±0.686         | 4.23±0.465           |
| Live female pup delivered per dam| 3.93±0.551         | 2.81±0.380           |

*Signifies significant difference (P<0.05) when compared to controls by independent t-test. ASD=Autism spectrum disorder, SEM=Standard error of mean

Table 2: Spectrum of different types of malformations observed in our study

| Type of defects          | Control (n=142) | ASD group (n=183), n (%) | ASD group (malformations in male and female pups) |
|--------------------------|-----------------|--------------------------|--------------------------------------------------|
|                          | n=110 male pups, n (%) | n=73 female pups, n (%) | n=110 male pups, n (%) | n=73 female pups, n (%) |
| Tail abnormality         | 0               | 112 (61.2)*              | 83 (75.45)**                                       |
| Dental malformation      | 0               | 7 (3.82)*                | 7 (6.38)**                                        |
| Genital malformation     | 0               | 6 (3.28)*                | 6 (5.45)**                                        |
| Paw malformation         | 0               | 2 (1.1)                  | 2 (1.82)                                          |
| Hind limb paralysis      | 0               | 1 (0.55)                 | 1 (0.91)                                          |
| Total                    | 0               | 128 (69.9)               | 99 (90.0)                                         |

*P<0.05 when compared to control group. **P<0.05 when compared to ASD group (female pups). ASD=Autism spectrum disorder
Gender discrimination
Percentage of animals showing malformation was significantly higher ($P < 0.05$) among male pups than female (90% in male vs. 39.72% in female pups). Again tail malformation was significantly higher ($P < 0.05$) in male than female pups (75.45% in male vs. 39.72%). Dental and genital malformations were also higher among male autistic pups. Thus, male pups are more prone to malformations associated with ASD. It strengthens the hypothesis that ASD more commonly affects male gender.

Characterization of the most common deformity
Tail deformity
The results are shown in Tables 3-5. As per classification by Saft et al., simple bent tail in the middle (69.6%) was the most common deformity. According to our classification, simple bent tail (V/U/S shape, i.e., single or double bending) was the most common tail malformation found (82.1%).[11]

Discussion
The VPA model is a widely used validated model of ASD. Regarding construct validity, autism is a multifactorial disease and VPA mimics one component of its etiology. Although the complete pathogenesis of autism is not clear until now, VPA mimics many metabolic and molecular alterations in autism such as oxidative stress,[12-14] excitatory-inhibitory imbalance, and hyperserotonemia are common to both human autism and VPA model of autism.[15]

Pretreatment with antioxidants, for example, Vitamin C and E and ascorbic acid decreases these manifestations suggesting an important role of oxidative stress.[16,17] Another important pathway affected by VPA is inhibition of histone deacetylase.[15]

VPA model exhibits good face validity manifesting two important characters of the disease, i.e., (a) persistent social communication and interaction deficits and (b) restricted, repetitive patterns of behaviors, interests, or activities. Like their human counterpart, VPA-treated ASD rats show features of social communication and interaction deficit,[18-22] and repetitive behavior.[22-26] This model also has reasonable predictive validity.[15] Hence, it can be assumed that the VPA model of autism is a reasonably good valid model of autism, mimicking human disease to a reasonable extent.

Now coming to findings in our study, in this study we found that litter size (number of pups born per mother) was significantly less in the ASD group. The similar finding regarding pregnancy outcome in prenatally VPA exposed rats was also described by Favre et al. The incidence of different congenital malformations was significantly higher in the ASD group compared to controls.[27]

Table 3: Morphological characterization of tail deformities in valproic acid -treated rat by Saft et al. (2014)

| Type of deformity                  | Number of animals in which the malformation was present (total tail malformation n=112), n (%) |
|-----------------------------------|---------------------------------------------------------------------------------------------|
| Short tail                        | 3 (2.67)                                                                                    |
| Simple bent in the middle         | 78 (69.6)                                                                                   |
| Short and bent tail               | 2 (1.78)                                                                                    |
| Double flexure tail               | 29 (25.89)                                                                                  |

Table 4: As per location of bending

| Type of deformity     | Number of animals in which the malformation was present (total tail malformation n=112), n (%) |
|-----------------------|---------------------------------------------------------------------------------------------|
| Proximal bending      | 12 (10.71)                                                                                  |
| Bending in mid portion of tail | 80 (71.42)                                      |
| Bending in distal tail | 20 (17.85)                                                                                  |

Table 5: Morphological classification suggested by us depending on extent and severity of tail malformation

| Type of deformity                                | Number of animals in which the malformation was present (total tail malformation n=112), n (%) |
|--------------------------------------------------|---------------------------------------------------------------------------------------------|
| Simple bent tail (V/U/S shape i.e., single or double bending) | 92 (82.14)                                                                                  |
| Grossly dysmorphic banding (>3 banding)          | 15 (13.39)                                                                                  |
| Short deformed tail (without kink)               | 3 (2.67)                                                                                    |

Figure 1: Bar diagram showing pregnancy in valproic acid and control group
Figure 2: Different type of gross congenital malformation encountered in our study
Studies report dysmorphisms and minor physical malformations in patients with ASD.[28‑33] However, many of the studies suffer from methodological issues such as small sample sizes, lack of appropriate control, no definition of a birth defect, and potential confounding factors little or no consideration of potential confounding variables.[34] Hence in this study, we give additional evidence regarding congenital malformations in ASD from studies on an animal model. It seems that congenital malformation is a common associated feature of Autism. Again, males were more commonly affected. This strengthens the earlier hypothesis that ASD more commonly affects males. However, most of the guidelines do not recommend it specifically that screening for birth defects is a must for patients with ASD or vice versa. It seems that screening for birth defects should be a routine part in children diagnosed with ASD. Even in children with a birth defect, a screen for symptoms of ASD will be reasonable and this point should be considered while preparing guidelines.

In our study, the most common gross malformation was a tail deformity. It was present in 61.2% of autistic pups both in male and female. In rats, embryologically tail develops from different structures like the tail bud then neural tube, notochord, and tail gut by the process of secondary body formation without formation of germ layers.[35] During secondary neurulation, multipotential cell population in the tail bud becomes organized in the dorsal part of the tail bud; the process of canalization (neuroepithelium surrounding central cavity) gives rise to a sacrocaudal neural tube whose lumen is continuous with the lumen of the primary neural tube more rostrally. During the subsequent embryologic development process, caudal-most of the neural tube undergoes apoptosis. However, the closure of neural tube is rather a discontinuous process.[36‑38] Combined development and differentiation of all these structures ultimately leads to the development of the tail.[39,40] In our VPA model of autism, VPA was administered on 12.5 days of pregnancy. Again, it is the period of development of the tail notochord. Notochord of rat tail develops between day 12 and 13 from a common condensed mesenchymal cell mass located ventrally to the secondary neural tube. This condensed mesenchymal cell mass gives rise to a thin cord (origin of notochord) and a thick cord (origin of the tail gut).[41,42] Hence, there is a strong possibility that deformities of tail observed in VPA model of autism may be due to its effect on notochord differentiation and development. This area needs further evaluation.

Valproate inhibits folate receptors noncompetitively and thus, in utero VPA exposure could be disrupted developmental processes giving rise to a wide range of defects.[43] Valproate affects axial structure development[35,44] and neural tube development.[44‑46]

Other mechanisms implicated in valproate-induced defects are altered cell proliferation, differentiation and apoptosis,[47] DNA damage and the consequent increase of embryonic p27(KIP1) and caspase-3 expression,[48] epigenetic modifications during early organogenesis expression,[49] and histone deacetylase inhibition.[50]

The tail is an important organ for thermoregulation and balance.[51,52] It has no fur, surface to volume ratio is large and well perfused with blood vessels.[53] The surface area of rat's tail is only 5% of its total body surface area, but yet it can dissipate almost 17% of the total body heat.[49] The rate of heat loss in tailless rats is less compared to tailed rats. The core body temperatures of tailless rats rise higher, and when high, it takes longer time for their high temperatures to come down.[36,54] Hence, this tail malformation may affect the normal body physiology of the animal. This issue needs further evaluation.

Another congenital malformation observed in this study was teeth overgrowth. Incisor malocclusion and overgrowth are common among rodents, reaching an incidence of approximately 1% in 2-year-old Wister rats.[55,56] In our study, seven cases (3.82%) of dental malformation (teeth overgrowth leading to a malocclusion) were found in the ASD group. The incisor teeth of rodents grow continuously throughout their lives. Optimal occlusion of the incisors is necessary for a balance between their rapid extrusive growth and normal attrition. To maintain normal occlusion of their incisors, rodents require material to gnaw on. Insufficient wear of rodent incisors is a cause of rapid tooth elongation and ultimately malocclusion. Dental overgrowth and malocclusion can further lead to chronic difficulty in feeding causing malnutrition and also trauma to surrounding tissues. It can sometimes be the cause of death of the rodent.[55,56]

Another gross abnormality noted is genital overgrowth. The genital abnormality is reported among 22% of those exposed to valproate.[57] Other abnormalities noted in our study were paw malformation and hind limb paralysis.

Our observation supports that males are more frequently affected by developmental malformations associated with autism. Tail, dental, and genital malformations were significantly high among male pups when compared to female pups. This observation supports the earlier hypothesis that males are more commonly affected by ASD than females.[58‑60]

Conclusion

The incidence of congenital malformations is quite high among patients with ASD. In our study with in uteri
VPA exposure model of ASD, we found that prevalence of congenital malformations was quite high in the ASD group. This provides us additional information regarding the link between autism and congenital malformations. Congenital malformations may be a manifestation of the autism spectrum of disorders itself. Further study is required to find out the common thread between these two manifestations of the same spectrum of the disease. Furthermore, we suggest that birth defect evaluation should be a part of routine evaluation of patients with ASD and vice versa, i.e., all patients with a birth defect should be routinely screened for ASD.

In our study, the most common anomaly observed was a tail deformity. Saft et al. characterized the spectrum of tail malformations in VPA-exposed rats.[11] In this study, we have proposed our own system of classification of tail malformations. The different patterns of malformation show the wide range of effects of valproate during organogenesis at a different stage and different time points.

Acknowledgment
This research has received research grant from the Department of Biotechnology (DBT), India.

Financial support and sponsorship
This research was supported by a grant from the Department of Biotechnology, Government of India.

Conflicts of interest
There are no conflicts of interest.

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