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*Human and Animal Physiology*

# Sex-Related Behavioral Abnormalities and Neuroanatomical Changes in a Rat Model of Autism

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**Abstract.** This study examined sex-specific behavioral characteristics of social behavior, as well as anatomical changes in the medial prefrontal cortex (mPFC) in a rat model of autism. For experimental purposes on the 12.5th day of gestation, half of the pregnant rats received a single intraperitoneal injection of 500 mg/kg sodium valproate (valproic acid - VPA) dissolved in 0.9% saline, while the other half (control group) was administered by saline alone. The 6-month-old offspring (males – M, females – F) from both the saline and VPA-exposed dams were assigned to one of four experimental groups before undergoing behavioral experiments: Contr(M) and Contr(F) representing prenatally saline-injected male and female groups, VPA(M) and VPA(F) – prenatally VPA-injected male and female groups. Social behavior was assessed using a three-chamber social interaction testing apparatus. Immunohistological experiments were conducted to determine the effect of VPA on the number of NeuN-immunoreactive (NeuN-ir) cells in the mPFC. The results indicate that male rats prenatally exposed to VPA demonstrate deficit in social behavior – reduced social exploration and impaired social novelty preference. Female rats exposed to VPA did not show such marked changes in social behavior. Immunohistochemical evaluation shows a significant decrease in the number of NeuN-ir neurons in the mPFC in male rats exposed to VPA, whereas cell loss is non-significant in females. It can be assumed that observed anatomical changes in the mPFC may be responsible for deficit in social behavior in male rats exposed to VPA. © *2026 Bull. Natl. Acad. Sci. Georg.*

**Keywords:** rat model of autism, sex, social behavior, medial prefrontal cortex

## Introduction

Autism spectrum disorder (ASD) is a developmental disorder characterized by deficits in social reciprocity and communication, along with restricted interests and repetitive behaviors. ASD is more common in males than in females, with a male-to-female ratio of 4.3:1 (Markram et al., 2007). The neurobiological basis of behavioral dysfunction and the high male prevalence in ASD remain poorly understood.

As human autistic tissue samples are limited, animal models can be used to study the neurobiological mediators in more detail and in a more controlled environment. The most used environ-

mentally triggered model of autism results from embryonic exposure to valproic acid (VPA) in the rat (Fombonne, 2003). In humans, VPA is widely used as an antiepileptic, and also prescribed against migraines (Wieck, & Jones, 2018). VPA exposure in the first trimester of gestation represents the highest risk for the child to develop autism (Rapin, & Tuchman, 2008). The idea of early embryogenesis as the critical time for autism led to development of the VPA rat model. Previous animal studies also reported that VPA induced more extensive behavioral and molecular alterations in males than in females (Kim et al., 2013). However, little attention has been paid to how ASD in males differs from those in females, particularly how neurobiological differences contribute to the experience of autism in both males and females.

It is suggested that changes in the functioning of cortical areas play an important role in the pathophysiology of autism (Lord et al., 2006). The experimental studies reveal that the medial prefrontal cortex (mPFC) is heavily involved in ASD associated brain functions: in rodents, acute optogenetic manipulation within mPFC disrupts normal social exploration (Yizhar, et al., 2011); in monkeys, lesions of the mPFC cause loss of social skills (Bachevalier, & Mishkin, 1986).

Studying sex-specific behavioral characteristics of social behavior, as well as anatomical changes in cortical tissues, specifically in the mPFC- in rats prenatally exposed to VPA will enhance our understanding of the neuropathology of autism and help clarify how differences in neurobiology may underlie differences in behavior.

## Materials and Methods

***Animals and ethics.*** Outbred albino rats were procured from the Laboratory Animal Division of the Ivane Beritashvili Center of Experimental Biomedicine. All experimental procedures were conducted in accordance with the European Communities Council Directive Guidelines for the care and use of Laboratory animals (2010/63/EU – European Commission) and approved by the animal care and use committee at the Ivane Beritashvili Center of Experimental Biomedicine. The rats were housed under standard laboratory conditions, with controlled temperature (22 ± 2°C), relative humidity (25 ± 5%), and a 12-hour light-dark cycle, and provided with ad libitum access to food and water.

**The VPA rat model of autism.** For experimental purposes, male and female rats were allowed to mate overnight. The morning following mating, vaginal secretion was collected, and if spermatozoa were present, it was designated as day 0.5 of pregnancy. On the 12.5th day of gestation, half of the pregnant rats received a single intraperitoneal injection of 500 mg/kg sodium valproate (Sigma-Aldrich) dissolved in 0.9% saline, while the other half (control group) was administered by saline alone. Females were left undisturbed to rear their offspring until postnatal day 20, at which point the male and female pups were weaned. Subsequently, the 6-month-old offspring (male – M, female – F) from both the saline and VPA-exposed dams were assigned to one of four experimental groups before undergoing behavioral experiments: Contr(M) and Contr(F) – prenatally saline-injected male and female groups, VPA(M) and VPA(F) – prenatally VPA-injected male and female groups.

**Three chamber social interaction test.** Social behavior was assessed using a three-chamber social interaction testing apparatus (measuring 60 cm x 120 cm x 50 cm) following previously established protocols (Mirza, & Sharma, 2018) with some modification. The testing procedure comprised three sessions: 5-minute habituation phase, 10-minute sociability phase (a stranger rat1-S1 was randomly placed in one side chamber under a small wire cage) and the final 10-minute social novelty preference phase (a novel stranger rat 2 – S2 was introduced into the previously empty chamber). The time the rat spent in each chamber and the time spent sniffing each cage was recorded. For both phases were quantified the social

exploration indexes which in sociability phase was calculated as time spent sniffing the S1 cage divided by time sniffing the empty cage. In the social preference phase the social exploration index was calculated as the time spent sniffing the S2 cage divided by the time spent sniffing the S1 cage.

**Immunohistochemistry.** At the end of the behavioral experiments, a random sample (n = 4) for each group of animals was used for histological studies to determine the effect of VPA on the number of NeuN-immunoreactive (neuronal nuclear antigen – a biomarker for neurons) cells in the mPFC. All reagents and buffers were purchased from Abcam and immunostaining was conducted under the appropriate protocols (https://www.abcam.com/en-us/technical-resources/protocols/ihc-with-samples-in-paraffin). A total of 6-10 sections of PFC levels from experimental and control animals were selected to assess the effects on NeuN cells in the PFC. Cell counting was performed blindly, using systematic random sampling. A 2-dimensional counting grid (1000 μm x 1000 μm) was used at 40x magnifications.

**Statistical analysis.** Statistical analysis was performed using SigmaStat statistical software. All the data were expressed as a mean ± standard error of the mean (SEM). Differences were considered significant when $p<0.05$. Data for all behavioral parameters as well as immunohistochemical results were statistically analyzed by two-way ANOVA followed by post hoc comparisons. Student's t-test was used to compare the mean values of two independent groups.

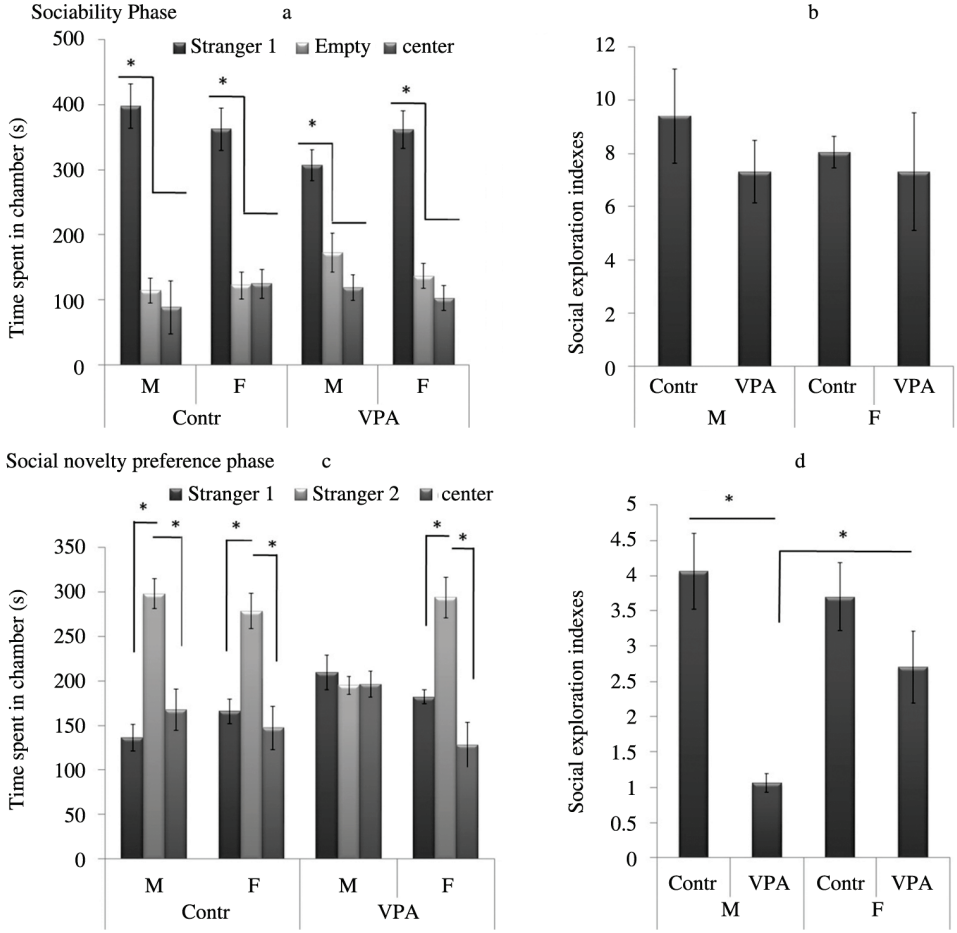


**Fig. 1.** Sex-related social behavior abnormalities in a rat model of autism induced by VPA. Three chamber social interaction test encompassing (a, c) stay duration in each chamber and (b, d) social exploration indices in the sociability and social novelty preference phases. Data are expressed as the mean ± SEM. * $p < 0.001$.

## Results

### Social behavior

***Sociability test.*** In a three-chamber apparatus for testing social behavior, male and female rats exposed to VPA, as well as rats in the control group, spent more time in the chamber with a stranger than in the empty chamber, indicating normal social behavior. Two-way ANOVA showed no significant effect of group ($F_{3,137}$ = 0.0145, $p$ = 0.998) but showed a significant effect of chambers ($F_{2,137}$ = 102.529, $p < 0.001$) and no significant interaction between group and chambers ($F_{6,137}$ = 1.719, $p$ = 0.122). Post hoc analysis revealed a significant difference between the time spent in the chamber containing the stranger rat and in an empty chamber in all groups ($p < 0.001$; Fig. 1a). Two-way ANOVA showed no significant difference in social exploration indices between control and VPA-exposed groups of rats ($F_{1,45}$ = 0.427, $p$ = 0.517) as well as between male and female rats ($F_{1,45}$ = 0.104, $p$ = 0.749). There is not a statistically significant interaction between group and sex factors ($F_{1,45}$ = 0.0952, $p$ = 0.759; Fig 1.b).

***Social novelty preference test.*** In the social novelty preference phase, social preference between the familiar rat (S1) and the novel rat (S2) was assessed by measuring staying time in each chamber (Fig. 1c). Two-way ANOVA showed no significant effect of group ($F_{3,137}$ = 0.0334, $p$ = 0.992), a significant effect of chamber ($F_{2,137}$ = 37.991, $p < 0.001$) and interaction between group and chamber ($F_{6,137}$ = 6.370, $p$ = 0.001). Post hoc analysis revealed a significant difference between the time spent in the chamber containing the S2 rat versus the chamber containing the S1 rat in male and female rats from control group ($p < 0.001$, in both cases) as well as in VPA- exposed female rats ($p < 0.001$). No significant differences were found between the time spent in the side chambers in male rats exposed to VPA ($p$ = 0.815).

For social exploration indices, a two-way ANOVA showed a significant effect of group ($F_{3,137}$ = 22.601, $p < 0.001$) as well as a significant interaction between group and sex factors ($F_{1,45}$ = 5.758, $p$ = 0.021). Post hoc analysis revealed a significant difference between male and female rats in the VPA-exposed rats ($p$ = 0.008), whereas no difference between male and female rats was identified in the control group ($p$ = 0.519) Post hoc analysis revealed also a significant difference between control and VPA-exposed male rats ($p < 0.001$), whereas no significant difference ($p$ = 0.101) between control and VPA-exposed female rats was observed (Fig. 1d).

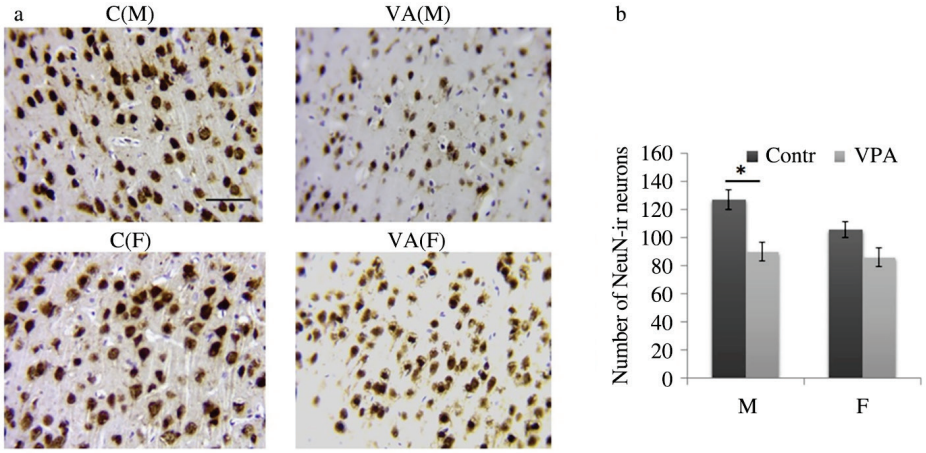


**Fig. 2.** Effect of prenatal exposure to VPA on the number of NeuN-ir cells in the mPFC in male and female rats. a) The representative microphotographs of the NeuN-ir staining in the mPFC in control and VPA-exposed groups of rats, b) the mean number of NeuN-ir neurons in the mPFC in control and VPA-exposed groups of rats. Data are given as mean ± SEM. * $p < 0.002$. Scale bar 40 μm.

**Number of NeuN-immunoreactive neurons in the PFC.** The number of NeuN-immunoreactive (NeuN-ir) neurons in the mPFC was counted in all groups of rats. The t-test revealed a significant difference between control male rats and males exposed to VPA ($P = 0.005$), whereas no significant difference was observed between control and VPA-exposed female rats ($P = 0.056$). Representative micrographs and the mean number of NeuN-ir neurons in the mPFC in male and female rats from the control and VPA-exposed groups are shown in Fig. 2.

## Discussion

The results of the present study indicate that prenatal administration of VPA to rats can induce sex-specific ASD-like behavioral patterns accompanied by some anatomical changes in the mPFC. In particular, the results of the experiments presented indicate that prenatal exposure to VPA on the12.5th day of pregnancy has a long-term effect on the postnatal behavior of male offspring. Compared with control group, male rats prenatally exposed to VPA demonstrate deficit in social behavior - reduced social exploration and impaired social novelty preference assessed in a three-chamber task. Female rats exposed to VPA did not show such marked changes in social behavior. The loss of cells in the mPFC also differed between the sexes. Immunohistochemical evaluation shows a significant decrease in the number of NeuN-ir neurons in the mPFC in male rats exposed to VPA, whereas cell loss is non-significant in females. These findings suggest that female rats are less vulnerable to VPA exposure. Overall, these findings revealed sex-related anatomical changes in the mPFC that may be responsible for sex-dependent social behavior alterations in rats exposed to VPA.

It is interesting to note that most autism studies tend to include only males in neurobiological research, which is explained by the variability of data in females caused by hormonal fluctuations associated with the female reproductive cycle. Researchers know that the female reproductive cycle introduces variability into certain physiological measures, and this can be seen as a confounding factor in studies seeking to identify stable neurobiological markers of autism. However, a growing body of research is challenging gender bias in autism research and emphasizing that hormonal fluctuations, once considered a serious complication, do not necessarily interfere with neurobiological research as previously thought. A study by Becker et al. (2016) investigated whether female rats are more variable than male rats in scientific reports of neuroscience-related traits. They concluded that even when female rats were used in neuroscience experiments without taking into account the stage of the estrous cycle, their data were no more variable than that of males. As they note, this is true for behavioral, electrophysiological, neurochemical, and histological measures. A study by Markham et al. (2007) compared social recognition memory in young sexually mature female rats (aged 3–5 months) during proestrus and estrus and found that performance remained stable throughout all phases of the estrous cycle. Therefore, the present study did not take into account hormonal variability associated with the female reproductive cycle and the experiments were conducted without considering the estrous cycle phases in female rats.

Another topic requiring discussion concerns the debate over the existence of anatomically comparable structures and functions of the mPFC in rodents and humans. In humans, the mPFC is identified as a crucial brain region of social cognition and behaviors (Bicks, et al., 2015). Patients with lesions of the mPFC exhibit severe social impairment (Forbes, & Grafman, 2010). Neuroimaging studies have revealed altered PFC activity in individuals with ASD during social tasks (Sumiya, et al., 2020).

However, there is some controversy regarding the existence of an anatomically comparable PFC structure in rodents (Wise, 2008), and the association between mPFC activity and abnormal social

behavior in rodents (Wang, et al., 2014). Nevertheless, some evidence suggests that there is significant functional homology between human and rodent mPFC structures. Bicks et al. (2015) suggest in their review article that comparable brain regions and neural circuits generally contribute to common social behavior in rodents and humans and note that translatability of rodent behavioral patterns is achieved by using ethologically relevant behavioral paradigms and measuring behavior-induced brain activation (Bicks, et al., 2015; Kim, et al, 2015). In this regard, it is interesting to note that, using a three-chamber behavioral paradigm that assesses social behavior in rodents researchers showed that neural activity in the mPFC correlates with social-approach behavior in mice (Lee, et al., 2016). Another study examined whole brain c-Fos activity in a social context and found that the mPFC in mice was activated during social interactions (Kim, et al, 2015), suggesting a relevant involvement of the PFC in social behavior. Therefore, it is reasonable to postulate that comparable brain regions and neural circuits generally contribute to common social behaviors in rodents and humans (Bicks, et al., 2015). Accordingly, it can be assumed that the more pronounced social behavior abnormalities observed in male rats in this study may reflect the higher prevalence of autism among males in humans.

Our immunohistochemical experiments showed a reduced total number of NeuN-ir cells in the mPFC in male rats prenatally exposed to VPA compared to females and unexposed control males. Our results are in accordance with a number of other studies that consistently have found that VPA exposure causes a significant reduced brain mass and cortical neurons loss (Favre, et al., 2013). However, our findings contrast with studies showing a significantly higher total number of neocortical neurons in VPA-exposed pups compared with controls (Sabers, et al., 2014). The difference from our results may reflect the fact that in this study assessment was performed at postnatal day 23, while in our study immunohistochemical evaluations were conducted in adult rats. Additional difficulty for interpretation is due to the difference in VPA administration protocols. The study of Sabers et al. (Sabers, et al., 2014) used doses of VPA that were significantly lower (20 and 100 mg/kg/day) and were administered chronically, whereas our study used a single acute high dose of VPA (500mg/kg) administered on day 12,5 of gestation. In addition, longitudinal and cross-sectional MRI studies of brain size in autistic humans over a life span of 2 to 50 years have shown age-related anatomical abnormalities in autism, characterized by early brain overgrowth in infancy and early childhood followed by an accelerated rate of decline in size, degeneration, atrophy and neuronal loss of certain structures from adolescence to middle age (Courchesne, et al., 2011). It should be noted that in our experiments the number of neurons in the PFC was estimated in male rats at the age of 6 months, which corresponds to human adulthood. Accordingly, the decrease in the number of neurons in male rats prenatally exposed to the VPA compared to the control group, is consistent with the data of age-related neuroanatomical changes described in autistic humans.

## Conclusion

The experiments in this study revealed that male rats prenatally exposed to VPA demonstrate pronounced social behavior abnormalities accompanied by a significant decrease in the number of NeuN-ir neurons in the mPFC, compared with males from control group or VPA-exposed females. It can be assumed that the observed anatomical changes in the mPFC may be responsible for deficit in social behavior in male rats. However, further studies focusing on the role of biological sex are necessary to better understand how male and female neurobiological signatures differ, and how these differences translate into abnormal ASD-like behavior.

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*ადამიანისა და ცხოველთა ფიზიოლოგია*

# სქესთან დაკავშირებული ქცევითი დარღვევები და ნეიროანატომიური ცვლილებები აუტიზმის ვირთაგვას მოდელში

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**წარმოდგენილ ნაშრომში შეისწავლებოდა სოციალური ქცევის სქესთან დაკავშირებული სპეციფიკური ქცევითი მახასიათებლები, ასევე ანატომიური ცვლილებები მედიალურ პრეფრონტალურ ქერქში (mPFC) აუტიზმის ვირთაგვას მოდელში. ექსპერიმენტული მიზნებისთვის, ორსულობის მე-12,5 დღეს, ვირთაგვების ნახევარს უტარდებოდა 500 მგ/კგ ნატრიუმის ვალპროატის (ვალპროის მჟავა – VPA), ხოლო მეორე ნახევარს (საკონტროლო ჯგუფი) მხოლოდ ფიზიოლოგიური ხსნარის ერთჯერადი ინტრაპერიტონეალური ინექცია. ამ ორი ჯგუფის 6 თვის შთამომავლობა ქცევითი ექსპერიმენტების ჩატარებამდე განაწილდა ოთხ ექსპერიმენტულ ჯგუფში: პრენატალურად ფიზიოლოგიური ხსნარის ზემოქმედების მქონე მამრი (male – M) – Contr(M) და მდედრი (female – F) – Contr(F) ვირთაგვების ჯგუფები და პრენატალურად VPA-ს ზემოქმედების მქონე მამრი – VPA(M) და მდედრი – VPA(F) ვირთაგვების ჯგუფები. სოციალური ქცევა შეფასდა სამკამერიანი სოციალური ურთიერთქმედების ტესტირების აპარატის გამოყენებით. VPA-ს ზემოქმედების ეფექტები mPFC-ში NeuN-იმუნორეაქტიული (NeuN-ir) უჯრედების რაოდენობაზე შეფასდა იმუნოჰისტოლოგიურ ექსპერიმენტებში. შედეგებით გამოვლინდა სოციალური ქცევის დეფიციტი – სოციალური კვლევის შემცირება და სოციალური სიახლისადმი უპირატესობის დარღვევა პრენატალურად VPA-ს ზემოქმედების მქონე მამრ ვირთაგვებში. VPA-ს ზემოქმედების მქონე მდედრ ვირთაგვებს სოციალურ ქცევაში მნიშვნელოვანი ცვლილებები არ აღენიშნებოდათ. იმუნოჰისტოქიმიურ ექსპერიმენტებში mPFC-ში NeuN-ir უჯრედების რაოდენობის მნიშვნელოვანი შემცირება გამოვლინდა პრენატალურად VPA-ს ზემოქმედების მქონე მამრ ვირთაგვებში, მაშინ როდესაც NeuN-ir ნეირონების რაოდენობის შემცირება VPA-ს ზემოქმედების მქონე მდედრებში უმნიშვნელოა. მიღებული შედეგების საფუძველზე შეიძლება ვივარაუდოთ, რომ mPFC-ში გამოვლენილი ცვლილებები პასუხისმგებელია სოციალური ქცევის დარღვევაზე VPA-ს ზემოქმედების მქონე მამრ ვირთაგვებში.**

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