

Age-Related Behavioral and Neuroanatomical Changes in a Rat Model of Autism

Khatuna Rusadze^{*}, Temur Naneishvili^{}, Maia Burjanadze[§],
Gela Beselia^{§§}, Nino Chkhikvishvili[§], Mariam Chighladze[§],
Barbare Nozadze[§], Manana Dashniani[§]**

^{*} Department of Biology, Akaki Tsereteli State University, Kutaisi, Georgia

^{**} Academy Member, Department of Behavior and Cognitive Function, Ivane Beritashvili Center of Experimental Biomedicine, Georgia

[§] Department of Behavior and Cognitive Function, Ivane Beritashvili Center of Experimental Biomedicine, Georgia

^{§§} Department of Physiology and Pharmacology, Petre Shotadze Tbilisi Medical Academy, Georgia

Abstract. In this study, a valproic acid (VPA) rat model of autism spectrum disorder (ASD) was used to investigate how deficits in social behavior manifest differently during two life stages corresponding to adolescence (30 ± 5 postnatal days) and adulthood (120 ± 10 postnatal days), and whether these behavioral changes are in line with anatomical alterations in the medial prefrontal cortex (mPFC). For experimental purposes, half of the pregnant rats received a single intraperitoneal injection of 500 mg/kg VPA, while the other half was administered by saline alone on the 12.5th day of gestation. Social behavior was assessed using the three-chamber social interaction testing apparatus. Immunohistochemical analyses were conducted to determine the effect of VPA exposure on the number of NeuN-immunoreactive cells in the mPFC. The results showed that during the sociability phase, rats of both ages exposed prenatally to VPA displayed the same level of interest in social interaction as control rats. However, during the social novelty preference phase, adult rats prenatally exposed to VPA exhibit a deficit in preference for social novelty. Immunohistochemical experiments revealed a significant reduction in the number of NeuN-immunoreactive neurons in the mPFC of adult rats exposed to VPA, whereas no cell loss was observed in adolescents. Overall, these findings revealed age-related anatomical changes in the mPFC that may be responsible for age-dependent social behavior alterations in rats exposed to VPA. These findings highlight the importance of the mPFC in social behavior and may contribute to our understanding of the dysfunctional processing of social information that underlies neurodevelopmental disorders. © 2026 Bull. Georg. Natl. Acad. Sci.

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

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by early-onset difficulties with social communication, repetitive behaviors and stereotypies. Although the etiology of autism remains unclear, emerging evidence from

clinical and experimental studies suggests that early alterations that change over time may be responsible. Environmental factors are known to influence brain development (MacNeil et al., 2009) and induce alterations at neuroanatomical, neurochemical and behavioral levels.

Animal models generated by exposure to environmental factors are important tools for understanding the pathogenic mechanisms of autism. The idea of early embryogenesis as the critical time for autism led to the development of the valproic acid (VPA) induced rat model. In humans, VPA is widely used as an antiepileptic drug, and is also prescribed for the treatment of bipolar disorders and migraines (Nevitt, et al., 2017; Wieck, & Jones, 2018). The possible association between *in utero* VPA exposure and autism was first reported by Christianson et al. (1994). Prenatal exposure to VPA is associated with an increased risk of developing ASD in humans and the development of autistic-like behaviors in rodents. Strong clinical validation of the model is given by observation that offspring of rats exposed to a single dose of VPA on embryonic day 12.5, present neuroanatomical and behavioral characteristics similar to human autism (Rodier, 1997). Taking into account striking behavioral and anatomical parallels between the VPA model and human autism, the VPA-induced rat model is well suited to study structural, morphological and neurochemical alterations in order to elucidate the potential neuropathology of autism.

Certain ASD behavioral symptoms and neurobiological parameters can persist, whereas others may improve throughout the lifespan (Lian, et al., 2025), but the specific patterns of changes have not been clearly delineated. According to a patch-clamp study, VPA treatment induces age-related changes (in offspring ranging from adolescence to adulthood) in the neuronal activity of pyramidal neurons in the medial prefrontal cortex (mPFC) (Májer, et al., 2024). In this study, rats prenatally exposed to VPA pass from an enhanced long-term potentiation (LTP) phenotype in early life to a LTP deficit phenotype in adulthood. It is suggested that the mPFC is critical in behaviors related to ASD. Deficits in social interaction are more clearly linked to abnormalities in mPFC function and these are consistently reported in the VPA rat (Roullet, et al.,

2013). Following damage or stimulation in this area, impaired social skills were observed (Yizhar, et al., 2011; Bachevalier, & Mishkin, 1986).

In this study, a VPA rat model of ASD, was used to investigate how social behavior deficits manifest differently during two life stages corresponding to adolescence (30 ± 5 postnatal days) and adulthood (120 ± 10 postnatal days), in line with anatomical changes in the mPFC.

Materials and Methods

Animals and ethics. Outbred albino rats were obtained from the Laboratory Animal Division of the Ivane Beritashvili Center of Experimental Biomedicine. Rats of the same age were housed in groups (three to four per cage) in cages in a room with a 12-hour light/dark cycle. The room temperature was maintained at $23 \pm 2^\circ\text{C}$. Throughout the study, the rats were given unlimited access to food and water. All experimental procedures adhered to the European Communities Council Directive Guidelines for the care and use of laboratory animals (2010/63/EU – European Commission) and received approval from the animal care and use committee at the Ivane Beritashvili Center of Experimental Biomedicine.

The VPA rat model of autism. Time-mated female rats received a single intra-peritoneal dose of 500 mg/kg sodium valproate (Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline at gestational day 12,5 (Schneider, & Przewłocki, 2005). The other half (control group) was administered by saline alone. Females were left undisturbed to rear their offspring until postnatal day (PD) 21, at which point the pups were weaned. Subsequently, the prepubertal adolescent (1 month old, PD 30 ± 5) and young adult (3 months old, PD 120 ± 10) offspring males, from both the saline and VPA-exposed dams were assigned to one of four experimental groups before undergoing behavioral experiments: Control1 and Control3 - prenatally saline-injected adolescent and adult groups, VPA1

and VPA3 – prenatally VPA-injected adolescent and adult groups.

Three chamber social interaction test. The three-chamber social interaction test was conducted to assess sociability and social novelty preference (Mirza, & Sharma, 2019). The testing apparatus consisted of a rectangular three-chambered box (measuring 60 x 120 x 50 cm) and a lid with a video camera. The dividing walls of the chamber were made of transparent plastic, with small opening doors allowing access to each chamber. The testing procedure comprised three sessions. In the initial session the test rat were first placed in the middle chamber and allowed to explore the entire test chamber for 5 minutes. After the 5-minute period, the test rat was placed in a holding cage, and a male rat (stranger 1 – S1) with no prior contact with the test rat was enclosed in one of the wire cages. Next, the test rat were returned to the middle chamber and allowed to explore for 10 minutes (sociability test). In the final 10-minute social novelty preference phase, a second unfamiliar rat (stranger 2 – S2) was enclosed in the wire cage on the opposite side. The time the rat spent in each side compartment was recorded. The time during which the rat explored the wire cage from a distance of up to 1 cm, referred to as sniffing time, was also recorded.

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 (NeuN – 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 mPFC levels from experimental and control animals were selected to assess the effects of VPA exposure on the number neurons in the mPFC. Cell counting was performed blindly, using systematic random

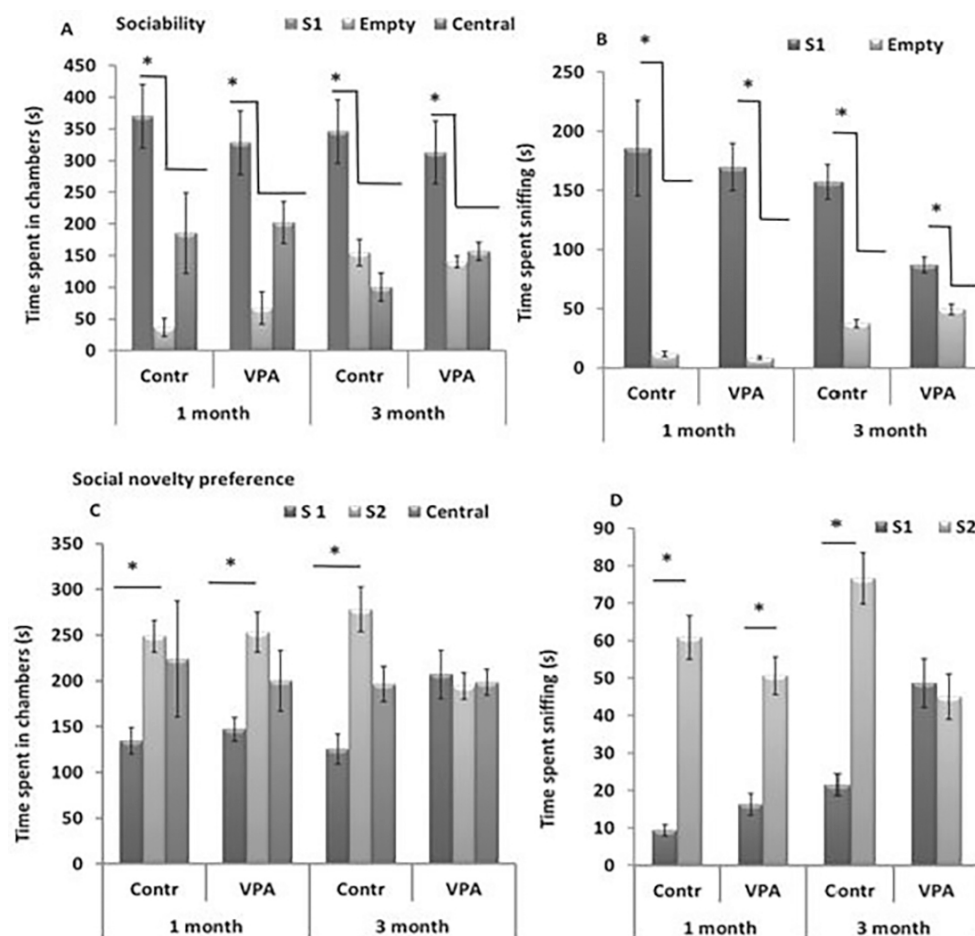
sampling. A 2-dimensional counting grid (1000 μm x 1000 μm) was used at 40x magnifications.

Statistical analysis. Statistical analysis was conducted using SigmaStat statistical software. One- or two-way analysis of variance (ANOVA) was used to analyze the effects of VPA exposure and age on behavior, followed by post-hoc comparisons where necessary and appropriate. Differences were considered significant when $p < 0.05$. The values in the graphs are expressed as the mean $\pm$ SEM.

Results

Three chamber social interaction test.

Sociability test. To assess social behavior, we first compared the performance of rats of all groups (Contr1, VPA1, Contr3 and VPA3) in a three-chamber test of sociability, which examines the preference for a chamber containing a stranger rat versus an empty chamber by measuring staying time in each compartment. Two-way ANOVA showed no significant effect of group ($F_{3,167} = 0.00967, p = 0.999$), and significant effect of chamber ($F_{2,167} = 39.958, p < 0.001$). There is no significant interaction between group and chamber ($F_{6,167} = 1.854, p = 0.092$). Post hoc analysis revealed a significant difference between the time spent in the chamber containing the stranger rat and in an empty and central chamber in all groups ($p < 0.001$; Fig. 1A). Two-way ANOVA for sniffing time (exploration the wire cages from a distance of up to 1 cm) showed no significant effect of group ($F_{3,111} = 0.145, p = 0.933$), and significant effect of chamber ($F_{1,111} = 104.723, p < 0.001$). There is no significant interaction between group and chamber ($F_{3,111} = 0.986, p = 0.402$). Post-hoc analysis revealed a significant difference in the time spent sniffing the cage containing an unfamiliar rat versus the empty cage across all groups ($p < 0.001$; Fig. 1B). The results showed that control rats and rats exposed prenatally to VPA of both ages spent more time in the chamber with an unfamiliar rat compared to an empty wire cage and spent more



* $p < 0.001$. Data are expressed as the mean $\pm$ SEM.

Fig. 1. Social behavior in the sociability and social novelty preference phases of a three-chamber task in control rats and rats prenatally exposed to VPA at two life stages corresponding to adolescence and adulthood: (A, C) stay duration in each chamber and (B, D) time spent sniffing each cage.

time sniffing the wire cage with rat than the empty wire cage. At this stage, the VPA treated both groups showed the same interest in social contacts as the control groups.

Social novelty preference test. In the social novelty preference phase a new rat was added in the previous empty chamber and social preference between familiar rat (S1) and novel rat (S2) was assessed by measuring staying time in each chamber. Two-way ANOVA showed no significant effect of group ($F_{3,167} = 0.00493$, $p = 1.000$), and no significant effect of chamber ($F_{2,167} = 0.464$, $p = 0.630$). There is no significant interaction between group and chamber ($F_{6,167} = 11.552$, $p < 0.001$). Post hoc analysis revealed a significant difference between

the time spent in the chamber containing the familiar rat (S1) and the novel rat (S2) in the Contr1 ($p < 0.001$), Contr3 ($p = 0.013$) and VPA1 ($p = 0.010$) groups, while difference between the time spent in the chamber containing S1 and S2 rat no significant difference was detected in VPA3 group ($p = 0.969$). Two-way ANOVA for sniffing time showed significant effect of group ($F_{3,111} = 5.071$, $p = 0.003$) and chamber ($F_{1,111} = 89.689$, $p < 0.001$), as well as statistically significant interaction between group and chamber ($F_{3,111} = 11.477$, $p < 0.001$). Post-hoc analysis revealed a significant difference in the time spent sniffing the cage containing an unfamiliar rat (S2) versus the cage containing a familiar (S1) rat in the Contr1, Contr3 and

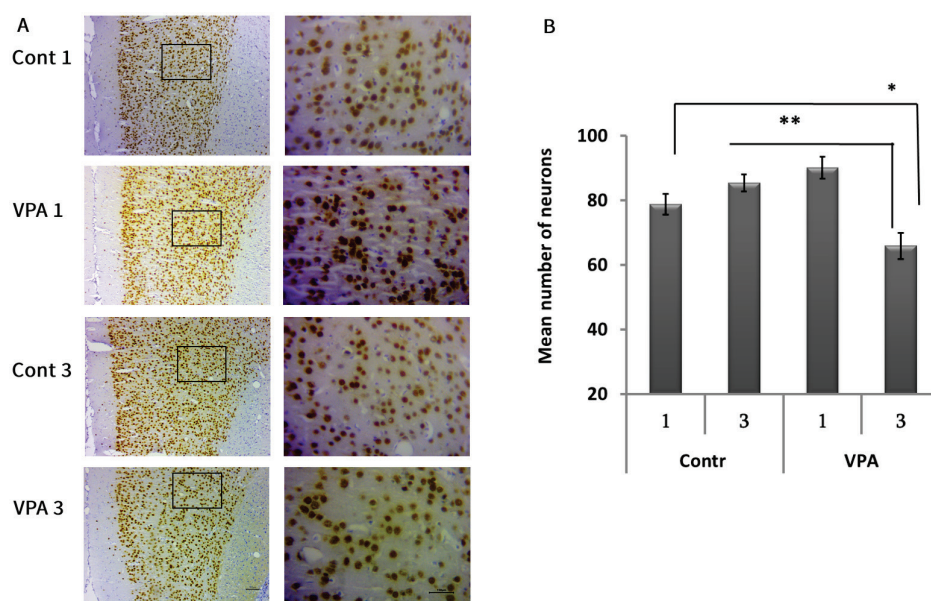


Fig. 2. The effect of prenatal VPA exposure on the number of NeuN- immunoreactive neurons in the mPFC in adolescent and adult rats. (A) Representative microphotographs of NeuN-ir cells in the mPFC of control adolescent and adult rats, as well as rats exposed to VPA. The left panels show low-magnification images (10 μ m), whereas the right panels show high-magnification images (40 μ m) (B) Mean number of NeuN-immunoreactive neurons in the mPFC in the control group and the group of rats exposed to VPA. Data are presented as the mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.001$.

VPA1 ($p < 0.001$) groups. In the VPA3 group, no significant difference was observed in the time spent sniffing the unfamiliar rat (S2) compared to the familiar rat (S1) ($p = 0.651$). The results showed that control rats of both ages and adolescent rats exposed prenatally to VPA spent more time in the chamber with an unfamiliar (S2) rat compared to chamber with familiar (S1) rat and spent more time sniffing the wire cage with unfamiliar rat than the wire cage with familiar rat. Adult rats exposed to VPA prenatally spent equal amounts of time in both chambers (Fig. 1C) and spent equal amounts of time sniffing the cages with familiar and unfamiliar rats (Fig 1D). At this stage, adult rats that received VPA did not show a preference for social novelty.

Number of NeuN-immunoreactive neurons in the mPFC. NeuN-immunoreactive cells numbers in the mPFC were counted in all groups of rats. The one-way ANOVA analysis showed a statistically significant difference between groups ($P = <0.001$). Post-hoc analysis revealed a significant difference between adult rats exposed to VPA and control

adult ($P < 0.001$) and adolescent rats ($P = 0.039$), as well as adolescent rats exposed to VPA ($P < 0.001$). In adolescents cell number in the mPFC was increased compared to age much controls but difference between groups did not reach statistical significance ($P = 0.082$). Figure 2 shows the average number of NeuN-immunoreactive neurons in the mPFC, as well as representative micrographs of control rats and rats exposed to VPA, from both age groups.

Discussion

Using a VPA rat model of ASD, the present study examined how deficit in social behavior manifested differently in two periods representing adolescent, and adulthood life stage in line with anatomical changes in the mPFC. The results showed that, during the sociability phase, rats of both ages that had been exposed to VPA in utero displayed the same level of interest in social interaction as control rats of the same age. However, during the social novelty preference phase, a difference was observed between the adult control rats and rats exposed to

VPA. At this stage, adult rats that had received VPA did not show a preference for social novelty. Immunohistochemical experiments revealed a significant reduction in the number of NeuN-immunoreactive neurons in the mPFC of adult rats exposed to VPA, whereas no cell loss was observed in adolescents. Overall, these findings revealed age-related anatomical changes in the mPFC that may be responsible for age-dependent social behavior alterations in rats exposed to VPA.

Our anatomical findings are consistent with the results of MRI studies of individuals with autism, which have identified age-related anatomical abnormalities in autism characterized by excessive brain growth in infancy and early childhood, followed by accelerated atrophy and loss of neurons in certain structures from adolescence to middle age (Courchesne, et al., 2011). The decrease in the number of neurons in male rats prenatally exposed to the VPA compared to the control groups, is consistent with the data of age-related neuroanatomical changes described in autistic humans.

It is interesting to note that in the present study, adolescents cell number in the mPFC was increased compared to age-matched controls but the difference between groups did not reach statistical significance, possibly due to the relatively small number ($n = 4$) of animals in each age group. Since ASD is associated with excessive early brain overgrowth and an excess of neurons, Courchesne et al. (2024) conducted interesting experiments. They investigated whether variation in patient-derived embryonic brain cortical organoids (lab-grown tissue model derived from human pluripotent stem cells) enlargement is correlated with variations in ASD social severity and ASD social subtypes at early ages. The ASD brain cortical organoids grew at an accelerated rate, nearly 3 times faster than controls.

In addition to quantitative changes in the neurons of the mPFC, other neuroanatomical changes have been reported in animals exposed to VPA. Codagnone, et al. (2015) found that in the mPFC

the typical organization of cortical columns was preserved in animals exposed to VPA, but the inter-neuronal space was wider than in the control group. Using the *in utero* VPA exposure model, local changes in principal neuron connectivity have been found in the rat mPFC (Rinaldi, et al., 2008a,b). Changes in local and distant connectivity in the brain have been proposed as a possible cause of autistic behavior (Geschwind & Levitt, 2007).

There is some controversy regarding the association between mPFC activity and abnormal social behavior in rodents (Wang et al., 2014). However, there is growing evidence that the mPFC plays a key role in both the formation of social behavior and recognition memory. The ability to distinguish between familiar and unfamiliar conspecifics, known as social recognition memory, is a fundamental ability for social species such as rodents and humans. It is known that the activity of glutamatergic ionotropic receptors mediates synaptic plasticity and the consolidation of various types of memory. Marcondes, et al. (2020) demonstrated that blocking glutamatergic receptors by administering NMDA and AMPA receptor antagonists to the mPFC prevents the long-term consolidation of social recognition memory.

Conclusion

The results of this study revealed an age-related neuronal loss in the mPFC, corresponding to deficits in social behavior in rats exposed to VPA during the prenatal period. These findings highlight the importance of the mPFC in social behavior and may contribute to our understanding of the dysfunctional processing of social information underlying neurodevelopmental disorders.

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ადამიანისა და ცხოველთა ფიზიოლოგია

ასაკთან დაკავშირებული ქცევითი და ნეიროანატომიური ცვლილებები აუტიზმის ვირთაგვას მოდელში

ხათუნა რუსადე*, თემურ ნანეიშვილი**, მაია ბურჯანაძე§, გელა ბესელია§§, ნინო ჩხიკვიშვილი§, მარიამ ჭილაძე§, ბარბარე ნოზაძე§, მანანა დაშნიანი§

* აკაკი წერეთლის სახელმწიფო უნივერსიტეტი, ბიოლოგიის დეპარტამენტი, ქუთაისი, საქართველო

** აკადემიის წევრი, ივანე ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ქვევისა და კოგნიტურ ფუნქციათა ლაბორატორია, თბილისი, საქართველო

§ ივანე ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ქვევისა და კოგნიტურ ფუნქციათა ლაბორატორია, თბილისი; საქართველო

§§ პეტრე შოთაძის თბილისის სამედიცინო აკადემია, ფიზიოლოგიისა და ფარმაცოლოგიის დეპარტამენტი, საქართველო

წარმოდგენილ კვლევაში, აუტისტური სპექტრის აშლილობის (ASD) ვალბროის მკვას (VPA) ვირთაგვას მოდელის გამოყენებით, შესწავლილ იქნა სოციალური ქცევისა და მედიალური პრეფრონტალური ქერქის (mPFC) ანატომიური ცვლილებები სიცოცხლის ციკლის ორ ეტაპზე, რომლებიც შეესაბამება მოზარდობის (დაბადებიდან 30 ± 5 დღე) და ზრდასრულობის (დაბადებიდან 120 ± 10 დღე) პერიოდებს. ორსულობის მე-12,5 დღეს, ვირთაგვების ნახევარს ჩაუტარდა 500 მგ/კგ VPA-ს, ხოლო მეორე ნახევარს მხოლოდ ფიზიოლოგიური ხსნარის ერთჯერადი ინტრაპერიტონეალური ინექცია. VPA-ს ზემოქმედების ეფექტები სოციალურ ქცევაზე შეფასდა სამკამერაიანი სოციალური ურთიერთქმედების ტესტირების აპარატის გამოყენებით, ხოლო mPFC-ში NeuN-იმუნორეაქტიული უჯრედების რაოდენობა ფასდებოდა იმუნოჰისტოქიმიურ ექსპერიმენტებში. მიღებული შედეგების თანახმად სოციალურობის ფაზაში, ორივე ასაკის VPA-ს ზემოქმედების მქონე და შესაბამისი ასაკის საკონტროლო ჯგუფის ვირთაგვებს შორის განსხვავება არ გამოვლინდა, თუმცა, სოციალური სიახლის უპირატესობის ფაზაში, განსხვავება დაფიქსირდა ზრდასრულ საკონტროლო ვირთაგვებსა და VPA-ს ზემოქმედების მქონე ვირთაგვებს შორის. ამ ეტაპზე, VPA-ს ზემოქმედების მქონე ზრდასრულ ვირთაგვებში გამოვლინდა სოციალური სიახლისადმი უპირატესობის დეფიციტი. იმუნოჰისტოქიმიურმა ექსპერიმენტებმა აჩვენა NeuN-იმუნორეაქტიული ნეირონების რაოდენობის მნიშვნელოვანი შემცირება ზრდასრული VPA-ს ჯგუფის ცხოველების mPFC-ში, მაშინ როცა მოზარდებში უჯრედების დაკარგვა არ გამოვლინდა. ამრიგად, ასაკთან დაკავშირებული ანატომიური ცვლილებები mPFC-ში შესაძლოა პასუხისმგებელი იყოს ასაკთან დაკავშირებულ სოციალური ქცევის ცვლილებებზე VPA-ს ზემოქმედების მქონე ვირთაგვებში. მიღებული შედეგები ადასტურებს mPFC-ის მნიშვნელობას სოციალურ ქცევის ორგანიზაციაში და ხელს შეუწყობს ნეიროგანვითარების დარღვევების განმაპირობებელი სოციალური ინფორმაციის გადამუშავების პროცესების შეცნობას.

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