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Experimental Pathology of Higher Nervous Activity

Disorders of Motor Activity in a Model of Autism Spectrum Disorders

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Autism, or autism spectrum disorder (ASD), is a multifactorial disease characterized not only by disturbances in psychoemotional status and social interaction, but also by somatic dysfunctions. A number of studies have also reported changes in the musculoskeletal system in patients with ASD. We report here studies using video movement analysis demonstrating decreases in horizontal and vertical motor activity; in addition, deviant movements were recorded, indicating deranged locomotor activity and increased anxiety in rats with the valproate model of autism. However, a mechano-myographic study did not reveal any significant changes in the contractility parameters of isolated skeletal muscles of rats with the model of ASD. This leads to the conclusion that general differences in movement may be an independent factor in the diagnosis of autism. A more rigorous study using a larger group and detailed kinematic analysis may help with further evaluation of motor variability as a potential diagnostic and prognostic marker for ASD.

Keywords: autism, autism spectrum disorder, ATP, P2 receptors, skeletal muscles, neuromuscular junction, neurotransmission.

Introduction. The US Autism and Developmental Disabilities Monitoring Network estimated that one in 36 eight-year-old children (approximately 4% of boys and 1% of girls) had an autism spectrum disorder (ASD) in 2020 [Maenner et al., 2023]. ASD is a neurodevelopmental disorder characterized by impairments in social interaction, a predominance of stereotypical behavior patterns, and a narrowed range of interests [Widiger and Hines, 2022]. Despite its increasing prevalence, ASD remains a disorder whose pathophysiology is poorly understood and the search for drug therapies is slow. The etiology of ASD remains unknown; some authors associate the occurrence and progression of ASD with the influence of genetic predisposition and environmental factors [Taylor et al., 2020]. Among the main pathophysiological mechanisms of the development of ASD are oxidative stress, neuroinflammation, various immune disorders, and mitochondrial dysfunction [Saffari et al., 2019; Citrigno et al., 2020; Doi et al., 2022; Singh et al., 2023].

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The role of animal models has been decisive in making significant progress in understanding the complex pathophysiology of ASD [Qi et al., 2021]. A useful model, which is supported by exhaustive animal studies, is based on exposing rodents to valproic acid (VPA); this shows striking similarities to the behavior, anatomy, and cellular and molecular changes observed in patients with autism. Many anatomical studies have also demonstrated that the VPA model provides good reproduction of central nervous system dysplasia in ASD, providing a valuable tool for studies of the underlying mechanism of ASD [Mabunga et al., 2015].

Published studies have demonstrated the involvement of the purinergic signaling pathway in the development of the nervous system, mediated by its influence on mechanisms such as cell proliferation, differentiation, formation of neuron-glial cell interactions, migration of neuronal precursors, and neurite outgrowth [Burnstock et al., 2011], though the link between anomalous purine metabolism and the etiology of ASD is still unclear. However, ontogenetic theory indicates that defects in early developmental processes contribute to the onset of various mental illnesses later in life [Ren et al., 2016; Williams et al., 2018; Courchesne et

al., 2019]. Additional evidence has emerged showing that purines, purine-metabolizing ectoenzymes, and purinoceptors are also involved in the pathophysiological processes of neural development [Fumagalli et al., 2017] and psychiatric disorders [Cieslak et al., 2016].

Depending on ligand, purinergic signaling receptors are divided into two main classes: P1 (adenosine receptors) and P2 (ATP/ADP and UTP/UDP receptors) [Burnstock, 2007]. The latter group includes P2X and P2Y receptors, which mediate hyperactivation of glial cells and the onset of inflammatory responses in the central nervous system (CNS) [Abbracchio and Ceruti, 2006; Inoue, 2008; Huang et al., 2019]. In addition, the expression of P2X7 receptors, which play a key role in the pathophysiology of CNS disorders and mediate the most severe signs of neuroinflammation, has been shown to be decreased in children with ASD [Lister et al., 2007; Naviaux et al., 2013].

Dysfunction of the purinergic signaling system is associated with the initiation of ASD, which in turn allows this signaling system to be seen as a potential therapeutic target. There is evidence that treatment of rats with the ASD model with suramin (20 mg/kg, i.p.) restores their communication abilities and reduces anxiety as measured using the elevated plus maze [Hirsch et al., 2020]. Treatment with suramin does not affect valproic acid-induced activation of P2X4 and P2Y2 receptors in the hippocampus or P2X4 receptor expression in the medial prefrontal cortex, though it normalizes elevated interleukin 6 (IL-6) levels [Smith et al., 2007].

Comorbidity has long been recognized in children with developmental disorders such as autistic disorder and attention deficit hyperactivity disorder [Gillberg et al., 1995; Watson et al., 2003]. And although ASD is regarded as a mental disorder, dysfunctions of other internal organs and systems may also be associated with ASD. Such features include, in particular, sensory abnormalities [Kern et al., 2006], sensory-motor deficits [Piek and Dyck, 2004], problems with fine and gross motor skills [Provost et al., 2007], movement disorders/motor skills [Green et al., 2009], balance problems [Minshew et al., 2004], muscle weakness [Hardan et al., 2003], and hypotonia [Ming et al., 2007].

Motor abnormalities have been recognized as an integral part of autism spectrum disorders [Ghaziuddin and Butler, 1988]. Moreover, observations have shown that movement abnormalities in autism are diverse and can cause disturbances in different parts of the central nervous system. The involvement of multiple structures is expected, because of the complex distribution of the motor system at the levels of the spinal cord, brainstem, cerebellum, and subcortical and cortical areas of the nervous system [Kingsley, 2000]. We suggested that in addition to the core behavioral symptoms of ASD, other neurodevelopmental problems associated with motor dysfunction might also be apparent in rats.

These observations made the purpose of this study to evaluate the general nature of changes in the motor activity of skeletal muscles in rats with a model of ASD.

Methods. ***Study object.*** The experiments used mongrel male laboratory rats weighing 160–240 g. Animals were kept with free access to food and water in conditions of natural alternation of daily illumination.

The following experimental groups were used:

1) control group (n = 12);

2) a group with the model of autism (MA, n = 12).

Rat model of ASD. High-dose valproic acid blocks enzymes involved in the deacetylation of histone proteins, which affects the expression of certain genes, modifying their functioning. Administration to pregnant females induces fetal valproate syndrome in the offspring of laboratory animals, this producing manifestations similar to those of ASD [Zheng et al., 2019]. Fetal valproate syndrome in the offspring was induced by giving female rats single s.c. injections of valproic acid sodium salt (500 mg/kg) in the shoulder area on days 12–13 of pregnancy. There was no increase in fetal deaths, no increase in postimplantation losses, and no decrease in litter size or fetal weight. Rats born after this exposure method were used in experiments at age six months. Pups of the same age, born to rats not exposed to drugs, were used as controls.

Open field test. The open field was a white square arena. The floor was divided into 25 rectangles of identical area to support visual recording of the animals' horizontal motor activity at the periphery, in 2/3 of the arena, and its center.

The open field (OF) test is used to study the innate characteristics of orientational and exploratory behavior and stress resistance [Kozlovsky and Kenunen, 1992]. This method was used to assess the orientational-exploratory reaction (OER) in terms of the number of squares crossed within the arena and the number of rearings onto the hind legs (vertical motor activity, VMA) under artificial lighting.

An animal placed in an unfamiliar open area displays orientational-exploratory reactions such as characteristic freezing, which is required for assessment of the level of risk [Blume et al., 2018].

Experimental protocol. An animal was placed with all four paws in the central square of the arena and its movements in the arena were recorded using a video system for 3 min. The arena was treated with water to remove odor after each animal was tested.

The following indicators were recorded:

1) horizontal motor activity (HMA). The main criterion for HMA was involvement of all four paws in the animals' movement. One square crossed with all paws was taken as a unit of movement. HMA was recorded at the periphery, in 2/3 of the arena, and at the center;

2) vertical motor activity (VMA). This consists of two types of rearing: the animal's hind legs remaining on the floor of the arena with the front legs resting against the wall of the field (wall rearing) and with the paws remaining suspended (free rearing). The numbers of rearings in the open and by the wall were counted separately.

Video movement analysis on the Vicon platform. The gait of rats of the control group was compared with that of

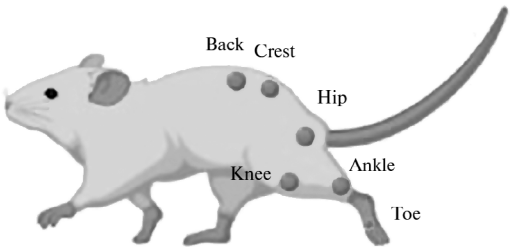

Fig. 1. Positioning of markers for video motion analysis.

rats of the MA group by video movement analysis. 3D data were captured using six Vicon MX cameras (Vicon Motion Systems, Oxford, UK) placed on special mounts in a semicircle. An Active Wand calibration marker (Vicon Motion Systems, Oxford, UK) was used to calibrate and synchronize the cameras. A Sony video camera was used to capture standard video images. Ten passive reflective markers were placed on the back muscles, sacral bone, knee joints, and ankle joints, as shown in Fig. 1.

During video capture, the rats began to move freely in the open field under artificial lighting. Spline interpolation was used to resample the Vicon data to 30 Hz before analysis. Gait cycle phases were defined with time stamps for gait events: foot liftoff and renewal of contact with the surface. Kinematic analysis was performed for the complete gait cycle of each rat tested.

Captured data were processed in Vicon Nexus 2.9 software to complete the 3D motion model manually and remove artifacts from recordings.

Data captured by Vicon Nexus 2.9 were converted into text format using the ASCII module and then processed using MATLAB software; the calculation method is described in detail in [Smirnova et al., 2022]. For each group, curves were obtained by averaging 30 steps by angle into the phases of a single step. This yielded data in the form of angulograms: the kinematic profiles of the knee joints of rats normalized by step phase. Angulograms were used to calculate the flexion angles of the knee joints as the difference between the angle at the beginning of the swing phase and the angle at the beginning of the push phase. Foot movement trajectories were constructed to determine the range of motion of the limb and the point of maximum footraise, as well as step length.

Data were processed statistically in SPSS Statistics. The compliance of the experimental data with the normal distribution was tested using the Kolmogorov test. Data on the parameters of motor activity in the open field and the step characteristics on execution of movement are presented as means and standard deviations $M \pm SD$. Kinematic analysis parameter data are presented as medians and lower and upper quartiles (Me; Q1; Q3). Independent samples were compared using the Mann–Whitney U test. Calculated Mann–Whitney U values were compared with critical values at a significance level of $p < 0.05$: if the calculated U value was equal to or less than the critical value, the difference was taken as statistically significant.

Mechano-myographic method for recording muscle contractile responses. Before experiments, animals were anesthetized by i.p. injection of sodium ethaminal solution at a dose of 40 mg/kg and exsanguinated, and soleus and extensor digitorum longus muscles with nerve stumps were harvested and placed in a suction electrode of an original design [Grishin et al., 2023]. Myoneural preparations were fixed at the tendon ends and then immersed in 10-ml reservoirs filled with Krebs solution (NaCl 118.0 mM, KCl 4.75 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.8 mM, KH_2PO_4 1.18 mM, $MgSO_4 \cdot 7H_2O$ 1.18 mM, glucose 11 mM), pH 7.4, $t = 37$°C.

Contractions were induced in muscle samples using a Digitimer MultiStimul D330 (UK) electrical stimulator; rectangular impulses of amplitude 10 V and duration 0.5 msec were applied at a frequency of 0.1 Hz for 2 min. Muscle contraction force was recorded using a Linton FCG-01 motor activity sensor (UK) and the analog signal was converted by a Biopack MP100MSW data acquisition system (USA).

The initial load on myoneural preparations was 1 g on soleus muscles and 0.5 g on EDL muscles. After adaptation of muscle preparations to the environment for 30 min, the stability of contractile responses was assessed twice with an interval of 5 min.

The effects of purinergic agonists and antagonists were assessed by adding 100 μM ATP to the vessel, followed by assessment of muscle mechanical responses 7 min later. Muscles were then washed with Krebs solution and incubated with suramin solution (100 μM) for 20 min, which was followed by addition of 100 μM ATP and repeat recording of muscle mechanical responses.

All responses captured within two minutes (12 contractile responses) were averaged and processed as a single result. Results were then calculated as percentages relative to the baseline results obtained at the beginning of the experiment.

Statistical data were processed in SPSS Statistics. Compliance of data with the normal distribution was tested using the Kolmogorov test. The arithmetic means of the parameters analyzed and standard errors were calculated. The statistical significance of changes was assessed using Student's t test for independent and pairwise matched samples. Differences were taken as significant at $p < 0.05$.

Results. Analysis of orientational-exploratory activity in the open field test showed that horizontal motor activity (HMA) in animals of the MA group was reduced as compared with the control group (Fig. 2).

The number of squares crossed was 30.5 ± 7.5, compared with 41 ± 3.6 in the control group. Once they had left the central zone of the field, the animals did not return to it, indicating a higher anxiety level in animals of this group. Vertical motor activity (VMA), reflecting both motor and exploratory reactions, was lower in animals of the MA group, at 3.2 ± 1.6 vertical rearings ($p < 0.05$) (Fig. 2). The MA group showed a tendency towards decreased motor activity, as well as increased anxiety.

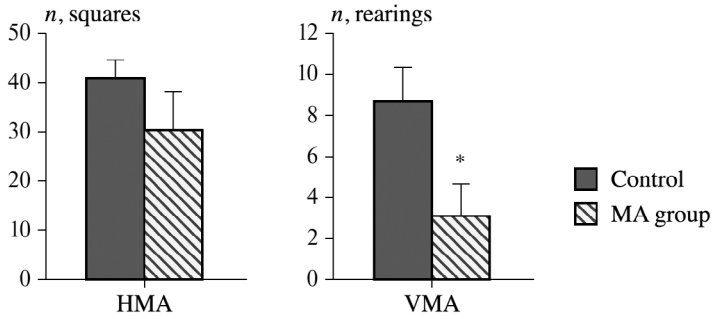

Fig. 2. Measures of horizontal (HMA) and vertical (VMA) motor activity in animals of the control group and the group with the model of autism (MA) in the open field test. Data presented as means; error bars show standard deviations. Significant differences compared to the control group, $p < 0.05$.

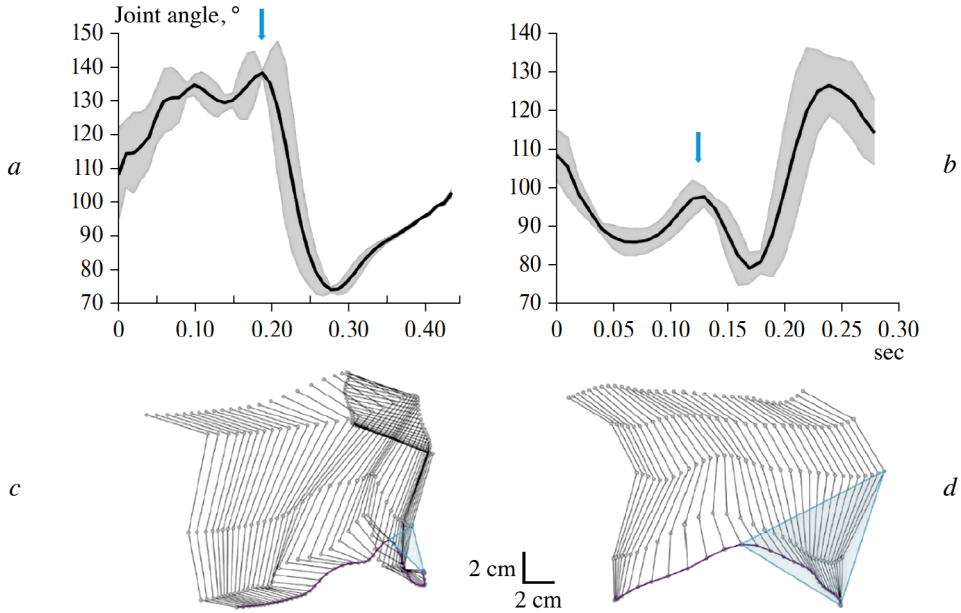

Fig. 3. Knee joint angulograms of rats of the group with the model of autism (MA) (*a*) and the control group (*b*). Representative images of hindlimb position of rats during the step phase in rats of the MA group (*c*) and the control group (*d*). Purple lines show the trajectory of the foot and blue triangles show the ranges of motion of the hindlimb. Arrows show the moment at which the push phase ends.

Angulograms of the hindlimb joints were constructed using video recordings of movement, as shown in Fig. 3, *a*, *b*. The angulograms show increases in the angle in the push phase in rats of the MA group as compared with the control group.

In the first third of the cycle, inappropriate movements were observed at the end of the push phase during movement initiation in animals of the MA group – the rat squeezed its hind paw, which was followed by a low-amplitude push (Fig. 3, *c*, *d*). The body transfer phase was shorter in the MA group. This type of movement may indicate the anxiety and reduced motor function characteristic of this group. However, changes in the ranges of motion of the knee and hip joints were not statistically significant (Fig. 4).

Rats in the MA group showed a significant decrease in step length and a significant increase in step duration as compared with the control group: 131 ± 38 mm ($p < 0.05$) and 0.63 sec ($p < 0.05$), respectively. Leg raise height was the same in both groups (Fig. 5).

The abnormality in locomotor activity described above in animals with ASD prompted us to run the next series of experiments. Evidence supporting the involvement of the synaptic component of the peripheral nervous system in various models of disorders has been reported [Khairullin et al., 2023a, 2023b]. Purinergic transduction is a key element of plasticity in neuromuscular transmission [Ziganshin et al., 2020]. We therefore elected to compare the mechanical activity of the lower leg muscles in rats from the control group and with that of rats with the model of ASD in conditions of purinergic modulation.

The data obtained here show that there were no significant changes in the contraction parameters of the muscles studied (Table 1). ATP retained significant modulating ability; no differences were found between the groups studied.

Discussion. A number of rodent models of ASD have been established as an approach to developing new therapeutics, these recapitulating many of the behavioral phenotypes observed in humans with ASD [Pardo and Meffert,

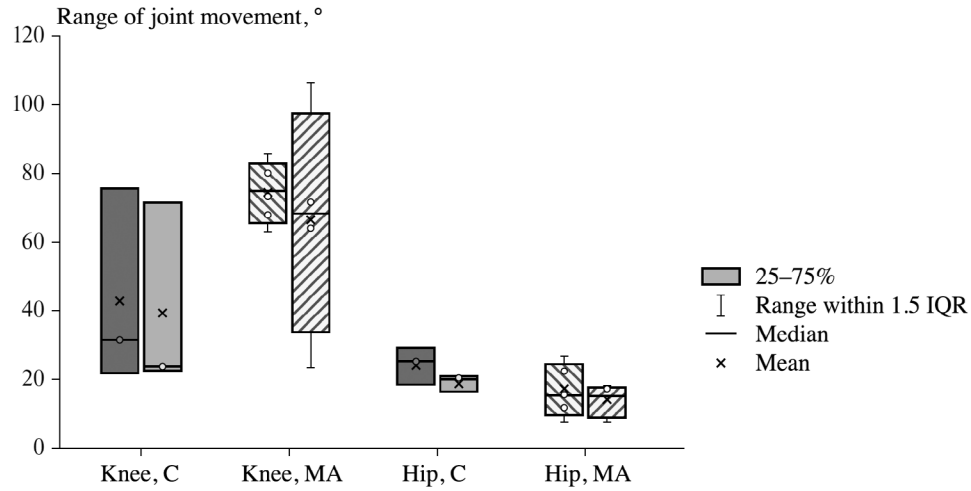

Fig. 4. Ranges of joint motion: left and right knees and hips, in the control group (C) and the group with the model of autism (MA). Data are presented as medians and spreads within groups are shown as interquartile ranges.

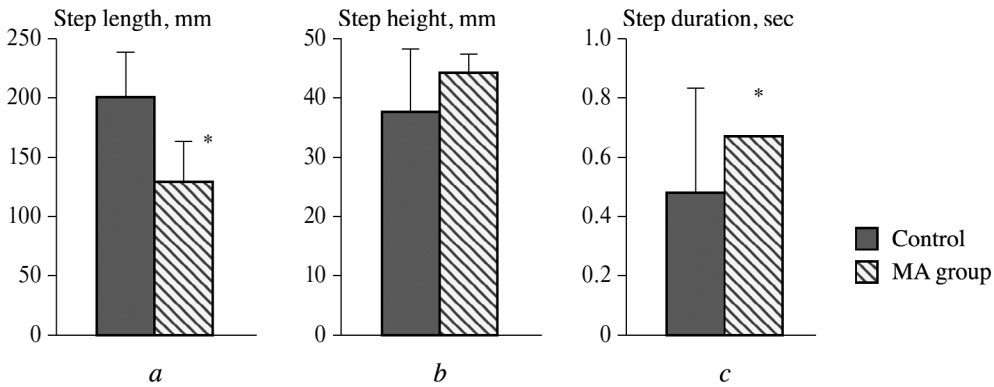

Fig. 5. Stepping characteristics on performance of movements in the open field identified using a video motion capture system (Vicon) in rats of the control group and the group with the model of autism (MA): (*a*) step length, mm; (*b*) step height, mm; and (*c*) step duration. Data are presented as means and standard deviations. Statistically significant difference between groups, $*p < 0.05$.

2018; Chaliha et al., 2020]. Valproic acid (VPA) is currently the most relevant pharmacological model for ASD in animal models, as administration of VPA in early pregnancy has been shown to lead to various abnormalities in brain development, including hyperactivity, attention deficit disorder, and ASD [Wood, 2014; Christensen et al., 2019].

As long ago as 1996, Rodier et al. discovered morphological changes in the brain occurring as a result of administration of VPA to pregnant rats: the number of neurons in the nuclei of the cranial nerves decreased and there were abnormalities in cerebellar development [Rodier et al., 1996]. Later studies demonstrated derangements in the behavior of rats with the valproate model of ASD, apparent as an increase in the threshold of pain sensitivity, a decrease in social exploratory activity, an increase in motor activity, and hyperactivity manifest as stereotypical behavior [Schneider and Przewłocki, 2005].

Animal models of neuropsychiatric and neurodevelopmental disorders, including autism, have yielded valuable data on the neural circuits and target receptors involved in the etiology and pathophysiology of altered behavior [Gandhi and Lee, 2021]. One of the neurotransmitter systems involved in the pathophysiology of mental disorders is the purinergic system [Cheffer et al., 2018]. Pathologies of purine and pyrimidine metabolism are known to damage the nervous system (producing developmental delay, epileptic seizures, autism).

P2 receptors are closely associated with the embryonic development of the nervous system, and any disruption of purinergic signaling could be the underlying process leading to mental illness in general [Oliveira et al., 2016].

P2X and P2Y receptors are known to control a wide range of biological characteristics relevant to autism; for example, purinergic signaling modulates normal synaptogenesis and brain development [Pan et al., 2020], innate and adaptive immune responses, chronic inflammation [Lee et al., 2015], neuroinflammation, antiviral signaling [Mitchell et al., 2017], activation of microglia, neutrophil chemotaxis, autophagy, intestinal motility [Talos et al., 2012], intestinal permeability [Amiet et al., 2008], chemosensory taste trans-

TABLE 1. Relationship between Contractile Parameters of the Study Muscles in Rats Induced by Electrical Stimulation and Experimental Conditions

Experimental conditions	Parameter	Baseline	ATP (100 μM)	Suramin (100 μM)	Suramin + ATP (100 μM)
Soleus					
Control (n = 12)	CS	100.0 ± 3.8	73.1 ± 6.4*	102.7 ± 4.4	96.4 ± 6.5
	CT	0.083 ± 0.005	0.082 ± 0.004	0.081 ± 0.005	0.080 ± 0.003
	RT/2	0.090 ± 0.006	0.104 ± 0.010	0.092 ± 0.004	0.094 ± 0.011
MA (n = 12)	CS	98.6 ± 5.1	74.8 ± 5.9*	103.2 ± 6.3	98.7 ± 5.3
	CT	0.081 ± 0.004	0.079 ± 0.006	0.080 ± 0.004	0.078 ± 0.006
	RT/2	0.091 ± 0.011	0.110 ± 0.013	0.093 ± 0.009	0.095 ± 0.009
Extensor digitorum longus					
Control (n = 12)	CS	100.0 ± 4.8	85.9 ± 3.7*	101.9 ± 4.6	99.2 ± 4.5
	CT	0.055 ± 0.006	0.058 ± 0.007	0.060 ± 0.007	0.059 ± 0.005
	RT/2	0.068 ± 0.004	0.070 ± 0.006	0.066 ± 0.005	0.069 ± 0.004
MA (n = 12)	CS	98.5 ± 3.6	84.2 ± 7.1*	97.9 ± 6.3	96.1 ± 5.2
	CT	0.059 ± 0.006	0.060 ± 0.005	0.061 ± 0.005	0.061 ± 0.004
	RT/2	0.070 ± 0.005	0.072 ± 0.008	0.071 ± 0.007	0.070 ± 0.005

CS – contraction force, CT – contraction time, RT/2 – half-relaxation time. Significant differences compared with baseline, $*p < 0.05$; significant differences compared with control, $^{\#}p < 0.05$.

duction [Besag, 2018], and chronic pain syndrome [Lamb et al., 2019]. There is no doubt that the causes of most of these disorders lie within the central nervous system, though the possibility that disturbances in the functioning of the peripheral nervous system (including neuromuscular synapses) also contribute cannot be excluded.

Although autism is diagnosed on the basis of three main characteristics – social deficits, communication impairments, and repetitive or stereotyped behavior – other behavioral features, such as sensory and motor impairments, are present in more than 70% of people with ASD [Bhat, 2021]. Autism-related characteristics such as sensory processing disorders and motor coordination deficits are common but have received less attention from the research community. For example, there are a number of qualitative and quantitative reports on ASD describing impairments to visuomotor and manual dexterity, limb coordination in tasks requiring balance, agility, and speed, as well as gait impairments and ataxia [Fatemi et al., 2012]. In addition, motor impairment may be among the earliest signs of some forms of ASD [Ozonoff et al., 2008]. Assessment of movement disorders may therefor aid in the early and quantitative diagnosis of pathology and in identifying dysfunctional brain regions and circuits in ASD. We showed that animals of the MA group displayed decreased vertical and horizontal motor activity in the open field. This is consistent with previous results obtained in animals showing decreased locomotor activity after pre- and postnatal administration of VPA [Gedzun et al., 2020; Kataoka et al., 2013; Mabunga et al., 2015] and with clinical studies showing that children with ASD spend less time actively exploring their environment [Elandaloussi et al., 2023]. ASD is also associated with anxiety disorders, and estimates of the prevalence of anxiety in people with ASD vary widely, from 22% to 84% [Nimmo-Smith et al., 2020]. We also found increased anxiety in rodents with prenatal exposure to VPA, as indicated by a decrease in VPA, changes in gait structure, and a lack of exploration of the central area in the open field test, which is consistent with other studies [Kataoka et al., 2013; Cartocci et al., 2018; Servadio et al., 2018]. Changes in exploratory behavior may reflect developmental disorders of the central nervous system. One possible explanation for the decrease in exploratory behavior in rats with VPA may be a decrease in the number of Purkinje cells in the cerebellar lobules of the vermis [Fatemi et al., 2012]. Similarly, decreases in the lobules of the cerebellar vermis correlating with decreased exploratory activity have been observed in children with autism [Pierce and Courchesne, 2001]. Furthermore, impaired cerebellar activity may also be indicated by a significant decrease in step length and an increase in step duration in MA rats as compared with the control group [Main and Kulesza, 2017]. A second possible explanation could be changes in neural structures involved in the regulation of fear. These include the medial prefrontal cortex and the amygdala. Abnormalities in these structures have been seen both in rats with a VPA model [Sui and Chen, 2012] and in autistic people [Bachevalier and Loveland, 2006; Arutiunian et al., 2023]. Schneider and Przewłocki [2005]

found that reduced exploratory behavior in adult rats in the VPA model is likely to be mediated by fear-related inhibition of exploratory behavior.

The group differences seen in locomotor activity demonstrate that rats in the MA group performed stepping less efficiently. Rats in the MA group required more paw movements and exhibited more atypical sensorimotor behavior (e.g., sudden paw lifts, short swing phase); these rats evidently had greater difficulty with the finer aspects of motor control and/or difficulty coordinating limb functions. Sensorimotor changes of this type have been associated with an imbalance between synaptic excitation and inhibition in the CNS due to dysfunction of GABAergic signaling, widespread changes in neuron morphology, and local derangements of neocortical microcircuits [Banerjee et al., 2013; Lee et al., 2017; Jiang et al., 2022]. As optimal motor performance is determined by the accurate and efficient reception and processing of sensory information, impairment of somatosensory processing provides a possible explanation for the motor deficits seen. Gross motor clumsiness and disturbances in gait and balance have frequently been reported in both children and adults with ASD [Fournier et al., 2010]. In addition to delayed onset of walking, children with autism also lack a mature heel-to-toe pattern and have a more waddling gait as compared with age-matched controls [Esposito and Venuti, 2008].

The present work demonstrated the absence of any significant changes in the strength of muscle contractions combined with a tendency to reduced motor activity in the group with the autism model, which contrasted with control animals; animals with the autism model also displayed increased anxiety, which correlated with the results of another study of peripheral cholinergic neurotransmission in rats with the model of ASD [Arkhipov et al., 2021]. Interestingly, changes in the mechanical activity of smooth muscle organs and increases in parasympathetic influences in rats with ASD have been demonstrated [Ziganshin and Ivanova, 2021].

Our study had a number of limitations. First, our experiments used male rats. Some investigations have found evidence of sex-related differences in social and communicative skills and restricted repetitive behavior in animal models of ASD. Secondly, the study addressed a limited cohort size. Furthermore, attention at the task-setting stage was focused on the effects of neuromuscular transmission in skeletal muscle, so a limited battery of behavioral tests was used. Future studies will expand the behavioral assessment and add detailed kinematic analysis.

Conclusions. The results obtained here suggest that general differences in movement may be an independent factor in the diagnosis of autism. A more rigorous study using a larger sample and detailed kinematic analysis may help further evaluate motor variability as a potential diagnostic and prognostic marker for ASD. Motor impairment is a highly underappreciated area for assessment and intervention in ASD. Motor skills should be routinely included in comprehensive screening, assessment, and treatment planning for ASD, especially after the early developmental period when parental attention often shifts to other manifestations of ASD.

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