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RAT TRANSTIBIAL HINDLIMB AMPUTATION MODEL: CHARACTERISTIC OF THE CENTRAL NERVOUS SYSTEM FUNCTIONAL STATE, PHYSICAL ENDURANCE AND BODY MASS IN DYNAMIC

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Limb loss is a relevant medical and social problem. In a full-scale war environment, amputation became a frequent occasion, which requires adequate rehabilitation measures in preparation for prosthetic procedures. The post-traumatic period's peculiarities, specifically the central nervous system (CNS) functional characteristics and physical endurance, must be known. Experimental studies on animal models play an important role; however, there is no information available about such studies.

***The aim.** To determine the influence of hindlimb loss on the state of the central nervous system in terms of behavioural responses, physical endurance, and body mass dynamics of rats in the early and late post-amputation period. To characterise the indicated amputation model for further use in experimental studies.*

***Materials and methods.** The experiment was conducted on white male rats. Transtibial amputation at the level of the lower third of the hind limb was performed in aseptic conditions in 6 animals under thiopental-sodium anaesthesia (40 mg/kg intraperitoneally). The state of CNS and physical endurance was determined during the early (3–4 days after amputation) and long-term (2 months after surgery) periods. Locomotor and exploratory activity, emotional responses and vegetative support were determined in the Open field test, and anxiety was determined in the Light-dark box test. The coordination of animal movement was measured in the rotarod test, and the depression level was measured in the Porsolt swim test. The physical endurance of the rats was studied using the forced swimming test with a load (5 % of the body mass at the base of the tail) as a control were used rats with comparable body mass. STATISTICA 12.0 was used to process the results.*

***Results.** In the early post-amputation period, exploratory behaviour and emotional results of rats in the Open field test were significantly suppressed, while in the long-term period of significance, only the reduction of emotional responses. In the Light-dark box test, the latency to enter the darkened compartment of the chamber increases reliably in both periods of observation (especially in the early period). Collectively, these results indicate changes in the reaction of animals with amputation of the hindlimb to stressful experimental conditions, in particular, an anxiety reduction, which requires further research. In the rotarod test, a significant deterioration of movement coordination of rats with amputation was found in both observation periods. After amputation, the manifestations of depressive behaviour in the Porsolt swimming test progressed, and the physical endurance in the forced swimming test with a load was significantly reduced. The body mass of rats with hindlimb amputation was significantly increased after 2 months (22 % average increase vs 4 % average increase in control, p<0.01). Results are important for experimental-based optimal rehabilitation programs after lower limb amputation.*

***Conclusion.** An easy-to-perform model of transtibial amputation at the level of the lower third of the hindlimb in rats is proposed. It is concluded that after amputation, exploratory behaviour and emotional responses were suppressed, which indicates changes in the responses of animals with amputation of the hindlimb to stressful experimental conditions, in particular, an anxiety reduction, which differentiates this animal amputation model from human amputation. After amputation, the depressed behaviour of rats progresses, movement coordination steadily worsens, and physical endurance is significantly reduced. The body mass of rats with hindlimb amputation increases considerably*

***Keywords:** transtibial hindlimb amputation, behavioural responses, physical endurance, body mass, rats, experiment*

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1. Introduction

Limb amputation leaves a lasting impact on the quality of life, physical endurance and psychological state of humans. Lower limb amputation is the most common type (up to 85 % of all amputation cases). It is estimated that quantity of humans with amputated limbs will increase more than two-fold before 2050 [1]. The main reasons for amputation may vary depending on social, economic, political and regional conditions and the level of medical care. Most reasons for amputation are vascular diseases, diabetes mellitus and limb injuries [2], among which, due to war, particularly due to Russian aggression against Ukraine, gunshot wounds and mine-explosive traumas have gained especial significance. There is no accurate statistic related to the number of soldiers who suffered lower limb wounds. According to the Health Care Department of Kyiv data, there were 15,000 people with amputated limbs in the first half of 2023 [3].

To properly plan the rehabilitation process and prosthetics, it is necessary to know the peculiarities of the post-traumatic period, specifically the functional characteristics of the central nervous system (CNS) and physical endurance. It is known that patients with traumatic amputation quite regularly have depression and post-traumatic stress disorder, characterised by fear and anxiety. To correct this condition, psychotherapy is offered in combination with antidepressants of various groups: tricyclic, serotonin and noradrenaline reuptake inhibitors, selective serotonin or noradrenaline reuptake inhibitors, and MAO inhibitors [4]. There is no information about physical endurance. To substantiate the choice of certain rehabilitation methods and schemes of optimal pharmacological correction of the specified functions in the post-amputation period, it is appropriate to use not only clinical but also experimental studies. However, no information about such studies was found in the special literature.

In clinical studies of recent years, the most discussed topic is the prevention of post-amputation thromboembolism using fractionated heparin (enoxaparin) [4, 5]. Much attention is paid to the pharmacotherapy of post-amputation pain. For short-term use for this purpose, an opioid agonist, morphine, is offered, as well as antagonists of NMDA receptors – ketamine, memantine, and dextromethorphan. The effectiveness of calcium channel blocker nifedipine, beta blocker propranolol, α2-receptor agonist clonidine, TNF-α inhibitor etanercept is less grounded; the possibility of reducing the central activation of neurons with the help of salmon calcitonin is considered. Amitriptyline and gabapentin are recommended for the treatment of phantom pain [6]. Recently, the use of regeneration stimulators – ReGeneraTing Agents (RGTAs) – in the post-amputation state has been discussed [4]. These agents are polysaccharides designed to replace degraded heparan sulfate in damaged tissues. They are resistant to degradation, able to bind to the structural and signalling proteins of the extracellular matrix and protect them, and able to stimulate regeneration [7].

Also, an important consequence of losing a limb is a change in body mass. Mostly, people with a lost limb are overweight, but it is believed that this does not affect the results of functional tests after rehabilitation and generally does not interfere with the rehabilitation process in a hospital [8]. However, body mass loss has been reported in the period from 4 weeks to 6 months after amputation. However, within a year, the weight is usually restored to the preoperative level or increases [9].

To prepare for prosthetics, it is necessary to substantiate the effectiveness of certain rehabilitation measures in the field of both the physical and psychological state of the victims. Unfortunately, modern methodological approaches, in particular ex vivo studies on cell cultures of neurons, osteoblasts, chondrocytes, myocytes, etc., do not allow determining the impact at the level of the whole organism, in which neurohumoral regulatory and counterregulatory mechanisms simultaneously function, and the central nervous system interacts with the musculoskeletal system, which has undergone significant biomechanical and energy changes after amputation. It is important to understand how the victim's organism will function in the conditions of the above-mentioned changes in body weight, which are not always easy to correct. Even for simple movement at the stage of preparation for amputation and after prosthetics, patients spend a lot of energy, which requires optimisation of physical endurance, and neuropsychological diagnostics and treatment remain an important direction of their rehabilitation [10]. Therefore, research at the whole-body level is needed.

To successfully solve the problem of rehabilitation after the loss of a limb, it is advisable to use an optimal experimental animal model of amputation. This allows to standardise the studied parameters and assess psychophysiological and biomechanical capacity, which requires determining the state of the central nervous system, physical endurance, etc.

In experimental medicine, amputation models are used in various animal species (dogs, sheep, etc.). Without dwelling on these models, it should be noted that for a long time, models of amputation of the fore or hind limbs in rats (as the most accessible species of laboratory animals) have also been used for this purpose. In particular, there is currently a renaissance of research on the electrical stimulation effect on regeneration after limb amputation in rats [11], which was initiated over 50 years ago by R.O.Becker. Recently, the state of ischemic-reperfusion injury of the limbs [12], loss of bone tissue, changes in the microstructure of trabecular bone and muscle atrophy using computed tomography and transmission electron microscopy [13], the efficiency of muscle reinnervation [14], etc. have been studied in models of amputation of the hind limbs of these animals. No studies have been found on the functional state of the CNS, physical endurance, and changes in body weight in rats with amputation models. However, given the uniqueness of humans as the only biological species that moves on two limbs and the interspecific biomechanical, biochemical, and psychophysiological differences, it is important to find out to what extent the limb amputation model in rats corresponds to or differs from the condition of humans with a lost limb. Animal models of amputation are also important to use to assess the condition of the skin, muscles, and bone of the stump, the course of regeneration processes, adaptation of the stump to the prosthesis receiving socket or to osteointegration, protection of the stump skin and bone from ascending infections, etc., and the condition of the spine, which is significantly affected by amputation due to impaired biomechanics of movement and load redistribution [10].

Thus, the idea of limb amputation models in experimental animals, particularly rats, has existed for a long time and has recently gained new development. However, in important terms of neuropsychological studies and assessment of physical endurance and body weight in dynamics, animal models of limb amputation have not been used.

The aim. To determine the influence of limb loss on the state of the central nervous system in terms of behavioural responses, physical endurance, and body mass dynamics of rats in the early and late post-amputation period. To characterise the indicated amputation model for further use in experimental studies.

2. Research design (methodology)

At the first stage, it was necessary to choose a model of limb amputation in rats that would preserve the mobility of animals and make it possible to determine behavioural responses in standard psychopharmacological tests and endurance to physical exertion. Amputation of the distal segment of the hindlimb, in particular the leg, better meets this requirement. Therefore, amputation of the lower third of the hindlimb was chosen. In the second stage, to determine the functional state of the animals' central nervous system in dynamics, the early (3–4 days after amputation) and chronic (2 months after surgery) periods were chosen. This would make it possible to choose the optimal targets of the rehabilitation influence at different times after the loss of a limb.

To characterise the state of the central nervous system, a complex of standard research methods was chosen: the Open field test (motor and oriented research activity, emotionality), the Light-dark box test (anxiety), the Porsolt swim test (depressive behaviour), the rotarod test (coordination of movements, muscle tone), swimming with load (physical endurance). To track gradual changes in body mass, was reasonable to weigh rats the day before and at different times after amputation.

Stages of research:

1. Analysis of publications on limb amputation, including animal models.
2. Selection of the optimal model of rat amputation.
3. Surgical intervention – transtibial amputation of the lower third of the hindlimb.
4. Detection of behavioural responses and physical endurance in the early (3–4 days) and late (2 months) post-amputation periods.
5. Processing and analysis of the obtained results.
6. Identification of promising directions for further research.

3. Material and methods

The study was conducted at the Educational and Scientific Institute of Applied Pharmacy of the National Pharmaceutical University (NUPh). The protocol of the experiments was approved by the bioethics commission of the NUPh (protocol No 13 dated 13.03.2024) and meets the requirements of the Helsinki Declaration on the humane treatment of animals (2000) and the Directive of the Council of the European Union on the protection of animals used for scientific purposes (2010) [15]. Experiments were performed on adult white random-bred male rats aged 7–8 months, weighing 230–310 g (determined using electronic scales with an accuracy of 1 g). The animals were kept on a standard vivarium diet with free access to water, constant humidity, and a temperature regime of +22–23 °C.

Transtibial amputation at the level of the lower third of the hindlimb was performed in aseptic conditions in 6 animals under thiopental-sodium anaesthesia (40 mg/kg intraperitoneally). Miniature (ophthalmological) surgical instruments were used, and an electrocoagulator was used for hemostasis. The nerves were cut under slight tension to minimise the risks of neuropathic pain. After cutting, the edge of the bone was carefully smoothed. A conical stump was formed. A myofasciocutaneous flap was formed to close the wound, muscles (myoplasty technique was used), and skin was sutured. Ceftriaxone (250 mg/kg) was administered intraperitoneally to the rats at the end of the operation to prevent infectious contamination. After surgery, rats were placed in individual standard plastic boxes with free access to pelleted feed and drinking water. A limited number of amputated rats (6) was taken for bioethical reasons.

The number of control animals was larger to reduce the dispersion of the results. In the early post-amputation period (3–4 days after surgery), 9 rats of similar age and initial body mass were used. They were injected with an anaesthetic agent and ceftriaxone in the same doses without surgical intervention to ensure the same pharmacological effects. In the chronic period (after 2 months), 11 control rats were studied. They were kept in similar conditions and fed in the same way as animals with amputations.

Research on the state of the central nervous system and physical endurance in each period was conducted over two days in such a sequence that the animals were not exhausted, and the previous test did not interfere with the application of the next one. In the Open field test, a brightly light platform measuring 75×75 cm with 30 cm sides was used, divided into 25 squares of 15×15 cm with 2 cm diameter holes at their corners. Locomotor (the number of crossed squares) and exploratory activity (the number of rearings and inspected holes – exploratory nose-pokes, and their sum), emotional responses (the number of acts of grooming), and their vegetative support were determined separately and together (the number of faecal boli, urinations), the sum of all activities in 3 minutes was counted [16, 17]. Later, the level of anxiety was investigated in the Light-dark box test [18]. The device is a rectangular box measuring 60×30×30 cm divided by a wall into two equal compartments of 30×30 cm. One of the compartments is closed with a removable lid. The other one is open; above it is a source of bright light used to create conditions of moderate stress for the animals. There is a hole in the wall between the light and dark boxes. Rats were placed in the brightly lit part of the device with their head away from the opening, and the time to enter the dark box was measured. Additionally, behavioural patterns such as rearings, grooming, defecation, and urination while on the illuminated platform, were measured. The observation time was 3 minutes. Next, the coordination of animal movements was measured in the rotarod test (rod rotation speed set at 10 rpm) [19]. In this test, rats had 3 tries, which allowed them to adapt to the difficult testing conditions, especially in the absence of a limb; the best result was accounted for. At the end of the first experimental day, the level of depression of the animals was determined in the Porsolt swim test [20]. The animals were placed in a pool with a diameter of 50 cm, a depth of 60 cm and a height of borders above water of 10 cm (escape is impossible) with water at room temperature (21–22 °C). A decrease in the latent time of the first episode of immobilisation (absence of any swimming movements), an increase in the number of episodes and the time of immobility of animals in water were considered markers of depressive behaviour [20].

On the second day, the physical endurance of the rats was studied by the forced swimming test with a load (5 % of the body weight at the base of the tail). In this test, a load of 10 % of the body weight is used, but given the specifics of this experiment – the reduced capabilities of animals with limb amputation – the weight of the load was reduced to 5 %. The pool described above was used. Swimming time was measured until the moment of exhaustion when the rats dived underwater and could not float back to the surface for at least 10 seconds [21]. A stopwatch was used. The experiments were recorded.

STATISTICA v12.0 and Excel programs were used for statistical processing of quantitative data. The statistical significance of intergroup differences was determined by the non-parametric Mann-Whitney test since the distribution test by the Shapiro-Wilk test showed that it was not normal. The paired Wilcoxon test was used to evaluate the body mass dynamic within the group. Changes with $p<0.05$ were considered statistically significant. The results are presented as M±m and Me [Q25; Q75]. For accounting results in an alternative form (presence/absence of a feature), the results were presented in %, and Fisher's angular transformation was used.

4. Results

All animals tolerated the surgical intervention well and quickly recovered from anaesthesia (Fig. 1). Early and late complications were not observed. Right after coming out of anaesthesia, the animals began to move, and on the second day after the amputation of the hindlimb, all rats freely moved in their cages. The stump in all rats in the early post-amputation period was in satisfactory condition while primary healing of the surgical wound was taking place. The wool cover of the stump was restored (Fig. 2, *a*). At the same time, muscle hypotrophy and flexural contracture of the hip joint gradually developed, and already a few days after the operation, the stump in some animals appeared to be shortened and significantly pulled up to the pelvis. Over time, these changes intensified (Fig. 2, *b*).

Behavioural patterns in the Open field test. On the 3rd day, in the Open field test, a declining trend in motor activity was observed (number of crossed squares), averaging a 33 % decrease (Table 1). Orientation and explorational responses have changed: the number of examined holes has decreased by 4 times ($p<0.05$), the number of rearings has decreased by 3.2 times, and the sum of orientation and explorational responses has decreased by 3.3 times ($p<0.05$). The number of acts of grooming (a behavioural marker of emotional responses) also decreased statistically significantly by 6.8 times ($p<0.01$), and vegetative manifestations of emotions (number of boli, urinations) did not undergo significant changes. The sum of indicators of all subtests of the Open field test tends to decrease in the early post-amputation period by an average of 40.9 %.

Fig. 1. Rat after transtibial amputation of the hindlimb. Getting out of anaesthesia

a

b

Fig. 2. Appearance of rats 2 months after amputation at the level of the lower third of the right hindlimb: *a* – stump with minor; *b* – with significant muscle atrophy

Table 1

Effect of transtibial amputation of the hindlimb on the behavioural responses of rats in the Open field test in the early and long-term periods

Period of research, indicator		Control		Amputation	
		M±*m*	Me [Q25; Q75]	*M*±*m*	Me [Q25; Q75]
Early period (3–4 days after amputation)		*n*=9		*n*=6	
Locomotor activity (squares crossed)		24.36±7.88	19 [7.75; 31.75]	16.33±7.38	8 [4; 30]
Exploratory activity	Rearings	6.88±2.10	6.5 [3.25; 8.5]	2.17±0.98	1.5 [0.25; 3.5]
	Exploratory nose-pokes	2.00±0.53	2 [1; 2.25]	0.50±0.50	0 [0; 0]*
	The sum	8.88±2.29	9 [4.25; 10.5]	2.67±1.26	1.5 [0.25; 5.0] *
Emotional responses and their autonomic support	Groomings	2.25±0.75	2 [1; 2.25]	0.33±0.21	0 [0; 0.75]**
	Fecal boli	1.50±0.50	1 [0.75; 2.25]	2.33±0.56	2 [1.25; 3.5]
	Urinations	0.50±0.19	0.5 [0; 1]	0.50±0.19	0.5 [0; 1]
	The sum	4.25±0.90	4.5 [2.5; 5.5]	3.17±0.54	3 [2.0; 4.0]
The sum of all activities		37.49±9.35	31.5 [20.25; 43.75]	22.17±8.81	12 [8.5; 38.75]
Long-term period (2 months after amputation)		*n*=11		*n*=6	
Locomotor activity (squares crossed)		27.36±6.78	15 [10; 42.5]	21.63±7.58	20 [6.25; 30]
Exploratory activity	Rearings	4.36±1.06	4 [2; 6]	2.00±0.73	1.5 [1; 2.75]
	Exploratory nose-pokes	2.27±0.67	2 [1; 3]	1.67±1.09	1 [0.25; 1]
	The sum	6.64±1.5	6 [2.5; 8]	3.67±1.52	2.5 [1.25; 5.25]
Emotional responses and their autonomic support	Groomings	1.54±0.20	1 [1; 2]	0.17±0.17	0 [0; 0] **
	Fecal boli	1.18±0.63	1 [0; 1]	1.00±0.63	0.5 [0; 1]
	Urinations	0.73±0.21	1 [0.5; 1]	0.67±0.21	0.5 [0; 1]
	The sum	3.45±0.45	3 [2.5; 4.5]	1.84±0.87	1 [1; 1.75] *
The sum of all activities		37.45±7.97	28[16.5; 55]	27.17±8.99	22 [9.25; 43]

*Note: statistically significant differences compared to the control: * – $p<0.05$, ** – $p<0.01$ (Mann-Whitney criterion).*

In the long-term post-amputation period (2 months), the locomotor activity of animals with amputation also tended to be lower (by 21 %) compared to control. The decrease in the number of orientation and explorational responses was also preserved, but now it was expressed weaker and had a tendentious character in all subtests.

Suppression of emotional responses also continued: the number of acts of grooming decreased by 9 times ($p<0.01$) compared to the control, and the decrease in the sum of markers of emotionality by 1.9 times became reliable ($p<0.05$). The sum of all activities of the Open field test remained tendentially reduced compared to the control by an average of 27.4 %.

The level of anxiety in the Light-dark box test. As can be seen from the Table 2, all animals of the control group quickly (within half a minute) entered the dark compartment of the box. This is due to the inherent rodents' instinctive fear of brightly lit spaces, which has an anxiogenic effect on rats. On the way to the dark box, they mostly did not examine the lightbox (there were no stances) and, for the most part, did not show emotional responses (grooming, defecation, urination). On the contrary, in rats with amputation in the early period, a significant (on average 7.1 times) increase in the latent period of entering the dark box was observed ($p<0.05$), which may mean a decrease in anxiety level. During this time, they all moved around the lighted platform, examined it, made stances, managed to reveal a small number of emotional responses (no more than intact rats revealed in seven times shorter time), and even looked into the opening to the dark compartment, but did not hurry to hide there. Only 33.3 % of rats with amputation versus 100 % in control ($p<0.01$) entered the dark box within 3 minutes of observation. At the same time, there were no changes in the number of stances and emotional responses during the stay in the lightbox compared to the control.

Similar, though less pronounced, differences took place in the long-term period. In animals with amputation, the latent period of entering the dark box remained increased compared to the control (by 4.2 times, $p<0.05$), while only 83.3 % of them entered within 3 minutes against 100 % in the control ($p<0.05$). The number of stances and displays of emotionality during the stay in the light box, as in the early post-amputation period, remained unchanged.

Coordination of movements in the rotarod test. Rats with amputation both in the early and in the remote period were kept on the rotating rod much worse than the control animals (Table 3). In both terms of the study, they all fell from the rod within 30 seconds, while in the control, at this time, the least coordinated animals were just starting to fall. A significant number of rats with amputation did not last even 5 seconds on the rod, while most control rats lasted 1 minute. The average time it took rats with amputation to fall was statistically significantly ($p<0.01$) inferior to the control value by 14 times on the 3rd day and by 16 times after 2 months. Therefore, the coordination of movements after the amputation of the hindlimb was persistently disturbed.

Table 2

Effect of transtibial amputation of the hindlimb on the behavioural responses of rats in the Light-dark box test in the early and long-term periods

Period of research, indicator		Group, number of animals			
		Control		Amputation	
		M±m	Me [Q25; Q75]	*M±m*	Me [Q25; Q75]
Early period (3–4 days after amputation)		*n*=8		*n*=6	
Latent period before entering, seconds		17.50±6.21	10 [5; 22.5]	124.00±35.46	180 [59.25; 180]*
Exploratory activity	Rearings	0.13±0.13	0 [0; 0]	0.33±0.21	0 [0; 0,75]
Emotional responses and their autonomic support	Groomings	0±0	0 [0; 0]	0.17±0.17	0 [0; 0]
	Fecal boli	0±0	0 [0; 0]	0.50±0.34	0 [0; 0,75]
	Urinations	0±0	0 [0; 0]	0.33±0.21	0 [0; 0,75]
Number of rats that entered in 3 minutes, %		100		33.3 ^^	
Long-term period (2 months after amputation)		*n*=11		*n*=6	
Latent period before entering, seconds		11.00±4.65	5 [3.5; 11.0]	46.00±27.12	24 [11.5; 31.25]*
Exploratory activity	Rearings	0.09±0.09	0 [0; 0]	0.33±0.21	0 [0; 0,75]
Emotional responses and their autonomic support	Groomings	0.09±0.09	0 [0; 0]	0.17±0.17	0 [0; 0]
	Fecal boli	0±0	0 [0; 0]	0.17±0.17	0 [0; 0]
	Urinations	0.09±0.09	0 [0; 0]	0.17±0.17	0 [0; 0]
Number of rats that entered in 3 minutes, %		100		83.3 ^	

*Note: Statistically significant differences compared to the control: * – p<0.05 (Mann-Whitney test); ^ – p<0.05, ^^ – p<0.01 (Fisher's test).*

Table 3

Effects of transtibial hindlimb amputation on the time it took rats to fall in the rotarod test in the early and long-term periods

Period of research, indicator		Group, number of animals			
		Control		Amputation	
		M±m	Me [Q25; Q75]	*M±m*	Me [Q25; Q75]
Early period (3–4 days after amputation)		*n*=8		*n*=6	
Time to fall, seconds		69.88±24.26	37.5 [29; 82.5]	5.00±0.86	4.5 [3.25; 6.5]**
The number of rats that fell from the rod, %	<5 sec	0		50 ^^	
	<30 sec	25		50 ^	
	<1 min	50		0 ^^	
	<5 min	25		0 ^	
Long-term period (2 months after amputation)		*n*=11		*n*=6	
Time to fall, seconds		97.27±18.70	67 [50; 160]	6.17±1.14	6.5 [3.75; 7.75]**
The number of rats that fell from the rod, %	<5 sec	0		33.3 ^^	
	<30 sec	9.1		66.7 ^^	
	<1 min	18.2		0 ^	
	<5 min	72.7		0 ^^	

*Note: Statistically significant differences compared to the control: * – p<0.05, ** – p<0.01 (according to the Mann-Whitney criterion); ^ – p<0.05, ^^ – p<0.01 (according to Fisher's angular transformation).*

Depression behaviour in Porsolt swimming test. During swimming, rats used a stump as much as possible. In the early post-amputation period, the latent period of immobility decreased significantly (on average, 1.7 times compared to the control, $p<0.05$) in rats with amputation (Table 4). The number and total duration of episodes of immobility tended to increase, and the average duration of such an episode was almost the same. In the remote period, an even more pronounced decrease in the latent period of immobility was observed (an average of 6 times compared to the control, $p<0.01$), and there was a statistically significant increase in the number ($p<0.01$) and duration of immobility episodes ($p<0.05$). These results indicate an increase in depressive behaviour in rats with hindlimb amputation over time.

Physical endurance in the weighted swimming test. During the test, the rats tried to use the stump to swim. A statistically significant ($p<0.05$) decrease in swimming time until exhaustion was observed in rats with amputation of the hindlimb in both terms of the study (Table 5). In the early period, this time was reduced by an average of 1.6 times, and in the late period – by 1.8 times. These results indicate that after transtibial hindlimb amputation in rats, there is a progressive decline in physical endurance.

Dynamics of body mass. As can be seen from Table 6, the initial body mass of animals with amputation was 11 % greater than that of controls, but the difference did not reach a statistically significant level. After 2 months, the average weight of control animals tended to increase by 10 g (4 %), and the weight of rats with amputation increased by 62.3 g (22.1 %), which is significantly higher compared to their initial weight ($p<0.05$) and to the control indicator for synchronous weighing by 31 % ($p<0.01$). Therefore, after amputation, there is a significant increase in body mass.

Table 4

Effect of transtibial amputation of the hindlimb on the behavioural responses of rats in the Porsolt swimming test in the early and long-term periods

Period of research, indicator	Group, number of animals			
	Control		Amputation	
	M±m	Me [Q25; Q75]	*M±m*	Me [Q25; Q75]
Early period (3–4 days after amputation)	*n*=8		*n*=6	
Latent period of immobility, sec	86.13±17.49	85.5 [67.25; 93]	52.00±9.11	53.5 [49.75; 63.25]*
Total time of immobility, sec	17.63±6.90	10 [5.75; 19.5]	26.00±15.79	8 [2.5; 30.75]
Number of immobility episodes	11.38±3.88	8.5 [5.75; 11]	13.50±6.94	6.5 [2.25; 17.5]
The average duration of immobility episode, sec	1.41±0.14	1.40 [1.08; 1.61]	1.43±0.20	1.27 [1.05; 1.72]
Long-term period (2 months after amputation)	*n*=11		*n*=6	
Latent period of immobility, sec	186.73±32.22	160 [112.5; 246]	31.33±8.99	22 [20; 30]**
Total time of immobility, sec	13.18±4.41	5 [2.5; 22.5]	55.83±19.57	44 [28; 64.5]*
Number of immobility episodes	4.18±1.19	3 [1; 7]	28.00±7.25	27.5 [15.25; 34.5]**
The average duration of immobility episode, sec	2.30±0.40	2.5 [1.83; 3.22]	1.80±0.19	1.79 [1.57; 1.99]

*Note: statistically significant differences compared to the control: * – p<0.05, ** – p<0.01 (according to the Mann-Whitney test).*

Table 5

The effect of transtibial amputation of the hindlimb on the duration of swimming with a load until exhaustion in early and remote periods

Period of research, indicator	Group, number of animals			
	Control		Amputation	
	M±m	Me [Q25; Q75]	*M±m*	Me [Q25; Q75]
Early period (3–4 days after amputation)	*n*=9		*n*=6	
Swimming time, sec	377.4±32.3	382 [290; 402]	240.2±34.4	273 [212.25; 291.75] *
Long-term period (2 months after amputation)	*n*=11		*n*=6	
Swimming time, sec	365.1±66.6	298 [201; 470]	204.7±64.8	123.5 [96.25; 319.5]*

*Note: statistically significant differences compared to the control: * – p<0.05, ** – p<0.01 (according to the Mann-Whitney test)*

Table 6

Dynamics of body mass in rats with transtibial amputation of the hindlimb

Period of research, indicator	Group, number of animals			
	Control (*n*=9)		Amputation (*n*=6)	
	M±m	Me [Q25; Q75]	*M±m*	Me [Q25; Q75]
Initial body mass, g	253.0±4.07	257.0 [242.0; 260.0]	281.7±15.83	290.0 [275.5; 307.5]
Body mass after 2 months, g	263.0±7.91	260.0 [247.5; 268.5]	344.0±5.89#	345.5 [335.5; 351.0] **
Increase in bode mass, g, %	10.0/4.0		62.3/22.1	

*Note: statistically significant differences with the initial state within the group: # – p <0.05 (even Wilcoxon criterion); with a synchronous control indicator: ** – p<0.01 (Mann-Whitney criterion).*

5. Discussion

As already mentioned, there is no information in the literature about changes in the state of the central nervous system and physical endurance of animals with limb amputation. The importance of such research is explained by the need to improve the complexity of rehabilitation measures and their experimental justification. Therefore, this study can be considered one of the first scientific explorations in this direction. On the other hand, a standardised animal model of amputation is important for studies of the condition of the stump, particularly for the development of means that improve the regeneration of skin and other structures and reduce muscle hypotrophy.

When discussing the results of behavioural tests, it should be noted that for an adequate assessment, it is important to analyse them comprehensively. The number of crossed squares in the Open field test is considered a marker of the level of motor activity and animal anxiety [22]. Contrary to expectations, the results show that amputated rats retain the ability to move quite intensively. In the absence of a prosthesis of the lost limb, locomotor activity only tended to decrease by an average of a quarter in the first days and by 21 % after 2 months. Considering these features, the absence of significant changes compared to the control allows us to state that the locomotor component of the stress reaction of rats does not change significantly after amputation. This is explained by the ability of animals to compensate for the absence of one limb by active use of three intact ones for movement, and this distinguishes the animal model of amputation from the course of the post-amputation period in humans, in which locomotor activity in the absence of a prosthesis and the use of crutches sharply decreases. The Open field test allows us to identify the features of various behavioural patterns under stress conditions, particularly the ability to orient and explore. It is believed that an increase in the number of rearing when exposed to intense lighting is a manifestation of anxiety in rodents [23]. Despite the absence of part of one supporting limb, the rats performed vertical rearings (mostly around the sides of the box), although their number tended to be less than in controls. This may indicate some anxiety reduction, although it is problematic to state this unequivocally given the certain biomechanical difficulties of standing on the hindlimbs in the partial absence of one of them.

It is more difficult to unequivocally interpret the changes in another component of the exploratory activity – the examination of the holes in the arena's floor (nose-pokes). This behaviour is based on the unconditional reflex of rodents to look into burrows and explore terrain [24]. This indicator also decreased in rats with amputation, although, unlike vertical rearings, this type of activity does not require the participation of the hindlimbs. In the early post-amputation period, the reduction in the inspection of the holes is statistically significant; in the long term, it is a trend. Although there is an opinion that anxiety can suppress the desire of rodents to explore a new environment, and less anxious animals, on the contrary, can explore it more intensively [25], in comparison with changes in other subtests of the Open field test (grooming) and indicators of the Light-dark box test, a significant reduction in the number of examined holes is logical to consider in the context of reducing anxiety. Also, these results prove that the absence of a limb causes changes in the functioning of the central nervous system at the level of unconditioned reflexes that are not directly related to the locomotor system. This phenomenon may be of fundamental importance and requires further study.

Grooming (washing, scratching) in rodents is considered an adaptive behaviour -under stress, a protective reaction to distraction [26], and intensive grooming (frequent washing) is considered a sign of concern and anxiety. A steady decrease in the number of acts of grooming, which we observed in both periods after amputation, especially in the later period, can be interpreted as a marker of a decrease in emotional responses, particularly anxiety. At the same time, the autonomic support of emotional responses (the number of defecation and urination) did not change compared to the control.

Relatively low anxiety in rats after amputation is also indicated by the results of the Light-dark box test, which is specific for this type of emotional disturbance. It is known that the time spent in the light part of the box depends on the level of anxiety of the animals: the higher the anxiety, the more animals tend to hide in the dark, closed space [18]. After amputation, rats spent significantly more time in the brightly lit compartment of the device than control rats, and a significant proportion of these animals did not enter the dark box during the entire 3 minutes of observation. This behaviour cannot be explained by the difficulty of movement, which prevents the rats from demonstrating a typical reaction – to quickly hide in the dark from the anxiogenic effect of bright light: their locomotor activity, as evidenced by the previous Open field test, is little different from that of control animals. An increase in the time spent in the illuminated compartment of the box indicates a change in the response to stressful conditions in rats with amputation. They can be interpreted as a reduction of anxiety, which confirms the findings of the Open field test.

So, concerning changes in the emotional state of the central nervous system after amputation of the hindlimb in rats, in two adjacent tests, Open field and Light-dark box, changes in response to stressful conditions, namely a decrease in anxiety, were first discovered. This distinguishes the emotional state of rats and humans after losing a limb. An increase in anxiety is more typical for humans [5, 26]. Anxiety in a person with a lower limb amputee is largely associated with impaired erticalsocial functioning, self-care ability, significant increase in energy expenditure for movement, which causes constant fatigue, stigmatisation, etc [1]. After losing one of them, animals that use 4 limbs for locomotion obviously have much fewer physiological limitations than humans and better compensate for impaired locomotor function. However, these differences do not allow us to fully explain the change in response to stressful conditions revealed in our study, which can be considered as a decrease in anxiety. The nature of this phenomenon needs further clarification.

The observed increase in body mass, which was found in rats, is also observed in a significant number of people after amputation of the lower limb. According to data [27], it occurs in 68.5 % of patients for the first year after amputation of lower limbs at hip level. In the increase in body mass, 4 trajectories of changes can be distinguished; 47.3 % of patients had a stable weight in the first year, and in 2.4 years, changes in body mass amounted to 4.2±11.5 kg [28]. The increase in the body weight of animals after amputation can be explained by a surplus of energy against the background of a decrease in the mobility of animals that were in conditions of normal spontaneous physical activity without physical training. These rats, as demonstrated by the results of the Open field test, retained the ability to move freely, but their locomotor activity decreased.

Although animals and humans move differently biomechanically, the proposed model of amputation in rats can be used to assess the state of metabolism and regeneration processes, create an experimental foundation for the optimal regime of physical rehabilitation and its pharmacological support, improve the functional state of the stump, and develop drugs for the preparation of prosthetics.

Practical relevance. The results of this study experimentally substantiate the directions of targeted correction of disorders of the functional state of the CNS, body weight, and physical endurance after lower limb amputation. This may contribute to the successful solution of the problem of rehabilitation after limb loss.

Study limitation: The study was performed on a limited number of animals (6) with amputation. A limited range of indicators of the functional state of the central nervous system was clarified. The increase in body mass is characterised phenomenologically without elucidating the peculiarities of animal metabolism.

Further research prospects. Increased number of markers of the functional state of the central nervous system, in particular, the study of the effect of amputation on the cognitive functions of animals. Study of possible sexual dimorphism of the central nervous system and body weight dynamics after amputation. Identification of metabolic features of the post-amputation period, as well as the functional state of the stump and its skin. Evaluation of the effect of rehabilitation measures.

6. Conclusion

1. An easy-to-perform model of transtibial amputation at the level of the lower third of the hindlimb in rats is proposed.

2. It was found that in the early post-amputation period (3–4 days after surgery) in rats, exploratory behaviour (primarily examination of hole, or exploratory nose-pokes) and emotional responses (grooming) in the Open field test are statistically significantly suppressed in the remote period (2 months) of significance only the reduction of emotional responses. In the Light-dark box test, the latency to enter the dark compartment increases reliably in both periods of observation (especially in the early period). Collectively, these results indicate changes in the reaction of animals with amputation of the hindlimb to stressful experimental conditions, in particular, an anxiety reduction, which requires further research.

3. In the rotarod test, a significant deterioration of movement coordination was found in rats with amputation both in the early and in the long term.

4. After amputation of the hindlimb in rats, the manifestations of depressive behaviour in the Porsolt swimming test progressed, and the physical endurance in the forced swimming test with a load was significantly reduced.

5. The body mass of rats after amputation of the hindlimb under conditions of usual physical activity significantly increases.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available at a reasonable request.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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