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Hypothalamic proline-rich polypeptide improves behavioral outcomes in rat model of focal cerebral ischemia

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Stroke is one of the leading causes of morbidity and mortality worldwide and according to the World Health Organization Statistics in 2030 stroke will be the leading causes of death in the world [14]. It is among the leading causes of adult long-term disability and loss of quality-adjusted life span [21]. Cerebral ischemia is the most common type of strokes, making 87% of all strokes [19]. Neurobehavioral consequences of cerebral ischemia includes wide spectrum of disturbances, such as paralysis, apathy, irritability, learning and memory difficulties, depression and anxiety. Post-stroke anxiety disorder is especially widely recognized in clinical field, with a prevalence of 12 - 28% [16]. Limited numbers of therapeutic treatments are available clinically and these treatments are only applicable to high selective groups. Thus, there is an urgent need to have a safe and effective treatment, and the development of new agents for stroke treatment is essential.

In this view the effects of the recently discovered PRP-1 on neurobehavioral changes caused by cerebral ischemia were investigated. The novel hormonal brain system of proline-rich polypeptides, consisting of 10–15 amino acids and four proline residues discovered by Galoyan et al. [13,15] are of special interest. It has been shown that one of them, PRP-1, consists of 15 amino acids and has the following primary structure: Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-GLn-Pro-GLy-Val-Tyr (AGAPEPAEPAQPGVY). It is produced by neurosecretory cells of hypothalamic nuclei (*nucleus paraventricularis* [NPV] and *nucleus supraopticus* [NSO]). There have been shown the beneficial effects of PRP-1 [1-2, 6-12, 20], including neuroprotective properties [5]. It was demonstrated cerebrovascular activity of PRP-1, which was displayed by increasing of local cerebral blood flow [3].

Materials and Methods

Animal preparation

Adult (4-5 months) male Albino rats, weighing 180-240g, were used. All animal's manipulations were approved by the Institutional Committee for the Humane Use of Animals (Ethics Committee) at Yerevan State Medical University, in accordance with the guidelines established by European Convention for Animal Care and Ethical Use of Laboratory Animals. Animals were housed in standard laboratory conditions with relative humidity of 40-70%, at a room controlled temperature of 25□2°C, under natural 12h light : 12h dark cycle. Rats were kept in standard laboratory cages (no more than 6 rats per cage) with free access to water and food. All the experiments were carried out between 9:00 and 18:00. The animals were allowed to acclimatize for 1 week prior the experiments.

Surgical procedure: left middle cerebral artery occlusion (MCAO)

Rats were anesthetized with chloral hydrate (dissolved in distilled water) at the dose of 400 mg/kg, intraperitoneally (i/p). The animal was placed on an elevated platform and its head was secured in a steriotaxic frame. The instrument used during the operation was a neurochemical Binocular Magnifier LBVO, with a fiber illuminator (Saint Petersburg). An incision of 1.5cm between the left eye and ear was made. The temporalis muscle was reflected from the underlying cranium. The zygoma was removed and the masseter muscles were retracted. The following procedure included a left middle cerebral artery occlusion, which was done by using a microsurgical microscope (OGME-PZ) set at a wide distance (f=190mm) and under a high focus (14x3.3). A small burr-hole (2mm in diameter) directly overlying the MCA was made using a high-speed dental drill under continuous saline irrigation. Care was taken to avoid thermal or physical injury to the dura. The 10-0 Ethicon suture was inserted under the left middle cerebral artery by sticking a needle through the dura mater. We used an adapted version of the Tamura et al. [22] method, modified by Topchyan A.V. [23]. According to this modified method, we did not remove the dura mater, instead we encircled it and then performed ligation on the middle cerebral artery. Furthermore, by Tamura et al. [23] method the ligation of the middle cerebral artery was done distal to the origin of the lenticulostriate artery, proximal to the inferior cerebral vein. In our experiments the ligation was done on the basis of middle cerebral artery, in order to enhance damage to the cerebral ischemia. After ligation of the middle cerebral artery (MCA) the blood flow was interrupted, which could be noticed under a surgical microscope. The temporalis muscle and skin were allowed to fall back and were then sutured separately. After MCAO the animals were divided into the following groups:

I group – occluded animals injected 0,9% NaCl twice daily (MCAO+saline treated rats),

II group – occluded animals injected PRP-1 20μg/kg twice daily (MCAO+PRP-1 treated rats). All rats were tested in EPM and Rota-rod tests before being subjected to MCAO and after divided into groups consequently on the 3rd, 6th and 12th day after MCAO.

Measurement of anxiety-related behavior

Evaluation of anxiety-like behavior was assessed in elevated plus-maze (EPM) test model, which is one of the most widely used animal models in contemporary preclinical anxiety and is based on unconditioned fear [17, 25]. The plus-maze was made of black wood and consisted of a central platform (10 x 10 cm), two opposite arms (50 x 10 cm) and enclosed arms (50 x 10 x 40 cm), which were elevated 50 cm above the floor [4]. To prevent rats from falling off, both sides of the arms were equipped with white rims (0.5 cm high). The EPM was situated in a bright light room, and the placement and lighting conditions were identical for each trial. At the beginning of each session, rats were placed in the center of the maze facing an open arm and were allowed to explore the maze for 5min. Thus, it was examined: percent of the time spent on the open arms, the open arm entries, percent of the time spent on the closed arms, total arm entries and the center square time. The animal was scored as having entered the arm when all four paws were inside the arm. Testing equipment was thoroughly cleaned up after each animal.

Evaluation of motor coordination

Maximum running capacity was measured using a motor coordination test [18, 26], the Rota-rod treadmill for rats (accelerating model 7750, *Ugo Basile, Varese, Italy*). The rats were placed on the rotating drum with an accelerating rotor mode (10 speeds from 4 to 40 rpm for 5 min). The apparatus recorded the time till the animal's fall from the rotating shaft as a performance time. The animals were trained for 5 trails per day for 2 days before MCAO to obtain stable baseline values.

Data analysis

The obtained values were expressed as the mean ± S.E.M. Student's paired t-test was performed to test for significant differences within the groups. One-way analysis of variance (ANOVA) was used to determine significant differences between the experimental groups. Statistically significant differences were inferred for $P<0.01$ and $P<0.05$. Statistical analyses were performed by computer software.

Results and Disscussion

One of the important neurobehavioral consequences of cerebral ischemia is anxiety. That is why one of the most implemented methods used for assessment of drug's efficacy and treatment outcome is the evaluation of anxiety. In view of this it was investigated rats behavior in EPM test, as this test

was undoubtedly one of the most widely used animal models of anxiety in the last two decades [17] in contemporary preclinical research on anxiety.

The EPM data are shown on Fig.1, 2, and 3. The carried out experiments demonstrate that MCAO is accompanied by anxiety development in ischemic rats. It is obvious that on the 3rd post-ischemic day (Fig.1), behavioral changes in saline treated rats (MCAO+saline) are noticeable: the time spent on open arms is decreased for 67.5% (A) ($F_{(1;38)}$=16.2 (P<0.01)), percent of the open arms entries – 27% (B) ($F_{(1;38)}$=3.4 (P>0.05)), total entries – 36.2%(C) ($F_{(1;38)}$=21.7 (P<0.01)), the center square time – for 68.2% (D) ($F_{(1;38)}$=25.7 (P<0.01)) compared with the controls. It was revealed that the injection of PRP-1 (MCAO+PRP-1 in the dose of 20μg/kg 2 times daily) prevents anxiety development, as there is a noticeable confidential enhancement of the time spent on the open arms – for 51.7 % (A) ($F_{(1;28)}$=7.3 (P_1<0.05), percent of the open arms entries – for 47.5% (B) ($F_{(1;28)}$=10.6 (P_1<0.01)), the center square time – more than twice compared with saline treated rats (D) ($F_{(1;28)}$=18 (P_1<0.01)). Though, for the total entries (C) there was registered a decrease – for 19.6% ($F_{(1;28)}$=3.2 (P_1>0.05)) compared with saline treated rats.

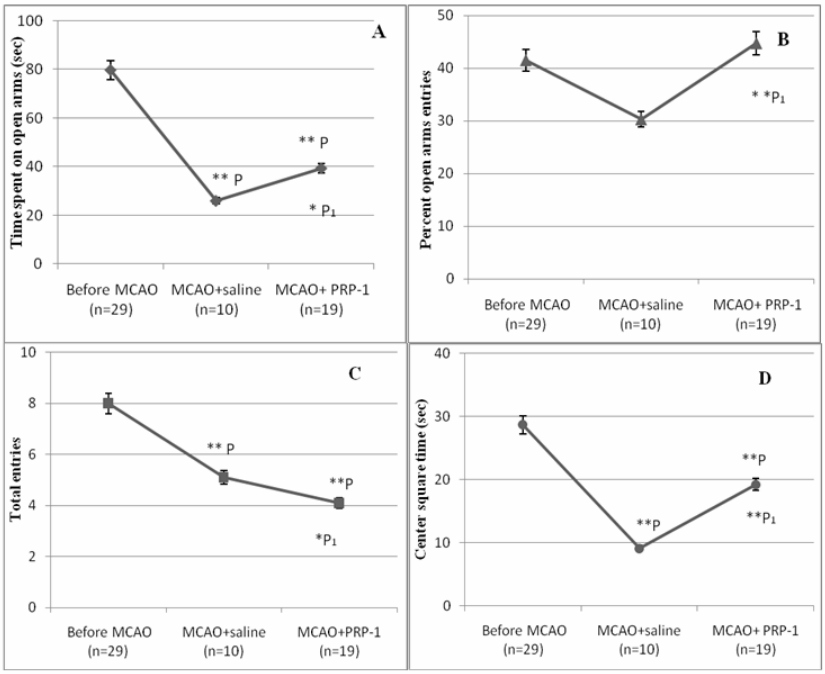

Fig. 1. Anxiety-related behavior of rats in the EPM on the 3rd post-ischemic day. A–time in seconds spent on the open arms, B–percent of the open arms entries, C–total entries, D–the center square time in seconds. Each value indicates the mean ± S.D. Significant differences: P–in comparison with the pre- MCAO rats, P_1–in comparison with the MCAO+saline treated rats, * -P, P_1 □ 0.05; ** - P, P_1 □ 0.01.

On the 6th post-ischemic day it was obtained further development of anxiety, which was displayed by decrease of all parameters characterizing the rat's behavior on EPM test. Thus, on Fig. 2 it is noticeable that on the 6th post-ischemic day, in saline treated rats the time spent on the open arms is decreased for 79.5% (A) ($F_{(1;38)}$=21.9 (P<0.01)), percent of the open arms entries – for 52% (B) ($F_{(1;38)}$=3.4 (P>0.05)), total entries – 37.5% (C) ($F_{(1;38)}$=23.3 (P<0.01)), the center square time – 64.2% (D) ($F_{(1;38)}$=21.5 (P<0.01)) compared with the controls. The injection of PRP-1 during 6 days after MCAO (immediately after ligation, twice a day, 20μg/kg) showed more extended anxiety. Thus, it is noticeable an enhancement of the time spent on the open arms more than 7 times ($F_{(1;28)}$=12.5 (P_1<0.01)) (A), and the percent of the time on the open arms about 2 times (B) ($F_{(1;28)}$=12.3 (P_1<0.01)), exceeding the pre-MCAO values. The center square time (D) on the 6th post-ischemic day is increased for 69% ($F_{(1;28)}$=3.0 (P_1>0.05)) compared with MCAO+saline group rats, though the total entries (C) is decreased for 8% ($F_{(1;28)}$=4.8 (P_1<0.05)) compared with the saline treated rats.

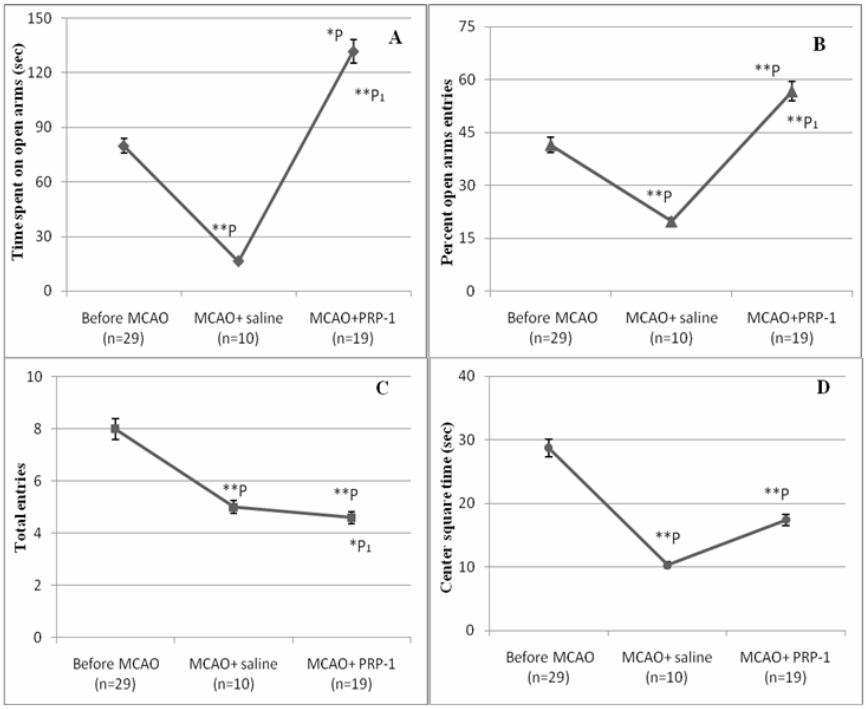

Fig. 2. Anxiety-related behavior of rats in the EPM on the 6th post-ischemic day. A – time in seconds spent on the open arms, B – percent of the open arms entries, C – total entries, D – center square time in seconds. Each value indicates the mean ± S.D. Significant differences: P – in comparison with before MCAO rats, P_1 – in comparison with MCAO+saline group rats. * -P, P_1 □ 0.05; ** - P, P_1 □ 0.01.

The same analysis of rat's behavior was done after 12 days of MCAO. Thus, on Fig. 3 it is demonstrated that on the 12th post-insult day all parameters of the EPM in MCAO+saline group rats are decreased as follows: time spent on open arms in seconds for 73.9% (A) ($F_{(1;38)}$=18.8 (P<0.01)), percent open arms entries for 40.9% (B) ($F_{(1;38)}$=9.0 (P<0.01)), total entries for 26.25 %(C) ($F_{(1;38)}$=20.2 (P<0.01)), center square time for 59.2% (D) ($F_{(1;38)}$=19.2 (P<0.01)) compared with control group rats. As it is shown on the above mentioned figures, the parameters characterizing rats' behavior in EPM test are decreased compared with 3rd and 6th days after MCAO. Though, i/p. injection of PRP-1 during 12 days after MCAO shows more extended anxiety. Thus, in case of MCAO+PRP-1 treatment all the values are increased, even exceeding the ones before MCAO values. The time spent on the open arms (A) compared with both the MCAO+saline and the control group rats was increased in the appropriate way: for 484% ($F_{(1;28)}$=14.4 (P_1<0.01)) and for 52.4% ($F_{(1;47)}$=5.4 (P<0.05)), the percent open arms entries for 129%(B) ($F_{(1;28)}$=32.7 (P_1<0.01)) and for 35.4%($F_{(1;47)}$=35.7 (P>0.01)), the center square time (D) for 202% ($F_{(1;28)}$=149.7 (P_1<0.01)) and for 23.3%($F_{(1;47)}$=5.5 P<0.05)), the total entries (C) for 33% ($F_{(1;28)}$=39.4 (P_1<0.01)) compared with saline treated group rats, and was about of the same value as it was in case of the controls ($F_{(1;47)}$=0.01 (P>0.05)).

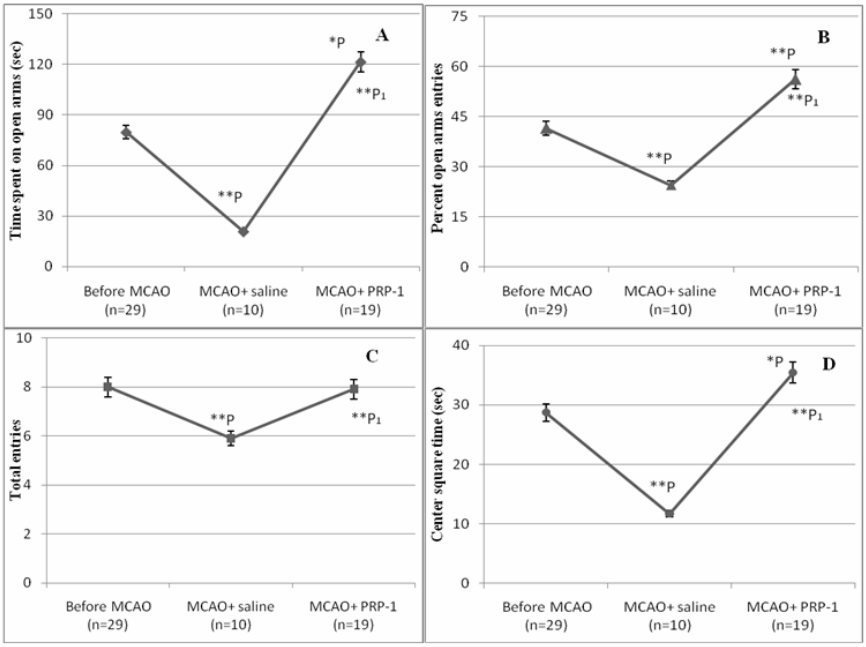

Fig. 3. Anxiety-related behavior of rats in the EPM on the 12th post-ischemic day. A – time in seconds spent on the open arms, B – percent of the open arms entries, C – total entries, D – center square time in seconds. Each value indicates the mean ± S.D. Significant differences: P – in comparison with before MCAO rats, P_1 – in comparison with MCAO+saline group rats, * -P, P_1 □ 0.05; ** - P, P_1 □ 0.01.

Thus, our investigation indicates that the local ischemic damage of the brain induced in particular by the long-term exposure (6, 12 days) of MCAO, within the selected EPM model for the assay of anxiety-related behavior, results in an expressed anxiety development: decrease of the time spent on the open arms, the open arm entries and the center square time with the locomotor activity. Obtained data indicate the anxiolytic effect of the PRP-1: increasing the time spent on the open arms, the open arms entries, center square time, total entries respectively. For evaluation of potential therapeutic efficacy of neuroprotection and assessment of the treatment outcome, besides the cognitive and behavioral characteristics, motor disbalance is important as well. The Rota-rod test is an established motor test to evaluate the balance and coordination aspects of rats before MCAO and on the 3rd, 6th and 12th post-ischemic days.

The experiments have shown that before MCAO animals had a moderate level of motor coordination which was counted by the failing frequency from the revolving rod. From Fig.4 it is obvious that the control group value of fall-down time was equal to 43.9±18.4 (n=40)**.** After MCAO on saline treated rats the fall-down time is decreased consequently: on the 3rd day it is equal to 29.1± 17.1 (for 33.7%) ($F_{(1;59)}$=8.99 (P<0.01)), on the 6th day – 16.9± 15.8 (for 61.5%) ($F_{(1;59)}$=31.28 (P<0.01)), and on the 12th day – 8.6 ± 8.5 (for 80.4%) ($F_{(1;59)}$=65.8 (P<0.01)). PRP-1 treated rats have a shown reduced fall-down time compared with saline treated group – for 36.4% (39.7 ± 23.6 (($F_{(1;39)}$=2.61 (P_1>0.05)) on the 3rd day post-insult day. On the 6th and 12th post-ischemic days of PRP-1 treatment there is observed a noticeable improvement of the value in comparison with both the saline and control group rats, appropriate by for 319% (70.9 ± 79.8) ($F_{(1;39)}$=8.8 (P_1<0.01)and for 61.5% ($F_{(1;59)}$=4.1 (P<0.05) on the 6th day after MCAO, and for 91,6% (87.4 ± 92.1) ($F_{(1;39)}$=14.5 (P_1<0.01) and 99% ($F_{(1;59)}$=8.3 (P<0.01)) – on the 12th day after MCAO.

Our data show that MCAO occlusion leads to development of motor discoordination, especially after 12 days. The mentioned disorder has been prevented by a long-term (during 12 days) administration of PRP-1.

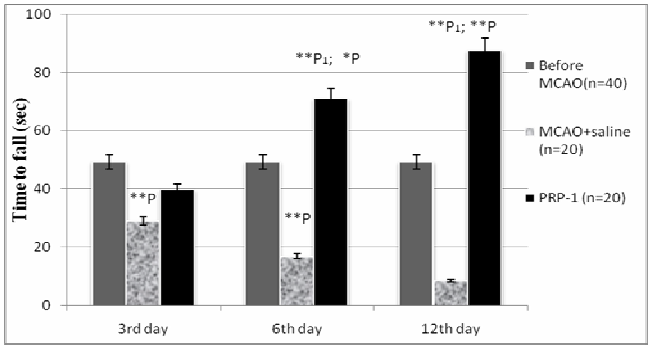

Fig. 4. Motor learning and coordination in the Rota-rod test. Each data point represents the mean ± S.D. Significant differences. P – in comparison with pre-MCAO rats, P_1-in comparison with MCAO+saline group rats, * -P, P_1 □ 0.05; ** - P, P_1 □ 0.01.

Thus, investigation of PRP-1, discovered as a unique mediator of the hypothalamus – neurohypophysis – bone marrow – thymus axis with immunotropic, neuroprotective, anti-ischemic, antitumor properties and its cerebrovascular effect [3], demonstrated that the mentioned peptide has the ability to protect ischemic brain tissue [24], which is displayed by improvement of behavioral outcomes after MCAO. PRP-1 prevents the development of anxiety and corrects motor coordination caused by local ischemic injury.

Hence, the obtained data could serve as a basic platform for development of PRP-1 as a potential protective agent for stroke management.

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Պրոլինով հարուստ պոլիպեպտիդ-1-ը բարելավում է առնետների վարքագծային փոփոխությունները ֆոկալ իշեմիայի պայմաններում

Է.Լ. Երիցյան

Նոր հայտնաբերված պրոլինով հարուստ պոլիպեպտիդ-1-ը (ՊՀՊ-1), որն արտադրվում է *N. supraopticus* and *N. paraventricularis*-ում և անջատվել է խոշոր եղջրավոր անասունների նեյրոհիպոֆիզի նեյրոսեկրետոր բջիջներից, հանդիսանում է բազմաբնույթ կենսաբանական հատկություններով միջնորդանյութ: Ցուցադրվել է վերջինիս հակամանրէային, հակաօքսիդանտային և նյարդապաշտպան ազդեցությունը: ՊՀՊ-1-ը բարելավում է նաև ուղեղի արյան շրջանառությունը՝ առանց զարկերակային ճնշման փոփոխության, որը հիմք հանդիսացավ ուսումնասիրել նրա ազդեցությունը առնետների իշեմիայով հարուցված վարքագծային փոփոխությունների վրա: Առնետների մոտ ուղեղի լոկալ իշեմիան մոդելավորվել է միջին ուղեղային զարկերակի կապումով (ՄՈւԶԿ): Կոորդինացիոն փոփոխությունները գնահատվել են *պտտվող-ձող* (Rota-rod), իսկ տագնապի զարգացումը՝ *բարձրացված խաչաձև լաբիրինթոս* թեստերում:

Ինչպես ցույց են տվել հետազոտության արդյունքները, ուղեղի ֆոկալ իշեմիան հանգեցնում է կոորդինացիոն խանգարման և տագնապի զարգացման: Մինչդեռ, ՄՈւԶԿ ենթարկված առնետների մոտ ՊՀՊ-1-ի 6 և 12 օր տևողությամբ ն/ո ներարկումը (օրական 2 անգամ 20մկգ/կգ դեղաչափով) կանխում է իշեմիայով պայմանավորված տագնապի և կոորդինացիոն խանգարումների զարգացումը:

Гипоталамический пролином богатый полипептид-1 улучшает поведенческие результаты у крыс в модели фокальной ишемии головного мозга

Э.Л.Ерицян

Новый пролином богатый полипептид-1 (ПБП-1) – медиатор, выделенный из нейросекреторных гранул нейрогипофиза *N. supraopticus* и *N. paraventricularis* крупного рогатого скота, проявляет широкий спектр биологической активности, включающий антиоксидантное, нейропротективное и антимикробное действие. ПБП-1 способствует также улучшению кровоснабжения мозга, не вызывая особых изменений в системном артериальном давлении. Принимая во внимание описанные свойства ПБП-1, было исследовано его влияние на изменения поведения крыс с церебральной ишемией.

Фокальная мозговая ишемия была смоделирована окклюзией средней мозговой артерии (ОСМА) у крыс. Поведение животных оценивалось в тесте *приподнятого крестообразного лабиринта*, координация движений – в тесте *Rota-rod*.

Показано, что ОСМА у крыс сопровождается развитием двигательной дискоординации и тревожности. В/б введение ПБП-1 крысам с ОСМА в дозе 20 мкг/кг в течение 6 и, особенно, 12 суток (два раза в день, сразу после перевязки) препятствует развитию тревожности и двигательного дисбаланса, вызванных локальным ишемическим поражением мозга.

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