

Comparison of the Behavior of Rats after Immobilization with Structural Changes in the Motor Cortex

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Behavioral and neuronal-glia changes after emotional stress induced by discontinuous (7–8 h per day for one week) immobilization were compared in Wistar rats ($n = 20$). Immobilization led to increases in horizontal and vertical activity and the duration of “comfort” grooming in the open field test. Morphometric measurements demonstrated significant increases in the density of hypoxic neurons in the motor area of the right hemisphere of experimental animals as compared with measures in controls. Hypoxic changes in neurons were functional in nature. Experimental rats can be regarded as a model of the redistribution of brain functional activity with a preferential increase in the role of the left hemisphere.

KEY WORDS: open field, behavior, rats, immobilization, motor cortex, neurons, glia.

Behavioral manifestations and their associated biochemical processes in the brain in stress loadings have been studied in some detail [2, 3, 8, 10, 12, 22, 24]. The question of possible morphological neuronal-glia changes in the brain accompanying stress is much more complex. Modeling of stress often involves creating situations in which there is either a pain component (electrical pain stress) or extreme loadings (swimming, running on a treadmill), which prevent the effects of the emotional component of stress from being detected. Models of emotional stress without pain sensations are generally based either on species-specific unconditioned reactions (cat-dog, emotional resonance using the Simonov method) or reactions to novelty – the open field test (OFT), elevated plus maze, or some kind of change in the experimental context (an unexpected sound, flashes of light, etc.). Combinations of different elements can be used. The limitations of these models include the relatively rapid acclimation of the animals (rats), high individual sensitivity, and difficulty in dosing the exposure. Our study aimed to provide a comparison of behavioral and neuronal-glia changes after emotional stress induced by discontinuous (7–8 h per day)

immobilization of the animal. The version of immobilization used here, i.e., restriction of the animal's ability to move, does not prevent movement of the head or tail, and allows paw movement; only freedom of movement is restricted. In the physiology of higher nervous activity, resistance of the animal to attempts to restrict its movement activity was termed, by Pavlov, “the freedom reflex” [16]. Pavlov noted that the freedom reflex is “a reaction general to animals, one of the most important innate reflexes; the absence of this reflex would interrupt the animal's life at the smallest obstacle” [16, p. 345]; furthermore, it is difficult to eliminate this reflex in animal studies. These points provide grounds for regarding immobilization as a powerful emotional stressor for animals. The effects of immobilization on the animal's behavior are assessed using the open field test.

Our task was to study the state of nerve and glial cells in the motor cortex after one week of discontinuous immobilization of rats. This part of the brain was selected as functionally related to the nature of the treatment used.

METHODS

Studies were performed using 20 male Wistar rats weighing 220–250 g. Rats of the experimental group (EG,

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seven animals) were subjected to daily immobilization for 7–8 h using special perforated plastic tubes, which allowed the animal's tail and head to remain free. This prevented overheating of the rat and compression of blood vessels running to and from the head. During immobilization of the experimental rats, control animals were kept in the same room, in their home cages. The control group consisted of 13 rats: six were "intact" controls, not subjected to any treatment, and seven were control rats, which were tested twice in the OFT simultaneously with six experimental rats. Testing in the OFT, verification of the rats' responses to the approach of the experimenter's hand, and suspension of the animals were performed before the beginning of immobilization and one day after the end of immobilization. Experiments were performed in accordance with humanitarian principles as laid out in European Community Directives (86/609EC).

The open field was an arena 1 m in diameter divided into 32 squares. During the experiment, the field was illuminated at about 30 Lx. The rat was placed in the center of the field. The observation time was 5 min. The whole experiment was recorded using a video camera and the recordings were analyzed. The number of squares crossed (horizontal activity) was measured, along with increases in the numbers of squares crossed in the internal sectors of the open field, the total number of rearings (with and without support from the wall; vertical activity), the duration of grooming (substitution activity), and the duration of active behavior in the OFT.

Analysis of the results consisted of assessing the behavior of each rat individually with identification of the differences (with signs) between measures in the preliminary and final tests for the individual animal; group mean values were then calculated. Comparison of the results of behavioral tests was performed using the nonparametric Wilcoxon test.

One day after the behavioral experiments, animals were decapitated, brains were removed and fixed in 4% paraformaldehyde solution and, after 14 days, were embedded in paraffin-celloidin using a standard method. Brains from a control animal and an experimental animal were placed in a single block. This ensured identical conditions (temperature, chemical, vibration) during embedding and preparation of sections. The coordinates of the motor zone of the neocortex were taken from a stereotaxic atlas of the brain [25]: bregma from 1.7 mm to 0.4 mm; 1 mm from the midline, and 2 mm across the surface.

A microtome was used to prepare frontal sections of the motor cortex of the brain of thickness 8 μm ; every tenth section was placed on a slide and stained by the Nissl method. It should be emphasized that brain sections from control and experimental animals were mounted on the same slide, so all stages of staining were performed simultaneously. Classical hypoxic neurons were regarded as pathological: these were hyperchromic neurons with whole nuclei, well stained apical

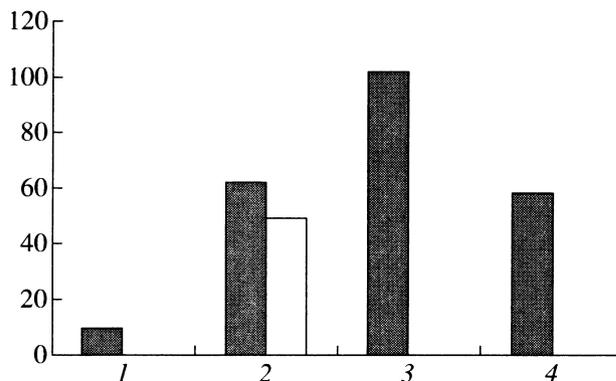


Fig. 1. Increases in measures of the activity of rats in the open field test after one week of immobilization. The vertical axis shows group mean increases in behavioral parameters (during 5-min tests): 1) number of rearings; 2) number of squares crossed (dark columns show the total numbers of squares; white columns show numbers of squares in the inner sectors of the open field); 3) total duration of grooming (sec); 4) duration of activity, sec. There were significant increases in 1–3, $p = 0.043$ (Wilcoxon's test). There was a tendency to an increase in the duration of activity (4), $p = 0.08$.

dendrites, and a characteristic corkscrew sinuosity [19, 23]. On each fifth section, an ocular grid with a square side of 130 μm in the $\times 20$ ocular with a $\times 40$ objective was used to count the total numbers of neurons and pathological neurons, the numbers of satellite glial cell nuclei (astrocytes and oligodendrocytes), and the numbers of nuclei of so-called free glia, separated from neurons by distances of more than the diameter of a glial cell (astrocyte, oligodendrocyte) nucleus. Counts on sections were performed separately in each hemisphere, the right and the left, in 25 fields on five sections i.e., 50 fields per whole brain.

Neuron body area was also determined. This was done by measuring (in μm) the transverse (a) and longitudinal (b) diameters of the cell and calculating the area as $S = \pi/4(ab)$.

Mean parameters of nerve and glial cell elements were calculated for the motor cortex of each brain, separately for each hemisphere and as a total of both. Morphometric data obtained in animals of different groups were compared using the Mann–Whitney test run on the standard program bundle Statistica.

RESULTS

The results presented in Fig. 1 show that after one week of discontinuous immobilization, rats showed increased vertical activity in the OFT, along with increased horizontal activity, and increased total grooming time (changes in these parameters were significant at the level of $p = 0.043$, Wilcoxon test). The increase in the total duration of activity was a trend. In the control group, there were no significant changes in behavioral measures.

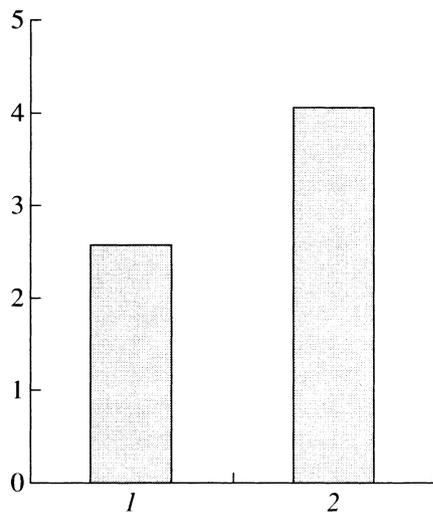


Fig. 2. Numbers of pathological neurons in layer V of the motor cortex per unit area summed for the hemispheres (field of $130 \times 130 \mu\text{m}$). Mean values in control (1) and experimental (2) groups. There was a significant difference in the mean numbers of pathological neurons in each hemisphere between the control group and the immobilized group: $p < 0.01$ (Mann–Whitney test).

The increase in the number of rearings in experimental rats resulted from an increase in the proportion of rearings without the support of the wall. In control rats, grooming was generally restricted to brief washing of the snout or short periods of scratching with hindpaw. The duration of grooming in rats of the experimental group increased due to increases in the duration of grooming of the whole body (“comfort grooming”), which could occur even in the center of the field. Control rats moved predominantly along the walls of the open field; of six rats, three made excursions to the center of the field (one of them twice). Rats of the experimental group moved freely over the whole of the field, changing movement direction at any point; these animals did not remain mainly by the walls.

After 3–4 days from the beginning of immobilization sessions, rats easily entered the tube, though after fixation of the animal, vocal reactions and attempts to escape persisted to the end of the experiments. After release from the tube, the rats immediately started grooming; they showed no aggressive reactions to the experimenter’s hand.

Morphometric measurements showed that the density of pathological neurons, in terms of the mean for the two hemispheres, was significantly greater in experimental animals (Fig. 2, 2) as compared with control rats (Fig. 2, 1; $p < 0.01$). The densities of pathological neurons in the motor zone of the cortex in the two hemispheres are shown for control and experimental rats in Fig. 3. It is important to note that there was a significant increase ($p < 0.02$) in the density of pathological neurons in the motor zone of the

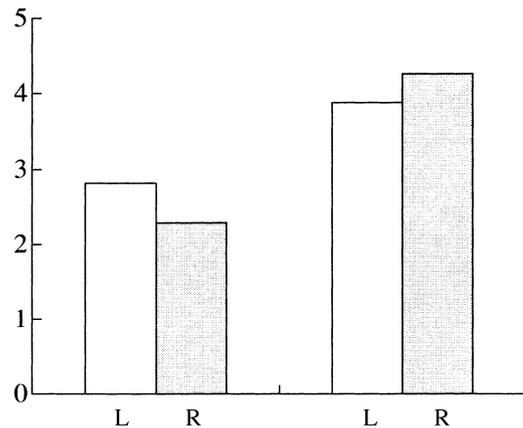


Fig. 3. Numbers of pathological neurons in layer V of the motor cortex in fields of $130 \times 130 \mu\text{m}$. White columns show controls; dark columns show experimental rats. L = left; R = right hemisphere. A) Controls; B) after immobilization. There were significant differences (Mann–Whitney test) in the numbers of pathological neurons in the right hemisphere (dark columns) between rats of the control and experimental groups: $p < 0.02$. There was no difference between the numbers of pathological neurons in the left hemisphere (white columns): $p = 0.34$.

cortex in the right hemisphere in experimental rats as compared with values in control animals (Fig. 3). This plot also shows that in terms of the density of pathological neurons, differences in zones of the motor cortex between the right and left hemispheres represented a trend in control animals ($p = 0.15$); this trend was stronger in rats tested after immobilization ($p < 0.11$).

Determination of the density of glial elements, neuron body areas, and glial cell nuclei revealed no significant differences when mean values were compared between groups of rats. The only feature was individual variability, both among control animals and among experimental animals.

There were no significant changes in body weight or the state of the fur.

DISCUSSION

Differences in behavioral measures in the OFT between initial and final testing in rats of the control group, i.e., before and after the period of immobilization of rats of the experimental group, were not significant, so changes in behavioral measures in rats of the experimental group can be attributed to the treatment applied. Our version of immobilization did not subject the animal to pain; furthermore, it has been shown to modify pain sensitivity in rats, such that there are increases in the pain sensitivity threshold at six days of immobilization [20]. Restriction of movement activity is a stimulus inducing the “freedom reflex.” Hindrance to

the animal's movement activity is as adequate a stimulus as food is for food-procuring reactions and pain for defensive reactions [16, 18]. Each "session" of immobilization was accompanied by clear signs of defensive and aggressive behavior: the rats squeaked and twisted their heads and tails, and tried to bite any object brought to the snout. During a week of experiments, these behavioral features persisted (i.e., there was no extinction of the "freedom reflex") and provided evidence of the negative emotional state of the animal. Pavlov [16] emphasized that resistance became all the stronger as the duration of lack of freedom increased. The model used here is close to the situation in which a human has to maintain forced immobility, for example, in limb fractures (constant immobilization) or, a more stringent variant, in solitary confinement. The process of suppression of the "freedom reflex" as a method amounts to an emotogenic (psychogenic) stressor, reactions to which differ from reactions to physical stresses [21, 24, 26, 27].

The end of immobilization induced a significant increase in horizontal and vertical activity in rats in the OFT. The observations were consistent with Lorentz's brief formulation: the mere termination of dissatisfaction is perceived as marked satisfaction [14]. However, this increase in activity was specific in nature. Experimental rats changed their behavioral strategy in the OFT: they shortened their paths along the walls and lengthened them in the intermediate and central parts of the OFT (Fig. 1, 2); "comfort grooming" became predominant in place of simple washing; the number of rearings without using the wall for support increased. All these observations indicate that these animals lacked anxiety or fear in the situation which prior to immobilization had seemed dangerous. Rats of the control group retained the former qualitative and quantitative behavioral features.

Comparison of the picture of the behavior of rats of the experimental group with changes in morphological characteristics of nerve elements in the motor cortex demonstrated a notable relationship. It follows from our results that there was a significant increase in the density of pathological neurons seen in the motor cortex of the right hemisphere of experimental rats as compared with the counterpart in control animals. The decrease in activity in the right hemisphere due to exclusion of a proportion of the neurons was of equal strength to the increase in functional activity in the left hemisphere. The literature contains extensive material on the behavioral consequences of artificial exclusion of the right hemisphere. This applies particularly to the studies of Bianki et al. [6], who showed that inactivation of the right hemisphere in male rats by spreading depression produced sharp changes in their behavior in the open field, with significant increases in motor and orientational activity and the number of grooming reactions. Belenkov noted that a characteristic feature of cold exclusion of the motor and sensorimotor areas of the cat brain consisted of the appearance of marked excitation, evaluated by behavioral methods [5], as

well as increases in neuron activity on the symmetrical side [1]. Simonov wrote that exclusion of the right hemisphere simplifies the situation and that this leads to positive emotions [18]. The mainly right-sided nature of changes is also due to the anatomical features of the connections of the right and left hemispheres. The left hemisphere has greater connections than the right with the activatory stem formations, while the leading connections of the right hemisphere are diencephalic [7].

It is known that the hemispheres can mount different responses to acute stress; this has particularly been demonstrated in terms of the lipid peroxidation (LPO) reaction and probably depends on initial significant differences in LPO levels and on the fundamentally different responses of the right and left hemispheres due to different levels of LPO and neuron membrane lipid composition [9, 10, 12].

The literature contains reports of the biochemical and microcirculatory measures of the state of the brain after immobilization for one week using a scheme analogous to that used here [11]: immobilization sessions increased glucose utilization and decreased capillary blood volume, with increases in blood lactate levels; functional hypoxia predominated in the frontal and parietal cortex, thalamus, hypothalamus, midbrain, cerebellum, pons Varolii, and medulla oblongata. The role of circulatory cerebral hypoxia in the post-stress development of neurosis-like states in animals is well known [3]. In the present study, the nature of pathological changes to neurons corresponded to known biological shifts in the brains of experimental rats in the stress protocol used here.

We found no significant changes in the density of glial elements or nuclear protoplasm size in light and dark neurons and glial cells, indicating the absence of serious abnormalities in the functioning of the motor cortex. Glial function is associated with involvement in the regulation of neuron activity; quantitative and qualitative changes in glial cells in stress conditions are more marked than changes in neurons [13, 15]. Persistence of cortical function is also indicated by the fact that short-lived, interrupted immobilization (for one week) allows the rats to acquire operant defensive reflexes (albeit with some defects: the reflex is acquired at later time points, there is a decreased proportion of reflex performances, and reduced retention of the temporal association) [17].

Activation of behavior in the OFT as an effect of discontinuous immobilization may point to compensatory processes and provide evidence of adaptation of the animals to changing conditions. The characteristics of adaptation on fractionated stressing have been described in some detail in the literature [2, 3, 8, 20, 22]. In particular, studies reported in [8] showed that the sequelae of single exposures to stress induced by "mild" (non-traumatic) immobilization lasted more than one day. This was apparent as increases in the release of noradrenaline (NA), adrenaline (A), and dopamine (DA). Repeated immobilization significantly

increased the NA reaction, while the DA tendency continued and release of A decreased. The increase in dopamine activity in mild stress was regarded as compensatory, increasing the tone of the emotionally positive system in novel conditions. The consequences of discontinuous stress also included increased serotonin release in the hypothalamus [8]. It has been suggested that the increase in the dopamine concentration in the intercellular environment can reduce serotonin release into the same environment in conditions of emotional stress [4]. In any case, the behavioral measures obtained in our studies indicate that the final balance is probably determined by dopamine: if the term "euphoria" can be applied, then, most precisely, it determines the visible behavior of the animals after immobilization ends. The decreased functional activity of neurons in the right hemisphere may be one of the routes to adaptation (training) of the body to stress loadings.

CONCLUSIONS

The data obtained here show that hypoxic changes in neurons in the motor cortex following one week of discontinuous immobilization are functional in nature. In this type of emotional stress, significant increases in the densities of hypoxic neurons were seen in the motor zone of the right hemisphere in experimental animals as compared with control animals. The experimental rats can be regarded as a natural model of the redistribution of the functional activity of the brain, with a predominant role for the left hemisphere.

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