Effects of Diazepam, Piracetam, and Mexidol on Passive Avoidance Response

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Abstract—Effects of the anxiolytic diazepam and nootropics piracetam and mexidol on passive avoidance conditioning (PAC) in rats were compared in experiments using a three-compartment apparatus. The latter consisted of a central, brightly lit compartment, a noxious dark compartment in which footshock was delivered to a rat, and a safe dark compartment, where the rat was not exposed to electric shock. Footshock during the acquisition of passive avoidance response in the control animals caused an abrupt increase in the latency to escape from the central compartment during testing but did not result in preference for a safe compartment. On the basis of these data on differential effects of footshock on PAC, we suggested that learning processes, which determined the motor response delay and the safe compartment preference, had a diverse associative nature. An increase in latency is associated with the classical fear conditioning regardless of the place of electric shock exposure. In contrast to this, the safe compartment preference is associated with the formation of the memory trace about the location of the footshock exposure. The use of pharmacological substances that affect fear and memory in different ways provided additional arguments in favor of the assumption about various associative processes determining passive avoidance learning. Reduction in the level of fear using diazepam decreased the latency of motor response compared to the control value but did not affect the preference for a safe compartment. In contrast, the mnemotropic properties of piracetam and mexidol increased the preference for a safe compartment without increasing the latency. These differential pharmacological effects confirm that PAC is based on the fear conditioning, which causes an increase in the latency to escape from the central compartment, and on the memory of the location of shock exposure, which provides the preference for the safe compartment.

Keywords: modified passive avoidance, memory, diazepam, piracetam, mexidol, conditioned fear, safe compartment preference.

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Passive avoidance conditioning (PAC) is widely used to study the effect of various factors on learning and memory. In particular, the technique in experimental psychopharmacology is used as a basic model for the analysis of newly synthesized nootropic substances due to advantages such as rapid learning and the possibility of differential effects on different phases of memory, which is not burdened by subsequent learning. A chamber consisting of dark and illuminated compartments connected by a door is usually used. Due to the hole-exploratory behavior, the animal placed in the illuminated compartment scurries quickly to the dark compartment, where it receives an electric footshock. This leads to a sharp increase in the latency to enter it during subsequent testing, which is considered to be an indicator of learning and memory.

There are few works devoted to the analysis of the nature of the memory trace formed during the passive avoidance learning, despite the importance of this problem. According to the postulate, the memory trace contains the characteristics of the place of application of pain stimulation and provides “anticipation of the dangerous consequences of entering it” [1]. To analyze this postulate, passive avoidance response was studied in a three-compartment apparatus, which consisted of an illuminated compartment, a noxious dark compartment in which the rat was shocked, and a safe dark compartment in which it was not exposed to painful electric shock [2]. This postulate suggested that the animals would enter the safe compartment.

However, it was found that an increase in the response latency did not lead to the preference for the safe compartment not only after one but also after three testing trials (electric shock in a noxious compartment after entering during retesting). On this basis, it was suggested that the learning processes that caused an increase in the latency and the preference for a safe compartment had a different associative
nature. An increase in the time spent in the central compartment is due to a nonspecific defensive freezing behavior that is not linked to the place of electric shock exposure. The preference for a safe compartment is associated with the need to remember the place of shock application and development of spatial differentiation between it and the noxious compartment. In our opinion, the analysis of the role of these associative processes can be facilitated by studying the effects of neurotropic substances that selectively affect learning and memory or fear.

Modern experimental psychopharmacology possesses these possibilities. Nootropics with a wide range of pharmacological effects (piracetam and mexidol, in particular) are drugs affecting learning and memory [3]. Anxiolytics are drugs that affect fear and anxiety [4]. The modulation of the GABAergic system using agonists and antagonists of GABA receptors in the central nervous system is an important mechanism of their action [5–9]. Diazepam is a widely known agonist of GABA receptors used to affect fear, during PAC, in particular [10–12].

According to the mentioned above, the effects of piracetam, mexidol, and diazepam on PAC using a three-compartment apparatus were compared to analyze the memory trace formed.

MATERIALS AND METHODS

This study was carried out using 83 white outbred male rats weighing 180–200 g. The rats were kept in plastic cages under the conditions of 12-h daylight and unlimited access to water and food. Three series of experiments with both control and experimental groups were carried out (Table 1). Thirty minutes prior to the experiment, tested animals of the first series were intraperitoneally injected with 300 mg/kg of piracetam (125 mg/mL), while 0.5 mg/kg of diazepam (2.2 mg/mL) and 50 mg/kg of mexidol (50 mg/mL) were administered to the animals of the second and third series. At the same time, the control animals were injected with an equivalent volume of physiological saline.

The experiments were carried out in a chamber with dimensions $60 \times 30 \times 35$ cm, which was divided into three compartments. The small doors connected an illuminated central compartment with the side dark compartments, in one of which the rat was shocked during passive avoidance response (hereinafter referred to as the “noxious compartment”), while no electric shock was given in another one (the “safe compartment”). During the first session, spontaneous motor activity was assessed for 3 min in the chamber with open doors. In the second test session, the animal acquired PAC when it was placed into the illuminated compartment with its tail toward the far wall; and the latency to enter one of the dark compartments was recorded. After that, the door was closed, and an electric shock (0.7 mA) was applied for 10 s through a grid floor. A day after, during the third session, the rat was placed into a lit compartment and tested for passive avoidance response. If the animal did not leave the central compartment, its behavior was recorded for 5 min.

To evaluate PAC, in addition to the latency to escape from the central illuminated compartment, the preference for one of the compartments by the rats was recorded. The statistical significance of the increase in the latency during testing (in comparison with the initial value) was determined using the Wilcoxon signed-rank test. The difference between the experimental and control groups was determined using the unpaired two-sample Wilcoxon test, while the significance of the frequency of entries into the compartments was assessed using the criterion of frequency agreement [13].

RESULTS AND DISCUSSION

According to the experimental data shown in Fig. 1, the electric shock delivered to the rat entering one of the dark compartments from the starting compartment during the passive avoidance training resulted in a statistically significant increase in the latency of this response in the first two series of experiments in control animals. This suggests that, according to the conventional indicator of the successful acquisition of passive avoidance conditioning (i.e., the latency), learning was observed. However, this did not lead to a preference for entering the safe compartment (Table 1). In the first series of experiments, the number of control rats entering the safe compartment was four times lower than the number of animals remaining in the central compartment and entering the noxious compartment. In the experiments of the second series, the number of such animals was even less than the number of those who preferred only the noxious compartment.

The difference in the latency values within the group to which piracetam was administered and in the corresponding control group was not statistically significant. This may indicate the absence of the effect of the drug on learning and memory in this model. However, in contrast to the control animals, the number of experimental rats entering the safe compartment was several times higher than the number of animals entering the noxious compartment and remaining in the central compartment. The lack of preference for a safe compartment in control animals described here and the positive effect of piracetam on its choice agree with the previously obtained data [14], which indicates good reproducibility of the experimental results.

In control animals of the third series, the latency also abruptly increased (seven times), although this increase did not reach a statistically significant level ($p = 0.056$), which can be explained by the wide range...
of values in this group due to the presence of untrained animals, whose latency remained low. In contrast to the abovementioned increase in the latency in the control animals and the animals affected by piracetam, the latency in the tested rats affected by mexidol did not increase compared to the initial level; moreover, it was less than the value that was recorded in the control animals during PAC tests. According to the conventional belief, these results could indicate that the drug did not improve memory and even suppressed it.

However, this, first of all, contradicts the previously shown positive effect of mexidol on training and memory [15]. Second, more than 85% of the rats affected by mexidol preferred a safe compartment during this experiment, and no single experimental animal remained in the central compartment. This indicates the high efficiency of mexidol in the formation of the memory trace of the painful stimulation place and the previously mentioned “prediction of the consequences of entering it.” The short latency to enter the safe compartment in most rats indicates efficient action of the drug on the spatial component of memory in this model. Mexidol possesses antioxidant activity, which can provide the preference for a safe compartment, as was found in the case of using the antioxidant carnosine [16].

Diazepam injection reduced the latency to escape from the central compartment compared to that in control rats. According to this, this agonist of benzodiazepine receptors is believed to suppress rodents’ memory [11, 12]. Our data on the lack of preference for the safe compartment (Table 1) agree with this conclusion.

According to the obtained results, the values of the passive avoidance latency and the number of entries into the safe compartment provide multidirectional characteristics of PAC acquisition. These data agree with the previously obtained results indicating that these characteristics obey various quantitative laws [14]. In the cited study, the latency characterizing time spent in freezing behavior in the central compartment showed a linear dependence on the electric current intensity and had the highest values under greater

![Fig 1. Latencies to escape from the central compartment. The left bars in each pair are the latencies before training, and the right bars are the latencies during testing. * A significant increase compared with the latency during learning according to the Wilcoxon signed-rank test (p < 0.05); #, a tendency to an increase compared to the latency during learning according to the Wilcoxon signed-rank test (p < 0.07); v, significant difference from the control group according to the Wilcoxon criterion (p < 0.05).]

Table 1. Preference for the compartment by the animal during the testing of passive avoidance conditioning

| Series | Substance          | Noxious compartment | Central compartment | Safe compartment | Z    | p*   |
|--------|--------------------|---------------------|---------------------|------------------|------|------|
| 1      | Saline (n = 10)    | 5                   | 3                   | 2                | 3.13 | 0.002|
| 2      | Piracetam (n = 10) | 1                   | 2                   | 7                | 2.236| 0.025|
| 3      | Diazepam (n = 20)  | 16                  | 0                   | 4                | 4.111| 3.984E–5|
| 4      | Saline (n = 16)    | 10                  | 2                   | 8                | 1.581| 0.114|
| 5      | Mexidol (n = 7)    | 4                   | 3                   | 9                | 1.061| 0.289|

Note: * The values of the criteria for the agreement of frequencies and significance levels in relation to the total value of the preference for the noxious and central compartments are shown.
exposure. Other authors reported a similar linear dependence of time spent freezing upon electric current when using the model of posttraumatic stress disorder [17]. On the contrary, the preference for a safe compartment was least observed with greater exposure, and most animals remained in the starting compartment [14]. This indicates enhanced freezing behavior that complicates the preference for a safe compartment. Therefore, the obtained data confirmed the previous hypothesis [2] that the learning processes, which caused a delay in the motor response and determined the preference for a safe compartment, had a different associative nature.

An increase in the latency and, therefore, freezing after a single training trial indicated the suppression of the innate response of the rodent to escape from the light compartment into the dark one. This is explained by the rapid learning of the conditioned emotional response of fear based on classical conditioning, which was repeatedly shown in different experiments [6–12, 17, 18]. On the basis of the test results and literature data, we may conclude that conditioned fear can be increased by enhanced painful shock stimulus and reduced by anxiolytics; this confirms its role in PAC.

It is important to note that the footshock delivered during the passive avoidance training increased freezing behavior and the latency of the motor response not only in the same apparatus but also in a dimly lit shuttle box, where this shock was not delivered, which indicates the nonspecific nature of the conditioned fear [2]. A similar increase in freezing in one chamber due to electrodermal stimulation in the second one was also observed by other authors, who also suggested the nonspecific nature of the conditioned fear [17, 19].

According to the results of this and previous experiments [2], the rapid acquisition of the conditioned emotional fear response, providing for a dramatic suppression of innate behavior, did not result in the rapid formation of the memory trace, which reflected the spatial characteristics of the experiment. This probably suggests that remembering the place of shock application, spatial differentiation, and the preference for a safe compartment are complex processes and difficult tasks compared to classical conditioning acquisition.

The necessity to develop spatial differentiation complicates the task; it is used to reveal neuropharmacological effects on learning and memory [20, 21]. The change in the location of the hole in the shuttle-box apparatus during the avoidance response training abruptly decreases the response reproducibility. Moreover, nootropics that do not affect the acquisition of the avoidance response contributed to the learning of a spatially modified skill [21, 22]. In our opinion, this complication of the task associated with the need to identify a safe compartment explains why piracetam and mexidol did not increase the latency (an increased latency indicates the acquisition of the classical fear conditioning) but proved to be more effective when the proposed modified method was used during our experiments.

To our knowledge, the most interesting fact obtained during this study was not previously noted in the literature. As mentioned above, an increase in the latency is due to the fear caused by footshock. Therefore, the absence of such an increase in the latency to escape from the central compartment observed in the rats affected by mexidol indicates the absence of fear, despite the previous exposure to electric footshock. At the same time, all animals left the central compartment, and six of seven rats entered the safe compartment. A possible explanation is based on bilateral interrelations that exist between the problem-solving ability and emotional tension of animals [23]. From this point of view, fear reduces the ability to solve a problem, while the availability of information necessary to solve a problem reduces the basis for fear. We have previously shown that, in addition to improved reproducibility of the avoidance response under the conditions of its functional impairment, nootropics, including piracetam and mexidol, also reduced the emotional tension, which causes intersignal responses, defecation, urination, and other responses [15, 21].

In our experiments, a memory trace was rapidly formed under the effect of mexidol. This memory trace reflected the place of footshock exposure. As a result, the rats did not enter the noxious compartment and preferred the safe one. Due to this, fear did not increase in comparison with the initial value, and the latency after the footshock did not increase either, in contrast to the control animals.

Thus, the results obtained during this study confirmed the previous assumption [2] about the difference in associative processes that caused an increase in the latency and the preference for a safe compartment. Inhibition of the motor response, leading to an increase in the latency to escape from the central compartment, is associated with the conditioned emotional fear response, which is not linked to the place of electrodermal stimulation. The preference for a safe compartment is associated with the acquisition of spatial memory, which provides differentiation between the noxious and safe compartments. For further anal-

**Table 2. Effect of drugs on the latency to escape from the central compartment and the preference for the safe compartment**

| Drug     | The effect of the drug on: | latency | preference for the safe compartment |
|----------|-----------------------------|---------|-------------------------------------|
| Piracetam| Absent                       | Positive| Absent                              |
| Mexidol  | Negative                    | Positive| Absent                              |
| Diazepam | Negative                    | Positive| Absent                              |

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ysis of the role of these associative processes, we used drugs of the class of nootropics developed to stimulate cognitive and mnestic processes as well as anxiolytics that reduce anxiety and fear. Table 2 summarizes our results on the effect of these drugs on PAC. According to these data, under the effect of nootropics, the preference for a safe compartment increased, while the latency did not increase compared to the control values. On the contrary, the anxiolytic diazepam reduced the latency to escape from the central compartment but did not affect the preference. These differential pharmacological effects confirm that passive avoidance response is based on the acquisition of conditioned fear, which causes an increase in the latency to leave the central compartment, and the memory of the place of painful electric shock exposure, which results in the preference for a safe compartment.

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**COMPLIANCE WITH ETHICAL STANDARDS**

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. The experiments were carried out in compliance with the ethical standards for the care and use of animals established by the Moscow State University Bioethics Committee. The Principles of Good Laboratory Practice (National Standard of the Russian Federation GOST R 53434-2009) and European Communities Council Directives (November 24, 1986; 86/609/EEC) were followed.

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