Active Control and Its Stress Effects

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The effects of 5-fold injections of normal saline were studied in Wistar rats in two conditioned reflex situations: defense and alimentary. Intact animals developed conditioned pain avoidance reflex in repeated tail-flick test after 60 presentations over 3 days. Two injections of normal saline were sufficient for the formation of a stable negative stress status in experimental rats, which manifested by a drastic increase in tactile sensitivity persisting for 5 days after discontinuation of injections. Only 15% experimental rats *vs.* 40% intact animals developed the food-procuring response in a multialternative maze. Presumably, repeated injections of normal saline should be regarded as a potent negative stress factor modulating animals behavior, and these signs should be taken into consideration, when interpreting the effects observed in pharmacological investigations.

Key Words: *normal saline; tail-flick test; training; Wistar rats*

Adequate and correct control is a central problem in biomedical studies. A group of intact animals (passive control) and a group of animals injected with the solvent, usually normal saline (NS) (active control), are commonly used in the evaluation of drug effects in pharmacology. The use of active control in the experimental protocol is explained by the need of discriminating between the effects of the test drug and solvent, because the procedure of injection is associated with painful sensations during introduction of the needle and during penetration of NS into tissues. The effects of repeated injections now attract special attention [1-3].

Here we studied the effects of 5-fold injections of NS to Wistar rats in two opposite psychological situations: 1) during defense reaction (formation of avoidance reaction to pain — tail-flick test with thermal irritation) and 2) during training (attaining food reward under conditions of free choice in a multialternative maze).

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MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (n=80) weighing 200-230 g kept in groups of 10 animals in a laboratory vivarium.

The nociceptive sensitivity was tested using an Analgesiatest analgesiometer in a tail-flick test. The test was modified as follows: before injection of NS the rats (n=40) developed pain avoidance reflex (tail-flick reaction to thermal irritation with a light beam). Ten measurements of the tail flick latency (TFL) were carried out every 60 sec at 10.00 and 16.00. After stable reaction was observed for 3 days, 0.85% NS was injected during the subsequent 5 days (0.3 ml once a day intraperitoneally). TFL was measured 1, 5, and 24 h after injection of NS; the animals were observed for 5 days after the injections were discontinued. Intact animals (n=40) served as controls. The testing was carried out under conditions of 23-h alimentary deprivation (the animals were fed after the evening measurement of TFL at 19.00). The access to water was free.

Intact (n=20) and preinjected (after 5 injections of NS, n=20) rats were trained in a multialternative maze, in which the animals were to find (without the experimentator's assistance) the conditions of repeated food

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getting (sunflower seeds) from 2 of the 4 feeders in the maze. The locomotor activity, number of excursions into the maze, number of confirmations and errors during the experiment were recorded.

Psychoemotional status of rats during training was analyzed by counting the unconditioned reactions, which were then divided into active and passive [1,4]. Active reactions included ticks, jumps, scratching, sneezing, tooth clapping, shaking off, fear, jumping from the cage; passive reactions included grooming, sitting, stupor, peeping. The training was carried out daily by the free choice method for 13 min after 23-h food deprivation.

The results were statistically processed using Kolmogorov—Smirnov's test and Student's t test.

RESULTS

In intact animals the conditioned pain avoidance reflex in response to thermal exposure (light beam) formed during 3 days (30 tests during the morning and evening sessions), the time course of TFL did not depend on the time of the day. TFL gradually decreased from 5.63 to 3.59±0.06 sec during testing and persisted at this level for 3 days, the coefficient of variations did not surpass 1.35±0.7% (Fig. 1). TFL sharply decreased immediately after the first injection of NS, but during the evening session (7 h after the injection) this parameter did not differ from the initial level (Table 1, Fig. 1). Starting from the 2nd injection of NS a stable regularity formed, manifesting during subsequent injections: TFL during the morning session preceding NS injection (24 h after the previous injection) was significantly shorter than during the evening session (3.24± 0.04 sec; $p<0.05$). TFL decreased after each injection (1 h after NS injection); during the evening sessions they increased again, but did not returned to the initial level. Changes in the mean TFL induced by injections

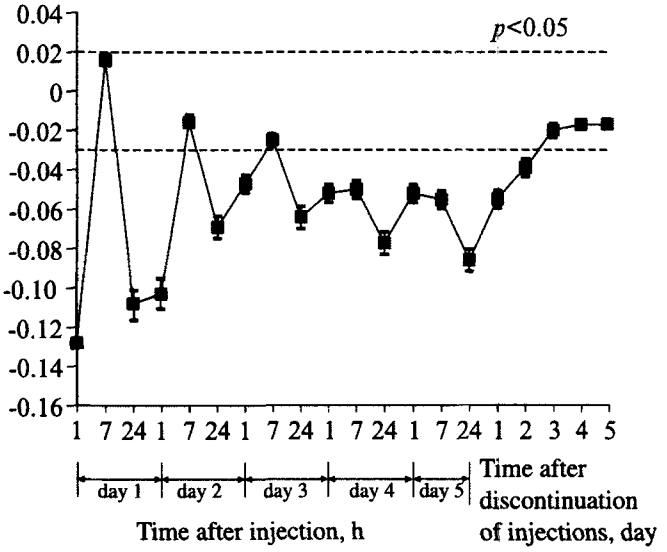

Fig. 1. Latency of tail flick response after injection of normal saline.

of NS, persisted for 2 days after the injections were discontinued (Fig. 1). However, statistical analysis showed that deviations in the distribution of TFL values (normal reaction, asymmetry and excess parameters) induced by injection of NS persisted during all 5 days of testing after the injections were discontinued; a total of 80 measurements were made during this period (Table 1).

Hence, 2 injections of NS were sufficient for the formation of a regularity presenting as regular increase of the nociceptive sensitivity before regular injection, which increased still more during the injection. The time course of these changes indicates the central conditioned-reflex origin of this reaction as a mental phenomenon of pain associated with injection, but not with the tail-flick test procedure, as during the evening sessions the nociceptive sensitivity corresponded to the initial level.

TABLE 1. Distribution of TFL Values One Hour before Injection of NS and after Discontinuation of Injections ($M±m$)

Experiment conditions	Day of testing	Latency	Range	Excess	Asymmetry
Before injections	4-6	3.59±0.06	4.38±0.06	0.95±0.05	0.85±0.06
NS injections	1	3.17±0.04**	2.53±0.04**	-0.42±-0.04**	2.02±0.03**
	2	3.20±0.03**	2.98±0.06**	-0.52±-0.03**	2.22±0.06**
	3	3.34±0.03**	2.45±0.03**	-0.46±-0.03**	2.14±0.04**
	4	3.36±0.04*	2.52±0.04**	-0.26±-0.02**	1.98±0.03**
	5	3.31±0.03**	2.51±0.02**	-0.21±-0.05**	1.76±0.05**
Injections discontinued	1	3.28±0.04**	2.53±0.06**	-0.11±0.06**	0.31±0.03**
	2	3.39±0.04*	2.43±0.04**	0.06±0.02**	0.43±0.03**
	3	3.45±0.05	2.62±0.05**	0.43±0.03*	0.56±0.04*
	4	3.52±0.04	2.68±0.05**	0.34±0.05**	0.42±0.04*
	5	3.53±0.05	2.76±0.04**	0.52±0.06*	0.61±0.05*

Note. *$p<0.05$, **$p<0.001$ compared to TFL before injections.

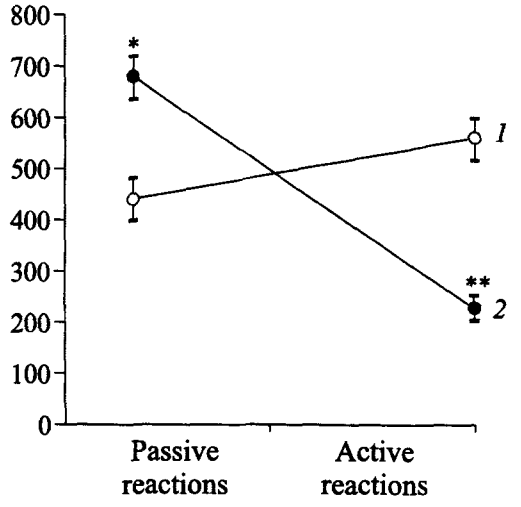

Fig. 2. Ratio of passive and active unconditioned manifestations in two groups of Wistar rats. Ordinate: number of unconditioned reactions during 15 experiments. *1*) intact rats; *2*) rats injected with normal saline before training. *$p<0.05$, **$p<0.001$compared to intact rats.

Behavioral studies showed that the percentage of rats refusing from training increased to 85% (n=17) after intraperitoneal injections of NS (*vs.* 60% in the group of intact rats, n=12). Active controls (injections of NS) exhibited predominantly passive defense reactions (defecation, urination, stupor, peeping, long sitting) during the entire experiment, this indicating psychoemotional stress (Fig. 2).

Similar effects were observed previously after intramuscular injections of NS [1] and in many studies using the open field test [2,3]. The fact of refusal from training and high percentage of passive defense reactions demonstrated during training indicate that 5-fold injection procedure led to the formation of a stable defense status in animals, which suppressed the subsequent motivated and cognitive activity in the alimentary situation. Our findings and published data indicate that the procedure of NS injection is far from the common notion of a "control" and it is more justified to regard it as a model of negative stress exposure. As active control is inevitable in pharmacological studies, one should not neglect its negative characteristics when evaluating the drug effects.

The results of comparison of drug-induced changes will be largely determined by the difference in the range of values after injections of normal saline and the test drug.

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