Central Injection of Caspase Inhibitors Facilitates the Formation of Freezing Behavior in Rats

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We studied the effects of central administration of pancaspase inhibitor z-VAD-fmk and specific caspase 3 inhibitor z-DEVD-fmk on learning and memory in rats. The inhibitors selectively facilitated the formation of freezing behavior without modulating consolidation and retrieval of passive defense behavior in the model of conditioned freezing.

Key Words: *apoptosis; caspase 3; freezing behavior; training; memory*

Caspases, a family of cysteine proteases, are an important component of programmed cell death process. Fourteen caspases involved in apoptosis were identified. In the nervous tissue, caspase 3 attracts special attention, because this enzyme is assumed to play the major role in the terminal stage of apoptosis in brain cell [6].

New data on the non-apoptotic functions of caspase 3 were recently reported: it is involved in biochemical cascades linked with neuroplasticity underlying integrative activity of the nervous system [1]. However, experimental data on the role of caspases in the mechanisms of learning and memory are scanty and contradictory [2,4,5,8].

We studied the involvement of brain cysteine proteases in the formation, consolidation, and reproduction of conditioned freezing behavior in adult rats.

MATERIALS AND METHODS

Experiments were carried out on 84 adult male Wistar rats (280-300 g) kept 4 per cage with free access to water and food at 21±1°C and 12:12 day:night schedule. Caspase inhibitors z-VAD-fmk (specific for all caspases) and z-DEVD-fmk (specific caspase 3 inhibitor) were used. z-VAD-fmk dissolved in 0.09% NaCl to a concentration of 40 μg/μl was injected (2 μl) unilaterally into the brain ventricles through steel cannula guides inserted 3 days before the experiment according to stereotaxic coordinates (AP 1.2, L ±1.5, H 4.0). Caspase inhibitor z-DEVD-fmk dissolved in 0.5% DMSO to a concentration of 10 nmol/μl was injected (2 μl) similarly. Controls received 2 μl of the corresponding solvents.

The rat was placed into experimental box (25×40×25 cm) with loudspeakers for acoustic signal. Electric current (0.4 mA) was delivered to the wire floor of the box. The training session lasted for 3 min. During the first 2 min the animals were allowed to move free in the box and explore it. During the next 30 sec an acoustic signal was presented, electrocutaneous painful stimulation was performed during the last 2 seconds of acoustic stimulation. The animal was left in the experimental box for 30 sec. The time of freezing was recorded during each stage of learning. After 24 h the rat was again placed into the experimental box for 3 min and total freezing time (situation testing) was recorded. After 24 h testing with acoustic stimulation was carried out in an experimental box, which differed from the previous box by illumination, floor, and wall color. During the first 2 min the animal was allowed to explore the new box; during the next 30 sec an acoustic signal not combined

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with electrocutaneous painful stimulation was presented, after which the rat was left in the experimental box for 30 sec more. The freezing duration was recorded at all stages of testing with acoustic stimuli [5].

In series I, caspase inhibitors were injected 30 min before training in order to evaluate their effect on the formation of freezing behavior. In series II, caspase inhibitors were injected 30 min after training in order to characterize their effects on the consolidation processes. In series III, only caspase 3 inhibitor was used, which was injected 24 h after training (1 h before situation testing) in order to evaluate the effect of the inhibitor on reproduction.

The results were statistically processed using Student's *t* test and ANOVA/MANOVA analysis of dispersions.

RESULTS

The initial (on the day of training) freezing time in the experimental box before and during acoustic signal characterizes the orientation and exploratory behavior of animals, while prolongation of this parameter during testing under similar conditions in comparison with the day of training characterizes long-term memory of defense behavior. Pancaspase inhibitor z-VAD-fmk injected 30 min before training did not change the initial time of freezing before and during acoustic stimulation. Freezing time during testing with acoustic stimulus in experimental animals was significantly longer than in controls (Student's test, Table 1). Analysis of the data by the ANOVA/MANOVA also showed significant differences in the dynamics of conditioned fear formation in response to the key stimulus between experimental and control rats ($F=7.32$, $p<0.03$). No appreciable difference between the groups was detected during situation testing ($F=0.34$, $p=0.58$; Table 1).

Hence, injection of z-VAD-fmk 30 min before training facilitated the formation of freezing behavior in response to the key stimulus (sound) and had no effect on the orientation and exploratory activity of animals and formation of long-term situation memory of defense behavior.

Similar results were obtained in experiments with specific caspase 3 inhibitor z-DEVD-fmk injected 30 min before training (Table 1). Two-way analysis showed significant differences in the dynamics of the formation of freezing behavior in response to acoustic signal between the groups ($F=5.61$, $p<0.04$). z-DEVD-fmk injected 30 min before training did not modify the formation of freezing behavior in response to conditioned situation stimulus.

Caspase inhibitors z-VAD-fmk and z-DEVD-fmk injected into the brain ventricles 30 min after training did not modify the storage/consolidation of situation freezing (Table 2).

Analysis of the effects of z-DEVD-fmk injected 30 min before testing also revealed no significant differences between the experimental and control groups during testing with acoustic signal and situation, which suggest that caspase 3 inhibitor did not modify reproduction of the conditioned freezing behavior (Table 3).

The results of our experiments indicate selective effects of caspase inhibitors on training and

TABLE 1. Effects of Caspase Inhibitors z-VAD-fmk and z-DEVD-fmk on the Formation of Freezing Behavior in Rats

Conditioned stimulus		Control (NaCl)	z-VAD-fmk	Control (DMSO)	z-DEVD-fmk
Sound	initial value	3.5±1.1	0	3.8±1.1	3.2±1.4
	during testing	6.2±3.7	19.0±4.3*	7.7±2.6	16.3±3.3*
Situation	initial value	0	2.0±0.8	1.8±1.1	2.0±1.1
	during testing	62.5±24.2	44.1±20.1	62.0±16.7	54.0±8.8

Note. *$p<0.05$ compared to the control. Here and in Table 2: freezing time, sec.

TABLE 2. Effects of Caspase Inhibitors z-VAD-fmk and z-DEVD-fmk on Consolidation (Retention) of Freezing Behavior in Rats

Conditioned stimulus		Control (NaCl)	z-VAD-fmk	Control (DMSO)	z-DEVD-fmk
Sound	initial value	2.50±1.04	2.0±0.9	1.2±1.0	2.5±2.2
	during testing	17.8±4.7	8.30±4.01	12.5±5.9	10.0±3.5
Situation	initial value	3.5±3.2	21.0±20.1	0	2.50±2.02
	during testing	68.0±28.7	78.0±26.8	42.0±3.5	39.0±5.7

TABLE 3. Effect of Caspase 3 Inhibitor z-DEVD-fmk on the Reproduction of Freezing Behavior in Rats

Conditioned stimulus		Control	z-DEVD-fmk
Sound	initial value	3.3±3.3	0.0
	during testing	11.0±3.0	6.7±2.5
Situation	initial value	3.3±3.3	3.2±2.0
	during testing	12.8±1.9	8.4±1.5

memory processes: z-DEVD-fmk and z-VAD-fmk facilitated the formation of freezing behavior in response to presentation of the key stimulus (sound) without modifying the formation of situation memory, consolidation (storage) and reproduction of passive defense behavior in rats during conditioned freezing. Similar unidirectional effects of pancaspase inhibitor z-VAD-fmk and caspase 3 specific inhibitor z-DEVD-fmk observed in experiments indicate the key role of caspase 3 in these processes, which is confirmed by experiments on other models [4,8].

The facilitating effect of caspase inhibitors injected into the brain ventricles on the formation of long-term memory of the key stimulus deserves special attention. These results contradict published data on the predominantly suppressive effects of caspase inhibitors on the mechanisms of learning and memory. It was shown that caspase inhibitors injected into rat hippocampus after training in Morris water maze blocked the consolidation of long-term spatial memory, but did not change the short-term memory [4]. Study of the central effects of z-DEVD-fmk on the active avoidance model showed that specific caspase 3 inhibitor significantly reduced the total number of avoidance reactions and disordered the formation of escape reaction, a component of avoidance response related to spatial orientation. In addition, a decrease in the total number of responses to conditioned stimulus (light) and in the level of interstimulus reactions was observed, this indicating the effect of the test inhibitors on the mental status and orientation and exploratory activity of animals [8]. We consider that the disagreement in experimental data is caused by the characteristics of experimental models and differences in the protocols of training and caspase treatment. Despite certain differences, these studies also detected selective effects of caspase inhibitors on various types of memory and behavior. The authors of these reports attribute the detected behavioral effects of caspase inhibitors to the "non-apoptotic" function of cysteine proteases. It was hypothesized that caspases activated during training modified specific proteins in certain synapses, thus causing their structural modification underlying the formation of long-term memory [1,4]. Possible effects of inhibitors on caspases involved in apoptosis in various brain compartments were not the objects of these studies and parameters of cell death were not estimated. In our experiments caspase inhibitors stimulated the formation of conditioned freezing response to the trigger signal (a form of memory associated with the function of the amygdaloid-hippocampal complex), which was presumably caused by inhibition of caspase 3 and suppression of apoptosis processes in these brain structures. This interpretation is in line with the data on reduced level of apoptosis in some brain structures, *e.g.* in the hippocampus of animals placed into enriched medium and during training [2,7].

We think that caspase inhibitors modify the "apoptotic" and "non-apoptotic" functions of caspases in the brain, while their behavioral effects largely depend on specific conditions of the experiment. Hence, investigating the effects of caspase inhibitors on training and memory, it is essential to register not only changes in activity of these enzymes, but also apoptosis markers in different brain structures.

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