DIFFERENCES IN THE ACTION OF TYPICAL AND ATYPICAL TRANQUILIZERS ON MITOCHONDRIAL STRUCTURE AND FUNCTION DURING EMOTIONAL-PAINFUL STRESS

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The study of mechanisms of the psychotropic action of tranquilizers is unusually important not only from the standpoint of knowledge of the pathogenetic steps in the development of diseases, but also the standpoint of the search for and creation of new preparations with a particular action. The discovery of benzodiazepine receptors and proof of the participation of the GABA-ergic system in the realization of the psychotropic effect of typical (benzodiazepine) tranquilizers has considerably broadened our ideas on the mechanisms of their effects, whereas for the large group of atypical (nonbenzodiazepine) tranquilizers these still remain completely unexplained.

The problem is even more important with the appearance of new groups of tranquilizers based on natural metabolites, and with a powerful effect on brain metabolism. These include fenibut, mebicar, litonit,* and various other preparations.

The aim of this investigation was to study differences in the action of typical and atypical tranquilizers on structure and function of the brain mitochondria of rats during chronic emotional-painful stress (EPS).

EXPERIMENTAL METHOD

Experiments were carried out on 260 male Wistar albino rats weighing 180-200 g, kept on the standard animal house diet. Highly emotional animals were selected [1] by preliminary testing. Chronic EPS was produced in the form of an anxiety neurosis [14] in the writer's modification [7]. Chlordiazepoxide (2 mg/kg was used as the typical and litonit (10 mg/kg) as the atypical tranquilizer. The preparations were administered prophylactically in a course lasting 2 weeks (intraperitoneally in average therapeutic or anxiolytic doses). EPS was produced against the background of administration of the drugs. Concentrations of oxidized and reduced nicotinamide coenzymes were determined in brain homogenates, and the coefficient of correlation was calculated [13]. Activity of tissue respiration enzymes was determined in mitochondrial fractions isolated by differential

* Litonit is a Soviet preparation of lithium nicotinate (translator).

TABLE 1. Changes in Activity of Tissue Respiration Enzymes and Concentration of Nicotinamide Coenzymes in Brain of Rats with Chronic EPS after Preliminary Administration of Tranquilizers

Parameter studied	Control		Stress		Litonit + EPS		Chlordiazepoxide + EPS	
	$M \pm m$	%	$M \pm m$	%	$M \pm m$	%	$M \pm m$	%
MDH	2,10±0,091	100,0	1,15±0,057*	54,8	2,94±0,131*	140,0	0,91±0,038*	43,3
GDH	7,39±0,45	100,0	8,86±0,42	119,9	7,69±0,46	104,1	6,20±0,61	83,9
SDH	42,50±0,43	100,0	37,20±1,24*	87,5	21,44±0,69*	50,4	26,83±0,85*	63,1
CO	172,5±12,9	100,0	301,8±11,2*	174,9	175,2±2,5	101,6	161,2±5,7	93,4
$NAD^+ + NADP^+$	188,0±5,5	100,0	69,0±3,0*	36,7	205,7±13,0	109,4	88,0±2,5*	46,8
NADH + NADPH	70,0±2,5	100,0	60,5±2,0*	86,4	75,5±3,0	107,9	55,4±2,7*	79,1
$\frac{NAD^+ + NADP^+}{NADH + NADPH}$	2,68±0,12	100,0	1,14±0,04*	42,5	2,72±0,13	101,5	1,59±0,05*	59,3

Legend. * $P < 0.05$. There were 10 experiments in each series. Here and in Tables 2 and 3 comparison was with control.

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TABLE 2. Changes in Ultrastructural Parameters of Mitochondria in Sensomotor Cortex of Rats during Chronic EPS Preceded by Administration of Tranquilizers

Experimental conditions	Statistical index	N_{cr}	$V_{v_{mc}}$	$S_{v_{om}}$	$S_{v_{im}}$	$\frac{V_{v_{mc}}}{S_{v_{om}}}$	K_f^{cr}
Control	$M \pm m$	22,4 0,73	0,467 0,022	5,52 0,10	23,21 0,86	0,085 0,009	2,41 0,11
Stress	$M \pm m$	7,4 0,21	1,171 0,040	6,89 0,17	9,03 0,37	0,170 0,016	3,36 0,19
	%	33,0	250,7	124,8	38,9	202,4	139,4
	P	<0,001	<0,001	<0,001	<0,001	<0,001	<0,001
Litonit (10 mg/kg) + EPS	$M \pm m$	15,8 0,98	0,552 0,079	5,63 0,54	17,10 1,09	0,098 0,013	2,86 0,17
	%	70,5	118,2	102,0	73,7	115,3	118,7
	P	<0,001	>0,2	>0,5	<0,001	>0,2	>0,1
Chlordiazepoxide (2 mg/kg) + EPS	$M \pm m$	9,4 0,70	1,304 0,106	7,33 0,80	9,78 0,73	0,179 0,035	3,48 0,29
	%	42,0	279,2	132,8	42,1	210,6	144,4
	P	<0,001	<0,001	<0,05	<0,001	<0,001	<0,001

Legend. Number of observations in each series was 30-50.

TABLE 3. Effect of Prophylactic Course of Typical and Atypical Tranquilizers on State of Mitochondrial Membranes during Chronic EPS Shown by Fluorescent Probe Data

Experimental conditions	K_b		K_d		N		F_{mol}	
	M^{-1}	%	M	%	mmoles/mg protein	%	abs	%
Control	$1{,}6 \cdot 10^{-2}$	100,0	63,2	100,0	$1{,}4 \cdot 10^{-4}$	100,0	$2{,}1 \cdot 10^{6}$	100,0
Stress	$2{,}6 \cdot 10^{-2***}$	162,5	15,6***	24,7	$2{,}5 \cdot 10^{-4***}$	178,6	$2{,}2 \cdot 10^{5***}$	10,5
Litonit (10 mg/kg) + stress	$1{,}5 \cdot 10^{-2*}$	93,7	50,0**	79,1	$3{,}1 \cdot 10^{-4***}$	221,4	$3{,}4 \cdot 10^{5***}$	16,2
Chlordiazepoxide (2 mg/kg) + stress	$2{,}8 \cdot 10^{-2***}$	175,0	26,7***	42,2	$5{,}2 \cdot 10^{-4***}$	371,4	$1{,}5 \cdot 10^{5***}$	7,1
Control	$4{,}8 \cdot 10^{4}$	100,0	$2{,}1 \cdot 10^{-5}$	100,0	$6{,}3 \cdot 10^{-4}$	100,0	$3{,}4 \cdot 10^{5}$	100,0
Stress	$7{,}6 \cdot 10^{4***}$	158,3	$1{,}3 \cdot 10^{-5***}$	61,9	$4{,}8 \cdot 10^{-4***}$	76,2	$4{,}7 \cdot 10^{5***}$	138,2
Litonit (10 mg/kg) + stress	$4{,}3 \cdot 10^{4*}$	89,6	$1{,}4 \cdot 10^{-5***}$	66,7	$6{,}5 \cdot 10^{-4*}$	103,2	$2{,}9 \cdot 10^{5***}$	85,3
Chlordiazepoxide	$8{,}4 \cdot 10^{4***}$	176,1	$1{,}2 \cdot 10^{-5***}$	57,1	$5{,}7 \cdot 10^{-4*}$	90,5	$2{,}7 \cdot 10^{5***}$	79,4

Legend. Asterisks denote values of coefficient of correlation: *) 0.30 (weak correlation), **) 0.30-0.69 (moderately close correlation), ***) 0.70 and over (high degree of correlation).

centrifugation [12]. Glutamate dehydrogenase activity (GDH) was expressed in μmoles $NADP^+$/mg protein/min [5], malate dehydrogenase (MDH) in μmoles NADH/mg protein/min [15], succinate dehydrogenase (SDH) in nanomoles succinate/mg protein/min [3], and cytochrome oxidase (CO) in nanomoles oxidized dimethyl-p-phenylenediamine/mg protein/min [11]. Mitochondrial protein was determined by the usual method. The ultrastructure of the mitochondria in the sensomotor cortex was studied with the HU-II-E-I Hitachi electron microscope (Japan). Material obtained was subjected to morphometric analysis [4]. The number of cristae N_{cr}, the relative volume of the mitochondria ($V_{v_{mc}}$), the surface area of the outer membrane ($S_{v_{om}}$), the surface area of the inner membrane ($S_{v_{im}}$), the degree of swelling of the mitochondria $\left(\frac{V_{v_{mc}}}{S_{v_{om}}}\right)$, and the coefficient of fragmentation of the cristae (K_f^{cr}) were determined. The state of the mitochondrial membranes was studied with the aid of fluorescent probes (FP): 1-anilinonaphthalene-8-sulfonate (ANS) and N-phenyl-1-naphthylamine (PNA), by successive titration operations, titration of probes with membranes and titration of membranes with probes. Fractions were separated by automatic pumping, in conjunction with the Uvicord III-2089 spectrophotometer (Beckman, USA) and fluorescence was excited and recorded on a spectrofluorometer (Opton, West Germany). The results were processed by the double reciprocal method [2] and the binding constant of FP (K_b), the specific number of binding sites (N), the dissociation constant of the probe – membrane complex (K_d), and the molar fluorescence or quantum yield F_{mol} were calculated, followed by calculation of the coefficient of correlation. The state of the membranes was studied with fluorescent probes in the Institute of Pharmacology, Academy of Medical Sciences of the USSR.

EXPERIMENTAL RESULTS

Previous investigations showed that litonit, a nicotinic acid derivative has a marked tranquilizing action unaccompanied by muscle relaxation, ataxia, drowsiness, or other side effects [6, 10]. It was shown at the same

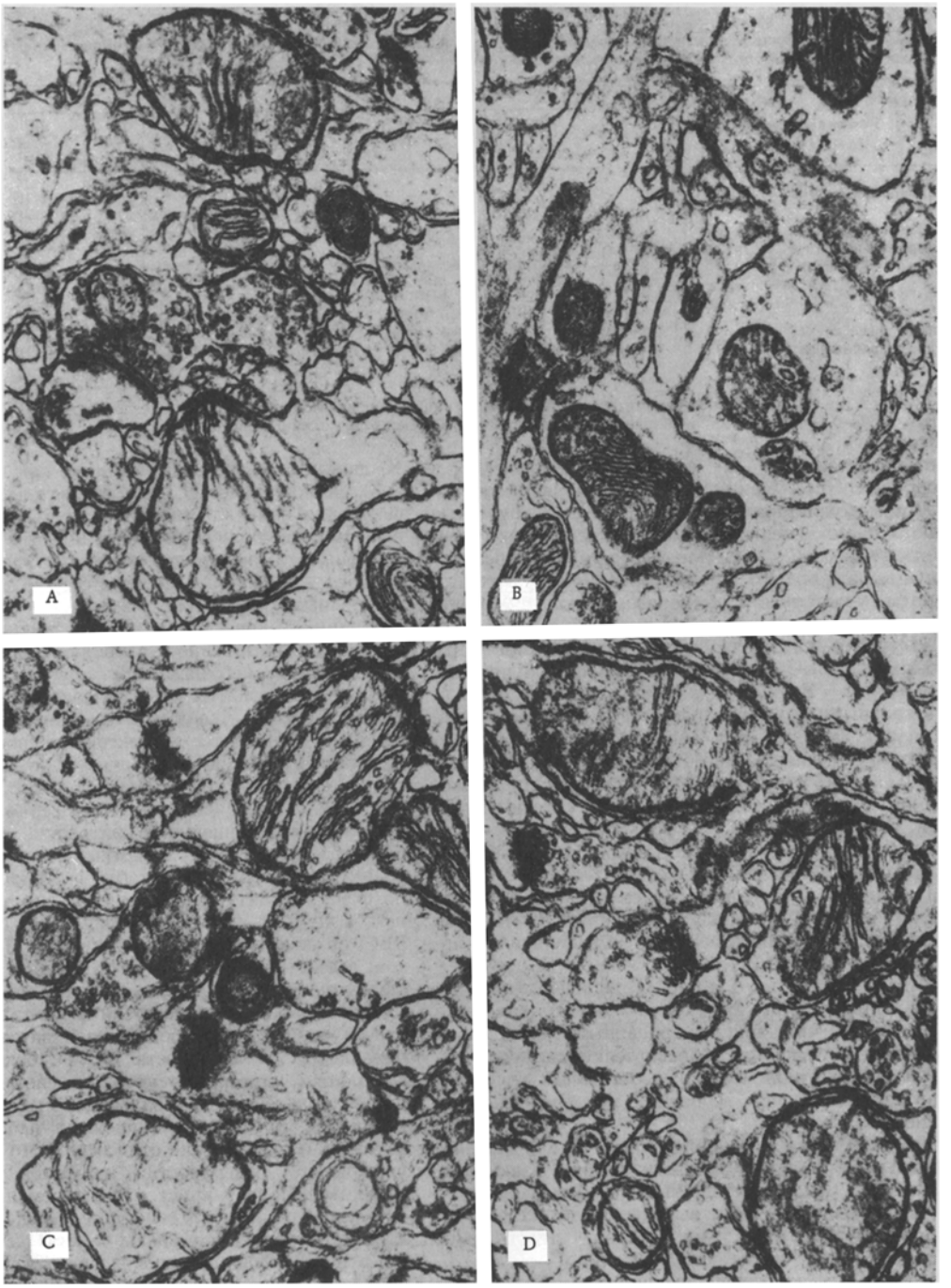

Fig. 1. Ultrastructure of mitochondria of sensomotor cortical neurons of rats in different physiological states (80,000×). A, C) Chronic EPS; B) EPS after a prophylactic course of litonit (10 mg/kg); D) the same after administration of chlordiazepoxide (2 mg/kg).

time that chronic EPS causes inhibition of brain energy metabolism by lowering the concentration of high-energy phosphates, and by inhibiting respiration and oxidative phosphorylation, and uncoupling the latter. Against this background litonit and chlordiazepoxide had a stress-protective action [8, 9]. Considering the leading role of mitochondria in the synthesis of high-energy adenosine phosphates, their structure and function were studied.

EPS in the excess-catabolic stage caused marked discoordination of activity of the leading enzymes of tissue respiration located in the mitochondria. Activity of MDH, one of the key enzymes of the Krebs' cycle, was inhibited by half (Table 1). Such marked inhibition of an NAD-dependent dehydrogenase can be explained by a sharp fall in the content of nicotinamide coenzymes. Whereas the content of reduced forms fell by 13.6% ($P <$

0.05), the content of oxidized forms fell by more than 2.7 times, and this led to a significant decrease in the coefficient of association from 2.68 ± 0.12 to 1.14 ± 0.04 ($P < 0.001$). The decrease in the content of oxidized "starter" forms is evidently the reason for inhibition of NAD-dependent dehydrogenases. This conclusion is confirmed by the results of a study of activity of another enzyme, namely SDH, transferring electrons directly to FAD. SDH proved to be more resistant to the action of EPS: The fall in its activity was not more than 12.5%. The twofold increase in lactate concentration in the brain and its threefold increase in the liver, with a simultaneous decrease in the liver glycogen content is evidence that when tissue respiration and oxidative phosphorylation are inhibited the anaerobic pathway of replenishment of high-energy phosphates is activated, although its energy efficiency is comparatively low.

The study of GDH activity, with an important role in not only carbohydrate, but also protein metabolism, showed that in this stage of EPS no significant change in amino-acid metabolism has yet taken place, for activity of the enzyme was virtually unchanged. The "bottleneck" in the chain of biological oxidation is CO. Such a marked increase in its activity (almost twofold) is evidence of the intensity of tissue respiration in the region of electron transfer from cytochrome c to cytochrome *a*, and thereafter to a_3, located on different sides of the mitochondrial membranes. The fall in oxygen consumption of the brain mitochondria evidently activates not only the anaerobic pathway, but also cytochrome oxidase activity, which is an important adaptive mechanism under conditions of EPS. The study of activity of mitochondrial enzymes, including so highly specific an enzyme for the mitochondria as SDH, showed that in EPS there is marked discoordination between enzyme systems participating in tissue respiration and oxidative phosphorylation.

The study of mitochondrial ultrastructure in the cerebral cortex during EPS showed a decrease in the number of mitochondria. Whereas in intact neurons, besides oval mitochondria rectangular forms also were found, in EPS they became round in shape. The relative volume of the mitochondria was increased by 2.5 times (Table 2). At the same time the surface area of the outer membrane increased significantly (by 24.8%) and the surface area of the inner membrane decreased by 2.5 times. As a result of the increase in relative volume of the mitochondria and surface density, the degree of their swelling was doubled, the number of cristae was reduced by 3.3 times, and their coefficient of fragmentation increased from 2.41 ± 0.11 to 3.36 ± 0.19 ($P < 0.001$). It will be clear from Fig. 1A, B, which shows mitochondrial ultrastructure in EPS, that besides the changes described above, translucency of the matrix and disturbances of the outlines of the membranes in some regions also were found, and are probable evidence of damage to their membranes.

Proof of the hypothesis enunciated above was given by the results of a study of interaction of mitochondrial membranes with FP. Addition of ANS to a suspension of mitochondria isolated from stressed animals showed considerable (by 89.5%) quenching of fluorescence (Table 3). The change of fluorescence was the result of an increase (by 78.6%) in N and K_b (by 62.5%), whereas K_d, as a reciprocal of K_b, fell by 75.3%. By contrast, when PNA was added in EPS, F_{mol} rose by 38.2%, N fell by 23.9%, and K_b rose by 58.3%, just as in the case of ANS, but K_d fell by 29.1%. Consequently, EPS significantly changed intermolecular relations between the mitochondrial membranes, and this evidently led to disturbance of the structure of the phospholipid bilayer and, consequently, of activity of the enzymes built into it. Changes in the mitochondrial membranes took place at different depths in the bilayer, corresponding to the depth of penetration of the FP chosen for testing into them.

The study of the development of EPS against the background of a prophylactic course of tranquilizers revealed not only their protective properties, but also differences in their action. Whereas in the case of litonit, MDH activity was actually increased a little, with chlordiazepoxide it was significantly reduced compared not only with the control, but also with the group of stressed animals not receiving tranquilizers (Table 1). Meanwhile, after a course of litonit the nicotinamide pool remained unchanged, whereas after chlordiazepoxide, just as during EPS, it remained persistently low. A clear line of stimulation of tissue respiration was thus noted in the action of litonit, but inhibition in the action of chlordiazepoxide. SDH activity was inhibited by both drugs about equally, but CO activity was indistinguishable from the control. The stress-protective action of the preparations was characteristically exerted on mitochondrial structure. Litonit prevented swelling of the mitochondria and an increase in their relative volume, the increase in surface area of the outer membrane, and fragmentation of the cristae. At the same time it restored close to normal the surface area of the inner membrane and the number of cristae (Fig. 1B). Chlordiazepoxide had no such action on the mitochondrial ultrastructure. Moreover, with respect to some parameters it impaired mitochondrial structure (Fig. 1D) and significantly increased the relative volume of the mitochondria and coefficient of fragmentation compared with the group of stressed animals not receiving tranquilizers.

It can be concluded from a comparison of the effect of these two compounds, derivatives of different classes, on mitochondrial membrane function that both litonit and chlordiazepoxide have marked membranotropic action. During interaction with ANS (in the surface layers of the membrane) litonit showed a distinct ten-

dency toward normalizing F_{mol}, whereas chlordiazepoxide aggravated the effects of stress. For PNA both preparations (but litonit to a rather greater degree) approximated the quantum yield towards its initial values (Table 3). The preparations had different effects on K_b: Litonit normalized it for ANS and PNA, but chlordiazepoxide increased it compared both with the control and with the group of stressed animals not receiving tranquilizers. For PNA both drugs returned N close to its initial values, but for ANS they considerably increased N. A tendency was observed in the action of both drugs to normalize K_d, but this tendency was stronger in the case of litonit.

These facts are all evidence that litonit has a stronger stabilizing effect on intermolecular interrelations in membranes, and improves not only the ultrastructure but also the function of mitochondria, which is primarily linked with energy generation. With respect to these same parameters chlordiazepoxide either has little effect or impairs mitochondrial structure and function. Its stress-protective action is evidently realized through different mechanisms.

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