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COLD STRESS RELATED ALTERATION OF RNA BIOSYNTHESIS IN BRAIN CORTEX OF MOTHER-DEPRIVED NEWBORN RATS

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Summary

We studied the influence of maternal deprivation on the RNA biosynthesis in the brain cortex of 10 day-old rats. Mother-deprived pups, placed at 25°C showed a reduction in body temperature of 6 +/- 1°C. After mother retrieval, RNA biosynthesis decreased 27% and 34% in total brain cortex and in isolated neurons, respectively. This fall is proportional to the body temperature reduction and can be avoided placing the pups at 37°C immediately after the separation. Rethermostatization of offsprings, after one hour at 25°C, showed an overshoot of RNA biosynthesis (145%) with further stabilization of synthesis rates to normal levels after 100 min. This classical physiological mechanism was further studied *in vitro*. Comparing *in vivo* and *in vitro* experiments, it is concluded that overshooting can not be observed *in vitro* if temperature reduction was not previously performed *in vivo*. Thus, this phenomenon seems to respond to humoral factors in order to be triggered. Afterwards, *in vitro* overshooting following cold stress *in vivo,* demonstrates that the depressed tissue by itself has the capability to turn back to normal RNA levels in the same way as observed *in vivo*.

Key Words: brain development, brain cortex, maternal deprivation, cold stress, RNA biosynthesis

The results of investigations carried out in newborn rats subject to maternal deprivation may have important clinical implications for humans. The accute maternal deprivation induces physiological changes in the rat offspring, such as variations in body temperature, cardiac rhythm, endorphin levels, endocrine response and motor activity. Retardation in growth and development has been reported for longer separations (1-9).

It has been observed that the accute maternal deprivation generates an increase in the levels of plasmatic corticosterone and ornithin-decarboxylase (ODC) activity in brain, liver and heart of the offspring. Normal levels of these parameters can be restored simulating the licking of the young by the mother with a wet brush (10). The activity of ODC, the first enzyme involved in the biosynthesis of polyamines, has been extensively related with the synthesis rates of proteins and nucleic acids (11). Also, the role of glucocorticoids in stress situations (12,13) and its inhibitory effect on the synthesis of RNA in lymphocytes (14) are well established. These biochemical changes are initial steps after maternal deprivation and could affect RNA metabolism.

On the other hand, mother deprivation induces a fall in body temperature since offspring thermoregulatory system is not fully mature at the early postnatal period (14). Temperature variation could also be implicated in metabolic changes and this alteration could affect nervous system development of pups.

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In fact, cold-related stress induced during early postnatal life could take part on behavioral changes in adulthood (9,16,17). Thus, elucidation of the biochemical events triggered after cold stress could be relevant to understand neurochemical alterations during postnatal development. The purpose of the present work was to investigate the effect on brain cortex RNA biosynthesis of mother-deprivation and acute cold stress.

Methods

Wistar 10 day-old rats were used. All the experiments were performed between 10 a.m. and 2 p.m. Mother-deprived pups were retrieved from the mother and placed for one hour in cages at different temperature according to the experiment. After the cold stress the animals were sacrified at distinct intervals of times. In some experiments the animals were rethermostatized in a bath at 37°C or placing them again with the mother.

The rectal temperature of the animals was registered with a type J probe connected to a thermocouple Digi-sense (Cole Palmer, Chigaco, IL).

***In vivo* RNA biosynthesis rates:** the animals received intrathecally a 15 min pulse of 20 µCi ^{3}H-uridine (20 Ci/mmol, Radiochemical Center, Amersham)and were sacrified at the times indicated on the corresponding experiments. RNA biosynthesis was determined in total brain cortex and in neuronal bodies. Brain cortex was dissected and homogenized (10% p/v) in buffer A (50 mM Tris-HCl, 0.32 M sucrose, 1 mM EDTA, 0.5 mM PMSF, pH 7.4) in a glass-tefflon Potter-Elvehjem tissue homogenizer, with three series of 15 strokes at 1400 rpm, clearence 50 µm. Neuronal bodies were isolated as described previously by Azcurra and Carrasco (18). Briefly, brain cortex was transferred to ice cold solution B (7.5% PVP, 10 mM Ca^{++}, 1% BSA fraction V) and sieved successively through 330, 110 and 73 µm meshes. The volume of the resulting suspension was then adjusted with solution B to 20 ml per initial brain cortex and layered over a four-step sucrose gradient consisting of 5 ml of 1.65 M; 4 ml of 1.4 M; 4 ml of 1.25 M and 3 ml of 1.0 M. Centrifugation was performed at 40.000 g for 30 min. Neuronal cell bodies were recovered more than 96% pure in the pellet as checked by phase contrast microscopy. Neuronal pellets were homogenized in buffer A.

***In vitro* RNA biosynthesis rates:** experiments were carried out in brain cortex prisms which were obtained as described elsewhere (19). Briefly, dissected brain cortex was decanted into the barrel of a 20 ml plastic syringe whose tip was sawed off, and was pushed through a 300µm nylon mesh stretched tightly over its truncated end. Tissue prisms were then transferred to 10 ml flasks containing 3 ml Hank's medium and preincubated at 37°C for 10 min. Prisms were decanted and medium was replaced by 3 ml of supplemented Hank's medium containing the radioactive precursor. The cultures received a 15 min pulse of ^{3}H-uridine (1 µCi/ml). Incubations were performed at 37°C in a giratory shaker under 95% O_2:5% CO_2 atmosphere. Reaction was stopped by addition of 5 ml cold Hank's and tissue prisms were recovered by centrifugation at 1000 g for 5 min. The activity remaining in the culture medium was also measured as an internal control of the experiments. Prisms were washed three times with cold Hank's, homogenized in buffer A and saved for assays.

In both, *in vivo* and *in vitro* experiments, the radioactive pulse was given immediately before the measurements were performed.

RNA quantitation: RNA biosynthesis rate was determined in aliquots of both, total brain cortex and neuronal bodies homogenates after acid-precipitation by the method of Fleck and Munro (20). RNA was quantified spectrophotometrically at 260 nm and the RNA biosynthesis rate was measured as pmoles of ^{3}H-uridine per mg RNA in the acid-precipitable fraction. Non-acid precipitable radioactivity was evaluated in order to determine the size of the precursor pool.

Nucleoside diphosphate (NDP) kinase activity: was assayed in brain cortex homogenates by the pyruvate kinase-lactate dehydrogenase-coupled enzyme system. Results are expressed as enzyme units per mg of protein. One unit is defined as the amount of enzyme causing the oxidation of 1 µmol of NADH per min at 25°C.

Results

To determine if the cold stress that follows maternal deprivation influences RNA biosynthesis in the brain cortex of 10-days old rats, we divided the offspring in three groups. The first stayed with the mother (control group), while the second and third groups were retrieved from the mother and kept at 25°C and 37°C, respectively. After 60 min, the animals were killed and RNA biosynthesis was measured in brain cortex.

Table 1

	Brain cortex	**Neuronal bodies**
	pmoles of ^{3}H-uridine per mg RNA	
Control group	28,8 +/- 4,2	66,5 +/- 11,8
Mother-deprived group (25°C)	21,1 +/- 2,3*	43,9 +/- 6,5*
Mother-deprived group (37°C)	27,3 +/- 4,7	64,4 +/- 10,2

Effect of mother-deprivation and cold stress on overall RNA biosynthesis in total brain cortex and neuronal bodies. Control group was kept with the mother. Mother-deprived groups were kept at the temperature showed in brackets. Data were analyzed by analysis of variance procedures (ANOVA) with the level of significance at $p < 0,01$. Values represent the mean +/- SEM of 6 pups/group. A posteriori Scheffe F test was performed independently for brain cortex and neuronal bodies comparing mother-deprived groups against the respective control. *Significant difference of mother-deprived group kept at 25°C, $p < 0,01$.

Table 1 shows that mother-deprived animals kept at 25°C suffered a 27% decrease in RNA biosynthesis in total brain cortex with respect to the control group. A 34% decrease was obtained when this parameter was measured in isolated neuronal pericarions. On the other hand, if the mother-deprived animals were immediately thermostatized at 37°C, no differences in RNA biosynthesis were observed with respect to the controls.

These results suggest that temperature is the only sensorial stimulus delivered by the mother that triggers the observed decrease in RNA biosynthesis. An additional evidence of the influence of temperature is the fact that the mother deprived group, kept at 25°C, was the only one that showed shivers. Other experiments show that the magnitude of the fall in RNA synthesis increases when the room temperature decreases (figure 1, *in vivo* slope) with a maximum decrease of 70% in animals kept at 10°C (23°C of body temperature).

Further, we studied the kinetics of the fall in RNA biosynthesis by placing the pups at 10°C. Figure 2 shows that the drop takes place in the first 20 min, after which the RNA biosynthesis levels remain depressed for the following 40 min during which the cold stress was mantained. In the same figure, the results obtained with a group of rats that were kept at 10°C for 60 min and then rethermostatized to 37°C are shown. RNA biosynthesis was measured at different times after rethermostatization. It can be observed that normal levels of synthesis are recovered during the first 20 min, after which the animals showed an overshoot in RNA synthesis that last approximately 60 min after the shift to 37°C. The greatest synthesis rate was observed after 40 min of rethermostatization and represents a 144.8% increase of RNA biosynthesis respect to controls. The results of rethermostatization was identical if the temperature shift to 37°C was reached placing the pups again with the mother or in a temperature controled environment in the absence of the mother.

The changes in RNA biosynthesis could represent thermodinamic conditions (kinetic alterations by temperature shifting), a physiological response to temperature variation with humoral or intracellular effectors, or both. To evaluate these hypothesis, we performed *in vitro* experiments with brain cortex prisms of 10 days-old rats not maternally-deprived. The tissue was subjected *in vitro* to a similar pattern of

temperature variation as performed *in vivo*. *In vitro* temperature units were comparable to body temperature units of cooled pups. In these experiments, we observed that *in vitro* RNA synthesis kept a direct relationship with temperature (figure 1, *in vitro* slope). The magnitude of the fall is twice as large *in vivo* than *in vitro*. Figure 2 shows that when the tissue prisms are incubated at 23°C and then rethermostatized, they keep a reduced RNA biosynthesis level of 40% with respect to their controls). After temperature shift to 37°C, RNA biosynthesis levels restore to the control level but in any case it was detected RNA oversynthesis. Therefore, this experiment does not allow to reproduce the overshoot phenomenon observed *in vivo*.

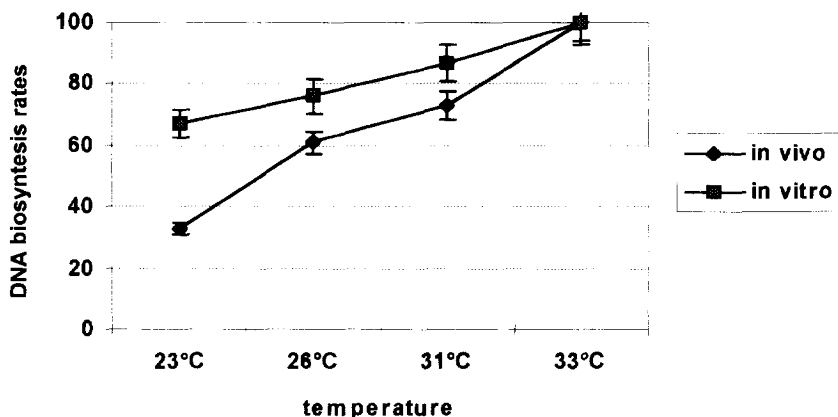

Fig. 1

Relation of RNA biosynthesis rates and temperature. The RNA biosynthesis rate was calculated for distinct temperatures and expressed as a percentage of biosynthesis at 37°C. Temperatures of *in vivo* determination refers to animal body temperature. *In vitro* temperatures refers to incubation temperature of prisms cultures. Each point n= 3.

We further investigated the nature of the biosynthesis recovery phase in an *in vivo/in vitro* experiment. We exposed 10 day-old rats to a period of one hour at 10°C. Animals were sacrified immediately, brain cortex prisms were isolated, incubated at 37°C and *in vitro* RNA biosynthesis was measured after 15 min pulse. Animals kept with the mother were used as controls. Figure 3 shows that after *in vivo* cold stress, an increased RNA synthesis is detected *in vitro*, which is not proportional to the change in temperature. Measurement of timepoint was performed after 15 min *in vivo* intrathecally pulse.

The similar availability of the nucleotide precursor pool for the different experimental groups during *in vivo* and *in vitro* experiments was checked by evaluating the intracellular radioactivity remained after acid-precipitation. These experiments failed to detect any significant differences for this variable (data not shown). The comparison of NDP Kinase activity in brain cortex of controls and mother-deprived animals (1 hour at 10°C) further support that there exists no such differences (controls: 0.11 +/- 0.03 U/mg; deprived animals: 0.09 +/- 0.02; n: 4, p > 0.1).

Discussion

It has been reported that the stress produced by the sensorial deprivation that results from the mother-offspring separation produces changes in different biochemical parameters of the pups (7-9,21). In this

report, we demonstrate that one hour maternal deprivation induces a 27% decrease in RNA biosynthesis in total brain cortex of 10 day-old rats. When this measurement was carried out in isolated neurons, the fall observed was of 34% approximately. This reflects the differential sensitivity of neurons with respect to the unfractionated tissue, as we have also shown in the case of amphetamin-induced hyperthermia (18).

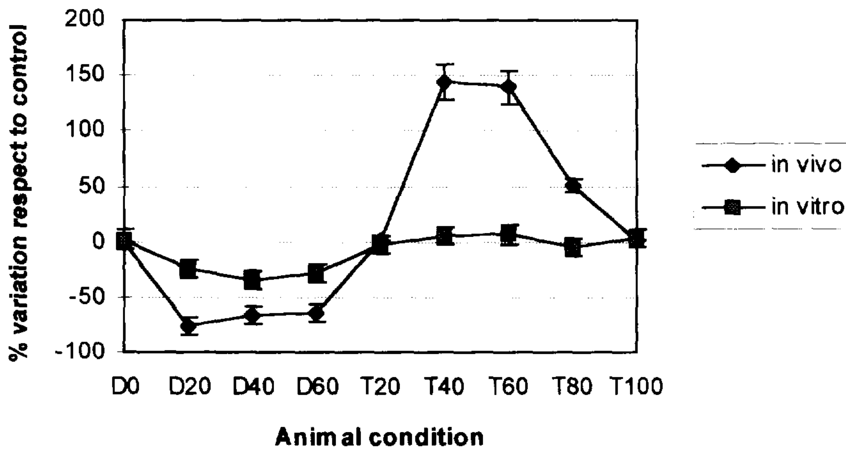

Fig. 2

Fall and recovery of RNA biosynthesis after cold stress and rethermostatization. Each value plotted represents the mean of 3 pups/group. Mother deprived pups were placed at 10°C (23 +/- 1 °C body temperature). *In vitro* temperature was set at 23°C. Percentage of variation in biosynthesis rate was calculated taking the respective control group as 0% of variation. The x axis indicates the condition (D: deprived, T: rethermostatized) and the corresponding accumulative time at that condition. For example, D40 indicates 40 min of maternal-deprivation; T20 indicates 20 min of rethermostatization (that animal/tissue was previously kept cooled for 80 min). Each point n= 3.

We have made distinct observations that remark the critical influence of temperature on RNA biosynthesis: i) offsprings that have been separated from their mother is the only experimental group that shows shivers, ii) immediate thermostatization to 37°C avoid RNA biosynthesis reduction, (iii) rethermostatization of pups restores the normal levels of RNA biosynthesis of mother-deprived pups and iii) there is a strong correlation between RNA biosynthesis fall and temperature reduction.

Other authors have found that ODC activity, a marker of mother deprivation in rats, markedly decreases in the first half hour after the young have been separated from their mother (7). They have also reported that the long-term negative effects of mother deprivation on protein synthesis and weight gain can be blocked simulating the mother's licking with vigorous strokes of the young with a wet brush. Apparently, the pressure and the massage exerted by the mother's tongue on the offspring is biologically important.

The biological phenomenon observed in our laboratory does not seem to be related to the one described above, since brushing the mother-deprived rats does not restore normal levels of RNA biosynthesis (data not shown). In addition, it has been observed that the ODC activity decrease associated to maternal deprivation does not depend on changes in body temperature (20).

The differential sensitivity of RNA biosynthesis fall under *in vivo* and *in vitro* experiments, clearly indicates alternative events of RNA response in the different experimental conditions. The lack of *in vitro* overshoot further support the need for humoral factors that trigger the RNA biosynthesis reduction as was observed *in vivo*.

Moreover, normalization of RNA biosynthesis after rethermostatization did not show a temperature-dependent curve. Instead of that, it was observed an overshoot before stabilization at the normal levels. Overshoot is a clear indicator of the presence of physiological factors involved in this phenomenom.

The experiments that combine *in vivo* and *in vitro* conditions showed that the overshoot in RNA biosynthesis needs *in vivo* conditions only during a first step. After this, metabolic recovery of biosynthesis in the depressed tissue depends exclusively on intracellular conditions.

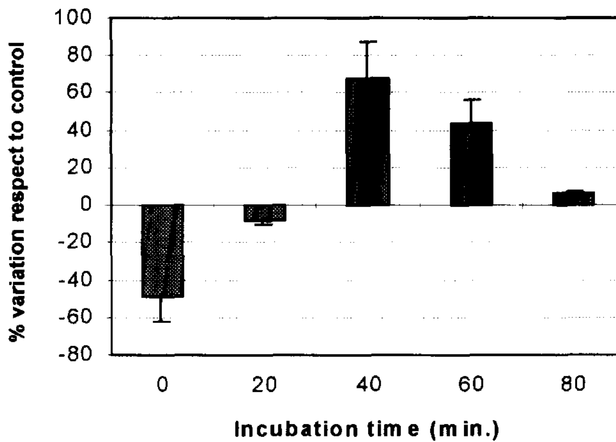

Fig. 3

In vivo / in vitro experiment. Animals were exposed to cold stress *in vivo*. After prisms isolation, the tissue received *in vitro* a 15 min pulse (with the exception of timepoint 0). RNA biosynthesis rates was determined as described elsewhere. Each point n= 3.

We can not completely reject the possibility that differences in the availability of precursors could partially explain the observed fall in RNA biosynthesis. However, we could not find differences in the intracellular pool of ^{3}H-uridine for the different groups. This measurement is of importance since the availability of intracellular ^{3}H-uridine that could be incorporated to RNA depends directly on the size of this pool (21). The activity of NDP Kinase, the enzyme that catalyzes the conversion of nucleosides diphosphates to triphosphates, was also unchanged among groups.

Taking into account the rol of glucocorticoids in stress situation, we evaluate corticosterone as a putative humoral factor involved on the response to temperature shift. The increase in corticosterone levels in the blood of mother deprived rats is well-known (7), as well as the role of this hormone in the determination of glucose levels. This hormone has also been postulated to be an inhibitor of glucose transport and RNA biosynthesis (10). Therefore, a possible explanation for our results could involve a variation in the plasmatic levels of corticosterone. Nevertheless, the i.p. injection of corticosterone to 10-day rats yielded dose-dependent increases in the RNA biosynthesis in brain cortex (data not shown). Thus, the mechanism triggered by cold stress remains obscure. Given the known relationship between cathecolamines and stress (22,24), and that the changes we observed in RNA biosynthesis are strongly related to cold stress, we are presently investigating the role of cathecolamines in the mechanisms involved in the response to mother deprivation.

On the other hand, Zymmerberg and Shartrand found that young rats subjected to cold stress were developmentally delayed and had lower body and brain weight. These animals also showed altered drug responsiveness and dopaminergic system function in relation to pups kept with the mother or separated but placed at nest temperature (9). Furthermore, Kehoe and Shoemaker showed that midbrain endorphin levels were decreased after isolation (8). These findings, together with our observations, suggest that isolation and the modification of temperature environment could affect several neurochemical parameters (such as dopaminergic system, endorphin levels and total RNA synthesis) which could trigger profound behavioral alterations not only during the early life but also in adulthood.

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