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Short Administration of ZnTe to Male and Female Rats Previous to Mating and Fertilization Affects Behavioural Responses of Litters During the Prepuberal Period

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Trace elements are an omnipresent group of chemical elements that are present practically in all types of environments sustaining life. Previous evidence from our laboratory has shown that chronic administration of ZnTe to pregnant rats affected several behavioural parameters related to motivated, lateralized exploration, and defensive behaviour in litter rats. In the present work, the possible effect of trace elements administration in a time period before fertilization to parent rats was investigated in its litter rats. Three experimental groups were formed: parent rats receiving water, as control; parent rats receiving ZnTe (0.3 μg/L, "ZnTe Pre") during a short period before mating, and parent rats receiving receiving ZnTe (0.3 μg/L) after mating in the same schedule than previous research ("ZnTe Post"). Results showed that in group "ZnTe Pre," trace elements treatment increased significantly some parameters of motor activity in litter rats, and a similar finding also was observed in motivated exploration. In lateralized exploration as measured in the Lateral Double Hole-board Labyrinth, both "ZnTe Pre" and "ZnTe Post" groups showed absence of the natural left-biased exploration of litter rats, decreasing the proportion of animals exploring the left side of the labyrinth. Defensive behaviour was affected in minor degree in the "ZnTe Pre" group and was significantly different both from Control and "ZnTe Post" group. Other behaviours, such as social interaction was not affected in the "ZnTe Pre" group, while as found previously social parameters were significantly altered in the "ZnTe Post" group. Present data are in agreement with previous results, and support the concept that trace elements can act on epigenetic mechanism regulation occurring prior to the ontogenetic development.

Keywords: Defensive Behaviour, Lateralization, Trace Elements, ZnTe.

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1. INTRODUCTION

Recently a growing interest has been raised to determine the probable role of trace elements on metabolic and internal homeostatic processes in the cell. The most marked point regarding these chemical elements is its ubiquitous presence in practically all types of environments in the world. For instance, selenium, that initially was considered a toxic element for human health is present in a special type of proteins, the selenoproteins, where it is specifically incorporated into the amino-acid cysteine, forming selenocysteine.[8] It is interesting to note that several selenoproteins are expressed in the brain, suggesting that this type of proteins might be participating in selected brain functions, not specifically elucidated at the present time.[8, 19] Tellurium, another trace element frequently found associated with copper and sulfur-bearing ores,[18] not recognized actually as an essential element to living beings is present in some plants of the *Alium* family, and specific disorders have been associated to ingestion of the metalloid in animals, suggesting a possible role in biological systems.[6, 11, 20] Previous evidence presented by our laboratory has shown that ZnTe in non-toxic concentrations administered in the drinking water to pregnant rats along all gestation, delivery, lactation, weaning and preadolescent periods affected the behavioural responses in litter rats in several different situations.[21] Contrasting behavioural effects were observed in the ZnTe treated animals. On one hand, treated young rats displayed excitatory motor and selective motivated exploration responses in a behavioural automatic activity measuring device, and on the other hand, impairments in motivated and lateralized behavioural display in a lateralized exploratory labyrinth were found. Furthermore, changing the environmental conditions and challenging stimuli, defensive behavioural responses were

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attenuated in ZnTe treated rats compared to control rats.[21] All these behavioural alterations were found to be associated with changes in methylation patterns of DNA in the hippocampus but not in the prefrontal cortex of ZnTe-treated animals.[21] ZnTe is composed of two active trace elements, Zinc and Tellurium, and both elements in principle could affect organisms.[6, 10, 11, 20] Previous work has shown that Te specifically increased motivated behaviour, blocked spontaneous left-biased exploration, escape and social responses related to territorial challenges.[22] On the other hand, Zn increased ambulatory activity, rearing and focalized motivated behaviour.[22] Thus, changes due to ZnTe treatment can be considered as a complex resultant interaction of both trace elements in the brain.[22] However, one important question regarding the biological effects after trace elements exposition that has not been investigated is the time indispensable to produce a biological change in the animals. Several possibilities there exist in order to study this question. In the present report, the possible effect of trace elements administration to parent rats before mating was studied in its prepuberal litter rats.

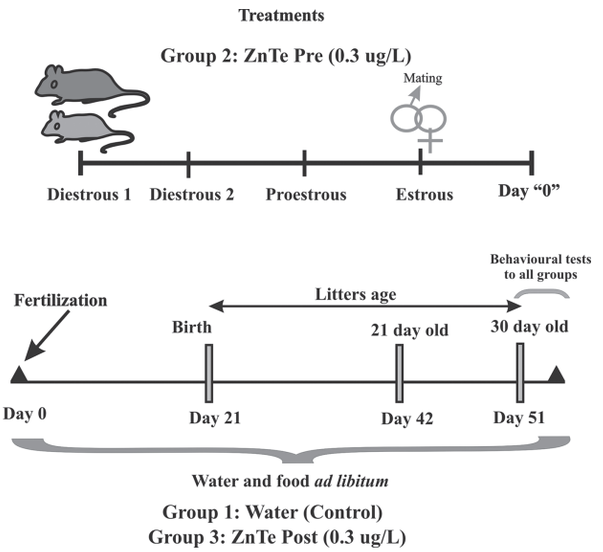

Fig. 1. General protocol of ZnTe treatments to parent rats and its litters. Three groups were formed: Group 1 (Water Control); Group 2 (ZnTe administration during estrous cycle and ending after mating, "ZnTe Pre"), and Group 3 (ZnTe chronic administration after mating, "ZnTe Post"). At time "0", groups 1 and 2 received water during all the experiment, meanwhile Group 3 received ZnTe. Further details, see Materials and Methods Section.

2. MATERIAL AND METHODS

2.1. Animals

Rats of a Holzman-derived colony, weighing 250–300 g, 90 days old and maintained in thermoregulated (22–24 °C) and controlled light conditions (06.00 on–20.00 h off) were used. Standard rat chow and water were available ad libitum for animals after mating. Trace elements treatment was applied only during the diestrum-estrous stages of female rats caged together with one male. Zinc Telluride (0.3 μg/L) was administered in drinking water (Fig. 1).

2.2. Experimental Design

The experimental protocol used is shown in Figure 1. Three experimental groups were formed: Group 1: "Water Control" (parent rats receiving only water administration during all the experiment, $n = 2$); Group 2: "ZnTe Pre" (parent rats receiving short administration of 0.3 μg/L of ZnTe before and during mating, $n = 2$, and Group 3: "ZnTe Post" (parent rats that received chronic administration of 0.3 μg/L of ZnTe after mating, $n = 2$, during all pregnancy, delivery, lactation and prepuberal stages of litters). Trace element administration in Group 2 lasted the few days of the cycling female rat (diestrous 1, diestrous 2, proestrous and estrous). When sperms were found in the vaginal smears (Day 0), trace elements administration stopped and drinking solution was changed to tap water. From there on, food and water were available *ad libitum* for Group 2 and 3.

Zinc telluride was from Sigma-Aldrich Co., U.S.A. At birth, pups were standardized to 10 animals per litter trying to maintain whenever possible the relationship of 1:1 of male to female rats. When maturing rats were 21 day-old (Day 42 of treatment), young rats were weaned and separated from their mothers. At 30 day-old (Day 51 of treatment) young rats of both sexes were subjected to a battery of behavioural tests in order to evaluate general motor activity, motivated exploration, lateralized, social, and defensive behaviour in the same way as previously described.[21, 22] Total number of young rats used in all experiments was 11–20, since some animals were lost for reasons not related to experimental treatments. After ending the behavioural tests, all animals were sacrificed by decapitation.

2.3. Behavioural Tests

The following behavioural tests were used to evaluate exploration of novel environments, lateralization, defensive behaviour, and social interaction.

2.3.1. The General Activity and Exploratory Behaviour Detector (OVM)

It consists of rectangular open-field with acrylic walls, equipped with infra-red detectors and digital counting devices for measuring animal activity (Optovarimex instruments, U.S.A). Device was enriched with holes in the floor, and a tube rack as novelty object as described in detail previously.[21]

Five variables were selected. Two of them are general estimates of motor and ambulatory activity displaying the general state of animals, and the other three are indices of motivated exploratory activity. Variables were:

(1) Ambulatory behavioural activity, the motor activity displayed by animals while they move in any direction

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of the arena during exploration, as measured by automatic digital counting proportional to the time of active movement.
(2) Non ambulatory behavioural activity, all movements that animals display remaining in one position, without displacement as measured by automatic digital counting proportional to the time of behavioural movement. Grooming and sniffing are the main behavioural components of this variable.
(3) Head-dipping, counted as frequency of head dips into any of the four holes of the OVM hole-board when this animal behaviour lasted at least 2 seconds.
(4) Rearing, counted as frequency of animal's rears, standing still on his rear feet and leaned on the walls of the OVM hole-board cage, sniffing to the air for at least 2 seconds.
(5) Focalized exploration, measured by digital counting proportional to time at a rate of 2 Counts/sec when the animal sniffs, touches with its front feet, climbs over the tube rack or explores the holes of the rack.

Variables (3), (4) and (5) were measured by an expert observer unaware of treatments. Test was applied to single animals and had a total duration of 5 min.

2.3.2. The Double Lateral Hole-Board Labyrinth (DHBL)

This labyrinth evaluates motivated exploration that can be expressed in lateralized form, as described previously.[1, 21]

DHBL is made of wood and is composed by a rectangular cage 39 cm wide, 70 cm length and 15 cm height. Inside there are two compartments disposed in 90° each. The first compartment (Initial) has 39 cm length and 15 cm wide with a central entrance to the second compartment (Corridor). Corridor has 55 cm of length, 17 cm wide, and on its side walls there are 4 lateral holes, each 3 cm in diameter. In this test behavioural activity of animals was driven only by exploratory motivation induced by novel environments. The following variables were measured:
(1) Corridor behavioural activity. All behaviours displayed by rats while they are in the corridor of the labyrinth, such as walking, rearing, head-dipping, and sniffing on the left or right side walls, including non-exploratory behaviours such as grooming and immobilization measured by a digital automatic counter (counting rate 2 counts/sec) monitored by an observer unaware of treatments.
(2) Initial Compartment behavioural activity. It is included in this measure all the behavioural activity displayed by rats while they were in this compartment. This activity was not measured directly and was calculated by subtracting corridor behavioural activity counting from the total counting of the test (3 min = 360 counts).
(3) Lateralized exploration. It is included in this variable all behaviours related to exploration displayed when the animal chooses one side of the corridor during exploration. Behaviours included:

(i) Walking nearby the left or right wall of the corridor, at constant speed, with vibrissae touching the wall.
(ii) Lateral head-dipping.
(iii) Rearing against the left or right walls of the corridor. This score was measured in the same way than Corridor Behavioural Activity.

(4) Non-exploratory activity. It is included in this variable the following behaviours:

(i) Immobilization at any site of the corridor; walking at the center of the corridor not approaching to any side wall.
(ii) Grooming.

Non-exploration activity was calculated by subtracting the lateralized exploratory activity from the corridor behavioral score.

In this test, behavioural laterality was considered to be present when the median of lateralized exploration on one side of the walls statistically outnumbers the opposite exploration.

Test was applied to single animals and had a total duration of 3 min.

2.3.3. Forced Swimming Test

This test measures the defensive behavioural response of animals subjected to a stressful situation represented by active swimming in a closed environment having no escape.[21] Device consists of a transparent acrylic tube measuring 50 cm height by 12 cm diameter (internal diameter), filled with water at room temperature up to half of the cylinder height. Two variables were measured.
(1) Active swimming activity, all the vigorous swimming movements displayed by animals involving all four extremities at approximately constant rate, and motor activity showed during immersion looking for a escape. Activity was measured by digital automatic counting at a rate of 2 Counts/sec monitored by an expert observer unaware of treatments.
(2) Immobilization, the time lapse where animals do not swim, floating without movements or displaying slow motion of its extremities enough to avoid sinking into the water. Since test had a total duration of 3 min (360 Counts), this behavioural activity was obtained by subtracting the active swimming activity from total counting.

2.3.4. The Social Interaction Test

This test (intruder-host territorial test) measures the social display of two interacting rats in a determined territory challenge by an intruder. Test was performed in a rectangular steel cage (26 cm width, 42 cm long and 20 cm height) with wood shavings in the floor. Total duration of testing was 5 min. In the two initial min the testing animal (host rat) was put alone in the arena in order to familiarize

with the cage. At the beginning of min 3, a different and new rat the same size and sex (intruder rat) was put in one corner of the cage. Behavioural display was recorded until testing period was finished. The following variables were measured:

(1) Latency to interact, time measured by digital counting that the host animal takes to face the intruder. Sniffing, touching, gentle biting, and dragging the intruder were recorded as social behavioural display.

(2) Number of contacts, frequency of contacts displayed by the interacting animals. A contact was defined when the host or the intruder animal displayed any of the behaviours above mentioned during the social interaction.

(3) Percentage of "α" contacts, number of "α" episodes displayed by the host to the intruder. An alpha episode is defined as an interaction initiated and addressed to the intruder by the host animal. These include biting, sniffing, touching and dragging. When the behaviour is reversed, the behavioural display of the host is considered as submissive acceptance and named "β" activity. Percentage was calculated by dividing α activity by total number of contacts ($\alpha+\beta$). This variable measures the natural capacity of coping and territorial defensive behaviour of animals under testing.

(4) Duration of α contact, time measured by digital counting of the duration of α social interaction displayed by the host animal in the test.

All behavioural tests were filmed by with a digital video camera, and recorded in a DVD player/recorder Phillips, model DVDR3455H.

2.4. Experiments

The following experiments were performed.

2.4.1. Effects of Short Administration of ZnTe to Parent Rats on General Motor and Motivated Behaviour of Its Litter Rats

In this experiment the influence of ZnTe treatment before fertilization (Group 2), and after fertilization (Group 3) to parent rats on general motor and motivated behaviour induced by novelty in its litter rats was evaluated. Measuring of the behavioural activity was performed using the OVM device as described previously.[21,22]

2.4.2. Effects of Short Administration of ZnTe to Parent Rats on Lateralized and Motivated Behaviour of Its Litter Rats

In this experiment the influence of ZnTe treatment before fertilization (Group 2), and after fertilization (Group 3) to parent rats on lateralized and motivated behaviour induced by novel environment in its litter rats was evaluated. Measuring of the behavioural activity was performed using the LDHB.

2.4.3. Effects of Short Administration of ZnTe to Parent Rats on Defensive Behaviour of Its Litter Rats

In this experiment the influence of ZnTe treatment before fertilization (Group 2), and after fertilization (Group 3) to parent rats on defensive behaviour of its litter rats was evaluated. Measuring of the behavioural activity was performed by the forced swimming test.

2.4.4. Effects of Short Administration of ZnTe to Parent Rats on Social Behaviour of Its Litter Rats

In this experiment the influence of ZnTe treatment before fertilization (Group 2), and after fertilization (Group 3) to parent rats on social behaviour of its litter rats was evaluated. This behavioural activity was measured by the intruder-host territorial test.

2.5. Statistical Analysis

Multiple comparisons for behaviours between experimental groups, was made by the Non Parametric Test of Dunn.[9] When comparisons involved paired groups, the Mann-Whitney Test was used. The significance of single percentage differences was analyzed by the Binomial Distribution (The Sign Test). A p value of less than 0.05 was considered as statistical significant. Results are presented as the mean $\pm$ standard error of the mean in the horizontal, ambulatory and non ambulatory activities; the median $\pm$ standard error the median in head dipping, rearing and focalized exploration in Experiment 1. With exception of percentage of animals in Experiment 2 (Fig. 3(C)), in all the other experiments data are expressed as the median $\pm$ standard error of the median.

2.6. Ethical Care of Animals

The present experimental protocol was revised and approved by the Comité Institucional de Cuidado de Animales de Laboratorio (Institutional Committee of Care and Welfare of Experimental Animals) of the Faculty of Medical Sciences, Universidad Nacional de Cuyo (CICUAL).

3. RESULTS

As it was previously described, ZnTe solutions did not affect daily water consumption nor induce any malformations in newborn rats.[21]

3.1. Experiment 1

The general motor activity and motivated behaviour of litter rats for all three groups is shown in Figure 2. Horizontal motor and ambulatory activity of rats in Group 2 and Group 3 were significantly increased in the 5 min test in the OVM (Fig. 2(A)). No differences were found in the score of non ambulatory activity of the ZnTe treated rats compared to control (Fig. 2(A)).

Regarding the motivated behaviour (Fig. 2(B)), results show that head-dipping was not changed by the short ZnTe

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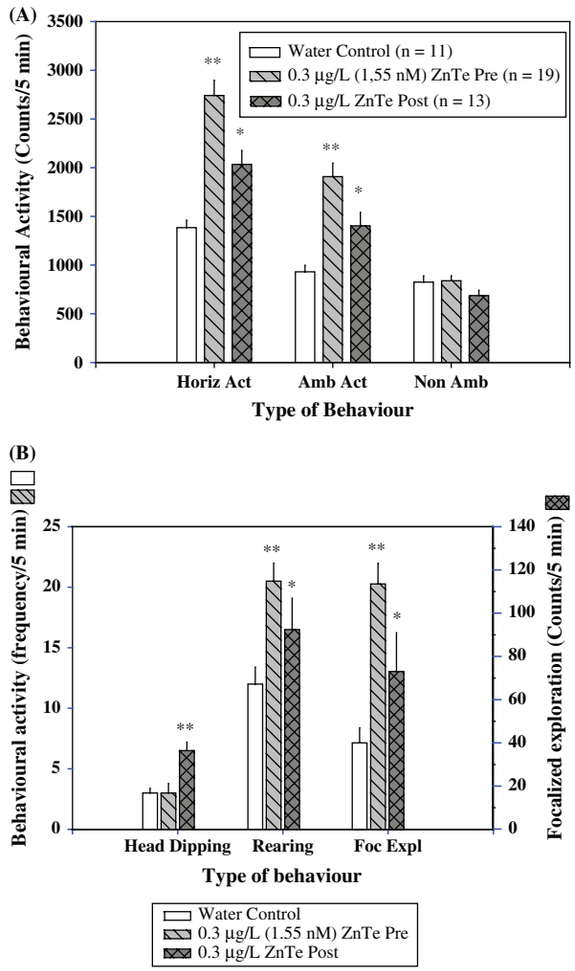

Fig. 2. Behavioural parameters displayed in the OVM during a total time of 5 minutes of litter rats from Groups 1–3. (A): General motor behaviours. Horiz Act = horizontal activity; Amb Act = ambulatory activity; Non Amb = non ambulatory activity. $^{*}p < 0.05$ versus Control; $^{**}p < 0.01$ versus Control. For "Horiz Act" and "Amb Act" score, comparisons between Group 2 and 3 were significantly different, $p < 0.01$. (B) Motivated exploratory activity. Foc Expl = focalized exploratory activity. $^{*}p < 0.05$ versus Control; $^{**}p < 0.01$ versus Control. In head dipping behaviour, score from Group 3 significantly different from groups 1 and 2, $p < 0.05$.

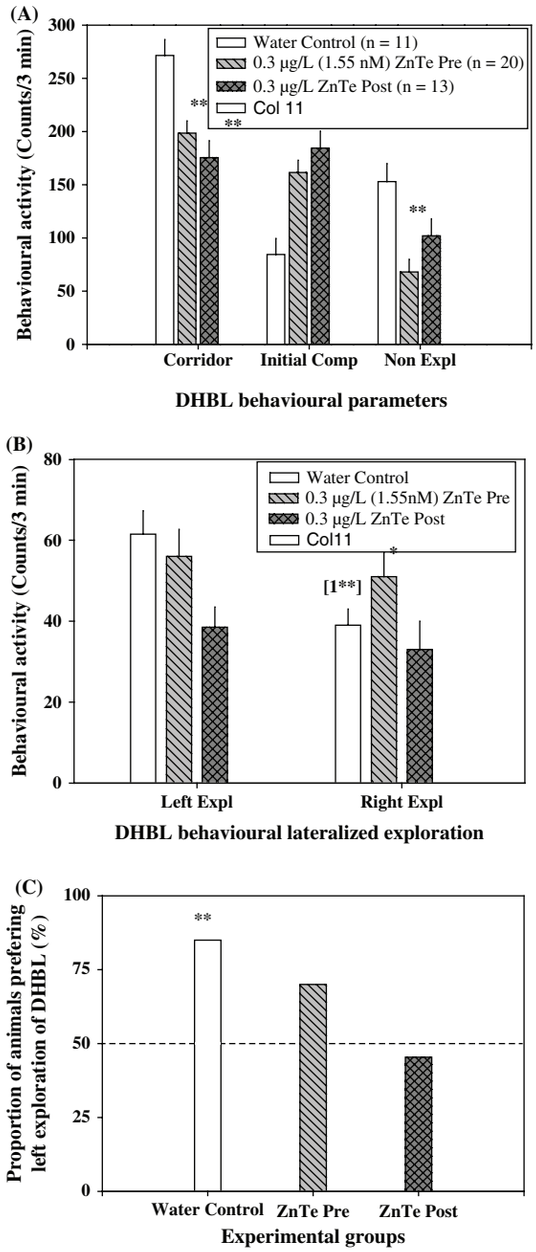

Fig. 3. Behavioural parameters displayed in the DHBL during a total time of 3 min of litter rats from Groups 1–3. (A) Initial Comp = Total score measured in the initial compartment; Non Exptl = total score not related to exploration measured in the corridor compartment. $^{**}p < 0.01$ versus Control. (B) Lateralized exploration of the corridor compartment. [1**] $p < 0.01$ versus left exploration in Control group. $^{*}p < 0.05$ versus right exploration of Control. (C) Proportion of animals showing left-biased exploration of the corridor. $^{**}p < 0.01$ versus 50% random exploration.

treatment to parents, but rearing and focalized exploration was significantly affected (Fig. 2(B)).

3.2. Experiment 2

The motivated and lateralized behaviour induced by novelty of litter rats with parents acutely or chronically exposed to ZnTe or water is shown in Figure 3. Behavioural activity displayed by animals from Group 2 in the corridor was significantly decreased compared to Group 1 (Fig. 3(A)). The same decrease was also observed in animals from Group 3. At the same time, behavioural activity in the initial compartment was significantly increased (Fig. 3(A)). Non exploratory activity in Group 2 and Group 3 compared to control was significantly decreased (Fig. 3(A)).

Lateralized exploratory behaviour showed a statistically bias to the left in Group 1 animals (Fig. 3(B)). The short treatment with ZnTe to the parents (Group 2) abolished

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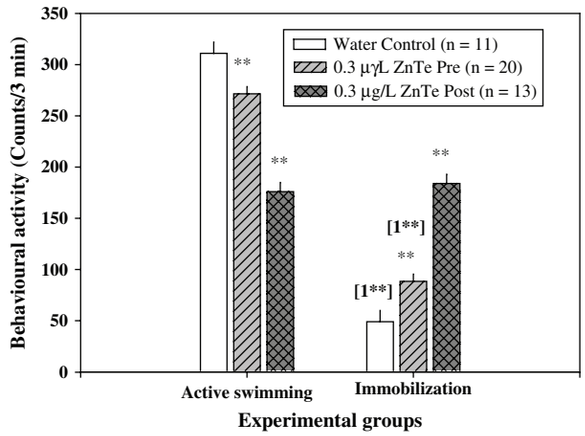

Fig. 4. Defensive behaviour displayed in the forced swimming test of litter rats from groups 1–3. $^{**}p < 0.01$ versus Control; [1**] $p < 0.01$ versus Active swimming in the corresponding group.

completely this left biased exploration (Fig. 3(B)). A similar finding was also observed in animals from Group 3.

When proportion of animals showing the left lateralized exploration was analyzed in Group 1, a significant increased proportion of left exploration rats (85%) was observed (Fig. 3(C)). Proportion of rats in Group 2 and Group 3 animals was not statistically different from 50%, and significantly lower than 85% found in Group 1 animals (Fig. 3(C)).

3.3. Experiment 3

The defensive behavioural pattern induced by a forced swimming challenge in rats from Groups 1, 2 and 3 is shown in Figure 4. Control group showed an active swimming activity about six times the immobilization period. This difference was statistically significant (Fig. 4). Short ZnTe treatment in Group 2 reduced significantly the active swimming activity, but the chronically ZnTe treatment in Group 3 reduced even greater this behaviour (Fig. 4). Immobilization time was significantly increased in Groups 2 and 3, compared to control. However, only in animals from Group 3 active swimming and immobilization activities were equalized (Fig. 4).

Table I. Behavioural parameters displayed during the Intruder-host-territorial Test of litter rats from groups 1–3. Additional details see Materials and Methods Section.

	Experimental groups		
Behavioural activity	Water control ($n = 21$)	0.3 μg/L ZnTe Pre ($n = 20$)	0.3 μg/L ZnTe Post ($n = 13$)
Latency to confront the intruder	13 ± 2.1	8.5 ± 3	$74 \pm 34^{(1)}$
Number of α Contacts	10 ± 0.8	10 ± 0.8	$2 \pm 0.5^{(1)}$
Duration of α contacts (Counts/3 min)	88 ± 5	$108 \pm 7.6^{(2)}$	$19 \pm 7^{(1)}$
% of α contacts during the test	76.4 ± 3.3	75 ± 2.5	$26.8 \pm 7^{(1)}$

Notes: (1) Significantly different from Control group, $p < 0.01$. (2) Significantly different from Control group, $p < 0.05$.

3.4. Experiment 4

The social behaviour (intruder-host territorial challenge) in rats from Groups 1–3 is shown in Table I. Latency to interact with the intruder, number of α contacts and percentage of α contacts were similar in groups 1 and 2 (Table I). Only a small but significant increase in duration of α contact was observed in Group 2; while animals from Group 3 showed scores significantly different from Control in all the behaviours measured (Table I).

4. DISCUSSION

In previous studies from our laboratory, trace elements administration to rats was performed in a chronic modality.[21,22] Since this working scheme extended in time during many important functional stages of the animals, such as pregnancy, delivery, lactation, and juvenile period after weaning of litters, there arise several questions about its possible intrinsic mechanism. On one hand, it is the problem of the necessary time to induce the observed biological effects in the offspring. Time course of events can predict or give an approximate idea about what sequential steps in the cell internal regulation must be followed in order to set a change in the molecular and neural mechanisms necessary to induce a macroscopic behavioural effect. On the other hand, the marked change in the ratio of non-methylated cytosine to methylated cytosine in the DNA of hippocampal neurons in the trace element-treated animals found previously;[21] put into perspective an epigenetic mechanism linked to trace elements. Epigenetic changes appear to depend on time,[5] and clear differences in DNA methylation marks are evident in the preimplantation and post-embryonic development periods.[2] Thus, a comparison between two strategic timing periods, such as before and after fertilization was expected to give an additional insight on the epigenetic changes induced by trace elements.

As results show (Figs. 2–4, and also in Table I), three out of four general behavioural patterns were significantly affected by the short treatment of ZnTe (Group 2). Then, it is evident that a prolonged administration of trace elements is not a strict requirement in order to induce behavioural changes in litters. In spite that many affected behaviours in the short treatment parallel those found with the chronic administration (Figs. 2 and 3), differences, and however were observed in some others (Fig. 4 and Table I). These results suggest that diverse molecular targets or sites in the cell are involved when chemical elements interact with the physiological processes before and after fertilization. The precise molecular mechanism of action of ZnTe is not known. Apparently, an intermediate or an advanced step in

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its course of action could be a modification of the dynamics of the cell DNA methyltransferases that modulate the methylation patterns of DNA expression,[2] since animals treated with the chronic regimen showed altered ratios of methylated cytosine to non methylated cytosine.[21] Present results also revealed that whatever the intrinsic molecular mechanism that might be involved in trace element modulation; there are two groups of behavioural expressions that appear to be differentially affected, according to the time of stimulation. General motor activity, motivated exploration (Fig. 2), and lateralized exploration (Fig. 3) appear to be the most susceptible brain processes affected; while defensive and social behaviour (Fig. 4 and Table I) more resistant to changes. Perhaps, the different brain structures that control these two general behaviours[12, 14] can have distinct sensitivity to external chemicals, such as Zn or Te.

In general perspective, the most significant finding found with the short trace element administration, is that behavioural changes linked to ZnTe treatment to parent rats appeared later in the descendants, when exposition to these chemicals were no longer present (Group 2). This result suggests that trace element affected cell mechanisms in the parent rats that involved epigenetic changes manifested in the descendent rats. Although previous evidence showed that the brain structure with altered patterns of DNA methylation in the ZnTe treated rats was the hippocampus and not the prefrontal cortex; the rich behavioural spectrum affected by treatment is difficult to be due to a unique activation of only one brain structure. Trace elements must be acting on other brain neuronal circuits not presently explored. Although, the interactions between trace elements and DNA have been described,[20, 23, 17, 10, 7, 3, 24, 25, 15] still its precise molecular mechanism is speculative.

5. FINAL REMARKS

Confirming previous results from our laboratory,[21] present data show that alterations in behavioural responses induced by trace elements are not only diverse and selective, but clearly depending on the time of stimulation. This means that the effects of these chemical elements can not be explained as random unspecific actions on brain tissue. DNA methylation changes in normal animals occur during embryonic development, and DNA epigenetic marks are largely erased in early embryonic development stages.[2] However, at later stages during embryonic development, de novo DNA methylation marks are made. Since it is thought that these two molecular processes depend on two different enzyme systems; DNMT1 which maintains the DNA methylation, and DNMT3a, DNMT3b which are required for de novo methylation,[4, 16] it is possible to speculate that trace elements could be affecting these complex enzyme systems in a differential manner. Of course, these are not the only possible molecular mechanisms whereby ZnTe could be exerting these behavioural effects, since epigenesis can be regulated by several others, such as participation of sirtuin enzymatic proteins that are involved in the modification of histone proteins.[13] Whether these proteins might be implicated in the biological effects detected in the present study is actually unknown.

Finally, present results show a new and outstanding feature about the biological effects of trace elements as acute environmental influence to living systems: induction of epigenetic molecular events in cells that affect the subsequent progeny.

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