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Rat embryo exposure to all-trans retinoic acid results in long-term cognitive deficits

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Abstract. – AIM: The present study examines in particular associative learning and aversive memory abilities in adult Sprague Dawley rats exposed to all-trans retinoic acid (ATRA) in the period spanning gestational days (GD) 11-13.

MATERIALS AND METHODS: The ATRA dosage of 2.5 mg/kg compatible with high neonatal survival, sufficient to supply offspring for later behavioral testing, was used.

RESULTS: The results show that the GD 11-13 ATRA exposure compromises the ability of rats to learn an active avoidance task. Indeed, unlike control rats, the ATRA-treated rats did not improve in performance over blocks of training, the number of attempts they made to avoid foot shock being significantly affected. The memory ability, assessed with the passive avoidance paradigm, was not affected by ATRA exposure.

CONCLUSIONS: The results provide further evidence that, beyond gross CNS malformations, gestational ATRA exposure induces long-term cognitive deficits in the offspring, thus raising further warning for better control of retinoid safety during pregnancy, an aspect relevant to human health protection within the regulatory environment.

Key Words:
All-trans retinoic acid, Development, Active avoidance task, Aversive memory task, Rat.

Abbreviations

RA = retinoic acid
RAR = retinoic acid receptor
ATRA = all-trans RA
GD = gestational day
PND = postnatal day
CAR = conditioned avoidance response
CNS = central nervous system
GE = ganglionic eminence

Introduction

Retinoic acid (RA), the active form of the nutrient vitamin A, modulates neurogenesis, neuronal survival, and helps maintain neuronal plasticity and cognitive functioning in adulthood acting via specific retinoic acid receptors (RARs)[1]. The receptors RARα, β and γ exhibit different subcellular locations implying their potential regulation of both transcriptional and non-genomic actions[2]. During embryo development, RA regulates axial and regional patterning, organogenesis, limb formation, and neurogenesis[3-5]. The pathways on which RA acts require balanced concentrations of this retinoid, and deviation of RA levels from normal results in abnormal growth and development[6].

Over the time, the actual or potential use of retinoids, such as all-*trans*-RA (ATRA), 9-*cis*-RA and 13-*cis*-RA, in the treatment of clinical disorders has progressed significantly, and indeed several studies have shown their benefit in malignancy[7-9], immune[10-12] and neurodegenerative disorders[13-15]. Because of their teratogenicity, however, management of pregnant patients with retinoids is actually a challenge, thus raising warning for better control of the therapeutic use of retinoids in pregnancy.

Besides birth defects, there is evidence that maternal RA exposure during gestation may also hesitate in neurofunctional deficits in the offspring with no apparent malformations at birth[16], thus, raising concern about cognitive and psychiatric sequelae associated with RA excess during development. Therefore, there is a growing interest in applying methods known to index the many categories of behavior in laboratory animals, an aspect of great relevance to human health protection within the regulatory environment[17].

Previous studies have shown that prenatal ATRA exposure causes, in the rat, long-lasting impairment in motor skills associated with transient morphological alterations[18-20] and dysfunction of the mitochondrial respiratory chain in the cerebellum[21]. In the present study we have examined in particular associative learning and aversive memory in 180-day old rats exposed to ATRA in the period spanning gestational days

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(GD) 11-13. The ATRA dosage of 2.5 mg/kg was used based on previous observations[18-20] that this schedule is compatible with high neonatal survival, sufficient to supply offspring for later behavioral testing.

Materials and Methods

Animals, Animal Husbandry, and Dosing

Pregnant (GD 7) Sprague-Dawley rats (n=37) were purchased from Harlan (San Pietro al Natisone, Italy). On the day of arrival, they were housed individually, allowed free access to food and water, kept under controlled environmental conditions (ambient temperature 24-25°C, humidity 50-60%, 12-h light/dark cycle, light on at 6:00 a.m.), and randomly assigned to two experimental groups: (1) 2.5 mg/kg ATRA (Sigma-Aldrich, Milan, Italy) in sesame oil (n=19, initial body weight 254.08±2.82 g); (2) sesame oil (control, n=18, initial body weight 259.65±4.84 g). Both groups were gavaged once daily for three consecutive days between GD 11 and 13. Dose volume was 1 ml of oil solution per kg of body weight. The ATRA suspensions were prepared fresh daily under dim illumination and kept in amber bottles to prevent photo-degradation. Body weight gain of control and ATRA-treated pregnant rats was monitored every day. No difference in final (GD 20) body weight gain was found between control and ATRA-treated rats (CTRL: 359.3 ± 5.26 g; ATRA: 352.1 ± 7.52 g).

On the day of birth (designated postnatal day or PND 1), all pups were weighed, checked for any external malformations, sexed and then randomly culled to eight pups per litter. Pups were weaned at 21 days of age and group-housed. One male rat from each litter was used for behavioral studies assuming the pregnant female as being the experimental unit. This procedure allows a "per litter" analysis, thus preserving the correct significance level of statistical test[22]. To escape the problem of litter confounding only one rat from each litter was assigned to each test. With this procedure, the litter random factor and the subject random factor become one and the same thing, eliminating a serious bias and greatly simplifying data analysis[23].

Experiments were conducted in accordance with guidelines released by the Italian Ministry of Health (D.L. 116/92), and the "Guide for the Care and Use of Laboratory Animals" as adopted and promulgated by the USA National Institutes of Health. All efforts were made to minimize the number of animals used and their suffering.

Reproduction Data

The number of dams giving birth, pregnancy length, litter size and mortality at birth were determined. Body weight gain of pups before and after weaning have been extensively studied in a previous work[18]. Body weight monitored at the time of behavioral testing was not altered by ATRA exposure (CTRL 405.8±3.55g; ATRA 397.4±2.49 g).

Passive Avoidance Task

Experiments were performed as described previously[24] using the passive avoidance apparatus (Ugo Basile, Comerio, Varese, Itay). Passive avoidance is a 2-d task assessing aversive memory. In the training session (d 1), rats were individually placed in the lighted chamber for 10 s and the latency to enter the dark chamber (approach latency) was measured as a control for visual ability and motor activity. Immediately after the rat entered the dark chamber, a 0.8 mA, 2-s foot shock was delivered. Rats remained in the dark compartment for a further 10 s to allow them to associate the dark compartment with the received foot shock. In the retention test session (d 2), rats were placed in the lighted compartment and the door was opened. The latency to enter the dark compartment (avoidance latency) was measured (cut-off time 180 s).

Active Avoidance Task

Experiments were performed as described previously[24] using a device consisting of a 2-way avoidance box (Ugo Basile, Comerio, Varese, Italy). Two-way active avoidance is a 1-d task assessing associative learning. Essentially, rats learned to avoid a 0.5-mA, 2-s foot shock (unconditioned stimulus), signaled by a 80-dB, 10-s tone (conditioning stimulus), moving into the opposite chamber [conditioned avoidance response (CAR)]. The test consisted of a 100-trial session scheduled in 4 blocks, each consisting of 25 trials with a 50-s inter-trial interval.

Statistical Analysis

General reproduction data were analyzed by Student's *t*-test. Fisher's exact test was used for evaluation of mortality at birth. Concerning the passive avoidance task, differences in the acquisition or retention trials were analyzed using the non parametric Mann-Whitney U test, because

Table I. Effects of the GD 11-13 ATRA treatment on reproductive parameters.

Treatment	Dams giving birth, %	Pregnancy length (days) (means±SEM)	Litter size at birth (means ± SEM)	Mortality at birth
Control (sesame oil)	100	21.4 ± 0.17	13.8 ± 0.5	5/234
Retinoic acid (2.5 mg/kg)	100	21.5 ± 0.16	11.4 ± 1.3	80/228*

*Different from control, $p < 0.0001$ (Fisher's-exact test). GD: gestational day.

variances were unequal. Concerning the active avoidance task, differences in CAR and latencies to escape the aversive stimulus were first analyzed using repeated-measures ANOVA (treatment x blocks, with block as a repeated measure). Within-group comparisons were performed by Dunnett's multiple comparison test. Differences between CTRL and ATRA-treated rats within each block were analyzed using the Student's *t* test.

Results

Reproduction Data

The GD 11-13 ATRA treatment did not affect pregnancy length, litter size at birth and number of dams giving birth, whereas it caused a significant increase in mortality at birth (Table I).

Passive Avoidance Task

Neither the approach latencies (acquisition trial) nor the avoidance latencies (retention trial) differed between controls and ATRA-treated rats, thus suggesting that prenatal ATRA treatment did not impair the ability of rats to perform this aversive memory task (Figure 1).

Active Avoidance Task

CAR, i.e. attempts to avoid foot shock, increased significantly over blocks of training in both control and ATRA-treated rats ($p < 0.05$, Dunnet's multiple comparison test), thus suggesting that both groups were able to learn this task over training. Despite that, a significant difference in conditioned avoidance responses was found between the two groups at the 3rd and 4th blocks of training (Figure 2). This result indicates that the learning ability of ATRA-treated rats was however impaired over training since they did not achieve the same performance level as the untreated rats. Neither escape nor avoidance latencies were different between controls and ATRA-treated rats (*data not shown*).

Discussion

The present findings show that rat embryo exposure to ATRA results in long-term impairment in learning ability, whereas memory ability appears to remain unaltered. In particular, the ATRA-treated rats do not achieve the same performance level over blocks of training as control rats, the number of avoidance responses being significantly reduced. Moreover, a high mortality at birth occurs in the ATRA-treated group (35.8%) in comparison with the untreated one (2.13%), thus, replicating previous data[18,25-28].

The compromised ability of ATRA-treated rats to learn an active avoidance task is a finding consistent with literature data showing that exposure to ATRA or 13-*cis* RA on GD 11-13 disrupts learning ability measured under shock avoidance (28). An intriguing aspect concerns, however, the apparent lack of ATRA effect on memory. In this regard we could infer that, generically, different behavioral domains are subserved by different brain areas, and developmental pattern as well as temporal windows vary within different regions of

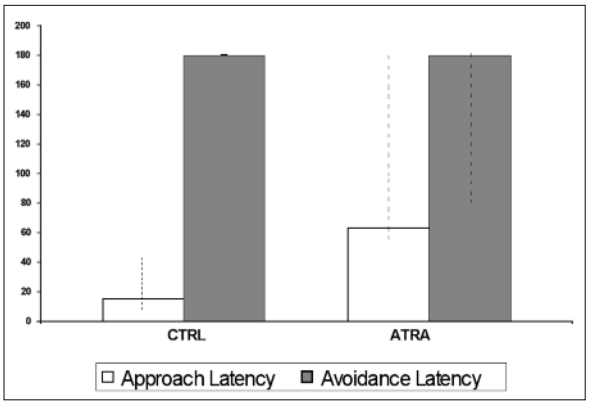

Figure 1. Effects of prenatal ATRA exposure on approach latency (acquisition trial) and avoidance latency measured 24 h later (retention trial) in 180 day-old rats subjected to the passive avoidance task. Data represent median values and interquartiles (dashed line). Control (CTRL): n=10; ATRA: n=9.

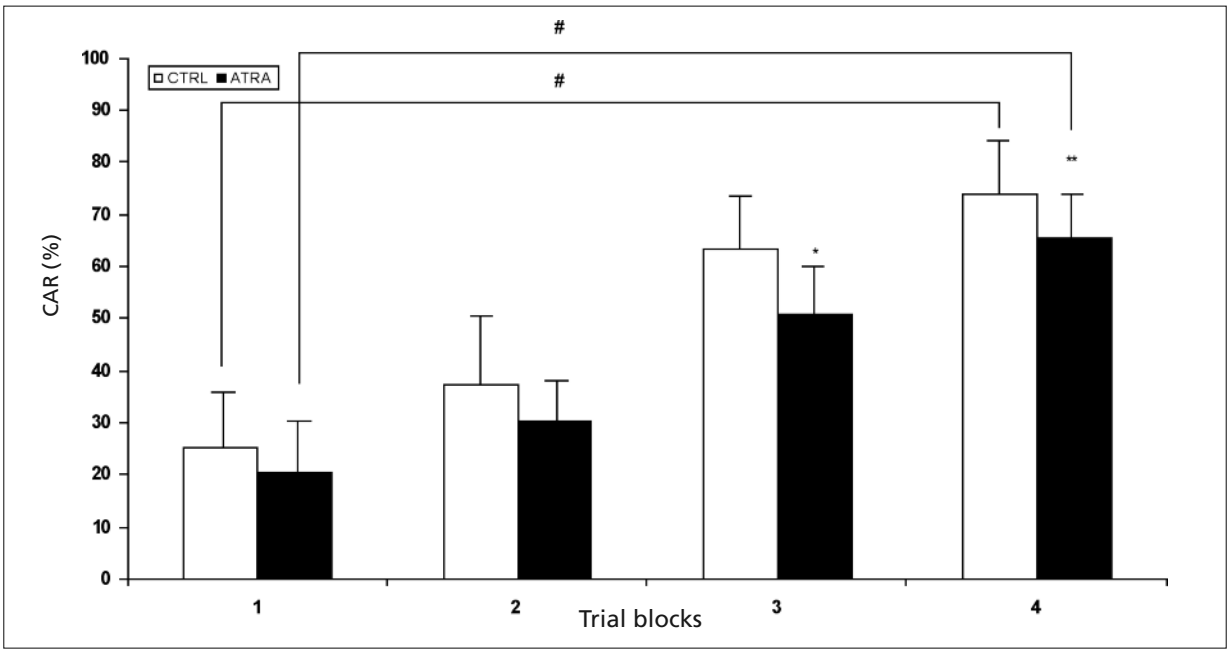

Figure 2. Effects of prenatal ATRA exposure on active avoidance learning in 180 day-old rats. The rats were subjected to 100 trial sessions (4 blocks of 25 trials each), with a 50-s inter-trial interval. The conditioning stimulus was an 80-dB tone for 10 s. The unconditioned stimulus was a 2-s positive half-wave constant current of 0.5 mA intensity. The number of conditioned avoidance responses (CAR) during each block of training was normalized assuming the 25 value as the maximum number of CAR attainable. Data represent mean values (%) ± S.E.M. Control (CTRL) n=8; ATRA n=10. ≠Different from first block, $p < 0.05$ (Dunnett's multiple comparison test). *Different from CTRL, $p < 0.05$; **Different from CTRL, $p < 0.002$ (Student's t-test).

the brain. Depending on the temporal and regional emergence of critical developmental processes, the ATRA effects may, therefore, differ in relation to the embryonic stage when ATRA exposure occurs. Estimated timelines of regional neurogenesis in the rat, show that an exponential proliferation of a multipotent population of pseudostratified cells localized in the ventricular zone occurs around GD 12, and shortly thereafter, the anatomically superficial subventricular zone is distinguishable[29]. Therefore, at the embryonic stage of ATRA exposure, a burst of cell proliferation contributing to the ultimate cell population of the mature neocortex was taking place.

Moreover, at this preplate stage, the two main neuronal populations of the mammalian cortex, namely excitatory glutamatergic pyramidal cells and GABAergic interneurons, are generated in different sectors of ventricular zones and migrate to the cortex along different routes[30,31]. An effective influence of RA on tangential neuronal migration from the ganglionic eminence (GE) to the cerebral cortex with a decline in the cortical GABAergic interneuron population has been recently demonstrated[32]. Further, in adult mice, 13-*cis* RA reduces cell proliferation in the subventricular zone, a brain region that continues to generate new neurons, and severely disrupts capacity to learn a spatial radial maze task[33]. Moreover, the GE is a region of very high neuronal RA synthesis[34], and RA is also instrumental for inducing a network of dopaminergic signal transductions in the GE[35]. Thus, one could speculate that the GD 11-13 ATRA exposure may interfere with the developmental pattern of the neocortex through impairment in cell proliferation and/or migration. It is, however, clear that all brain areas have multiple reciprocal connections forming circuits subserving various functions. Damage to a particular structure in the circuit or a connecting pathway may produce structural or functional changes upstream or downstream and results in behavioral changes that are a consequence of damage to the circuit as a whole.

Although there are differences between rats and humans in cognitive repertoire, these and previous results suggest that a high risk exists for long-term impairment in cognition following inadvertent human exposure to RA during gestation. Comparative neuroanatomical studies[36] have shown that the developing rat brain morphology between embryonic days 11-21 is comparable to

human brain morphology at embryonic weeks 4-16. This timing correspondence strongly supports that RA exposure during gestation can be detrimental to neurodevelopmental events in humans. In fact, women of childbearing age are advised to use an adequate contraception at the time of treatment with retinoids for skin diseases or cancer, and to delay conception for at least 1 month after termination of therapy owing to RA accumulation after multiple doses[37]. The recommended dosage range for retinoids is higher than the dosage range used in the current study: 0.5-1 mg/kg/day for 15-20 weeks (Accutane®) or 45 mg/m^2/day for 30 days (Vesanoid®) versus 2.5 mg/kg for 3 days (current study). This is of great concern since, besides the prescription drugs whose pregnancy risk may be successfully managed with more restrictive programs for preventing foetal exposure and RA birth defects[38], dietary intake of vitamin A and nutritional supplements may also pose a risk, and indeed no increment of this vitamin is recommended during pregnancy[39].

Conclusions

The present work provides further evidence that the GD 11-13 ATRA exposure, even at a dose level devoid of gross malformations, can induce long-term detrimental effects on cognition, thus raising warning for better control of the therapeutic use of retinoids in pregnancy, mainly considering that all endogenous mechanisms in retinoid homeostasis protect against vitamin A deficiency, whereas no defence mechanisms have evolved to protect from RA excess[6].

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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