ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1999, 59: 115-122

Limited changes in handedness and morphine reactivity in CD-1 mice after pre- and postnatal ozone exposure

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Abstract. Outbred CD-1 mice were either not exposed (control group) or exposed to ozone (O_3) (0.3, 0.6, or 0.9 ppm), during foetal and neonatal life until the time of weaning (postnatal day (PND) 26). On PND 70 the subjects were tested for handedness using a paw preference task assessing both the animals' capability to reach a food pellet in a feeding tube and the individual preference for the use of one of the other forepaw. O_3 exposure did not affect the animals' capability to learn the task but caused changes in handedness. Specifically, females exposed to the intermediate O_3 concentration showed a reduced preference for the right paw than both their same-sex controls and 0.6 ppm males. On PND 100, mice underwent a hot plate test after IP treatment by either saline or morphine HCl (10 mg/kg). The results were generally in the direction of reduced drug sensitivity after exposure to the highest concentration. The evidence for this effect was more robust in the case of an organised avoidance response (wall-rearing) than in the case of a reflexive response (limb withdrawal); in the case of the former, latency data showed an effect on both males and females while frequency data showed an effect only in females. Overall, the O_3 effects are suggestive of subtle CNS changes affecting mouse behavioural responses.

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Key words: developmental exposure, paw preference test, hot plate test, ozone, mouse

INTRODUCTION

Recent studies have shown that early exposure to ozone can modify somatic and neurobehavioural development.

In rats, exposure to 1.0 or 1.5 ppm O_3 during mid- and late gestation led to reduced neonatal growth rates. Late gestation exposure also produced a delayed appearance of several reflexes and responses such as righting, rearing, grooming, and horizontal movement in a open field (Kavlock et al. 1980).

A study performed in our laboratory on CD-1 mice exposed to ozone (0, 0.4, 0.8, or 1.2 ppm) during mid- and late pregnancy showed a depression of offspring postnatal body weight gain at the highest concentration. Negative results, however, were obtained by an extensive assessment of neural and behavioural functions in the postnatal period and at the young adult stage (Bignami et al. 1994). More extended exposure (0, 0.2, 0.4, or 0.6 ppm from 10 days before mating until gestational day 17) also failed to alter postnatal neurobehavioural development but produced some "borderline" deficits in social interactions and radial maze learning at the young-adult and adult stages (Petruzzi et al. 1995).

More substantial changes occurred when O_3 exposure (0.6 ppm) was prolonged, from before mating until pups' weaning. Specifically, this treatment caused a marked reduction of postnatal body weight gain and selective effects on various behavioural parameters, including an attenuation of sexual dimorphism in several non-sexual responses (particularly rearing and sniffing in the open-field and activity in the final conditioned place preference test session) and an impairment of passive avoidance acquisition in the initial period of training (Dell'Omo et al. 1995a). Another experiment using the same exposure and time schedules, showed in O_3 mice a slight impairment in the Morris maze test during the last day and a strong tendency to make turns to the left in the maze, whereas the controls preferred clockwise turns (Dell'Omo et al. 1995b).

This O_3 effect on laterality could be viewed in the light of the modifications produced by O_3 in several immunological parameters (see review by Jakab et al. 1995). In fact, besides the well documented asymmetries in the relations between the brain and the immune system (Neveu et al. 1988, 1989, 1991, Betancur et al. 1991) there is a direct evidence that immune mechanisms are involved in the production of laterality phenomena in a sex-specific fashion. For example, in females but not in males of the NZB mouse strain handedness appears to be associated with the timing of production of anti-erythrocyte and anti-DNA antibodies (Neveu et al. 1989; for other examples of sex-specific association between immune processes and laterality phenomena see Neveu et al. 1988, Betancur et al. 1991).

The present study was designed to test behaviourally a "changed laterality" phenomenon upon O_3 exposure by the analysis of ozone effects on handedness by the use of a paw preference task. This test, besides assessing the animals' capability to extract a food pellet from a feeding tube, allows to quantify their preference for the use of one or the other forepaw (Collins 1968, Neveu et al. 1988, Betancur et al. 1991).

The study was also aimed at assessing whether developmental O_3 exposure modifies pain reactivity and response to an opiate analgesic (morphine) in the adult. In fact, recent studies suggest an effect of O_3 on pain mechanisms showing a stimulation of C fibers during O_3 inhalation correlated with the enhanced airway responses induced by the pollutant (Coleridge et al. 1993), and a reduced airway hyperresponsiveness to O_3 following capsaicin treatment (Jimba et al. 1995). In addition, opioid peptides have been implicated in the mediation of immune changes, including those associated with laterality phenomena (e. g. suppressive effects of stress on natural killer cell cytotoxicity, Neveu et al. 1994).

METHODS

Animals and breeding procedures

Mice of an outbred Swiss-derived strain (CD-1) weighing 27-30 g were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival at the laboratory, the animals were housed in an air conditioned room (temperature 21 ± 1°C, relative humidity 60 ± 10%) with lights on from 9.30 p.m. to 9.30 a.m. Adult males and females were housed in same-sex pairs in 33 x 13 x 14 cm Plexiglas boxes with a metal top and sawdust as bedding. Pellet food (Enriched Standard Diet purchased from Mucedola, Settimo Milanese, Italy) and water were continuously available. After one week of acclimatisation, 10 males and 10 females were randomly assigned to the control group and to each of the three O_3 exposure conditions (see below). Breeding pairs were formed after a 6-day exposure period (for rationale see Petruzzi et al. 1995). The study was removed ten days after discovery of the plug.

Exposure apparatus and procedures

The apparatus (custom made by A.and L. CO. Industries, Segrate, Italy) was the same as that used in previous studies (Bignami et al. 1994, Musi et al. 1994, Dell'Omo et al. 1995 a,b, Petruzzi et al 1995); it included a device for O_3 production, a control system for continuous delivery of O_3 (with monitoring and recording of concentrations) and four stainless-steel exposure chambers (52 x 135 x 113 cm) with a hatch glass in the front door. The chambers were located in an air-conditioned room, adjacent to the housing room, and were equipped with racks which could hold up to 24 Plexiglas housing boxes (33 x 13 x 14 cm). Temperature and humidity conditions were as indicated above.

Ozone was produced with an electric arc discharge O_3 generator (ozonator). Different O_3 concentrations were obtained by varying the airflow directed to the ozonator (Regulator 44-2200, Drager Tescom GMBH, Lubeck, Germany) and by subsequent regulation by flow regulators-meters (model RMA-13-SSV, Dwyer Instruments, Inc., Michigan City, USA) of the appropriate volume of ozonized mixture to be added to the clean air directed into each chamber. Ozone levels were monitored in samples drawn at the bottom of the chamber (close to exhaust) every 18 min for a 4.5 min period, after extensive preliminary verification that O_3 levels in these samples were representative of those in different parts of the chamber. Ozone was measured by an ultraviolet (UV) O_3 analyser (O_3 41 M, Environnement SA, Poissy, France) equipped with a control system for the repeated check of the calibration between successive measure cycles, based on the measurement of known O_3 concentrations. Actual concentrations were recorded during the last 30 sec of each 4.5 min period. Ozone concentration in the room was generally below 0.02 ppm and never exceeded 0.04 ppm. Chamber concentrations of O_3 deviated less than 15% from the stated value. The ozonized air passed through three filters before being discharged into the outdoor environment.

Ten males and 10 females were randomly assigned to a no-exposed control group (hereafter 0.0 ppm) and to each of three O_3 exposure conditions (0.3, 0.6, or 0.9 ppm). The former group was maintained in the same exposure chamber as the three remaining groups, but the ozone producing apparatus was switched off. Exposure lasted from 6 days prior to the formation of breeding pairs until PND 26. Exposure was essentially continuous except for the brief interruptions required for animal maintenance and weighing.

Other general procedures

At birth, proportion of pregnancies carried to term, litter size, sex ratio, and neonatal mortality were noted to assess any effect on reproductive performance. The number of litters in each exposure condition was reduced from 10 to 7 due to some instances of pregnancy failure or neonatal mortality in the 0 and the 0.9 ppm groups (2 and 3 cases, respectively), which led to random discarding of other litters in order to achieve a balanced design. All litters were culled to eight pups (four females and four males) that were raised by their biological mothers. On PND 21 pups were weaned and reduced to three pups per sex in each litter. The mice were left in the exposure chambers until PND 26 and then transferred to the housing room where they were caged in same-sex pairs after randomly discarding of another pup per sex in each litter. All animals were weighed on PNDs 2, 7, 13, 19, 27, 40, and 100.

Paw preference test at PND 70

This test was performed following a procedure reported by other authors (Collins 1968, Neveu et al. 1988, 1989, 1991; Betancur et al. 1991). Mice were deprived of food for 20 h and then put into a testing cubicle in which a pellet was available in a feeding tube. The sequence of right or left paw used to reach food was scored. In every testing session, 50 paw searches were routinely observed for each mouse. Animals were tested three times (approximately every 5 days) over a 2-week period and the mean score of these three sessions was used for the statistical analysis. In addition, the latency to learn the task was measured for each animal (i. e. the time needed to reach the food pellet for the first time).

Hot plate test at PND 100

At the beginning of the test, half of the subjects ($n = 7$ in each combination of sex and exposure condition) were injected IP with 10 mg/kg of morphine HCl and the other half with saline (NaCl 0.9%). After 20 min, each animal was placed at the center of a glass cylinder-covered (diameter 19 cm) hot plate (model D837 Socrel Comerio, Italy) maintained at 52 ± 0.1°C. All animals were used once. The animals were videorecorded using a

Sony Videocassette recorder VO-5800 apparatus and their behaviour subsequently scored by a trained observer using an Esterline Angus Operation Recorder (model A 620X, Indianapolis, IN). Latency (to the nearest millisecond) and frequency of wall-rearing, hindlimb licking, and hindlimb withdrawal reflex were scored, cutoff time being 1 min.

Statistical analysis

Data on pups' weight from PND 2 to 40 were subjected to a mixed-model analysis of variance (ANOVA) with litters as random factor nested under exposure condition (exposure), sex as within-litter factor, and days as repeated measures within subject. The same design (except for the absence of repeated measures) was used in a separate ANOVA on weight data for PND 100. The weights were averaged within litters and sexes; therefore, all ANOVAs were based on one female and one male value in each litter for each test day.

The data on paw preference were analysed by ANOVAs considering exposure as grouping factor and sex as within-litter factor. In the ANOVA on hot plate data a second within-litter factor was pre-test treatment (morphine vs. saline).

Post-hoc comparisons within logical sets of means were performed by the Tukey's HSD test, the use of which is permissible or even recommended in the absence of significant main or interaction effects in the ANOVA, in order to minimize frequencies of both Type I and Type II errors (Wilcox 1987).

RESULTS

Effects on reproductive performances and postnatal body weight gain

O_3 exposure at any concentration did not affect either the proportion of successful pregnancies, litter size, sex ratio, and neonatal mortality. The data on postnatal weight need not to be reported in detail, since they replicated the effect found in a recent experiment with the same exposure schedule (Dell'Omo et al. 1995 a). In brief, O_3 produced the expected retardation of postnatal body growth (PNDs 2-40, exposure x repeated measures, $F_{15,290} = 2.1$, $P < 0.01$; by posthocs, differences between 0 and 0.9 mice significant from PND 19 onwards, $P < 0.05$). In addition, the ANOVA on PND 100 data confirmed that the O_3 reduction of body weight gain is not compensated after an extended period of time after exposure discontinuation (exposure, $F_{3,108} = 3.09$, $P<0.05$; 0-0.9 ppm difference, $P < 0.05$).

Paw preference test

The data shown in Table I indicate that prolonged O_3 exposure failed to affect learning to reach the food pellet in the test tube but produced some concentration- and sex-dependent effects on paw preference. The latter was apparently shifted to the right in O_3 males and, *vice versa*, to the left in O_3 females (sex x exposure $F_{3,95} = 7.17$, $P<0.01$; post-hocs: 0.6 ppm females *versus* 0.6 ppm males, Ps<0.05).

TABLE I

Paw preference test at PND 70 of CD-1 mice exposed pre- and postnatally to ozone

	0 ppm		0.3 ppm		0.6 ppm		0.9 ppm	
	M	F	M	F	M	F	M	F
Latency (min.) to first response[§]	23.50 (6.00)	17.38 (6.36)	12.77 (4.93)	6.69 (2.60)	13.87 (4.03)	16.92 (6.93)	33.73 (12.43)	16.75 (2.14)
Paw preference[o]	23.78 (1.67)	28.00 (2.33)	21.31 (2.34)	27.70 (1.84)	30.33 (2.25)	19.33* (2.44)	27.81 (2.02)	22.08 (2.67)

M, males; F, females. n = 11-15; 0.0 ppm, control, no-exposure group. [§] Indicates the mean time (SEM) employed to reach the food pellet for the first time. *Significantly different from 0.6 ppm males ($P<0.05$). [o]Expressed as mean number (SEM) of right paw entries over the three sessions.

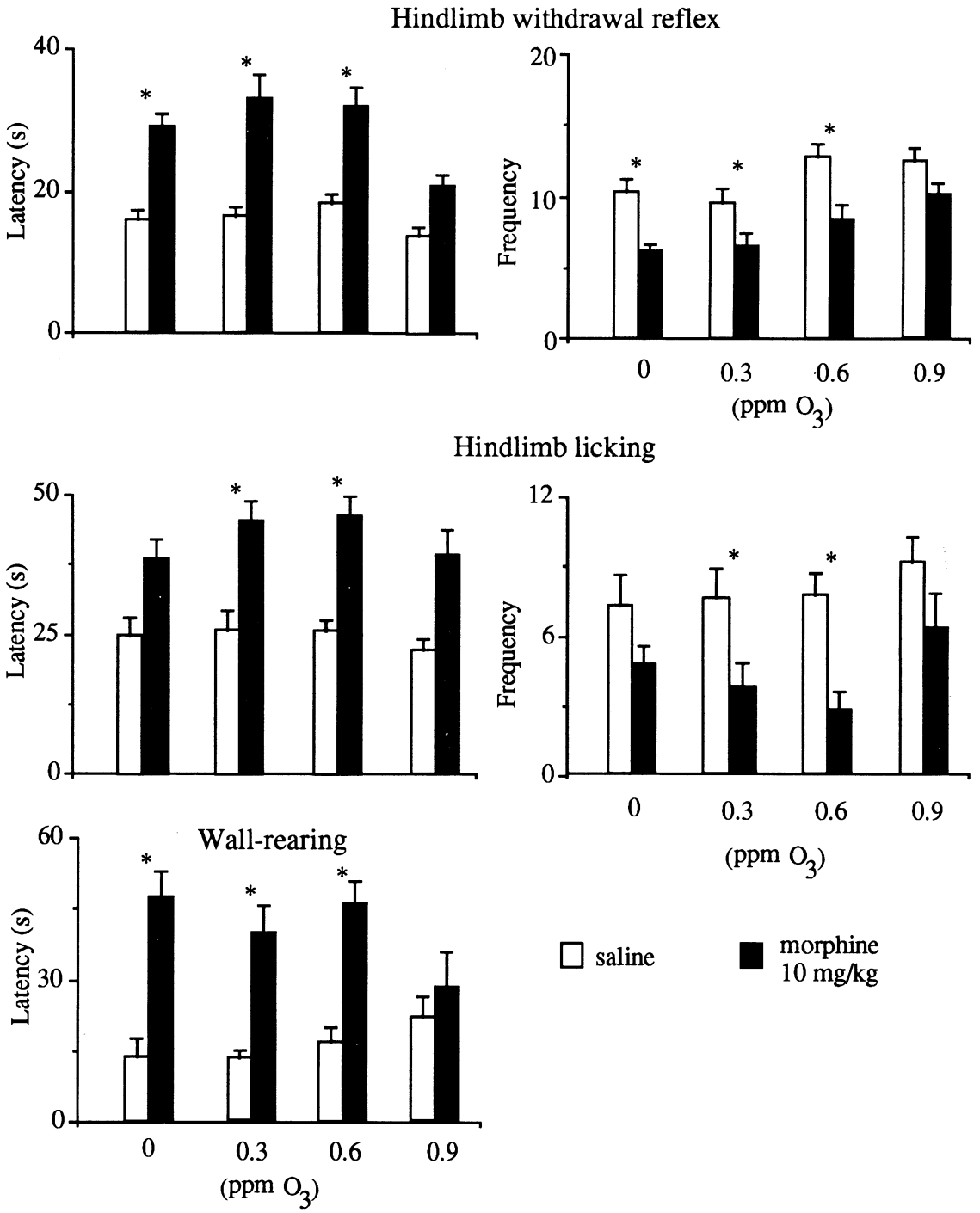

Fig. 1. Hot plate responses of CD-1 mice exposed pre- and postnatally to ozone (O_3) and receiving either a morphine challenge (10 mg/kg) or a saline control injection 20 min before the test. Data represent mean latency and frequency +SEM (data pooled over sexes in each combination of exposure and treatment; 0.0 ppm refers to the control, no-exposure group; $n = 14$). For wall-rearing frequency requiring consideration of the sex variable see Table II. *Significantly different from the corresponding saline group ($P<0.05$).

TABLE II

Mean frequency (SEM) of wall-rearing responses in the hot plate test of adult CD-1 mice exposed pre- and postnatally to ozone

	0 ppm		0.3 ppm		0.6 ppm		0.9 ppm	
	S	M	S	M	S	M	S	M
Males	15.85 (3.02)	3.00* (1.94)	11.85 (1.82)	3.57* (1.82)	12.29 (1.84)	1.42* (0.81)	8.85 (1.63)	7.14 (1.10)
Females	7.00 (1.35)	0.71* (0.47)	9.57 (1.10)	2.57* (1.88)	6.57 (0.97)	0.85* (0.46)	10.71 (1.85)	2.71* (1.10)

S, saline; M, morphine; 0.0 ppm, control, no-exposure group. * Significantly different from the corresponding saline group ($P<0.05$). $n = 7$.

Hot plate

The data shown in Fig. 1 (pooled over sexes in the absence of interactions between sex and exposure) indicate that mice exposed to the low and intermediate O_3 concentrations (0.3 and 0.6 ppm) showed morphine effects which were indistinguishable from those of control animals. Morphine analgesia was apparently reduced in 0.9 ppm mice, as it is suggested by the lack of significant saline-morphine differences at this concentration in all graphs concerning limb withdrawal and licking. The corresponding ANOVAs, however, showed only a main effect of morphine, without interaction of exposure and pre-test treatment. The data on wall-rearing latency (Fig. 1 and Table II) provided more definite evidence of an attenuation of morphine effect after exposure to 0.9 ppm O_3. In the case of wall-rearing latencies, the ANOVA yielded a significant interaction between exposure and pre-test treatment ($F_{3,96} = 3.33$, $P < 0.05$). In the case of wall-rearing frequency, the attenuation of morphine effects was clearly limited to males (exposure x pre-test treatment x sex ($F_{3,96} = 3.07$; $P < 0.05$; for posthocs see Table II).

DISCUSSION

The present data indicate that extended pre- and postnatal O_3 exposure may affect the development of brain mechanisms responsible for handedness. This partially confirms previous findings concerning the development of a laterality bias in O_3-treated mice tested in a Morris maze apparatus (Dell'Omo et al. 1995b). In the present experiment, however, the change in paw preference was limited to 0.6 ppm females which showed a preference for the use of the left paw when compared with 0.6 ppm males; in the Morris maze test the change was not sex dependent and consisted of a strong tendency of O_3-treated mice to make turns to the left when compared to controls (Dell'Omo et al. 1995b). On the other hand, the different role of sex in the two situations could be ascribed to O_3 effects on interaction between sex hormones and immune mechanisms which in turn could have been responsible for the changes in handedness. In fact, the association between immune reactivity and laterality is a reportedly sex-dependent phenomenon, as shown by the varying relations between immune parameters and handedness phenomena (see the review by Neveu 1993 and the example given in the Introduction). The fact that intermediate O_3 exposure may exert the maximal efficacy may tentatively be explained by the subtle interplay within a complex neuro-endocrine-immune system in which a particular dose, when given at a critical developmental stage, is particularly disruptive.

The O_3-induced effects on handedness could be viewed in light of the changes produced by early stress and those suggesting that the effects of early O_3 exposure be due at least in part to a stress response. Specifically, mild to moderate postnatal stress has been shown to increase the frequency of lateralization and to produce differential effects on various immune functions (Neveu et al. 1994) and extended developmental exposure to O_3 resulted in an attenuation of sex differences in several responses (Dell'Omo et al. 1995b, for the effects of early stress on sexual dimorphism see the reviews by Weinstock

et al. 1988 and Ward 1992). However, the present data do not allow to discriminate between the different role played by immune mechanisms or early stress on the changes observed since evaluation of the effects on immune parameters was not the scope of the study.

The data concerning effects on morphine reactivity in the hot plate test showed a general tendency towards a reduced drug sensitivity after exposure to the highest concentration (0.9 ppm). This was evidenced by the lack of saline-morphine differences in the 0.9 ppm group in five of the six measures taken which included both organised and avoidance responses. This do not suffice, however, to conclude that the O_3 exposure exerted a strong impact on the development of the pain system (involving for example changes in both supra spinal and spinal mechanisms). In fact in two cases (hindlimb licking latency and frequency) the lack of saline-morphine difference was also present in the controls and we can not exclude that reduction in saline/morphine difference in some cases can be caused by any stress due to the injection of saline solutions. Moreover Watkinson et al. (1995, 1996) demonstrated hypothermic response in adult rats and mice following 5-days exposure to O_3, but animals rapidly recovered after cessation of exposure. Long-term effects following developmental exposure cannot be totally excluded.

In any event, the present data and those obtained in studies using the same schedule of O_3 exposure (Dell'Omo et al. 1995a, b) suggest that more attention should be given to possible neurobehavioral effects of developmental O_3 exposure in humans. In fact, a recent study has shown a low threshold (0.05 ppm) for the production of other (mainly respiratory) adverse effects of O_3 in children (Observatoire régional de santé d'Ile-de-France 1994; summary in Boissavy-Vinau 1995), while there is considerable controversy concerning possible relations between handedness, dyslexia and immune system alterations (for references and discussion see Betancur et al. 1991).

ACKNOWLEDGEMENTS

This study has been supported as a part of the Subproject on Behavioural Pathophysiology (Project of Non-Infectious Pathology) of the Istituto Superiore di Sanitá (ISS) Italy, and financed by a grant from joint programme ISS- Ministero dell'Ambiente "PA PR22-1S, Animal models of neurobehavioural risk following developmental exposure to gaseous environmental pollutants" to Enrico Alleva. The authors gratefully thank Prof. Giorgio Bignami for critical reading of an early version of the manuscript and Mr. Luigi Amorico for data collection.

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Received 18 December 1998, accepted 19 April 1999