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Developmental exposure to xenoestrogen enhances spatial learning in male rats

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Abstract

Steroid hormones have profound effects on the development and function of the nervous system. Environmental estrogens or xenoestrogens are manmade or are natural compounds, which mimics the action of estrogen hormones. The experimental evidence for impairment of cognitive functions in humans and mammals following exposure to xenoestrogens has been fiercely debated. The strongest arguments against such studies have been that the route, time course, and intensity of exposure did not simulate environmental exposure, and that the chemicals tested have additional, non-estrogenic toxic effects, hindering a generalization of actual "xenoestrogenic" effects. Here we show that an environmental-like exposure to the pure estrogen, 17α-ethynylestradiol (EE2) during development enhances spatial learning abilities in adult male Sprague-Dawley rats. To simulate an environmental exposure, we used a very low dose (4 ng/kg/day) of EE2 equivalent to concentrations measured in European and US streams which was given orally with a non-invasive method, and we extended the treatment for the entire course of development, from conception to puberty. The animals were tested in a Morris water maze protocol at 6 months of age. Male rats treated with EE2 during development showed a faster learning during the training phase, and remembered better the position of the hidden platform in the short term. Our study demonstrates that actual levels of exposure to xenoestrogens can permanently alter cognitive abilities of a mammalian species.

Keywords: Spatial learning; Water maze; Estrogen; Xenoestrogen; Endocrine disruptor

Introduction

There is increasing public concern for the contamination of food and water with endocrine-disrupting compounds (EDCs), chemicals that alter the functions of the endocrine system and cause reproductive disorders (Anway et al., 2005; Colborn et al., 1993; Guillette and Gunderson, 2001; Timms et al., 2005). A large class of EDCs are called xenoestrogens because they mimic the actions of estrogen hormones naturally produced by the organism (Fisher, 2004). Besides their importance for reproduction, estrogens play a critical role in many cognitive abilities of higher vertebrates including humans (McEwen, 2002; Rapp et al., 2003; Scharfman and MacLusky, 2005). Although several studies reported alterations of cognitive functions in humans and laboratory animals resulting from exposure to xenoestrogens (Jacobson and Jacobson, 1996; Lephart et al., 2004; Ryan and Vandenbergh, 2006; Schantz and Widholm, 2001; Wisniewski et al., 2005), the significance and environmental relevance of these studies for human and wildlife have been questioned because of methodological and conceptual issues (Rice et al., 2003; Safe, 2005; Zala and Penn, 2004). The use of relatively high doses given via injections make difficult to predict effects of low doses on natural populations exposed through contaminated water and food. In fact, some authors have wondered in the past whether the experimental effects of estrogen on cognitive behavior are pharmacological in nature (Luine et al., 1998). A major criticism concerns the generalization of results from studies on specific compounds, because non-estrogenic, additional toxic properties of xenoestrogens may act as confounding variables (Fisher, 2004; Schantz and Widholm, 2001; Zala and Penn, 2004).

In this study, we addressed these issues by simulating an environmental exposure to 17α-ethynylestradiol (EE2), a pure, synthetic estrogen that is the main component of the anticonceptional pill. Because of its large use, EE2 is found in polluted

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surface waters worldwide (Nash et al., 2004). Previous work has shown that EE2 has an estrogenic potency similar to that of the natural hormone 17β-estradiol (Folmar et al., 2002). Pharmacological doses of EE2 have been used to study estrogenic effects on development (Arabo et al., 2005; Dugard et al., 2001; Sawaki et al., 2003a,b) or as positive control in studies on environmental estrogens (Carr et al., 2003; Della Seta et al., 2006). A recent study on mice reported effects of relatively high doses of EE2 (5 μg/kg/day) on mice behavior, with a masculinization of both short-term spatial memory and anxiety (Ryan and Vandenbergh, 2006). So far, no effects of EE2 at true environmentally relevant doses have been reported. In the present study, male rats were exposed to low doses throughout development with non-invasive methods. One group received a very low dose of EE2 (4 ng/kg/day) which represents concentrations actually measured in contaminated European and US streams (Nash et al., 2004) and is 1000 to 10,000 times lower than the doses used in previous studies (Arabo et al., 2005; Dugard et al., 2001; Ryan and Vandenbergh, 2006; Sawaki et al., 2003a,b). Another group was given a higher dose (400 ng/kg/day) which corresponds to that received by women who take the anticonceptional pill. The animals were exposed to EE2 during embryonal and postnatal development via the placenta and the milk by giving the chemical orally to their mothers during gestation and lactation. After weaning and until puberty, EE2 was given orally to the experimental animals. At 5 months of age the males were tested with a Morris water maze protocol, a powerful tool to study spatial memory in rats (D'Hooge and De Deyn, 2001; Morris, 1984). Spatial memory is sensitive to estrogen in a variety of species including humans (reviewed by Lephart et al., 2004) and is therefore an ideal system to study the effects of environmental estrogens on cognitive behaviors. We found that male rats exposed to EE2 learned the position of the hidden escape platform faster than controls. Our study shows that a true environmental-like exposure to a 'pure' xenoestrogen during development alters spatial memory abilities in rats.

Materials and methods

Animals and treatment

Thirty-six Sprague-Dawley male rats were used, which were born and raised in the Physiology Department, University of Siena. They were the offspring of 36 females and 20 males purchased from Harlan Italy. Starting from 5 days before pairing the mothers had been trained to drink from a pipette the vehicle (peanut oil, Sigma-Aldrich), a non-stressful treatment protocol. From gestation day 5 to weaning of the pups at postnatal day 21 (PND 21) the mothers received orally 4 ng/kg/day (EEL, $N=12$) or 400 ng/kg/day (EEH, $N=12$) of 17α-ethinylestradiol (EE2, Sigma-Aldrich), or the vehicle alone (OIL, $N=12$). EE2 is passed to the fetus via the placenta and the milk (Landrigan et al., 2002; Slikker et al., 1982). The dose received by the EEL group represents concentrations actually measured in contaminated European and US surface waters, where EE2 is one of the most common hormonally active pollutants (Nash et al., 2004). The EEH dose is equivalent to that of most anticonceptional estrogenic or estrogen plus progestin pills. At PND 2 we weighed the pups and one operator measured the anogenital distance, an index of morphological sex differentiation. The litters were then reduced to 5 male and 5 female pups. Body mass was measured again at PND 7, 14, 22 and 32. The young rats were separated from the mother at weaning (PND 22), and received the treatment orally until puberty (PND 32). Thus, experimental rats received the treatment indirectly through the placenta and the milk from conception to weaning, and directly until puberty. On PND 32, juveniles were randomly assigned to unisexual groups of 4 so that no cage contained siblings and all animals in a cage had received the same treatment. Only one male per litter was used for the present study, therefore the final sample size was $N=12$ for all groups, i.e. 3 cages of 4 unrelated males per treatment. Breeders and experimental rats were housed in Plexiglas cages (Tecniplast, Italy, 60×37×20) with metal tops and a sawdust bedding at 21±1 °C, with a relative humidity of 60±10% and a 12 h light/12 h dark cycle (lights off: 07:30). Water and food (Harlan Teklad rat chow) were available *ad libitum*. Experimental procedures and animal care adhered to EU and institutional guidelines.

Behavioral testing

Adult rats were tested with a Morris water maze protocol at 6 months of age, i.e. 5 months after the end of treatment. Until testing the animals had remained in the groups of 4 formed at PND 32. The tests were performed during the dark phase (09:00–17:00) under dim red light combined with low indirect white light. A circular tank with a diameter of 180 cm and a circular escape platform of 12 cm was placed in a 5×7 m^2 room with several extra maze cues. A videocamera placed above the tank was used to videorecord all tests. The water was kept at 25±1 °C and was made opaque with skimmed milk. The animals were tested with a protocol that was completed in one day. The hidden platform acquisition training was carried out in two series of 8 trials, with a 4-h interval between the beginning of the first series and the beginning of the second one, during which the animals were returned to their cage group. The starting position was pseudorandomized and the platform was 2 cm under the water level and remained always in the same quadrant during all 16 trials. The rats were released in the water with their head pointing to the tank wall. After they reached the submersed platform, they were allowed to rest on it for 15 s, after which they were returned to a warm box for 60 s until the following trial. If a rat did not reach the platform within 60 s, it was guided to it by the observer. For each trial, we measured the latency to reach the platform and the path length. The latter was measured on drawings of the path followed by the animals traced by one observer during the test, which were later compared with videorecordings. The last hidden platform trial was immediately followed by a probe trial without platform (60 s). In this test, we measured the time spent by the animal in each quadrant using the software Observer and the videotapes. After the probe trial, we performed a cue test in which the platform, raised 2 cm above the water level, was moved between quadrants in a pseudorandomized order in 8 trials, whereas the starting position was fixed. Between trials the rats were kept in a warm box for 60 s. For each trial, we measured the latency to reach the platform and the path length as described for the place test above.

Statistical analysis

The escape latency and swimming path length were compared between groups and blocks (mean of 4 consecutive trials) with a two-way repeated-measure ANOVA, with the treatment as between-subjects factor and the blocks as within-subjects factor. For the probe test, the analysis was performed with the treatment as between-subjects factor and the maze quadrant as within-subjects factor. Post-hoc comparisons were performed with a Tukey's Honest Significant Difference test. Data were tested with a Mauchly's Test of Sphericity prior to ANOVA. All statistical analyses were done with SPSS for Windows 11.0.1.

Results

Survival and morphological parameters

There was no evident effect of the treatment on survival and physiological parameters. There was no difference between treatments for the mean pup body mass, total litter mass, anogenital distance, and number of pups, both for males and females analyzed separately or together (Table 1). We also found

Table 1
Morphological parameters of pups measured at PND 2

	Sex	OIL (N=12)	EEL (N=12)	EEH (N=12)	F	P
Mean pup mass (g)	M	6.40±0.12	6.60±0.20	6.56±0.13	0.49	0.61
	F	6.04±0.09	6.19±0.15	6.14±0.15	0.36	0.70
	M+F	6.21±0.11	6.39±0.17	6.36±0.13	0.51	0.61
Total litter mass (g)	M	45.03±3.75	43.96±3.44	40.03±3.09	0.59	0.56
	F	40.74±3.19	42.98±2.08	41.20±4.33	0.13	0.88
	M+F	85.78±4.84	86.94±3.18	81.23±6.02	0.39	0.68
Anogenital distance (mm)	M	3.79±0.08	4.09±0.17	4.09±0.13	1.74	0.19
	F	1.31±0.04	1.41±0.06	1.40±0.10	0.54	0.59
	M+F	2.58±0.09	2.71±0.12	2.71±0.13	0.40	0.68
Number of live pups	M	7.00±0.52	6.75±0.60	6.17±0.52	0.60	0.55
	F	6.75±0.54	7.00±0.43	6.75±0.70	0.07	0.94
	M+F	13.75±0.66	13.75±0.70	12.92±1.02	0.35	0.70

Data shown (means±SEM) were calculated on litter means. The last two columns report the result of a one-way ANOVA (F) and relative significance (P). No significant effect of the treatment was found for any of the measured variables.

no effect of the treatment on the growth rate of the experimental males from PND 2 until they reached puberty at PND 32 (Fig. 1).

Morris water maze

Males exposed to both doses of EE2 performed better than controls in a protocol where they had to memorize the position of a hidden, submerged platform, showing a faster acquisition of the spatial information (Figs. 2a, b). In the hidden platform training, the analysis revealed a significant effect of the block for the latency and the path length ($F_{3,99}$=56.81 and $F_{3,99}$=76.38, $P<0.0001$), a significant effect of the treatment for the latency ($F_{2,33}$=3.38, P=0.046) and a marginally significant effect of the treatment for the path length ($F_{2,33}$=3.04, P=0.061). The largest differences between group means were found at block 2 (trials 5–8) and 3 (trials 9–12). Therefore, we performed a two-way repeated-measures ANOVA limited to these two blocks. In the second and third block of training trials, animals exposed to either 400 ng EE2/kg/day (EEH) or 4 ng EE2/kg/day (EEL) during development remembered the position of the platform better than control animals (OIL), as shown by the shorter latency to reach the platform (two-way repeated-measures ANOVA, $F_{2,33}$=5.359, P=0.01; Tukey's post-hoc tests: EEL vs. OIL, P=0.031; EEH vs. OIL, P=0.015; EEL vs. EEH, P=0.95; Fig. 2a) and the shorter swimming path length ($F_{2,33}$=5.911, P=0.006; EEL vs. OIL, P=0.017; EEH vs. OIL, P=0.012; EEL vs. EEH, P=0.99; Fig. 2b). The treatment affected specifically the speed at which the animals learned the position of the submerged platform:

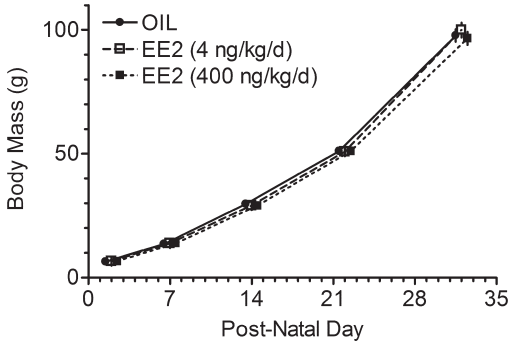

Fig. 1. Body mass of male rat pups exposed to 4 ng/kg/day or 400 ng/kg/day of the synthetic estrogen 17α-ethynylestradiol (EE2) during development. The animals had been exposed to EE2 in utero and continued to receive it via milk until weaning (PND 21) and then directly until they reached puberty at PND 32. There was no effect of the treatment on pups growth.

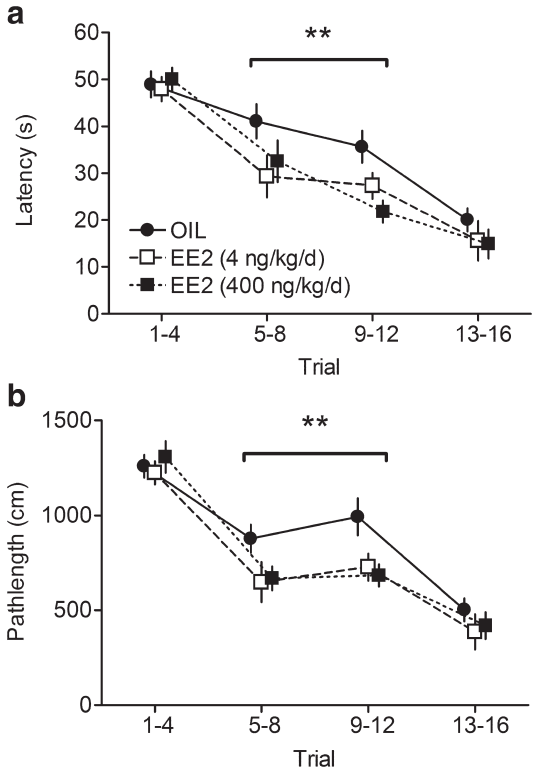

Fig. 2. Male rats exposed to 4 ng/kg/day or 400 ng/kg/day of EE2 during development show improved spatial memory abilities in adulthood. In a Morris Water Maze acquisition protocol, both EEL and EEH animals learned the position of a submerged, hidden escape platform faster than controls: in the second and third block of trials, they found the hidden platform in a shorter time (a) and by swimming a shorter distance (b) compared to controls. Trials 9–16 were started 4 h after trials 1–8. Data are shown as means±S.E.M. **$P<0.01$ (a) and **$P<0.006$ (b), two-way repeated-measures ANOVA followed by Tukey's HSD post-hoc tests.

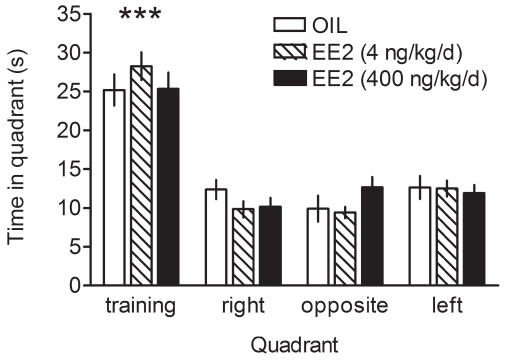

Fig. 3. Male rats exposed to 4 ng/kg/day or 400 ng/kg/day of EE2 during development do not show changes in spatial reference memory in adulthood. After training had been completed, the animals underwent a 60-s probe test during which the platform was removed from the training position. All animals spent more time in the training quadrant than in the other quadrants, however there was no effect of the treatment. ***$P < 0.001$, two-way repeated-measures ANOVA.

when the platform was removed in the probe test performed at the end of the training trials, the animals spent more time in the maze quadrant where the platform had been located during the training trials ($F_{3,96}=60.794$, $P<0.0001$) but there was no difference between treatment groups ($F_{2,32}=0.525$, $P=0.596$; Fig. 3). In addition, the treatment did not affect sensory or motor capacities because there was no difference between groups in a cue test where the escape platform was visible (latency: $F_{2,33}=0.088$, $P=0.916$; path length: $F_{2,33}=0.168$, $P=0.846$; Fig. 4).

Discussion

Xenoestrogen and cognitive functions

We found that an environmental-like exposure of male rats to the xenoestrogen ethynylestradiol during development enhances the acquisition of spatial information in a Morris water maze protocol. Our results provide a clear demonstration that exogenous estrogens entering the diet at actual environmental concentrations can affect mammalian cognitive functions. Effects of endocrine-disrupting compounds (EDCs) including xenoestrogen on animal and human cognitive functions have been described previously in both epidemiological and experimental studies (reviewed by Schantz and Widholm, 2001; Zala and Penn, 2004). Conclusions drawn from experimental studies, however, have been often questioned when the experimental design failed to replicate natural modalities of exposure, which for terrestrial vertebrates generally means long-term, low-dose, oral exposure through food and water. A second series of criticisms concerned the generalization of the effects of few substances as "xenoestrogenic" effects, i.e. due to the estrogenic action of the chemical. For this reason, two recent reviews conclude that to date there is no evidence that the effects of pollutants on cognition result directly from estrogenic action (Schantz and Widholm, 2001; Zala and Penn, 2004). In our study, both lines of criticism were addressed. First, we used a treatment protocol that did not differ in principle with an environmental exposure: The animals were treated during development by administering ethynylestradiol orally to the mothers, and after weaning the oral treatment was given directly to the experimental animals. Second, we used a compound that is a 'pure' estrogen, in that it is a synthetic derivate of natural estrogens, has been developed for oral administration, and has no known toxic, unspecific effects at the highest dose used in this study (Huezo, 1998).

Environmental effects on brain and behavioral differentiation

Previous studies had shown organizational effects of exogenous estrogen on spatial abilities (Carr et al., 2003; Isgor and Sengelaub, 1998; Ryan and Vandenbergh, 2006; Williams et al., 1990). We need to stress, however, that so far few studies (if any) had examined the effects on brain and behavioral differentiation of exogenous estrogen in an environmental-like context: *very low, oral doses throughout development*. Ryan and Vandenbergh (2006) used an environmental-like treatment with EE2 in an experiment with mice, however they used a dose of 4 μg/kg, i.e. respectively 10- and 1000-fold higher than our high and low doses. Thus, in the latter studies, the treatments were more pharmacological in nature and had major consequences on physiological parameters. Our lowest dose not only match the concentrations actually measured in polluted rivers and streams (Nash et al., 2004) but was also well within the physiological range of estrogen concentration in rat fetuses (Montano et al., 1995; vomSaal et al., 1997). Higher doses given for shorter periods of time can produce very different effects. For example, exposure during development to 0.015 μg/kg EE2 resulted in increased testicular and epididymal weights in adult rats whereas doses from 0.15 to 150 μg/kg had no such effects (Putz et al., 2001). In an experiment that replicated the treatment protocol of the present study, a 0.4 μg/kg dose caused a complete disruption of the estrus cycle, whereas the 0.004 μg/kg dose had little effects on the reproductive physiology (Fusani et al., in press). Large effects from small exposures are typical of hormonally active substances such as endocrine disruptors (reviewed by Welshons et al., 2003).

The direction of the effects suggests that estrogen exposure hypermasculinized the neural circuits underlying spatial

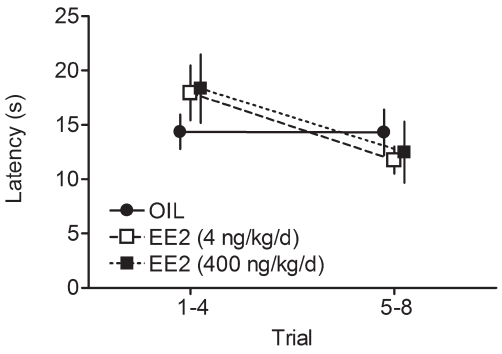

Fig. 4. Male rats exposed to 4 ng/kg/day or 400 ng/kg/day of EE2 during development do not differ from controls (OIL) in a Morris Water Maze cued task in which the platform was elevated 2 cm above the water.

memory abilities. Although the effects of the treatment were not studied on females, several observations support this interpretation. Both EE2-treated groups learned the position of the hidden platform faster than control animals, and remembered the position better after a 4-h pause. Thus, their spatial acquisition and short-term memory were enhanced by the treatment. The existence of a male advantage in spatial learning and memory tasks, shown by a series of classical studies (reviewed by Williams et al., 1990), has been object of debate, particularly after a study showing that pretraining (i.e. familiarization with the water maze) reduces or eliminates sexual differences (Perrot-Sinal et al., 1996). In addition, both male and female advantages were found, depending on the type of task and testing protocol (Dohanich, 2003). However, a recent meta analysis shows a consistent male advantage in hippocampal tests, which is particularly robust for Sprague-Dawley rats tested in the water maze (Jonasson, 2005). Postnatal androgen and/or estrogen treatment masculinize spatial abilities of female rats (Isgor and Sengelaub, 2003). Some of the effects of androgen such as testosterone on spatial abilities are probably mediated by its conversion into estrogen by the enzyme aromatase (Isgor and Sengelaub, 1998, 2003), and both aromatase and estrogen receptors are expressed in glial cells and neurons of the rat hippocampus (reviewed by Rune and Frotscher, 2005). The origin of the sexual differences in spatial abilities could lie partly in the transient elevation in the concentration of estrogen receptors in the perinatal period (MacLusky et al., 1979; O'Keefe and Handa, 1990). During development, estrogen masculinizes the volume of the CA1 layer and the CA1 soma size (Isgor and Sengelaub, 1998). Thus, although we do not provide evidence that the behavioral effects found in the present study are due to an amplification of the masculinizing processes described above, this seems to be a reasonable interpretation of our results. The fact that we did not find any similar effect for sexually dimorphic morphological variables such as the anogenital distance does not contrast with this view, because behavioral endpoints are often more sensitive to endocrine modulators than morphological ones (Dessi-Fulgheri et al., 2002; Farabollini et al., 2002). In addition, a treatment similar to the one used in the present study induces masculinization of sexual behavior in female rats (Della Seta et al., unpublished data). Nevertheless, we cannot rule out that other factors may have mediated the effects of the EE2 treatment. For example, estrogen can affect the HPA axis and corticosteroids are known to influence water maze performances (McEwen, 1999; Sandi, 2004).

Conclusions

Our study demonstrates that exposure to the estrogen 17α-ethynylestradiol during development alters permanently adult cognitive functions in rats. Because EE2 is a pure estrogen, the effects can be attributed unquestionably to the estrogenic action of the chemical. In addition, the modalities and the doses of the treatment did not differ substantially from an exposure through contaminated food and/or water. These findings show that environmental concentrations of estrogenic contaminants are sufficient to induce significant modifications of cognitive abilities in a mammalian species. Because estrogenic mechanisms are highly conserved within mammals and vertebrates in general, this study suggests that other species including humans can be affected by xenoestrogen contamination.

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