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Neonatal Treatment With Tamoxifen Causes Immediate Alterations of the Sexually Dimorphic Nucleus of the Preoptic Area and Medial Preoptic Area in Male Rats

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ABSTRACT Tamoxifen is an antiestrogen widely used for the treatment of breast cancer. Current evolutions in preventive strategies to include healthy premenopausal women warrant the study of its developmental toxicity. Perinatal treatment of male rodents with tamoxifen caused reproductive tract lesions and sexual behavior deficits similar to those induced by diethylstilbestrol (DES). Those abnormalities could originate, at least in part, from lesions of the hypothalamic-pituitary axis. The initial alterations caused by tamoxifen in the hypothalamic medial preoptic area (MPOA) and sexually dimorphic nucleus of the preoptic area (SDN-POA) were studied in 6-day-old male rat pups treated with 100 μg tamoxifen (group 1), 1 μg DES (group 2) or vehicle (group 3) from PN1 to 5. In situ hybridization was performed to analyze the expression of the GAP-43 gene, a marker of neuronal differentiation, and morphometry was used to study the neuronal density in the SDN-POA and MPOA and the volume and number of neurons in the SDN-POA. Tamoxifen reduced severely the volume and neuron numbers in the SDN-POA (46.1% and 47.8% of controls, respectively). The neuronal density of the MPOA was not modified. GAP-43 gene expression was decreased as demonstrated by a greater percentage of unlabeled neurons (grade 0) mirrored by a lesser percentage of intensely labeled ones (grade 2) in the SDN-POA and MPOA. In contrast to the effects of the antiestrogen, DES did not affect the above endpoints. These data indicated that developmental exposure of male rat pups to tamoxifen-induced immediate neuronal loss in one and altered differentiation in two hypothalamic areas crucial to reproduction. How those initial alterations contribute to the pathogenesis of the reproductive disorders observed in the adult male needs further investigation. *Teratology 56:220–228, 1997.* © 1997 Wiley-Liss, Inc.

Tamoxifen is a triphenylethylene antiestrogen widely used for the treatment of breast cancer in women and in clinical trials as a preventive agent for the same disease (Morrow and Jordan, '94; Jordan, '95a). Worldwide, approximately four to five million women are currently undergoing tamoxifen therapy (Jordan, '95b). Recently, following the evolution of therapeutic and preventive strategies, a significant proportion of tamoxifen patients have been of childbearing age (Vancutsem and Williams, '93; Clark, '93; Jordan, '95b), raising concerns about in utero exposure to the drug. Significantly, despite manufacturer's warnings, several pregnancies have been recorded during the course of tamoxifen treatment (Cullins et al., '94; Clark, '93).

The teratogenic potential of tamoxifen in humans is largely unknown. However, tamoxifen is structurally related to the synthetic estrogen diethylstilbestrol (DES), a compound notorious as a developmental toxicant causing reproductive abnormalities (Gill, '88; Newbold and McLachlan, '88). Clinically, in utero exposure of male fetuses to DES has been linked in men to increased frequencies of primary sterility, testicular hypoplasia, and semen pathologies (for review, see Gill, '88). These deficits were reproduced experimentally in male rodents exposed perinatally. In this model, tamoxifen and DES induced strikingly similar lesions of the genital tract, including aspermia, hypogonadism, and cryptorchidism (for review, see Newbold and McLachlan, '88; Iguchi and Ohta, '95). Additionally, in utero exposure of men to exogenous estrogens had been previously associated with a decrease in masculinity (Yalom et al., '73) and the effects of DES on the development of psychosexual behavior in men have been a source of concern (Gill, '88; Wilcox et al., '95). In adult rats, neonatal tamoxifen and DES treatment decreased male sexual behavior (Döhler, '91; Csaba et al., '86). Sexual behavior in this species has been closely associated with the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA), a cluster of neurons located in the medial preoptic area (MPOA)

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(Jacobson and Gorski, '81; Döhler, '91). The SDN-POA is three to five times larger in adult male rats than in females, and its size in the adult is largely determined by the hormonal environment of the neonate (MacLusky and Naftolin, '81; Döhler, '91). Animal data and the increased risk of in utero exposure to tamoxifen recently prompted the National Toxicology Program to focus on the developmental toxicity of tamoxifen.

The teratogenic effects of DES are thought to result, at least in part, from lesions incurred by the developing hypothalamic-pituitary axis (Gill, '88). Indeed, the normal sexual differentiation of the brain is under estrogenic control during a critical developmental period. This period takes place perinatally in rodents (MacLusky and Naftolin, '81) and could start during the first trimester of gestation in humans (Kaplan et al., '76). It is well demonstrated that neonatal exposure of male rodents to exogenous estrogens leads to abnormal development of the hypothalamic-pituitary axis, emphasizing the sensitivity of the developing neuroendocrine system to hormonal imbalance (Pinilla et al., '95). Accordingly, decreases in luteinizing hormone releasing hormone (LHRH) preoptic area (POA) content and in luteinizing hormone (LH) basal secretion were observed in adult male rats after neonatal exposure to estradiol and DES, respectively (Elkind-Hirsch et al., '84; Register et al., '95). In the developing POA, axogenesis, a step necessary to neuronal differentiation, is modulated by steroids (Toran-Allerand, '91). The gene expression of GAP-43, a protein crucial to axogenesis during brain development (Meiri et al., '86), is estrogen responsive (Shugrue and Dorsa, '93; Shugrue and Dorsa, '94) and mirrors neuronal differentiation (Sumantran and Feldman, '93; Zawia and Harry, '96). Preliminary evidence demonstrated that DES inhibits growth cone extension in vitro, a measure of axogenesis (Janevski et al., '93).

In a first step to study the alterations of the SDN-POA and MPOA caused by tamoxifen in the male and compare them to those induced by DES, we have characterized the early (postnatal day 6 [PN6]) changes in neuron numbers and differentiation occurring in the SDN-POA and MPOA of male Sprague Dawley rats treated neonatally with tamoxifen or DES from PN1 to PN5. Specifically, at PN6, we have (1) measured the volume and neuron number in the SDN-POA; (2) determined the neuronal density of the SDN-POA and MPOA; and (3) studied, in the same regions, the gene expression of GAP-43 in individual neurons by in situ hybridization (ISH) using digoxigenin-labeled riboprobes.

MATERIALS AND METHODS

Animals

Timed-pregnant Sprague Dawley rats were obtained at postconception day 14 from Harlan Sprague Dawley (Indianapolis, IN) and housed individually in a temperature-, light-, and humidity-controlled room with free access to water and laboratory rodent chow (PMI Feeds, St Louis, MO). The rats were randomly allocated to three experimental groups and checked twice daily to determine the time of delivery. On the day of birth (PN0), the pups were weighed, their sex determined by measuring the anogenital distance and the litters were culled to 10 pups each. All neonatal male pups were given daily subcutaneous injections of 100 µg tamoxifen (group 1) or 1 µg DES (group 2) in 50 µl peanut oil from PN1 to 5 according to well-established protocols (for review, see Iguchi and Ohta, '95). Control animals (group 3) received equal volume of vehicle. Injection sites were immediately covered with Vetbond™ (3M Animal Care Products, St. Paul, MN) to prevent seepage. Twenty-four hours after the last injection (PN6), the pups were euthanized by CO_2 overdose, weighed and the brain and both testes carefully dissected and weighed. The brains and testes of 6 pups per group were fixed overnight in 10% neutral buffered formalin. The following morning, the brains were placed in a stainless steel brain slicer (RBM-2000C, Activation Systems, Warren, MI) and coronal sections made rostrally to the optic chiasma and at the level of the mammillary bodies. The brain samples containing the hypothalamic POA and the testes were then short-processed for routine paraffin embedding. Testes were stained with hematoxylin and eosin (H&E) and examined under light microscopy.

Morphological analysis of the SDN-POA and MPOA

Serial 5-µm coronal sections were made through the POA and every tenth section stained with cresyl violet acetate, examined by light microscopy on an Olympus AH-2 microscope and captured at the same magnification (100×) through a high-resolution 3CCD DKC-5000 digital camera. All analyses were made using NIH-Image 1.60. Size calibration of the sections was performed using images of a stage micrometer captured during the same session. The sections were number coded and analyzed in random order by an investigator blind to the treatment the animals received. On each section, the boundaries of a vertical rectangle (450 µm × 700 µm) were drawn immediately besides the third ventricle to encompass the SDN-POA, the entire medial preoptic nucleus (MPN), and included a laterocentral and laterodorsal margin of surrounding MPOA. The area delimited by the rectangle was named MPOA. Whenever present, the SDN-POA was identified as an intensely stained cellular region, dorsal to the optic chiasma, immediately lateral to the third ventricle, and equidistant from the top and infundibulum of the latter. The contour of the SDN-POA was drawn at a magnification of 100×, the borders corrected slightly at a magnification of 200× and the area measured. An error of 7.2% was estimated by measuring several sections twice. The volume of the SDN-POA was calculated by summing the serial cross-sectional areas and multiplying them by the thickness of the sections and the interval between each section. At 300× magnification,

morphologically identifiable neurons were counted within the SDN-POA and MPOA. Cell counts were corrected using the Abercrombie method (Abercrombie, '46). A counting error of 5.5% was estimated by counting several sections twice. The volume of the SDN-POA was subtracted from that of the MPOA, and neuron densities for each region were determined by dividing the corrected number of neurons by the calculated volumes of the SDN-POA and MPOA.

In situ hybridization for GAP-43 mRNA

The 800-base pair (bp) coding region of the GAP-43 rat cDNA inserted in a pBluescript SK+ plasmid (generous gift from Dr. Dorsa, University of Washington, Seattle, WA) (Shugrue and Dorsa, '94) was used to generate sense and antisense complementary RNA probes. After linearization of the template with *Hind*III or *Eco*RI, labeled antisense transcripts, complementary to mRNA, and sense riboprobes were synthesized for in situ hybridization (ISH) by in vitro transcription with, respectively, T3 or T7 RNA polymerase using a digoxigenin RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Direct immunological detection was used to compare the labeling efficiency of both riboprobes to that of serial dilutions of the digoxigenin-labeled control RNA supplied by the manufacturer. The sensitivity of the method allowed for the detection of 0.1–1 pg of homologous sequences.

Brain sections corresponding to map 20 of Swanson's atlas (Swanson, '92) were deparaffinized with xylene and rehydrated through a series of graded ethanol washes to distilled water. Prehybridization treatments included digestion with 5 µg/ml proteinase K at 37°C for 30 min, rinsing twice for 5 min each in 10 mM Tris–HCl (pH 7.5), 0.3 M NaCl, refixation in 4% neutral buffered paraformaldehyde at room temperature for 5 min, and acetylation with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The slides were prehybridized at 45°C for 4 hr in 50% formamide, 1× Denhardt's (0.02% bovine serum albumin [BSA], 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400), and 200 µg/ml *Escherichia coli* tRNA. Hybridization was carried out overnight in a humidified chamber at 45°C with approximately 400 ng/slide of T3 or T7 riboprobe in 50% formamide, 1× Denhardt's, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.5), 0.5 mM EDTA and 500 µg/ml *E. coli* tRNA. Following hybridization, the slides were washed twice at 45°C for 30 min each in 2× SSC, 0.1% Triton X-100, 1 mM EDTA, and 30 min in 0.1× SSC, 0.1% Triton X-100, 1 mM EDTA. Unhybridized single-stranded RNA probes were digested with 60 µg/ml RNase A, 10 U/ml RNase T1 in 10 mM Tris–HCl (pH 7.5), and 0.3 M NaCl for 40 min. The detection of the digoxigenin-labeled nucleic acids was carried out using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The slides were rinsed in 100 mM Tris–HCl (pH 7.5), 0.15 M NaCl for 1 min and blocked in 1% blocking reagent, 100 mM Tris–HCl (pH 7.5), 0.15 M NaCl for 1 hr. The anti-digoxigenin antibody (1/500 in 100 mM Tris–HCl (pH 7.5), 0.15 M NaCl) was applied for 1 hr in a humidified chamber. The slides were then washed twice in 100 mM Tris–HCl (pH 7.5), 0.15 M NaCl for 15 min each and equilibrated for 10 min in 100 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 50 mM $MgCl_2$ and revealed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as color substrate. The slides were kept in the dark and the reaction monitored under a microscope. The intensity of labeling in the cerebral cortex was used as a reference since tamoxifen was previously shown not to modify GAP-43 gene expression in this region (Shugrue and Dorsa, '94). The reaction was stopped in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA for 5 min at room temperature when the staining of laminae II and III of the cerebral cortex appeared similar. Control slides included (1) rat uterus (unrelated tissue), (2) omission of the digoxigenin-labeled probe, (3) serial sections processed with similarly labeled T7 sense riboprobe, as well as (4) RNase A treatment (100 µg/ml) before hybridization with the T3 antisense riboprobe. Sharp, finely granular, brownish purple intracytoplasmic deposits were interpreted as positive signal. Per section, approximately 250–300 neurons of the SDN-POA and MPOA were graded individually under a light microscope at a magnification of 400× according to the presence, intensity and homogeneity of a brownish purple precipitate in their cytoplasm. Grade 0 corresponded to the absence of cytoplasmic labeling, grade 1 to the presence of a light to moderate and/or nonhomogeneous precipitate, and grade 2 to the presence of a dark, homogeneous precipitate. A grading error of 8.2% was estimated by grading several sections twice.

Statistical analysis

Differences between groups were evaluated by a one-way analysis of variance (ANOVA) followed when appropriate by a Student's t-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Body and organ weights

The effects of neonatal treatment with tamoxifen or DES on body and organ weights of male pups are presented in Table 1. At PN6, the pups treated with tamoxifen or DES weighed respectively 12.5% ($P \leq 0.01$) and 7.5% ($P \leq 0.05$) less than controls. Absolute brain weight was not affected by either treatment, but relative brain weight (mg/g) was increased 12.2% and 6.9% after tamoxifen- and DES treatment, respectively ($P \leq 0.01$). Compared to controls and tamoxifen, DES induced a 14.3% and 14.0% decrease in absolute testes weight, respectively ($P \leq 0.01$). This decrease remained significant when corrected for differences in body weight. In contrast, tamoxifen did not modify the absolute weight of the testes but significantly increased their relative weight to 112.0% of the control values ($P \leq 0.05$).

TABLE 1. Body and organ weights of 6-day-old male rat pups treated from postnatal day 1 to 5 with vehicle, 100 μg tamoxifen, or 1 μg diethylstilbestrol[1]

		Brain wt		Testes wt[2]	
Treatment	Body wt (g)	Absolute (mg)	Relative (mg/g)	Absolute (mg)	Relative (mg/g)
Control	14.7 ± 0.3	596.3 ± 9.7	40.8 ± 0.8	29.4 ± 0.7	2.0 ± 0.1
Tamoxifen	13.0 ± 0.4*	594.8 ± 10.0	45.8 ± 0.9*,‡	29.3 ± 0.5†	2.3 ± 0.1**,†
Diethylstilbestrol	13.7 ± 0.4**	594.6 ± 14.0	43.6 ± 0.5*	25.2 ± 0.9*	1.8 ± 0.1**

[1]Values represent means ± S.E.M. of n = 12 (control), n = 11 (tamoxifen) and n = 9 (diethylstilbestrol).
[2]Both testes were weighed together for each animal.
*Statistically different from controls ($P \leq 0.01$).
**Statistically different from controls ($P \leq 0.05$).
†Statistically different from diethylstilbestrol ($P \leq 0.01$).
‡Statistically different from diethylstilbestrol ($P \leq 0.05$).

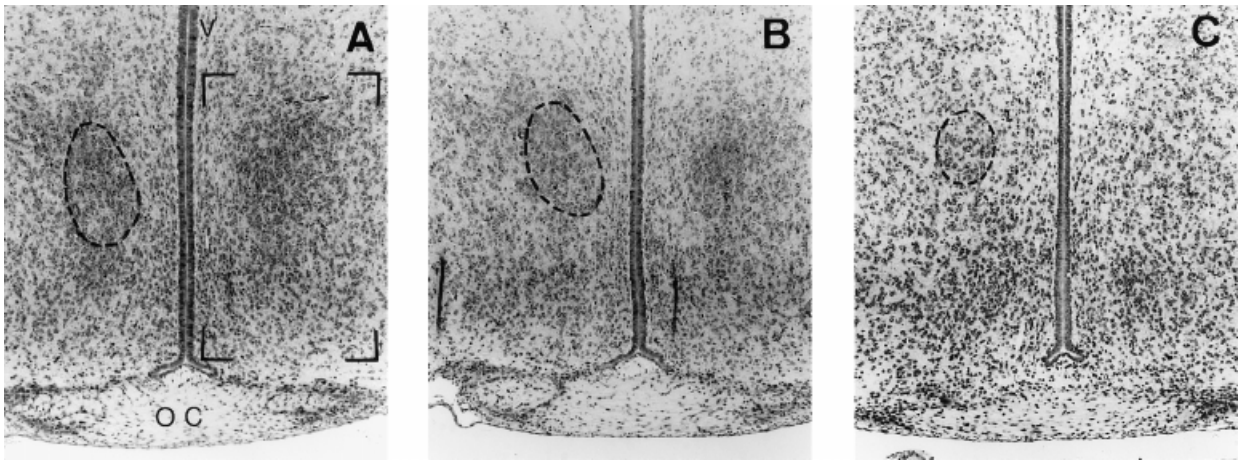

Fig. 1. Representative coronal sections through the medial preoptic area (MPOA) of 6-day-old male rat pups treated from postnatal day (PN) 1 to 5 with vehicle **(A)**, 100 μg tamoxifen **(B)**, or 1 μg diethylstilbestrol (DES) **(C)**. Corner brackets define the studied MPOA region and dotted lines the sexually dimorphic nucleus of the preoptic area (SDN-POA). OC, optic chiasma; V, third ventricle. Cresyl violet acetate staining. ×57.

Microscopic analysis

Histological examination of the testes did not reveal the presence of significant lesions (data not shown). The results of the microscopic analysis of the SDN-POA and MPOA are presented in Figures 1 and 2. Tamoxifen induced a sharp decrease both in the volume and the number of neurons in the SDN-POA, reaching values of, respectively, 46.1% and 47.8% of controls ($P \leq 0.01$). In contrast, DES did not modify the volume or neuron number in the SDN-POA. The neuronal densities, calculated as the number of neurons per volume, of the SDN-POA or the MPOA were not affected by either treatment. In all cases, the neuronal density of the MPOA remained, as expected, lower than that of the SDN-POA.

In situ hybridization analysis

Positive hybridization signal was observed as brownish-purple precipitates in the cytoplasm of neurons containing GAP-43 transcripts. No signal was detected in any of the negative controls, ascertaining the specificity of the T3 antisense riboprobe (Fig. 3). Excellent preservation of the cellular morphology permitted the identification of individual cells and faint positive signal could be recognized allowing for grading of signal intensity because the background staining using riboprobes under stringent conditions was inexistent. As an internal control for every section, the ependymal cells remained unstained. The effects of neonatal treatment with tamoxifen and DES on GAP-43 gene expression in the SDN-POA and MPOA neurons are presented in Table 2. Within each treatment group, the mean percentage of neurons in each category of staining intensity was the same in both the SDN-POA and the MPOA, demonstrating the homogeneity of the GAP-43 expression within the entire region.

At PN6, tamoxifen administration decreased GAP-43 gene expression in neurons from both the SDN-POA and MPOA. Indeed, the examination of individual neurons revealed that the levels of GAP-43 transcripts

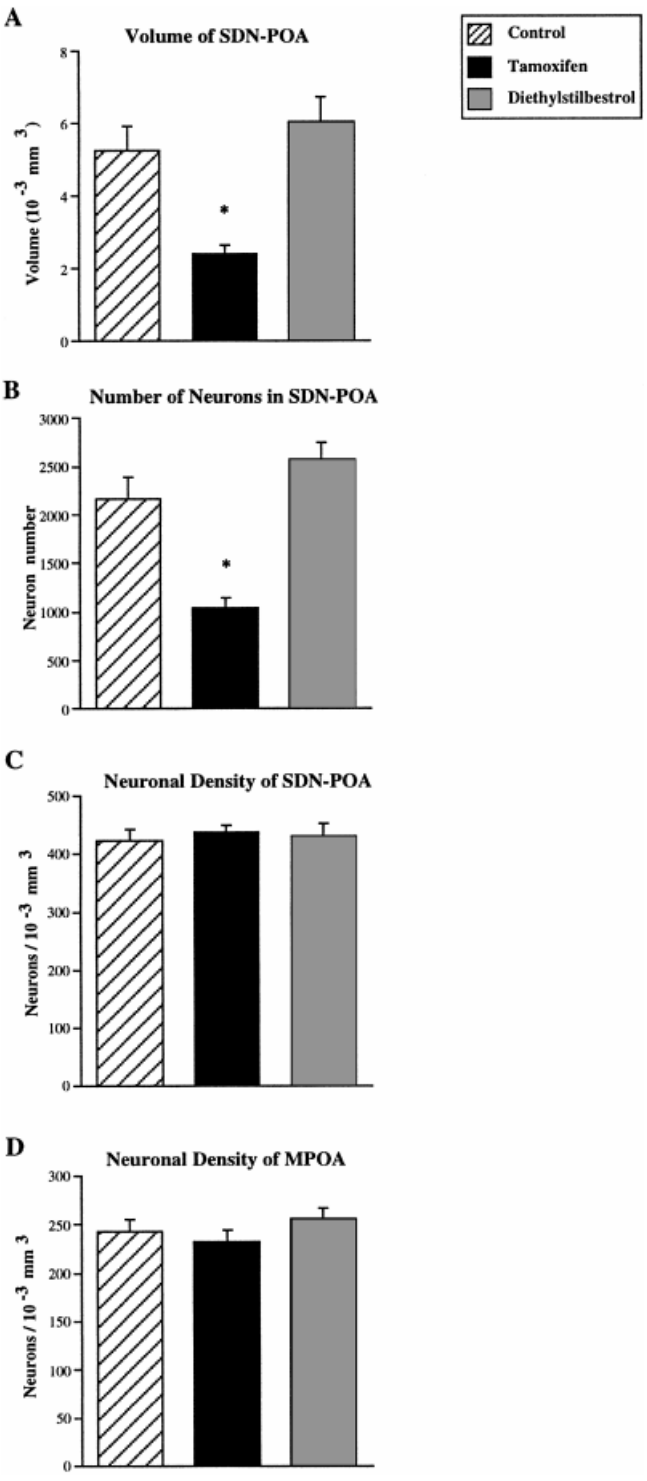

Fig. 2. Volume of the SDN-POA **(A)**, number of neurons in the SDN-POA **(B)**, neuronal density of the SDN-POA **(C)**, and neuronal density of the MPOA **(D)** in 6-day-old male rat pups treated from PN1 to 5 with vehicle, 100 µg tamoxifen or 1 µg DES. Bar graphs represent means ±S.E.M. *Statistically different from controls and diethylstilbestrol ($P \leq 0.01$).

were overall lower in tamoxifen- than in vehicle- and DES-treated pups. After tamoxifen treatment, 23.7% of SDN-POA and 24.9% of MPOA neurons were unlabeled (grade 0) while only 14.9% of SDN-POA and 20.9% of MPOA neurons were intensely labeled (grade 2). The percentage of grade 2 neurons in the SDN-POA were 40.2% and 44.1% in controls and DES-treated pups and that of the MPOA neurons 39.2% and 40.9%. Therefore, in the SDN-POA of the tamoxifen group, unlabeled neurons augmented 3.4-fold from control values ($P \leq 0.01$), and this change was mirrored by a comparable 2.7-fold decrease in the percentage of grade 2 neurons. DES did not modify GAP-43 gene expression by individual SDN-POA neurons.

In the MPOA, the percentage of unlabeled neurons was 8.9% and 9.3% in controls and DES-treated pups and increased to 24.9% after treatment with tamoxifen. This represented a 2.8- and 2.7-fold augmentation from controls and DES values, respectively ($P \leq 0.01$). Again, the percentage of intensely labeled neurons decreased from 39.2% and 40.9% in control and DES-treated groups, to 20.9% after tamoxifen treatment ($P \leq 0.01$), which reflected the increased percentage of unlabeled neurons after tamoxifen treatment.

DISCUSSION

In this study, we have characterized the initial alterations caused by tamoxifen in the SDN-POA and MPOA of neonatal male rats and compared these effects with those induced by DES. Our data demonstrated that, at PN6, major and specific tamoxifen-induced changes in neuron numbers and differentiation were already present in both regions while none were discernable after DES treatment. Male pups were given 100 µg tamoxifen from PN1 to PN5 according to a commonly used protocol (for review, see Iguchi and Ohta, '95)) and available human and rat pharmacokinetics data (Lien et al., '91; Robinson et al., '91). The chosen dose of DES respected the relative potency of the diethylstilbene and tamoxifen to displace [^{3}H] estradiol from the estrogen receptor (Skidmore et al., '72; Korach et al., '79).

At PN6, the volume of the SDN-POA decreased to 46.1% after tamoxifen treatment. This change represented most likely irreversible and nonprogressive tamoxifen-induced alterations as similar volume loss, ranging from 40% to 63% of controls, were noted in the SDN-POA of adult male rats treated perinatally with tamoxifen (Döhler et al., '84, '86). This is not surprising in view of the fact that the neurogenesis of the SDN-POA is completed by PN10 (Jacobson et al., '80) and underlines the importance of the initial alterations caused by tamoxifen in the maturing hypothalamus. Tamoxifen caused a 2.09-fold decrease in SDN-POA neuron numbers that paralleled closely the observed volume loss, demonstrating that the diminution in SDN-POA volume occurring after tamoxifen treatment reflected a decrease in neuron numbers. Compared to the male, the smaller volume of the female SDN-POA has been proposed to originate from a net cell loss due

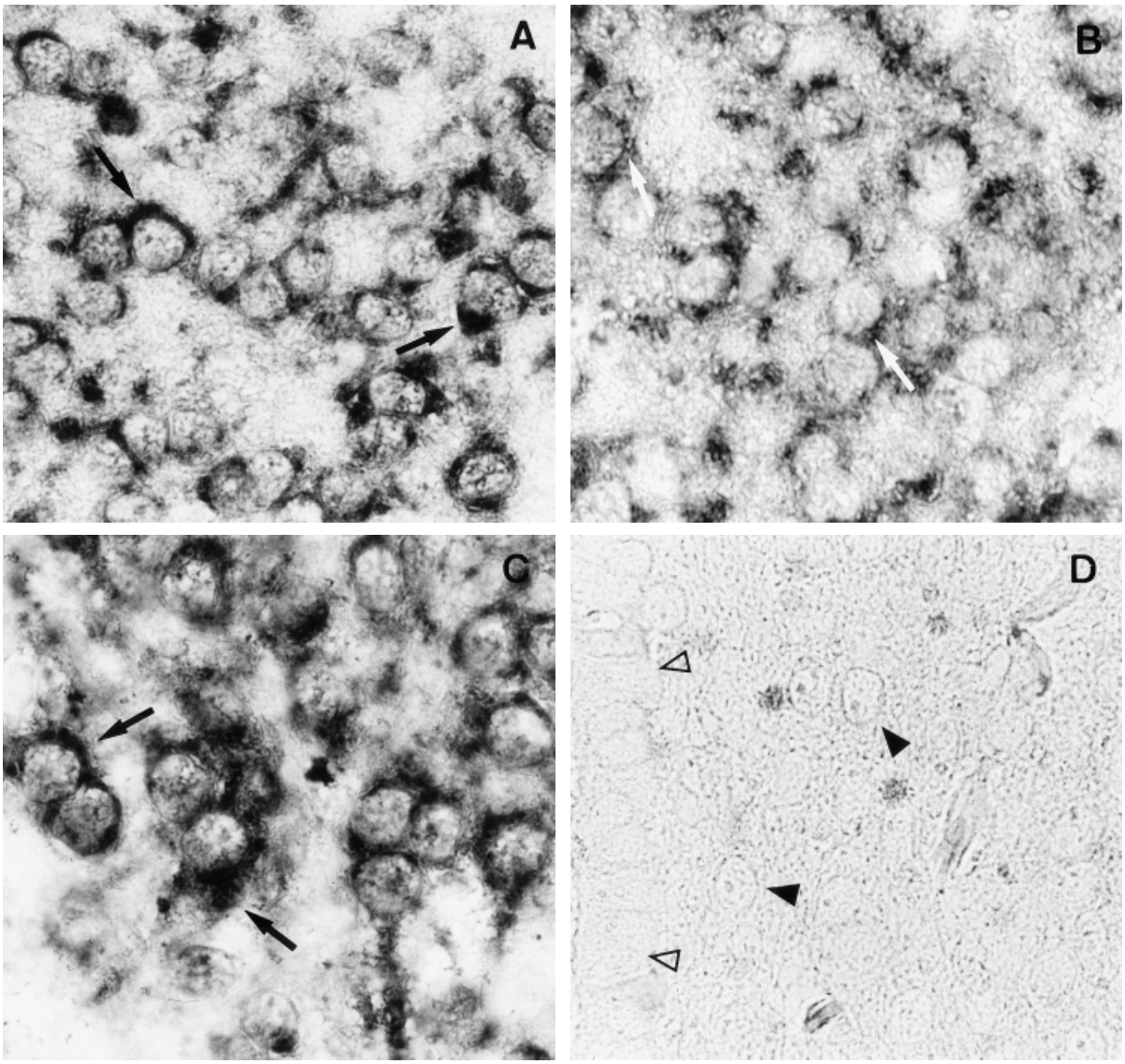

Fig. 3. In situ hybridization analysis of the MPOA of 6-day-old male rat pups treated from PN1 to 5 with vehicle **(A)**, 100 μg tamoxifen **(B),** or 1 μg DES **(C)**. **A–C:** Sections hybridized with a digoxigenin-labeled T3 antisense riboprobe complementary to GAP-43 transcripts. Labeling is indicated by the presence of dark intracytoplasmic deposits. Black arrows, intensely labeled neurons (grade 2); white arrows, moderately stained neurons (grade 1). **D:** Section from a vehicle-treated animal processed with sense noncomplementary T7 riboprobe. No labeling is present. Plain arrowheads, neuron nuclei. Open arrowheads, ependymal cells. No counterstaining. ×848.

to the lower estradiol levels in the developing female brain (Dodson et al., '88; Döhler, '91). Furthermore, the ability of tamoxifen to antagonize the estradiol-induced survival of embryonic hypothalamic neurons has been demonstrated in vitro in previous reports (Rasmussen et al., '90; Robbins et al., '90). To our knowledge, this is the first in vivo study that demonstrated the perinatal diminution of neuron numbers following tamoxifen treatment. Tamoxifen, acting as an antiestrogen, could have antagonized the survival of SDN-POA neurons in vivo, according to the hypothesis that estradiol encourages their maintenance. Alternatively, differences in cell migration, rather than in viability, cannot be excluded at this point.

In the rat, reduction in SDN-POA volume has been regularly associated with an inhibition of male sexual behavior (Döhler, '91). It is widely admitted that, during development, estrogens are necessary to masculinize the SDN-POA. Therefore, the volume decrease observed in the present study could be interpreted as a demasculinizing effect of tamoxifen, which would have repercussions on the sexual behavior of the adult. Interestingly, sexual behavior has been reported to be altered in male rats treated neonatally with tamoxifen (Döhler, '91). In contrast to tamoxifen, DES did not modify the volume of the SDN-POA of male pups at PN6 or affected the number of neurons in the SDN-POA. Those results are in agreement with the theory

TABLE 2. GAP-43 gene expression in the sexually dimorphic nucleus of the medial preoptic area and the medial preoptic area of 6-day-old male rat pups treated from postnatal day 1 to 5 with vehicle, 100 μg tamoxifen, or 1 μg diethylstilbestrol[1]

	Staining intensity expressed in percentage of neurons					
	SDN-POA			MPOA		
Treatment	Grade 0	Grade 1	Grade 2	Grade 0	Grade 1	Grade 2
Control	7.0 ± 1.7	52.9 ± 2.4	40.2 ± 3.1	8.9 ± 2.2	51.9 ± 2.7	39.2 ± 1.4
Tamoxifen	23.7 ± 2.7*	61.4 ± 4.5**	14.9 ± 4.9*	24.9 ± 1.1*	54.2 ± 5.7	20.9 ± 5.9*
DES	7.9 ± 1.7	48.0 ± 2.0	44.1 ± 2.8	9.3 ± 2.2	49.8 ± 1.9	40.9 ± 1.8

[1]Values represent means ± S.E.M. of n = 6 (control) and n = 5 (tamoxifen and diethylstilbestrol).
*Statistically different from controls and diethylstilbestrol ($P \leq 0.01$).
**Statistically different from diethylstilbestrol ($P \leq 0.05$).

that estrogenic stimulation of the SDN-POA reaches its maximum effect in the untreated male (Döhler, '91), which implies that a strong estrogen agonist, such as DES, does not modify the volume of the male SDN-POA. Surprisingly, while according to our study and previous reports (Tarttelin and Gorski, '88; Register et al., '95), DES administered perinatally fails to modify the morphology of the male SDN-POA, it has nevertheless been reported to induce demasculinization of sexual behavior in the adult rat (Csaba et al., '86). Taken together, those data support that demasculinization could also result from modifications of other brain centers (Albinsson et al., '96). The possibility, however, that undetected changes in the SDN-POA contribute to behavioral demasculinization must still be entertained. The MPOA studied in the present report corresponded approximately to the "surround" area previously described by Dodson et al. ('88). In the latter area, the neuronal density is about one third higher in 30-day-old male Sprague Dawley rats than in females of the same age. At PN6, tamoxifen did not decrease the neuronal density of the surrounding MPOA, suggesting that it had not demasculinized this area.

Tamoxifen inhibited GAP-43 mRNA expression in the SDN-POA and the MPOA of 6-day-old pups. By assessing GAP-43 gene expression in individual neurons, we were able to demonstrate that this tamoxifen-induced decrease resulted from both a diminution of the number of neurons in the SDN-POA and of the ability of some of the neurons in the SDN-POA and MPOA to express GAP-43 mRNA, as evidenced by their redistribution from grade 2 to grade 0. This latter finding is particularly interesting because it raises the possibility that a subset of the SDN-POA and MPOA neuronal population is selectively targeted by the antiestrogen. Dual labeling for specific neurotransmitters and GAP-43 should allow to determine whether one type of neurons is specifically affected. The SDN-POA and MPOA seemed to react differently to tamoxifen treatment. Indeed, GAP-43 gene expression decreased similarly in neuron populations from both areas while neuronal loss occurred only in the SDN-POA. Dodson et al. ('88) showed that a large number of neurons forming the SDN-POA divide and migrate late during development. As estrogens are thought necessary to maintain developing neurons, those late-arising neurons could account for the tamoxifen-induced neuronal losses in the SDN-POA. In contrast, the expression of GAP-43 gene would have been inhibited similarly in the more mature neurons of both regions.

GAP-43 gene expression has been previously used as a marker of neuronal differentiation in vitro (Robbins et al., '90; Sumantran and Feldman, '93) and in vivo (Zawia and Harry, '96) and its decrease suggested that tamoxifen could have altered the ability of neurons to differentiate and generate axons. Support for that theory is provided by the reduction, after neonatal tamoxifen treatment, of the density of synapses in the ventrolateral part of the hypothalamic ventromedial nucleus (Pozzo Miller and Aoki, '91). This nucleus receives axons from the center of the medial preoptic nucleus (MPNc), which is responsible for most of the sexual dimorphism of the SDN-POA (Simerly and Swanson, '88). The possibility of tamoxifen causing a premature peak in the mRNA expression of GAP-43 in the SDN-POA and MPOA cannot be excluded, however, and the sequence of alterations in GAP-43 mRNA expression caused by the triphenylethylene needs to be characterized in temporal studies to determine whether the decrease observed at PN6 reflects a downregulation or follows an ill-timed increased expression. Nevertheless, this altered differentiation could have repercussions on the mature neurons of the MPOA. LHRH neurons, which are particularly abundant in the MPOA, might potentially be affected as suggested by one report that chronic administration of tamoxifen caused changes in their morphology (Foster et al., '93). DES treatment did not modify GAP-43 gene expression in male rat pups. Similarly, the SDN-POA volume and neuron number as well as the MPOA neuronal density were unaffected by the strong estrogen agonist. Tamoxifen exerted opposite effects on those parameters, which suggested that it acted as an antiestrogen on the developing SDN-POA and MPOA. An indirect effect of tamoxifen through the inhibition of testosterone synthesis seemed unlikely because previous studies have demonstrated that testos-

terone concentrations were unchanged in 2- and 5-day-old male rat pups after perinatal administration of tamoxifen (Döhler et al., '84, '86).

Perinatal administration of DES and tamoxifen results in similar lesions of the genital tract and comparable decreases in male sexual behavior in rodents (Döhler, '91; Csaba et al., '86; for review, see Newbold and McLachlan, '88; Iguchi and Ohta, '95). The origin of these reproductive tract lesions is multifactorial and tought to involve the hypothalamic-pituitary axis (Gill, '88). Strikingly, the morphology of the SDN-POA and GAP-43 mRNA expression in the SDN-POA and MPOA of 6-day-old male rats was altered by tamoxifen, but not DES, suggesting that the two compounds might act through different mechanisms in these areas. Alternatively, as of yet undiscovered alterations of the SDN-POA and MPOA themselves or of other hypothalamic centers involved in the control of reproduction might uncover a mechanism of action common to both drugs.

The increases of relative testes weight after tamoxifen and of brain weight after tamoxifen and DES treatments resulted most likely indirectly from the changes in body weight. Indeed, tamoxifen- and DES-treated pups weighed less than controls. This decrease might be attributed to an estrogenic-like anorectic action (Wade and Heller, '93) or inhibition of growth hormone and insulin-like growth factor-I (IGF-I) secretions (Tannenbaum et al., '92; Huynh et al., '93). Systemic toxicity is considered unlikely as no clinical signs were observed after treatment or, to the extent of our knowledge, have been reported previously. No significant testes changes were observed at PN6 after tamoxifen treatment. Divergent results have been obtained in earlier studies, reflecting the complexity of the system. In one report, aspermia and a 5-fold decrease in testes weight were observed in 2-month-old Sprague Dawley rats treated perinatally, while another study reported no difference in 3-month-old Lewis rats treated with 100 µg tamoxifen at PN1 only (Döhler et al., '84, '86), suggesting that prolonged treatment might be an important factor. In contrast to tamoxifen, DES induced a 14.3% decrease in testes weight at PN6. Testicular lesions, including male hypogonadism, have been described in adult mice after neonatal DES treatment (Newbold and McLachlan, '88) but, to our knowledge, no alterations had been reported previously in neonates.

Preventive treatment with tamoxifen could span over a significant portion of the patient's reproductive years (Jordan, '95b), therefore increasing the probability of fetal exposure. Because tamoxifen can remain in human tissues for weeks, discontinuation of treatment as early as the first missed period would likely result in continued exposure through the first trimester of pregnancy (Iguchi and Ohta, '95). Significantly, exposure to DES during the first trimester of gestation was correlated with the occurrence of genital malignancies (Herbst et al., '71). Therefore, the clinical importance of tamoxifen and its widespread usage warrant investigation into its effects on the developing organism. Further studies should determine the potential contributions of tamoxifen-induced alterations in the SDN-POA and MPOA to the genesis of reproductive lesions in the adult.

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