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COMPARATIVE STUDY OF THE NEUROPROTECTIVE EFFECT OF DEHYDROEPIANDROSTERONE AND 17β-ESTRADIOL AGAINST 1-METHYL-4-PHENYLPYRIDIUM TOXICITY ON RAT STRIATUM

M. TOMAS-CAMARDIEL,[a] M. C. SANCHEZ-HIDALGO,[a] M. J. SANCHEZ DEL PINO,[b] A. NAVARRO,[b] A. MACHADO[a] and J. CANO[a]*

[a]Departamento de Bioquímica, Bromatologia y Toxicologia, Facultad de Farmacia, Universidad de Sevilla, C/Prof. Garcia Gonzalez s/n, Sevilla, Spain

[b]Departamento de Bioquímica, Facultad de Medicina, Universidad de Cadiz, Cadiz, Spain

Abstract—The effects of dehydroepiandrosterone, estradiol and testosterone on 1-methyl-4-phenylpyridium (MPP^+)-induced neurotoxicity of the nigrostriatal dopaminergic system were examined in rat. They were subjected to a unilateral intrastriatal infusion of the following treatment conditions: MPP^+ alone or co-injection of MPP^+ plus each hormone. Four days after injection, concentrations of dopamine and their metabolites were determined from the corpus striatum. To corroborate the neurochemical data an immunohistochemical analysis of tyrosine hydroxylase-immunoreactive fibers and acetylcholinesterase histochemistry in the striatum was performed. Moreover, we performed a dose–response study of the three hormones on the high-affinity dopamine transport system in rat striatal synaptosomes.

Rats co-injected within the striatum with MPP^+ and either dehydroepiandrosterone or estradiol had significantly greater concentrations of dopamine and less tyrosine hydroxylase-immunoreactive fibers and acetylcholinesterase fiber density loss compared with their respective controls. In addition, 4 days after injection, the brain was fixed and cut into coronal sections, and was immunostained with major histocompatibility complex class II antigens for activated microglia, and glial fibrillary acidic protein for activated astrocytes. Dehydroepiandrosterone also attenuated microglial cell activation. In contrast, testosterone showed reductions in dopamine concentrations similar to those obtained by MPP^+. The protective effect of dehydroepiandrosterone against the MPP^+ neurotoxic dopaminergic system may be produced by its partial prevention of MPP^+ inhibition of NADH oxidase activity, whereas the estradiol may function as a neuroprotectant by reducing the uptake of MPP^+ into dopaminergic neurons.

Our findings we suggest indicate that dehydroepiandrosterone and estradiol by a non-genomic effect may have an important modulatory action, capable of attenuating degeneration within the striatum, and in this way serve as neuroprotectants of the nigrostriatal dopaminergic system. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: testosterone, dopamine, glia, glial fibrillary acidic protein, OX-6.

Increasing data indicate that several steroids can modify neuronal vulnerability to different insults. Several epidemiological reports suggest that estrogen replacement therapy in postmenopausal women may be protective against Alzheimer's disease (Henderson, 1997) and even on specific neurological symptoms such as cognitive deficits (Schmidt et al., 1996). Estradiol may also protect the brain against neurodegenerative disease and it may play a critical role in preventing the decline in cognitive function associated with normal aging. 17β-Estradiol (E2) may exert trophic and protective effects by influencing the expression of genes that encode survival factors in the brain. It was first suggested that gonadal steroid hormones affect the nigrostriatal dopaminergic system when cases of chorea were reported during pregnancy and following the administration of oral contraceptives (Gamboa et al., 1971; Barber et al., 1976). Since it was first reported (Bedard et al., 1977) that estrogen administered orally could improve the condition of patients suffering from DOPA-induced dyskinesia, the biochemical and behavioral effects of these hormones on the female nigrostriatal dopaminergic system have been studied extensively (Van Hartesveldt and Joyce, 1986). It is interesting to note that the striatal concentration of E2 is second only to that found within the hypothalamus (Bixo et al., 1986), and is significantly higher than that reported for the rest of the brain.

The action of other hormones on the striatum has also been reported. High doses of testosterone increase striatal dopaminergic metabolism in male rats (Thiblin et al.,

*Corresponding author. Tel.: +34-95-4556751; fax: +34-95-4556752.

E-mail address: josefina@cica.es (J. Cano).

Abbreviations: 5-HIAA, 5-hydroxyindolacetic acid; 5-HT, serotonin; AChE, acetylcholinesterase; ANOVA, analysis of variance; DA, dopamine; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide; DOPAC, 3,4-dihydroxyphenylacetic acid; E2, 17β-estradiol; EDTA, ethylenediaminetetra-acetate; GFAP, glial fibrillary acidic protein; HVA, homovanillic acid; MHC-II, major histocompatibility complex class II antigens; MPP^+, 1-methyl-4-phenylpyridium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

1999). The human body contains an affluent adrenal steroid, dehydroepiandrosterone (DHEA), which is the precursor of both male and female sex hormones. DHEA and its sulfate derivate are the most abundant steroids produced by the human adrenal, but no receptors have been identified for these steroids. However, the cellular origin and pathway by which DHEA is synthesized in brain are not yet known. Recently, there is evidence that astrocytes and neurons express P450 17α-hydroxylase and synthesize DHEA from pregnenolone (Zwain and Yen, 1999). DHEA is the most abundant steroid in the blood of young adult humans. Levels in humans decline with age and during certain types of illness or stress. Therefore, a decrease of DHEA levels may contribute significantly to the increased vulnerability of the aged or stressed human brain to such damage. It has been reported that DHEA possesses antioxidant properties (Boccuzzi et al., 1997; Aragno et al., 1999). Likewise, DHEA protected mice from lipopolysaccharide and this protective effect appears to be mediated by mechanisms that block the toxin-induced production of tumor necrosis factor (TNF)-α and interleukin-1 (Ben-Nathan et al., 1999).

Several models for Parkinson's disease (PD) have been developed over the last 20 years. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a form of parkinsonism clinically indistinguishable from the common forms of the disease (Calne and Langston, 1983). Much evidence indicates that the sequence of steps leading to dopamine (DA) neuron degeneration begins with the oxidation of MPTP into its active metabolite 1-methyl-4-phenylpyridium (MPP^+) which is actively accumulated in dopaminergic neurons via the high-affinity DA uptake system (Hirsch et al., 1998). Intracerebral administration of MPP^+ was shown to be neurotoxic in rats (Heikkila et al., 1985), a species that is virtually insensitive to peripherally administered MPTP (Chiueh et al., 1984). Dluzen et al. (1996a,b) have demonstrated that estrogen protects against the parkinsonism-inducing drug MPTP in the striatum of female mice. Since the incidence of PD is higher in males than in females (Marder et al., 1996) along with that testosterone did not protect against MPTP (Dluzen, 1996) and that DHEA declines with age and during certain types of illness or stress (Kimonides et al., 1998; Mao and Barger, 1998), we have undertaken a comparative study among these three hormones. In this experiment, we investigated the potential protective activity of E2, testosterone and DHEA on the parkinsonism-inducing drug MPP^+ in the striatum of the rats.

We combined a neurochemical analysis of DA and its metabolites along with an immunohistochemical analysis of tyrosine hydroxylase (TH)-immunoreactive fibers and acetylcholinesterase (AChE) histochemistry in the striatum. Moreover, we performed a dose–response study of the three hormones on the high-affinity DA transport system in rat striatal synaptosomes.

In addition, we also performed an immunohistochemical study of reactive astroglia and microglia. Astrogliosis, as assessed by an increase in the astrocyte-localized protein, glial fibrillary acidic protein (GFAP), was used as a quantitative and independent biochemical marker of neural injury in the striatum. Microglia have similar properties to those of peripheral macrophages in many aspects and may be responsible for a number of immunological events in the brain (Ling and Wong, 1993; Gehrmann et al., 1995). Therefore, we have studied the microglial reaction using a specific monoclonal antibody that recognizes microglia and to determine their state of activation of major histocompatibility complex class II antigens, MHC-II antigen (OX-6) (Watanabe et al., 1999).

EXPERIMENTAL PROCEDURES

Animals and treatment

Female Wistar rats (200–250 g) born and housed in our laboratory were used for these studies. Rats were anaesthetized with chloral hydrate (400 mg/kg) i.p. and positioned in a stereotaxic apparatus (Kopf Instrument, Tujunga, CA, USA) to conform with the brain atlas of Paxinos and Watson (1986). The solutions were injected with a Hamilton syringe positioned at 0.5 mm caudal, 2.5 mm lateral and 6.5 mm ventral to bregma at a rate of 0.5 μl/min. Monastral Blue B suspension (Sigma, St. Louis, MO, USA) served as an inert tracer of the injection site. Animals received unilateral striatal injections using dimethyl sulfoxide (DMSO, 2 μl) as vehicle with all compounds. The animals received unilateral striatal injections containing (1) 2 μl of DMSO (Sigma); (2) 10 μg of E2, testosterone or DHEA (Sigma) in 2 μl of DMSO; (3) 1 μg of MPP^+ (RBI, Natick, MA, USA) in 2 μl of DMSO; (4) 10 μg of each hormone+1 μg of MPP^+ in 2 μl of DMSO. Animals were left for recovery and killed by decapitation 4 days after surgery. The brain was quickly removed and the striatum dissected out on a cold plate. The tissue was frozen at −80°C until used.

Experiments were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals. All efforts have been made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data, and to utilize alternatives to *in vivo* techniques, if appropriate. The Scientific Committee of the University of Seville approved the experiments carried out in the present study.

For each hormone used for intrastriatal injections animals were divided into two groups: six animals processed for quantification of DA 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and four animals processed for immunohistological evaluation.

Furthermore, we analyzed the effect of the local intrastriatal injection of E2 during the estrous cycle in the dopaminergic system. Four groups (one for each phase of the estrous cycle) of five rats were injected with E2 and E2+MPP^+ and processed for quantification of DA and their metabolites.

Measurement of catecholamines and their metabolites

Analyses were performed by high-performance liquid chromatography with electrochemical detection. A Merck L-6200 pump was used in conjunction with a glassy carbon electrode (Antec EC detector) set at 0.8 V (vs. Ag/AgCl reference electrode). A Merck Lichrocart cartridge (125×4 mm) column filled with Licrospher reverse-phase C18 5-μm material was used. The mobile phase consisted of a mixture of 100 mM formic acid, 0.36 mM octane sulfonic acid, 1.0 mM citric acid, 0.1 mM EDTA, 5.0% (v/v) acetonitrile and 0.25% (v/v) diethylamine adjusted to pH 3.1 with KOH and was thoroughly degassed. Analyses were performed in the isocratic mode, at a flow rate of 1 ml/min and at room temperature. Standards were prepared in 0.1 M perchloric acid/1 mM sodium bisulfite and stored at

4°C for up to 2 months. The detection limit of the assay was 50–100 pg/sample. Tissue was homogenized in 0.1 M perchloric acid containing 1 mM sodium bisulfite by ultrasonic disintegration over ice using a Labsonic 1510. Tissue was centrifuged at $12\,000 \times g$ for 15 min at 4°C and the supernatant was then filtered through a 0.2-μm filter. Concentrations in brain samples were calculated with the aid of (linear) calibration curves obtained after the injection of pure standards.

Statistical analysis

Results are typically expressed as means ± S.D. Observed mean differences were analyzed by one-way analysis of variance (ANOVA) followed by the parametric Tukey test.

Measurement of DA uptake

Rat synaptosomes were prepared and incubated in Krebs medium as described previously (Lapchak and Hefti, 1992). In brief, striatal tissue was homogenized in 0.32 M ice-cold sucrose (10 strokes with a ground glass homogenizer) followed by centrifugation in an Eppendorf microfuge (3400 r.p.m., 10 min) at 4°C. The supernatant was then collected and recentrifuged (14 000 r.p.m., 20 min) at 4°C. The pellet was collected and used as the P2 synaptosomal fraction. After resuspension with 100 μl of normal Krebs medium, 50 μl of this solution was preincubated at 37°C for 5 min with increasing doses of E2, DHEA and testosterone respectively (100 nm–30 mM) in 0.45 ml of normal Krebs medium containing pargyline (10 μM) and imipramine (10 μM). For [^{3}H]DA uptake, 2 μCi of [2,5,6-^{3}H]DA (12.5 Ci/mmol) was added to the solution containing the synaptosomes and incubated for another 5 min, a time allowing maximization of DA uptake (Schoemaker and Nickolson, 1983). Following the incubation, the synaptosomes were pelleted, solubilized and the content of [^{3}H]DA was determined by liquid scintillation counting. The protein content of striatal synaptosomes was determined using the procedure of Lowry et al. (1951).

Assuming that the uptake of DA into an intracellular compartment is a posterior event (in time) to the synaptic uptake we also measured the uptake of DA after 12 min of incubation in order to include the vesicular transporter in the measurement of DA uptake (Lui et al., 1992).

Measurement of NADH oxidase activity

Rats were killed by decapitation and brains without cerebellum were rapidly excised and homogenized in 0.23 M mannitol, 0.07 M sucrose, 1 mg/ml bovine serum albumin, 1 mM EDTA, 15 mM Tris–HCl, pH 7.3, at a ratio of 1 g of tissue/9 ml of homogenization medium in a Teflon/Potter homogenizer at 0–2°C. The homogenate was centrifuged at $700 \times g$ for 10 min and mitochondria precipitated by centrifugation at $8000 \times g$ for 10 min and washed in the same conditions. Mitochondrial suspensions were frozen and thawed three times and homogenized by passage through a tuberculin needle. MPP^+ and/or DHEA were added to submitochondrial particles. Proteins were determined by using the Folin phenol reagent (Lowry et al., 1951). NADH oxidase mitochondrial activities were assayed spectrophotometrically at 340 nm ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) in 100 mM phosphate buffer (pH 7.4) at 30°C in submitochondrial particles. Enzyme activity was expressed in nmol NAD/min/mg of protein.

Immunohistological evaluation: TH, GFAP and OX-6

Rats were perfused through the heart under deep anesthesia (chloral hydrate) with 150–200 ml of 4% paraformaldehyde in phosphate buffer, pH 7.4. The brains were removed, and then cryoprotected serially in sucrose in phosphate-buffered saline, pH 7.4; first in 10% sucrose for 24 h and then in 30% sucrose until sunk (2–5 days). The brains were then frozen in isopentane at −15°C, and 25-μm sections were cut on a cryostat and mounted in gelatin-coated slices.

Primary antibodies used were: mouse-derived anti-TH (Boehringer-Mannheim, Germany; 1:200), anti-GFAP (Chemicon International; 1:300), and mouse-derived OX-6 (Serotec, Oxford, UK; 1:200). OX-6 is directed against a monomorphic determinant of rat MHC-II antigens. All incubations and washes were in Tris-buffered saline (TBS), pH 7.4 unless otherwise noted. All work was done at room temperature. Sections were washed and then treated with 0.3% hydrogen peroxide in methanol for 30 min, washed again, and incubated in a solution containing TBS and 1% horse serum for 60 min in a humid chamber. Slices were drained and further incubated with the primary antibody in TBS containing 1% horse serum and 0.25% Triton X-100 for 24 h. Sections were then incubated for 2 h with biotinylated horse anti-mouse IgG (Vector, 1:200) followed by a second 1-h incubation with ExtrAvidin®–peroxidase solution (Sigma, 1:100). The antibody was diluted in TBS containing 0.25% Triton X-100, and its addition was preceded by three 10-min rinses in TBS. The peroxidase was visualized with a standard diaminobenzidine/hydrogen peroxidase chromogen reaction for 5 min.

To perform quantitative measurement of the lesion, the areas lacking TH-immunopositive terminals were drawn with the aid of a drawing tube at ×20 magnification, and the area was calculated using image analysis software (Visionlab; Biocom). Areas were measured in striatum of 10 alternative sections for each animal, corresponding to coronal sections where the lesion was maximal (from 1.1 to 0.1 mm anteroposterior to bregma).

Data are expressed in mm^2 as mean ± S.D. values of four animals for each treatment. The difference between areas was compared by paired two-tailed Student's *t*-test, comparing each treatment with the DMSO and the MPP^+ groups.

AChE histochemistry

AChE staining was chosen because it provides an indication of the extent of damage to fiber systems containing acetylcholine or monoamines (Butcher and Marchand, 1978; Emson et al., 1979). Sections were rinsed in sodium acetate buffer and incubated at room temperature for 45 min with 4 mM acetylthiocholine, 2 mM $CuSO_4$,10 mM glycine, 0.2 mM ethopropazine in 50 mM sodium acetate buffer. After washing they were exposed to 1.25% NaS, H_2O and 1% $AgNO_3$, dehydrated and mounted.

Identical quantitative measurements for areas lacking AChE staining with those described for areas lacking TH-immunoreactive terminals were performed.

RESULTS

Effect of estradiol, testosterone and DHEA on DA depletion induced by MPP^+ in rat striatum

Vehicle-injected animals. Animals injected with vehicle (DMSO) (1% Monastral Blue in DMSO) into the left striatum did not show significant change in the levels of DA, serotonin (5-HT) and their metabolites as compared with the contralateral side. The contralateral side of the striatum was used as control. We have previously reported that DA levels in the hemisphere contralateral to transection of the medial forebrain bundle did not differ from those measured in unoperated control animals or in sham-operated animals (Venero et al., 1997).

Levels of DA, DOPAC and HVA. We assumed that if massive DA loss after MPP^+ treatment is an index of dopaminergic cell disruption, the toxicity of MPP^+ could be measured by quantification of the remaining DA in the tissue. Levels of DA, DOPAC and HVA were mea-

Table 1. Concentrations of DA, serotonin and their metabolites in rat striatum 4 days after their respective treatments

Treatment	DA	DOPAC	HVA	5-HT	5-HIAA
Control	7583 ± 500[b]	825 ± 92	335 ± 32	611 ± 85	256 ± 128
DMSO	6957 ± 311[b]	809 ± 189	340 ± 82	524 ± 83	449 ± 94
MPP^+	5797 ± 500	698 ± 68	294 ± 69	521 ± 129	314 ± 141
E2	7215 ± 326[b]	835 ± 102	331 ± 41	565 ± 106	207 ± 74
E2+MPP^+	6771 ± 178[a,b]	806 ± 114	316 ± 19	595 ± 51	254 ± 114
DHEA	7274 ± 454[b]	513 ± 81[a]	282 ± 68	416 ± 143	306 ± 48
DHEA+MPP^+	6899 ± 104[b]	548 ± 80	384 ± 1	378 ± 128	655 ± 248[a,b]
T	5649 ± 688[a]	457 ± 86[a,b]	256 ± 86	437 ± 103	455 ± 34
T+MPP^+	5279 ± 555[a]	527 ± 77[a]	299 ± 113	431 ± 72	489 ± 20

Female Wistar rats were treated with 2 µl of DMSO (vehicle) and their respective drugs: MPP^+ and the hormones E2, DHEA and testosterone alone or with MPP^+. Numbers are expressed as ng/g of wet tissue, and are mean ± S.D. of six independent experiments. Statistical analysis: one-way ANOVA followed by Tukey's test; $P < 0.05$ (a: compared to control; b: compared to MPP^+). T, testosterone.

sured in lesioned striatum within 4 days after intrastriatal injection of either DMSO+MPP^+ or co-injection of MPP^+ and either E2, testosterone or DHEA. Intrastriatal injection of 2 µl of DMSO did not show significant changes in DA and their metabolites with respect to control striatum (Table 1). In lesioned rats, 4 days after striatal injection of MPP^+ (1 µg) in 2 µl of DMSO the decline in the concentration of DA was −25%, −16% for DOPAC and −14% for HVA. It is interesting to note that intrastriatal injection of MPP^+ (1 µg) in 2 µl of Ringer produced a decrease of DA −40.8%, DOPAC −27.9% and −24.4% for HVA (Vizuete et al., 1997). This smaller depletion with DMSO could be due to that DMSO may protect against MPP^+ toxicity through the inhibition of $^{\bullet}OH$ radical-mediated oxidative injury (Wu et al., 1994). Intrastriatal co-administration of MPP^++E2 partially attenuated the acute DA, DOPAC and HVA depleting effect induced by MPP^+. Co-administration of MPP^++DHEA partially attenuated the acute DA, HVA depleting effect induced by MPP^+ whereas the DOPAC decreased significantly with DHEA administration (Table 1). In contrast, testosterone alone produced a decrease of DA and DOPAC compared with control and with MPP^+. Co-injection of MPP^+ and testosterone produced DA concentrations that were even lower than MPP^+-treated animals (Table 1).

When levels of DA were measured along the four phases of the estrous cycle, no statistically significant differences were found, neither in the E2+MPP^+-treated animals for each phase nor MPP^+ ones (Table 2). However, another neurotoxin, methamphetamine, produced different neurotoxicity in the striatum as a function of the day of the estrous cycle (Yu and Liao, 2000). Even though this was obtained using systemic injections into mice.

Levels of 5-HT and 5-hydroxyindolacetic acid (5-HIAA). No statistically significant differences were obtained for striatal 5-HT and 5-HIAA concentrations among the control and treatment groups (Table 1). Except after co-injection DHEA and MPP^+ the 5-HIAA was significantly increased when it was compared with control and with MPP^+.

DA uptake in control and E2-, DHEA- and testosterone-treated rats: effects of intrastriatal injection of MPP^+

To further characterize the protective effect rendered by E2 and DHEA on MPP^+ depleting effect on DA levels, we measured the effect of increasing concentrations of E2, DHEA and testosterone on DA uptake in the P2 fraction. As seen in Fig. 1, in all cases DA uptake was highly inhibited in a dose-dependent manner. The resulting IC_{50} values were: E2 (12.5 µM), DHEA (52.5 µM) and testosterone (56.2 µM) (Fig. 1).

Effect of MPP^+ and DHEA on the NADH oxidase activity

In order to evaluate the effect of MPP^+, DHEA and the both compounds together submitochondrial particles were used to measure the NADH activity. The maximal activity found for these was 277 ± 6 nmol NAD/min/mg of protein. In these conditions we also tested the effect of MPP^+ and/or DHEA.

It is known that MPP^+ inhibits the mitochondria respiratory rate by acting on complex I of the respiratory chain (Singer et al., 1987; Bougria et al., 1995; Ramsay

Table 2. Concentrations of DA and its metabolites in rat striatum along the four phases of the estrous cycle 4 days after a single intrastriatal injection of the next treatments

Phase of cycle	DA	DOPAC	HVA
(A) E2+MPP^+			
Diestrous	8828 ± 240	921 ± 54	372 ± 31
Proestrous	9066 ± 548	973 ± 122	410 ± 52
Estrous	8614 ± 224	824 ± 89	334 ± 56
Metaestrous	9083 ± 766	1013 ± 102	370 ± 39
(B) MPP^+			
Diestrous	6497 ± 636	732 ± 116	325 ± 54
Proestrous	6692 ± 232	791 ± 83	367 ± 50
Estrous	6658 ± 370	676 ± 29	305 ± 31
Metaestrous	6750 ± 642	786 ± 53	360 ± 47

Female Wistar rats were treated with E2+MPP^+ or MPP^+ alone. Numbers are expressed as ng/g of wet tissue, and are mean ± S.D. of five independent experiments. No statistically significant differences were found along the four estrous phases. Statistical analysis: one-way ANOVA followed by Tukey's test.

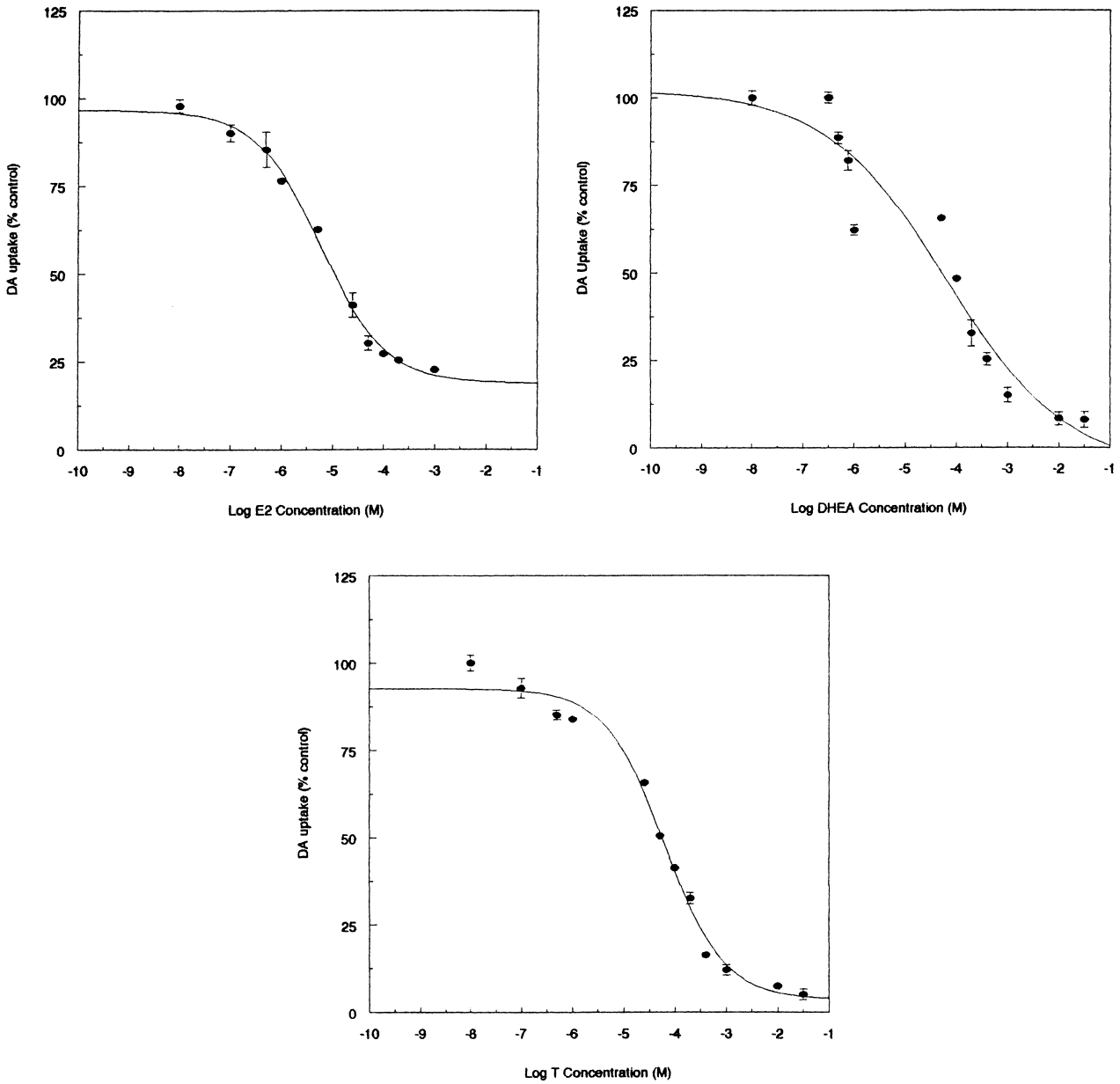

Fig. 1. Inhibition of [^{3}H]DA uptake by E2, DHEA and testosterone (T) in striatal synaptosomes. Synaptosomes were preincubated at 37°C for 10 min in 0.5 ml of normal Krebs medium containing ascorbate (1 mg/ml), pargyline (10 μM), imipramine (10 μM) and increasing concentrations of E2 or DHEA or testosterone (100 nM–3 mM). Results are expressed as percentage of control. Each point represents the results from independent experiments with duplicate determinations.

et al., 1991; Bowling and Beal, 1995). Here we study this inhibitory effect on NADH oxidase activity. As can be see in Fig. 2A, this enzymatic activity was clearly inhibited by MPP^+ in a dose-dependent manner. This inhibition seems to be similar to that reported for the maximal respiratory rate (Absi et al., 2000).

The DHEA showed a slightly inhibitory effect on the NADH oxidase activity. However the concentration used in the experiment of protection against MPP^+ did not have an inhibitory effect on NADH oxidase activity.

In order to point out the effect of DHEA on the inhibition of NADH oxidase activity, DHEA was added before the neurotoxin. Figure 2B shows that 3 μM of DHEA prevents significantly the MPP^+ inhibition.

AChE histochemistry

Labeling of the AChE enzyme histochemistry was diminished in all the lesioned area when compared with the DMSO-injected side (Fig. 3). The loss of the striatal dopaminergic afferents was observed by anti-TH antibody. The pattern of AChE staining in all conditions studied was very similar to that of the anti-TH antibody (Figs. 3 and 4).

Quantitative measurement of the area lacking AChE staining showed a greater loss when MPP^+ was injected alone (0.469 ± 0.004) than when it was co-injected with E2 (0.342 ± 0.003, $P = 1.2 \times 10^{-8}$) or DHEA (0.343 ± 0.010, $P = 5.4 \times 10^{-7}$) (Fig. 5).

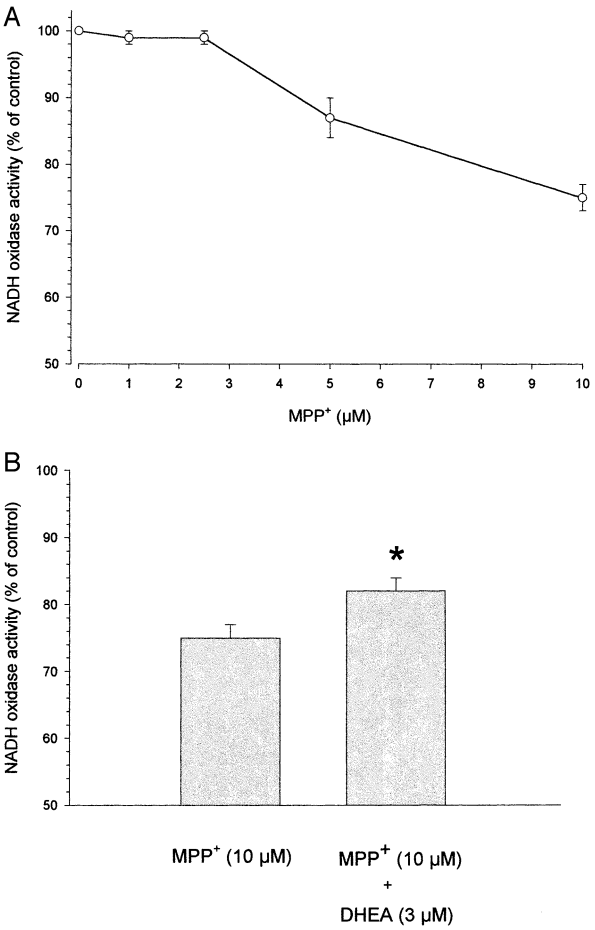

Fig. 2. Effect of MPP^+ and DHEA on the NADH oxidase activity. (A) Effect of MPP^+ on NADH oxidase activity. (B) DHEA prevention of MPP^+ inhibition of NADH oxidase activity. The results are expressed as percentage of NADH oxidase activity in the absence of inhibitor and are mean ± S.E.M. of five independent experiments. Lines were drawn only for clarity and are not the real fit of the curve. $*P = 0.00055$.

Effect of intrastriatal injection of MPP^+ on TH immunohistology

Dopaminergic terminal degeneration consequent to the intrastriatal injection of 1 μg of MPP^+ was assessed by TH immunoreactivity. Figure 3 shows a tissue section of an animal intrastriatally injected with DMSO solution alone; the general appearance of the tissue was normal, even around the injection site. In contrast, inclusion of 1 μg of MPP^+ produced a high loss of TH-positive fibers around the injection site (Fig. 3). Intrastriatal co-injection of 10 μg of E2 plus 1 μg of MPP^+ produced a lesser decrease of TH-positive fibers around the injection site (Fig. 3). A similar result was found with co-injection of 10 μg of DHEA plus 1 μg of MPP^+ (Fig. 4). On the contrary, the loss of TH immunoreactivity after co-injection of 10 μg of testosterone plus 1 μg of MPP^+ was similar in size to that produced by MPP^+ alone (data not shown).

Quantitative analysis of the lesion showed that the area lacking TH-immunoreactive terminals was significantly smaller when MPP^+ was co-injected with E2 (0.342 ± 0.005, $P = 3.5 \times 10^{-8}$) or DHEA (0.355 ± 0.006, $P = 9 \times 10^{-8}$) than when MPP^+ was injected alone (0.454 ± 0.002) (Fig. 5).

Astroglial response to E2, testosterone and DHEA injection

We observed an area lacking GFAP staining after MPP^+ injection (Fig. 6B) with respect to DMSO injection (Fig. 6A). The extension of this area coincides with the loss of TH fibers (Fig. 6A, B compared with Fig. 3). Surrounding the area lacking GFAP-positive cells, reactive astrocytes, with more abundant and larger processes than resting astrocytes, were observed. These cells were clearly reactive astrocytes with thick and darkly stained processes oriented towards the depleted area. In addition, a strong accumulation of these reactive astrocytes was observed around the empty area (Fig. 6D) as compared with injection of DMSO (Fig. 6C). As can be seen in Fig. 7, counting of reactive astrocytes in striatum after different treatments was performed. The quantitative evaluation of the number of GFAP staining-positive cells per area was performed counting in each section six different locations around to injection site (Fig. 6A). For each experimental condition we used four animals and six sections for each animal were counted. A significant decrease in the density of GFAP-reactive astrocytes was found after intrastriatal injection of E2 (15%) and DHEA (25%) and an increase after testosterone injection (39%) with respect to DMSO, being most significant with DHEA treatment (Fig. 7). MPP^+ injection produced an increase of astrocytes around the injection site. When MPP^+ was co-injected with either E2 or DHEA, the increase in the number of astrocytes was reduced 25% and 35% respectively (Fig. 7) (see also Fig. 6D compared with Fig. 6F).

Microglial response to E2, DHEA and testosterone injection

We performed OX-6 immunostaining to investigate the reactive changes in microglial cell population. Microglial cells are characterized by a high degree of morphological and functional plasticity. 'Activated' cells arise in adult brain after injury and are characterized by thicker processes and larger cells bodies than cells in control brains.

As seen in Fig. 8, counting of reactive microglia (OX-6) was performed in all experimental conditions studied. For each experimental condition we used four animals and five sections for each animal. We counted all positive OX-6 cells around the injection site and the results were expressed as cells/mm^2. MPP^+ injection produced a significant increase of reactive microglia as compared with DMSO injection. This increase produced by MPP^+ was significantly either decreased or increased when MPP^+ was co-injected with DHEA or testosterone respectively; in contrast, the E2 had no statistically significant effect (Figs. 8 and 9). In addition, the increase of microglia produced by testosterone was accompanied by morphological changes, large cell bodies and thick processes (Fig. 10).

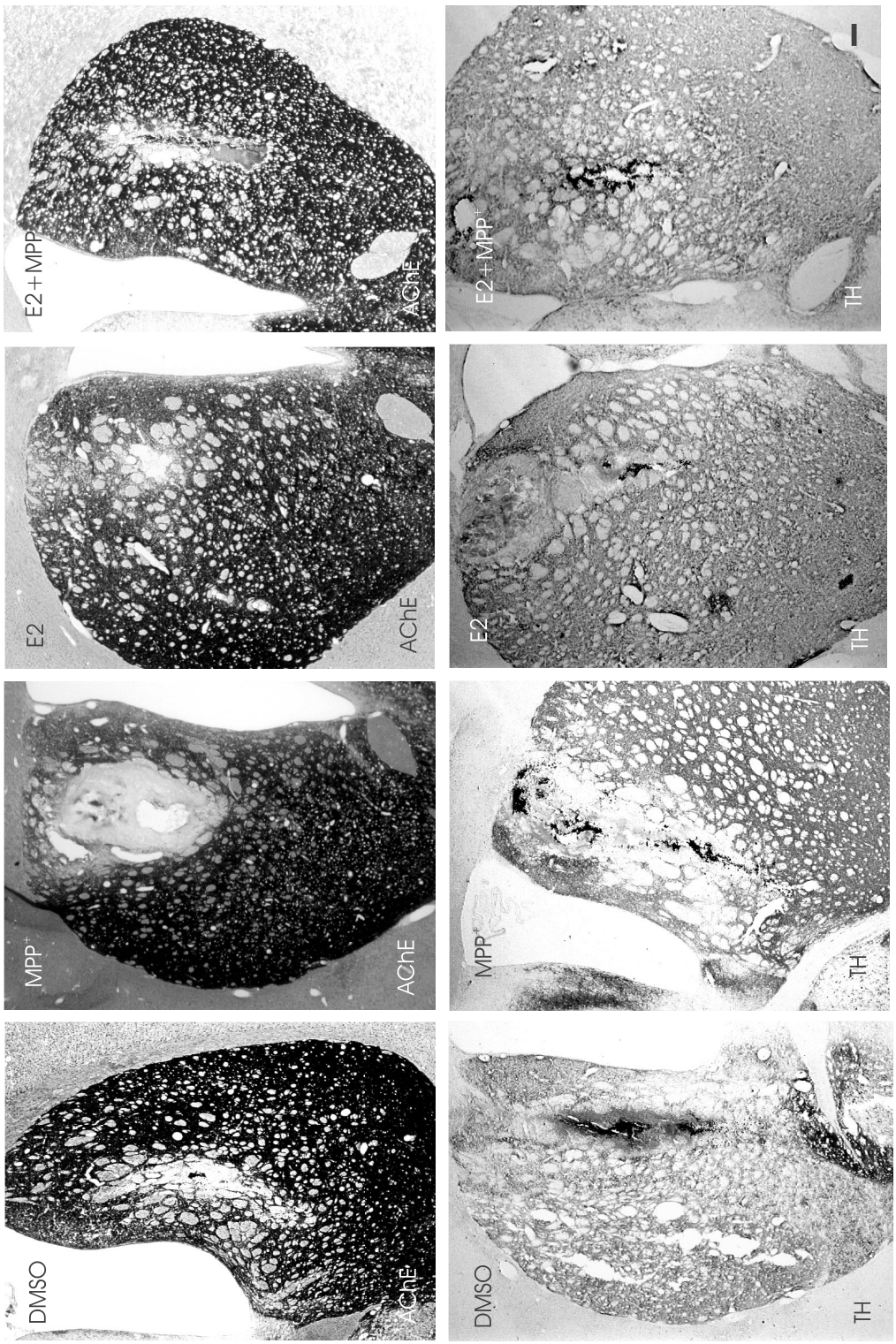

Fig. 3. Photomicrograph of coronal sections showing AChE histochemistry and TH immunoreactivity in striatum 4 days after different treatments. Degeneration of DA fibers correlates with the pattern of AChE histochemistry. Scale bars = 200 μm.

DISCUSSION

Our data show that the striatum is partially protected by DHEA and E2 against damage on dopaminergic terminals induced by intrastriatal injection of MPP^+. In contrast, testosterone did not provide any neuroprotection. The protective effect of E2 fits with the view that estrogen replacement in ovariectomized mice reduced the DA, DOPAC and HVA depletions induced by MPTP treatment, which indicates that estrogen has neuropro-

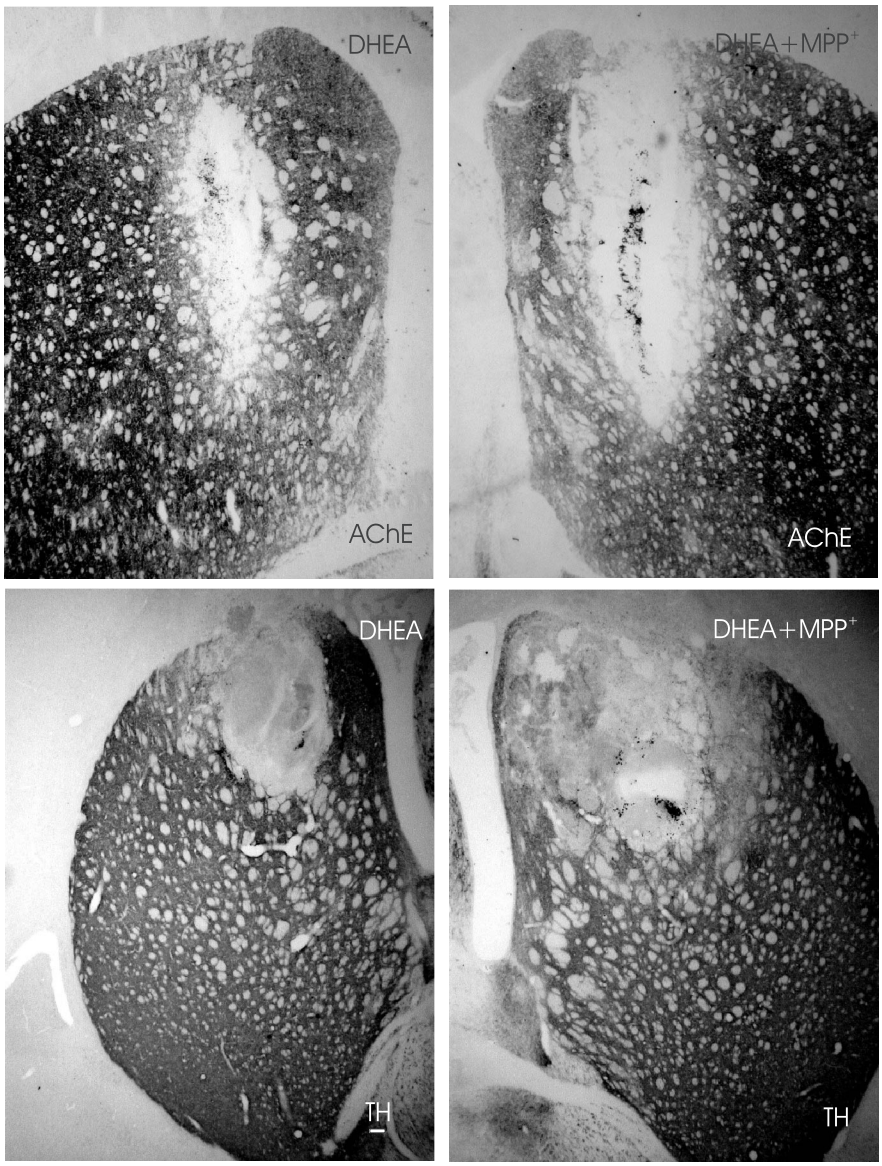

Fig. 4. Photomicrograph of coronal sections showing AChE histochemistry and TH immunoreactivity in striatum 4 days after different treatments. Degeneration of DA fibers correlates with the pattern of AChE histochemistry. Scale bars = 150 μm.

tective properties in this model of striatal dopaminergic neurotoxicity (Dluzen et al., 1996a,b; Miller et al., 1998; Callier et al., 2000; Grandbois et al., 2000; Sawada and Shimohama, 2000). However, this is the first report to demonstrate the protective effect of DHEA against MPP^+ in this system. The question then is which mechanism accounts for the partial protective effect of E2 and DHEA on the DA depleting effect elicited by MPP^+ in rat striatum.

Intrastriatal injection of MPP^+ produces a significant decrease of DA levels along with the specific absence of TH immunoreactivity around the injection side, demonstrating the extent of TH loss. Moreover, other signals of damage have been studied. In the mammalian CNS, glial cells are in a key position to support neurons and maintain a functional environment. Rapid activation of GFAP after injury implies that glia fulfills important early functions in wound healing and in supporting neuronal cell survival (O'Malley et al., 1992; McGeer et al., 1989). Conversely, increased expression of GFAP is a major contributor to CNS scarring, resulting in a physical barrier in the proximity to damage neurons. The GFAP accumulation is a prominent feature of astrocytic gliosis and reactive astrocytes have been found in affected areas of PD patients (Yamada et al., 1992). In addition astrocytic reaction has been reported after administration of MPTP (O'Callaghan et al., 1990; Kohutnicka et al., 1998). We have performed an immunohistochemical analysis of reactive astrocytes following injection of MPP^+. In our lesion model, treatment with MPP^+ alone caused a specific absence of GFAP immunolabeled around the injection site.

Microglia are the principal immune cells in the brain originating from mesodermally derived macrophages

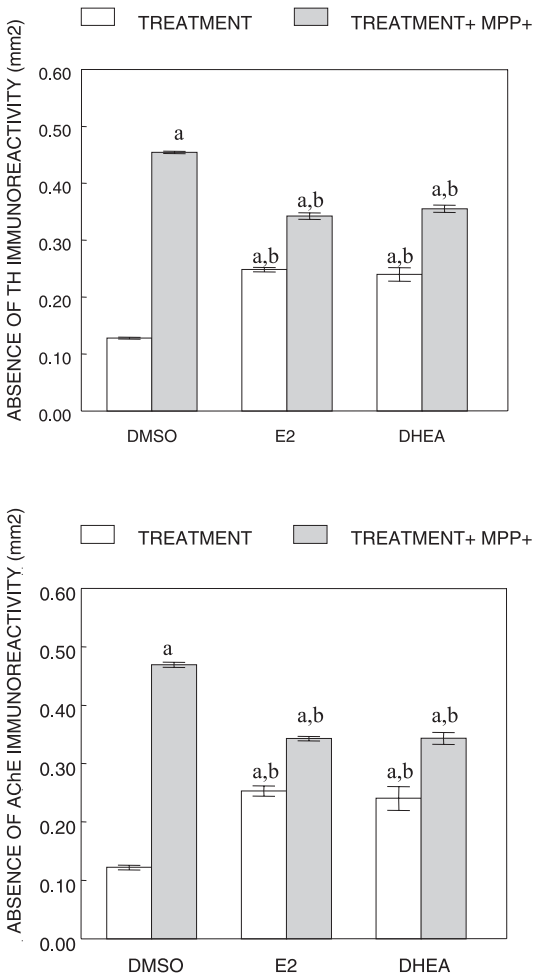

Fig. 5. Quantitative analysis of the lesion showed the areas lacking in TH-immunoreactive terminals and AChE immunoreactivity. Statistical significance: one-way ANOVA followed by the Tukey test: a, $P < 0.05$ as compared with DMSO and b, $P < 0.05$ as compared with MPP^+.

that become permanently resident in the brain during development (Streit and Kincaid-Coton, 1995). Like macrophages, microglia respond to various stimuli by acquisition of a reactive phenotype as evidenced by the elevated expression of a number of cell surface molecules, including MHC-II antigens (Kalaria, 1999). Microglia cells are normally beneficial, producing growth factors, protecting the CNS against pathogens, and phagocytosing cellular debris from neurons and glia that are removed during development. However, because microglia are rapidly activated after CNS injury and can be surprisingly damaging, it is important to selectively control their detrimental functions (for review, see Gehrmann et al., 1995; Streit and Kincaid-Coton, 1995; Kreutzberg, 1996). Activation of microglia has been described in an animal model of PD produced by MPTP (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Kurkowska-Jastrzebska et al., 1999). Expression of MHC-II antigen-positive reactive microglia has been observed in the substantia nigra (McGeer et al., 1988a,b) and following 6-hydroxydopamine-induced lesions to the nigrostriatal tract (Akiyama and McGeer, 1989). Moreover, a marked increase of MHC-II antigen expression on microglia and T-cell recruitment in substantia nigra was found (Kurkowska-Jastrzebska et al., 1999). These authors suggest a possible immune involvement in the pathological changes that leads to dopaminergic neuronal damage after MPTP toxicity. Recently, McNaught and Jenner (1999) have reported that altered glial function causes neuronal death and increases neuronal susceptibility to MPP^+ toxicity in primary ventral mesencephalic co-cultures. The injection of MPP^+ produced a significant increase of reactive microglia, measured as positive OX-6 cells.

When MPP^+ was co-injected with E2 or DHEA, the decrease in DA levels and the halo produced by TH immunohistology were smaller than those found with MPP^+ alone. These results corroborate the neurochemical data of E2 and DHEA. In contrast, testosterone was neurotoxic based upon the similarity of reductions in DA concentrations obtained between testosterone and MPP^+ groups. The E2 protective effect against MPP^+ neurotoxicity is likely related to the action of this hormone and MPP^+ on the DA transporter. As it has been previously reported (Disshon and Dluzen, 1999; Arvin et al., 2000), the estrogen would have the effect of blocking DA uptake, thereby decreasing MPP^+ toxicity. It has been reported that estrogen significantly diminished the depletion of striatal DA resulting from the neurotoxin 6-OHDA administration to rats (Dluzen, 1997). Although, some controversies exist about the effect of the E2 on DA transporter. It has been previously demonstrated that estrogen can increase the density but not the affinity of striatal DA uptake receptor sites (Morissette et al., 1990; Morissette and Di Paolo, 1993). Ovariectomy produces a time-dependent decrease of the DA transporter density in the striatum of the rat (Bosse et al., 1997). In contrast, E2 inhibits striatal DA uptake by decreasing the affinity of the transporter for DA (Disshon et al., 1998). Our results showed that E2 inhibited [^{3}H]DA uptake in a dose-dependent manner whereas the testosterone and DHEA inhibited [^{3}H]DA uptake to a concentration much higher than that required to E2. Therefore, the protective effect of E2 against DA depletion by MPP^+ could be ascribed to DA transporter inhibition; by preventing DA uptake through actions on the DA transporter, estrogen may block MPP^+ uptake and its resultant neurotoxicity.

DHEA is a widely studied steroid hormone with multifunctional properties. Reports suggest that some of the many activities of DHEA are due to its protective effect against oxidative stress: oxidative stress induced by virus infections (Araghi-Niknam et al., 1998), by H_2O_2 and sodium nitroprusside (Bastianetto et al., 1999), by cumene (Gallo et al., 1999), by CCl_4 (Aragno et al., 1993), by the streptozotocin treatment in rats (Aragno et al., 1999), by acute hyperglycemia (Aragno et al., 1997), by ischemia/reperfusion (Lohman et al., 1997; Aragno et al., 2000), by trauma hemorrhage (Jarrar et al., 2001). It is interesting to note that many of these protective effects are also produced by melatonin. The infection of C57BL/6 mice by murine retrovirus produced a physical inactivity, which is prevented by mela-

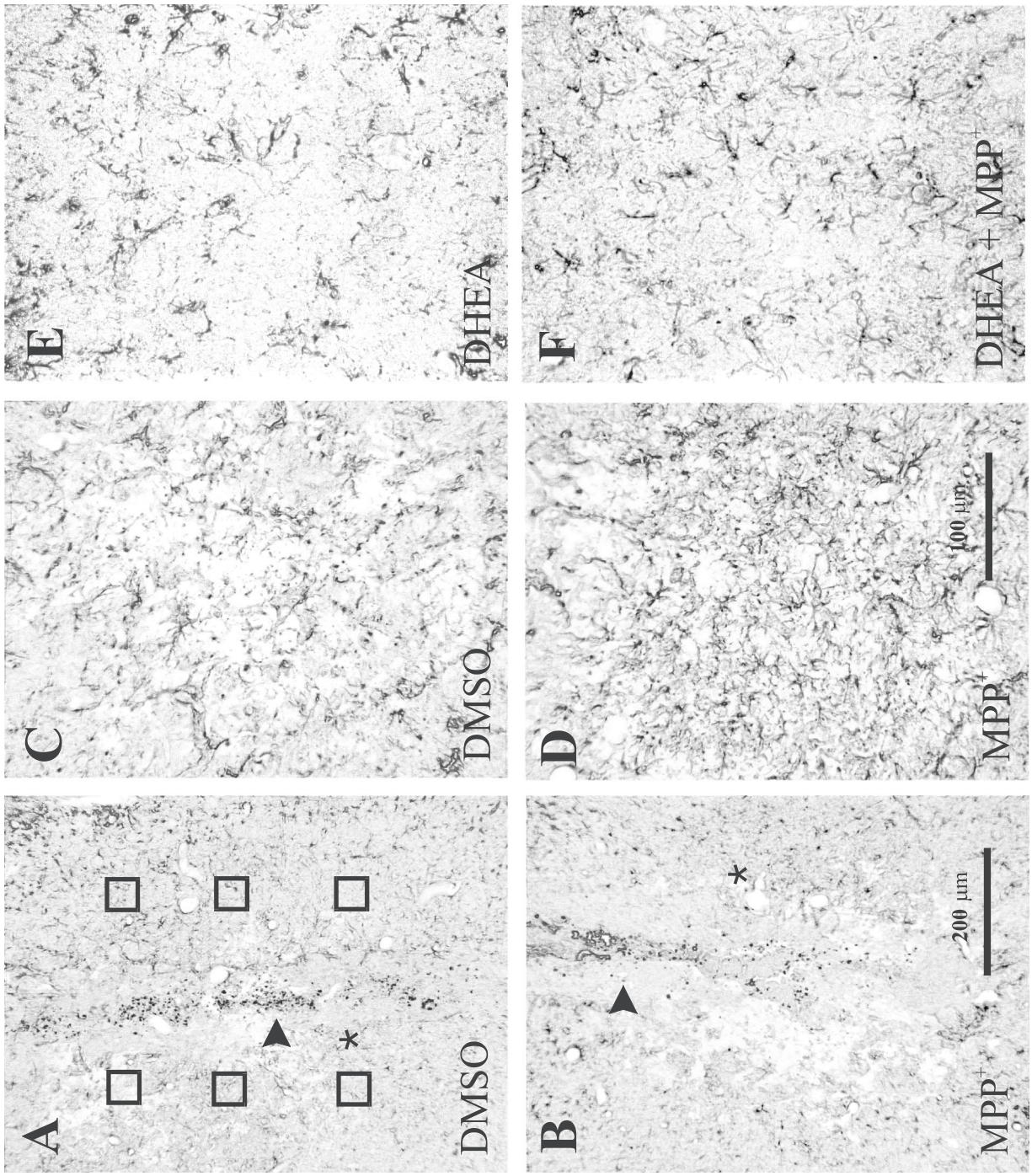

Fig. 6. Photomicrographs of GFAP-immunostained tissue sections from the striatum 4 days after injection of Monastral Blue in DMSO (A) and in MPP^+ (B). The tracer Monastral Blue is clearly visible throughout the injection tract (arrowheads). (C, D) High-powerbright-field photomicrographs of the areas denoted by asterisks in A and B respectively. High-power-bright-field photomicrographs after injection of DHEA (E) and DHEA plus MPP^+ (F). Note that the number of GFAP-positive cell bodies was decreased with respect to DMSO (compare E to C) and MPP^+ (compare F to D). The insets in A illustrate the location of the areas used to obtain the data described in Fig. 7. Scale bar = 200 µm (A, B) and 100 µm (C–F).

tonin and DHEA (Araghi-Niknam et al., 1998). DHEA and melatonin prevent *Bacillus anthracis* lethal toxin-induced TNF production in macrophages (Shin et al., 2000). DHEA and melatonin prevent lipid peroxidation produced in C57BL/6 mice infected with the LP-BM5 leukemia retrovirus (Zhang et al., 1999). Melatonin and DHEA show protective effects against septic shock (Maestroni, 1996; Schurr et al., 1997; Ben-Nathan et al., 1999; Crespo et al., 1999). The mechanism of the protective effect of melatonin seems to be produced by its antioxidant properties (Poeggeler et al., 1994), scavenger of hydroxyl (Tan et al., 1993) and peroxyl radicals (Pieri et al., 1994). Similar properties have been described for DHEA (Tamagno et al., 1998). In many of the oxi-

dative stresses there is a mitochondria damage and in some of them the mitochondria increase the free radicals production. Melatonin has been described as protective against the mitochondria damage, and this effect could be related with new actions of melatonin in mitochondria (Acuña-Castroviejo et al., 2001). Melatonin is also able to protect against the MPTP/MPP+ toxicity (Acuña-Castroviejo et al., 1997; Iacovitti et al., 1997; Jin et al., 1998). Absi et al. (2000) show that the protective effect of melatonin against MPP+ is produced by the fact that melatonin prevents the MPP+ inhibition of complex I of the mitochondrial respiratory chain. We studied this possibility for DHEA, finding that it shows a similar behavior as melatonin: DHEA produced a significant protection against the MPP+ inhibition of NADH oxidase activity. Taking into account these data, it may be suggested that DHEA could be able to prevent or protect against the damage caused by MPTP/MPP+ through free radicals production (Smith et al., 1994; Pong et al., 2000). The inhibition of LPS-induced reactive oxygen species by DHEA has also been suggested (Wang et al., 2001). For that reason we may suggest that at least this is one of the protective actions of DHEA. It could also be important in the other conditions where DHEA has been reported as protectant. However, further studies are required to address these important issues. At the same time, we must take into account that DHEA could also produce some actions through its binding – with low affinity – to estrogen receptors (Nephew et al., 1998), or through its conversion to E2, but the amounts of E2 produced by DHEA are below the limit of detection (Marx et al., 2000). Another possibility of action could be through some other metabolites such as androstenediol, which confers protection against lethal infection with influenza A virus (Padgett et al., 2000). Nevertheless, some DHEA effects have been pointed out only after a preincubation period (Tamagno et al., 1998).

Our results also show that the reactive astrocytes in terms of GFAP-immunopositive cells surrounded the lesioned cavity decreased by co-injections of MPP+ with E2 or DHEA. These results are related at least in part to the finding that E2 and DHEA produced a protective effect against MPP+ toxicity, however they could also be a direct effect of these hormones. The anti-inflammatory (Cuzzocrea et al., 2000) and immunosuppressive actions of these hormones could, therefore, mitigate these astrocytic responses to injury. The effect of neurosteroids in the formation of gliotic tissue has been assessed in adult male rats after a penetrating wound of the cerebral cortex and the hippocampal formation (Garcia-Estrada et al., 1999). They reported that 17β-E2, testosterone and DHEA decrease the accumulation of astrocytes in the proximity of the wound. In addition, when we study the MHC-II antigen expression on microglia, we found that only the DHEA produced a protective effect. The observed effect on microglia may result from a direct hormonal inhibition of local proliferation of microglia. The neuronal survival increase and differentiation and reduced astroglial proliferation rates in mouse brain cells in cultures by DHEA have been reported (Bologa et al., 1987). DHEA selectively inhibits production of TNF-α and interleukin-6 in astrocytes induced by an inflammatory signal (Di Santo et al., 1996; Zwain and Yen, 1999). This immunosuppressive action could protect against MPP+ injury as it has been reported that suppression of macrophage/microglia activity prevented the deterioration of hindlimb motor function after ischemic injury to the spinal cord (Giulian and Robertson, 1990). This inhibition might be useful in regulating astrogliosis following CNS injury and in some neurodegenerative diseases, in addition to PD. Marked astrocytic hypertrophy with attendant accumulation of GFAP occurs in human and experimental disease states linked to an inflammatory response (Aquino et al., 1988, 1990).

Our results demonstrate that intrastriatal injection of MPP+ has a specific effect on glial cells metabolism and it is under neuroendocrine control as indicated by a sig-

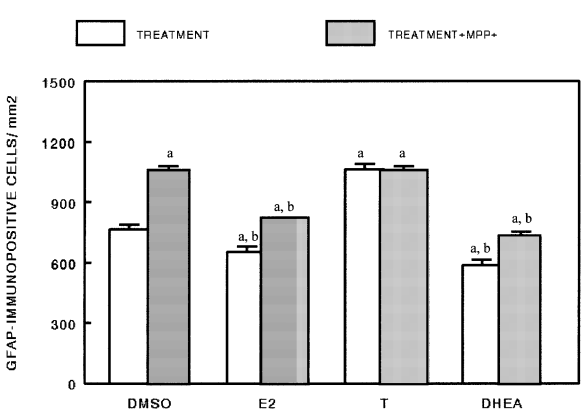

Fig. 7. Quantification of GFAP immunolabeled astrocytes in striatum after different treatments. Counts of immunolabeled cells were performed as described in Results. Results are expressed in cells/mm^2 and represent the mean ± S.D. of four animals. Statistical significance: one-way ANOVA followed by the Tukey test: a, $P < 0.05$ as compared with DMSO and b, $P < 0.05$ as compared with MPP+. T, testosterone.

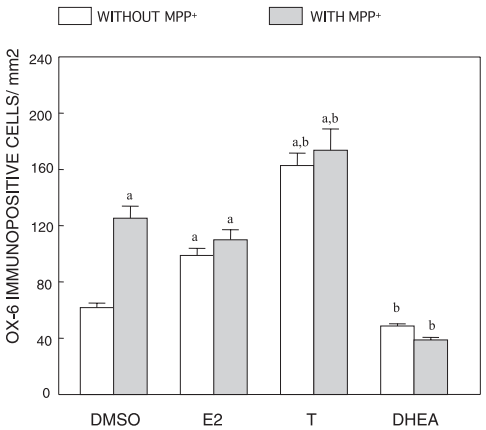

Fig. 8. Quantification of OX-6 immunolabeled microglia in striatum after different treatments. Counts of immunolabeled cells were performed as described in Results. Results are expressed in cells/mm^2 and represent the mean ± S.D. of four animals. Statistical significance: one-way ANOVA followed by the Tukey test: a, $P < 0.05$ as compared with DMSO and b, $P < 0.05$ as compared with MPP+. T, testosterone.

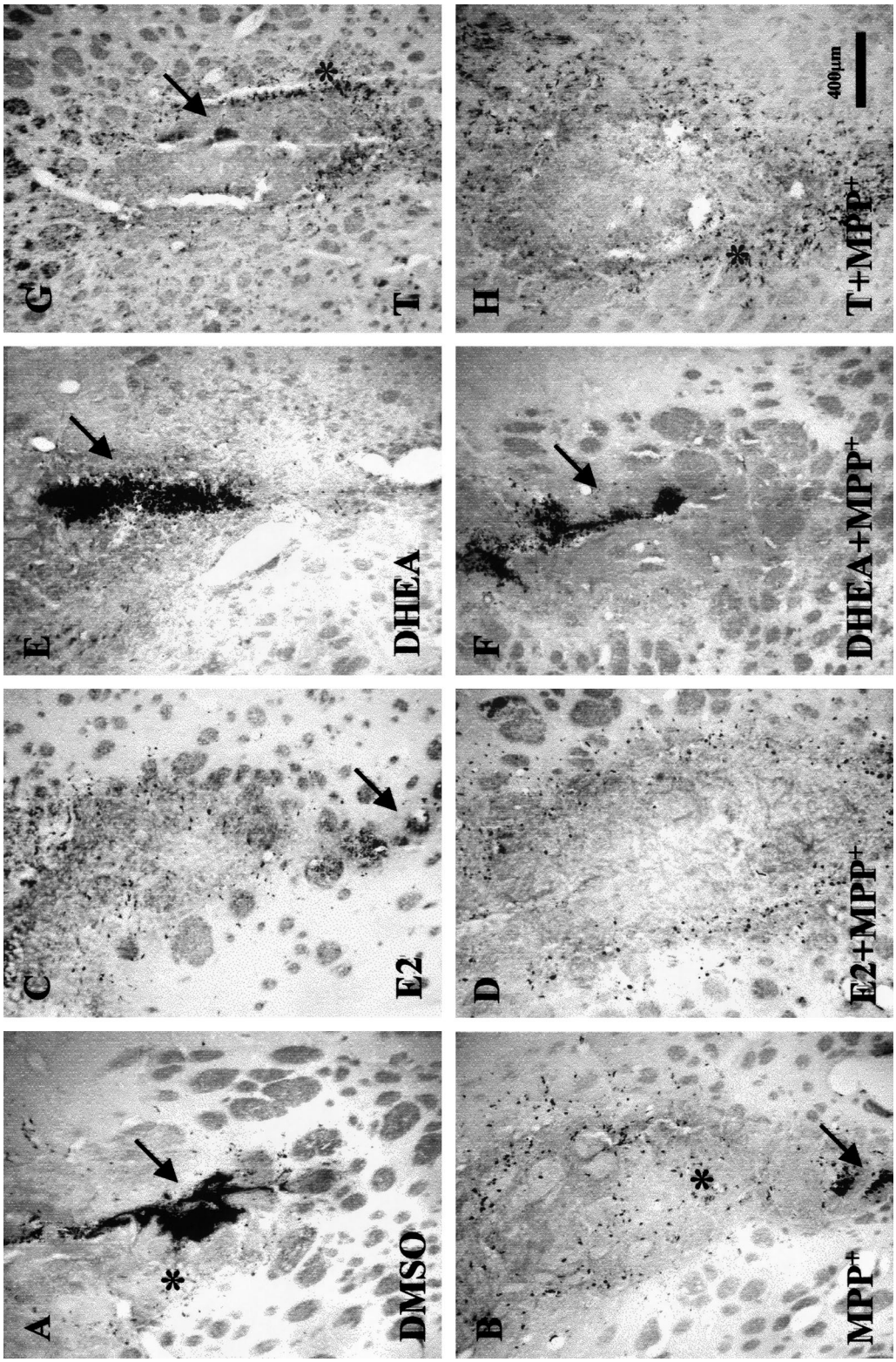

Fig. 9. Immunohistochemical staining for OX-6 in the striatum 4 days after injection of Monastral Blue in different compounds. The tracer Monastral Blue is clearly visible throughout the injection tract (arrow). Note the striking decrease of OX-6 immunoreactivity after injection of DHEA and the marked induction of MHC-II antigen expression, OX-6 after testosterone (T) injection. Scale bar = 400 µm.

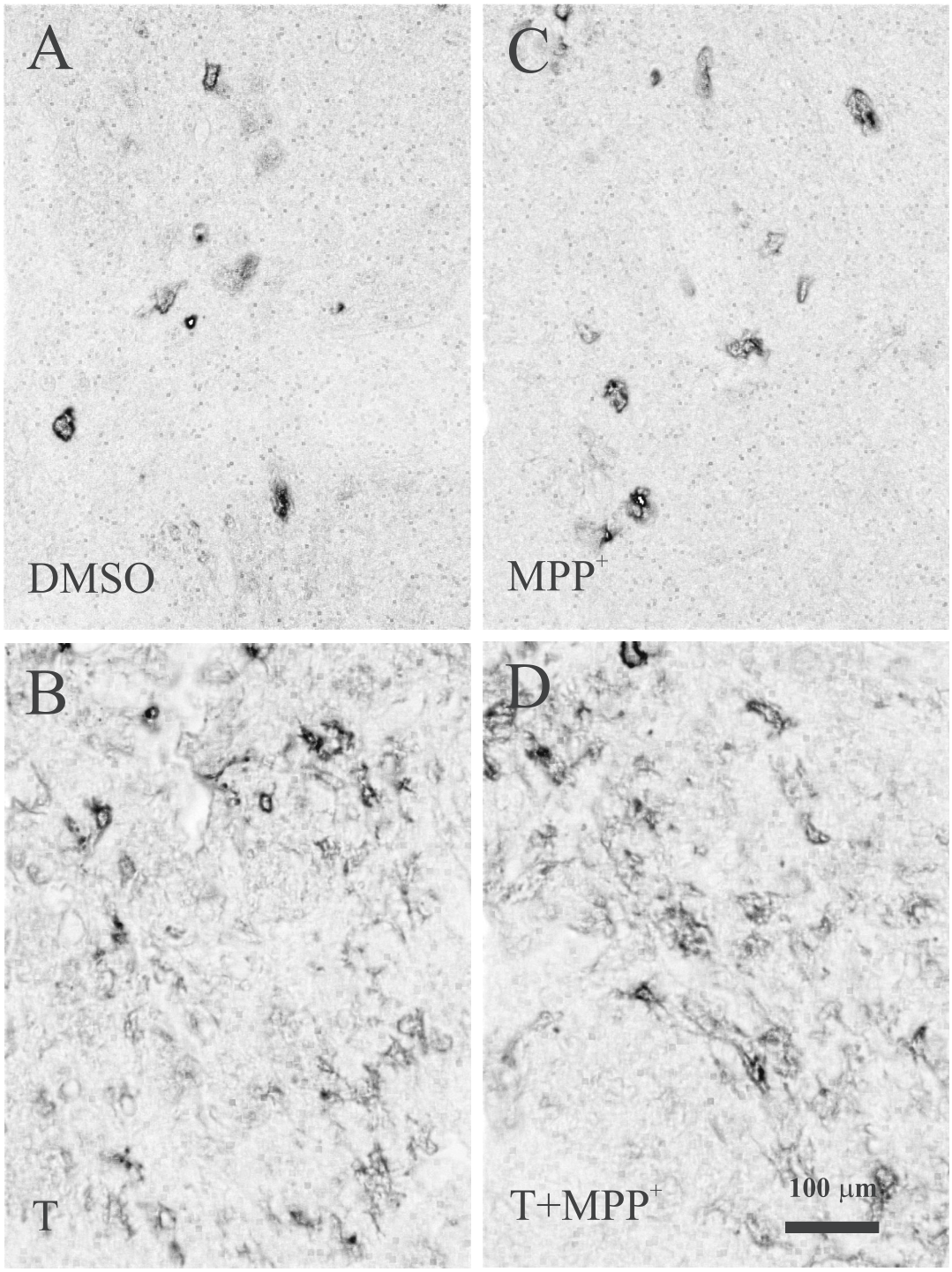

Fig. 10. High magnifications of the zones marked by asterisks in Fig. 9A, B from Fig. 9A, B respectively and Fig. 9C, D from Fig. 9G, H respectively. Note the striking increase in the number of microglia and they were mostly of ramified morphology. This change in microglia was not observed with any other hormones. Scale bar = 100 μm.

nificant reduction of the expression of GFAP by E2 and DHEA and in the MHC-II by DHEA. Thus, the ability to manipulate the extent and the occurrence of astroglial and MHC-II reactivity can have implications for CNS regeneration.

The potential for estrogen to act as a neuroprotectant of the dopaminergic system may be related to the epidemiological reports that show a sex difference in PD. It has been reported that males have anywhere from a 1.4 to 3.7 greater prevalence for PD (Diamond et al., 1990). However, the neuroprotective action of DHEA could be related to the increased prevalence of neurodegenerative diseases by aging. It has been postulated that decreases in plasma of DHEA may contribute to the development of some age-related disorders (Yen et al., 1995). DHEA age-related declines could exacerbate neurotoxicity, and

the data suggest that therapeutic gains may be obtained with pharmacological manipulation. A working hypothesis is that the decrease of DHEA which would result from aging could contribute to the impairments in advanced ages, i.e. PD. Treatment strategies that block the inflammatory response may halt the progression of PD. Pharmacological agents that suppress microglial activation or reduce microglial-mediated oxidative damage may prove useful strategies to slow the progression of PD.

CONCLUSION

All these data show that probably the principal neuroprotective capacity of E2 and DHEA against MPP^+ damage could be produced by: one direct effect, the E2 inhibition of the DA uptake system and the protection against MPP^+ inhibition of complex I along with its antioxidative capability by DHEA and another indirect effect, their anti-inflammatory capability, specially for DHEA that decreases the inflammatory process triggered by MPP^+ injection.

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