Brain Aromatase Is Neuroprotective

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ABSTRACT: **The expression of aromatase, the enzyme that catalyzes the biosynthesis of estrogens from precursor androgens, is increased in the brain after injury, suggesting that aromatase may be involved in neuroprotection. In the present study, the effect of inactivating aromatase has been assessed in a model of neurodegeneration induced by the systemic administration of neurotoxins. Domoic acid, at a dose that is not neurotoxic in intact male mice, induced significant neuronal loss in the hilus of the hippocampal formation of mice with reduced levels of aromatase substrates as a result of gonadectomy. Furthermore, the aromatase substrate testosterone, as well as its metabolite estradiol, the product of aromatase, were able to protect hilar neurons from domoic acid. In contrast, dihydrotestosterone, the 5α-reduced metabolite of testosterone and a nonaromatizable androgen, was not. These findings suggest that aromatization of testosterone to estradiol may be involved in the neuroprotective action of testosterone in this experimental model. In addition, aromatase knockout mice showed significant neuronal loss after injection of a low dose of domoic acid, while control littermates did not, indicating that aromatase deficiency increases the vulnerability of hilar neurons to neurotoxic degeneration. The effect of aromatase on neuroprotection was also tested in male rats treated systemically with the specific aromatase inhibitor fadrozole and injected with kainic acid, a well characterized neurotoxin for hilar neurons in the rat. Fadrozole enhanced the neurodegenerative effect of kainic acid in intact male rats and this effect was counterbalanced by the administration of estradiol. Furthermore, the neuroprotective effect of testosterone against kainic acid in castrated male rats was blocked by fadrozole. These findings suggest that neuroprotection by aromatase is due to the formation of estradiol from its precursor testosterone. Finally, a role for local cerebral aromatase in neuroprotection is indicated by the fact that intracerebral administration of fadrozole enhanced kainic acid induced neurodegeneration in the hippocampus of intact male rats. These findings indicate that aromatase deficiency decreases the threshold for neurodegeneration and that local cerebral aromatase is neuroprotective. Brain aromatase may therefore represent a new target for therapeutic approaches to neurodegenerative diseases.** J Neurobiol 47: 318–329, 2001

Keywords: **neurodegeneration; neuroprotection; androgen; estrogen; aromatase**

INTRODUCTION

The biosynthesis of estrogens from C19 steroids is catalyzed by aromatase, an enzyme that consists of two components: a cytochrome P450 (P450arom), the product of the *cyp19* gene, and the NADPH cytochrome P450 reductase. Aromatase is expressed in the brain (Naftolin et al., 1971), where it is involved in the regulatory effects of androgens, via conversion to estrogens, on neural differentiation, neural plasticity, neuroendocrine functions, and sexual behavior (MacLusky and Naftolin, 1981; Hutchison, 1991; Lephart, 1996). Many lines of evidence indicate that estrogens

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have neurotrophic and neuroprotective properties (Behl and Holsboer, 1999; Green and Simpkins, 2000; Garcia-Segura et al., 2001). Estradiol promotes viability and survival of neurons in primary cultures (Chowen et al., 1992), and reduces the secretion of Alzheimer's β-amyloid peptides (Xu et al., 1998). Furthermore, estradiol protects neurons from cell death induced by a variety of stressors *in vitro*, including oxidative stress (Behl et al., 1997), anoxia (Zaulyanov et al., 1999) and toxicity induced by AMPA (Zaulyanov et al., 1999), glutamate (Singer et al., 1996; Sawada et al., 1998; Zaulyanov et al., 1999), kainate (Regan and Guo, 1997), and NMDA (Regan and Guo, 1997; Weaver et al., 1997). There is also abundant evidence for neuroprotection by the hormone *in vivo*. Estradiol promotes synaptic plasticity (McEwen and Alves, 1999) and is neuroprotective in experimental models of forebrain ischemia in rats (Simpkins et al., 1997; Dubal et al., 1999), mice (Culmsee et al., 1999), and gerbils (Sudo et al., 1997). Neuroprotective effects of estradiol have also been described in basal forebrain cholinergic neurons (Rabbani et al., 1997) and the nigrostriatal system (Dluzen, 1997). Estradiol also protects hippocampal hilar neurons from excitotoxic insults *in vivo* (Azcoitia et al., 1999a,b; Reibel et al., 2000; Veliskova et al., 2000). These laboratory research findings are in good agreement with the estrogen treatment-associated improvement of memory performance in healthy elderly women (Wolf et al., 1999), as well as with the possible effect of estrogen in the prevention of Alzheimer's disease (Henderson et al., 1994; Fillit, 1994). Although recent clinical studies suggest that estrogen may not be effective for the treatment of diagnosed Alzheimer's disease (Mulnard et al., 2000; Henderson et al., 2000), this hormone may act as a preventive agent.

Neuroprotective properties have also been ascribed to estrogen precursors. Pregnenolone and dehydroepiandrosterone enhance memory and cognitive function in different animal models (Flood et al., 1992; Vallée et al., 1997), and dehydroepiandrosterone reduces neuronal death in brain cell cultures (Bologa et al., 1987; Kimonides et al., 1998). Testosterone, the direct precursor of estradiol and substrate of aromatase, also promotes neuroprotection in several experimental models. Testosterone promotes the survival of specific neuronal populations (Yu, 1982; Jones, 1994; Perez and Kelly, 1996; Rasika et al., 1999), induces motor axon regeneration in axotomized motoneurons (Yu, 1982; Jones, 1994; Perez and Kelly, 1996), prevents the hyperphosphorylation of the microtubule associated protein tau (Papasozomenos, 1997), which is abnormally hyperphosphorylated in Alzheimer's disease, and reduces neuronal secretion of β-amyloid peptides associated with Alzheimer's disease (Gouras et al., 2000).

The neuroprotective effects of pregnenolone, dehydroepiandrosterone, and testosterone may be exerted, at least in part, by their conversion to estrogens, either in the periphery or in the brain (Zwain and Yen, 1999). Aromatase, the final enzymatic step of the biosynthetic route of estrogens, may then play a crucial role in the neuroprotective effects of steroids. Under normal conditions, aromatase expression in the mammalian central nervous system is confined to neuronal cell bodies and neuronal processes (MacLusky and Naftolin, 1981; Hutchison, 1991; Lephart, 1996). However, we have shown that neurotoxic and mechanical lesions in the brains of rats and mice increase aromatase activity and induce *de novo* expression of the enzyme in reactive astroglia in all injured brain areas, including the cortex, corpus callosum, striatum, hippocampus, thalamus, and hypothalamus (Garcia-Segura et al., 1999a,b). Furthermore, Peterson et al. (2001) have recently shown that aromatase mRNA and protein are rapidly and locally up-regulated in glia following neural injury in the zebra finch brain. This finding suggests that injury-dependent up-regulation of aromatase may be a conserved characteristic of the vertebrate brain and an important component of the initial response of neural tissue to injury (Peterson et al., 2001). The increased expression of aromatase around the injury (Garcia-Segura et al., 1999 a,b; Peterson et al., 2001) suggests that this enzyme may be involved in the protection of injured nervous tissue by increasing local estrogen levels. As a first step to test this hypothesis, the present study was designed to determine whether brain aromatase activity is neuroprotective.

MATERIALS AND METHODS

Animals

Adult male C57BL6 mice, aromatase-deficient mice that lacked exons 1 and 2 and the proximal promoter region of the *cyp19* gene by homologous recombination (ArKO mice) (Honda et al., 1998), wild-type littermates, and Wistar albino adult male rats were maintained on 12:12 h light/dark cycle, with free access to food and water. Manipulation of the animals was performed following the European Union Normative (86/609/EEC), and special care was taken to minimize animal suffering and to set the number of animals to the minimum required.

Experiment 1: Effect of Castration and Hormonal Replacement of Male Mice

Male mice were bilaterally gonadectomized under 2,2,2, tribromoethanol anaesthesia (0.2 g/Kg b.w., Fluka Chemika

AG, Buchs, Switzerland) when they were 6 weeks old. Three weeks after surgery, 10 castrated males and seven sham-operated males received one 0.1 mL intraperitoneal (i.p.) injection of domoic acid (Tocris Cookson, Bristol, UK; 0.5 mg/Kg b.w., dissolved in phosphate buffered saline solution, 0.1 *M*, pH 7.4) plus one 0.1 mL i.p. injection of corn oil. Six castrated animals received one i.p. injection of domoic acid (0.5 mg/Kg b.w.) and one i.p. injection of testosterone (5 mg/Kg b.w., dissolved in corn oil). Four castrated animals received one i.p. injection of domoic acid and one i.p. injection of estradiol (5 mg/Kg b.w.). Four castrated animals received one i.p. injection of domoic acid and one i.p. injection of dihydrotestosterone (DHT; 5 mg/Kg b.w.). Control sham-operated males ($n = 5$) and control castrated males ($n = 9$) were injected with vehicle solutions.

Experiment 2: Aromatase-Deficient Mice

Three adult male aromatase knock-out (ArKO) mice and three male wild-type littermates received one i.p. injection of domoic acid (0.5 mg/Kg b.w.). Four intact male ArKO mice and four intact male wild-type littermates injected with vehicle were used as controls.

Experiment 3: Systemic Aromatase Inhibition in Intact Male Rats

Adult male rats were anaesthetized with tribromoethanol, and an osmotic minipump (Alzet 2002, Alza Corp., Palo Alto, CA; flow rate 0.5 μL/h) was subcutaneously implanted between the scapulae. Before implantation, the pumps were filled with either vehicle (0.9% NaCl) or the aromatase inhibitor fadrozole (4.16 mg/mL, CGS16949A, Ciba-Geigy, Basel, Switzerland). Six days after implantation of the minipumps, animals received one i.p. injection of kainic acid (Sigma, St. Louis, MO; 7 mg/Kg b.w., dissolved in 0.1 mL of phosphate buffer, 0.1 *M*, pH 7.4), one i.p. injection of estradiol (Sigma; 400 μg/Kg b.w. dissolved in corn oil), and/or one i.p. injection of vehicle solutions, according to the following experimental groups: control animals receiving vehicles ($n = 9$); animals receiving kainic acid ($n = 8$); animals receiving fadrozole ($n = 6$); animals receiving fadrozole and kainic acid ($n = 8$); and animals receiving fadrozole, kainic acid, and estradiol ($n = 7$).

Experiment 4: Systemic Aromatase Inhibition in Castrated Male Rats

Adult male rats were bilaterally gonadectomized under tribromoethanol anaesthesia when they were 7 weeks old. One month after surgery, the rats were subcutaneously implanted with an osmotic minipump filled with either vehicle or fadrozole as described above. Six days after implantation of the minipump, animals received one i.p. injection of kainic acid (7 mg/Kg b.w., dissolved in 0.1 mL of phosphate buffer, 0.1 *M*, pH 7.4), one i.p. injection of testosterone (400 μg/Kg b.w. dissolved in corn oil), and/or one i.p. injection of the vehicle solutions, according to the following experimental groups: animals treated with only vehicle solutions ($n = 6$); animals treated with kainic acid ($n = 4$); animals treated with kainic acid and testosterone ($n = 3$); and animals treated with fadrozole, kainic acid, and testosterone ($n = 3$).

Experiment 5: Intracerebral Aromatase Inhibition in Intact Male Rats

Adult male rats were anaesthetized with tribromoethanol, and an osmotic minipump (Alzet 2002, flow rate 0.5 μL/h) was subcutaneously implanted between the scapulae. Before implantation, the pumps were filled with either vehicle (0.9% NaCl) or fadrozole (0.13 mg/mL). The minipumps were then attached to a brain infusion cannula (Alza Corp., Palo Alto, CA) through polyethylene tubing and primed by immersion in normal saline at 40°C for 4 h under sterile conditions. The cannula was intracerebrally implanted with the tip in the right lateral cerebral ventricle (0.3 mm anteroposterior; 1 mm lateral; 4.5 mm dorsoventral), and secured to the skull with an anchoring screw and dental cement. Six days after implantation of the minipumps, the rats received either one i.p. injection of kainic acid (7 mg/Kg b.w., dissolved in 0.1 mL of phosphate buffer, 0.1 *M*, pH 7.4) or one injection of vehicle. In summary, four experimental groups were studied: control animals receiving intracerebral and systemic vehicles ($n = 6$); animals receiving intracerebral fadrozole and systemic vehicle ($n = 6$); animals receiving intracerebral vehicle and systemic kainic acid ($n = 6$); and animals receiving intracerebral fadrozole and systemic kainic acid ($n = 6$).

Histology

One week after injection of domoic acid, kainic acid, or vehicle, the animals were perfused under pentobarbital anaesthesia (100 mg/Kg b.w.) through the ascending aorta, first with saline and then with 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. The brains were removed and immersed in the same fixative for 4 h at 4°C and then rinsed in buffer. The hippocampal formation of the left hemisphere was dissected out and horizontal sections (30 μm thick) were cut on a Vibratome. Sections were Nissl stained with toluidine blue for morphometric analysis.

Morphometric Analysis

The number of Nissl stained neurons in the hilus of the dentate gyrus was estimated by the optical disector method using total section thickness for disector height (Hatton and von Bartheld, 1999) and a counting frame of 55 $\times$ 55 μm. A total of 120 counting frames were assessed per animal. Six sections per animal were assessed. Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221, Traunreut, Germany) attached to the stage of a Leitz microscope. Cell nuclei from Nissl-stained neurons that came into focus while focusing down through the disector height were

counted. All counts were performed blind. In rats the temporal portion of the hippocampus is more sensitive to systemic kainic acid than the septal portion (Buckmaster and Dudek, 1997), therefore counts were restricted to the left temporal hippocampus in a region delimited by the dorsoventral coordinates −4.5 mm to −7.5 mm relative to bregma. In mice, counts were also restricted to the left temporal hippocampus in a region delimited by the dorsoventral coordinates −2.5 mm to −4.0 mm. The cross-sectional area of the hilus was measured in each section with the aid of a camera lucida and using a magnetic tablet (Summa Sketch III) and the NIH Image analysis program. Because no significant differences in this parameter were observed among the different experimental groups, the changes in the number of neurons per unit volume obtained with the disector method should reflect modifications in neuronal content. Data within each experiment were analyzed using one-way analysis of variance followed by a Duncan test, with $p < .05$ considered to be significant.

RESULTS

The Aromatase Substrate Testosterone and the Aromatase Product Estradiol Attenuated Neuronal Death in Mice in Response to Excitotoxins, while the Nonaromatizable Androgen DHT Was Not Neuroprotective

The first experiment was designed to determine whether the aromatase substrate testosterone and the aromatase product estradiol were neuroprotective in the experimental model selected. The effect of these steroids was compared with the effect of the nonaromatizable androgen DHT, the reduced metabolite of testosterone. The number of hilar hippocampal neurons was assessed in intact male mice and in gonadectomized males that were treated either with domoic acid alone, or with domoic acid and one of the above mentioned steroids. Differences between the experimental groups in the number of neurons in the hilus of the dentate gyrus were recognized by qualitative inspection of Nissl-stained sections (Fig. 1).

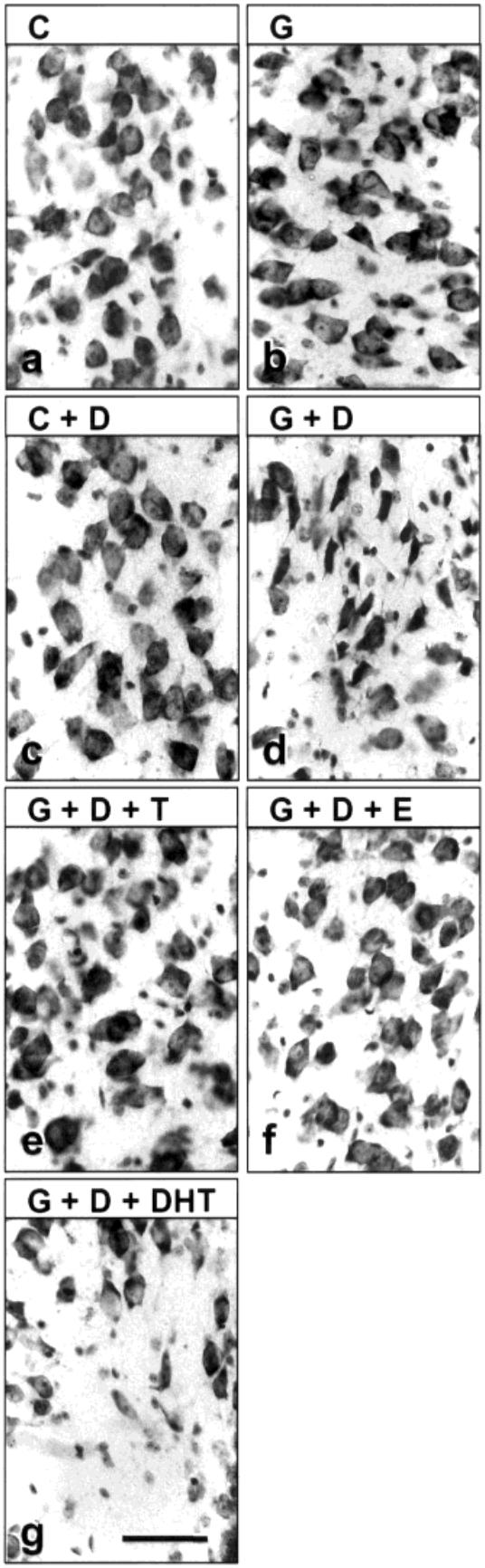
ANOVA revealed a significant effect of the treatment [$F(6, 38) = 23.01$; $p < .001$]. No significant ($p > .2$) neuronal loss was observed in the hilar dentate area of intact mice injected with the neurotoxin compared to intact mice injected with vehicle (Figs. 1 and 2). Furthermore, gonadectomy alone did not affect the number of hilar neurons ($p > .2$). However, a significant loss of hilar neurons was observed in gonadectomized males injected with domoic acid compared to gonadectomized males injected with vehicle, to intact males injected with domoic acid, and to intact control males ($p < .001$; Figs. 1 and 2). Testosterone replacement of gonadectomized mice prevented the effect of domoic acid (Figs. 1 and 2). The number of hilar neurons in gonadectomized animals injected with testosterone and domoic acid was significantly higher ($p < .001$) than the number of neurons in gonadectomized animals injected with domoic acid alone. No significant differences were observed between intact animals and gonadectomized animals injected with testosterone and domoic acid. Estradiol treatment also prevented the effect of domoic acid (Figs. 1 and 2). No significant differences were observed between intact animals and gonadectomized animals injected with estradiol and domoic acid ($p > .2$). In contrast, DHT, injected at the same dose as testosterone, did not prevent the neurodegenerative effect of domoic acid (Figs. 1 and 2).

Aromatase-Deficient Mice Lose More Neurons Than Wild-Type Following Excitotoxin

To determine whether aromatase deficiency increases vulnerability to neurotoxins, the effect of domoic acid was assessed in ArKO male mice and their wild-type male littermates (Figs. 3 and 4). The aromatase deficient state of these animals did not significantly alter the number of hilar neurons in the absence of the neurotoxin. No significant differences in neuronal numbers were detected between ArKO and wild-type mice ($p > .2$). However, the quantitative estimation of the number of hilar neurons revealed a significant effect of domoic acid treatment in ArKO mice, but not in the wild-type animals [ANOVA: $F(3, 10) = 9.15$; $p < .005$]. The number of hilar neurons in ArKO mice injected with domoic acid was significantly decreased compared to control ArKO mice ($p < .01$), to wild-type controls ($p < .05$), and to wild-type mice injected with domoic acid ($p < .01$). In contrast to the effect of domoic acid in hilar neurons of ArKO mice, no significant effect of the neurotoxin was observed in wild-type animals. The number of neurons in wild-type animals injected with domoic acid was not significantly different from the number of neurons in control wild-type animals ($p > .2$).

Systemic Aromatase Inhibition Enhanced Neurodegeneration in Response to Neurotoxin in Intact Male Rats and This Effect Was Counterbalanced by Estradiol

To further test the role of aromatase in neuroprotection, intact male rats were implanted with an osmotic minipump containing the aromatase inhibitor fadrozole. The number of hilar neurons in the left hippocampal formation was assessed after an i.p. injection of kainic acid. ANOVA revealed a significant effect of the treatment [$F(4, 33) = 10.70$; $p < .001$].



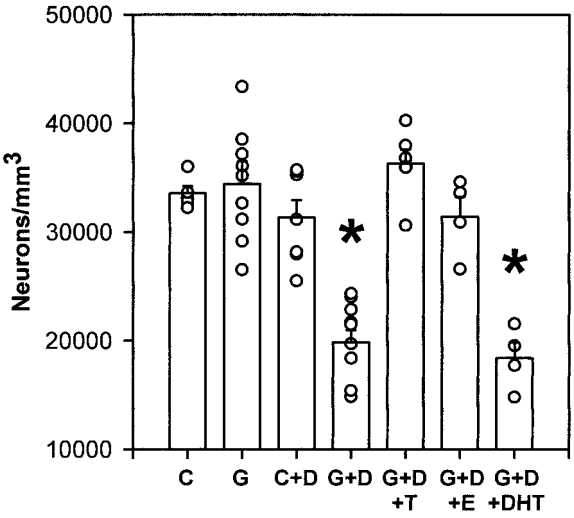


Figure 2 Number of Nissl-stained neurons (neurons/mm^3) in the hilus of the dentate gyrus of adult male C57BL6 mice. (C) Intact controls injected with vehicles ($n = 5$). (G) Gonadectomized animals injected with vehicles ($n = 9$). (C+D) Intact males injected with domoic acid ($n = 7$). (G+D) Gonadectomized males injected with domoic acid ($n = 10$). (G+D+T) Gonadectomized males injected with domoic acid and testosterone ($n = 6$). (G+D+E) Gonadectomized males injected with domoic acid and estradiol ($n = 4$). (G+D+DHT) Gonadectomized males injected with domoic acid and dihydrotestosterone ($n = 4$). Animals were killed 1 week after the injections and the number of hilar neurons was estimated with the optical disector method. Bars are mean ±S.E.M. Circles are individual values. Asterisks, significant difference ($p < .001$) compared to intact controls.

The number of hilar neurons was not affected by kainic acid in control animals (Figs. 5 and 6). Furthermore, fadrozole alone did not affect the number of hilar neurons (Figs. 5 and 6). However, animals that were treated with both fadrozole and kainic acid had a significant decrease in the number of hilar neurons compared to animals treated with vehicle ($p < .001$),

Figure 1 Effect of gonadectomy and hormonal replacement on the neurodegenerative effect of domoic acid on hilar neurons in the dentate gyrus of male C57BL6 mice. (a–g) Representative Nissl-stained histological sections of the hilus. (a) Intact male (C) injected with vehicles. (b) Gonadectomized animal (G) injected with vehicles. (c) Intact animal (C) injected with domoic acid (D). (d) Gonadectomized animal (G) injected with domoic acid (D). (e) Gonadectomized animal (G) injected with domoic acid (D) and testosterone (T). (f) Gonadectomized animal (G) injected with domoic acid (D) and estradiol (E). (g) Gonadectomized animal (G) injected with domoic acid (D) and dihydrotestosterone (DHT). All panels are at the same magnification. Scale bar, 50 μm.

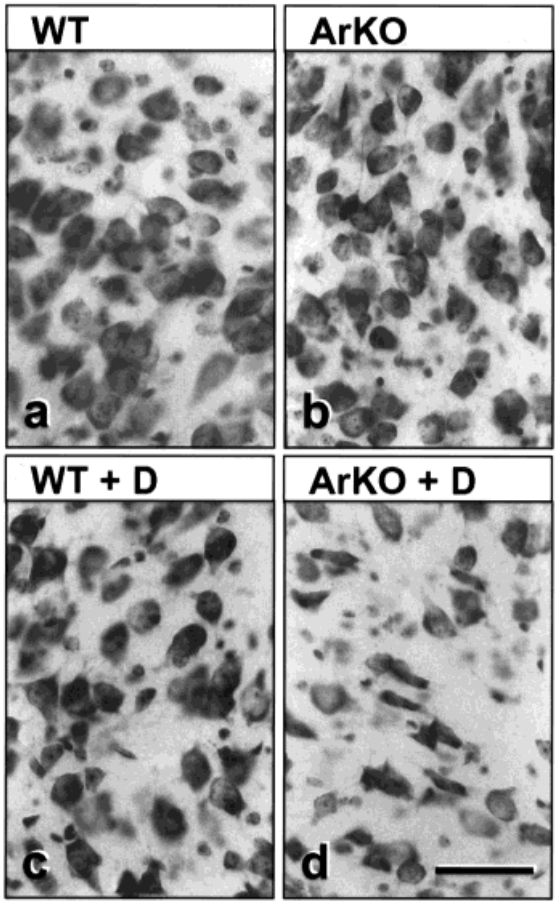


Figure 3 Effect of the systemic administration of domoic acid on hilar neurons in aromatase deficient mice. (a–d) Representative Nissl-stained histological sections from the hilus of the dentate gyrus of wild-type and aromatase knock-out adult male mice. (a) Wild-type animal (WT) injected with vehicle. (b) Aromatase knock-out mouse (ArKO) injected with vehicle. (c) Wild-type animal (WT) injected with domoic acid (D). (d) Aromatase knock-out animal (ArKO) injected with domoic acid (D). All panels are at the same magnification. Scale bar, 50 μm.

to animals treated with kainic acid alone ($p < .001$), and to animals treated with fadrozole alone ($p < .005$). Estradiol treatment prevented the neurodegenerative effect of kainic acid in animals treated with fadrozole. The number of hilar neurons in animals treated with fadrozole, kainic acid, and estradiol was not significantly different from control animals ($p > .2$), and was significantly higher than in animals treated with fadrozole and kainic acid ($p < .005$).

Neuroprotection by Testosterone in Castrated Male Rats Was Abolished by Systemic Aromatase Inhibition

To assess whether the neuroprotective effect of testosterone depends on its aromatization to estradiol, castrated male rats were treated with kainic acid in the presence or absence of the aromatase inhibitor fadrozole. Kainic acid resulted in significant neuronal loss in the hilus of the dentate gyrus of castrated male rats compared to animals treated with vehicles ($p < .005$; Figs. 7 and 8). Testosterone administration prevented the neurodegenerative effect of kainic acid (Figs. 7 and 8); the number of hilar neurons in animals treated with kainic acid and testosterone was not significantly different from the number of hilar neurons in animals treated with vehicle solutions ($p > .2$). However, fadrozole prevented neuroprotection by testosterone; animals treated with fadrozole, kainic acid, and testosterone had a significant decrease in the number of hilar neurons compared to animals treated with vehicle solutions ($p < .005$) and to animals treated with kainic acid and testosterone ($p < .05$).

Intracerebral Aromatase Inhibition Enhanced Neurodegeneration in Response to Neurotoxin in Intact Male Rats

To test the role of local brain aromatase activity in neuroprotection, the aromatase inhibitor fadrozole was infused into the right lateral cerebral ventricle of a group of rats. The number of hilar neurons in the left

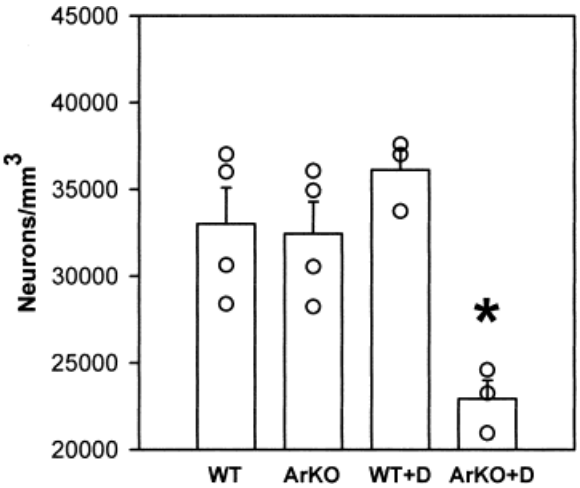


Figure 4 Number of Nissl-stained neurons (neurons/mm^3) in the hilus of the dentate gyrus of wild-type control mice injected with vehicle (WT, $n = 4$), aromatase knock-out mice injected with vehicle (ArKO, $n = 4$), wild-type mice injected with domoic acid (WT+D, $n = 3$), and ArKO mice injected with domoic acid (ArKO+D, $n = 3$). Bars are mean ±S.E.M. Circles are individual values. The number of neurons in ArKO mice injected with domoic acid (asterisk) is significantly decreased ($p < .05$) compared to the rest of the experimental groups.

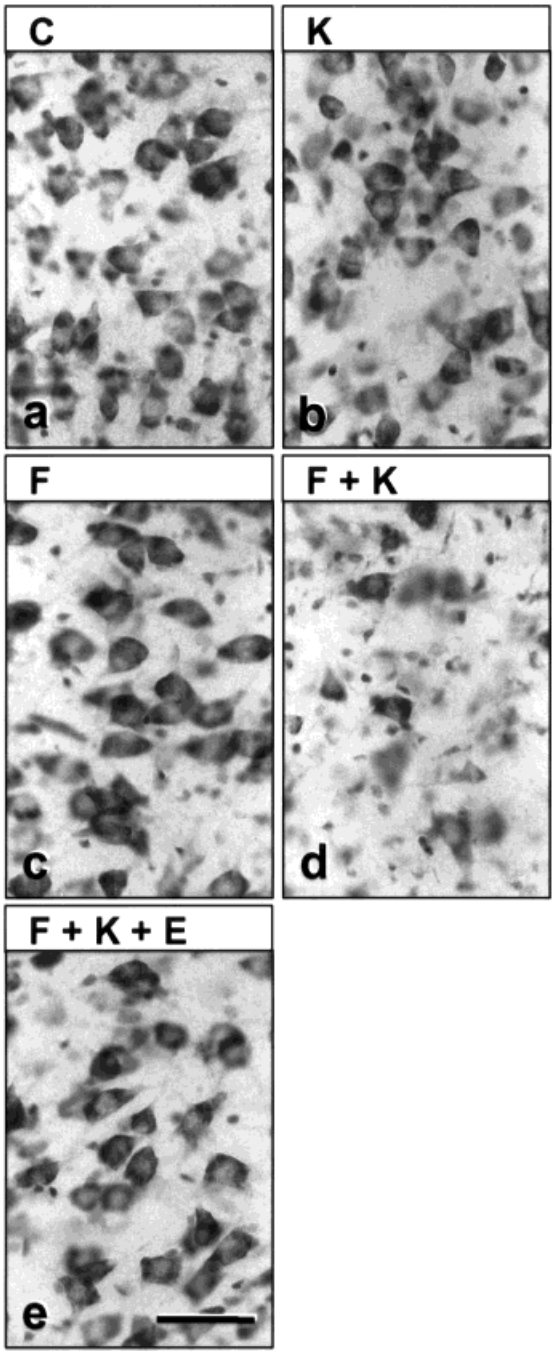


Figure 5 Effect of the systemic administration of the aromatase inhibitor fadrozole and the systemic administration of kainic acid on the number of hilar neurons in the dentate gyrus of intact male rats. (a) Control (C) treated with vehicles. (b) Rat treated with kainic acid (K). (c) Rat treated with fadrozole (F). (d) Rat treated with fadrozole (F) and kainic acid (K). (e) Rat treated with fadrozole (F), kainic acid (K), and estradiol (E). All panels are at the same magnification. Scale bar, 50 μm.

hippocampal formation was assessed after an i.p. injection of kainic acid (Figs. 9 and 10). ANOVA revealed a significant effect of the treatment [$F(3, 20) = 4.92$; $p < .05$]. The number of hilar neurons was not affected by i.p. injection of kainic acid in control animals ($p > .2$). Furthermore, the infusion of fadrozole in the cerebral ventricle did not affect the number of hilar neurons in animals that received an i.p. injection of vehicle ($p > .2$). However, animals that were treated with both fadrozole and kainic acid showed a significant decrease in the number of hilar neurons compared to animals treated with vehicle ($p < .05$), to animals treated with kainic acid alone ($p < .005$), and to animals treated with fadrozole alone ($p < .01$).

DISCUSSION

The present findings indicate that inactivation of aromatase, the enzyme that catalyzes the synthesis of estrogens, results in increased vulnerability to excitotoxic degeneration *in vivo*. We have studied the effect of domoic and kainic acid, two well characterized neurotoxins, on hippocampal hilar neurons. Systemic administration of domoic acid in mice results in neurodegenerative damage in the hippocampal formation (Strain and Tasker, 1991). The neurodegenerative effects of domoic acid are mediated by an increase in intracellular calcium, as well as by an increased release of glutamate that results in the activation of NMDA receptors (Berman and Murray, 1997; Nijjar

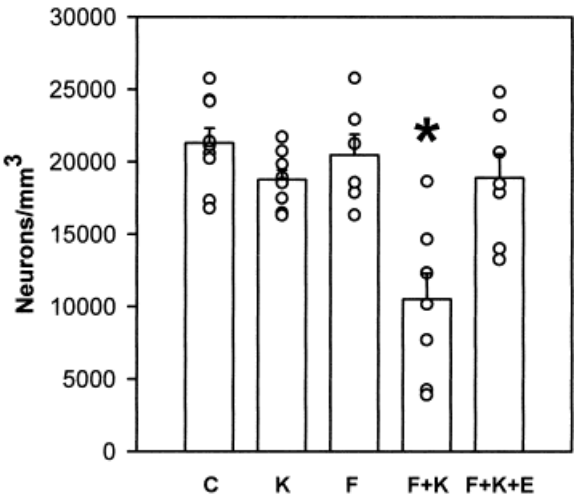


Figure 6 Number of Nissl-stained neurons (neurons/mm^3) in the hilus of the dentate gyrus of adult intact male rats. (C) Control adult males treated with vehicles ($n = 9$). (K) Animals injected with kainic acid ($n = 8$). (F) Animals treated with the aromatase inhibitor fadrozole ($n = 6$). (F+K) Animals treated with fadrozole and kainic acid ($n = 8$). (F+K+E) Animals treated with fadrozole, kainic acid, and estradiol ($n = 7$). Data are mean ±S.E.M. Circles are individual values. Asterisk, significant difference ($p < .05$) compared to the rest of the experimental groups.

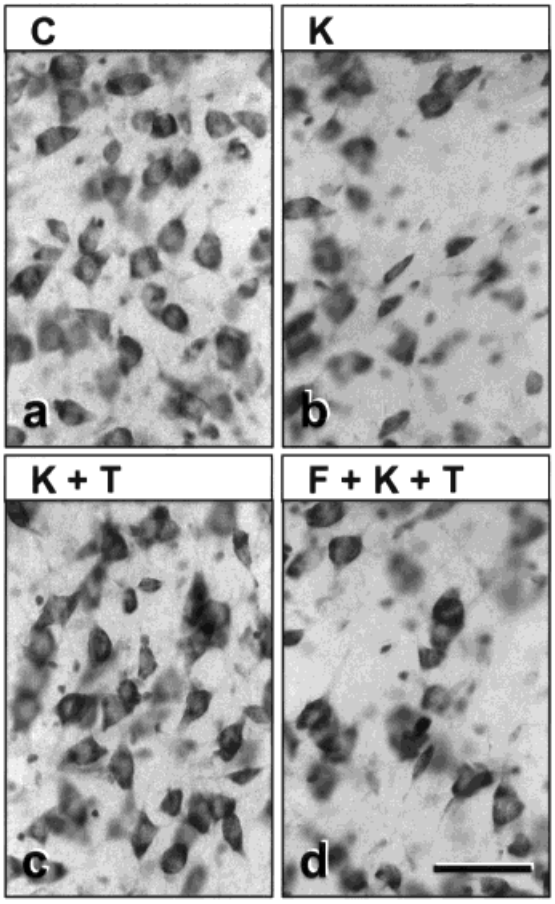


Figure 7 Effect of the systemic administration of the aromatase inhibitor fadrozole and the systemic administration of kainic acid on the number of hilar neurons in the dentate gyrus of castrated male rats. (a) Control (C) treated with vehicles. (b) Rat treated with kainic acid (K). (c) Rat treated with kainic acid (K) and testosterone (T). (d) Rat treated with fadrozole (F), kainic acid (K), and testosterone (T). All panels are at the same magnification. Scale bar, 50 μm.

and Nijjar, 2000). We used a low dose of domoic acid that did not affect hilar neurons in intact animals. However, the same low dose of domoic acid was able to induce significant neuronal loss in mice with reduced androgen levels as a result of castration, indicating that physiological levels of testicular secretions are neuroprotective. Furthermore, testosterone replacement prevented domoic acid-induced neuronal loss, indicating that this aromatase substrate protects hilar neurons from neurotoxic insults *in vivo*. Two metabolites of testosterone had different effects on hilar neurons. Estradiol, the product of the enzyme aromatase, was able to protect hilar neurons from domoic acid. In contrast, DHT, the 5α-reduced metabolite of testosterone and a nonaromatizable androgen, was not. These results are compatible with the possibility that the neuroprotective effect of testosterone in this experimental model may be, at least in part, due to its conversion to estradiol by the enzyme aromatase.

The role of aromatase in neuroprotection was directly tested using aromatase deficient mice. The number of hilar neurons was not significantly different between ArKO mice and their wild-type littermates, indicating that aromatase deficiency does not affect the normal development of the hilus or the maintenance of hilar neurons, at least in young adult animals. However, domoic acid, at a dose that was not effective in inducing neuronal loss in the hilus of wild-type mice, resulted in a significant decrease in the number of hilar neurons in ArKO mice. This finding indicates that aromatase deficiency increases the vulnerability of hilar neurons to neurotoxic degeneration.

Aromatase deficiency may increase neuronal vulnerability to degenerative stimuli either by preventing estradiol formation, by reducing testosterone degradation, or both. Indeed, ArKO mice have high serum levels of testosterone (7.06 $\pm$ 2.21 ng/mL in ArKO male mice versus 0.60 $\pm$ 0.25 ng/mL in wild-type males, unpublished results), and high testosterone levels may increase neuronal death in some experimental models of neurodegeneration *in vivo* (Hawk et al., 1998; Nishino et al., 1998; Garcia-Segura et al., 2001). To solve this question and to further test the

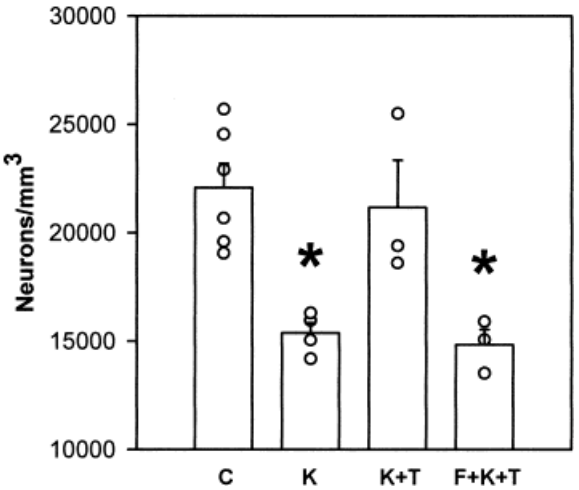


Figure 8 Number of Nissl-stained neurons (neurons/mm^3) in the hilus of the dentate gyrus of adult castrated male rats. (C) Control castrated males treated with vehicles (n = 6). (K) Animals injected with kainic acid (n = 4). (K+T) Animals treated with kainic acid and testosterone (n = 3). (F+K+T) Animals treated with the aromatase inhibitor fadrozole, kainic acid, and testosterone (n = 3). Data are mean $\pm$S.E.M. Circles are individual values. Asterisks, significant difference ($p < .05$) compared to the control group.

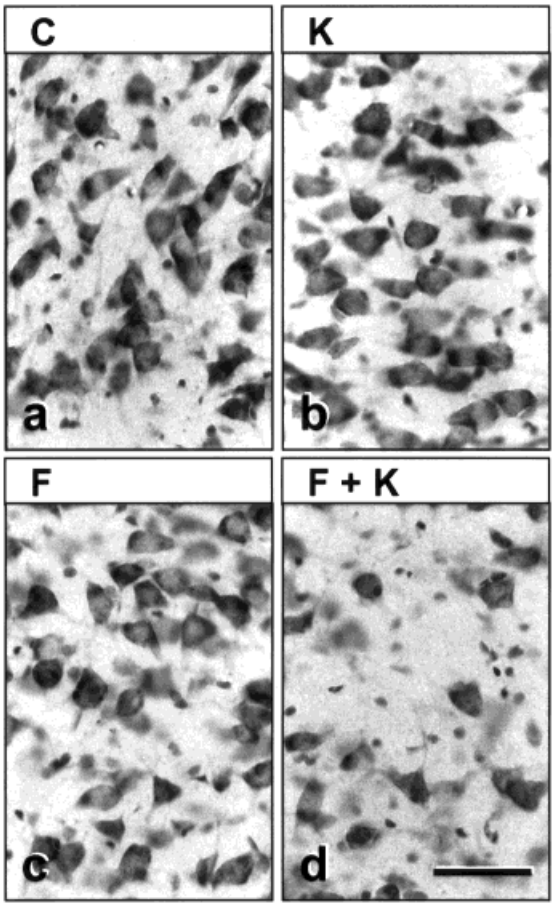


Figure 9 Effect of the intracerebroventricular administration of the aromatase inhibitor fadrozole and the systemic administration of kainic acid on the number of hilar neurons in the dentate gyrus of intact adult male rats. (a) Control rat (C) treated with vehicles. (b) Rat treated with kainic acid (K). (c) Rat treated with fadrozole (F). (d) Rat treated with fadrozole (F) and kainic acid (K). All panels are at the same magnification. Scale bar, 50 μm.

role of aromatase in neuroprotection by using an alternative model, rats were implanted with osmotic minipumps filled with the aromatase inhibitor fadrozole (Wozniak et al., 1992; Clancy et al., 1995) at a dose that has been shown to be effective in the inhibition of the enzyme (Clancy and Michael, 1994). To test the effect of inactivating aromatase in response to a neurotoxic stimuli, we used kainic acid, a well characterized neurotoxin for hilar neurons in the rat (Buckmaster and Dudek, 1997). Kainic acid was administered at a dose that does not affect hilar neurons in intact male rats, but results in significant neuronal loss in the hilus of castrated rats (Azcoitia et al., 1999a). As expected, the number of hilar neurons was not affected by kainic acid in control animals that were not treated with fadrozole. Furthermore, fadrozole alone did not affect the number of hilar neurons, indicating that fadrozole is not neurotoxic by itself. However, animals that were treated with both fadrozole and kainic acid showed significant neuronal loss, indicating that aromatase inhibition increases the neurodegenerative effect of kainic acid. This finding further supports the results obtained with the ArKO mice and confirms that aromatase is neuroprotective against excitotoxicity.

To determine whether the formation of estradiol is involved in the neuroprotective effect of aromatase, animals were treated with fadrozole, kainic acid, and estradiol. Estradiol treatment prevented the neurodegenerative effect of kainic acid in animals treated with fadrozole. This finding, showing that the neurodegenerative effect of aromatase deficiency is counterbalanced by the aromatase product estradiol, strongly suggests that the neuroprotective properties of aromatase lie in its ability to catalyze the formation of estradiol rather than in reducing testosterone levels.

To further test whether the aromatization of testosterone to estradiol is neuroprotective, castrated male rats were treated with kainic acid at a dose that causes neurodegeneration in the hilus (Azcoitia et al., 1999a). The neurodegenerative effect of kainic acid was blocked by testosterone, confirming the finding obtained in mice. However, testosterone was not able to block kainic acid-induced neurodegeneration in castrated male rats treated with fadrozole, suggesting

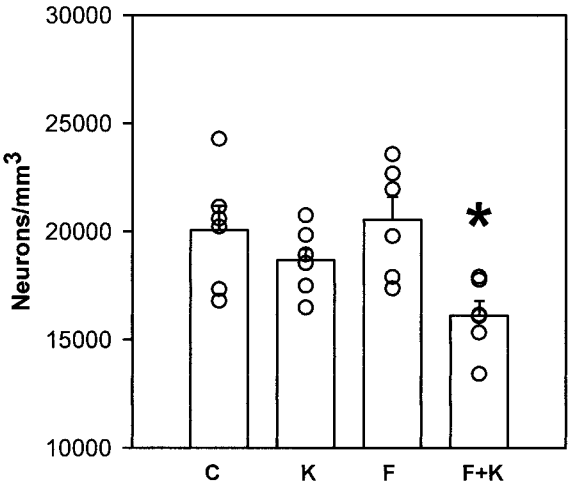


Figure 10 Number of Nissl-stained neurons (neurons/mm^3) in the hilus of the dentate gyrus of intact male rats. (C) Control adult male rats treated with vehicles (n = 6). (K) Animals treated with kainic acid (n = 6). (F) Rats infused in the lateral cerebral ventricle with the aromatase inhibitor fadrozole (n = 6). (F+K) Animals treated with fadrozole and kainic acid (n = 6). Data are mean ±S.E.M. Circles are individual values. The number of neurons in animals treated with kainic acid and fadrozole (asterisk) is significantly decreased ($p < .05$) compared to the rest of the experimental groups.

that testosterone exerts neuroprotective effects in this experimental model via conversion to estradiol. This finding gives additional support to the idea that aromatase plays a role in neuroprotection by promoting the formation of estradiol.

Because aromatase is up-regulated in the brain after a neurotoxic or mechanical lesion (Garcia-Segura et al., 1999a,b; Peterson et al., 2001), our hypothesis was that induction of brain aromatase may be part of the endogenous response by the neural tissue to reduce neurodegeneration. To determine whether local cerebral aromatase activity is involved in neuroprotection, fadrozole was infused in the lateral cerebral ventricle of male rats at a concentration within the range previously shown to inhibit aromatase in the rat brain (Bonsall et al., 1992; Clancy et al., 1995; Vagell and McGinnis, 1997). The aromatase inhibitor alone did not affect the number of neurons in the hilus, further indicating that aromatase activity is not indispensable to maintain hilar neurons under normal circumstances. As expected from our previous results, treatment with kainic acid did not affect the number of hilar neurons in animals infused in the lateral cerebral ventricle with vehicle. However, kainic acid resulted in significant neuronal loss in the hilus of animals infused in the cerebral ventricle with the aromatase inhibitor fadrozole. This finding indicates that local cerebral aromatase activity is involved in neuroprotection. Therefore, the increased expression of aromatase in the brain after injury (Garcia-Segura et al., 1999a,b; Peterson et al., 2001) may reduce neurodegeneration.

Although other possible mechanisms of action of brain aromatase cannot be excluded, our findings suggest that one of the main and most probable effects of the enzyme in preventing neuronal death is the conversion of androgens into estrogens, thereby increasing local estradiol levels in the damaged brain areas. Because aromatase is up-regulated in the adult central nervous system by different forms of lesions (Garcia-Segura et al., 1999b; Peterson et al., 2001), this enzyme may be a general neuroprotective factor in the brain. It is interesting to note that estrogen receptors are up-regulated as well in the brain after injury (Dubal et al., 1999). This suggests that the endogenous response of neural tissue to cope with neurodegenerative insults may include the induction of aromatase, the consecutive increase in the local production of estradiol, and the parallel increase in estrogen receptors, which are known to mediate neuroprotection by estradiol in several experimental models *in vivo* (Azcoitia et al., 1999b; Sawada et al., 2000; Veliskova et al., 2000; Wilson et al., 2000). In addition, aromatase may also promote neuroprotection by increasing local estradiol concentration to levels compatible with the antioxidant neuroprotective effects of the molecule (Behl and Holsboer, 1999). Further studies are necessary to extend neuroprotection by aromatase to alternative experimental models of neurodegeneration. Because aromatase is expressed in the adult human brain, including the hippocampus (Stoffel-Wagner et al., 1999), this enzyme may represent a new molecular target for the therapy or prevention of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases. The role of brain aromatase in neuroprotection calls for the development of new therapeutic strategies aimed at the up-regulation of the enzyme in the brain without affecting its expression in other tissues. The neuroprotective role of brain aromatase should also be taken into consideration when aromatase inhibitors are used for the treatment of certain forms of cancer.

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