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Endogenous Estrogen Formation Is Neuroprotective in Model of Cerebellar Ataxia

Amanda Sierra,[1] Íñigo Azcoitia,[2] and Luis Miguel Garcia-Segura[1]

[1]Instituto Cajal, C.S.I.C., Madrid, Spain; and [2]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain

The expression of aromatase, the enzyme that transforms testosterone into estradiol, was analyzed by reverse transcriptase polymerase chain reaction in the inferior olive of adult male rats. The expression of this messenger in the inferior olive suggests that this brain area may be able to synthesize estradiol. The neuroprotective role of estradiol in the inferior olive was then assessed in a model of cerebellar ataxia, achieved by the ip administration of 3-acetylpyridine (3-AP). In a first experiment, male Wistar rats were orchidectomized to diminish the plasmatic levels of testosterone, the direct precursor of estradiol. Inmediately after castration, animals were implanted with a silicone tube that was either empty or filled with estradiol. One week later, animals were injected with 3-AP. Estradiol treatment resulted in a significant reduction in neuronal death in the olive. In a second experiment, animals were treated with fadrozole, an aromatase inhibitor, to assess the role of endogenous estradiol formation in neuroprotection. The results show that the inhibition of aromatase activity, and therefore the decrease in endogenous estrogen formation, increases the death in inferior olive. In conclusion, this study indicates that the inferior olive is a steroidogenic tissue and that olivary neurons are protected by exogenous and endogenous estradiol.

Key Words: Aromatase; estradiol; neuroprotection; inferior olive.

Introduction

Decreasing levels of estradiol after menopause in women are associated with loss in cognitive function, the progression of neurodegenerative disorders, increased depressive symptoms, and other neurologic and psychologic disturbances *(1–10)*. This suggests that the decrease in estradiol levels after menopause may have a negative impact on brain function. In agreement with this assumption, massive evidence from animal studies indicates that estradiol is neuroprotective (for recent reviews see refs. *11–16*). Many studies have assessed the neuroprotective effect of estradiol either after the addition of the hormone to neuronal cultures or after its systemic administration to adult female rodents. However, the potential neuroprotective role of endogenous estradiol formation has been in general neglected.

Androgenic C19 steroids (testosterone and androstenedione) are transformed into estrogenic C18 steroids (estradiol and estrone) by the activity of the enzyme aromatase *(17)*. This enzyme consists on two proteins, the cytochrome p450Aro (CYP19) and NADPH cytochrome p450 reductase. We have recently reported that aromatase is neuroprotective in the hippocampus. Aromatase knockout male mice are more susceptible than wild animals to excitotoxic hippocampal neurodegeneration. Furthermore, the inhibition of aromatase activity in male rats enhances excitotoxicity, an effect that is reverted by the administration of exogenous estradiol *(18)*. These findings suggest that endogenous formation of estradiol may be protective for hippocampal neurons.

To determine whether aromatase and endogenous estradiol formation have neuroprotective effects in areas other than the hippocampus, we used a well-established model of cerebellar ataxia: the degeneration of rat inferior olivary nucleus after treatment with 3-acetylpyridine (3-AP), an antimetabolite of nicotinamide. The destruction of the inferior olive results in loss of climbing fiber input to cerebellar Purkinje neurons *(19)*, and this deafferentiation leads to ataxia *(20)*. In the present study, we assessed the expression of aromatase in the inferior olivary nucleus as well as the effect of estradiol and the aromatase inhibitor fadrozole (FAD) on the survival of inferior olivary neurons after 3-AP treatment.

Results

Inferior Olive Expresses Aromatase

The expression of mRNA for aromatase was assessed by reverse transcriptase polymerase chain reaction (RT-PCR). A band corresponding to the expected size for aromatase was obtained in the samples from the inferior olive (Fig. 1).

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Author to whom all correspondence and reprint requests should be addressed: Luis M. Garcia-Segura, Instituto Cajal, C.S.I.C., Avenida Dr. Arce, 37, E-28002, Madrid, Spain. E-mail: lmgs@cajal.csic.es

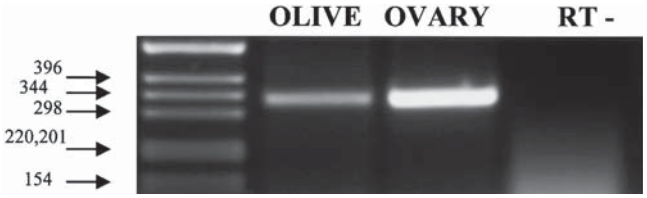

Fig. 1. Aromatase mRNA expression in inferior olive and ovary (as positive control). Numbers on the left represent known molecular weights, in base pairs. The expected PCR product for aromatase has 330 bp. The fourth lane is a negative control for genomic contamination, PCR amplification of RNA with no retrotranscription.

Additionally, the ovary, a steroidogenic tissue with high expression of aromatase, was positive for the amplification with the same protocol. Sequencing confirmed the specificity in relation to the sequence published in GeneBank. The percentage of alignment was 76.3% for the sense chain of aromatase and 87.3% for the antisense.

Estradiol Protects Inferior Olivary Neurons from 3-AP Toxicity

An experiment was designed to determine whether 17β-estradiol is able to prevent the cell loss caused by 3-AP in the inferior olive. Control animals (orchidectomized) were compared with animals treated with 3-AP or with 3-AP plus estradiol. The estradiol treatment protocol used resulted in serum levels of the hormone of 26.79 ± 7.51 pg/mL at the day of death, as measured by radioimmunoassay (RIA); estradiol was undetectable in untreated animals. In addition, RIA for testosterone revealed undetectable levels in the control and estradiol groups.

The visual inspection of inferior olive in Nissl-stained sections revealed an apparent neuronal loss in animals treated with 3-AP compared with control animals. The loss of Nissl-stained neurons was apparently reduced by the administration of estradiol (Fig. 2). This qualitative impression was confirmed by quantitative analysis. The quantification of neuronal numbers in the inferior olive using Nissl sections was extremely difficult and unreliable, given the high density of neurons in control animals. To avoid this problem, we used sections immunostained with the specific neuronal nuclear marker NeuN (Fig. 2). The different treatments resulted in a significant change in the number of neurons in the medial nucleus of the inferior olive (MIO) (χ^2 [2] = 12.490; p = 0.002). Treatment with 3-AP caused a 40% neuronal loss in castrated males. Chronic administration of estradiol prevented neuronal loss induced by 3-AP (Fig. 3).

In contrast to the effect of the treatments on the number of neurons, no apparent differences were observed in the intensity of NeuN staining among the experimental groups. No significant differences in the cross-sectional area of the medial inferior olive were detected (data not shown), indicating that the differences in neuronal counts reflect differences in the total neuronal number and not mere differences in cell density.

To evaluate cell death, sections were stained with Fluoro-Jade. The treatments had a significant effect on the number of Fluoro-Jade–stained neurons (χ^2 [2] = 11.649; p = 0.003) (Fig. 4). Fluoro-Jade staining was undetectable in the inferior olive of control animals. Animals treated with estradiol and 3-AP had a mean of 7 ± 4 stained neurons per olive. By contrast, animals injected with 3-AP in the absence of estradiol showed 73 ± 13 neurons/olive (p = 0.004 vs the control group and p = 0.005 vs estradiol plus 3-AP).

Aromatase Inhibition Increases Neurodegeneration after 3-AP Toxicity

To test the role of aromatase and estradiol synthesis in neuroprotection, animals were treated with FAD, a pure inhibitor of aromatase. FAD alone had no significant effect on neuronal number. The number of NeuN-immunoreactive neurons was significantly decreased in animals treated with 3-AP and FAD compared with animals treated with 3-AP alone (70 and 30% of neuronal loss with respect to the control group, respectively; χ^2 [4] = 27.806; $p < 0.001$) (Figs. 5 and 6). Furthermore, the administration of estradiol to animals treated with FAD and 3-AP reverted the effect of FAD.

In parallel to the decrease in the number of neurons stained with NeuN after treatment with FAD and 3-AP, there was an increase in the number of cells stained with Fluoro-Jade (χ^2 [4] = 16.534; p = 0.002) (Fig. 4). The number of Fluoro-Jade–stained neurons raised from 38 ± 21 in 3-AP–treated animals to 206 ± 40 in animals that received 3-AP plus FAD (p = 0.03). Estradiol-treated animals had a mean of 23 ± 8 neurons/olive (p = 0.02 vs FAD + 3-AP). Fluoro-Jade–stained neurons were detected neither in control animals nor in animals treated with FAD alone.

Correlation Between NeuN Immunostaining and Fluoro-Jade Staining

The apparent opposite relationship between the two variables measured (number of NeuN-immunoreactive neurons as a measure of neuronal survival and number of Fluoro-Jade–stained neurons as an indication of cell death) was confirmed by statistical analysis (Fig. 7). There was a relatively high correlation between the number of NeuN-positive cells in the medial inferior olive and the number of Fluoro-Jade–positive cells in the whole inferior olive. The correlation coefficient was R^2 = 0.8193. Analysis of variance for regression was significant ($p < 0.001$), and, as expected, the slope was negative ($p < 0.001$ vs being null).

Discussion

Previous studies have shown that inferior olivary neurons express estrogen receptors *(21)* and that inferior olive function is affected by estradiol *(22)*. These findings, together with

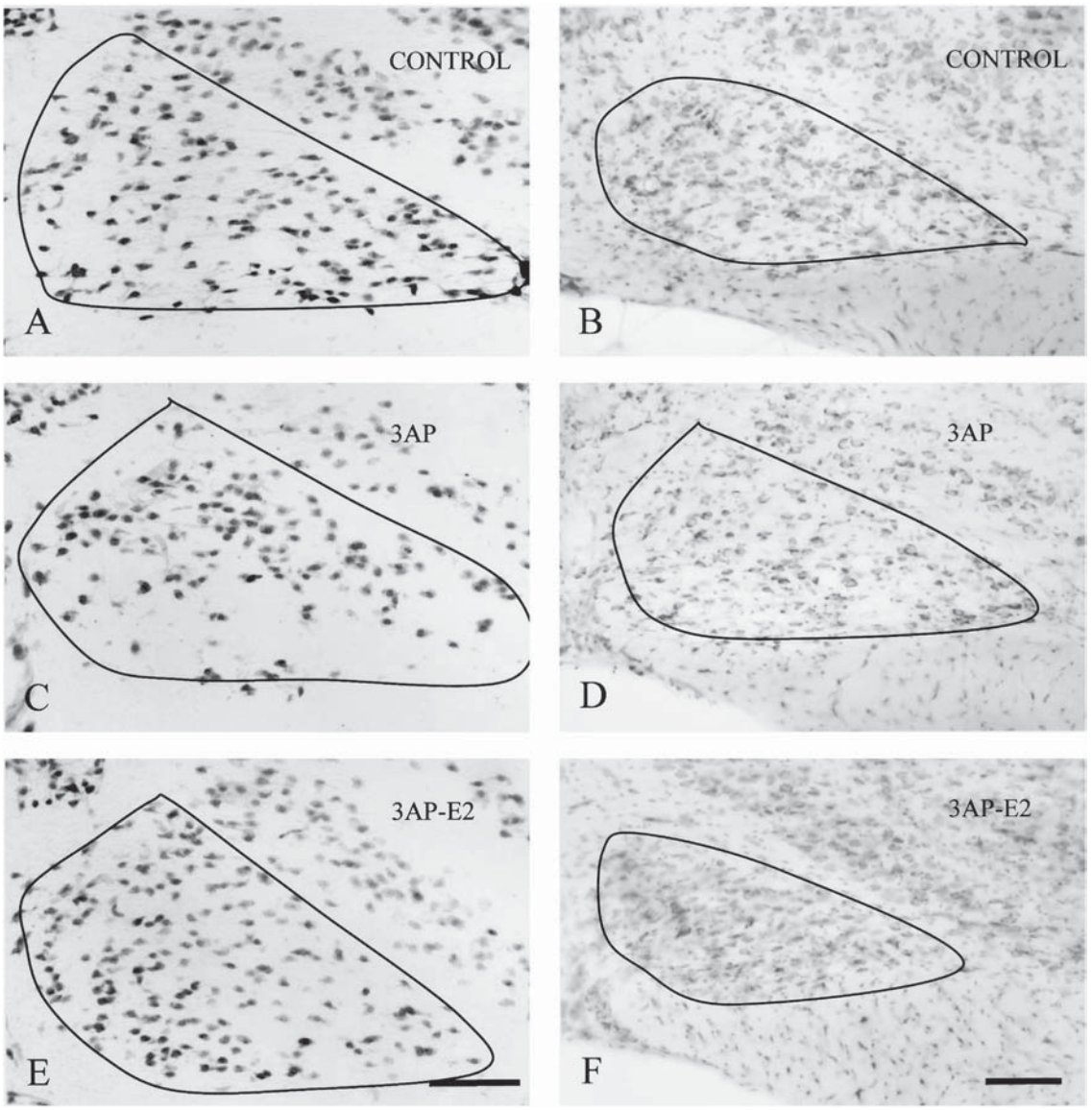

Fig. 2. Representative sections from medial nucleus of inferior olive of orchidectomized male rats. (**A,C,E**) Sections immunostained with NeuN; (**B,D,F**) sections Nissl-stained with toluidine blue. (**A,B**) Control rats were injected with vehicle. (**C,D**) Rats were injected with 3-AP. (**E,F**) Rats were treated with estradiol (E2) and 3-AP. Chronic administration of estradiol prevented the neuronal death produced by the neurotoxin. Bar = 200 μm.

the present observation of the expression of aromatase in the inferior olive of male rats, suggest that estradiol may play an important role in this nucleus. Here, we have assessed whether estradiol is a neuroprotectant for inferior olivary neurons in male rats. Systemic administration of 3-AP is a well-recognized model of inferior olive neurodegeneration and cerebellar ataxia *(23)*. This toxin produces metabolic stress because it interferes with the formation of NAD^+ (nicotinamide adenine dinucleotide). Olivary neurons have a very high metabolic rate and are therefore very sensitive to 3-AP toxicity. The effect of estradiol administration on the survival of inferior olivary neurons was assessed in male rats treated with 3-AP. In the present study, animals were castrated to reduce plasma testosterone levels and, therefore, to reduce the formation of estradiol from its circulating precursor. Indeed, castration resulted in undetectable levels of both hormones in plasma. Estradiol administration, under this condition, resulted in a significant protection of inferior olivary neurons against 3-AP.

The toxicity of 3-AP was qualitatively assessed by Nissl-staining and by a semiquantitative analysis of neurons stained with Fluoro-Jade, a marker of dying cells *(24)*. Previous studies have shown the validity of Fluoro-Jade as a stain suitable for detecting necrotic neurons in inferior olive after 3-AP administration *(25)*. Furthermore, the number of surviving inferior olivary neurons was assessed by unbiased morphometry using NeuN, a specific neuronal marker *(26)*. Our findings indicate that the number of NeuN-immunoreactive sur-

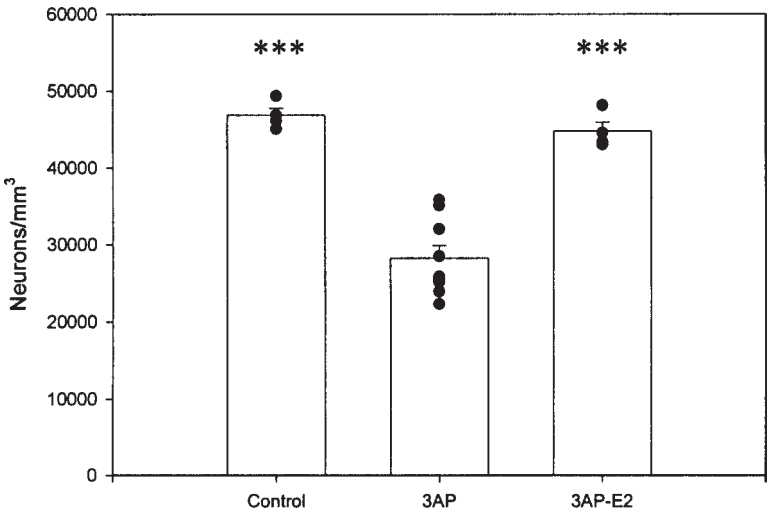

Fig. 3. Number of NeuN-immunoreactive neurons in medial inferior olive in castrated male rats treated with vehicles (Control, $n = 4$), 3-AP ($n = 9$), or 3-AP and estradiol (3-AP–E2, $n = 4$). 3-AP was neurotoxic for the olivary neurons and resulted in a 40% neuronal loss ($p < 0.001$ vs the control group) in castrated male rats. Estradiol administration abolished the effect of 3-AP ($p < 0.001$). Data represent individual values (dots) and means ± SEM. Asterisks indicate significant differences with respect to the 3-AP group.

viving neurons is inversely correlated with the number of Fluoro-Jade–stained dying cells in the inferior olive when animals from different groups are compared. This indicates that the combination of NeuN immunoreactivity and Fluoro-Jade staining is a reliable method to assess neuronal viability in the inferior olive after 3-AP toxicity.

Administration of 3-AP resulted in a significant loss of NeuN-immunoreactive neurons in male rats. The magnitude of neuronal loss in our study is in agreement with that observed in previous studies using calbindin-D28k immunoreactivity, another neuronal marker for inferior olivary neurons *(23)*. Since sex steroids are known to affect calbindin-D28k expression in the brain *(27)*, we decided to use a different neuronal marker, NeuN, for morphometric analysis. The number of NeuN-immunoreactive neurons that survived to 3-AP was significantly increased in estradiol-treated animals. The neuroprotective effect of estradiol was accompanied by a decrease in the number of Fluoro-Jade staining in the inferior olive.

Having determined that exogenous estradiol was neuroprotective for inferior olivary neurons, our next goal was to determine whether endogenous estradiol formation was neuroprotective as well. Estradiol may be formed from aromatization of testosterone in the periphery or in the brain *(18)*. As our present results indicate, the inferior olive is also a potential area for estradiol biosynthesis. To test the role of endogenous estradiol formation in neuroprotection against 3-AP, we used FAD, a pure and nonsteroidal inhibitor of the enzyme aromatase *(28)*. The dose used in our study is known to inhibit more than 90% of total enzyme activity including brain aromatase *(29)*. The inhibition of aromatase, and thus the blockade of estradiol biosynthesis, enhanced the injury produced by 3-AP in the inferior olive. FAD treatment decreased the number of NeuN-expressing neurons that survived to 3-AP, and increased the number of Fluoro-Jade–stained dying cells. Moreover, administration of exogenous estradiol was capable of reverting the toxicity produced by the combination of FAD plus 3-AP. This suggests that the toxic effect of FAD is owing to the inhibition of aromatase and not to another unknown effect of the drug. Therefore, we may conclude that endogenous estradiol is neuroprotective for inferior olivary neurons. Our results do not permit differentiation between brain and peripheral estradiol production, but the possibility exists that FAD inhibits aromatase in the inferior olive as well as in peripheral tissues.

In conclusion, our findings indicate that the inferior olive expresses the messenger for the enzyme involved in estradiol biosynthesis and that the inferior olive is a target for estradiol. Furthermore, our data indicate that endogenous estradiol in male rats exerts a neuroprotective effect on inferior olivary neurons. Therefore, estradiol may be important for the maintenance of motor function that is under the control of the olivocerebellar system. The decline in estrogen formation with aging may thus contribute to age-related disturbances in motor function by affecting inferior olivary nucleus. Moreover, the neuroprotective effects of estradiol in the olivocerebellar system may contribute to the decreased risk of falling and better postural balance observed in postmenopausal women receiving estrogens *(30,31)*.

Materials and Methods

Animals

Wistar albino male rats weighting 250–300 g were maintained on a 12:12 h dark:light cycle, with free access to chow and water. Manipulation of the animals was performed fol-

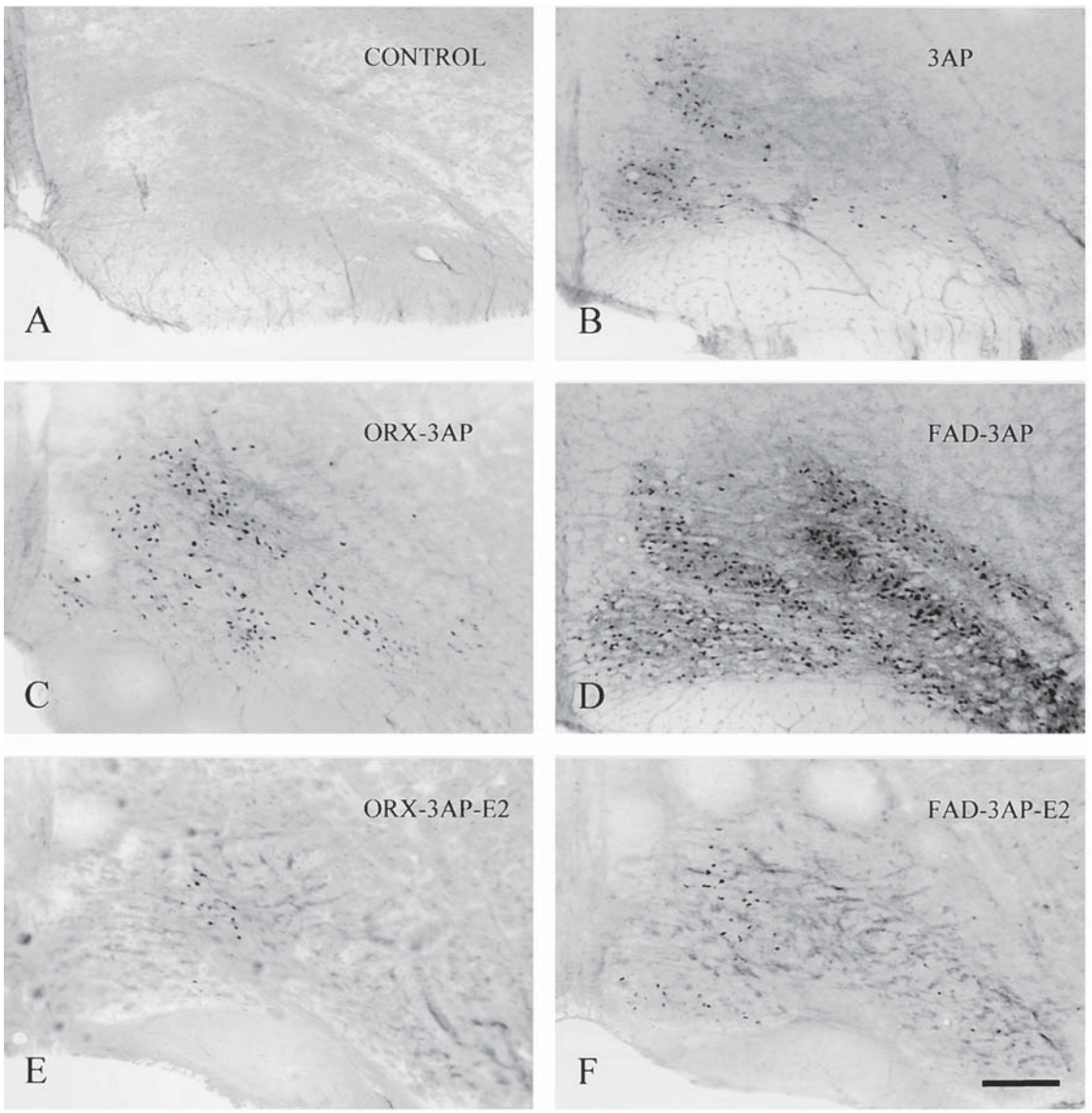

Fig. 4. Representative sections of inferior olive stained with Fluoro-Jade. (**A**) Control intact animal treated with vehicles; (**B**) intact animal treated with 3-AP; (**C**) orchidectomized animal treated with 3-AP; (**D**) intact animal treated with aromatase inhibitor FAD and 3-AP; (**E**) orchidectomized animal treated with 3-AP and estradiol (E2); (**F**) intact animal treated with FAD, 3-AP, and estradiol. In control and orchidectomized animals, 3-AP induced the appearance of acidic neurons stained with Fluoro-Jade. The administration of FAD increased the number of degeneration neurons. This situation is reversed in the presence of estradiol. Bar = 200 μm.

lowing 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.

RNA Isolation and RT-PCR Analysis

Total RNA from the inferior olive and surrounding tissue (pyramidal tracts and part of the reticular nucleus) and from the ovary were isolated with TRIzol Reagent (Gibco, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Five micrograms of the samples for the olive, and 2 μg for the ovary, were used for first-strand synthesis with M-MLV Reverse Transcriptase (Promega, Madison, WI) and random primers (Gibco) for 1 h at 37ºC, followed by inactivation at 70ºC for 15 min. Then, 4 μL was used for amplification by PCR with 0.3 μL of Taq DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 50 μL, in a thermal cycler (Mastercycler; Eppendorf, Hamburg, Germany). For aromatase amplification the primers used were TAT TGG AAA TGC TGA TTG CGG for sense and TTG GGC TTG GGG AAA TAC TCG for antisense (accession code for GeneBank M33986); the resulting fragment had 313 bp. Primers were used at a final concentration of 0.4 μ*M*. To ensure that no genomic amplification is detected, the pair of primers was designed to

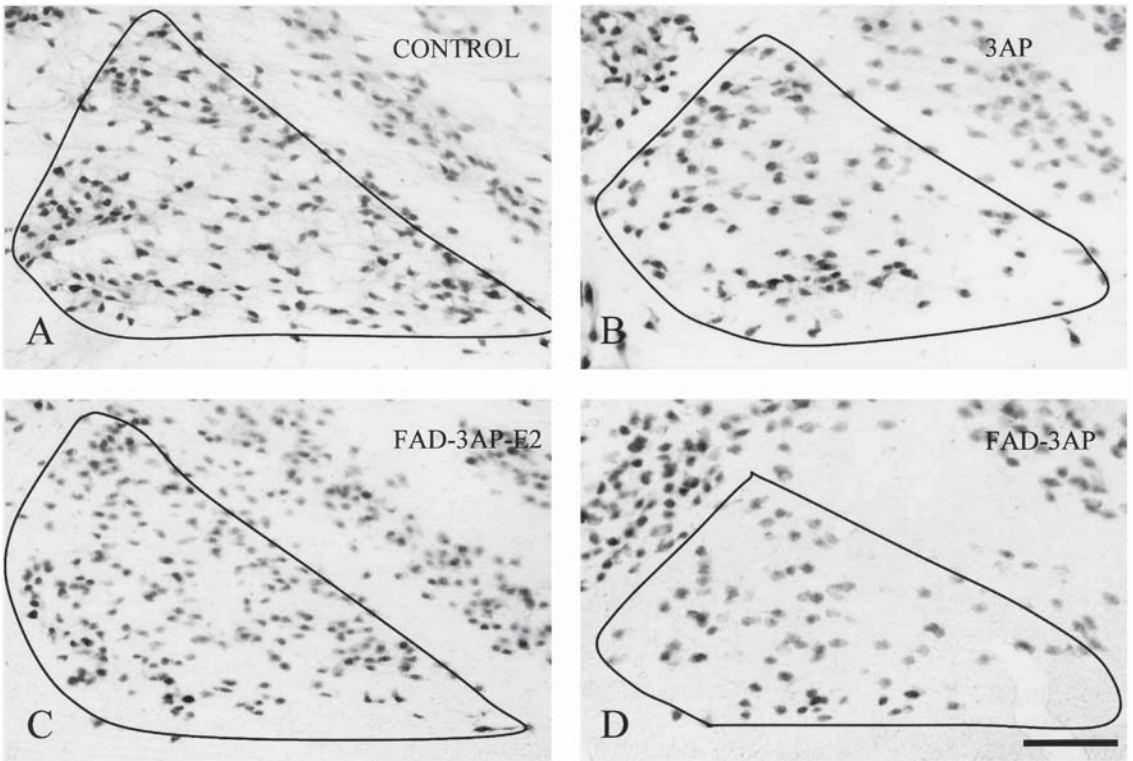

Fig. 5. Representative histologic sections of MIO showing immunostaining for NeuN. (**A**) intact control rat treated with vehicles; (**B**) rat treated with 3-AP; (**C**) rat treated with aromatase inhibitor FAD, 3-AP, and estradiol; (**D**) rat treated with FAD and 3-AP. The aromatase inhibitor enhanced neuronal death produced by 3-AP. Estradiol prevented neuronal death caused by 3-AP and FAD. Bar = 100 μm.

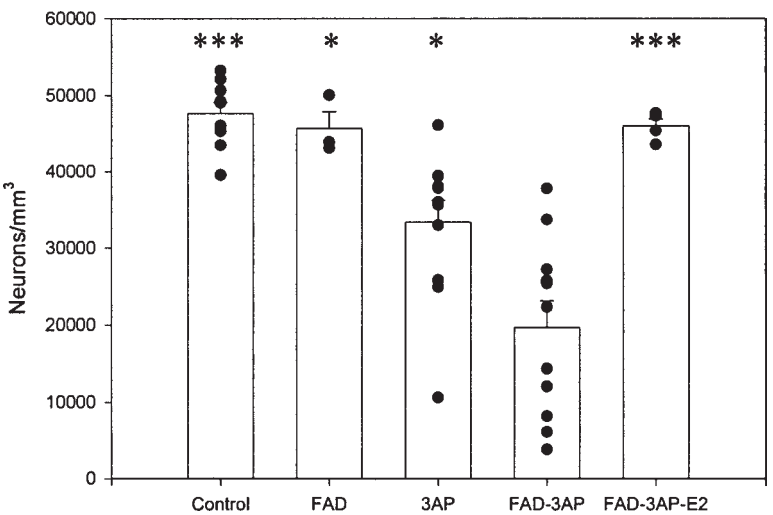

Fig. 6. Number of NeuN-immunoreactive neurons in medial olivary nucleus in intact male rats treated with vehicles (Control, $n = 9$); FAD ($n = 3$); 3-AP ($n = 11$); FAD and 3-AP (FAD–3-AP, $n = 11$); or FAD, 3-AP, and estradiol (FAD–3-AP–E2, $n = 4$). The toxic effect of 3-AP ($p = 0.003$ vs the control group) was enhanced by the chronic treatment with FAD ($p < 0.001$ vs the control group and $p < 0.05$ vs the 3-AP group). This effect was reverted by estradiol ($p < 0.001$ vs FAD plus 3-AP). Data represent individual values (dots) and means ± SEM. Asterisks indicate significant differences with respect to the FAD–3-AP group.

hybridize a fragment that includes some introns; in case of DNA presence, the amplified fragment would be much longer. The amplification protocol consisted of 35 cycles with 1 min of denaturation at 95ºC, 1 min of annealing at 65ºC, and 1 min of elongation at 72ºC. Images from 1.5% (w/v) ethidium bromide–stained agarose gels were captured with GelPrinter (TDI, Madrid, Spain).

To assess the reliability of the amplification products, bands were excised and DNA was purified using the QIAquick Gel Extraction Kit (Quiagen Gmbg, Hilden, Germany). One

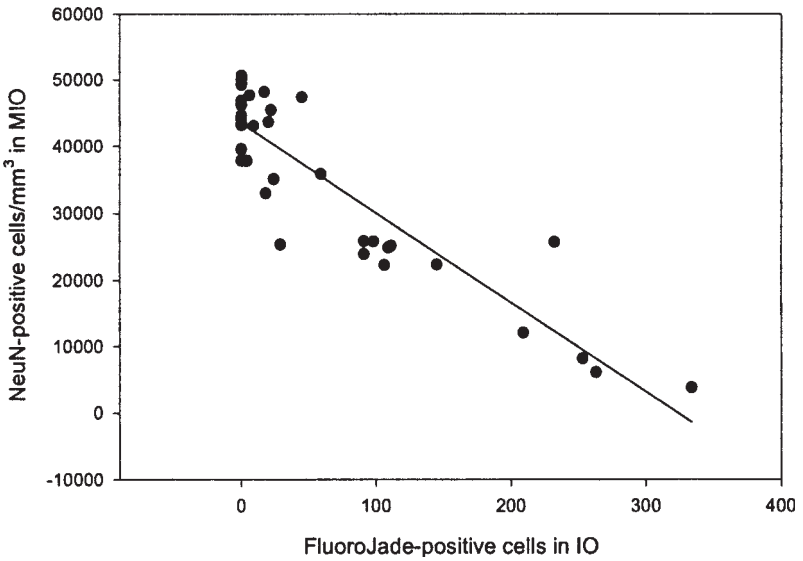

Fig. 7. Linear regression analysis between neuronal survival (NeuN-positive cells in MIO) and neuronal death (Fluoro-Jade–immunoreactive cells in inferior olive). The plot shows a high negative correlation between the two variables (R^2 = 0.8193).

hundred nanograms of each sample was sequenced (Automatic Sequencing Center, CSIC, Madrid, Spain) with the same sense and antisense primers. The obtained sequence was aligned with the expected sequence obtained from GeneBank.

Effect of Estradiol Administration in 3-AP–Treated Animals

To assess whether estradiol protects inferior olivary neurons, animals were bilaterally orchidectomized under 2,2,2-tribromoethanol anesthesia (0.2 g/kg; Fluka Chemika AG, Buchs, Switzerland) in order to reduce circulating testosterone that may be converted into estradiol. Immediately after castration, intrascapular silastic tubes (Nalgene 550 Silicone Tubing 8060-0020, 1.57 mm id, 0.317 mm od, Nalge, Rochester, NY) were implanted between scapulae. A group of animals was implanted with silastic tubes filled with 17β-estradiol (1:1 in cholesterol, 5 mm; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Another group of animals was implanted with empty tubes. One week later, animals were injected intraperitoneally with 3-AP (30 mg/kg in saline; Sigma) or with vehicle. This dose has been tested previously and causes a significant neuronal loss in the inferior olive *(32)*.

Effect of Aromatase Inhibition on 3-AP Toxicity

Intact male rats were anesthetized with tribromoethanol, and an osmotic minipump (flow rate of 0.5 μL/h, Alzet 2002; Alza, Palo Alto, CA) was subcutaneously implanted between the scapullae. Before implantation, the pumps were filled with either saline or the aromatase inhibitor FAD (4.16 mg/mL in saline, CGS16949A; Ciba-Geigi, Basel, Switzerland). Animals were implanted with estradiol tubes or with empty tubes. Seven days after implantation, animals received 30 mg/kg of 3-AP or vehicle.

Histology

One week after 3-AP administration, animals were submitted to cardiac puncture under pentobarbital anesthesia (75 mg/kg) to obtain blood samples. These samples were centrifuged twice at 1100*g* for 30 min at 4ºC to separate the sera and then kept at –20ºC until estradiol and testosterone RIA (Ultra-sensitive Estradiol RIA from Diagnostic Systems, Webster, TX; and Coat-A-Count Free Testosterone RIA from Diagnostic Product, Los Angeles, CA). The animals were then perfused through the ascending aorta with 50 mL of saline followed by 300 mL of 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 4ºC and rinsed in buffer. The rhombencephalon was dissected out and coronal Vibratome sections were cut at 50 μm.

To evaluate neuronal survival, three consecutive sections (taking as reference the coordinates –12.80 mm from bregma *[33]*; Fig. 8) for each animal were immunostained with NeuN, a specific neuronal marker. This protocol resulted in a high homogeneity in the data from animals of the same group. Sections were first quenched in 1% hydrogen peroxide, 50% methanol in phosphate-buffered saline (PBS) for 10 min, then rinsed with PBS and incubated overnight with the NeuN antibody (1:5.000 in PBS; Chemicon, Temecula, CA) diluted in PBS with 0.3% bovine serum albumin and 0.3% Triton X-100. Sections were then rinsed in the same medium and incubated for 2 h at room temperature in biotinylated goat anti–mouse IgG (Pierce, Rockford, IL) diluted 1:250. Immunostaining was amplified with the avidin-biotin

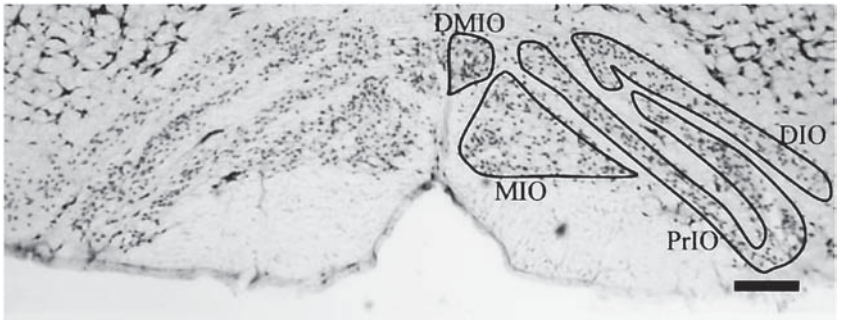

Fig. 8. Different nuclei of inferior olive, at Paxinos and Watson *(33)* coordinates: –12.80 mm from bregma, stained with NeuN antibody. DMIO, dorsomedial; MIO, medial; DIO, dorsal; PrIO, principal. Bar = 200 μm.

system (ImmunoPure ABC Peroxidase Staining Kit, Pierce) for 90 min, and peroxidase was detected using diaminobenzidine as chromogen. Other sections were Nissl stained with Toluidine blue.

Cell death was evaluated using Fluoro-Jade (Histochem, Jefferson, AR), an anionic dye reported to selectively stain degenerating neurons *(24)*, according to the protocol described by Poirier et al. *(34)*. Briefly, mounted sections were immersed for 17 min in 0.06% potassium permanganate, rinsed in water, and then stained at room temperature for 30 min with 0.001% Fluoro-Jade in 0.1% acetic acid.

Morphometric Analyses

The number of NeuN-positive cells in the MIO was estimated by the optical disector method as described by Howard and Reed *(35)*, using total section thickness for disector height *(36)* and a counting frame of 55 μm × 55 μm. A total of 100 counting frames were assessed per animal. Section thickness was measured using a digital-length gage device (Heidenhain-Metro MT 12/ND221; Traunreut, Germany), attached to the stage of a Leitz microscope. Cell nuclei that came into focus while focusing down through the disector height were counted. MIO areas were estimated by using a randomly translated point grid with a known area associated with each point *(35)*. All counts were performed blind. Microphotographs were taken with a Nikon digital camera.

Cell death was evaluated by simple count of Fluoro-Jade–positive cells in the whole inferior olive in a Leica Microscope under a 450-nm filter. It is not possible to evaluate Fluoro-Jade sections by the optical disector method because the areas cannot be fully delimited and the section thickness cannot be properly estimated. Two sections were evaluated by this method for each animal. These semiquantitative measures were used to have a correlation between neuronal survival and cell death for each animal.

Statistical Analyses

Data of each experiment were assessed for homogeneity of variances using Levene's statistic. Since the variances of the data were not homogeneous, analysis was carried out using a Kruskal-Wallis test followed by a Games-Howel test. In the evaluation of cellular death, linear regression analysis was done. Differences with $p < 0.05$ were considered significant. All analyses were done using the statistical program SPSS (SPSS, Chicago, IL). All values represent mean ± SEM. The number of animals studied for each experimental group is indicated in the figure legends.

Acknowledgments

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References

1. Fillit, H., Weinreb, H., Cholst, I., et al. (1986). *Psychoneuroendocrinology* **11,** 337–345.
2. Paganini-Hill, A. (1995). *Prog. Cardiovasc. Dis.* **38,** 223–242.
3. Paganini-Hill, A. and Henderson, V. W. (1996). *Arch. Intern. Med.* **156,** 2213–2217.
4. Haskell, S. G., Richardson, E. D., and Horwitz, R. I. (1997). *J. Clin. Epidemiol.* **50,** 1249–1264.
5. Saunders-Pullman, R., Gordon-Elliott, J., Parides, M., Fahn, S., Saunders, H. R., and Bressman, S. (1999). *Neurology* **52,** 1417–1421.
6. Sherwin, B. (1999). *J. Psychiatry Neurosci.* **24,** 315–321.
7. Strijks, E., Kremer, J. A., and Horstink, M. W. (1999). *Clin. Neuropharmacol.* **22,** 93–97.
8. Wolf, O. T., Kudielka, B. M., Hellhammer, D. H., Torber, S., McEwen, B. S., and Kirschbaum, C. (1999). *Psychoneuroendocrinology* **24,** 727–741.
9. Yaffe, K., Browner, W., Cauley, J., Launer, L., and Harris, T. (1999). *J. Am. Geriatr. Soc.* **47,** 1176–1182.
10. Asthana, S., Baker, L. D., Craft, S., et al. (2001). *Neurology* **57,** 605–612.
11. Behl, C. and Manthey, D. (2000). *J. Neurocytol.* **29,** 351–358.
12. Green, P. S. and Simpkins, J. W. (2000). *Int. J. Dev. Neurosci.* **18,** 347–358.
13. Wise, P. M., Dubal, D. B., Wilson, M. E., and Rau, S. W. (2000). *J. Neurocytol.* **29,** 401–410.
14. Garcia-Segura, L. M., Azcoitia, I., and DonCarlos, L. L. (2001). *Prog. Neurobiol.* **63,** 29–60.

15. Lee, S. J. and McEwen, B. S. (2001). *Annu. Rev. Pharmacol. Toxicol.* **41,** 569–591.
16. Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W., Bottner, M., and Rosewell, K. L. (2001). *Brain Res. Brain Res. Rev.* **37,** 313–319.
17. Lephart, E. D. (1996). *Brain Res. Brain Res. Rev.* **22,** 1–26.
18. Azcoitia, I., Sierra, A., Veiga, S., Honda, S., Harada, N., and Garcia-Segura, L. M. (2001). *J. Neurobiol.* **47,** 318–329.
19. Baetens, D., Garcia-Segura, L. M., and Perrelet, A. (1982). *Exp. Brain Res.* **48,** 256–262.
20. Fernandez, A. M., Gonzalez de la Vega, A. G., Planas, B., and Torres-Aleman, I. (1999). *Eur. J. Neurosci.* **11,** 2019–2030.
21. Shughrue, P. J., Lane, M. V., and Merchenthaler, I. (1997). *J. Comp. Neurol.* **388,** 507–525.
22. Smith, S. S. (1998). *Neuroscience* **82,** 83–95.
23. Fernandez, A. M., Gonzalez de la Vega, A. G., and Torres-Aleman, I. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 1253–1258.
24. Schmued, L. C., Albertson, C., and Slikker, W. Jr. (1997). *Brain Res.* **751,** 37–46.
25. Krinke, G. J., Classen, W., Vidotto, N., Suter, E., and Wurmlin, C. H. (2001). *Exp. Toxicol. Pathol.* **53,** 365–372.
26. Mullen, R. J., Buck, C. R., and Smith, A. M. (1992). *Development* **116,** 201–211.
27. Stuart, E. B., Thompson, J. M., Rhees, R. W., and Lephart, E. D. (2001). *Brain Res. Dev. Brain Res.* **129,** 125–133.
28. Bhatnagar, A. S., Hausler, A., Schieweck, K., Browne, L. J., Bowman, R., and Steele, R. E. (1990). *J. Steroid Biochem. Mol. Biol.* **37,** 363–367.
29. Clancy, N. and Michael, R. P. (1994). *Neuroendocrinology* **59,** 552–560.
30. Naessen, T., Lindmark, B., and Larsen, H. C. (1997). *Am. J. Obstet. Gynecol.* **177,** 412–416.
31. Randell, K. M., Honkanen, R. J., Komulainen, M. H., Tuppurainen, M. T., Kroger, H., and Saarikoski, S. (2001). *Clin. Endocrinol. (Oxf.)* **54,** 769–774.
32. Fernandez, A. M., Garcia-Estrada, J., Garcia-Segura, L. M., and Torres-Aleman, I. (1997). *Neuroscience* **76,** 117–122.
33. Paxinos, G. and Watson, C. (1986). *The rat brain in stereotaxic coordinates*. Academic: Sydney.
34. Poirier, J. L., Capek, R., and De Koninck, Y. (2000). *Neuroscience* **97,** 59–68.
35. Howard, C. V. and Reed, M. G. (1998). *Unbiased stereology: three-dimensional measurement in microscopy*. BIOS Scientific: Oxford.
36. Hatton, W. J. and von Bartheld, C. S. (1999). *J. Comp. Neurol.* **409,** 169–186.