

Brain Aromatase Activity is Inhibited by *in vivo* Treatment with
CGS 20267

A Research Report on an Experiment Studying the
Effects of CGS 20267 (letrozole) on Aromatase Activity in
Specific Brain Areas of the Adult Male Rat.

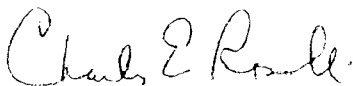
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Summary

To determine the effects of CGS 20267 (letrozole), a highly potent inhibitor of the aromatase enzyme, on aromatase activity *in vivo*, adult male rats were treated with Silastic implants containing crystalline letrozole. Three rats received one implant, three rats received two implants, and three rats remained untreated as controls. Six days later, the rats were sacrificed and tissues were obtained from specific brain areas: the preoptic area, cingulate cortex, medial basal hypothalamus, septum, and amygdala. Aromatase activity in each of the tissues was determined by a validated radiometric assay which measured the amount of $^3\text{H}_2\text{O}$ produced by the conversion of [1β - ^3H]androstenedione to estrone. Letrozole decreased aromatase activity in all brain areas, except the cortex, when compared to control ($p < 0.01$), indicating that the specific inhibitor significantly affects aromatase activity in tissues where the enzyme is present. Aromatase activity did not differ significantly between treatments (one implant or two implants) in any of the brain tissues ($p > 0.05$), suggesting that letrozole had no dose dependent effects at the doses tested.

Introduction

Aromatization of androgens to estrogens is important in many brain processes, including sexual differentiation and the expression of male sexual behavior¹⁴. The enzyme that catalyzes this reaction, cytochrome P₄₅₀ aromatase, has been localized to specific brain areas that are known to play a role in reproductive behavior, including the hypothalamus and limbic system⁸. Regulation of aromatase in the brain is tissue-specific: aromatase activity in the hypothalamus and preoptic area is androgen dependent, whereas in the amygdala and cerebral cortex it is not⁹. Previous studies have reported that castration decreases levels of circulating androgens, which in turn decreases aromatase activity, while exogenous androgen treatment restores aromatase activity^{8,10}. Aromatase activity is also affected by the *in vivo* administration of aromatase inhibitors^{5,7}. Many studies have employed steroidal aromatase inhibitors, such as 1,4,6-androstatriene-3,17-dione (ATD)^{2,5,12}. A study examining the effects of ATD suggests that ATD may bind to the androgen receptor as well as inhibit aromatase directly⁵. To eliminate the possibility of activating self-regulatory androgenic responses within the tissues, non-steroidal aromatase inhibitors have been developed, such as the highly specific inhibitor, CGS 20267 [letrozole; 4,4'-(1H-1,2,4-triazol-1-yl-methylene)-bis-benzonitrile]. Letrozole does not affect corticosterone or aldosterone production, making it a very selective inhibitor of estrogen synthesis¹.

This experiment was designed to evaluate the effects of letrozole on aromatase activity in specific brain tissues in adult male rats. A previous, unpublished study demonstrated that *in vivo* treatment with letrozole produced a paradoxical increase in aromatase activity in homologous brain areas of the male guinea pig³. The purpose of this study was to determine if letrozole would have a similar effect in male rats, and whether the drug has any dose dependent effects.

Materials and Methods

Animals and tissue collection

Nine adult male Sprague-Dawley rats (~250 g) were obtained from Simonsen Breeders (Gilroy, CA) and housed under a 12-h light:12-h dark schedule with food and water available *ad libitum*. Six days before the assay, 3 cm Silastic capsules (id, 1.58 mm; od, 3.18 mm, Dow Corning, Corp., Midland, MI) filled with crystalline letrozole were implanted subcutaneously between the scapulae of each treated animal. Three rats received one implant, three rats received two implants, and three rats remained untreated as control. After six days, the rats were sacrificed by decapitation and the brains were quickly removed and placed on ice. Brain dissection was performed freehand on a sheet of cold dental wax. The following areas were collected: preoptic area (POA), septum (Sept), cingulate cortex (Ctx), medial-basal hypothalamus (MBH), and amygdala (Amyg) (Fig. 1).

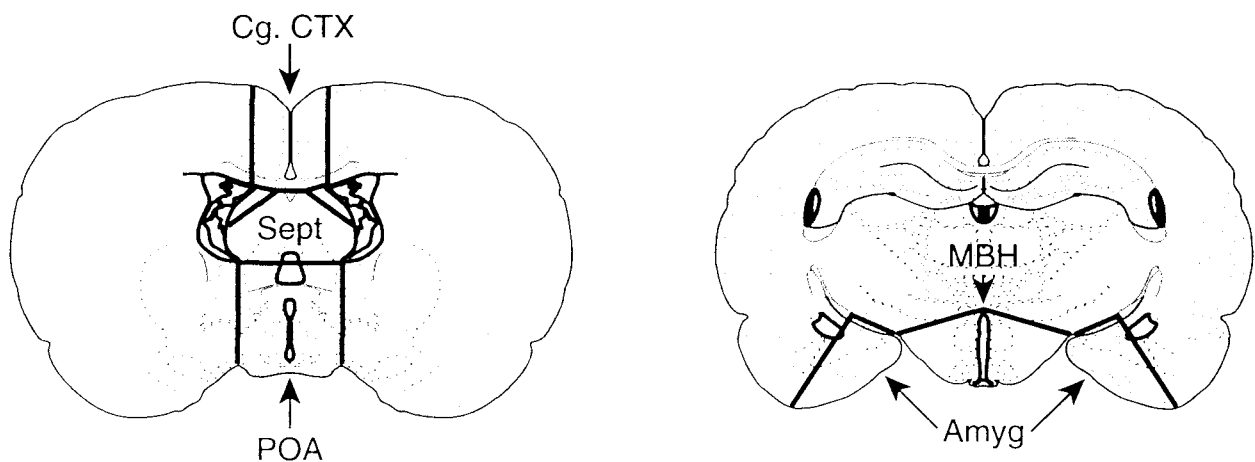


Fig. 1. Atlas of brain regions in the adult male rat. Abbreviations: POA (preoptic area), Sept (septum), MBH (medial basal hypothalamus), Amyg (amygdala) Ctx (cingulate cortex).

Tissue preparation

Tissues were homogenized in TEGMD buffer (10 mM Tris HCl, 1.5 mM EDTA, 25 mM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in cold homogenization tubes. To obtain microsomes from tissue samples, the homogenates were centrifuged for 10 minutes at 1000 x g (4°C), after which the supernatants were removed and centrifuged for 10 minutes at 106,000 x g (4°C). The final supernatant was removed and the 106,000 x g pellet (microsomes) was resuspended by sonication in 30 volumes of potassium phosphate buffer (10 mM KH_2PO_4 , 100 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, pH 7.4). Protein concentrations were determined by the method of Lowry⁶.

Aromatase Assay

Aromatase activity in specific brain areas was determined by a previously validated radiometric assay⁸ in which the amount of $^3\text{H}_2\text{O}$ produced from incubations with [1β - ^3H]androstenedione is equivalent to the amount of estrogen formed (Fig. 2). Reactions were initiated by adding 100 μl of the tissue samples to 100 μl of potassium phosphate buffer containing a NADPH-generating system (5.0 mM glucose-6-phosphate, 1.0 mM NADP^+ , 100U glucose-6-phosphate dehydrogenase/ml) and a saturating amount of [1β - ^3H]androstenedione (0.2 μM). Tubes were capped, vortexed and shaken in a water bath at 37°C for one hour. The reaction was terminated by adding 0.4 ml of an ice-cold solution containing 10% trichloroacetic acid and 2% activated charcoal. The solutions were centrifuged for five minutes at 1200 x g (4°C). The $^3\text{H}_2\text{O}$ generated from the substrate during the one hour incubation remained in the supernatant while the [1β - ^3H]androstenedione was trapped by the charcoal.

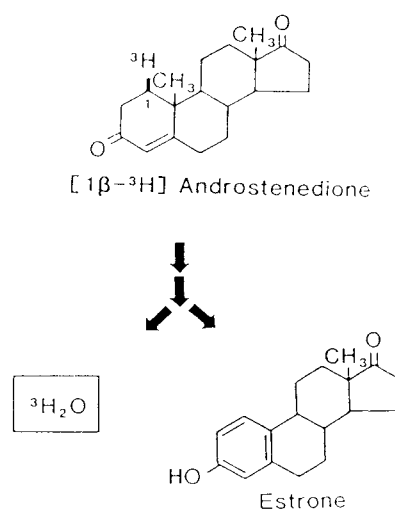


Fig. 2. Diagram of the conversion of radiolabelled steroid substrate, [1β - ^3H]androstenedione, to estrone and production of $^3\text{H}_2\text{O}$. Multiple arrows indicate multiple step conversion process.

The $^3\text{H}_2\text{O}$ was further purified by column chromatography. The chromatography columns (Pasteur pipets, 0.6cm x 10.0cm) were composed of two layers (Fig. 3). The bottom layer contained equal amounts of 50-100 and 100-200 mesh Dowex (Bio-Rad Laboratories, Richmond CA). The Dowex was washed, filtered, and resuspended in distilled water. Two milliliters of this mixture was added to each column. The top layer consisted of equal amounts of acid-activated charcoal and 100-200 mesh Dowex. The charcoal mixture was rinsed once in concentrated HCl and three times with distilled water, and centrifuged between washes at $2560 \times g$ for 10 minutes. The final pellet was resuspended in distilled water and 0.8 ml of this mixture was added to each column. The columns separated any unconverted [1β - ^3H]androstenedione remaining in the supernatant from the $^3\text{H}_2\text{O}$ which was eluted from the columns with 2 ml distilled water. Scintillation fluid was added to the eluent and radioactivity was counted on a Packard TriCarb 460 Scintillation Counter.

Statistical Analysis

Aromatase activity in the brain tissues was determined by the amount of $^3\text{H}_2\text{O}$ formed from [1β - ^3H]androstenedione during the one hour incubation period. The disintegrations per minute (DPM) of $^3\text{H}_2\text{O}$ were corrected for nonspecific conversion and recovery (experimental error), and expressed as fmol $^3\text{H}_2\text{O}/\text{h}/\text{mg}$ protein. Mean levels of aromatase activity in the control and treatment tissues were analyzed by a one-way analysis of variance, followed by post hoc analysis using Student-Newman-Keuls comparisons.



Fig. 3. Glass chromatography columns used to purify $^3\text{H}_2\text{O}$.

Results

Aromatase activity was measured in the POA, Sept, MBH, Amyg, and Ctx of all animals. Mean levels of aromatase activity of the treatment groups (one or two implants) in each brain area were obtained and compared to controls of same brain area (Fig. 4). One-way analysis of variance revealed that aromatase activity significantly decreased after treatment with letrozole in all brain areas ($p < 0.05$), except in the cortex ($p > 0.05$). On average, tissues exhibited a 70.5% decrease in activity when treated with one implant. Treatment with two implants produced an average decrease of 71.2% (Table 1).

Post-hoc analysis revealed significant differences between the control and treatment means of the individual experimental tissues ($p < 0.05$), but that there were no significant differences between treatment groups (one implant or two implants) ($p > 0.05$).

Fig. 4. Microsomal aromatase activity (mean \pm SEM, $n = 3$) in specific brain areas. Significant differences between groups are indicated above error bars (* = $p < 0.01$). Abbreviations: POA (preoptic area), Sept (septum), MBH (medial basal hypothalamus), Amyg (amygdala) Ctx (cortex).

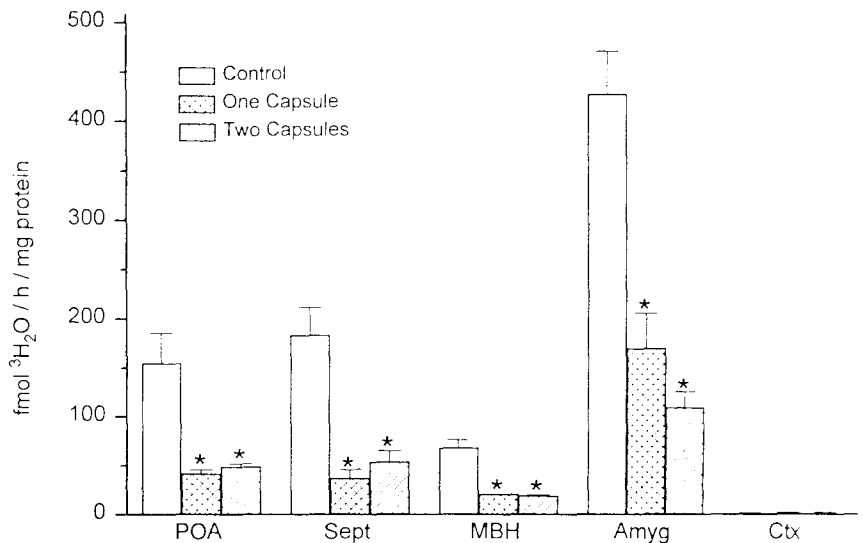


Table I. Percent decreases in aromatase activity after treatment with letrozole.

Tissue	Percent decrease in aromatase activity. One capsule.	Percent decrease in aromatase activity. Two capsules.
POA	72.0	68.2
Sept	79.6	70.4
MBH	69.4	71.9
Amyg	60.3	74.8
Ctx	-30.1	-19.5

Discussion

Aromatase activity (fmol $^3\text{H}_2\text{O}/\text{h}/\text{mg}$ protein) was measured in the POA, Sept, MBH, Amyg and Ctx of adult male rats. Enzyme activity decreased significantly in animals treated with letrozole as compared with control animals that did not receive the drug. Treatment with two implants did not appear to further inhibit aromatase activity more than treatment with one implant. Results from statistical comparison of aromatase activity levels between treatments of one and two implants confirmed the assumption that letrozole has no dose related effect on aromatase activity at the doses tested.

Previous studies have determined that aromatase activity depends highly on circulating androgens and that it is affected by treatment with steroid hormones ^{11,13}. Aromatase is also influenced by steroidal inhibitory substances, such as ATD⁵, or non-steroidal inhibitors, such as Fadrozole [4-(5,6,7,8-tetra-hydroimidazol[1,5,-a]pyridin-5-yl)]¹⁴. However, ATD binds to androgen

receptors and fadrozole affects corticosterone and/or aldosterone production¹ and may therefore have indirect and nonspecific effects on aromatase activity. This study employed a non-steroidal aromatase inhibitor to reduce the possibility of androgen receptor-mediated responses and limit extraneous effects on other steroidal metabolic pathways.

Letrozole decreases levels of aromatase activity *in vitro*³. In a recent study, *in vivo* treatment with letrozole implants increased aromatase activities in homologous brain areas in the male guinea pig³. Several explanations were proposed to explain these results. They include the possibility that locally produced estrogen controls enzyme production; that autoregulatory compensatory mechanisms exist within the cell; and that letrozole affects the turnover rate of the enzyme. In the current study, aromatase levels decreased with letrozole treatment, as expected. However, species differences appear to exist in the regulation of brain aromatase. It is possible that male rats lack one of the previously described compensatory or control mechanisms of the guinea pig. Additional studies are necessary to determine if the absence of one of these compensatory mechanisms may be responsible for the decrease in aromatase activity.

In summary, letrozole, an inhibitor of aromatase activity, significantly decreased aromatase activity in specific brain areas of the male rat. Letrozole appears to have no dose dependent effects. Treatment with letrozole increases in brain aromatase activity in the male guinea pig, but decreases brain aromatase activity in the male rat. The reasons for the different responses in the two animals remains to be determined.

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References

1. Bhatnagar, A.S., Häusler, A., Schieweck, K., Lang, M. and Bowman, R. Highly selective inhibition of estrogen biosynthesis by CGS 20267, a new non-steroidal aromatase inhibitor, *J. Steroid Biochem. Molec. Biol.* 37 (1990) 1021-1027.
2. Brueggemeier, R.W., Li, P.-K., Snider, C.E., Darby, M.V. and Katlic, N.E. 7α -substituted androstenediones as effective *in vitro* and *in vivo* inhibitors of aromatase, *Steroids*, 50 (1987) 163-178.
3. Choate, J.V.A. and Resko, J.A. Paradoxical effect of an aromatase inhibitor, CGS 20267, on aromatase activity in guinea pig brain, 1995, (submitted)
4. Clancy, A.N., Zumpe, D. and Michael, R.P. Intracerebral infusion of an aromatase inhibitor, sexual behavior and brain estrogen receptor-like immunoreactivity in intact male rats, *Neuroendocrinol.* 61 (1995) 98-111.
5. Kaplan, M.E. and McGinnis, M.Y. Effects of ATD on male sexual behavior and androgen receptor binding: A reexamination of the aromatization hypothesis, *Horm. Behav.* 23 (1989) 10-26.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein determination with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265-275.
7. Resko, J.A., Connolly, P.B., Roselli, C.E., Abdelgadir, S.E. and Choate, J.V.A. Differential effects of aromatase inhibition on luteinizing hormone secretion in intact and castrated male cynomolgus macaques, *J. Clin. Endocrinol. Metab.* 77 (1993) 1529-1534.
8. Roselli, C.E., Ellinwood, W.E. and Resko, J.A. Regulation of brain aromatase activity in rats, *Endocrinology*, 114 (1984) 192-200.
9. Roselli, C.E. and Resko, J.A. Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism, *Endocrinology*, 114 (1984) 2183-2189.
10. Roselli, C.E. and Resko, J.A. Effects of gonadectomy and androgen treatment on aromatase activity in the fetal monkey brain, *Biol. Reprod.* 35 (1986) 106-112.
11. Roselli, C.E. and Resko, J.A. Aromatase activity in the rat brain: hormonal regulation and sex differences, *J. Steroid Biochem. Molec. Biol.* 44 (1993) 499-508.
12. Roy, M.M. Effects of prenatal testosterone and ATD on reproductive behavior in guinea pigs, *Physiol. Behav.* 51 (1992) 105-109.

13. Santen, R.J. Is aromatization of testosterone to estradiol required for inhibition of luteinizing hormone secretion in men? *J. Clin. Invest.* 56 (1975) 1555-1563.
14. Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelson, C.R. and Bulun, S.E. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, *Endocrine Rev.* 15 (1994) 342-355.