Inhibition of Melatonin-Induced Ascorbic Acid and LHRH Release by a Nitric Oxide Synthase and Cyclic GMP Inhibitor

SHARADA KARANTH,[1] WEN H. YU, CLAUDIO A. MASTRONARDI, AND SAMUEL M. MCCANN
Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808

Melatonin (MEL), the principle secretory product of the pineal gland, has been shown to function as an antioxidant and free-radical scavenger. We previously showed that the release of ascorbic acid (AA) and luteinizing hormone releasing hormone (LHRH) from medial basal hypothalamus (MBH) was mediated by nitric oxide (NO) that released cyclic guanosine 3′5′-monophosphate (cGMP). Therefore, it was of interest to evaluate the effect of MEL on AA and LHRH release and study the effect of a nitric oxide synthase (NOS) inhibitor, 6-anilino-5,8-quinolinedione (LY 83583), and a guanylyl cyclase (GC) inhibitor, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (O.D.Q.), on the release process. Because NO has been shown to activate soluble guanylyl cyclase that elicited an elevation of cGMP in target cells, in the current investigation LY 83583, O.D.Q., or N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS, were used to evaluate their effects on MEL-induced AA and LHRH release. Medial basal hypothalami were incubated in 0.5 ml of Krebs-Ringer bicarbonate (KRB) buffer for 1 hr. Subsequently, the tissues were incubated with graded concentrations of MEL (10^{-8} to 10^{-4} *M*), MEL + NMMA (3×10^{-4} *M*), MEL + LY 83583 (10^{-6} *M*), or MEL + O.D.Q. (10^{-5} *M*) for 1 hr. Ascorbic acid and LHRH released into the medium were measured by high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA), respectively. Melatonin (10^{-6} and 10^{-5} *M*) significantly stimulated both AA and LHRH release, but the lower and the highest concentrations were ineffective. A combination of MEL + NMMA completely blocked both AA and LHRH release, supporting a role for NO in the releasing action. Both LY 83583 and O.D.Q. significantly suppressed MEL-induced AA and LHRH release, emphasizing the role of NOS, GC, and cGMP in mediating the action of MEL. The data of these *in vitro* experiments support a role for MEL in the hypothalamic control of AA and LHRH release. Exp Biol Med 229:650–656, 2004

This work was supported by National Institutes of Health Grant MH 51853 awarded to S.M.M

[1] To whom correspondence should be addressed at Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808-4124. E-mail: karants@pbrc.edu

Received November 21, 2003.
Accepted April 1, 2004.

1535-3702/04/2295-0001$15.00

Key words: melatonin; N^G-monomethyl-L-arginine (NMMA); LY 83583 (NOS inhibitor); O.D.Q. (GC inhibitor)

Melatonin (*N*-acetyl-5-methoxytryptamine; MEL), the principal secretory product of the pineal gland, is produced during the dark phase of the circadian cycle (1–3). In addition to the pineal gland, MEL is synthesized in other tissues such as the retina, Harderian gland, and gastrointestinal tract, but their contribution to the circulating plasma concentration is negligible (4–6). It is a highly conserved molecule and plays an important role in bio-rhythmicity of all living beings (1). Its secretion is photosensitive, exhibits circadian rhythmicity with the highest levels of the hormone occurring at night in darkness, and shows an age-related decrease in its secretion (7–9). It is a lipophilic hormone and easily passes through cell membranes and barriers (10–12). It accumulates in cell nuclei and protects DNA from oxidative damage and prevents lipid peroxidation both *in vivo* and *in vitro* (13–16). It is rapidly taken up by the brain and stimulates the main antioxidant enzyme of the brain, glutathione peroxidase (12, 17). Recently, MEL has been shown to function as an antioxidant and an effective free-radical scavenger (16–18). It is believed to scavenge the highly toxic hydroxyl radical, the peroxynitrite anion, superoxide, and singlet oxygen (18–21).

Melatonin plays a crucial role in several physiological functions such as the control of sexual maturation, the immune response, temperature regulation, aging, and senescence (22–26). Receptor binding sites have been detected in the brain and gonadal tissues of birds and mammals, suggesting that MEL exerts a direct effect on the vertebrate reproductive organs, seasonal reproduction of animals, and pubertal development in humans (22, 27–33). In addition, the existence of specific binding sites for MEL in lymphoid cells, thymus, and spleen emphasize its role in immunomodulation (34, 35). The development of a high-affinity high-specific-activity ligand, [^{125}I]iodomelatonin, has enabled the identification of MEL binding sites within the brain (29, 30). The highest concentrations of MEL

receptors were shown to occur in the pars tuberalis of mammals (36, 37). The cells of this structure showed ultrastructural characteristics considered necessary for endocrine secretion (38). Our laboratory reported that MEL stimulated luteinizing hormone releasing hormone (LHRH) release from median eminence pars tuberalis explant (39).

Ascorbic acid has been shown to regenerate MEL from MEL radical, illustrating a close association between the two antioxidants (40). Previously, we reported that ascorbic acid (AA) acts as an inhibitory transmitter in the hypothalamus (41). Therefore, it was of interest to study the effect of MEL on AA and LHRH release. The role of NO in MEL-induced AA and LHRH release was assessed by studying the effects of N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of nitric oxide synthase (NOS), 6-anilino-5,8-quinolinedione (LY 83583), a nitric oxide synthase (NOS) inhibitor (42), and 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (O.D.Q.), a selective inhibitor of the soluble guanylyl cyclase (GC; Ref. 43). The results support a role for NO and cGMP in the AA and LHRH-releasing action of MEL.

Materials and Methods

Animals. Adult male rats of the Sprague-Dawley strain (Holtzman, Madison, WI; 200–250 g) were housed two per cage under controlled conditions of temperature (23°–25°C) and lighting (on 0500–1700 hrs). The animals had free access to a pellet diet and tap water.

Chemicals. Sodium ascorbate, NMMA, LY 83583, and O.D.Q. were purchased from Sigma (St. Louis, MO).

***In Vitro* Studies.** *Incubation of Medial Basal Hypothalamus.* Animals were sacrificed by decapitation, and the brain was exposed by a dorsal incision. Medial basal hypothalami were dissected by vertical cuts along the lateral hypothalamic sulci, posterior edge of the optic chiasma, and the anterior edge of the mammillary bodies. A horizontal cut 1 mm from the base separated the island. Medial basal hypothalami (8–12 mg) were incubated *in vitro* as previously reported (41). In brief, one medial basal hypothalamus (MBH)/tube was placed in 0.5 ml of Krebs-Ringer bicarbonate (KRB) (pH 7.4) buffer supplemented with 20 μ*M* bacitracin (Sigma) in an atmosphere of 95% O_2 and 5% CO_2 in a Dubnoff shaker (50 cycles/min) for a period of 60 mins. After this preincubation, the tissues were incubated in 0.5 ml KRB or KRB containing various concentrations of MEL (10^{-8} to 10^{-4} *M*) for 1 hr. In order to assess the role of NO, the tissues were incubated with MEL or a combination of MEL + NMMA. Then, experiments were performed to study the effect of NOS and GC inhibitors. In these experiments, the tissues were incubated with MEL, LY 83583, or O.D.Q. or combinations of MEL + LY 83583 or MEL + O.D.Q. for 1 hr. At the end of this time, the medium was aspirated, and the medium and tissues were stored at –80°C. Ascorbic acid and LHRH released into the incubation medium were analyzed by HPLC and RIA, respectively.

Chromatography. Isocratic analysis were carried out with the Beckman system gold HPLC equipped with 126 module and diode array detector 168 operating at 254 nm (Beckman Instruments Incorporation, Fullerton, CA). The separation was carried out on a μ Bondapack Beckman ultrasphere C18 column (average particle size 5 μm, 25 cm × 4.6 mm). The mobile phase is a buffer consisting of 0.1 *M* sodium dihydrogen phosphate (NaH_2PO_4) and 0.2 m*M* Na_2 EDTA adjusted to pH 3.1 with orthophosphoric acid. The buffer was filtered through an 0.45-μm membrane filter (Gelman Sciences, Ann Arbor, MI) and degassed prior to use. The column was maintained at room temperature, and the mobile phase was used at a constant flow rate of 1.0 ml/min.

Preparation of Standard. A sample buffer consisting of 5 m*M* each of metaphosphoric acid and Na_2EDTA was prepared in HPLC grade water (V.W.R Scientific Products, Dallas, TX) and was used for preparing AA standards and MBH homogenates. The sample buffer had previously been shown to stabilize an AA solution for 3–4 hrs, and all the estimations were completed within this time (44). A standard curve for AA was prepared from a stock solution of 1 mg/ml and was found to be linear from 487.5 to 7800 ng. A standard curve was constructed with every batch of unknown samples. Ascorbic acid in standards, incubation medium, and homogenates were measured using 507 ASE auto sampler (Beckman), and samples were used at a volume of 30 μl. Each sample (unknown) was passed through syringe filters (Gelman Sciences) before being placed in the vial for counting. A standard calibration plot was obtained for AA concentrations (μg/ml) versus peak area (numerical units on the126 module).

LHRH Assay. LHRH was assayed as described (45) using highly specific antibody to LHRH kindly provided by Dr. A. Barnea (University of Texas, South Western Medical Center, Dallas, TX). The minimal detectable LHRH was 0.2 pg/tube, and the curve was linear up to 100 ng/tube. The inter- and intra-assay variations were 5% and 4%, respectively.

Statistics. Results were analyzed by one-way analysis of variance (ANOVA) or unpaired *t* tests wherever applicable, and $P < 0.05$ was considered significant.

Results

Effect of Graded Concentrations of MEL on LHRH Release. Incubation of MBH with different concentrations of MEL (10^{-8} to 10^{-4} *M*) for 1 hr significantly stimulated LHRH release (Fig. 1). The lowest effective concentration was 10^{-6} *M* and a similar increase was observed at 10^{-5} *M*. An increase observed for MEL (10^{-4} *M*) was not statistically significant.

Influence of NMMA on MEL-Induced LHRH Release. In order to assess the role of NO in MEL-induced LHRH release, the tissues were incubated with

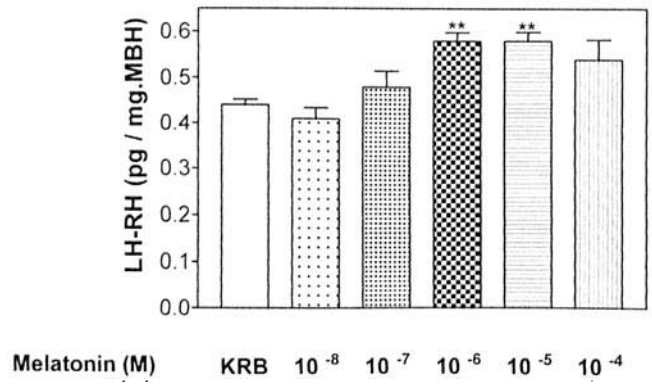

Figure 1. Effect of graded concentrations of MEL on LHRH release from MBH after 1 hr of incubation. In this and subsequent figures, the results are the mean ± SEM and the number of tissues for each group is 8. $^{**}P < 0.01$ versus KRB.

either NMMA, a competitive inhibitor of the enzyme, nitric oxide synthase, or a combination of NMMA (3×10^{-4} *M*) + MEL (1×10^{-6} *M*) for 1 hr. NMMA alone failed to alter LHRH release, and addition of MEL and NMMA together totally blocked MEL-induced LHRH release (Fig. 2).

Effect of MEL or a Combination of MEL + LY 83583 on LHRH Release. Medial basal hypothalami were incubated with either MEL or a combination of MEL (1×10^{-6} *M*) + LY 83583 (1×10^{-6} *M*), an inhibitor of NOS. LY 83583 failed to alter basal LHRH release, and a combination of MEL + LY 83583 totally blocked MEL-induced LHRH release (Fig. 3).

Effect of MEL or a Combination of MEL + O.D.Q. on LHRH Release. In order to assess the role of a specific GC inhibitor on MEL-induced LHRH release, MBH were incubated with MEL or a combination of MEL (1×10^{-6} *M*) + O.D.Q. (1×10^{-5} *M*). O.D.Q. alone failed to alter basal LHRH release (Fig. 4). A combination of MEL + O.D.Q. totally blocked MEL-induced LHRH release.

Effect of Graded Concentrations of MEL on AA Content in the Tissue and Medium. Incubation of MBH with graded concentrations of MEL (10^{-8} and 10^{-4} *M*) for 1 hr failed to alter AA content in the tissue but produced

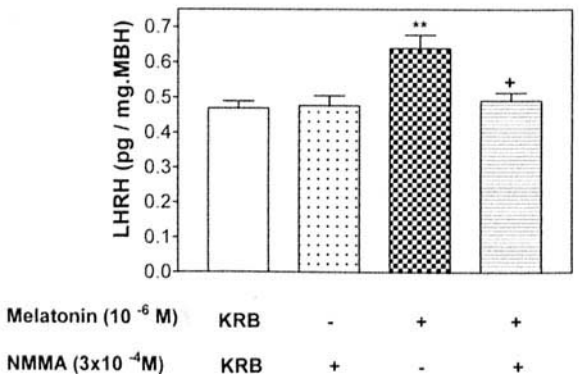

Figure 2. Influence of MEL or a combination of MEL + NMMA on LHRH after 1 hr of incubation. $^{**}P < 0.01$ versus KRB. $^{+}P < 0.05$ versus the group treated with MEL.

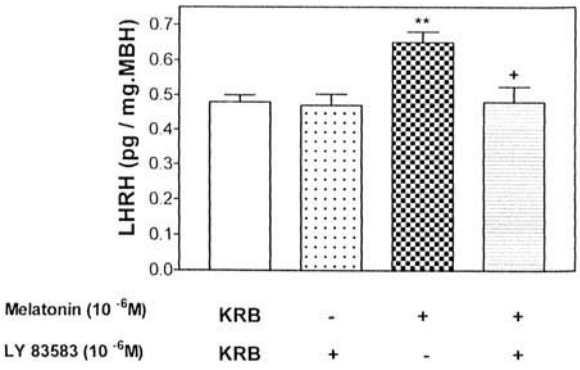

Figure 3. Influence of MEL or a combination of MEL + LY 83583 on LHRH release after 1 hr of incubation. $^{**}P = 0.05$ versus KRB. $^{+}P < 0.05$ versus the group treated with MEL.

a significant increase in the medium AA (Fig. 5A and B, respectively). The lowest effective concentration was 10^{-6} *M*, and the level remained high at 10^{-5} *M*. Comparison of AA content in the tissue and the medium revealed that the tissue AA level was significantly greater (100 times) than that of the medium, illustrating that a very small fraction of AA was released into the incubation medium.

Influence of NMMA on MEL-Induced AA Release. The role of NO in MEL-induced AA release was assessed by incubating MBH with MEL or a combination of MEL (1×10^{-6} *M*) + NMMA (3×10^{-4} *M*) for 1 hr. NMMA alone failed to modify AA release and a combination of MEL + NMMA totally blocked MEL-induced AA release (Fig. 6).

Effect of MEL or a Combination of MEL + LY 83583 on AA Release. Incubation of MBH with MEL (1×10^{-6} *M*) for 1 hr resulted in a significant increase in AA release and LY 83583 (1×10^{-6} *M*) alone suppressed basal AA release significantly (Fig. 7). A combination of MEL + LY 83583 totally suppressed MEL-induced AA release.

Effect of MEL or a Combination of MEL + O.D.Q. on AA Release. The role of the soluble GC inhibitor in

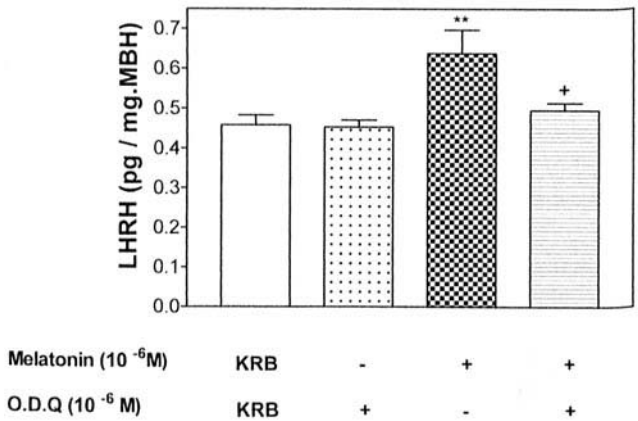

Figure 4. Influence of MEL or a combination of MEL + O.D.Q. on LHRH release after 1 hr of incubation. $^{**}P < 0.01$ versus KRB. $^{+}P < 0.01$ versus the group treated with MEL.

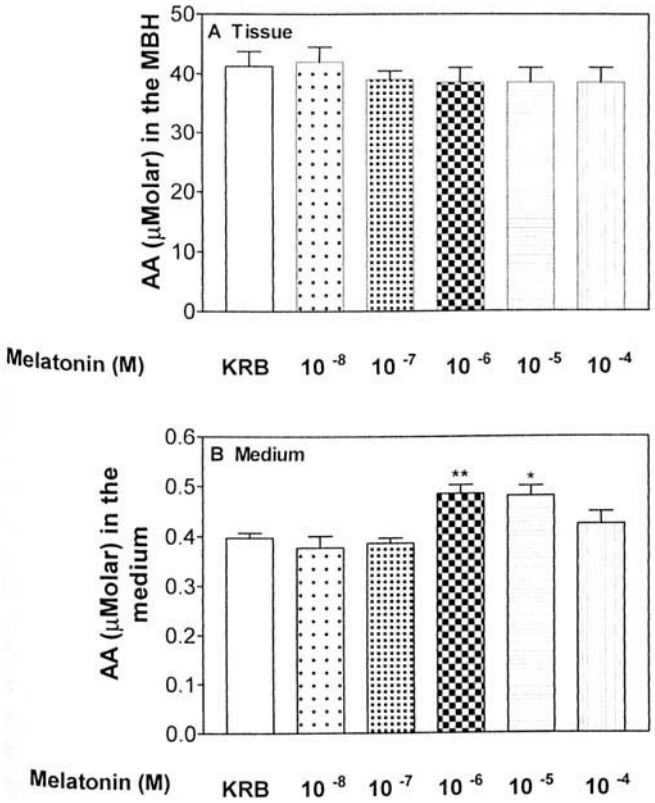

Figure 5. Effect of graded concentrations of MEL on AA content in the (A) tissue and (B) medium after 1 hr of incubation (A and B, respectively). $^{**}P < 0.05$ or $^{*}P < 0.01$ versus KRB.

MEL-induced AA release was assessed by incubating MBH with MEL or a combination of MEL (1×10^{-6} *M*) + O.D.Q. (1×10^{-5} *M*) for 1 hr. O.D.Q. alone failed to alter basal AA release (Fig. 8). A combination of MEL + O.D.Q. totally blocked MEL-induced AA release (Fig. 8).

Discussion

In the current investigation, we have used varying concentrations of MEL to evaluate its effect on AA and LHRH release. Melatonin produced a significant increase in both AA and LHRH release. Earlier reports have shown that perifusion of MBH with MEL stimulated LHRH release (46–49). Previously, our laboratory demonstrated that MEL stimulated LHRH release from median eminence pars tuberalis complex (39). A long exposure of hypothalamic explants from male mink to MEL also stimulated LHRH release, and this was accompanied by a decrease in the density of MEL binding sites in the pars tuberalis (PT; Ref. 49). In most mammals, high-affinity MEL receptors were found in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus that was believed to mediate the circadian effects of MEL (50–52).

Luteinizing hormone (LH)-containing cells in the pars tuberalis were demonstrated previously by immunocytochemistry (53). Melatonin has been shown to exert an inhibitory effect on LH release by median eminence pars tuberalis (ME-PT) explants (39). The largest amount of LH in the ME-PT complex was present in the gonadotrope-like cells in the pars tuberalis and in hypothalamic neurons (54).

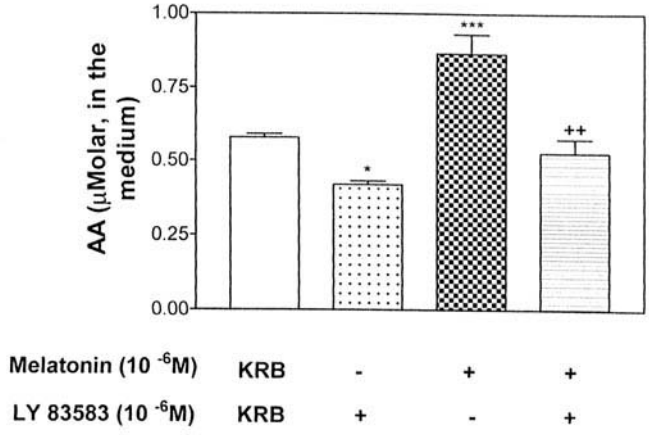

Figure 7. Effect of MEL or a combination of MEL + LY 83583 on AA release in the medium after 1 hr of incubation. $^{*}P < 0.05$ or $^{***}P < 0.001$ versus KRB. $^{++}P < 0.01$ versus the group incubated with MEL.

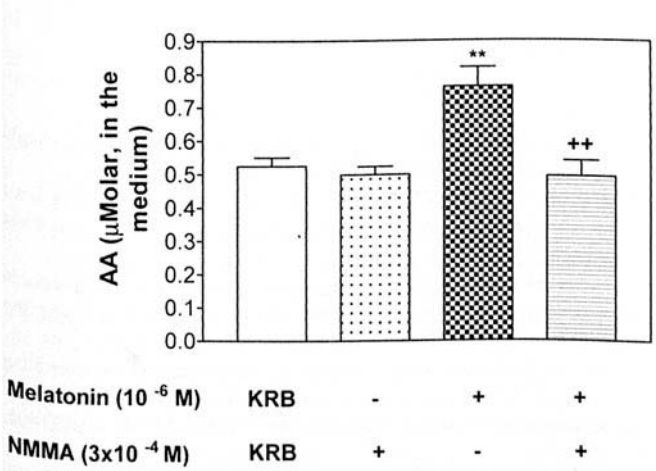

Figure 6. Influence of MEL or a combination of MEL + NMMA on AA in the medium after 1 hr of incubation. $^{**}P < 0.01$ versus KRB. $^{++}P < 0.01$ versus the group treated with MEL.

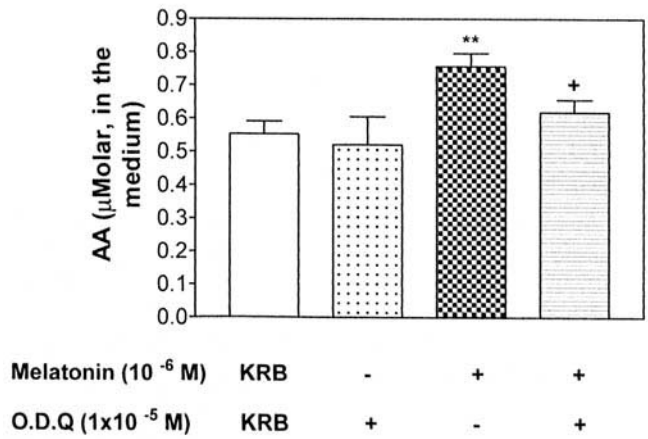

Figure 8. Effect of MEL or a combination of MEL + O.D.Q. on AA release in the medium after 1 hr of incubation. $^{**}P < 0.01$ versus KRB. $^{+}P < 0.05$ versus the group treated with MEL.

Melatonin exerted a dose-related suppression of the forskolin-induced release of cAMP from the pars tuberalis (37). It was hypothesized that MEL may act on its receptors on the gonadotropes of the pars tuberalis to lower cAMP, which may, in turn, inhibit LH release and remove the LHRH terminals from inhibition by LH, leading to an increase in LHRH release (39).

The current research suggests that MEL stimulated NO release that, in turn, stimulated LHRH release. Earlier studies have shown that NO stimulated LHRH release from the median eminence or from immortal cells of the hypothalamus (55–57). The participation of NO in MEL-induced LHRH and AA release was assessed by combined incubation with MEL and NMMA, a competitive inhibitor of NOS. Addition of NMMA resulted in a total blockade of MEL-induced LHRH and AA release, suggesting that the release process of both LHRH and AA was mediated by NO. Nitric oxide is an important neurotransmitter in the brain (58–60). It is synthesized from the substrate L-arginine, and the reaction is catalyzed by the enzyme NOS (61, 62). Three major types of NOS have been identified (63–65). These are neuronal NOS (n-NOS or type 1), macrophage (m) or inducible (I) NOS (type 2), and endothelial NOS (e-NOS, or type 3) (63–65). Of these, n-NOS and e-NOS, the constitutive isoforms, are calcium- and calmodulin-dependent, whereas i-NOS, the inducible isoform, is calcium- and calmodulin-independent (63–65). The close proximity of LHRH neurons to the anatomical localization of NO neurons in the hypothalamus further supports the important role of NO in the regulation of GnRH secretion (57, 66).

Earlier studies have shown that MEL (10^{-9} to 10^{-3} *M*) inhibited the constitutive form of NOS in both the cerebellum and the hypothalamus, suggesting that it played a role in the physiology of the hypothalamus-pituitary unit (67–69). The suppressive action of MEL on hypothalamic NOS was dependent on the homogenate concentration and was observed when the homogenate concentration was in the range of 0.5 to 2 mg/ml (67). However, in the presence of higher concentration of the homogenate (greater than 2 mg/ml), MEL failed to suppress NOS activity (67). Whether or not this is the case in the current investigation is not clear as one MBH/tube was incubated with MEL or other chemicals. Our results did not show an inhibitory action of MEL on NOS, and the reason for this discrepancy is not known.

LY 83583 has been shown to suppress NOS activity and reduced the levels of NO-dependent cGMP in tissues, implying that it inhibited NOS directly (42, 70, 71). On the other hand, O.D.Q. has been shown to act as a selective, reversible, and competitive inhibitor of the soluble GC targeted by NO both *in vivo* and *in vitro* (43, 72). However, it was not effective in suppressing particulate guanylyl cyclase or adenylyl cyclase, illustrating its specific action (43). In studies involving stimulation of brain slices with glutamate receptor agonist, NMDA, O.D.Q. neither interfered with any of the steps leading to NO synthesis nor inhibited NO release, supporting the view that its main target of action was soluble GC (43). In our experiments, LY 83583 and O.D.Q. significantly suppressed MEL-induced AA and LHRH release. This suggests that cGMP mediates both MEL-induced AA and LHRH release. However, LY 83583 was effective in lowering basal AA release, demonstrating that even basal AA release was mediated by cGMP.

It is most likely that LY 83583 may suppress neuronal NOS, resulting in lower NO release. This may decrease activation of soluble GC, resulting in lower cGMP, and which may, in turn, lower AA and LHRH release. O.D.Q., a selective inhibitor of the soluble GC, and LY 83583, an inhibitor of NOS, produced similar results. These observations suggest that the final effect with both the inhibitors was suppression of cGMP formation, although their targets were at different stages of the NO/cGMP signaling pathway. In our earlier experiment, AA inhibited NMDA-induced LHRH release but had no effect on the resting release (41). However, in the current investigation, AA had no effect on MEL-induced LHRH release. We conclude that MEL stimulates both AA and LHRH release and that the release process is mediated by NO and cGMP. MEL may play an important role in the hypothalamic control of AA and LHRH release. The antioxidant activity of MEL may be mediated via AA. We have obtained these results in a static incubation system, and the results have to be verified in animals that do not synthesize AA such as guinea pigs.

We would like to thank Judy Scott for her excellent secretarial assistance.

1. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal factor that lightens melanocytes. J Am Chem Soc 80:2587, 1958.
2. Hastings MH, Vance G, Maywood E. Some reflections on the physiology and function of the pineal. Experientia 45:903–909, 1989.
3. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocrinol Rev 12:151–180, 1991.
4. Zawilska JB, Novak JZ. Regulatory mechanisms in melatonin biosynthesis in retina. Neurochem Int 20:23–36, 1992.
5. Pang SF, Allen AE. Extra pineal melatonin in the retina: its regulation and physiological function. Pineal Res Rev 4:55–95, 1986.
6. Huether G. The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. Experientia 49:665–670, 1993.
7. Reiter RJ. Pineal function during aging, attenuation of the melatonin rhythm and its neurological consequences. Acta Neurobiol Exp 54(Suppl):31–39, 1994.
8. Nowak JZ, Zurawska E, Zawilska J. Melatonin and its generating system in vertebrate retina: circadian rhythm, effects of environmental lighting and interaction with dopamine. Neurochem Int 14:397–406, 1989.
9. Binkley S, Hryshchyshyn M, Reilly K. N-acetyl transferase activity responds to environmental lighting in the eye as well as in the pineal gland. Nature 281:479–481, 1979.

10. Shida CS, Castrucci AML, Lamy-Freund MT. High melatonin solubility in acqueous medium. J Pineal Res 16:198–201, 1994.
11. Costa EJ, Lopez RH, Lamy-Freund MT. Permeability of pure lipid bilayers to melatonin. J Pineal Res 19:123–126, 1995.
12. Mennendez-Pelaez AB, Poeggeler B, Reiter RJ, Burlow-Walden L, Pablos MI, Tan DX. Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. J Cell Biochem 53:572–582, 1993.
13. Mennendez-Pelaez AB, Reiter RJ. Distribution of melatonin in mammalian tissues: the relative importance of nuclear verses cytosolic localizations. J Pineal Res 15:59–69, 1993.
14. Tan DX, Poeggeler B, Reiter RJ, Chen LD, Chen S, Manchester LC, Barlow-Walden LR. The pineal hormone melatonin inhibits DNA-adduct formation induced by the chemical carcinogen safrole in vivo. Cancer Lett 70:65–71, 1993.
15. Reiter RJ, Pablos MI. Agapito TT, Guerrero JM. Melatonin in the context of free radical theory of aging. Ann N Y Acad Sci 786:362–378, 1996.
16. Reiter RJ, Tang L, Garcia JJ, Munoz-Hoyos A. Pharmacological actions of melatonin in oxygen radical patho-physiology. Life Sci 60:2255–2271, 1997.
17. Reiter RJ, Acuna-Castroviojo D, Tan DX, Burkhardt S. Free radical mediated molecular damage, mechanism for the protective actions of melatonin in the central nervous system (review). Ann N Y Acad Sci 939:200–215, 2001.
18. Hardeland R, Reiter RJ, Poeggeler B, Tan DX. The significance of the metabolism of the neurohormone melatonin: auto-oxidative protection and formation of bioactive substances. Neurosci Biobehav Rev 17:347–357, 1993.
19. Tan DX, Chen LD, Poeggler B, Manchester LC, Reiter RJ. Melatonin: a potent endogenous hydroxy radical scavenger. Endocrine J 1:57–60, 1993.
20. Gilad E, Cuzzocrea S, Zingarelli B, Salzman AL, Szabo C. Melatonin is a scavenger of peroxynitrite. Life Sci 60:169–174, 1998.
21. Zang LY, Cosma G, Gardner H, Vallyathan V. Scavenging of reactive oxygen species by melatonin. Biochem Biophys Acta 1425:469–477, 1998.
22. Pang SF, Pang CS, Pan AMS, Shiu SWY, Song Y, Lee PPN, Sho Y, Liu ZM. Pineal melatonin and its receptors in humans. In: Tang PL, Pang SF, Reiter RJ, Eds. Melatonin: A Universal Periodic Signal with Diverse Actions. Basal: Karger, Vol 21:pp133–146, 1996.
23. Caroleo MC, Frasca D, Nistico G, Doria G. Melatonin as immunomodulator in immuno deficient mice. Immunopharmacology 23:81–89, 1992.
24. Viswanathan M, Laitinen JT, Saavedra JM. Expression of melatonin receptors in arteries involved in thermoregulation. Proc Natl Acad Sci U S A 87:6200–6203, 1990.
25. Poeggeler B, Reiter RJ, Tan DX, Chen LD, Manchester LC. Melatonin hydroxyl radical-mediated oxidative damage and aging: hypothesis. J Pineal Res 14:151–168, 1993.
26. Pierpaoli W, Regelson W. Pineal control of aging: effect of melatonin and pineal grafting on aging mice. Proc Natl Acad Sci U S A 91:787–791, 1994.
27. Lincoln GA, Maeda K. Reproductive effects of placing micro-implants of melatonin in the mediobasal hypothlamus and preoptic area in rams. J Endocrinol 132:201–215, 1992.
28. Malpaux B, Daveau A, Maurice F, Gayrard V, Thiery JC. Short day effects of melatonin on luteinizing hormone secretion in the ewe: evidence for central sites of action in the mediobasal hypothalamus. Biol Reprod 48:752–760, 1993.
29. Duncan WJ, Takahashi JS, Dubokovitch ML. Characteristics and autoradiographic localization of I ^{125} Iodomelatonin binding sites in Djungarian hamster brain. Endocrinology 125:1011–1018, 1989.
30. Williams LM, Morgan PJ, Hastings MH, Lawson W, Davidson G, Howell E. Melatonin receptor sites in the Syrian hamster brain and pituitary. Localization and characterization using I^{125} Iodomelatonin. J Neuroendocrinology 1:315–320, 1989.
31. Vanecek J, Pavlik A, Illnerova H. Hypothalamic melatonin receptor sites revealed by autioradiography. Brain Res 435:359–362, 1987.
32. Ayre EA, Pang SF. I-[125] Iodomelatonin binding sites in the testis and ovary. Putative melatonin receptors in the gonads. Biol Signals 3:71–84, 1994.
33. Weaver DR, Reppert SM. The melatonin receptor gene is expressed in human suprachiasmatic nuclei. Neureport 8:109–112, 1996.
34. Pioli C, Carleo MC, Nistico G, Doria G. Melatonin increases antigen presentation and amplifies specific and non specific signals for T-cell proliferation. Int J Immunopharmacol 15:463–468, 1993.
35. Halder C, Singh R. Pineal modulation of thymus and immune function in a seasonally breeding tropical rodent Funambulus Pennanti. J Exp Zool 289:90–98, 2001.
36. Weaver DR, Reppert SM, Rivkees SA, Stopa EG. Putative melatonin receptors in a human biological clock. Science 242:78–81, 1988.
37. Morgan PJ, Williams LM, Davidson G, Lawson W, Howell E. Melatonin receptors on ovine pars tuberalis: characterization and autoradiographical localization. J Neuroendocrinology 1:1–4, 1989.
38. Fitzgerald KT. The structure and function of the pars tuberalis of the vertebrate adenohypophysis. Gen Comp Endocrinology 37:383–399, 1979.
39. Nakazawa K, Marubayashi U, McCann SM. Mediation of the short-loop negative feedback of luteinizing hormone (LH) on LH releasing hormone release by melatonin-induced inhibition of LH release from the pars tuberalis. Proc Natl Acad Sci U S A 88:7576–7579, 1991.
40. Mahal HS, Sharma HS, Mukherjee T. Antioxidant properties of melatonin: a pulse radiolysis study. Free Rad Biol Med 26:557–565, 1999.
41. Karanth S, Yu WH, Walczewska A, Mastronardi CA, McCann SM. Ascorbic acid acts as an inhibitory transmitter in the hypothalamus to inhibit stimulated luteinizing hormone-releasing hormone release by scavenging nitric oxide. Proc Natl Acad Sci U S A 97:1891–1896, 2000.
42. Kamagai Y, Midorikawa K, Nakai Y, Yoshikawa T, Kushida K, Homma-Takeda S, Shimojo N. Inhibition of nitric oxide formation and superoxide generation during reduction of LY 83583 by neuronal nitric oxide synthase. Eur J Pharmacol 360:213–218, 1998.
43. Garthwaite J, Southan E, Boulton CL, Nielsen EB, Schmidt K, Mayer B. Potent and selective inhibition of nitric oxide sensitive guanylyl cyclase by 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one. Mol Pharmacol 48:184–188, 1995.
44. Harapanhalli RS, Howell RW, Rao DV. Testicular and plasma ascorbic acid levels in mice following dietary intake, a high performance liquid chromatographic analysis. J Chromatography 614:233–243, 1993.
45. Dluzen DE, Ramirez VD. In vivo activity of the LHRH pulse generator as determined with push-pull perfusion of the anterior pituitary gland of unrestrained intact and castrate male rats. Neuroendocrinology 45:328–332, 1987.
46. Kao LW, Weisz J. Release of gonadotrophin-releasing hormone (Gn-RH) from isolated, perifused and medial-basal hypothalamus by melatonin. Endocrinology 100:1723–1726, 1977.
47. Bigdeli H, Snyder PJ. Gonadotropin releasing hormone release from the rat hypothalamus: dependence on membrane depolarization and calcium influx. Endocrinology 103:281–286, 1978.
48. Richardson SB, Prasad JA, Hollander CS. Acetylcholine, melatonin and potassium depolarization stimulate release of luteinizing hormone-releasing hormone from rat hypothalamus in vitro. Proc Natl Acad Sci U S A 79:2681–2689, 1982.
49. Messager S, Caillol M, Martnet L. Long-term exposure of hypothalamic explants to melatonin alters the release of gonadotropin releasing hormone and the density of melatonin binding sites in the pars tuberalis of the male mink (*Mustela vison*). J Pineal Res 26:17–27, 1999.

50. Dubosovich ML. Pharmacology and function of melatonin receptors. FASEB J 2:2766–2773, 1988.
51. Morgan PJ, Berrett P, Howell E. Melatonin receptors, localization, molecular pharmacology and physiological significance. Neurochem Int 24:101–146, 1994.
52. Zawilska JB, Nowak JZ. Melatonin receptors (Polish). In: Nowak JZ, Zawilska JB, Walszawa PWN, Eds. Receptors, Characteristics, Structure, Function. pp190–210, 1997.
53. Zawilska JB, Nowak JZ. Melatonin: from biochemistry to therapeutic applications. Pol J Pharmacol 51:3–23, 1999.
54. Emanuele BL, Anderson J, Baker G, McDonald D, Kirsteins L, Lawrence AM. Release of immuno reactive luteinizing hormone (LH) from rat hypothalamus-pars tuberalis explants. Brain Res 309:271–276, 1984.
55. Moretto M, Lopez FJ, Negro-Vilar A. Nitric oxide regulates luteinizing hormone releasing hormone secretion. Endocrinology 133:2399–2402, 1993.
56. Mahachoklertwattana P, Black SM, Kaplan SL, Bristow JD, Grumbach MM. Nitric oxide synthesized by gonadotropin releasing hormone neurons is a mediator of N-methyl-D-aspartate (NMDA) induced GnRH secretion. Endocrinology 135:1709–1712, 1994.
57. Rettori V, Belova N, Dees WJ, Nyberg CI, Gimeno M, McCann SM. Role of nitric oxide in the control of luteinizing hormone releasing hormone release in vitro. Proc Natl Acad Sci U S A 90:11543–11546, 1993.
58. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature 327:524–526, 1987.
59. Schumann EM, Madison DV. Nitric oxide and synaptic function. Ann Rev Neuro 17:153–183, 1994.
60. Garthwaite J, Charles SJ, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptor suggests role as intercellular messenger in the brain. Nature 336:385–388, 1988.
61. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142, 1991.
62. Knowles RG, Palacios M, Palmer RM, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylyl cyclase. Proc Natl Acad Sci U S A 86:5159–5162, 1989.
63. Dawson TM, Snyder SH. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 14:5147–5159, 1994.
64. Bredt DS, Snyder SH. Isolation of nitric oxide synthase, a calmodulin requiring enzyme. Proc Natl Acad Sci U S A 87:682–685, 1994.
65. Lamas S, Marsden PA, Li GK, Tempst P, Michel T. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc Natl Acad Sci U S A 89:6348–6352, 1992.
66. Bhat GK, Mahesh VB, Lamar CA, Ping L, Aguan K, Brann DW. Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin releasing hormone neurons and colocalization with N-methyl-D-aspartate receptors. Neuroendocrinology 62:187–197, 1995.
67. Bettahi I, Pozo D, Osuna C, Reiter RJ, Acuna-Castroviego D, and Guerrero JM. Melatonin reduces nitric oxide synthase activity in rat hypothalamus. J Pineal Res 20:205–210, 1996.
68. Bettahi I, Guerrero JM, Reiter RJ, Osuna C. Physiological concentrations of melatonin inhibit the norepinephrine induced activation of E_2 and cyclic AMP production in rat hypothalamus: a mechanism involving inhibition of nitric oxide synthase. J Pineal Res 25:34–40, 1998.
69. Pozo D, Reiter RJ, Calvo JR, Guerrero JM. Physiological concentrations of melatonin inhibit the nitric oxide synthase in rat cerebellum. Life Sci 55:455–460, 1994.
70. Diamond J, Chu EB. A novel cyclic GMP-lowering agent LY 83583, blocks carbachol-induced cyclic GMP elevation in rabbit atrial strips without blocking the negative ionotropic effects of carbachol. Can J Physiol Pharmacol 68:908–911, 1985.
71. Schmidt MJ, Sawyer BO, Truex LL, Marshall WS, Fleisch JH. LY 83583: an agent that lowers intracellular levels of cyclic guanosine 3′5′-monophosphate. J Pharmacol Exp Ther 232:764–769, 1985.
72. Fedele E, Jin Y, Varnier G, Raiteri M. In vivo microdialysis study of a specific inhibitors of soluble guanylyl cyclase on the glutamate receptor / nitric oxide / cyclic GMP pathway. British J Pharmacol 119:590–594, 1996.