

# Effects of Photoperiod and Androgen on Proopiomelanocortin Gene Expression in the Arcuate Nucleus of Golden Hamsters\*

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## ABSTRACT

In golden hamsters, seasonal changes in day length act via a pineal-dependent mechanism to regulate feedback and behavioral effects of androgen. Endogenous opiates participate in photoperiodically regulated neuroendocrine functions, but the effects of androgen on expression of the gene encoding POMC, the precursor of  $\beta$ -endorphin, have been controversial. We used quantitative *in situ* hybridization to examine regulation of POMC messenger RNA (mRNA) by testosterone and to test the hypothesis that short day lengths act through the pineal gland to amplify POMC mRNA expression. We studied intact hamsters and castrates with or without androgen treatment held in long (14 h of light, 10 h of darkness) or short (5 h of light, 19 h of darkness) days for 10 weeks.

POMC gene expression differed with rostral-caudal plane, photoperiod, and surgical treatment (castration and testosterone administration). Testosterone increased the number of silver grains in la-

beled cells throughout the arcuate nucleus, and short day castrates given androgen consistently had more silver grains per labeled cell than did their long day counterparts. Testosterone exerted an inhibitory effect, however, on the number of POMC mRNA-positive cells, and more POMC mRNA-labeled cells were found in the arcuate nucleus of long than short day castrates treated with testosterone. Photoperiod had no significant influence in castrates not receiving androgen. Testosterone treatment had generally similar effects whether it was begun at the time of castration or 5 weeks later. Pinealectomy blocked the influence of photoperiod on both the mean number of silver grains per labeled cell and the number of labeled cells.

The results indicate that day length regulates POMC gene expression when androgen levels are held constant, but that androgen is necessary for photoperiod effects to be expressed. (*Endocrinology* **140**: 197–206, 1999)

**D**AY LENGTH determines the neuroendocrine responses of seasonal breeders to gonadal steroid hormones (1, 2). This effect is particularly well documented in golden hamsters (*Mesocricetus auratus*), in which short photoperiods inhibit reproduction. During day lengths typical of the winter (nonbreeding season), gonadotropin secretion is suppressed even in the absence of androgen (3). The major physiological effect of short day length, however, is to increase the suppressive influence of testosterone (T) on LH and FSH secretion. Short days also reduce the activation of male sexual behavior by T (3–8). Removal of the pineal gland eliminates these effects of day length on reproductive function (1, 8–10).

Changes in opiate systems may mediate effects of photoperiod on hamster reproduction. Short days increase the

$\beta$ -endorphin content of the medial basal and anterior hypothalamus (11, 12), eliminate the ability of opiate receptor blockade to induce LH release (13, 14), increase the potency of opiate agonists in inhibition of copulatory behavior (15), and reduce opiate receptor concentrations in the medial amygdala, a region that regulates male sexual behavior (16, 17). Although the participation of  $\delta$ - and  $\kappa$ -receptors, enkephalins, dynorphin, melanocortins, or orphanin in photoperiodically triggered events have not been excluded, most research has focused on  $\mu$ -receptors and their principal ligand,  $\beta$ -endorphin. In previous work (18) we determined that castration reduces the abundance of the messenger RNA (mRNA) for POMC, the peptide precursor of  $\beta$ -endorphin and ACTH, in the hamster pituitary. Although we found that androgen replacement reverses this effect, there was no statistically significant effect of photoperiod on pituitary levels of POMC mRNA. As the critical effects of day length and androgen on the neuroendocrine axis occur in the brain, however, it is necessary to examine their effects on POMC gene expression in the arcuate nucleus, the exclusive locus of  $\beta$ -endorphin production in rodent forebrain (19, 20).

The effects of androgen on POMC gene expression in rat medial basal hypothalamus are controversial. Different investigators using different methods have reported that androgen increases (21, 22), decreases (23–25), or has no effect (26) on POMC mRNA levels. The effects of photoperiod on POMC gene expression have received little study in any seasonally breeding species.

Received May 5, 1998.

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\* This work was supported by NIH Grants MH-44132 and K02-MH-00914 (to E.L.B.).

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The present experiments investigated the control of POMC gene expression in the golden hamster by photoperiod and gonadal steroid hormones. We asked whether day length and/or castration regulate POMC expression in the arcuate nucleus. As the influence of photoperiod on gonadotropin secretion is mediated by the pineal gland, we determined whether effects of short days on POMC mRNA in gonad-intact hamsters are eliminated by pinealectomy. We determined whether photoperiod effects occur in castrated hamsters or in castrates treated with exogenous androgen to hold serum T at intermediate physiological concentrations. In light of previous findings that the efficacy of photoperiod and androgen may differ between acute and chronic castrates (6), we gave different groups of animals T capsules at the time of orchidectomy (a maintenance regimen) or 5 weeks later (a replacement regimen). In other species, the effect of androgen on POMC gene expression differs through the rostral-caudal extent of the arcuate nucleus (21, 27). We therefore also asked whether the effects of day length, castration, and androgen vary regionally in the hamster arcuate.

## Materials and Methods

### Animals

Adult golden hamsters (LVG strain) were obtained from Charles River Laboratories, Inc. (Lakeview hamstery). Animals were group housed on a light:dark cycle of 14 h of light and 10 h of darkness (14L:10D; lights on at 0630 h) and had *ad libitum* access to water and Purina chow (Ralston Purina, St. Louis, MO).

Eighteen hamsters remained intact (INT). Nine remained in the 14L:10D photoperiod throughout the experiment. An additional nine INT animals were transferred to short days (5L:19D; lights on at 0900 h) on day 0 of the study. Another group of eight gonad-intact hamsters was pinealectomized (PINX) by the technique of Hoffman and Reiter (28) and transferred to short days on day 0.

Seventeen hamsters were castrated (CAS) under sodium pentobarbital anesthesia (65 mg/kg) and received no androgen; 8 of these individuals remained in 14L:10D, and the remaining 9 were transferred to 5L:19D on the day of surgery. An additional 30 hamsters were castrated and designated to receive T in 1 of 2 dosing regimens. The first, designated T maintenance (CAS+T), consisted of capsule implantation at the time of orchidectomy. The CAS+T group consisted of 8 short day and 9 long day animals that were given sc 5-mm SILASTIC brand capsules (Dow Corning Corp., Midland, MI; no. 602-235; 1.47 mm id, 1.96 mm od) containing crystalline T (Sigma Chemical Co., St. Louis, MO) as soon as the testes were removed under sodium pentobarbital anesthesia. Capsules were soaked in distilled water for at least 16 h before implantation to insure steady release of T. The second androgen treatment regimen, designated T replacement (CAS... T), consisted of capsule implantation 5 weeks after castration. All of the 13 animals designated for the CAS... T treatment remained in long days for 5 weeks after orchidectomy, at which time each received 1 T implant under methoxyflurane anesthesia (Metofane, Mallinckrodt Veterinary, Inc., Mendelvin, IL), and 7 hamsters were moved to 5L:19D (day 0 of the experiment).

Ten weeks later, hamsters were decapitated between 0400–0500 h under dim red illumination. Serum was collected for RIA of T, LH, and PRL, and pituitaries were frozen for analysis of hypophyseal gene expression. Results of these assays have been reported previously (18). Brains were removed, rapidly frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ .

Frozen sections ( $-20^{\circ}\text{C}$ ) were cut in the coronal plane at a thickness of 12  $\mu\text{m}$  using a Harris wide range cryostat, mounted onto acid-alcohol-cleaned, baked, gelatin/chrome-alum-coated slides, and returned to  $-80^{\circ}\text{C}$ . On the day before hybridization, sections were postfixed for 5–10 min by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); rinsed in PBS; acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0); rinsed in  $2 \times \text{SSC}$  (standard saline citrate); dehydrated through 70%, 95%, and 100% ethanol (2.5 min each); diplicated in  $\text{CHCl}_3$  (5 min); and incubated in 100% and 95% ethanol

(2.5 min each). On the following day, slides were prehybridized in buffer containing 1 M NaCl, 0.2 M Tris (pH 7.5),  $10 \times$  Denhardt's solution (containing Ficoll, polyvinylpyrrolidone, and BSA), 50 mM EDTA, 2 mg/ml transfer RNA, 10 mM dithiothreitol, 50% deionized formamide, and 0.05% heat-denatured salmon sperm DNA under coverslips for 2 h at  $45^{\circ}\text{C}$ .

The sections were hybridized for 21 h at  $45^{\circ}\text{C}$  in the same buffer containing 10% dextran sulfate and boiled radioactive probe ( $\sim 1.5 \times 10^5$  cpm/ $70 \mu\text{l}$ /slide). The complementary DNA probe (gift from J. L. Roberts, Columbia University, New York, NY) (29) was prepared by nick translation using [ $^{35}\text{S}$ ]deoxy-CTP (New England Nuclear Corp., Boston, MA) and 100 ng of a 550-bp template that was excised from PBR322 and isolated on a low melting point agarose gel. The probe, which encodes most of the protein from the middle of the  $\text{NH}_2$ -terminal glycoprotein to the C-terminal of rat POMC, was purified on a G-50 column. Northern blots performed on hamster pituitary RNA have confirmed that this probe hybridizes to a single transcript of approximately 1.1 kb (18).

After hybridization, slides were rinsed at room temperature in  $4 \times \text{SSC}$ ,  $1 \times \text{SSC}$ , and  $0.1 \times \text{SSC}$  containing 10 mM dithiothreitol for approximately 1, 1, and 48 h, respectively. Slides were washed for several hours in  $0.1 \times \text{SSC}$  at  $45^{\circ}\text{C}$ , dehydrated in 70% and 90% ethanol in 300 mM ammonium acetate, rinsed in 100% ethanol, and air-dried. Slides were dipped in Kodak NTB3 emulsion (1:2; Eastman Kodak Co., Rochester, NY) in the dark and exposed for 9 days before developing in Kodak D-19 developer and fixer. Slides were counterstained with cresyl violet and coverslipped for microscopic analysis and quantification of grain counts.

Control sections were incubated in the same manner with a 1000-fold excess of unlabeled POMC complementary DNA. In addition, control experiments were performed in which sections were incubated with ribonuclease (100  $\mu\text{g}/\text{ml}$ ; Boehringer Mannheim, Indianapolis, IN) at  $37^{\circ}\text{C}$  before hybridization. In both cases, specific labeling of cells in the arcuate nucleus was absent.

Each section through the arcuate nucleus was analyzed using the NIH Image program (version 1.51) as described by Dolan *et al.* (30). Emulsion autoradiograms were analyzed using a camera attached to a Leitz microscope ( $\times 400$  magnification; Leitz, Rockleigh, NJ) and brightfield optics. Grain clusters were first visualized without a filter to confirm that they were localized over cell bodies. For estimation of single cell levels of POMC mRNA, clusters were visualized with a Bausch & Lomb no. 47 blue filter to eliminate differences in intensity of Nissl staining. Each labeled cell (grain density  $> 5$  times background) was outlined, and the density threshold was set to record the area covered by autoradiographic grains, generating an integrated density measure that corresponds to number of grains per cell. The analysis system was calibrated to ensure that light level and camera sensitivity were consistent for all measurements. The arcuate nucleus was divided into three levels along the A-P plane (rostral, middle, and caudal), corresponding to plates 25–28, 29–32, and 33–36 of the Paxinos and Watson atlas of the rat brain (31), respectively.

Treatment effects were assessed in three ways. First, we performed the Kolmogorov-Smirnov two-sample test (32, 33) to determine the effect of surgical and photoperiodic treatments on the frequency distribution of POMC-expressing neurons. The criterion level for accepting statistical significance was divided by the number of planned comparisons. Second, we performed two-way ANOVA to assess the effects of photoperiod and androgen, and their interaction, on the mean number of silver grains in labeled cells. The Kolmogorov-Smirnov analysis revealed the existence of two populations of POMC-expressing cells (see Fig. 1): a small population of cells with very low grain densities that, although higher than background, appears unaffected by the manipulations we employed, and a larger population with integrated densities exceeding 25. Accordingly, we used an integrated grain density value of 25 as a criterion for the ANOVA analyses. As the results from the different levels of the arcuate nucleus were collected from the same animals and were thus not independent values, this analysis was repeated for the rostral, middle, and caudal planes. Third, we used two-way ANOVA to examine the effects of photoperiodic and surgical treatments on the number of POMC-expressing cells labeled at or above the criterion value (integrated density,  $\geq 25$ ). Results were considered statistically significant in cases where  $P \leq 0.05$ .

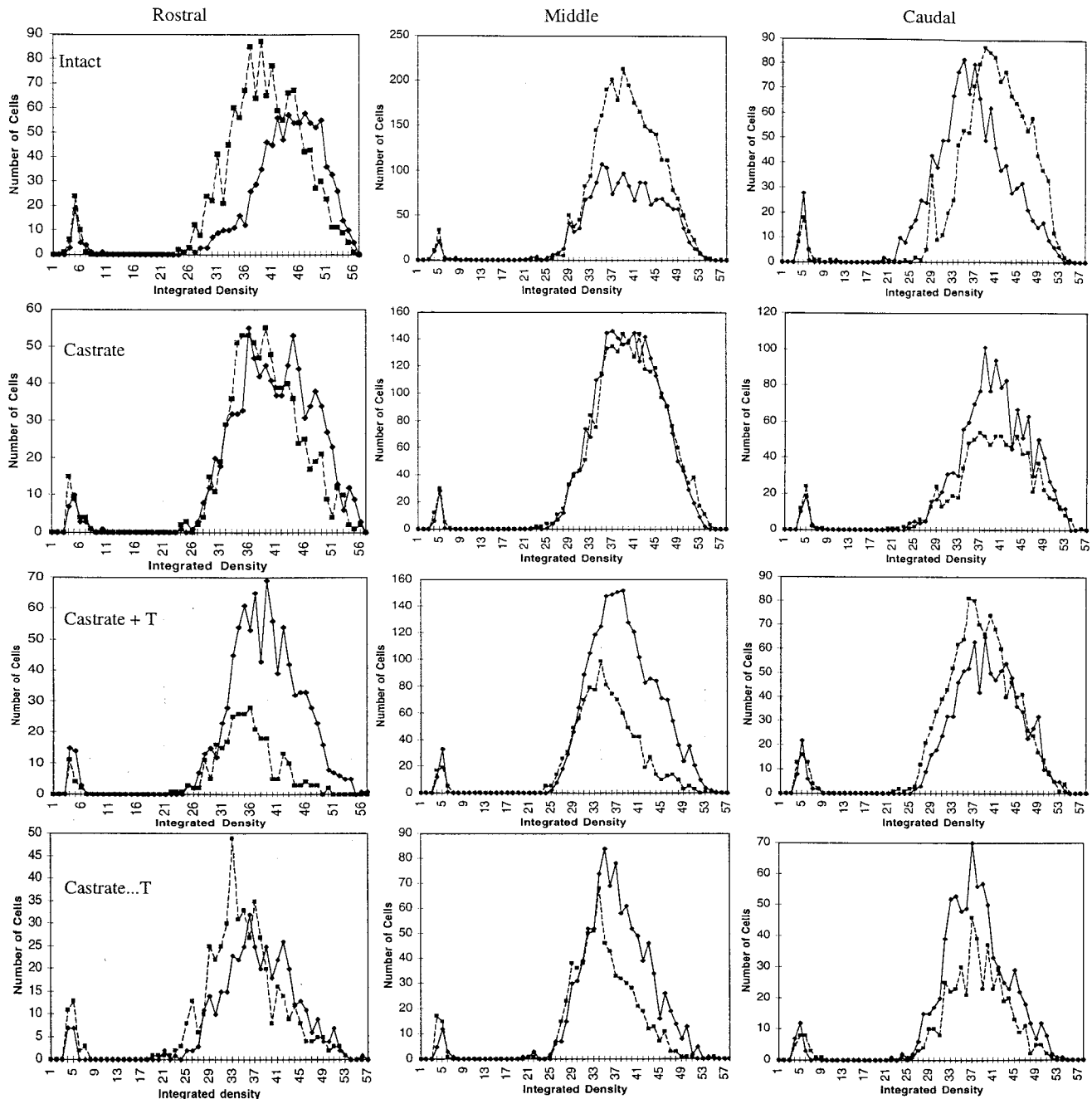


FIG. 1. Histograms illustrating effects of photoperiod on the number of POMC mRNA-positive cells in rostral (*left*), middle (*center*), and caudal (*right*) arcuate nucleus of male hamsters maintained in long (14L:10D; *solid lines and diamonds*) or short (5L:19D; *dashed lines and squares*) day lengths. *Top row*, Intact hamsters; *second row*, castrated hamsters; *third row*, CAS+T; *bottom row*, Cas. . . T. A statistically significant effect of photoperiod on distribution of cells by Kolmogorov-Smirnov test ( $P < 0.05$ ) was found in the rostral arcuate nucleus in each treatment group; in the middle arcuate nucleus in intact, CAS+T and CAS. . . T groups; and in caudal arcuate nucleus in intact and CAS+T hamsters.

## Results

### Gonadal and endocrine measures

As reported previously (17, 18), short days precipitated gonadal regression and significant declines ( $P < 0.01$ ) in serum testosterone, LH, and PRL concentrations in intact hamsters. Pinealectomy blocked these effects of short photoperiod on testis weight and serum hormone concentrations in hamsters with intact gonads ( $P < 0.01$ ). Serum LH and PRL

concentrations were significantly higher in long day than in short day castrates ( $P < 0.05$ ). Androgen treatments elevated serum testosterone concentrations in castrate hamsters to physiological levels (1.2–1.8 ng/ml), which did not differ with photoperiod or between maintenance and replacement regimens. Nevertheless, these treatments suppressed LH to significantly lower levels in short day castrates than in long day castrates ( $P < 0.05$ ). PRL values were lower in short day than in long day animals regardless of gonadal status and

were unaffected by castration or androgen replacement, but pinealectomy significantly elevated serum PRL concentrations above those in intact hamsters in the same short photoperiod ( $P < 0.05$ ).

#### POMC gene expression

**Effects of photoperiod and pinealectomy on POMC mRNA: mean numbers of silver grains per labeled cell.** When animals from all surgical conditions were considered, it was found that photoperiod regulated the abundance of POMC mRNA in the rostral (by overall two-way ANOVA,  $P = 0.0001$ ), but not in the middle or caudal arcuate nucleus. The interaction between photoperiod and surgical/endocrine treatment was significant in each area ( $P < 0.01$ ,  $P < 0.005$ , and  $P = 0.003$  in the rostral, middle, and caudal arcuate nucleus, respectively), indicating that the effect of day length depended upon the endocrine status of the animals.

Day length was without effect in the rostral arcuate nucleus of intact hamsters (Fig. 2, top). In the middle and caudal arcuate, however, long day intact animals had more silver grains per labeled cell than did their short day counterparts ( $P = 0.05$  and  $P < 0.01$ , respectively; Fig. 2). Photoperiod had no statistically significant effect on POMC mRNA in castrated hamsters not given T in any region of the arcuate nucleus. In contrast, short days consistently increased POMC mRNA when androgen levels were held constant by androgen maintenance or replacement. For example, in the rostral arcuate nucleus of T-treated castrates, the number of silver grains per labeled cell was greater in short day castrates regardless of whether the replacement or maintenance paradigm was used (short day vs. long day in CAS+T and CAS...T groups,  $P \leq 0.003$ ; Fig. 2). The same effect was evident in the middle arcuate, but the increase in the number of silver grains per cell in short day hamsters achieved statistical significance only in the CAS+T group ( $P = 0.002$ ; Fig. 2). In the caudal arcuate, short day CAS...T hamsters had more silver grains per labeled cell than their long day counterparts ( $P = 0.02$ ). Effects of short photoperiods on the mean number of silver grains per cell were consistently blocked by pinealectomy in the rostral and caudal arcuate nucleus ( $P \leq 0.02$ ). A similar trend in the middle arcuate nucleus was not significant ( $P = 0.08$ ).

**Frequency distribution of silver grain densities.** In addition to analyzing POMC mRNA abundance by using ANOVA to evaluate the mean number of silver grains per labeled cell, we examined the frequency distribution of grain densities using the Kolmogorov-Smirnov test. This analysis indicated effects of photoperiod that differed with rostro-caudal plane (Fig. 1). In the rostral arcuate nucleus of intact hamsters, long days shifted the distribution of POMC cells to the right, such that more cells with higher numbers of silver grains were observed ( $P < 0.05$ ). In the middle arcuate nucleus, however, photoperiod had no influence on the distribution of cells containing high levels of POMC mRNA, whereas in the caudal arcuate nucleus of intact hamsters, short days increased the proportion of cells with high numbers of silver grains ( $P < 0.05$ ).

Photoperiod had no effect in the middle or caudal arcuate nucleus of castrates not treated with androgen. In contrast,

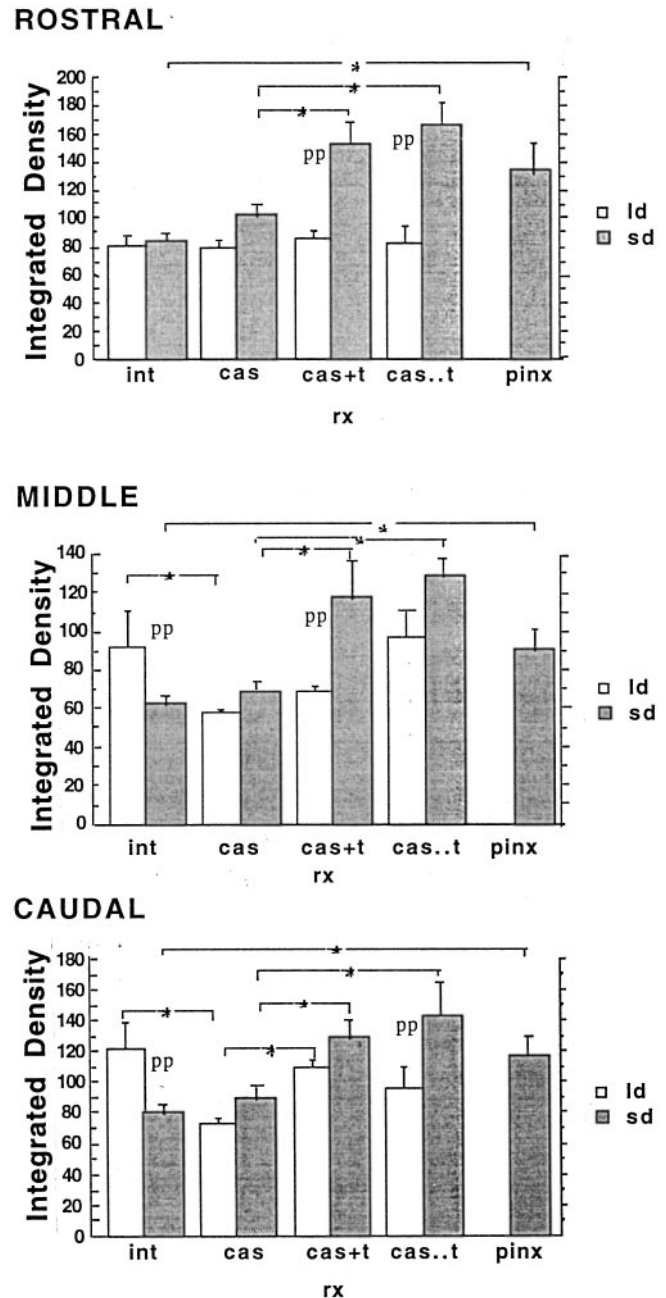


FIG. 2. Effect of photoperiod on mean number of silver grains in cells labeled for POMC mRNA (integrated density,  $>25$ ) in rostral (top), middle (center), and caudal (bottom) arcuate nucleus of hamsters exposed to long (14L:10D; open bars) or short (5L:19D; shaded bars) day lengths. Group designations: int, intact; cas, castrate; cas+t, implanted with SILASTIC T capsules at the time of castration; cas...t, implanted with T capsules 5 weeks after castration; pinx, pinealectomized. Asterisks indicate a significant effect ( $P < 0.05$ ) of surgical treatment among animals in the same photoperiod; groups under comparison are bracketed. pp indicates a significant effect ( $P < 0.005$ ) of photoperiod within a surgical group.

short days consistently reduced the proportion of intensely labeled cells throughout the arcuate nucleus in hamsters receiving exogenous T. This effect of photoperiod reached statistical significance in CAS, CAS+T, and CAS...T groups

in the rostral arcuate, in CAS+T and CAS...T groups in the middle arcuate, and in CAS+T animals in the caudal arcuate.

**Mean number of labeled cells.** We used ANOVA not only to examine the effects of photoperiod on the mean number of silver grains in labeled cells, but also to evaluate the effects of day length on the number of cells labeled above the criterion value. Only in the rostral arcuate nucleus did photoperiod significantly affect the number of labeled cells ( $P < 0.01$ ). There were significant interactions between photoperiod and treatment in both the middle and caudal arcuate nucleus ( $P \leq 0.01$ ), however, indicating that the effect of photoperiod depended upon gonadal status.

The effect of photoperiod on the total number of cells expressing POMC (Fig. 3) followed a completely different pattern than did the influence of day length on the mean number of silver grains per labeled cell. Short days increased the number of cells labeled above criterion grain density in the middle ( $P = 0.05$ ) and caudal ( $P = 0.005$ ) arcuate nucleus of intact hamsters. The change in the number of labeled cells upon short day exposure was blocked by pinealectomy (short day intact *vs.* PINX,  $P \leq 0.0001$ ; Fig. 3). Photoperiod had no significant effect in any portion of the arcuate nucleus in castrates not treated with androgen. In the rostral and middle arcuate, long day CAS+T hamsters had more labeled cells than did their counterparts in short days.

#### Effects of castration and androgen treatment on POMC mRNA

**Mean number of silver grains per labeled cell.** Castration and androgen treatment regulated the abundance of POMC mRNA in labeled cells in all three regions of the arcuate nucleus (by ANOVA of mean number of silver grains per labeled cell: rostral,  $P = 0.003$ ; middle,  $P = 0.002$ ; caudal,  $P = 0.007$ ). Furthermore, the significant interaction between photoperiod and surgical/endocrine treatment in each region indicated that the influence of androgen on POMC expression depended upon day length. Androgen generally increased the number of silver grains in arcuate nucleus cells. Thus, in labeled cells of the middle and caudal arcuate nucleus of long day hamsters, castration significantly reduced mean number of silver grains ( $P = 0.03$  and  $P = 0.003$ , respectively; Fig. 2). No such castration effect was evident in short day hamsters, which have low serum T concentrations in the intact condition.

Androgen treatment reversed the effects of castration. In the rostral arcuate nucleus, short day castrates receiving androgen either as replacement (CAS...T group) or maintenance (CAS+T group) had higher grain densities in the labeled cell population than did castrates not given androgen (CAS group;  $P \leq 0.003$ ). In the middle and caudal arcuate nucleus, androgen replacement significantly increased the mean number of silver grains in both photoperiods (CAS *vs.* CAS...T,  $P < 0.002$ ). Similarly, androgen maintenance significantly increased the number of silver grains per labeled cell in short days (CAS *vs.* CAS+T,  $P < 0.05$ ). In the caudal arcuate nucleus, androgen increased the mean number of silver grains in short day hamsters regardless of whether it

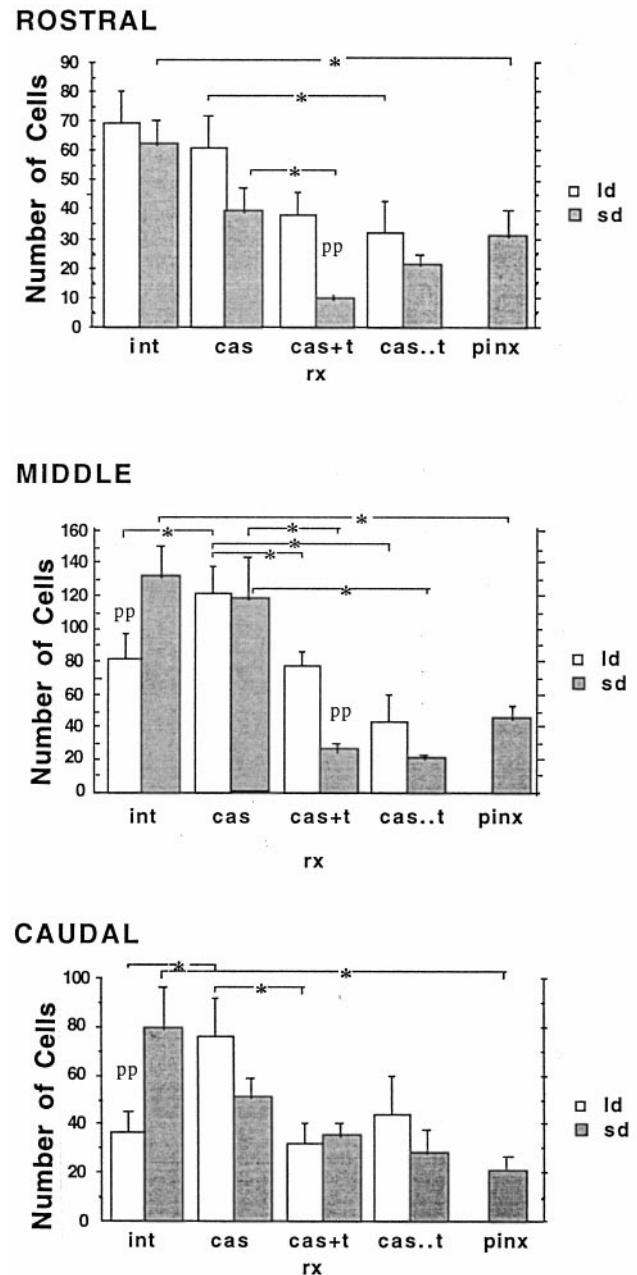


FIG. 3. Effect of photoperiod on the mean number of POMC mRNA-positive cells (integrated densities,  $>25$ ) in rostral (top), middle (center), and caudal (bottom) arcuate nucleus of hamsters exposed to long (14L:10D; open bars) or short (5L:19D; shaded bars) day lengths. Group designations are explained in Fig. 2. Asterisks indicate a significant effect ( $P \leq 0.05$ ) of surgical treatment among animals in the same photoperiod; groups under comparison are bracketed. pp indicates a significant effect ( $P < 0.005$ ) of photoperiod within a surgical group.

was given as a replacement or a maintenance regimen (CAS+T and CAS...T *vs.* CAS,  $P \leq 0.02$ ). Androgen maintenance increased the mean number of silver grains in long day castrates (CAS+T *vs.* CAS groups,  $P = 0.02$ ). A similar trend in long day hamsters receiving androgen replacement did not achieve statistical significance (CAS...T *vs.* CAS groups,  $P = 0.07$ ).

*Frequency distribution of silver grain densities.* Analysis of the frequency distribution of numbers of silver grains of arcuate nucleus neurons expressing POMC by the Kolmogorov-Smirnov test indicated effects of castration that differed with rostro-caudal plane, but showed consistently suppressive effects of T (Fig. 4).

In the rostral arcuate nucleus of long day hamsters, castration shifted the frequency distribution to the left, indicating a reduction of the proportion of cells with high levels of POMC mRNA. Nevertheless, castrated hamsters had more cells with high silver grain density than did either CAS+T or CAS. . . T animals. In short days, the Kolmogorov-Smirnov test indicated that castration had no significant effect on the distribution of silver grain densities. As in long days, however, T replacement or maintenance decreased the proportion of cells with high silver grain densities compared with castrate values. Pinealectomy also decreased the proportion of cells with high integrated densities in short day, gonad-intact animals.

In the middle arcuate nucleus castration had no significant effect on the distribution of cells bearing low or high numbers of silver grains in either photoperiod. T replacement or maintenance, however, shifted the frequency distribution to the left relative to that of CAS hamsters in both long and short days ( $P < 0.001$ ), indicating that fewer cells were heavily labeled in T-treated animals.

In the caudal arcuate, castration increased the proportion of cells with high numbers of silver grains in both photoperiods. As in INT animals, the frequency distribution of CAS. . . T hamsters was shifted to the left relative to that in the CAS group ( $P < 0.001$ ). In short days, T maintenance had a similar effect (CAS+T *vs.* CAS,  $P < 0.001$ ). Long day CAS+T animals did not differ significantly from untreated castrates in this regard.

*Mean number of labeled cells.* Castration and androgen treatment influenced the number of labeled cells throughout the arcuate nucleus (rostral,  $P = 0.0001$ ; middle,  $P = 0.0001$ ; caudal,  $P < 0.005$ ; Fig. 3). Castration increased the number of labeled cells in the caudal arcuate nucleus of long day hamsters ( $P < 0.02$ ). A similar trend in the middle arcuate nucleus was not statistically significant ( $P = 0.07$ ). Castration had no statistically significant effect on cell number in short day hamsters.

Androgen treatment had effects that were opposite those of castration, consistently decreasing the number of labeled cells. In the rostral arcuate, long day hamsters given T as either a replacement or a maintenance dose had fewer cells labeled to the criterion (CAS+T and CAS. . . T *vs.* CAS,  $P \leq 0.05$ ). In short days, the same effect was observed, but only for the maintenance paradigm (CAS+T *vs.* CAS,  $P = 0.01$ ). In the middle arcuate, androgen reduced the number of cells in both long ( $P \leq 0.05$ ) and short ( $P < 0.005$ ) photoperiods regardless of whether it was given as a replacement or a maintenance dose (CAS+T and CAS. . . T *vs.* CAS groups, Fig. 3). Androgen maintenance reduced the number of POMC-labeled cells in the caudal arcuate nucleus of long day hamsters (CAS+T *vs.* CAS,  $P < 0.005$ ); a similar trend in castrates receiving T replacement was not significant (CAS. . . T *vs.* CAS,  $P = 0.07$ ).

## Discussion

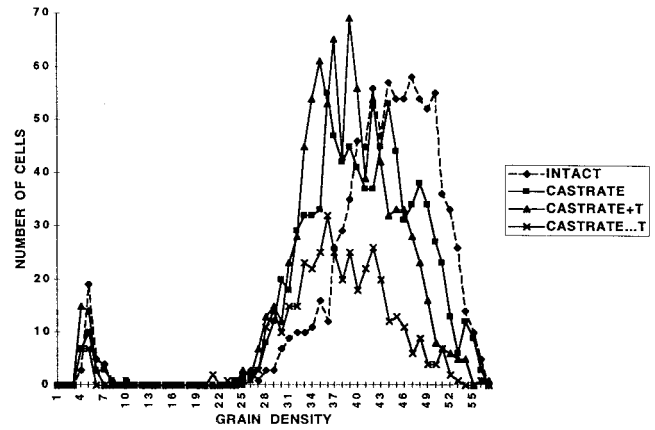
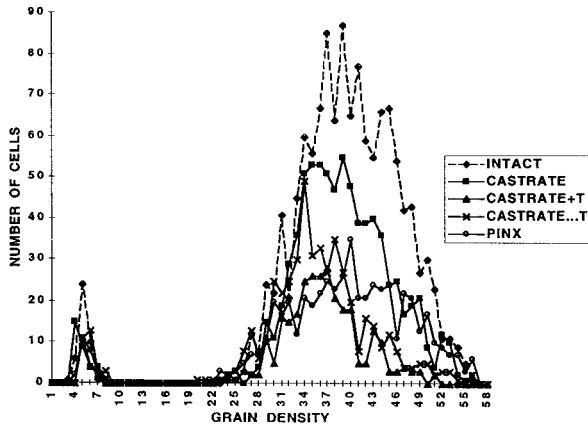
The present results comprise a clear demonstration that photoperiod regulates neuronal POMC mRNA abundance in a seasonal breeder. Our findings also confirm and extend work in other species which shows that androgens profoundly influence POMC mRNA levels. Short days suppress POMC mRNA expression in labeled cells of intact hamsters, but increase the number of these cells in the arcuate nucleus. Photoperiod has no effect on the abundance of POMC mRNA or the number of labeled cells in castrates, indicating that the influence of day length requires gonadal hormones. The most novel findings concern photoperiodic regulation of POMC mRNA when androgen levels are held constant. In T-treated castrates, short days reduce the number of cells with abundant POMC mRNA, but increase the number of silver grains per labeled cell. Thus, the impact of photoperiod is a compound result of both direct effects and those that are secondary to changes in androgen production. Our results also indicate that the pineal gland controls opiate gene expression and reveal a pattern of seasonal regulation in the brain distinct from that of expression of the same gene in the pituitary gland. Both the effects of androgen treatment and those of photoperiod are consistent with evidence that implicates opiates, particularly  $\beta$ -endorphin, in the suppression of gonadotropin secretion and male sexual behavior.

Our results indicate that the impact of day length and androgen on mean POMC mRNA levels per cell differs from their influence on the number of cells expressing POMC mRNA. Total POMC mRNA reflects both of these parameters, and modulation of not only the size of the population of cells expressing POMC, but also the number of copies of POMC mRNA per cell, is likely to have biological relevance. In fact, discrepant results of previous experiments on gonadal regulation of opiate gene expression in other species may result from the use of different methods that are influenced more by the number of cells expressing POMC or more by the intensity of expression (number of copies) within subpopulations of arcuate nucleus cells. The controversy surrounding regulation of  $\beta$ -endorphin content and POMC mRNA by the testes and by androgen is most apparent in studies of rats. Using *in situ* hybridization methods similar to ours, Chowen-Breed *et al.* (21, 22) found that acute castration reduces POMC mRNA in the rat arcuate nucleus and that T prevents this decline. The present finding that mean grain density per cell is suppressed by castration of long day hamsters is in agreement with these results. Our finding that this effect is absent in short day hamsters is most likely attributable to the low levels of endogenous androgens that result from gonadal regression. Also in agreement with Chowen-Breed *et al.* (21), we found that castrated hamsters receiving androgen had higher mean grain densities in labeled cells than did their untreated orchidectomized counterparts. Although the reduction in grain density we observed upon exposure of intact hamsters to short photoperiod was consistent with an inductive effect of androgen on POMC gene expression in individual neurons; this pattern was a complex combination of the effects of day length and T. Our analysis of the effects of androgen on the frequency distribution of POMC-expressing neurons was also in partial agreement

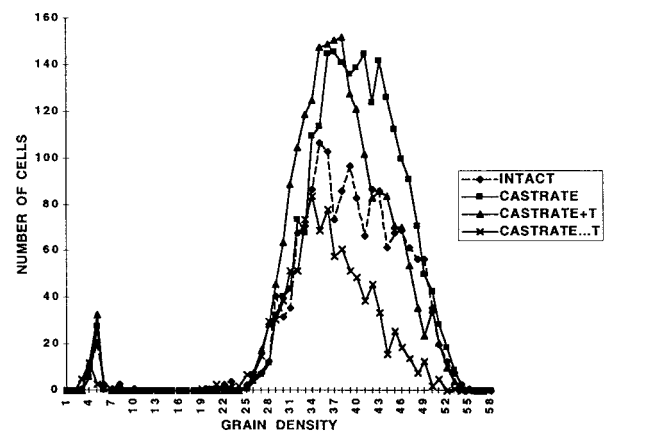
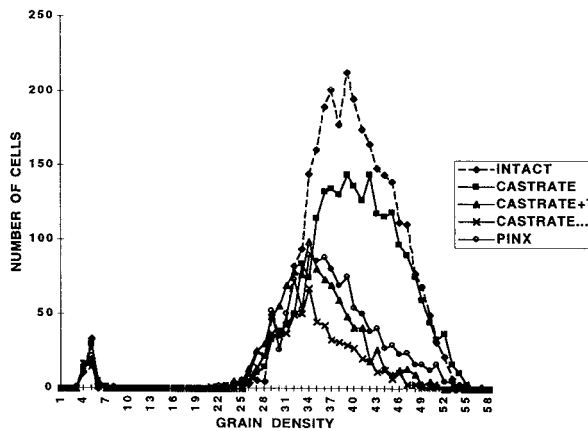
SHORT DAYS

LONG DAYS

rostral



middle



caudal

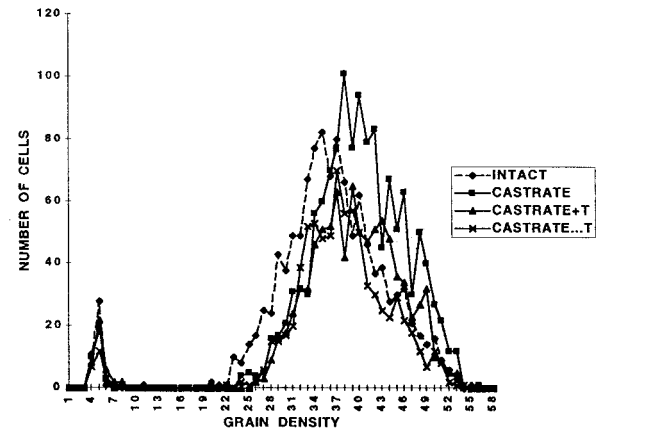
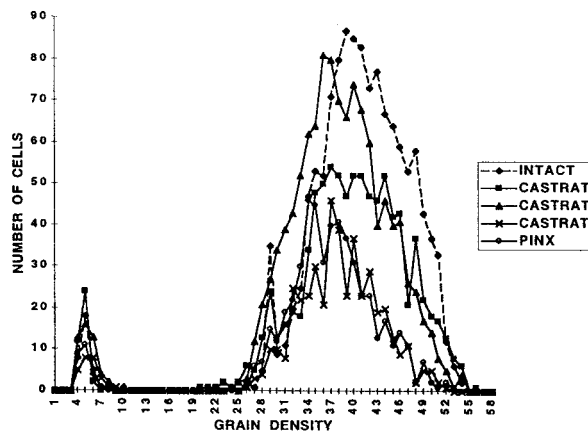


FIG. 4. Histograms illustrating the effects of surgical treatment on the number of POMC mRNA-positive cells in the rostral (top), middle (center), and caudal (bottom) arcuate nucleus of male hamsters maintained in long (14L:10D; left) or short (5L:19D; right) day lengths. See text for details of statistical comparisons.

with the findings of Chowen Breed *et al.* (21). In long day hamsters, castration shifted the frequency distribution in the rostral arcuate nucleus to the left, and the sharp reduction in circulating T levels experienced by short day hamsters was correlated with a corresponding shift in the frequency distribution.

In contrast, S1 nuclease protection assays indicate that castration increases POMC mRNA and that T reverses these effects in rats (23–25). The similarities and differences between these conclusions and ours probably reflect the differences in the methods used. Nuclease protection assays do not take into account anatomical differences across the arcuate, but are likely to be more sensitive than *in situ* hybridization to changes in gene expression in neurons that have low levels of POMC mRNA. Although the conclusions of these studies (23–25) differ from our analysis of mean grain density, they are entirely consistent with our finding that castration precipitates an androgen-reversible increase in the number of cells that met or exceeded the criterion silver grain density. Throughout the arcuate nucleus, we found that T shifted the distribution of cells to the left relative to that in castrates, indicative of a reduction in the proportion of neurons with high grain densities. The effects of short day exposure on POMC cell number are also consistent with an inhibitory effect of androgen; castration increased the number of cells expressing POMC in the midarcuate nucleus of long day hamsters. Furthermore, among intact animals, long day hamsters had fewer POMC-expressing cells than short day hamsters. Gonadal steroid hormones may suppress total POMC gene expression by reducing the number of cells showing hybridization signal throughout the arcuate nucleus, even though the abundance of POMC mRNA in individual cells that express high levels of this message is increased by androgen. As mRNA is extracted and pooled across all the cells in the medial basal hypothalamus in protection assays, the effects of treatment on the size of the population expressing POMC may outweigh influences on the abundance of POMC mRNA per neuron.

Our findings are less consistent with other experiments that used *in situ* hybridization to study gonadal regulation of POMC gene expression. Selmanoff *et al.* (26) found no effect of castration on periaruate POMC mRNA levels in rats. In addition to the difference in species, it is possible that the attention to regional differences among rostral, middle, and caudal arcuate nucleus allowed us to see effects of castration and androgen treatment that might not have been apparent in the earlier study. Hileman *et al.* (27) used *in situ* hybridization to examine effects of castration and androgen treatment, either as replacement or maintenance, on POMC gene expression in the arcuate nucleus of rams. They found that androgen suppresses the mean silver grain area per labeled cell. It is not clear whether this measure corresponds to our count of the integrated density of silver grains. Furthermore, Hileman *et al.* (27) found the suppressive effect of T given to chronic castrates to be greatest in the middle and posterior arcuate nuclei. Most recently, Hileman *et al.* (55) reported that T suppresses POMC mRNA in the arcuate nucleus of rams only under long day conditions. In agreement with our results, T reduced the number of POMC-labeled cells during long days. Although some discrepancies between these find-

ings and the present study may result from species differences, both experiments show conclusively that day length is a potent regulator of POMC gene expression in the arcuate nucleus of seasonal breeders.

Chronic castration of hamsters (11, 12) and rats (23, 34) induces an increase in  $\beta$ -endorphin concentrations within the medial basal hypothalamus that can be prevented by androgen maintenance. Exposure of hamsters to short days eliminated the influence of gonadectomy on hypothalamic opiate content (12). Although these findings are consistent with evidence in the present study, it is difficult to relate our findings on POMC mRNA levels to this report. If anything, androgen had a greater impact on the number of silver grains per labeled cell in short day hamsters. Effects of T on the frequency distribution and number of POMC-expressing cells were generally similar in long and short day hamsters. Discrepancies between our findings and studies of  $\beta$ -endorphin content (11, 12) may reflect photoperiodic influences on posttranscriptional control of POMC expression, including cleavage to  $\beta$ -endorphin as well as other products and transport to other areas of the brain.

Skinner and Herbison (35) recently reported a suppression of  $\beta$ -endorphin immunostaining in ewes during the breeding season compared with that in anestrous animals. Although differences in species and sex cloud the comparison, this finding appears consistent with the present results indicating a downward shift in the distribution of POMC mRNA-positive cells in the arcuate nucleus of short day hamsters. Despite the fact that long days are inductive of reproduction in hamsters and suppressive in sheep, such photoperiods increase PRL secretion in both species.  $\beta$ -endorphin release into the hypothalamo-hypophyseal portal system increases PRL secretion, and hyperprolactinemia elevates POMC mRNA in the rat periaruate region (26). The results of the present study make it unlikely, however, that effects of day length on PRL secretion are an important determinant of POMC gene expression in hamsters. The effects of short days on POMC mRNA could be dissociated from those on PRL by their dependence on androgens. Short days most consistently elevated the mean silver grain density in hamsters receiving androgen treatment, whereas the influence of photoperiod on PRL concentrations was similar in intact, castrated, and T-treated hamsters. The influence of PRL on POMC gene expression was also questioned by Wardlaw *et al.* (25), who found no effect of central or peripheral PRL treatment on the POMC mRNA content of the medial basal hypothalamus in S1 nuclease protection assays.

Behavioral and negative feedback effects of androgen may be more pronounced when T is administered immediately at the time of castration (androgen maintenance) than when it is given to chronic castrates several weeks after orchidectomy (androgen replacement). The efficacy of gonadal steroid hormones declines as a function of time since gonadectomy in several situations (36, 37). To determine whether the influence of T on POMC gene expression is similarly affected by the timing of androgen treatment after orchidectomy, we compared effects of CAS+T and CAS. . . T treatments. These treatments had generally similar effects on silver grain density and the number of cells labeled above the criterion value in both long and short day hamsters. Furthermore, the

present results demonstrated no noticeable influence of the interval between castration and T treatment on the enhancement of androgen's negative feedback effects by short days.

The present results indicate the existence of regional difference in the responses of the arcuate nucleus to environmental and hormonal input. In particular, the rostral arcuate nucleus seems the most highly sensitive to photoperiod. Nevertheless, consistent patterns were observed throughout the arcuate nucleus with respect to the effects of both day length and androgen. The heterogeneity within each third of the arcuate nucleus was apparent from histograms that revealed a consistent proportion of cells that were unresponsive to manipulation of gonadal status or day length and a larger proportion that appeared sensitive to either or both of these parameters. Further characterization of POMC-expressing cells, perhaps through the use of double labeling and/or tract tracing studies, would help to determine whether these proportions of cells reflect different functional populations. Our findings also address the tissue specificity of regulation of opiate gene expression by androgen and photoperiod. The pituitary POMC content of the same hamsters studied here was decreased after castration, but maintained by androgen (18). To the extent that the number of silver grains per labeled cell in the arcuate nucleus can be related to such results, the present findings suggest a similar response in both tissues. The restriction of influence of day length to the brain, however, indicates that effects of photoperiod are tissue specific.

Most, if not all, of the effects of photoperiod on behavioral and feedback effects of androgen depend upon the integrity of the pineal gland. We thus anticipated that pinealectomy would prevent the effects of short day exposure on POMC gene expression. This expectation was confirmed; in each region of the arcuate nucleus in which short days altered the grain density or the number of POMC mRNA-labeled cells, the photoperiodic effect was eliminated by pinealectomy. It will be desirable to compare POMC expression in pinealectomized and intact long day animals to determine whether the pineal may exert a photoperiod-independent influence. As photoperiod regulates reproduction through its control of the duration of nightly melatonin secretion (1, 38–41), it is likely that this pineal hormone is responsible for the effects of day length on POMC mRNA described here. Melatonin receptors are present in the medial basal hypothalamus, but their high concentration in the pars tuberalis of the pituitary has made it difficult to resolve their concentration in the arcuate nucleus. Lesion studies have suggested that a critical site of action of melatonin may reside in the dorsomedial hypothalamus, whereas intracranial administration of this substance indicates sites of action in basal anterior hypothalamus and midline thalamus (40, 41). It is not possible to resolve whether either of these sites may influence POMC gene expression through *trans*-synaptic mechanisms, or whether melatonin may act directly on arcuate neurons.

Photoperiodic regulation of POMC gene expression and its response to androgen may participate in seasonal changes in gonadotropin secretion and sexual behavior. The increased negative feedback effect of T in short day animals, as indicated by reduced LH secretion in CAS+T and CAS. . . T animals maintained in 5L:19D, was correlated with signifi-

cant increases in silver grain density per labeled arcuate cell. Day length may exert these effects by modifying the autoregulation of POMC mRNA by opiates (42). Photoperiod also regulates the metabolism of other neurotransmitters that may influence or respond to POMC-derived peptides. For example, photoperiod affects monoamine content and turnover in hamster medial basal hypothalamus (43). Short days increase the release of dopamine from terminals of arcuate neurons in the median eminence and decrease the number of neurons in the arcuate nucleus that contain immunoreactive amino acid decarboxylase (44, 45). Photoperiodic regulation of steroid-responsive dopaminergic neurons appears important to seasonal breeding in other species (46, 47), and reciprocal interactions between dopamine and  $\beta$ -endorphin may play a role in photoperiodic responses (48, 49). Excitatory amino acids may also participate in the regulation of reproduction by day length (50, 51), and these transmitters may regulate the expression or release of POMC-derived peptides (52, 53). The possibility that other modulators of POMC expression, including leptin, neuropeptide Y, tachykinins, galanin, and CRH, may be photoperiodically regulated remains unexplored (48, 54). Finally, day length may regulate posttranscriptional processing of POMC mRNA, posttranslational modification of POMC peptide, and expression of opiate and/or melanocortin receptor subtypes to effect seasonal changes in neuroendocrine function.

Nevertheless, the ability of photoperiod to modulate effects of androgen on POMC gene expression represents a level of environmental regulation of neuroendocrine function that has not been previously described. The pineal-dependent modulation of POMC expression in short day hamsters, which leads to an increase in the number of silver grains, but a decrease in the number of labeled cells, may contribute to the multiple physiological and behavioral influences of winter photoperiods.

### Acknowledgments

We thank Donald Fletcher for invaluable assistance in quantitative imaging of autoradiograms, Richard Hurlbut for excellent animal care, Drs. Joseph McCabe and Christine Wagner for advice on statistical analysis of grain distribution by the Kolmogorov-Smirnov test, and Dr. Sandra L. Petersen for critical reading and suggestions on the manuscript.

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