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Increased VIP and Decreased GnRH Expression in Photorefractory Dark-Eyed Juncos (*Junco hyemalis*)

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Most temperate zone birds show dramatic seasonal cycles in responsiveness to light. In the spring the hypothalamo–pituitary–gonadal axis of photosensitive birds is stimulated by long days. In the late summer birds no longer respond to long days, their gonads regress, and they are said to be photorefractory. After several weeks of refractoriness birds regain photosensitivity. During refractoriness circulating concentrations of luteinizing hormone are low and prolactin levels are high. These fluctuations in peripheral hormones result from changes in the brain rather than in the pituitary and/or the gonads. In the present study we examined seasonal changes in expression of vasoactive-intestinal polypeptide (VIP) and gonadotropin-releasing hormone (GnRH) in the brain of dark-eyed juncos (*Junco hyemalis*). Birds were photosensitive and exposed to long photoperiod (20:4 LD) for 1 day, 45–60 days, or not at all, or they were photorefractory (housed in 20:4 LD). The results indicate that VIP expression was similar in all photosensitive birds. However, photorefractory birds had significantly higher numbers of VIP-positive neurons in the infundibulum compared to photosensitive birds. The number of GnRH-positive neurons in the preoptic area was significantly lower in photorefractory birds and significantly higher in long-term photostimulated birds. These results indicate that the inverse relationship between circulating prolactin (released by VIP) and luteinizing hormone (released by GnRH) during refractoriness may result from neural changes in VIP and GnRH expression, respectively.

Reproductive behavior and physiology are cyclic in most temperate zone birds. Both migratory and nonmigratory species reproduce almost exclusively during the spring and early summer and remain reproductively quiescent during the autumn and winter. In the spring the avian reproductive system is stimulated by increasing day length. Toward the end of the summer, however, birds undergo a rapid regression of the gonads despite a continued long photoperiod. This period of insensitivity to light is termed photorefractoriness. Responsiveness is regained following exposure to the short days of autumn and winter. Annual variations in gonadal state and responsiveness to light and their neuroendocrine bases have been the topic of numerous investigations (reviewed by Wingfield and Farner, 1980; Follett, 1984; Gwinner, 1986; Nicholls *et al.*, 1988).

Increases in gonadal size and weight occur within the first week of photostimulation in Japanese quail (*Coturnix coturnix japonica*; Follett and Robinson, 1980). Similar increases in gonadal development following exposure to a long photoperiod have been described in all species investigated (Wingfield and Farner, 1978; Dawson and Goldsmith, 1982; Dawson, 1983; Rohss and Silverin, 1983; Wilson, 1991) and result from elevated pituitary gonadotropin secretion. Increases in plasma luteinizing hormone (LH) and follicle-stimulating

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hormone are seen prior to increases in gonadal steroids. In quail transferred from LD 8:16 to LD 20:4 significant increases in plasma LH are evident after exposure to 1 long day (Nicholls *et al.*, 1983; Creighton and Follett, 1987). Gonadotropin levels remain elevated until refractoriness sets in.

The onset of refractoriness is characterized by a rapid collapse at all levels of the hypothalamo–pituitary–gonadal axis (HPGA). Lipid deposits are visible in testicular Leydig cells, suggesting decreased steroid synthesis (Lam and Farner, 1976), and plasma gonadal steroid concentrations decrease (Rohss and Silverin, 1983; Dawson, 1983). This gonadal regression is preceded by decreases in plasma gonadotropin concentrations in all species investigated including canaries (*Serinus canarius;* Nicholls, 1974), tree sparrows (*Spizella arborea;* Wilson and Follett, 1984), the willow-ptarmigan (*Lagopus lagopus lagopus;* Stokkan and Sharp, 1980), and starlings (*Sturnus vulgaris;* Dawson and Goldsmith, 1982). Investigations of onset and maintenance of refractoriness, reestablishment of photosensitivity, and photoinduction reveal that the peripheral hormonal fluctuations which accompany these transitions in responsiveness to photoperiod in birds reflect changes in the brain rather than autonomous responses to light at the level of the pituitary and/or the gonads (Perera and Follett, 1992).

In the central nervous system (CNS) changes in gonadotropin-releasing hormone (GnRH) expression as a function of the photosensitivity have been investigated in the starling and the quail. Photosensitive photostimulated starlings have greater numbers of GnRH-like immunoreactive (li ir) fibers in the median eminence (ME; Foster *et al.*, 1987) and elevated hypothalamic GnRH content (Dawson *et al.*, 1985) compared to nonphotostimulated birds. Perera and Follett (1992) have shown that quail hypothalami release significantly higher concentrations of GnRH *in vitro* after just 1 day of photostimulation relative to unstimulated controls. The data suggest that the CNS of photoperiodic birds responds rapidly to increases in photoperiod with elevation of GnRH, while photorefractory birds show the opposite patterns of GnRH expression and content. Thus, seasonal changes in responsiveness to light are mediated by neural mechanisms, which in turn mediate pituitary and gonadal hormonal variations.

The pituitary hormone, prolactin (PRL), also increases during the spring and summer (Goldsmith, 1983). The highest concentrations of plasma PRL coincide with the occurrence of parental behaviors (Goldsmith, 1985); however, this increase is superimposed upon an underlying seasonal rhythm. Indeed, increases in plasma PRL are seen during the spring and summer in nonbreeding starlings (Ebeling *et al.*, 1982) and canvas back ducks (*Aythya valisineria;* Bluhm *et al.*, 1989). The annual waxing of PRL secretion occurs late in the breeding season relative to the gonadotropins (Dawson and Goldsmith, 1982; Goldsmith, 1983). Additionally, a surge in plasma PRL always accompanies the onset of refractoriness in all species investigated (Goldsmith, 1985; Nicholls *et al.*, 1988).

While GnRH expression, content, and release have been investigated at different phases of the photoperiodic cycle in birds, annual changes in vasoactive-intestinal polypeptide (VIP), the PRL-releasing hormone (Macnamee *et al.*, 1986) have not been explored. In the present study, we compared the central expression of VIP and GnRH in photorefractory juncos to that in photosensitive birds exposed to stimulating photoperiod for 1 day, 45–60 days, or not at all.

METHODS

Dark-eyed juncos from a local population in Fairbanks, AK were collected and housed indoors under artificial conditions. All subjects were adult males ranging in ages from 8 months to 2 years. Birds were

housed in individual cages in a room maintained at 20°. Food and water were available *ad libitum*.

The experimental design is depicted in Fig. 1. Juncos (n = 15) were collected in the field during the autumn of 1991 and housed under nonstimulating photoperiod (LD 10:14; lights on 0700 hr). On January 13, 1992, 10 birds were transferred to stimulatory photoperiod LD 20:4 (lights on 2100 hr). The following day 5 birds in the photosensitive unstimulated group and 5 birds of the photosensitive 1-day stimulated group were sacrificed. The remaining juncos remained under stimulatory photoperiod until May 6, 1992 (photorefractory; n = 5), at which time they were sacrificed. Long-term photosensitive photostimulated birds (n = 5) were collected in the field on May 14, 1992, when the local sunrise was 0430 hr, sunset 2300 hr, housed under LD 20:4 (lights on 2200 hr) for 4 days and subsequently sacrificed.

Just prior to sacrifice with an overdose of anesthesia (ketamine/xylazine) the body weight and diameter of cloacal protuberance of each subject was recorded, and a blood sample was drawn from the wing vein. An intracardial injection of heparin [0.3 ml; 1000 IU/ml in 0.1 *M* phosphate buffer (PB)] was administered, and subjects were perfused transcardially with 0.1 *M* PB (20 ml) followed by 4% paraformaldehyde (PARA; 25 ml). Testes weight was measured following the perfusions. The cranium was partially removed and the heads were immersed in PARA overnight at 4°. The next day the brains were removed from the cranium and placed in 0.001 *M* sodium azide in preparation for shipping and processing.

Brains were coated with 6% gelatin and then embedded in 12% gelatin. Gelatin blocks were immersed in 4% PARA for 48 hr. The blocks were mounted onto a vibratome and 50-μm sections were cut from the pan-olfactory region to the occulomotor nucleus. Free-floating sections were collected into 0.1 *M* PB, washed for 20 min in 0.1 *M* PB, and then treated with 0.05% hydrogen peroxide (H_2O_2) in distilled water (dH_2O) for 15 min to block endogenous peroxidases. Sections were washed three times in 0.1 *M* PB and immersed in 10% normal goat serum for 1 hr with gentle agitation. Every third section was treated with either an antibody against porcine VIP (Incstar; Stillwater, MN) or against GnRH (LR-1, gift of R. Benoit; LR-1 recognizes both chicken GnRHI and II). The third section was stained with various antibodies for other ongoing experiments in the laboratory.

Primary antibodies were made up in 0.3% Triton-X in 0.1 *M* PB (PBT) at 1:10,000 (anti-VIP) and 1:40,000 (LR-1). Sections were immersed in primary for 48 hr at 4°. They were then washed three times in 0.1% PBT for 15 min on the shaker, immersed in 1:250 biotinylated goat anti-rabbit immunoglobulin G (Sigma), washed three times in 0.1% PBT, and treated with 1:200 avidin–biotin (Vectastain) solution in 0.3% PBT. After three washes in 0.1% PBT, immunocytochemical product was visualized using 0.03% diaminobenzidene (DAB). Sections were mounted onto gelatin-subbed slides, air-dried coverslipped, and examined under a light microscope.

In order to control for variation in processing, each immunocytochemical run included one bird from two to four different experimental groups. The specificity of the antibodies was ascertained by preabsorption of

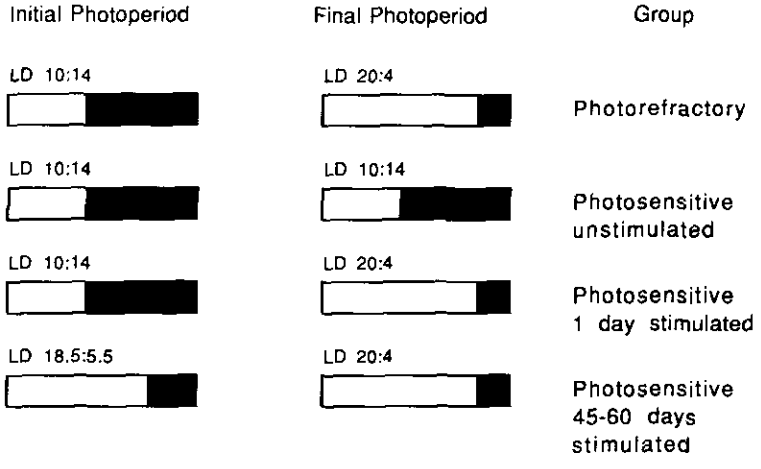

FIG. 1. Schematic depicting the experimental design. Nonbreeding male dark-eyed juncos were collected and placed under artificial lighting conditions indicated under "Initial Photoperiod." Prior to sacrifice subjects were held in conditions indicated under "Final Photoperiod" (see Methods for duration). Body mass, cloacal protuberance, testicular weight, and plasma LH were measured. The photorefractory group was exposed to a stimulatory photoperiod (LD 20:4) for approximately 5 months.

the primary antiserum with an excess of the appropriate antigen prior to exposure to the brain section. As a further control in some sections the primary antibody was omitted from the immunocytochemical protocol.

For VIP, immunoreactive neurons were counted in four sections in the septum lateralis (SL) and in five sections in the infundibulum (INF). For GnRH, neurons were counted in three sections through the preoptic area (POA). The POA was sampled from its most rostral aspect [at the point where the tractus mesencephalicus (TSM) extends from the midline to the ventral surface of the brain], to its caudal limit (where the third ventricle is enlarged dorsally), an extent of about 450 μm in the junco. Cell counts were done independently by two observers who were unaware of the experimental groups. Interrater agreement was high, $r^2 = 0.907$ (pairwise correlation). Since every third section was stained with each antibody, cell counts were multiplied by a factor of three. Because neurons can appear in more than one section, resulting in overestimation of cell number, the Abercrombie (1946) correction factor was applied. The correction factor was calculated as follows: $N/n = T/(T + D)$, where N is the corrected cell number, n is the uncorrected cell number, T is the section thickness, and D is the mean cell diameter. The mean cell diameter was measured on a sample of randomly picked VIP- and GnRH-positive neurons cut through the plain of the nucleus in the INF and POA, respectively. Comparisons were made using nonparametric Kruskal–Wallis and post hoc, two-way Mann Whitney U tests. All data are presented as means ± SEM.

Two plasma aliquots (20 μl each) from each bird were assayed for LH using the double-antibody radioimmunoassay developed by Follett *et al.* (1972) and adapted for passerine species (Follett *et al.*, 1975). The recognition specificity of this antibody for junco LH is unknown; however, this antibody has been used to measure plasma LH in a wide variety of avian species (Follett *et al.*, 1972; Silverin and Wingfield, 1982).

RESULTS

Long-term photostimulated juncos had significantly higher testicular weight (284.16 mg ± 62.6; $P < 0.001$, Kruskal–Wallis) and larger cloacal protuberances (5.1 ± 0.33 mm; $P < 0.05$, ANOVA) than other experimental groups. Photosensitive unstimulated, 1-day stimulated, and photorefractory juncos did not differ in testis weight or cloacal protuberance (2.85 ± 0.31 mg testes weight and 3.68 ± 0.17 mm cloacal protuberance; data reflect means ± SEM of all three groups). Long-term photostimulated juncos also had significantly higher plasma LH (2.26 ± 0.35 ng/ml) relative to other groups [0.93 ± 0.44 (photosensitive unstimulated); 1.08 ± 0.38 (photosensitive 1-day stimulated); 0.70 ± 0.07 (photorefractory); $P < 0.05$ ANOVA followed by Student–Newman–Keuls pairwise comparison].

Neurons expressing VIP-li ir were observed in the SL and the INF. Immunopositive fibers were observed in the SL, POA, lateral hypothalamus (LHN), periventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the hypothalamus (PHN), INF, *zona externa* of the ME, and the nucleus of Ruber. SL neurons were observed densely packed along the ventromedial aspect of the lateral ventricles beginning just prior to the decussation of the TSM and extending approximately 400 μm caudally. VIP-positive neurons in the SL appear pyriform in shape and they possess one or two major processes. The more rostral neurons (approximately 200 μm) are oriented perpendicular to the ventricular wall and emit processes which extend into the lateral ventricles and terminate in a knob-like structure in the cerebrospinal fluid (described in Silver *et al.*, 1988). As depicted in Fig. 2B, all four groups of birds had similar numbers of VIP-positive neurons in the SL.

INF VIP-positive neurons appear fusiform and do not appear to contact the CSF. Neurons are observed through the extent of the INF (approximately 800 μm along the rostro–caudal axis). Photorefractory juncos (Fig. 3A) had two to three times as many VIP-positive neurons (Fig. 2A) than did photosensitive birds (Figs. 3B–3D). Photosensitive unstimulated, 1-day stimulated, and long-term photostimulated birds did not differ in INF VIP-positive neuron number. For post hoc comparisons the photorefractory group was compared to pooled scores of all photosensitive birds, as their means were not significantly different.

GnRH-positive neurons were observed

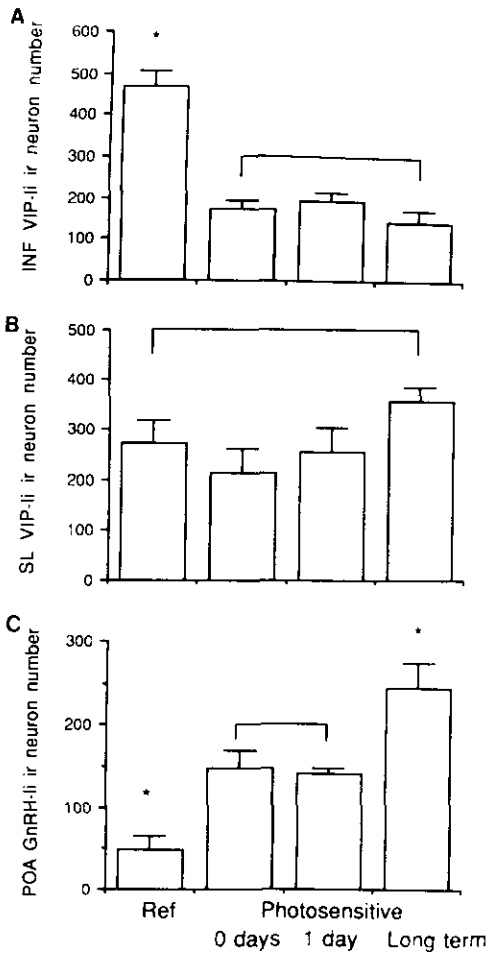

FIG. 2. Bar histogram depicting peptidergic expression in photorefractory ($N = 5$), photosensitive unstimulated ($N = 5$), 1-day stimulated ($N = 5$), and long-term photostimulated ($N = 5$) juncos. Immunoreactive neurons were counted by an observer unaware of the experimental treatments. (A) Photorefractory juncos had significantly greater numbers of VIP-positive neurons in the infundibulum ($P < 0.01$ Kruskal–Wallis; Mann Whitney U test $U = 0$, $P < 0.01$) than photosensitive birds. (B) Lateral septal VIP-li ir neuron number did not vary across experimental groups. (C) Photorefractory juncos had significantly lower, and photosensitive long-term stimulated juncos had significantly higher numbers of GnRH-positive neurons in the preoptic area ($P < 0.01$ Kruskal–Wallis; Mann Whitney U test $U = 2$, $P < 0.05$, two-tailed) as compared to photosensitive unstimulated and 1-day stimulated birds who did not differ from each other. *Statistically significant. Histograms marked by a common bracket do not differ from each other.

in the POA, nucleus of the pallial commissure, LHN, PHN, and the PVN. Immunopositive fibers were observed at the above loci and in the SL, INF, and *zona externa* of the ME. All immunoreactive neurons appeared fusiform with one or two major axes. Only POA neuron counts are reported here.

As depicted in Fig. 2C and Fig. 4 experimental groups of juncos differed in GnRH-li ir in the POA. Photorefractory juncos (Fig. 4A) had significantly fewer GnRH-positive neurons in the POA as compared to photosensitive unstimulated (Fig. 4B) and 1-day stimulated (Fig. 4C) birds. Long-term photostimulated juncos (Fig. 4D) had significantly greater numbers of GnRH-positive neurons in the POA than photosensitive unstimulated and 1-day stimulated conspecifics. For post hoc comparisons the cell counts of photosensitive unstimulated and 1-day stimulated birds were pooled, since they were not significantly different, and compared with photorefractory and long-term stimulated birds.

Preabsorption of the primary antibodies with 1:500 VIP and 1:10,000 chicken GnRH, respectively, completely abolished staining in the INF (for VIP) and POA (for GnRH). In addition, omission of the primary antibody from the immunocytochemical protocol resulted in complete absence of reaction product upon exposure to DAB.

DISCUSSION

The data suggest that VIP expression in the INF is higher in photorefractory than in photosensitive juncos, while there are no differences among photosensitive juncos exposed to stimulating photoperiod for 1 day, 45–60 days, or not at all. VIP expression in the SL, however, did not vary across the experimental groups, suggesting that the increase in INF VIP during refractoriness is specific to this brain region.

VIP is a potent PRL-releasing factor in birds. Administration of VIP to bantam hens (Macnamee *et al.*, 1986), ring doves

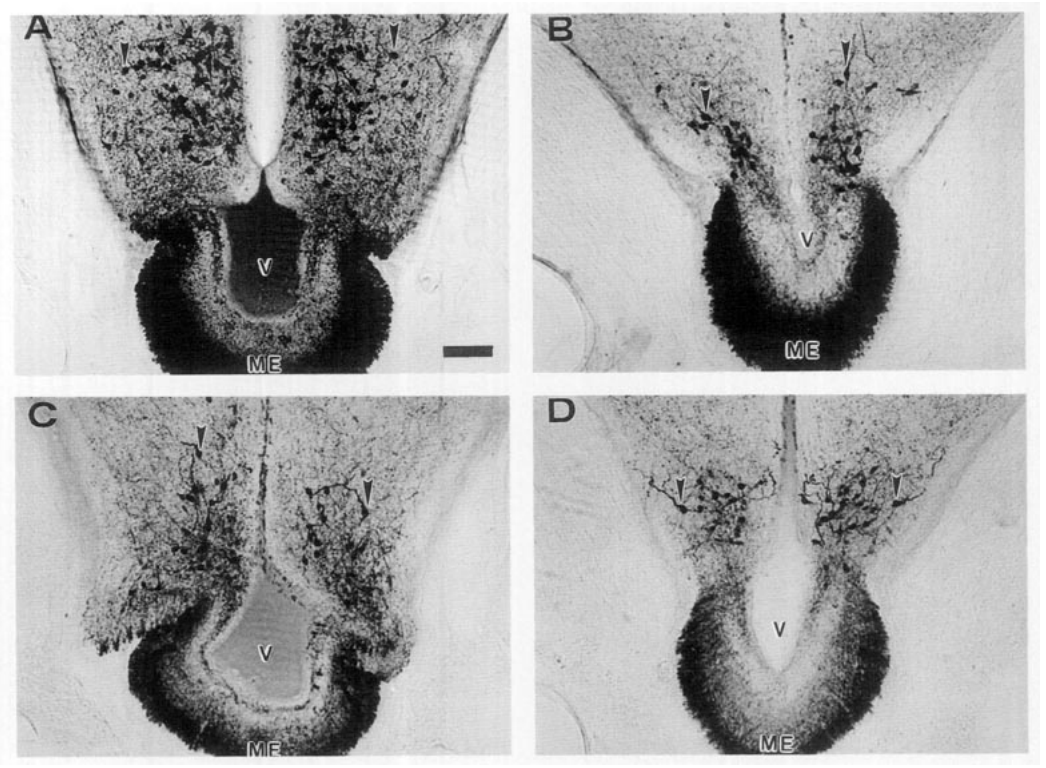

FIG. 3. Photomicrographs of VIP-li ir in the infundibulum of photorefractory (A), photosensitive unstimulated (B), 1-day stimulated (C), and long-term stimulated (D) juncos. Photorefractory juncos had significantly higher numbers of VIP-positive neurons in the infundibulum relative to photosensitive birds. V, third ventricle; ME, zona externa of the median eminence. Magnification bar, 50 μm. Arrows indicate ir neurons.

(Lea and Vowles, 1986), and turkeys (Opel and Proudman, 1988) elevates plasma PRL concentrations. *In vitro* administration of VIP to dispersed adenohypophyseal cells increases PRL secretion (Macnamee *et al.*, 1986). Additionally, VIP expression in the INF has been implicated in the control of PRL secretion in birds. The diameter of VIP-li ir INF neurons increases during incubation in ring doves (Clous *et al.*, 1990) and turkeys (Mauro *et al.*, 1989). This increase is positively correlated with peripheral PRL concentrations during incubation. Passive immunization of bantam hens with chicken VIP decreases both VIP-li ir in the INF and peripheral PRL levels (Sharp *et al.*, 1989). PRL secretion albeit typically associated with parental behaviors such as incubation and brooding in birds (Goldsmith, 1983; Silver, 1984; Lea, 1987; Silver, 1990), also varies seasonally in nonbreeding birds (Ebeling *et al.*, 1982; Bluhm *et al.*, 1989; see Introduction).

In temperate zone avian species photorefractoriness is characterized by a complete collapse of the HPGA. The present data suggests that during refractoriness POA GnRH expression detected by immunocytochemistry also decreases dramatically. Similarly, in starlings a decrease in hypothalamic GnRH has been reported using radioimmunoassay (Dawson *et al.*, 1985) and immunocytochemistry (Foster *et al.*, 1987). In juncos increases in hypothalamic GnRH expression are seen when photosensitivity is regained as evidenced by the fact that

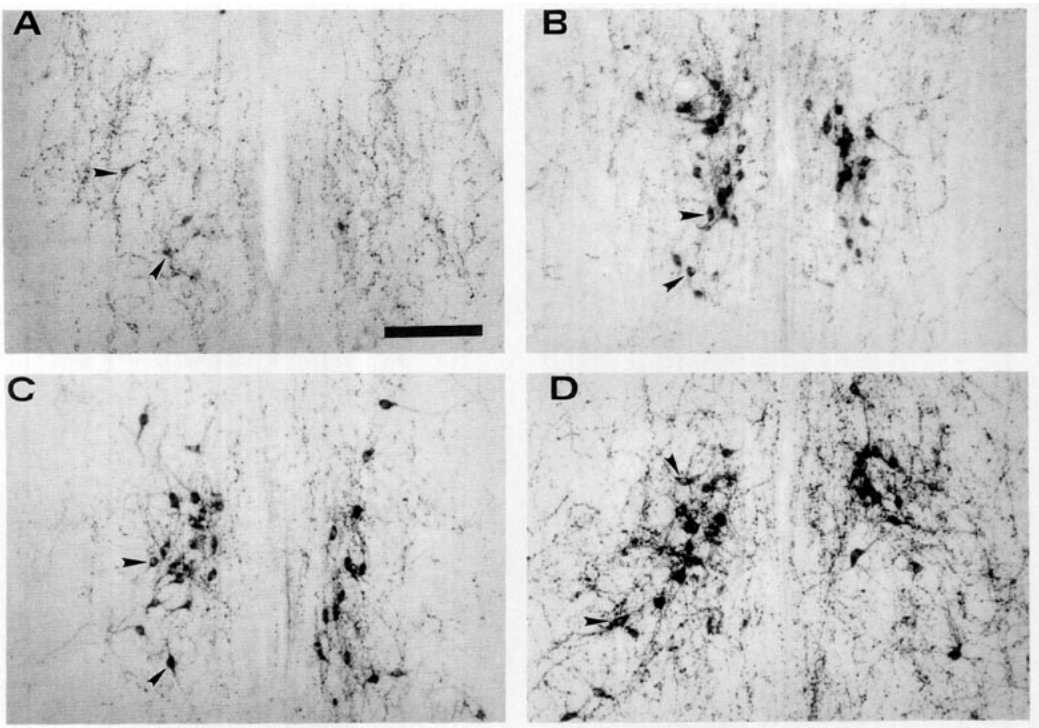

FIG. 4. Photomicrographs of GnRH-li ir in the preoptic area of photorefractory (A), photosensitive unstimulated (B), 1-day stimulated (C), and long-term stimulated (D) juncos. Photorefractory birds had significantly fewer and long-term photostimulated birds had significantly more GnRH-positive neurons in the preoptic area relative to other groups. V, third ventricle. Magnification bar, 50 μm. Arrows indicate ir neurons.

photosensitive unstimulated juncos have higher numbers of POA GnRH-positive neurons than refractory birds.

No difference in GnRH-li ir in the POA was observed between photosensitive unstimulated and photosensitive 1-day stimulated juncos. Earlier reports suggested that in the quail, increases in LH secretion are seen upon exposure to 1 photostimulatory day (Nicholls *et al.*, 1983; Creighton and Follett, 1987). Recently, the elegant experiments of Perera and Follett (1992) have shown that the *in vitro* release of GnRH by quail hypothalami increases after exposure to 1 long day. The clues suggest an increase in GnRH content within the brain after exposure to just 1 long day. This remains to be demonstrated *in vivo*.

Exposure to 1.5–2 months of stimulating photoperiod increases the number of POA GnRH-li ir neurons in the junco. Similar data have been reported in starlings (Foster *et al.*, 1987), in this case the increase in GnRH-li ir was limited to fiber number in the ME. In addition, plasma LH and testicular weight of long-term photo-stimulated juncos were significantly higher than other experimental groups. It should be noted that birds in this group were wild caught and were held in captivity for only a few days prior to sacrifice. Though unlikely, stress effects cannot be ruled in this paradigm.

Within a single breeding season, plasma concentrations of PRL and LH show an inverse relationship. Plasma LH levels are high early in the breeding season and then decrease when levels of plasma PRL are elevated (Goldsmith, 1983). Through the annual variations in sensitivity the avian

HPGA demonstrates several dramatic changes in content and secretion of hormones. Perhaps the most dramatic of these variations is the rapid decrease in LH secretion during photorefractoriness (Dawson *et al.*, 1985; Rohss and Silverin, 1983). Concomitantly, refractoriness is always accompanied by a transient surge in plasma PRL. Hence, the inverse relationship observed between PRL and LH within a breeding season is also observed during photorefractoriness. The present data suggest that this inverse relationship between plasma PRL and LH concentrations during photorefractoriness may originate in the brain. VIP and GnRH, the releasing factors for PRL and LH respectively, also exhibit an opposite pattern of expression in photorefractory juncos.

The photorefractory state is characterized by a nonresponsiveness of the HPGA to a stimulating photoperiod. Our data suggest that during this phase of the annual cycle INF VIP-li ir increases dramatically. This correlation may be causal. Although the critical experiments remain to be done, the latter hypothesis is suggested to explain the transient rise in PRL which always accompanies refractoriness in birds (Nicholls *et al.*, 1988). The present results strongly suggest that INF VIP neurons are intimately associated with the control of peripheral PRL secretion and may be involved in the rise of PRL seen during the onset of refractoriness.

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