Organization and Regulation of Hypothalamic Neurons

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Expression of D_1 and D_2 Dopamine Receptors in the Hypothalamus and Pituitary during the Turkey Reproductive Cycle: Colocalization with Vasoactive Intestinal Peptide

Yupaporn Chaiseha[a] Orlan Youngren[b] Khaled Al-Zailaie[b]
Mohamed El Halawani[b]

[a]School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand, and [b]Department of Animal Science, University of Minnesota, St. Paul, Minn., USA

Key Words

Birds · Catecholamine receptors · In situ hybridization · Prolactin · Vasoactive intestinal peptide · Arcuate nucleus · Ventromedial nucleus · Preoptic nucleus

Abstract

The regulation of avian prolactin (PRL) secretion and PRL gene expression is influenced by hypothalamic vasoactive intestinal peptide (VIP), the PRL-releasing factor in avian species. Recent evidence indicates that D_1 and D_2 dopamine (DA) receptors play a pivotal role in VIP and PRL secretion. The differential expression of DA receptors located on hypothalamic VIP neurons and anterior pituitary cells may affect the degree of prolactinemia observed during the turkey reproductive cycle. The relative expression of D_{1D} and D_2 DA receptor subtype mRNA was quantitated using in situ hybridization histochemistry (ISH). D_{1D} and D_2 DA receptor mRNA was found expressed throughout the hypothalamus and pituitary. The expression of D_{1D} DA receptor mRNA in the hypothalamus was found to be 6.8-fold greater than that of D_2 DA receptor mRNA. Higher D_{1D} DA receptor mRNA content was found in the anterior hypothalamus (3.6-fold), the ventromedial nucleus (2.0-fold), the infundibular nuclear complex (INF; 1.9-fold), and the medial preoptic nucleus (1.5-fold) of laying hens as compared to that of reproductively quiescent non-photostimulated hens. The levels seen in incubating hyperprolactinemic hens were essentially the same as in laying hens, except for the INF where levels were 52% higher. During the photorefractory stage (hypoprolactinemia), the D_{1D} DA receptor mRNA was at its lowest level in all areas tested. No differences were observed in hypothalamic D_2 DA receptor mRNA abundance throughout the reproductive cycle, except for an increase in D_2 DA receptor mRNA within the INF of photorefractory hens. Also, a marked reduction in D_2 DA receptor mRNA was observed in the pituitary of incubating hens. Pituitary D_{1D} DA receptor levels did not change when birds entered the incubating phase. Double ISH revealed that D_{1D} and D_2 DA receptor mRNAs were co-expressed within neurons expressing VIP mRNA, predominantly within the lateral hypothalamus and INF. D_{1D} DA receptor mRNA was more highly expressed than D_2 DA receptor mRNA. The present

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Mohamed El Halawani
495 Animal Science/Veterinary Medicine
1988 Fitch Avenue, University of Minnesota
St. Paul, MN 55108 (USA)
Tel. +1 612 624 9744, Fax +1 612 625 2743, E-Mail elhal001@tc.umn.edu

findings clearly demonstrate that the expression of stimulatory D_1 DA receptor mRNA in the hypothalamus increases in hyperprolactinemic incubating hens, whereas inhibitory D_2 DA receptor mRNA increases in the pituitary of hypoprolactinemic photorefractory hens.

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Introduction

Prolactin (PRL) secretion from the pituitary is closely correlated with the reproductive cycle in birds. During the reproductively quiescent stages of the cycle, plasma PRL levels are very low (5–10 ng/ml); however, during the laying and incubating stages, circulating PRL levels increase dramatically (500–1,500 ng/ml) [1]. In mammals, the regulation of PRL secretion is under the inhibitory control of tuberoinfundibular dopaminergic (TIDA) neurons in the hypothalamus [2, 3], which release dopamine (DA) that acts directly upon D_2 DA receptors located on pituitary lactotropes [4]. Removal of this dopaminergic inhibition results in increased PRL secretion and hyperprolactinemia [5, 6]. This is not the case in birds, where removal of hypothalamic inputs results in the complete cessation of PRL secretion [7, 8]. It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus [9, 10] and that the principal PRL-releasing factor is vasoactive intestinal peptide (VIP) [for review, see 11]. The role of DA in the regulation of avian PRL secretion is unclear as compared to the mammalian dopaminergic strategy for PRL control. In vitro studies on the effect of DA on chicken and turkey pituitary cells demonstrate inhibition of PRL release [12–16] similar to that seen in mammals. However, in vivo experiments have produced contradictory results. Intracerebroventricular (ICV) infusion of DA can either stimulate or inhibit PRL secretion, depending upon the dose infused [17]. Subsequent ICV infusion experiments suggest that different DA receptor subtypes with different affinities for DA may mediate the observed effects on PRL secretion [18]. It has been suggested that DA is inhibitory to the VIPergic system which stimulates PRL secretion in laying hens [19]. In vitro infusion of turkey hypothalamic explants has demonstrated that VIP secretion may be regulated by the opposing actions of stimulatory D_1 and inhibitory D_2 DA receptors in the hypothalamus, providing suggestive evidence for this hypothesis [20]. In addition, D_2 DA receptors also regulate PRL secretion in cultured turkey putuitary cells [16]. Three D_1 DA receptor subtypes (D_{1A}, D_{1B}, D_{1D}) have been cloned in chickens [21]. Recently, the cloning of cDNA from the turkey brain encoding D_1 and D_2 DA receptor subtypes has been reported [22, 23].

DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to release PRL [18]. Dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, dopaminergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator [for review, see 24]. The anatomical distribution of the avian dopaminergic system apparently resembles that of mammals [25, 26], as DA neurons are found throughout the avian hypothalamus [26–28]. The gross tissue distribution of D_1 and D_2 DA receptor subtypes in the turkey brain and pituitary has been determined [22, 23], but which receptors and which areas of the brain have a role in PRL secretion has not been established. The present studies were designed to localize and characterize DA receptors within individual areas of the hypothalamus and pituitary and to co-localize DA receptors with VIP neurons. D_{1D} DA receptor gene expression will be the focus of the study due to its demonstrated high levels of expression in the brain [22]. This study will attempt to provide further understanding of the interplay between DA receptors and of their role in the modulation of VIP and PRL secretion in birds.

Materials and Methods

Experimental Animals

Adult large white female Nicholas turkeys (54 weeks of age, 10–13 kg) were used. All birds were reared and housed in floor pens and feed and water were constantly available. Hens were divided into four groups according to their reproductive status [29]: non-photostimulated, reproductively quiescent (NPS), laying (LAY), incubating (INC), and photorefractory (REF).

Processing of Tissues for in situ Hybridization

Birds were killed by euthanasia injection (pentobarbital sodium, 6 g/ml; Anpro Pharmaceuticals, Arcadia, Calif., USA). A postmortem exam of each hen was performed to confirm its reproductive status. The brains, with attached pituitaries, were removed and immediately frozen on dry ice and stored at –80 °C. The brains were sectioned using a cryostat at a thickness of 15 µm and mounted onto microscope slides (Probe-On™, Fisher Scientific, Minneapolis, Minn., USA). The slides were stored desiccated at –80 °C until used.

Preparation of cRNA Probes

^{33}P-Labeled D_{1D} and D_2 DA Receptor Complementary RNA (cRNA) Riboprobes. 272- and 240-bp fragments of complementary DNA (cDNA) sequence coding for turkey D_{1D} and D_2 DA receptors, respectively, were provided [22, 23]. The cDNAs were subcloned

into XbaI/EcoRI-digested pBluescript SK(+) vectors (Stratagene, La Jolla, Calif., USA) and transformed into XL1-Blue cells according to the described methods [30]. Nucleotide sequence analysis of positive clones was performed on both strands by automated DNA sequencing (Advance Genetics Analysis Center, University of Minnesota, Minn., USA). In order to generate cRNA probes, the in vitro transcription and ^{33}UTP-radioactive labeling of the probes were performed as previously described [31, 32] by using a MAXIscript™ T_7/T_3 In Vitro Transcription Kit (Ambion Inc., Austin, Tex., USA). The cRNA probes were purified to obtain the full-length strand by polyacrylamide gel electrophoresis as previously described [30].

Digoxigenin-Labeled VIP cRNA Riboprobe. VIP cRNA probes were polymerase chain reaction (PCR) amplified using a GeneAmp PCR kit (Perkin-Elmer, Branchburg, N.J., USA) and generated from turkey cDNA sequence [33]. Primers complementary to bases 307–390 (encoding the 28 amino acids of the processed VIP peptide) of the turkey cDNA sequence were used to amplify a 216-bp VIP cDNA-specific fragment. The cDNA-specific fragment was subcloned into XbaI/EcoRI-digested pBluescript SK(+) vectors (Stratagene) and transformed into XL1-Blue cells as previously described [34]. VIP cRNA probe was generated by in vitro transcription and digoxigenin labeling of the probes was performed by using a MAXIscript™ T_7/T_3 In Vitro Transcription Kit (Ambion Inc). The VIP cRNA probe was purified by polyacrylamide gel electrophoresis [30] to obtain the full-length strand.

Single Label in situ Hybridization Histochemistry

In order to localize gene expression of D_1 or D_2 DA receptors within the brain and the pituitary, in situ hybridization (ISH) was performed as previously described [34]. Briefly, tissue sections were thawed to room temperature prior to use. The slides were then hybridized with 100 µl of hybridization solution containing ^{33}P-UTP-D_{1D}) or -D_2 DA receptor cRNA probes (0.5 × 10^6 cpm/slide), and incubated at 52 °C for 24 h in a humid incubator. The slides were paired together instead of using coverslips prior to incubation. The hybridization solution contained 50% formamide, 300 m*M* NaCl, 10 m*M* Tris HCl; pH 8.0, 1 m*M* EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin, 10 m*M* dithiothreitol, 500 ng/ml yeast tRNA, 1% SDS, and 10% dextran sulfate. After hybridization, the paired sections were separated by soaking in 2 × SSC at room temperature and then treated with RNase A solution (RNase A; Boehringer Mannheim, Indianapolis, Ind., USA; 20 µg/ml, 10 m*M* Tris-HCl; pH 8.0, and 0.5 *M* NaCl) for 30 min at 37 °C. The slides were washed once with 2 × SSC for 5 min at room temperature, once with 2 × SSC for 5 min at 55 °C, twice with 1 × SSC for 20 min at 55 °C, twice with 0.5 × SSC for 20 min at 55 °C, and 0.1 × SSC for 20 min at 55 °C. After the final wash, the sections were allowed to slowly cool to room temperature in the washing solution. The sections were then dehydrated through graded alcohol (50–100%) and quickly air-dried. Tissue sections were apposed to hyperfilm βmax autoradiograph film (Amersham, Arlington Heights, Ill., USA) for 1–2 days at –20 °C. X-ray films were developed and evaluated. The sections were then dipped in NTB_2 Nuclear track emulsion (Kodak, Rochester, N.Y., USA); diluted 1:1 with distilled water, dried overnight, and stored in a light-proof box at 4 °C until developed (4 days). The dipped sections were developed at room temperature with Kodak D-19 developer (Kodak); diluted 1:1 with water for 5 min, followed by 20 s rinse in distilled water, and fixed in a rapid fixer (Kodak) for 5 min. The sections were then rinsed with distilled water for 5 min. Following the development of autoradiographic grains, the sections were counterstained with the fluorescent dye Hoechst 33258 (0.001% bisbenzimide in 0.2 *M* KCl-HCl buffer; pH 2.0; Sigma, St. Louis, Mo., USA) as described [35] and coverslipped with DPX Mountant (Fluka Chemical, Ronkonkoma, N.Y., USA). Autoradiographic grains and counterstain were visualized with a fluorescent microscope equipped with a darkfield condenser and ultraviolet excitation using a UG1 filter set for excitation (Schott, Duryea, Pa., USA) and a 420-nm longpass emission filter.

Double Label in situ Hybridization Histochemistry

To identify VIP neurons that co-express D_1 or D_2 DA receptors, double label ISH was performed as described [36, 37] with slight modifications. In brief, sections were pretreated as described above for single label ISH. Sections were then hybridized with 100 µl of hybridization solution containing ^{33}P-UTP D_{1D} or D_2 DA receptor cRNA probes (0.5 × 10^6 cpm/slide), digoxigenin-labeled VIP cRNA probe (1.0 mg/ml), and incubated at 52 °C for 24 h in a humid incubator. The slides were paired together prior to incubation as described above. The hybridization solution contained 50% formamide, 300 m*M* NaCl, 10 m*M* Tris-HCl; pH 8.0, 1 m*M* EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin, 10 m*M* dithiothreitol, 1% SDS, 10% dextran sulfate, and 2.0 mg/ml yeast tRNA freshly denatured. After hybridization, the paired sections were separated by soaking in 2 × SSC at room temperature and then treated with RNase A solution for 30 min at 37 °C. The slides were washed twice with 2 × SSC for 5 min at room temperature, once with 2 × SSC for 5 min at 55 °C, twice with 1 × SSC for 20 min at 55 °C, twice with 0.5 × SSC for 20 min at 55 °C, and 0.1 × SSC for 20 min at 55 °C. The sections were then incubated for 60 min in blocking buffer (2 × SSC, 0.05% Triton X-100, 0.2% normal sheep serum), rinsed in buffer 1 (100 m*M* Tris-HCl and 150 m*M* NaCl, pH 7.4), and then incubated in buffer 1 containing antidigoxigenin fragments conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:1,000, 1% normal sheep serum and 0.3% Triton X-100 for 3 h at 4 °C overnight. Next, the sections were rinsed in buffer 1, rinsed in buffer 2 (100 m*M* Tris-HCl, 50 m*M* $MgCl_2$, and 100 m*M* NaCl, pH 9.5), and then incubated in buffer 2 containing 4-nitroblue tetrazolium-chloride (340 µg/ml; Boehringer Mannheim), 5-bromo-4-chloro-3-indolyl phosphate (175 µg/ml; Boehringer Mannheim), and levamisole (240 µg/ml; Sigma). When cells containing dark purple precipitate (corresponding to cells containing hybridized digoxigenin-labeled riboprobe) were clearly visible at the light microscopic level, the reaction was stopped by rinsing the sections in TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). Next, the slides were dipped in 70% ethanol, air-dried, and coated in 1.5% parlodion (Fisher Scientific) dissolved in isoamyl acetate. After air-drying, tissue sections were apposed to hyperfilm βmax autoradiograph film (Amersham) for 1 day at –20 °C. X-ray films were developed and evaluated. The sections were then dipped in NTB_2 Nuclear track emulsion (Kodak); diluted 1:1 with distilled water, dried overnight, and stored in a light-proof box at 4 °C until development (4 days). The dipped sections were developed at room temperature with Kodak D-19 developer (Kodak) and fixed in a rapid fixer (Kodak) and rinsed with distilled water as single labeled ISH. Following the development of autoradiographic grains, the sections were coverslipped with DPX Mountant (Fluka Chemical). Autoradiographic grains of D_{1D} and D_2 DA receptors and digoxigenin-labeled VIP were visualized with a fluorescent microscope equipped with a darkfield and a brightfield condenser.

Specificity of cRNA Probes Used for in situ Hybridization

The specificity of the D_{1D} and D_2 DA receptor cRNA probes used for ISH was previously verified by two methods [38]. First, ^{32}P-UTP-labeled antisense or sense cRNA probes were used to probe the total RNA from the turkey hypothalamus by Northern blot analysis. Second, ^{33}P-UTP-labeled sense and antisense cRNA probes of D_{1D} and D_2 DA receptor-specific fragments were hybridized to the brain sections. The labeled probes were applied to adjacent sections. Only the latter showed hybridization signals on tissue sections. No labeling on brain sections was observed with the sense cRNA probes (data not shown).

The specificity of VIP cRNA probe was previously verified [34]. The identity and integrity of the digoxigenin-labeled VIP cRNA probe was verified by digoxigenin-VIP single ISH. The anatomical distribution of digoxigenin-labeled cells observed in these studies agrees with previously published data on the localization of VIP cell bodies [34] and suggest that the VIP probe specifically hybridized to the correct mRNA.

In addition, RNase pretreatment of brain sections was performed as another control. RNase pretreatment of tissue sections in order to destroy target RNA is not an acceptable control for these probes, because trace amounts of RNase would likely also digest the cRNA probes [39]. The criteria used in the present study have been accepted as sufficient verification of specificity for probes utilized for ISH [39–42].

Image Analysis

Data were organized by coronal brain sections, which were taken from approximately 1.0 mm rostral to the optic chiasma to approximately 1.0 mm caudal to the median eminence and pituitary. To aid in the documentation of neuroanatomical results, nomenclature from an atlas of the turkey hypothalamus [Youngren, unpubl.], the chicken brain [43], and chicken hypothalamus [44] were used.

For the single label ISH, the autoradiograms of the brain sections were examined as previously described for comparison of the expression of D_{1D} and D_2 DA receptor mRNAs within different brain areas among reproductive groups. The microscopic images of the brain sections were visualized at 20 × magnification using a videocamera (CoolCam 2000 color) fitted to a fluorescent microscope (Nikon E800 Eclipe, Nikon, Japan). The images were then captured and stored by Image Pro Plus 1.3 software (Media Cybernetics, Silver Spring, Md., USA) with a fixed setting for the videocapture. Eight microscopic fields corresponding to the lateral septum (LS), medial preoptic nucleus (POM), anterior hypothalamic nucleus (AM), lateral hypothalamus (LHy), paraventricular nucleus (PVN), ventromedial nucleus (VMN), infundibular nuclear complex (INF), and pituitary (PIT) on pairs of adjacent sections (4 microscopic fields/section) from each bird were chosen for area imaging in each treatment group. The integrated density (arbitrary densometric unit; ADU) of hybridization signals was then analyzed on a pixel-by-pixel basis in the defined area using ImageQuant™ software (Version 1.1, Molecular Dynamics, Sunnyvale, Calif., USA). Background was measured from 8 nonhybridizing tissue fields, averaged, and subtracted from the integrated density of hybridization signals. Values corresponding to the expression of D_{1D} and D_2 DA receptors in the defined areas were calculated for each bird by summing the integrated density values from 8 microscopic fields from two consecutive sections as described [34].

For the double ISH, tissue sections were viewed under a bright-field microscope and the images were captured as described above. The hybridization signals of adjacent sections of VIP-D_{1D} DA receptor label and VIP-D_2 DA receptor label were observed and then compared in individual brain areas.

Table 1. Expression of D_{1D} DA receptor mRNA within individual areas of the turkey hypothalamus and pituitary at different reproductive stages

Area	Reproductive group			
	NPS	LAY	INC	REF
LS	12.4 ± 4.0^{a}	14.5 ± 3.9^{a}	6.8 ± 5.0^{a}	8.3 ± 2.7^{a}
POM	$14.1 \pm 2.6^{a,b}$	21.0 ± 4.2^{a}	24.4 ± 3.8^{a}	7.7 ± 2.3^{b}
AM	7.3 ± 1.8^{b}	25.8 ± 4.9^{a}	27.3 ± 4.4^{a}	13.3 ± 1.7^{b}
LHy	10.0 ± 3.2^{b}	20.2 ± 4.6^{a}	21.9 ± 4.7^{a}	8.0 ± 3.9^{b}
PVN	7.8 ± 3.5^{a}	5.6 ± 3.0^{a}	9.8 ± 4.1^{a}	6.2 ± 2.7^{a}
VMN	15.6 ± 4.3^{b}	31.0 ± 7.9^{a}	34.4 ± 6.1^{a}	14.4 ± 2.8^{b}
INF	18.8 ± 3.7^{c}	34.5 ± 6.1^{b}	52.6 ± 5.3^{a}	17.6 ± 2.0^{c}
PIT	12.7 ± 1.8^{a}	14.4 ± 2.5^{a}	17.0 ± 3.1^{a}	10.8 ± 4.5^{a}

Values represent mean ± SEM of arbitrary densometric units (ADU). Values with different superscripts on the same line are significantly different ($p < 0.05$, n = 6). See figure 6 for abbreviations.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis for D_{1D} DA and D_2 DA receptor gene expression by single ISH was performed employing the General Linear Model procedure of the Statistical Analysis System [45]. Significant differences in mean ± SEM among treatment groups were assessed using Tukey's Studentized Range Test at a significance level of $\alpha = 0.05$.

Results

D_{1D} DA Receptor mRNA Expression

D_{1D} DA receptor mRNA expression occurred throughout the turkey hypothalamus and pituitary, as revealed by single label ISH. D_{1D} DA receptor mRNA was found within the LS, POM, AM, LHy, PVN, VMN, INF, and PIT. Cells expressing the greatest density of D_{1D} DA receptor mRNA were located in the POM, AM, LHy, VMN and INF. Cells expressing the least D_{1D} DA receptor mRNA were found in the LS, PVN, and PIT (fig. 1, table 1). The expression of D_{1D} DA receptor mRNA was greater in the POM (1.5-fold), AM (3.6-fold), VMN (2.0-fold), and INF (1.9-fold) of LAY when compared to that of NPS (table 1). When birds made the transition from LAY to INC, the D_{1D} DA receptor mRNA levels seen in the AM remained essentially the same (fig. 2, 3A; $p > 0.05$), while levels in the INF increased 52% (fig. 3B, 4;

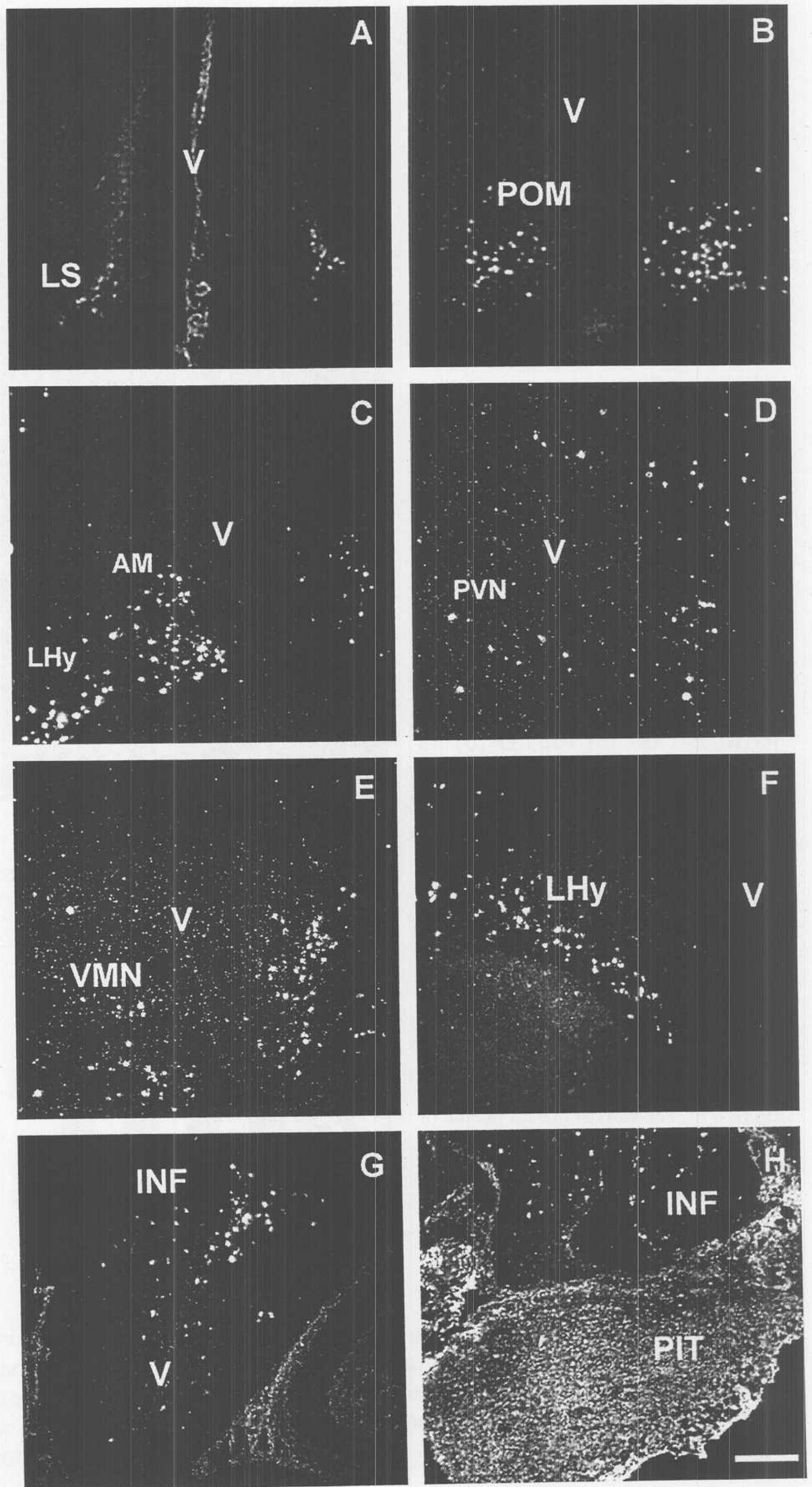


Fig. 1. Darkfield illumination photomicrographs of coronal sections illustrating the distribution of D_{1D} DA receptor mRNA in the hypothalamus and pituitary of the laying turkey. The specific hybridization binding of D_{1D} DA receptor cRNA probe was observed within the LS (**A**), POM (**B**), AM (**C**), anterior LHy (**C**), PVN (**D**), VMN (**E**), posterior LHy (**F**), INF (**G, H**), and pituitary (PIT) gland (**H**). All photographs are printed at the same magnification; magnification bar = 100 μm. See figure 6 for abbreviation definitions.

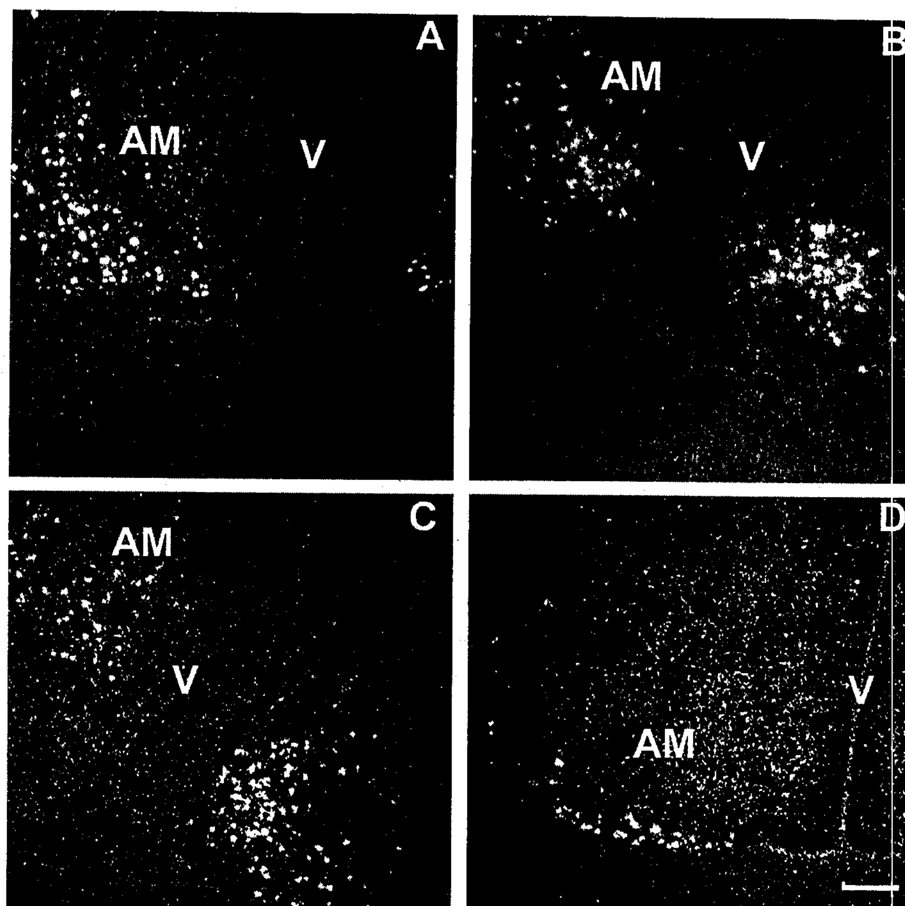
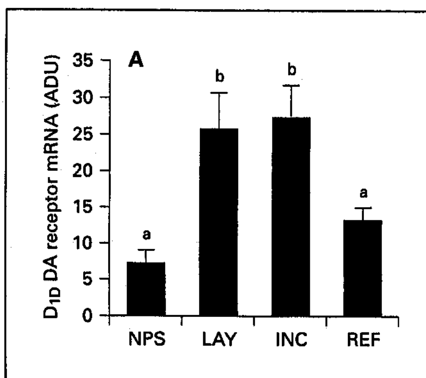


Fig. 2. Darkfield illumination photomicrographs of coronal sections demonstrating the distribution of D_{1D} DA receptor mRNA within the anterior hypothalamus (AM) of the turkey at different reproductive stages. NPS (**A**), LAY (**B**), INC (**C**), REF (**D**). All photographs are printed at the same magnification; magnification bar = 100 μm.



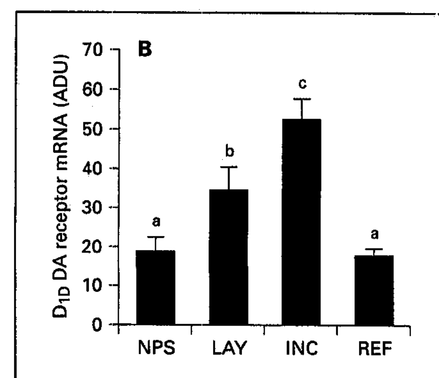


Fig. 3. **A** Changes in D_{1D} DA receptor mRNA expression within the anterior hypothalamus (AM) area of the turkey across reproductive stages. **B** Changes in D_{1D} DA receptor mRNA expression within the infundibular nuclear complex (INF) of the turkey hypothalamus at different reproductive stages. Values are presented as the mean ± SEM (n = 6). The vertical line on the top of the bar indicates the magnitude of the standard error. Within experiments, means with different letters are statistically significant ($p < 0.05$).

$p < 0.05$). Changes in the expression of D_{1D} DA receptor mRNA in the rest of the areas examined (LS, PVN, and PIT) were less dramatic. D_{1D} DA receptor mRNA levels in REF were the lowest of all areas tested.

D_2 DA Receptor mRNA Expression

Cells expressing D_2 DA receptor mRNA were also distributed throughout the turkey hypothalamus and pituitary. Expression of D_2 DA receptor mRNA was very low when compared to that of D_{1D} DA receptor mRNA, but was detectable within the hypothalamic areas tested. The

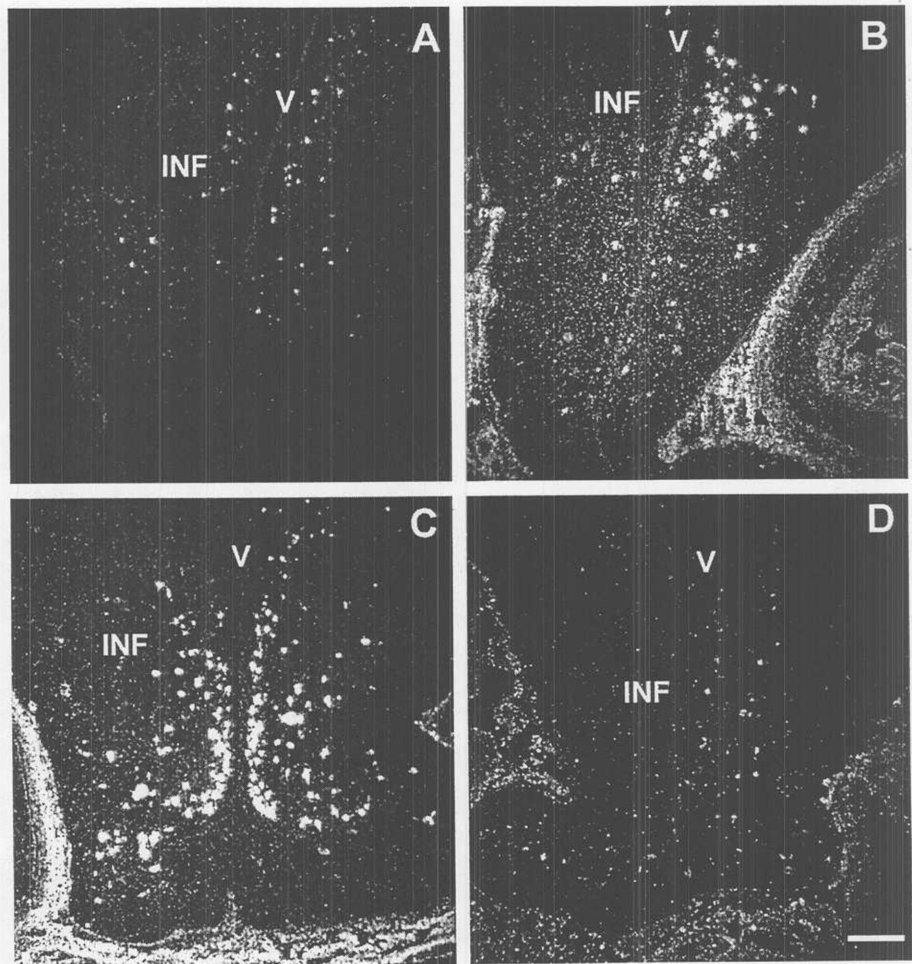


Fig. 4. Darkfield illumination photomicrographs of coronal sections demonstrating the distribution of D_{1D} DA receptor mRNA within the infundibular nuclear complex (INF) of the turkey at different reproductive stages. NPS (**A**), LAY (**B**), INC (**C**), REF (**D**). All photographs are printed at the same magnification; magnification bar = 100 μm.

expression of D_2 DA receptor mRNA in the hypothalamus was 6.8-fold less than that of D_{1D} DA receptor mRNA (fig. 5, 6, table 2). D_2 DA receptor mRNA was expressed within the same areas as D_{1D} DA receptor mRNA (fig. 6). No differences were observed in hypothalamic D_2 DA receptor mRNA abundance throughout the reproductive cycle of the turkey hen, except for a marked increase in D_2 DA receptor mRNA in the INF of REF hens (fig. 7, 8A). The D_2 DA receptor mRNA was predominantly expressed in the PIT (table 2), where a marked reduction in PIT D_2 DA receptor mRNA was seen in INC hens (fig. 8B, 9) when compared to other reproductive stages.

D_{1D} and D_2 DA Receptor mRNA Expression in VIP mRNA-Expressing Neurons

Neurons expressing VIP mRNA were found throughout the hypothalamus. D_{1D} and D_2 DA receptor mRNA expression was also found scattered within VIP neurons,

Table 2. Expression of D_2 DA receptor mRNA within individual areas of the turkey hypothalamus and pituitary at different reproductive stages

Area	Reproductive group			
	NPS	LAY	INC	REF
LS	4.9 ± 2.7^a	4.8 ± 2.6^a	5.2 ± 3.3^a	6.4 ± 3.4^a
POM	5.1 ± 2.8^a	2.0 ± 1.9^a	1.9 ± 1.1^a	6.5 ± 2.7^a
AM	11.1 ± 3.5^a	9.5 ± 2.5^a	3.2 ± 2.6^a	7.0 ± 2.5^a
LHy	11.3 ± 4.6^a	4.8 ± 2.4^a	5.3 ± 2.9^a	10.1 ± 3.3^a
PVN	4.3 ± 2.4^a	3.2 ± 1.7^a	5.5 ± 2.3^a	5.9 ± 3.8^a
VMN	15.2 ± 3.5^a	13.4 ± 1.5^a	14.0 ± 5.5^a	12.6 ± 4.0^a
INF	$14.3 \pm 3.3^{a,b}$	9.7 ± 2.9^b	9.7 ± 4.3^b	21.9 ± 5.1^a
PIT	23.7 ± 2.1^a	13.6 ± 1.9^b	9.4 ± 2.7^b	25.8 ± 8.9^a

Values represent mean ± SEM of arbitrary densometric units (ADU). Values with different superscripts on the same line are significantly different ($p < 0.05$, $n = 6$). See figure 6 for abbreviations.

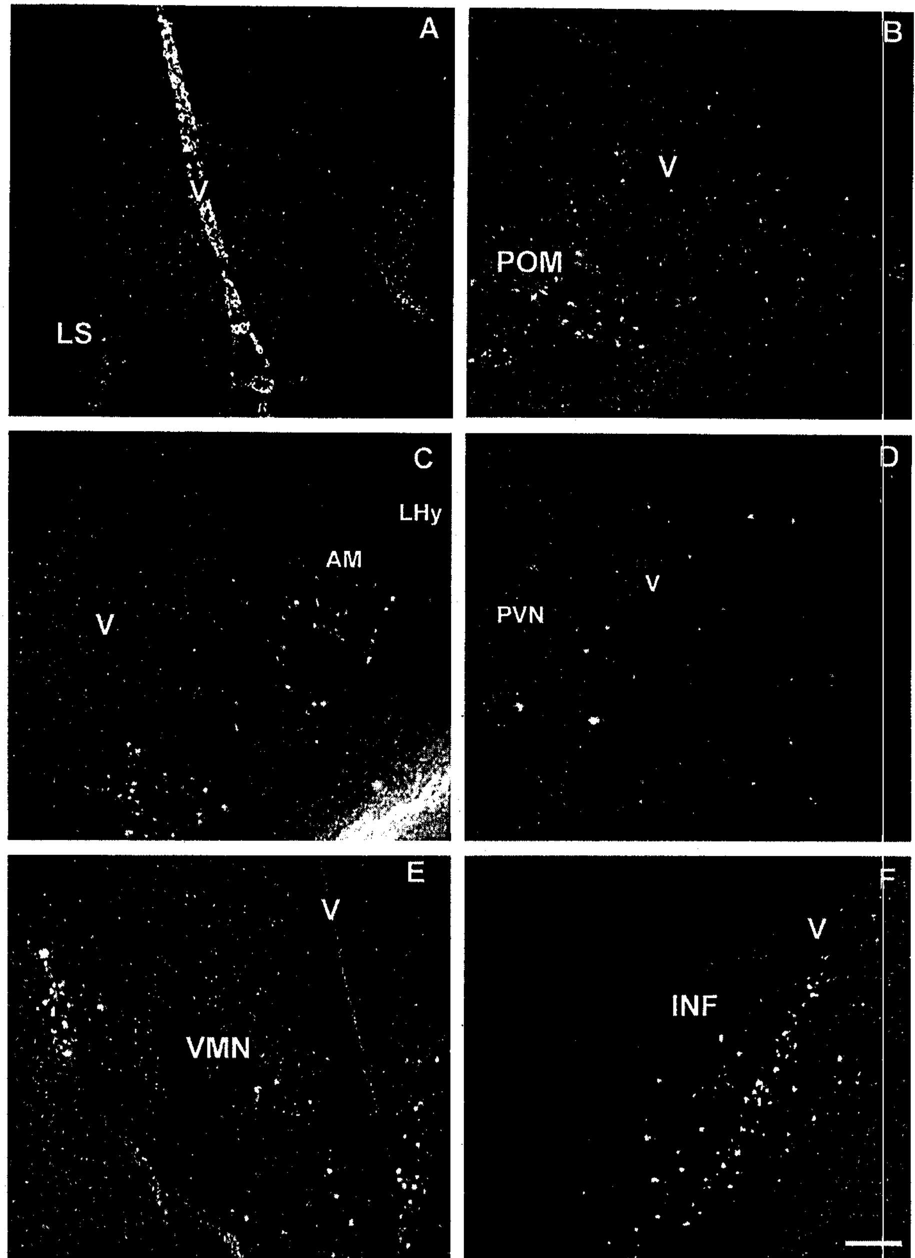


Fig. 5. Darkfield illumination photomicrographs of coronal sections demonstrating the distribution of D_2 DA receptor mRNA in the turkey hypothalamus. The neurons expressing D_2 DA receptor mRNA were observed within the LS (**A**), POM (**B**), AM (**C**), LHy (**C**), PVN (**D**), VMN (**E**), and INF (**F**). All photographs are printed at the same magnification; magnification bar = 100 μm. See figure 6 for abbreviation definitions.

as revealed by double label ISH. Within the LHy and INF, D_{1D} DA receptor mRNA was more highly expressed than D_2 DA receptor mRNA. Some neurons expressing VIP mRNA in the LHy and INF had overlying clusters of silver grains of D_{1D} receptor mRNA (fig. 10A, B) or D_2 DA receptor mRNA (fig. 10C, D), indicating that they co-expressed D_{1D} and D_2 DA receptor mRNA.

Discussion

The present study shows that D_{1D} and D_2 DA receptor gene expression is extensively distributed throughout the turkey hypothalamus and pituitary, and demonstrates for the first time that differential expression of DA receptor subtypes within specific areas of the hypothalamus and pituitary is correlated with the turkey reproductive cycle. Major expression of D_{1D} DA receptor mRNA is found within the POM, AM, LHy, VMN and INF. In contrast,

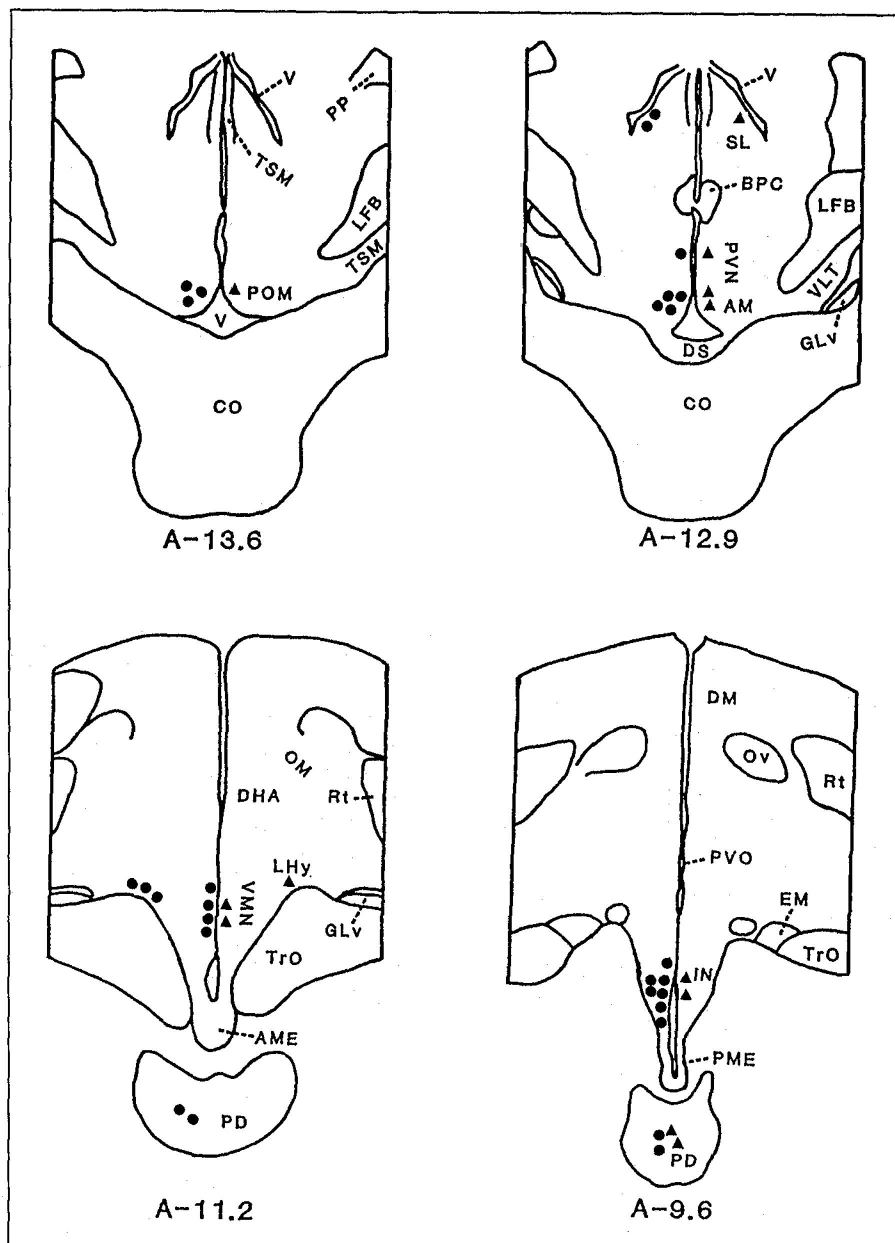


Fig. 6. Schematic coronal sections showing the distribution of D_{1D} (circles) and D_2 (triangles) DA receptor mRNA throughout the hypothalamus of the laying turkey hen. Coronal illustrations were redrawn from Mauro et al. [56] using an unpublished turkey atlas [O.M. Youngren, pers. commun.] with nomenclature from Kuenzel and van Tienhoven [44]. The following abbreviations are used in the figure legends: AM = nucleus anterior hypothalami; AME = anterior median eminence; BPC = bed nucleus pallial commissure; CO = chiasma opticum; DHA = area dorsalis hypothalami; DM = nucleus dorsomedialis hypothalami; DS = supraoptic decussation; EM = nucleus ectomamillaris; GLv = nucleus geniculatus lateralis, pars ventralis; IN = nucleus infundibuli; INF = infundibular nuclear complex; LFB = lateral forebrain bundle; LHy = lateral hypothalamic area; LS = organum septi laterale; OM = tractus occipitomesencephalicus; Ov = nucleus ovoidalis; PD = pars distalis; PME = posterior median eminence; POM = nucleus preopticus medialis; PP = paleostriatum primitivum; PVN = nucleus paraventricularis; PVO = paraventricular organ; Rt = nucleus rotundus; SL = nucleus septalis lateralis; TrO = tractus opticus; TSM = tractus septomesencephalicus; V = ventricle; VLT = nucleus ventrolateralis thalami; VMN = nucleus ventromedialis hypothalami.

the expression of D_2 DA receptor mRNA is found primarily in the pituitary. In addition, D_{1D} and D_2 DA receptor mRNA expression is co-localized with neurons expressing VIP mRNA in the LHy and INF.

D_{1D} DA receptor mRNA amounts are greater in LAY than in NPS, and when birds make the transition from LAY to INC, D_{1D} DA receptor mRNA in the INF increases by 50%. Also, when birds become REF, an increase in D_2 DA receptor mRNA is noted in the INF. These results correspond with a former study of D_{1D} and D_2 DA receptor gene expression in the entire hypothalamus and pituitary of the turkey hen, as measured by RT-PCR [22]. D_1 and D_2 DA receptors are stimulatory and inhibitory, respectively, to PRL release and expression in birds [18, 24]. The existence of specific DA-binding sites has been identified in the hypothalamus of the bantam hen [46] and D_1-like DA receptors exist in the brain of the pigeon [47, 48], the European starling [49], the quail [50], and the chick [51]. D_2-like DA receptors have been found in the pigeon [48] and quail brain [52] and D_1 and D_2 DA

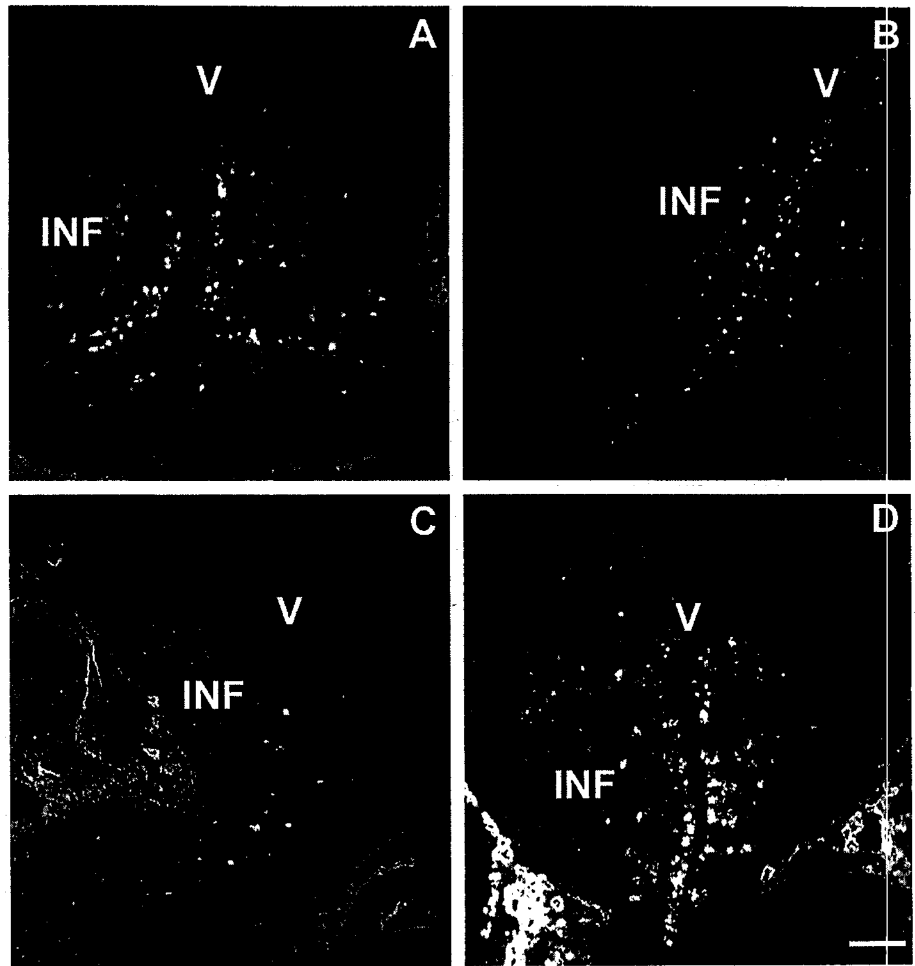
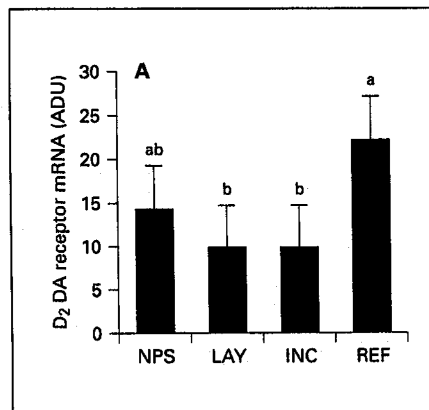


Fig. 7. Darkfield illumination photomicrographs of coronal sections demonstrating the distribution of D_2 DA receptor mRNA within the infundibular nuclear complex (INF) of the turkey across the reproductive stages. NPS (**A**), LAY (**B**), INC (**C**), REF (**D**). All photographs are printed at the same magnification; magnification bar = 100 µm.



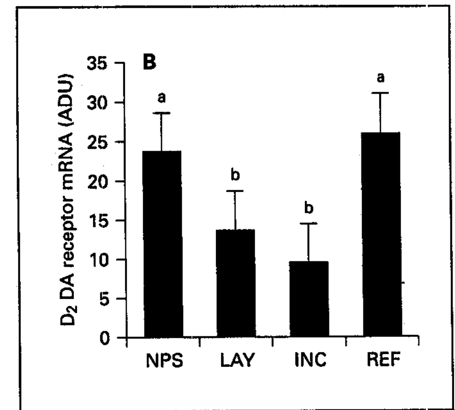


Fig. 8. A Changes in D_2 DA receptor mRNA expression within the infundibular nuclear complex (INF) of the turkey hypothalamus across reproductive stages. **B** Changes in D_2 DA receptor mRNA expression within the turkey pituitary at different reproductive stages. Values are presented as the mean ± SEM (n = 6). The vertical line on the top of the bar indicates the magnitude of the standard error. Within experiments, means with different letters are statistically significant ($p < 0.05$).

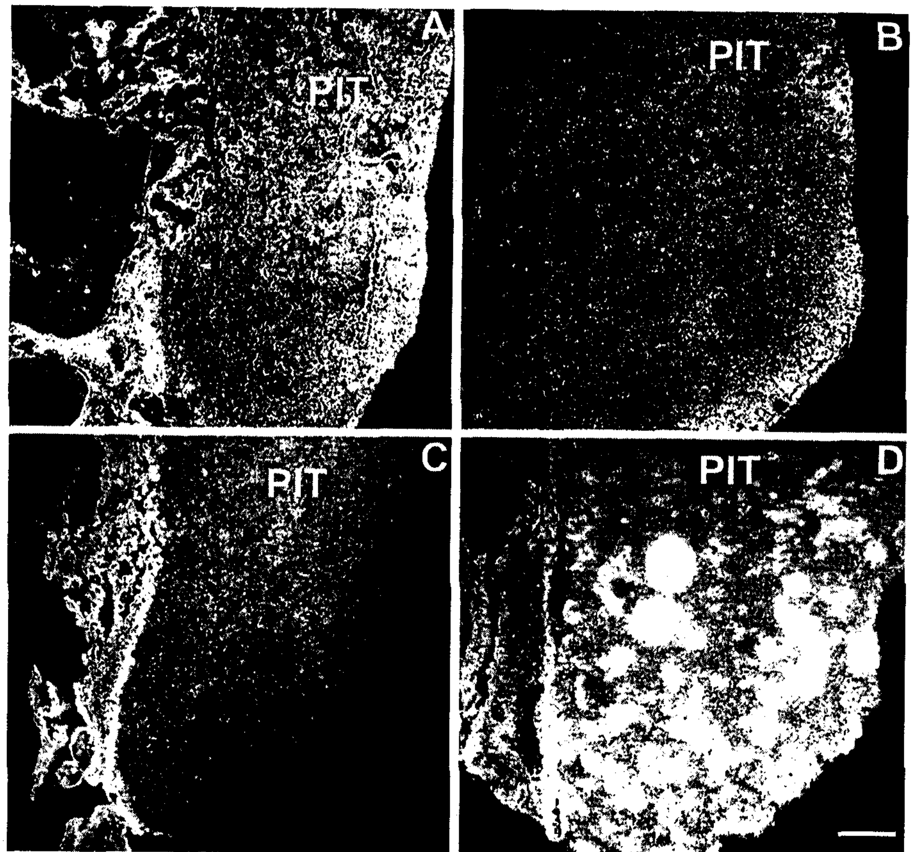


Fig. 9. Darkfield illumination photomicrographs of coronal sections showing the distribution of D_2 DA receptor mRNA within the turkey pituitary (PIT) at different reproductive stages: NPS (**A**), LAY (**B**), INC (**C**), REF (**D**). All photographs are printed at the same magnification; magnification bar = 100 µm.

receptor subtypes have been cloned from the hypothalamus of the chick [21] and turkey [23]. Recently, D_1 and D_2 DA receptor gene expression within the brain of the chicks [53] and turkey [22, 23] has been reported.

The present results agree with immunohistochemical studies indicating the distribution of DA neurons throughout the avian hypothalamus [28, 54, 55]. DA neurons project from the preoptic area to the INF [55]. The number of VIP-immunoreactive (VIP-ir) cells [56, 57] and VIP mRNA [34] dramatically increases in the INF of the incubating turkey hen. The number and size of VIP-ir neurons within the mediobasal hypothalamus of the turkey increases when plasma PRL is high, as it does in the domesticated pigeon and ring dove during the initiation of crop milk secretion and feeding of offspring [58, 59] and in the incubating bantam hen [60]. The current findings clearly indicate that the differential expression of stimulatory D_{1D} DA receptor mRNA in the hypothalamus relates to the degree of prolactinemia in turkey hens, and that the central site for DA mediation of VIP is located within the INF, in the same region as the VIP neurons known to be involved in PRL secretion [55].

In mammals, moderate to high levels of D_2 DA receptor message have been detected in many of the same hypothalamic nuclei [61, 62]. It has been suggested that diencephalic DA neuron groups (possibly in the LHy or INF) may regulate VIP secretion in birds via D_2 DA receptors [23]. The stimulatory effect of a D_{1D} DA agonist on hypothalamic VIP secretion is inhibited by a D_2 DA receptor agonist [20]. The results from previously studies report several dopaminergic cell groups localized in the LHy [25, 28]. In this study, the co-expression of D_2 DA receptor mRNA seen in VIP-expressing neurons within the LHy and INF supports this hypothesis. The preoptic area of the diencephalon has been shown to contain high densities of DA fibers and varicosities [25], and this study found D_2 DA receptor mRNA in this area.

D_2 DA receptors on pituitary lactotropes inhibit PRL release in both mammals [3] and birds [24] and DA-binding sites in the anterior pituitary of bantam hens decrease during incubation [46]. The present findings clearly indicate that the differential expression of inhibitory D_2 DA receptor mRNA in the turkey pituitary is correlated with the degree of prolactinemia, since D_2 DA receptor mRNA

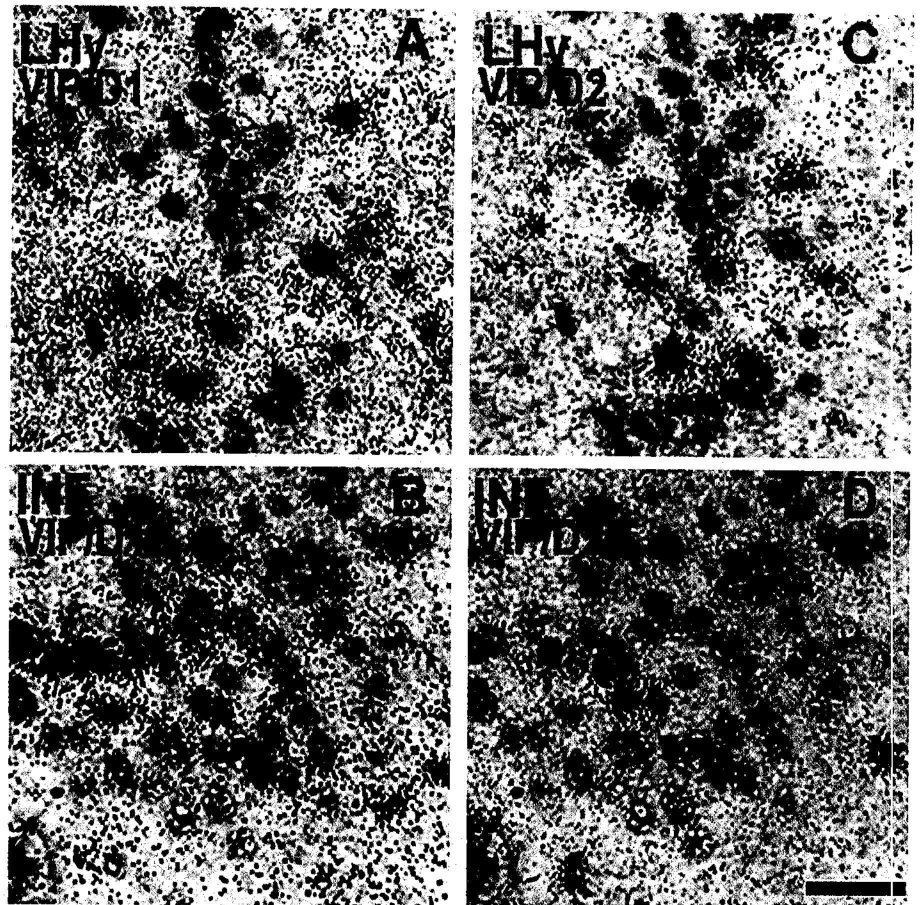


Fig. 10. D_{1D} DA receptor mRNA expression (clusters of black dots) within VIP mRNA-expressing neurons (cells containing purple-black precipitate) in the LHy (**A**) and INF (**B**) of the turkey hypothalamus. D_2 DA receptor mRNA expression (clusters of black dots) within VIP mRNA-expressing neurons (cells containing purple-black precipitate) in the LHy (**C**) and INF (**D**). All photographs are printed at the same magnification; magnification bar = 100 μm.

increases in the pituitary of hypoprolactinemic photorefractory hens. These findings are supported by the existence of dopaminergic fibers within the median eminence of the quail, chicken, and turkey [25, 54, 63] implicating this structure as a possible source from which DA could impact the avian pituitary and regulate PRL secretion.

D_1 and D_2 DA receptor mRNA is found in the LS and POM where VIP mRNA-expressing neurons are found [34]. A physiological role for VIP in the control of changes in gonadotropin-releasing hormone (GnRH) expression as a function of photosensitivity has been suggested [64] and a subset of VIP-ir neurons within the medial basal hypothalamus and septal region have been proposed as encephalic photoreceptor neurons [65]. VIP nerve terminals contact putative secretory GnRH neurons in the lateral septo-preoptic area [66–68]. This suggests that DA receptors may be involved in the VIP modulation of GnRH secretion in the septo-preoptic area and may influence interactions involved in mediating the photoperiodic response in birds. In mammals, it has been suggested that the dopaminergic system interacts with photoperiodic regulation of LH secretion [69, 70].

In summary, the present study provides robust evidence of the active participation of dopamine in the regulation of circulating PRL throughout the reproductive cycle of the turkey. When PRL increases as birds are photostimulated and begin to lay eggs, D_{1D} DA receptor mRNA also increases in hypothalamic areas known to be involved in reproduction. When plasma PRL reaches maximal levels during the incubation of eggs, D_{1D} DA receptor mRNA also increases dramatically within the INF, where high quantities of VIP, the avian PRL-releasing factor, are observed. When birds become photorefractory and the level of circulating PRL plummets, levels of D_{1D} DA receptor mRNA within the hypothalamus are also at their lowest levels. And the co-localization of dense D_{1D} DA receptor mRNA with hypothalamic neurons expressing VIP mRNA provides another link in the suspected DA/VIP interaction leading to the regulation of PRL. While the role of central DA in the stimulation of

PRL secretion is beginning to come into focus, its putative role as an inhibitor of PRL secretion remains hidden. D_2 DA receptor mRNA quantities in the hypothalamus are more than 6-fold lower than that of D_{1D} DA receptor mRNA. However, D_2 DA receptor mRNA does increase in the INF of photorefractory birds when PRL is low, suggestive of inhibitory activity. The avian pituitary contains high amounts of D_2 DA receptor mRNA, and D_2 DA receptors are known to antagonize the PRL-stimulating action of VIP at the pituitary level.

Acknowledgments

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