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Molecular Cloning and Tissue Distribution of an Avian D2 Dopamine Receptor mRNA From the Domestic Turkey (*Maleagris gallopavo*)

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ABSTRACT

The reverse transcriptase-polymerase chain reaction (RT-PCR), in combination with 5′ and 3′ rapid amplification of cDNA ends (RACE), was used to clone a G protein-coupled receptor from turkey brain mRNA. This cDNA clone has an open reading frame of 1,311 base pairs encoding a 436-residue protein with seven transmembrane-spanning domains and exhibits high homology with previously cloned mammalian D2 dopamine receptors. Northern blot analysis of turkey brain mRNA detected an approximate 2.4-kb transcript. RT-PCR and subsequent nucleotide sequence analysis of turkey brain and peripheral tissue mRNA also demonstrated the presence of an alternatively spliced mRNA corresponding to the predicted D2 short isoform. RT-PCR experiments demonstrated a widespread distribution of alternatively spliced D2 dopamine receptor transcripts throughout the turkey brain and in select peripheral tissues as well. In situ hybridization experiments detected strong autoradiographic signals over much of the turkey telencephalon, diencephalon, mesencephalon, cerebellum, pituitary, and pineal gland. Dopamine has several important functions as a neurotransmitter and hormone in mammals and may have similar actions in avian species. The cloning and tissue distribution of the D2 receptor subtype should enable the investigation of any functional role dopamine and dopamine receptors exert on the physiology and behavior of birds. J. Comp. Neurol. 407:543–554, 1999.

Indexing terms: reproduction; prolactin; bird; in situ hybridization; reverse transcriptase-polymerase chain reaction

Dopamine is a ubiquitous neurotransmitter that is found in both the central and peripheral nervous systems of many species and is involved in a wide variety of behavioral and physiologic functions (Bailhache and Balthazart, 1993; Crowley et al., 1991; Ferrari and Giuliani, 1993; Moons et al., 1994). The transduction of dopaminergic signals is mediated by several G protein-coupled receptor subtypes. These receptors are divided into two major subclasses: the D1-like (D1 and D5) and the D2-like (D2, D3 and D4) receptors, and are characterized further by their ability to stimulate or inhibit adenylate cyclase activity (Civelli et al., 1991; Jackson and Westlind-Danielsson, 1994). In addition to its role as a neurotransmitter, dopamine has been demonstrated to act as a hormone (Ben-Jonathan, 1985). In mammals, dopamine that is released from hypothalamic tuberoinfundibular (TIDA) neurons serves as the physiological inhibitor of prolactin secretion and is mediated through D2 dopamine receptors residing on pituitary lactotroph membranes. Recent evidence suggests that dopamine regulates prolactin secretion in birds as well (El Halawani et al., 1991; Youngren et al., 1995, 1996).

The anatomical distribution of the avian dopaminergic system apparently resembles that of mammals (Moons et al., 1994; Reiner et al., 1994). These studies demonstrated widespread distribution of dopaminergic perikarya and fibers throughout the avian brain. Notably, many dopamine-immunoreactive (ir) neurons and fibers can be found along the third ventricle in the periventricular nucleus (PHN) and infundibular nucleus (IN). These areas also

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have been shown to be immunoreactive for vasoactive intestinal peptide (VIP) (Hof et al., 1991; Mauro et al., 1989, 1992) and VIP mRNA (Kuenzel et al., 1997). It also has been shown that VIP, possibly released from the median eminence, is a known prolactin-releasing factor in turkeys (El Halawani et al., 1990a,b) and chickens (Sharp et al., 1989). The median eminence is the terminal field of some neurons originating from circumventricular hypothalamic nuclei. The median eminence is a region where hypophysiotropic factors (i.e., VIP) are released into the portal blood vessels and are ultimately responsible for the release of anterior pituitary hormones. Thus, it is possible that dopamine receptors, residing on hypothalamic VIP-secreting neurons, may indirectly mediate prolactin secretion. The recent demonstration that VIP secretion may be regulated by an opposing action of D1 and D2 dopamine receptors in the turkey hypothalamus provides suggestive evidence for this hypothesis (Chaiseha et al., 1997). In addition, D2 receptors can also regulate prolactin secretion in cultured turkey pituitary cells (Xu et al., 1996). It would be of interest then, to determine the distribution of dopamine receptors in the avian brain to provide more information for the functional analysis of dopaminergic control of prolactin secretion.

Recently, three D1 dopamine receptor subtypes from chickens were cloned (Demchyshyn et al., 1995); now, the cloning of a cDNA from turkey brain encoding a D2 dopamine receptor is reported here. The nucleotide sequence of the avian D2 dopamine receptor was shown to encode seven putative transmembrane-spanning domains and demonstrated 75% homology to known mammalian D2 receptors. The regional distribution of D2 receptor mRNA was semiquantified in turkey brain and peripheral tissues by using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Furthermore, the cellular distribution of D2 mRNA in turkey brain sections was examined by using in situ hybridization histochemistry. The putative structure of the turkey D2 dopamine receptor and the anatomic findings are compared with those of mammalian D2 receptor distribution and function.

MATERIALS AND METHODS

Animals

Nicholas Large White female turkeys that were reproductively active during the previous season were used in these experiments. Animals were killed with Euthanasia Injection (Anpro Pharmaceuticals, Arcadia, CA), tissue was collected and rapidly frozen in either liquid nitrogen (Northern blot and RT-PCR analysis) or on dry ice (in situ hybridization). The animal protocols described in this paper were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Cloning of a turkey D2 dopamine receptor cDNA

Total RNA was extracted from turkey brain by using TRIReagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocols and reverse transcribed as described below for semiquantitative RT-PCR. PCR primers, based on an analysis of conserved nucleotide sequence among many genera for D2 dopamine receptors (Bunzow et al., 1988; Macrae and Brenner, 1995; Martens et al., 1991; Stormann et al., 1990), were synthesized (Microchemical Facility, University of Minnesota core facility). The nucleotide sequences of the PCR primers were as follows: forward primer, 5′-ATCCACTGTGACATCTTTGT-CAC-3′; reverse primer, 5′-ACACCCAGGACAATAGCTAA-CAT-3′. PCR amplification of first-strand cDNA was subjected to a "touch-up" thermal cycling program: 20 cycles at 95°C for 1 minute, 42°C increasing per cycle to 55°C for 1 minute, and 72°C for 1 minute. A 932-base pairs (bp) fragment was fractionated on 3% agarose gels, excised, blunt-ended with T4 DNA polymerase/Klenow DNA polymerase (Promega, Madison, WI), and subcloned into SmaI-digested pBluescript SK(+) vector (Stratagene, La Jolla, CA) as described (Sambrook et al., 1989). Competent XL1-Blue cells (Stratagene) were transformed, and mini-preparations of plasmid DNA were prepared for insert sequencing. Positive clones were sequenced on both strands by automated DNA sequencing (Advanced Genetics Analy-

Abbreviations

APH	area parahippocampalis
CA	commissura anterior
CO	chiasma opticum
CP	commissura posterior
Cb	cerebellum
CDL	area corticoidea dorsolateralis
DA	tractus dorsoarchistriaticus
DL	nucleus dorsolateralis thalami; anterior or posterior part
DM	nucleus dorsomedialis thalami; anterior or posterior part
DLT	nucleus dorsolateralis
DS	decussatio supraoptica
DSD	decussatio supraoptica dorsalis
E	ectostriatum
FA	tractus frontoarchistriaticus
FLM	fasiculus longitudinalis medialis
GCt	substantia grisea centralis
GLv	nucleus geniculatus lateralis, pars ventralis
HA	hyperstriatum accessorium
Hp	hippocampus
HV	hyperstriatum ventrale
IN	nucleus infundibuli hypothalami
IP	nucleus interpeduncularis
LHy	area lateralis hypothalami
ME	median eminence
MnV	nucleus motorius nervi trigemini
N	neostriatum
NC	neostriatum caudale
NIII	nervus oculomotorius
OM	tractus occipitomesencephalicus
P	pineal gland
PHN	nucleus periventricularis hypothalami
PL	nucleus pontis lateralis
PMI	nucleus paramedianus internus thalami
POP	nucleus preopticus periventricularis
PVN	nucleus paraventricularis magnocellularis
Rt	nucleus rotundus
SAC	stratum album centrale
SCN	nucleus suprachiasmaticus
SGC	stratum griseum centrale
SGF	stratum griseum et fibrosum superficiale
SGFS	stratum griseum et fibrosum superficiale
SOe	nucleus supraopticus, pars externus
SN	substantia nigra
TeO	tectum opticum
TrO	tractus opticus
TSM	tractus septomesencephalicus
V	ventriculus
VLT	nucleus ventrolateralis thalami
VMN	nucleus ventromedianis hypothalami

sis Center, University of Minnesota core facility). Rapid amplification of cDNA ends (RACE) was performed with a RACE kit (Clontech, Palo Alto, CA) for amplification, subcloning, and sequence analysis of the turkey D2 dopamine receptor 5′ cDNA end by using the same turkey brain RNA described above. Amplification, subcloning, and sequencing of the 3′ portion of the coding region was obtained from a custom-made turkey pituitary cDNA library (Invitrogen, San Diego, CA). Nucleotide and amino acid analysis were performed by using GCG (Genetics Computer Group, Madison, WI) software.

Quantitative RT-PCR

RT-PCR was performed with a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT) according to the manufacturer's suggestions with slight modifications. Total RNA from selected turkey brain and peripheral tissues were pretreated with DNase I prior to first-strand cDNA synthesis to remove any contaminating chromosomal DNA (Huang et al., 1996). The DNase reaction consisted of (final concentrations): 2 µg total RNA, 1 U RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN), 1 U RNase inhibitor, 5 mM $MgCl_2$, and 1 × PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3). The DNase mixture was incubated at 37°C for 15 minutes, and the DNase was denatured at 65°C for 10 minutes and snap cooled on ice for 2 minutes. To the cooled DNA-free mixture, a reverse transcription master mix was added (final concentrations: 2.5 U murine leukemia virus reverse transcriptase, 2.5 mM oligo[dT], 1.5 mM of each dNTP) was added to the DNase-treated RNA, and one round of cDNA synthesis was performed in a thermal cycler (25°C for 5 minutes, 42°C for 1 hour, 95°C for 5 minutes, and 4°C soak).

Five microliters of RT reaction product were added to 20 µl of PCR master mixture (final concentrations: 2.5 U AmpliTaq DNA polymerase, 2 mM $MgCl_2$, 1 × PCR buffer II, and 40 pmol of each sense and antisense primer). Primers flanking the putative alternative splice sites for the turkey D2 dopamine receptor (forward primer, 5′-AATCTCATGGTGCTCCGCAGGC-3′; reverse primer, 5′-GTGCTGCTGCCTTGACAATGGTTC-3′) and a region flanking an intron for turkey β-actin (forward primer, 5′-ACCAGTAATTGGTACCGGCTCCT-C3′; reverse primer, 5′-TCTGGTGGTACCACAATGTACCCT-3′; You et al., 1995) were synthesized. Thermal cycling parameters were performed in the linear range of amplification and consisted of 32 (D2) or 26 (β-actin) cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Fifteen microliters of each PCR reaction product from separate D2 and β-actin amplifications were mixed, and 10 µl of this mixture was fractionated in 3% agarose gels. Bands were either excised and subcloned or Southern blotted onto nylon membranes according to manufacturer's suggestions (Micron Separations Inc., Westborough, MA). Membranes were then probed with subcloned nick-translated turkey D2 and β-actin cDNA fragments overnight at 42°C for 16 hours. The hybridization buffer was the same as that for Northern blots. The blots were washed once for 30 minutes with 2 × standard saline citrate (SSC), 0.5% sodium dodecyl sulfate (SDS) at 65°C and then once with 0.01 × SSC, 0.5% SDS at 65°C for 30 minutes. The blots were apposed to x-ray film for 5–15 minutes, then the x-ray film was developed.

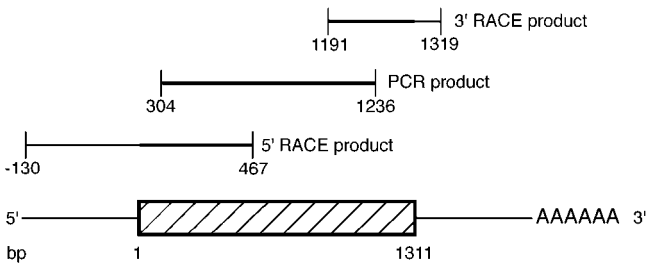

Fig. 1. Cloning strategy for reverse transcriptase-polymerase chain reaction (RT-PCR) cloning of the avian D2 dopamine receptor mRNA. Schematic diagram of the composite turkey D2 receptor cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) products, and original PCR product derived from oligonucleotide primers. The bottom diagram represents the putative full-length sequence of the turkey D2 dopamine receptor, including untranslated regions.

Steady-state quantification (from turkeys during the laying of eggs) of the alternatively spliced D2 dopamine receptor transcripts was performed by using computer-assisted densitometry (Ambis Inc., San Diego, CA). The band intensities of $D2_L$ and $D2_S$ were normalized to the band intensity of β-actin for each of the tissues examined. The results are expressed as arbitrary densitometric units (ADU) ± S.E.M. from five separate experiments.

In situ hybridization

Turkey brains were sectioned on a cryostat (Bright Instruments, Huntington, United Kingdom) to a thickness of 14 µm, thaw mounted onto microscope slides (Probe-On; Fisher Scientific, Minneapolis, MN), and stored desiccated at −20°C until used. Antisense ^{33}P-labeled RNA probe was synthesized by using the MaxiScript transcription kit (Ambion, Austin, TX), and the probe was purified by polyacrylamide gel electrophoresis, as described (Sambrook et al., 1989). Purified riboprobe was added to hybridization solution (50% formamide; 300 mM NaCl; 10 mM Tris, pH 8.0; 1 mM EDTA; 1 × Denhardt's reagent; 10 mM dithiothreitol; 500 ng/ml yeast tRNA; 1% SDS; and 10% dextran sulfate) to ≈5 × 10^5 CPM/slide and hybridized at 50°C overnight in a humid environment. The sections were then washed in two changes of 1 × SSC at 55°C for 40 minutes and once at 0.1 × SSC at 55°C for 20 minutes. After the final wash, the sections in buffer were allowed to cool to room temperature. The sections were then dehydrated through graded alcohols (50–100%) and quickly air dried.

The tissue sections were then either apposed to x-ray film or dipped in NTB-2 nuclear track emulsion diluted 1:1 with dH_2O.(Eastman Kodak Co., Rochester, NY). Sections dipped in emulsion were stored in a light-tight container at 4°C until developed (2–4 days). The dipped sections were developed with half-strength D-19 (Eastman Kodak Co.) for 5 minutes and fixed for 5 minutes with Fixer (Eastman Kodak Co.). 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) as described previously (Schnell and Wessendorf, 1995) and coverslipped with DPX Mountant (Fluka Chemical, Ronkonkoma, NY). Autoradiographic grains and counterstaining were visualized and imaged with a fluorescence microscope equipped with a

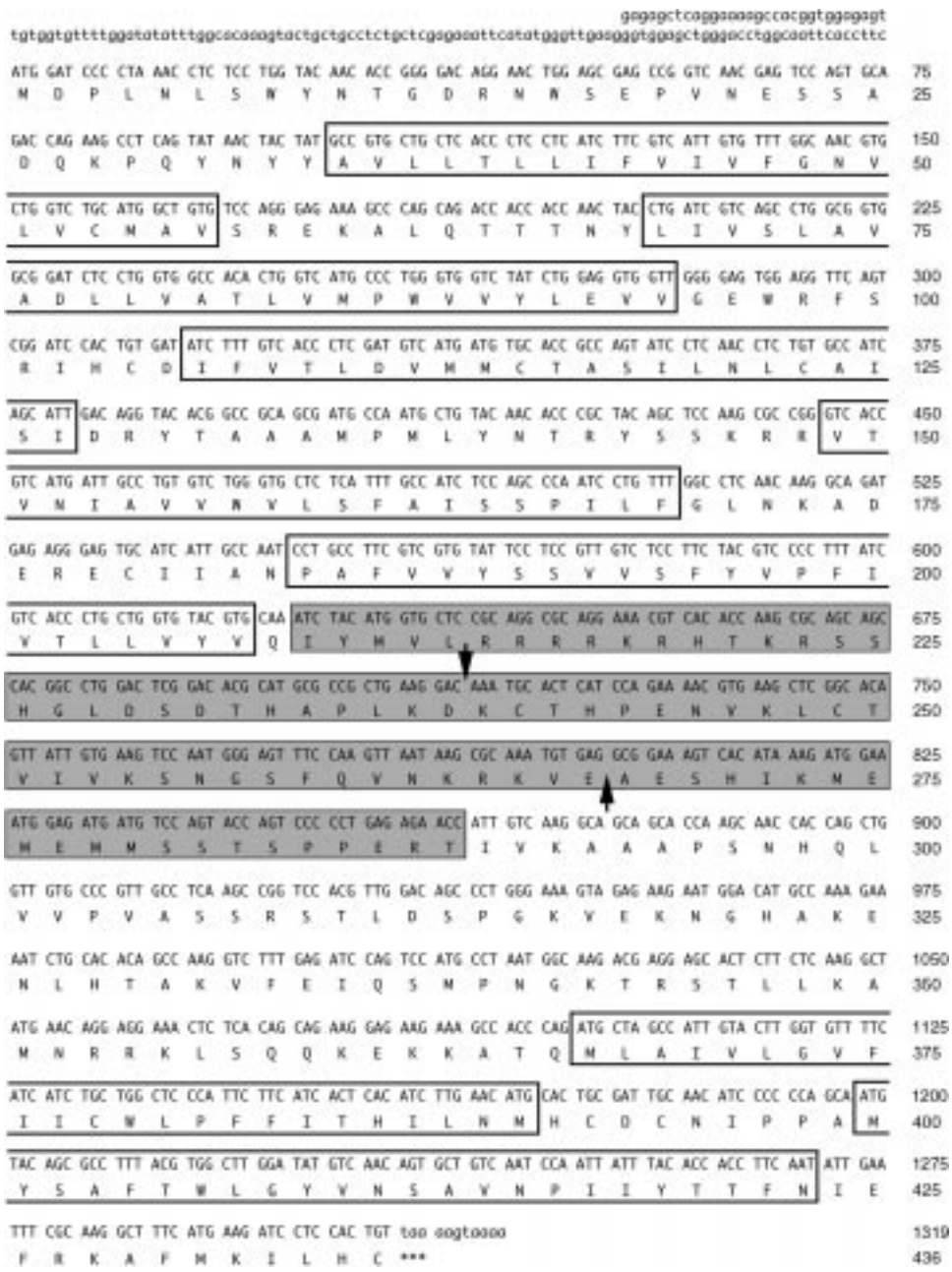

Fig. 2. Composite nucleotide and deduced amino acid sequences of three different turkey D2 dopamine receptor cDNA clones. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning at the presumptive start codon ATG. A single open reading frame of 1,311 base pairs (bp) is present, encoding a protein of 436 amino acids. Seven stretches of amino acid sequence predicted to encode transmembrane domains are boxed. The RT-PCR fragment and riboprobe used in Southern and Northern blotting and in situ hybridization histochemistry is indicated by a shaded box. Arrows indicate the beginning and end of the sequence that is alternatively spliced to produce the D2 short isoform.

darkfield condenser and ultraviolet excitation using a UG1 filter set (Schott, Duryea, PA) and a 420-nm long-pass emission filter. Imaging of autoradiographic grains and counterstaining was performed at the Bioimaging Processes Laboratory (Bioimaging Processes Laboratory; University of Minnesota core facility) by using a cooled, color CCD camera (Optronix, Schaumberg, IL) and processed with MetaMorph (Universal Imaging Corporation, West Chester, PA) and Photoshop 4.0 software (Adobe Systems, Mountain View, CA).

Northern blot analysis

Thirty micrograms of total RNA were fractionated by formaldehyde gel electrophoresis and transferred onto a positively-charged nylon membrane (Magnacharge; Mi-

cron Separations Inc., Westborough, MA) with 10 × SSC transfer buffer. D2 antisense RNA probe was labeled with ^{32}P-dUTP to ≈1.0 × 10^7 CPM/ml and hybridized to the RNA blot at 42°C overnight in buffer consisting of 50% formamide, 5 × SSPE, 0.1% SDS, and 10% dextran sulfate. The blots were washed twice with 2 × SSPE/0.5% SDS at 65°C and twice with 0.01 × SSPE/0.5% SDS at 65°C and apposed to x-ray film (Hyperfilm; Amersham, Arlington Heights, IL) for 7 days.

RESULTS

In an initial characterization of the 932-bp PCR fragment (Fig. 1), the nucleotide sequence was examined and compared with the sequences of known D2 dopamine receptors. This fragment demonstrated approximately 80% nucleotide homology to mammalian D2 receptors (Bunzow et al., 1988; Monsma et al., 1990) and corresponded to a region coding for the putative transmembrane regions 2–7 (TM II–VII). The next experiments were designed to obtain the 5′ and 3′ ends by using the RACE technique (Fig. 1). The 5′ cDNA end was cloned and included the D2 receptor ATG start codon through TM III as well as 130 bases of the 5′ untranslated region (UTR). The 3′ cDNA end was cloned from a turkey pituitary cDNA library and contained the D2 receptor TM VII through the termination codon. The nucleotide sequence of the cloned cDNA has an open reading frame of 1,311 bp, which is consistent with the D2 dopamine receptor. The deduced translated sequence is 436 amino acids (long isoform; $D2_L$) with an expected Mr of about 50 kDa. A short form ($D2_S$) also was isolated and sequenced and was identical to that of $D2_L$, except 87 nucleotides were deleted in the region coding for the putative third intracellular loop, which would produce a truncated protein of 405 amino acids.

The nucleotide and deduced amino acid sequence for the turkey D2 dopamine receptor mRNA is shown in Figure 2. Deduced amino acid homologies of the turkey D2 receptor aligned with the human, rat, frog, and fish D2 receptor sequences are compared in Figure 3. The overall nucleotide homology of the turkey $D2_L$ dopamine receptor to that of cloned mammalian $D2_L$ receptors was about 75%. The overall amino acid homology to that of cloned human D2 receptors was about 82%. A hydrophobicity plot (data not shown) demonstrated the existence of seven hydrophobic domains that most likely correspond to putative membrane-spanning regions.

RT-PCR analysis detected both a long ($D2_L$) and a short ($D2_S$) D2 receptor mRNA in turkey pallium, cerebellum, hypothalamus, pituitary, pineal gland, and spinal cord (Fig. 4A). Steady-state levels of $D2_L$ and $D2_S$ ratios differed in each brain region examined and are shown in Figure 4B. The distribution of peripheral tissue $D2_L$ receptor mRNA is shown in Figure 4A. $D2_L$ mRNA was detected in turkey heart, lung, liver, spleen, intestine, leg muscle, ovary, and oviduct; however, $D2_S$ was not detected in any of these tissues.

Specificity of probe used for in situ hybridization experiments

The specificity of the probe used for in situ hybridization experiments was tested by using two different methods. First, a ^{32}P-labeled antisense riboprobe fragment was used to probe a Northern blot, as described above. An approximately 2.4-kb fragment was observed (Fig. 5A); this value

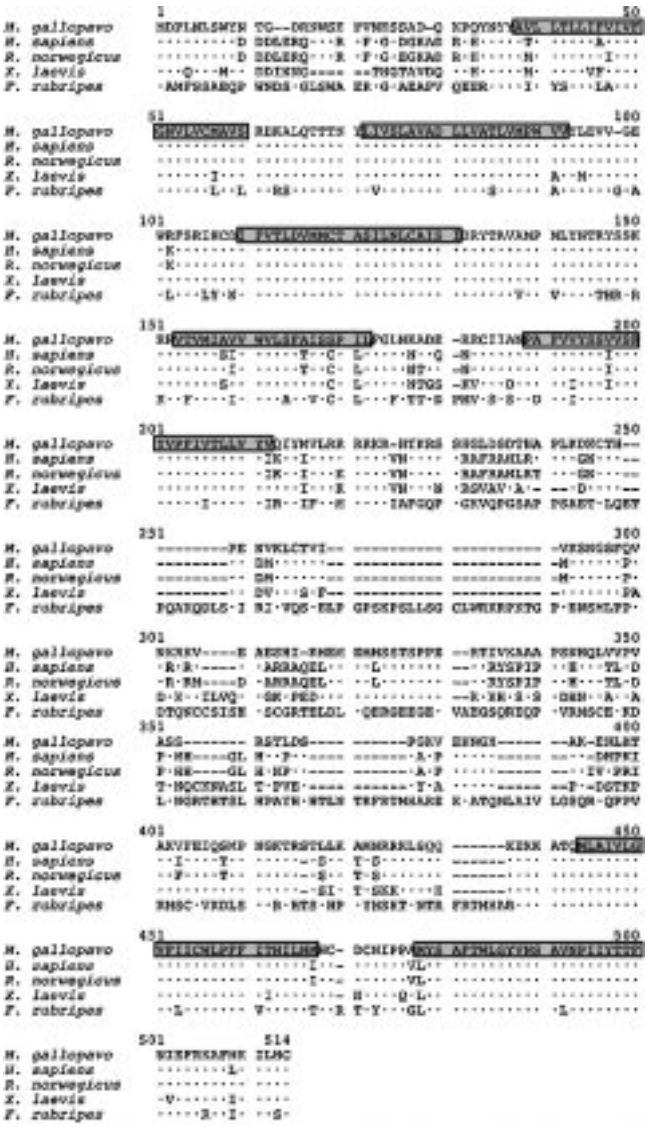

Fig. 3. Alignment of amino acid sequences encoding vertebrate D2 dopamine receptors. Alignment was performed by using amino acid sequences of the cloned turkey, human, rat, frog, and fish D2 dopamine receptor proteins (for references, see text). Residues matching the turkey sequences are indicated by bullets; residues spanning putative transmembrane domains are represented by shaded boxes drawn over the sequence.

is in close agreement with the values obtained from cloned mammalian D2 dopamine receptors (Bunzow et al., 1988; Gandelman et al., 1991; Mack et al., 1991). Second, ^{33}P-labeled sense and antisense riboprobes were applied to tissue sections (Fig. 5B). No labeling of neurons was observed with the sense riboprobe. RNase pretreatment of tissue sections to destroy target RNA is not an acceptable control for these probes, because trace amounts of RNase likely would destroy the RNA probe as well (Young, 1990). The criteria used in the present study have been accepted as sufficient proof of specificity for probes used for in situ hybridization (Chesselet et al., 1996; Emson, 1989; McFadden, 1995; Wilkinson, 1992; Young, 1990).

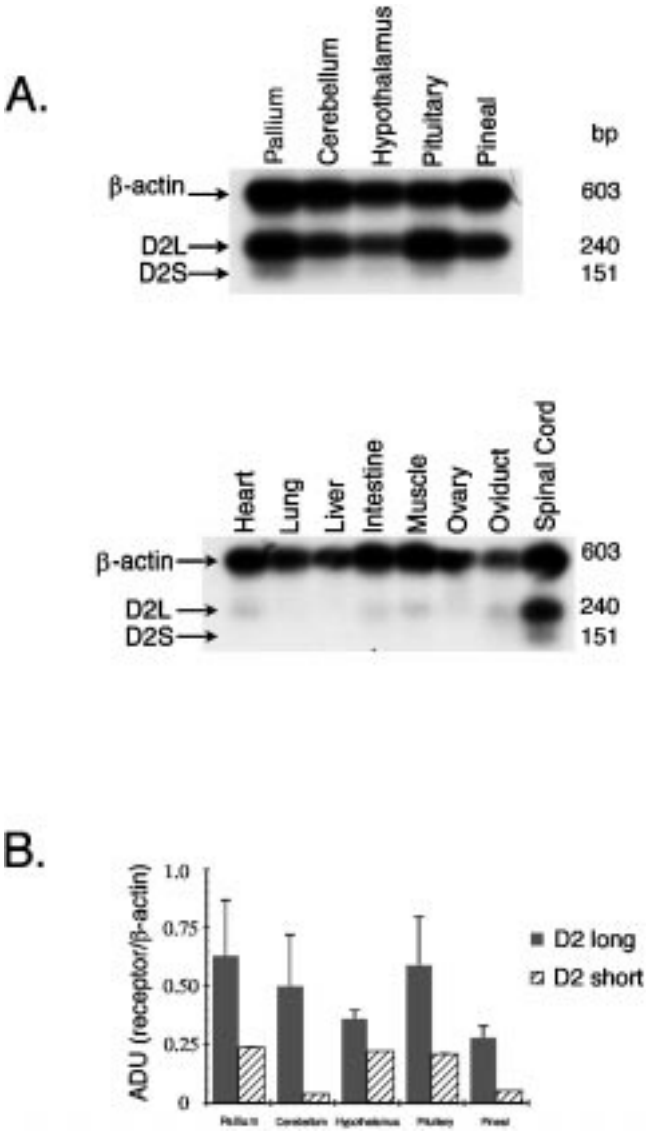

Fig. 4. RT-PCR analysis of D2 long ($D2_L$) and D2 short ($D2_S$) isoforms. **A:** Southern blot of RT-PCR products from selected brain and peripheral tissues from one turkey of steady-state levels of $D2_L$, $D2_S$, and β-actin. This blot was exposed to clearly show the presence of $D2_S$ mRNA. This results in overexposure of $D2_L$ and β-actin bands; properly exposed autoradiograms were used for semiquantitative densitometry. **B:** Semiquantitative densitometric analysis of $D2_L$ and $D2_S$ from RT-PCR Southern blots of select turkey brain regions. The results presented in this graph are the data from six turkeys.

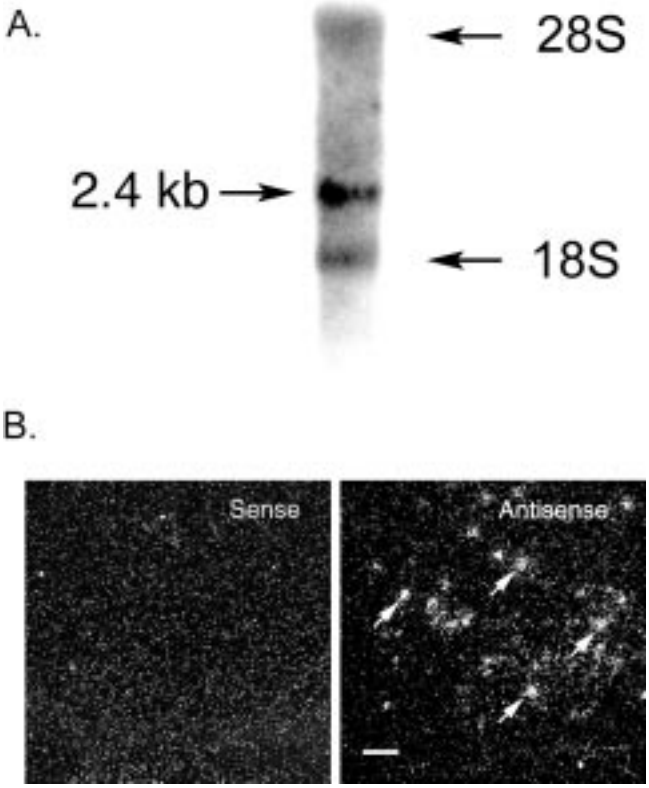

Fig. 5. Specificity of D2 probe used for in situ hybridization histochemistry. **A:** Northern blot of total turkey brain RNA using the D2 antisense RNA probe for in situ hybridization studies. A single 2.4-kb D2 mRNA transcript was detected. 28S (≈5 kb) and 18S (≈2 kb) ribosomal RNA bands were used to estimate the molecular mass of the D2 mRNA transcript. **B:** Darkfield photomicrographs of turkey brain sections processed for in situ hybridization with ^{33}P-labeled D2 sense (left) and antisense (right) RNA probes and dipped in photographic emulsion. Arrows indicate neurons in the lateral hypothalamic nucleus (LHy) labeled for D2 dopamine receptor mRNA. Scale bar = 100 μm.

In situ hybridization

Data are organized by coronal sections that were taken from approximately 1.0 mm rostral to the optic chiasm to approximately 1.0 mm caudal to the median eminence (see Figs. 12, 13). To aid in the documentation of neuroanatomic results, nomenclature from a published atlas of the chick brain (Kuenzel and Masson, 1988) and hypothalamus (Kuenzel and van Tienhoven, 1982) were used.

Telencephalon

Many neurons in the hyperstriatum accessorium (HA; Fig. 6), hyperstriatum ventrale (HV; Fig. 6), area parahippocampus (APH), neostriatum (N) and area corticoidea dorsolateralis (CDL) contained diffuse labeling for D2 mRNA.

Diencephalon

Consistently dense labeling for D2 mRNA was detected along the midline structures of the turkey brain diencephalon (i.e., flanking the third ventricle). Specifically, the nucleus preopticus pariventricularis (POP), nucleus paraventricularis (PVN), dorsolateral and dorsomedial thalamic nuclei (DL and DM), nucleus periventricularis hypothalami (PHN), nucleus paraventricularis magnocellularis (PVN), and nucleus infundibuli hypothalami (IN) were labeled heavily for D2 mRNA. Laterally, the nucleus supraopticus, pars externus (SOe), nucleus ventrolateralis thalami (VLT), nucleus rotundus (Rt), and nucleus geniculatus lateralis pars ventralis (GLv) demonstrated strong labeling for D2 mRNA expression. Labeling of many neurons within the lateral hypothalamus (LHy; Fig. 7) also was observed.

Mesencephalon

Dense labeling of neurons throughout the lateralmost regions of the mesencephalon for D2 mRNA was observed.

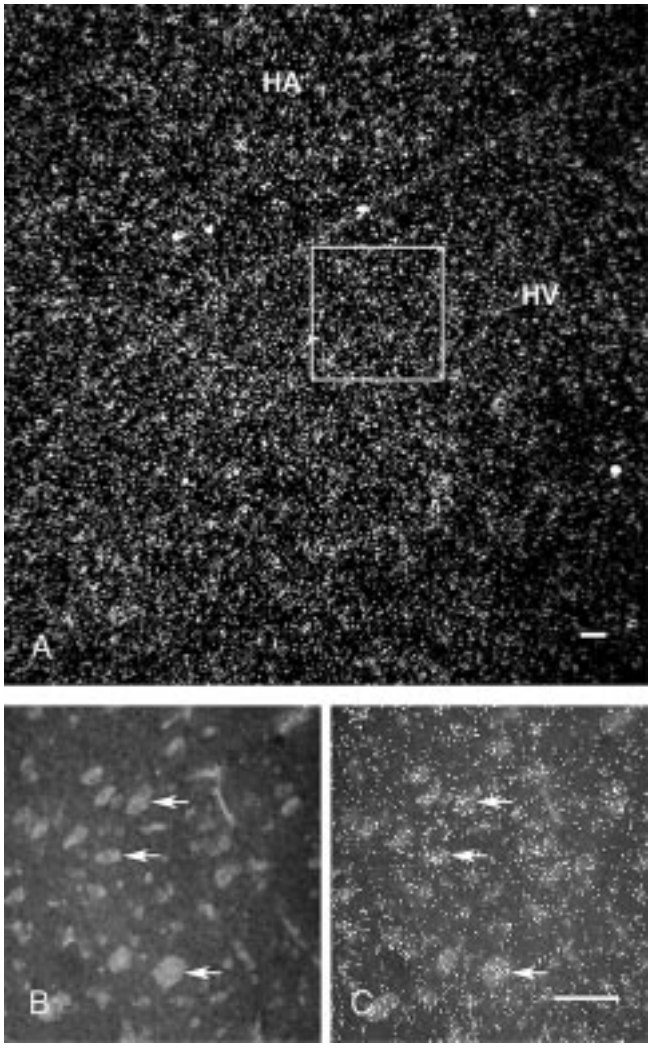

Fig. 6. Low- and high-power photomicrographs of emulsion-dipped sections through turkey telencephalon hybridized with D2 antisense probe. **A:** Low-power photomicrograph under darkfield (DF) illumination. **B:** High-power photomicrograph under ultraviolet (UV) illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate a few of several neurons in the HV labeled for dopamine receptor mRNA. Scale bars = 100 μm.

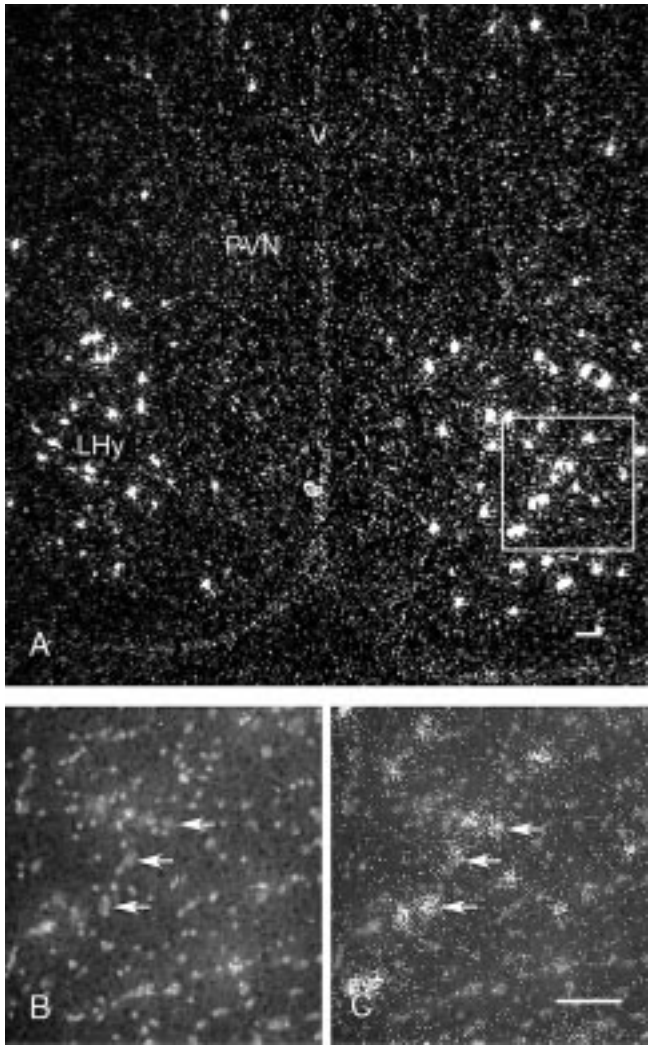

Fig. 7. Low- and high-power photomicrographs of emulsion-dipped sections through turkey hypothalamus hybridized with D2 antisense probe. **A:** Low-power photomicrograph under DF illumination. **B:** High-power photomicrograph under UV illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate a few neurons in the LHy labeled for D2 dopamine receptor mRNA. Scale bars = 100 μm.

High densities of D2 mRNA labeling were observed throughout the stratum griseum et fibrosum superficiale (SGFS), stratum griseum centrale (SGC), and associated structures of the optic tectum. The substantia grisea centralis (GCt), fasciculus longitudinalis medialis (FLM), nucleus nervi oculomotorii (OM), nucleus pontis lateralis (PL), and substantia nigra (SN; Fig. 8) also showed high levels of D2 mRNA expression.

Cerebellum, pituitary, and pineal gland

Within the cerebellum, dense labeling for D2 mRNA was observed over neurons comprising the granular and Purkinje cell layers (Fig. 9); no labeling over cells of the molecular layer was observed. In the pineal gland, very dense signal for D2 mRNA was observed (Fig. 10). Diffuse hybridization signals for D2 mRNA were observed over many cells of the pituitary (Fig. 11).

DISCUSSION

A cDNA corresponding to the turkey D2 dopamine receptor was cloned. Nucleotide and deduced amino acid analysis demonstrated that the sequence met several criteria common to members of the superfamily of G-protein-coupled receptors. Nucleotide and amino acid sequence alignments of the turkey $D2_L$ with humans (Stormann et al., 1990), rats (Bunzow et al., 1988), frogs (Martens et al., 1991), and fish (Macrae and Brenner, 1995) demonstrated a high degree of sequence homology with D2 dopamine receptors. The expected turkey D2 dopamine receptor protein (436 amino acids) was similar in size to that reported for rat (415 amino acids), frog (442

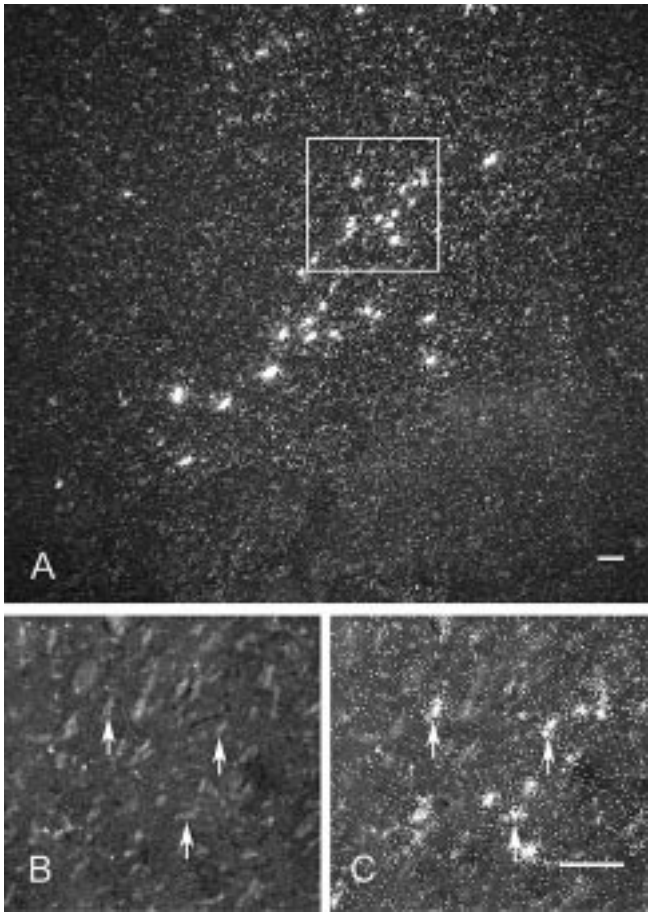

Fig. 8. Low- and high-power photomicrographs of emulsion-dipped sections through turkey mesencephalon hybridized with D2 antisense probe. **A:** Low-power photomicrograph under DF illumination. **B:** High-power photomicrograph under UV illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate a few neurons in SN labeled for D2 dopamine receptor mRNA. Scale bars = 100 µm.

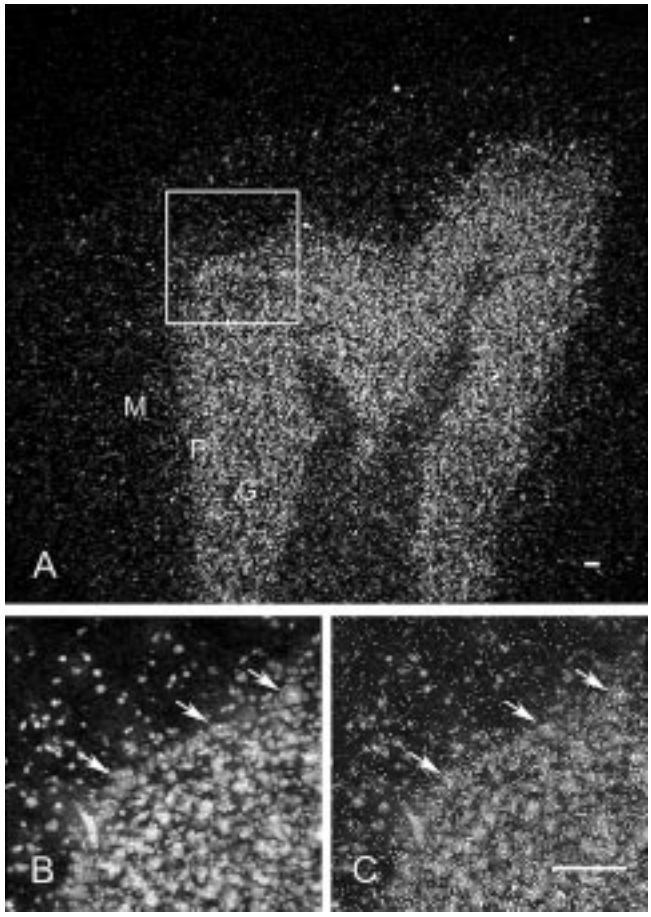

Fig. 9. Low- and high-power photomicrographs of emulsion-dipped sections through turkey cerebellum hybridized with D2 antisense probe. **A:** Low-power photomicrograph under DF illumination. M, molecular cell layer; P, Purkinje cell layer; G, granular cell layer. **B:** High-power photomicrograph under UV illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate Purkinje neurons labeled for D2 dopamine receptor mRNA. Scale bars = 100 µm.

amino acids), and fish (463 amino acids). Cysteine residues (C_{104} and C_{539}) are believed to form disulfide bonds to provide appropriate tertiary structure for the mature protein, and a terminal cysteine is believed to be involved in palmitoylation (Jackson and Westlind-Danielsson, 1994; Schmidt et al., 1988), a posttranslational modification that is important for anchoring the receptor to the plasma membrane and G-protein coupling.

It has been noted by other investigators studying other species (Chio et al., 1990; Giros et al., 1989; Monsma et al., 1989; Rao et al., 1990) that the turkey D2 receptor mRNA is alternatively spliced. The location of the alternatively spliced isoform ($D2_S$) is in the protein's putative third intracellular loop, a region thought to be an important site for interaction with G-proteins (O'Dowd et al., 1990). The physiologic significance of the alternative isoform is unknown, because both isoforms perform the same functions with nearly equal efficacy, although the $D2_S$ isoform may be coupled more efficiently to specific G proteins (Guiramand et al., 1995; Montmayeur et al., 1993). In the present study, both $D2_L$ and $D2_S$ isoforms were present in each of the brain tissues examined. The predominant isoform in all tissues was $D2_L$; however, the ratio varied between 2 and 3, depending on the brain region examined. These differences may be due to varying availability of splicing factors within these brain regions. $D2_L$, but not $D2_S$, was detected in several peripheral tissues as well.

The distribution of D2 receptor mRNA, as determined by in situ hybridization, histochemistry was widespread. Due to the paucity of information regarding the D2 dopamine receptor mRNA distribution in birds, little direct confirmation of our findings in the turkey brain exists. The following is a comparison of the present results with those of published accounts in mammals for D2 mRNA (Mansour et al., 1990; Weiner and Brann, 1989; Weiner et al., 1991).

The present study has demonstrated diffuse labeling for D2 mRNA throughout the turkey telencephalon. The labeling was observed over perikarya comprising HA, HV, and APH. In addition, autoradiographic grains were observed over N and NC. It is interesting to note that these areas are likely to be analogous to the mammalian cortex (Araki et al., 1992). In mammals, D2 mRNA has been observed in layers I–III of frontal, parietal, and cingulate

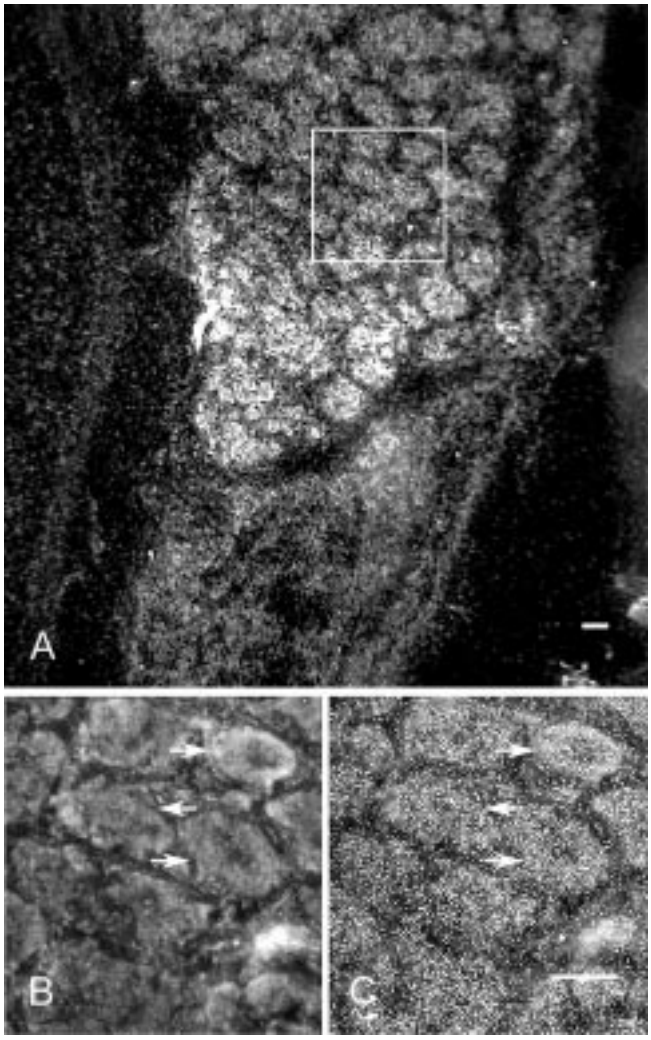

Fig. 10. Low- and high-power photomicrographs of emulsion-dipped sections through turkey pineal hybridized with D2 antisense probe. **A:** Low-power photomicrograph under DF illumination. **B:** High-power photomicrograph under UV illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate a few pinealocytes of many labeled for D2 dopamine receptor mRNA. Note that the area below the box contains unusually high amounts of hybridization. Hybridization experiments with other pineal glands from different animals showed a pattern similar to that observed in the boxed area. Scale bars = 100 μm.

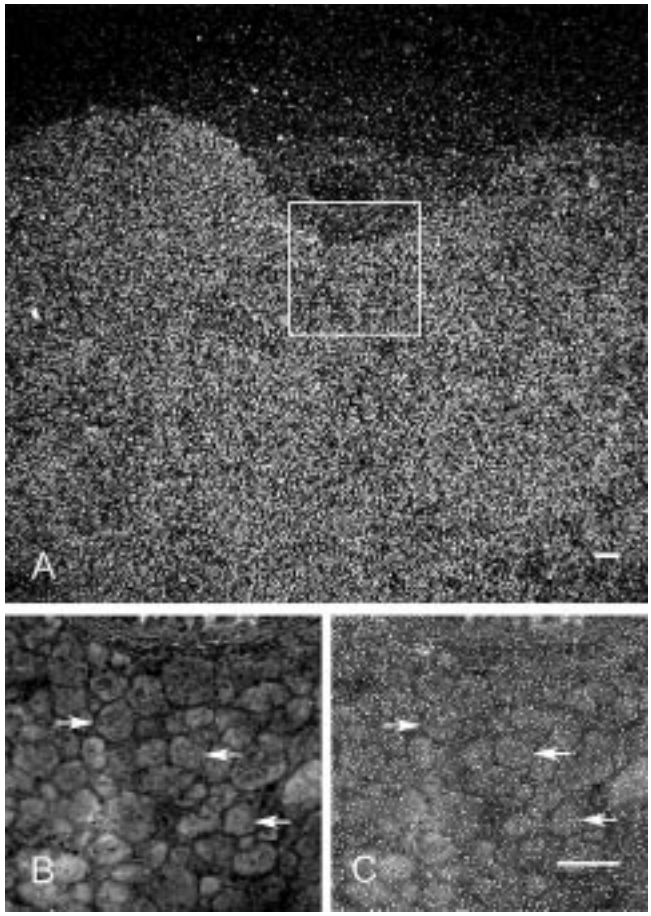

Fig. 11. Low- and high-power photomicrographs of emulsion-dipped sections through turkey pituitary hybridized with D2 antisense probe. **A:** Low-power photomicrograph under DF illumination. **B:** High-power photomicrograph under UV illumination. **C:** High-power photomicrograph under simultaneous UV and DF illumination. Arrows indicate cells in anterior pituitary labeled for D2 dopamine receptor mRNA. Note that the area in the upper part of the box contains nonpitutiary tissue, probably a blood vessel, that is unhybridized. Therefore, the hybridization pattern observed over pituitary cells probably is not due to nonspecific binding of probe to tissue. Scale bars = 100 μm.

cortices (Goldman-Rakic et al., 1990; Mansour et al., 1990; Weiner et al., 1991). The involvement of D2 receptors in turkey pallium may be to modulate excitatory dopaminergic input, as has been demonstrated in rats (Bradshaw et al., 1985).

Considerable mismatch exists between our localization of D2 mRNA and the distribution of tyrosine hydroxylase (TH), and dopamine β-hydroxylase (DBH) or dopamine and dihydroxyphenylalanine (L-DOPA) immunohistochemical labeling (Bailhache and Balthazart, 1993; Kiss and Peczely, 1987; Moons et al., 1994). Dopamine or its biosynthetic enzymes have be found in the portion of the avian telencephalon that is ventral to N. The present study has shown much D2 mRNA in the more dorsal HA and HV regions. Taken together, these data suggest that neurons in HA, HV, and APH synthesize D2 mRNA and then target mature D2 protein to other regions, perhaps where dopaminergic innervation is more dense (e.g., N and nucleus accumbens).

Expression of D2 mRNA in the turkey diencephalon was more restricted than the patterns observed elsewhere. Regions of the thalamus and hypothalamus that flank the third ventricle were densely populated with neurons expressing D2 mRNA. In rats, moderate to high levels of D2 message have been detected in many of the same hypothalamic nuclei as well (Weiner and Brann, 1989; Weiner et al., 1991). The PHN cell group of both species has been shown to be rich in VIP mRNA and protein (Hof et al., 1991; Kuenzel et al., 1997), a peptide known to be a potent prolactin-releasing factor in birds (El Halawani et al., 1990a,b, 1996). Data from the present study suggest the possibility that, through D2 receptors, dopamine may regulate VIP secretion from the PHN or from other diencephalic groups of neurons (e.g., LHy) in birds. The fact that

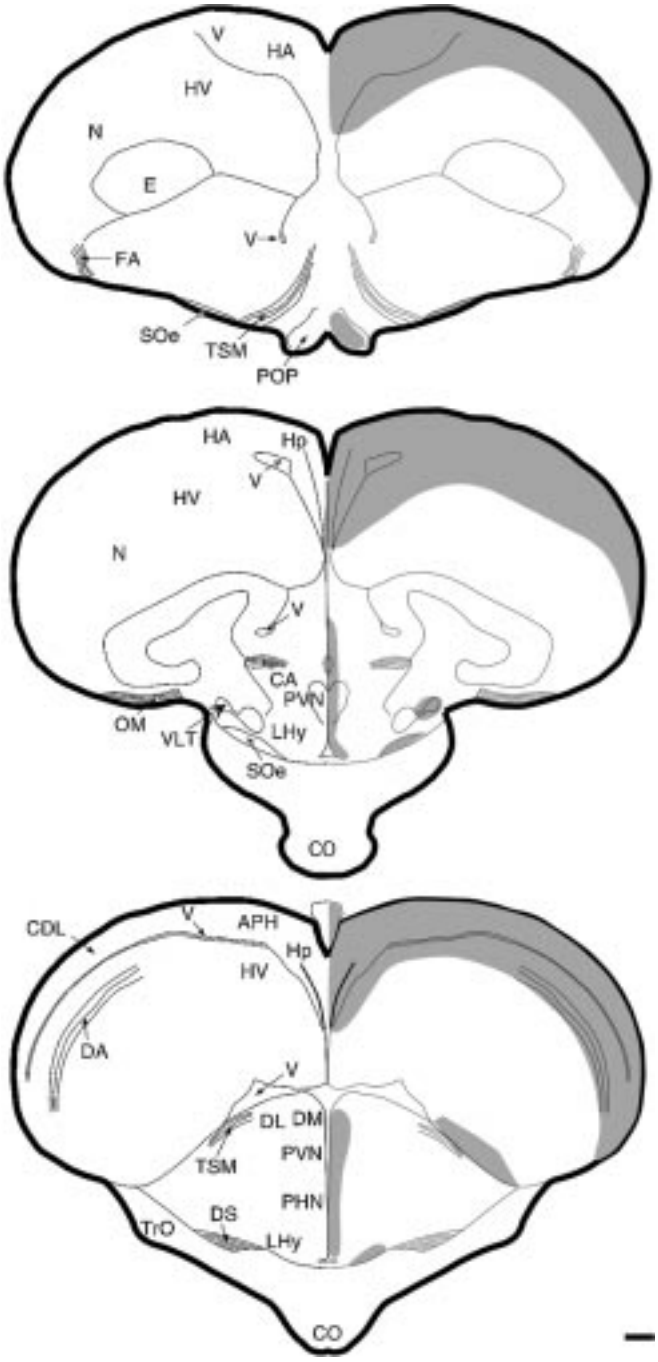

Fig. 12. Schematic diagrams of coronal sections of the turkey telencephalon and diencephalon. The shading represents areas where D2 mRNA was readily observed. The distance between diagrams is approximately 1 mm. For abbreviations, see list. Scale bar = 1 mm.

several dopaminergic cell groups have been localized to the PHN and LHy provides further evidence for this hypothesis (Moons et al., 1994). In addition, the preoptic area of the diencephalon has been shown to contain high densities of dopamine fibers and varicosities (Moons et al., 1994), and we similarly found much D2 mRNA in POP.

Of the medial mesencephalic structures, the SN, FLM, OM, and GCt have the highest densities of D2 receptor

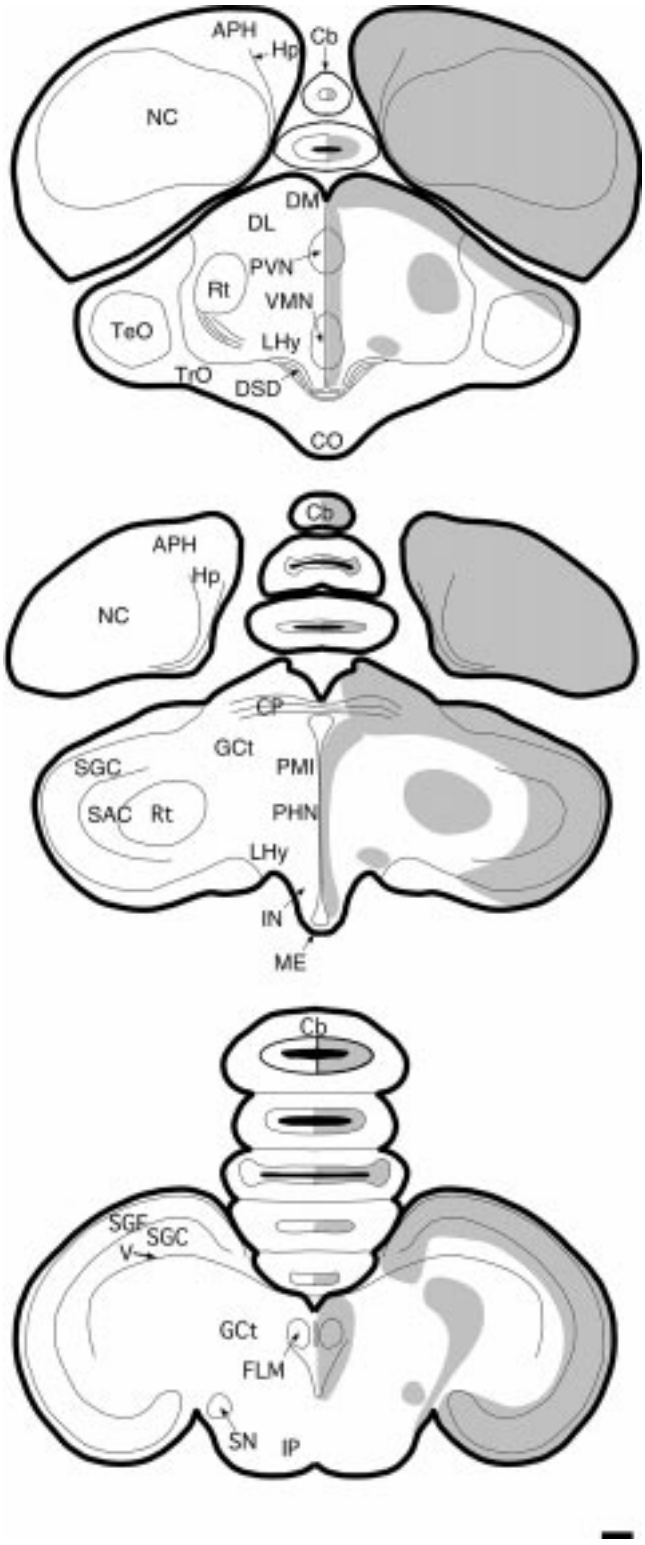

Fig. 13. Schematic diagrams of coronal sections of the turkey telencephalon, diencephalon, mesencephalon and cerebellum. The shading represents areas where D2 mRNA was readily observed. The distance between diagrams is approximately 1 mm. For abbreviations, see list. Scale bar = 1 mm.

mRNA. In mammals, SN is a hallmark for identification of D2 autoreceptors, because no D1 dopamine receptor mRNA has been found in this region (Weiner et al., 1991). Laterally, the subregions of the turkey stratum griseum (SGFS and SGC) and PL demonstrated strong D2 mRNA hybridization. The SGFS and SGC may be comparable to the mammalian superficial gray of the brainstem (Kuenzel et al., 1997). A mismatch between turkeys and rats appears to exist, because moderate levels of D2 mRNA have been detected in mammalian central gray matter but not in superficial gray matter (Weiner et al., 1991). Immunohistochemical evidence of dopaminergic innervation in the avian mesencephalon has shown considerable numbers of dopamine-containing cells in SN and PL (Moons et al., 1994).

The present study has demonstrated moderate-to-strong hybridization of D2 mRNA to the Purkinje and granular cell layers of the cerebellum. This is in contrast to that seen in rats, in which the presence of D2 receptors remains somewhat controversial. Some researchers have detected no D2 mRNA (Weiner et al., 1991) or only scattered labeling of Purkinje and granular cells (Brouwer et al., 1992). The observation that large amounts of D2 mRNA exist in the domestic turkey cerebellum indicates that this difference may be species specific. The cerebellum controls several aspects of motor activity through a series of inhibitory circuits; thus, it is possible that cerebellar D2 receptors are involved in coordinating motility in birds. The distribution of dopamine in the cerebellum of birds is restricted to very fine fibers innervating the Purkinje, molecular, and granular cell layers (Moons et al., 1994). If the abundance of an mRNA is a good indication of receptor numbers, then it is possible that high numbers of D2 receptors are a compensatory mechanism in response to low levels of dopamine in the avian cerebellum.

The present study has demonstrated the presence of D2 mRNA in turkey pineal gland. The pineal gland is the principal organ for melatonin secretion in mammals, and D2 dopamine receptors may regulate melatonin synthesis (Govitrapong et al., 1989). The present data suggest that D2 receptors may be important for regulation of melatonin synthesis and secretion in turkeys as well. We are unaware of any reports that describe the dopaminergic innervation of the avian pineal gland.

The distribution of D2 receptors in the turkey pituitary was different from that observed in rats (Bunzow et al., 1988). The pattern of hybridization in turkeys appeared homogeneous over most cells of the pituitary, whereas, in rats, the pattern of hybridization was restricted to the neural lobe. It is possible the labeling was nonspecific; however, the labeling that was observed by using a sense probe showed very faint hybridization. In mammals, D2 receptors on pituitary lactotrophs inhibit prolactin release (Ben-Jonathan, 1985). Dopamine also has been shown to inhibit prolactin release when it is applied to avian pituitaries in vitro (Xu et al., 1996). Perhaps the observed inhibition of prolactin by dopamine is due to the activation of D2 dopamine receptors. In the domestic turkey, the role of dopamine and dopamine receptors on the release of prolactin is currently under investigation (El Halawani et al., 1991; Youngren et al., 1995, 1996).

The present results provide important data that suggest a possible direct effect of dopamine on avian pituitaries. The existence of dopaminergic fibers in quail and chicken median eminence (Bailhache and Balthazart, 1993; Moons et al., 1994) implicates this structure as a possible source by which dopamine could come into contact with the pituitary. Thus, it is possible that avian prolactin secretion is regulated, at least in part, by a mechanism similar to that observed in mammals. The identification and cloning of the turkey D2 receptor mRNA presented here should enable the testing of this hypothesis as well as exploration of its role in other avian physiologic and behavioral functions.

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