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Increased abundance of alternatively spliced forms of D2 dopamine receptor mRNA after denervation

KIM A. NEVE*, RACHAEL L. NEVE†, SETH FIDEL†, AARON JANOWSKY*, AND GERALD A. HIGGINS‡

*Veterans Affairs Medical Center and Departments of Psychiatry and Pharmacology, Oregon Health Sciences University, Portland, OR 97201; †Department of Psychobiology, University of California, Irvine, CA 92717; and ‡National Institute on Aging, Gerontology Research Center, Baltimore, MD 21224

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ABSTRACT **The existence of two molecular forms of D2 dopamine receptors suggests that differences in the distribution or regulation of the two forms could be exploited for the pharmacological treatment of disease. Using probes selective for each alternatively spliced variant of D2 receptor mRNA, we determined that both variants were widely distributed in rat brain and pituitary but that the ratio of the forms varied among regions. mRNA for the 444-amino acid-long variant, $D2_{444}$, was the most abundant form in pituitary and neostriatum. Intermediate levels of both $D2_{444}$ mRNA and the short form, $D2_{415}$, were detected in midbrain, and low levels of $D2_{444}$ and $D2_{415}$ mRNAs were detected in all other regions examined, including hippocampus, cerebellum, and cortex. The $D2_{444}/D2_{415}$ ratio was generally lower in the regions of low expression than in pituitary and neostriatum. Dopamine-depleting lesions increased the density of D2 receptors in the denervated neostriatum by 29% without altering the affinity of the receptors for [^{3}H]spiperone. The proliferation of receptors appeared to be due to a lesion-induced increase of up to 120% in the abundance of both variants of mRNA in the neostriatum.**

When excitable tissue is denervated the tissue typically becomes supersensitive to the effects of neurotransmitters and drugs. Denervation supersensitivity has been proposed as a compensatory mechanism involved in recovery of function after nervous system damage (1) and as a mechanism of tolerance and withdrawal phenomena associated with chronic drug use (2). Damage to the rat dopaminergic mesotelencephalic projection results in heightened sensitivity of neostriatal neurons to dopamine agonists. One manifestation of denervation supersensitivity after unilateral damage to the dopaminergic system is rotation away from the damaged hemisphere in response to systemic administration of the dopamine agonist apomorphine (3, 4). Denervation-induced proliferation of D2 dopamine receptors is one potential cellular mechanism for behavioral supersensitivity to dopamine agonists (4–9).

The dopamine-depleted rat has been used as a model for parkinsonism, a disease in which degeneration of dopamine-containing neurons leads to a reduction in the dopaminergic innervation of the basal forebrain (10, 11). Since the concentration of dopamine may be decreased by 70% before patients exhibit clinical symptoms, and patients with parkinsonism appear to be supersensitive to the dopamine precursor L-dopa, it has been proposed that the development of denervation supersensitivity compensates for the loss of dopamine (12, 13). An increased density of D2 receptors may be one factor contributing to denervation supersensitivity in parkinsonism (14).

Two variants of D2 receptor mRNA that result from alternative splicing of a single gene product have been identified (15–17). The variants differ due to a stretch of 87 bases present in the third cytoplasmic loop of the long form ($D2_{444}$) but lacking from the short form ($D2_{415}$) described by Bunzow *et al.* (18). Studies of the distribution of D2 receptor mRNA have yielded contradictory results concerning the existence and distribution of $D2_{415}$ and $D2_{444}$ in rat brain (15–17, 19–22). We now describe the use of polymerase chain reaction (PCR) analysis, RNA slot blots, and *in situ* hybridization to define the distribution of each of the alternatively spliced variants in rat brain. Also, we report that denervation of D2 receptors increases the abundance of both forms of mRNA in rat neostriatum.

METHODS

Reverse Transcription (RT)–PCR. Single-stranded cDNA was produced from purified RNA by a primer extension/reverse transcriptase reaction. RNA (2 μg) was dissolved in 28.5 μl of RNase-free H_2O. After addition of 6.0 μl of 0.1 M MeHgOH, the RNA was incubated at room temperature for 7 min, and then 5 min after addition of 3.1 μl of 0.7 M 2-mercaptoethanol. For the RT reaction, the following components were then added: 10 μl of 5× RT buffer, 1.25 μl of RNasin (50 units, Promega), 1 μl of PCR primer (1 μg), 1 μl of 25 mM dNTPs, and 2.65 μl of avian myeloblastosis virus reverse transcriptase (50 units, Life Sciences, St. Petersburg, FL). MeHgOH, 2-mercaptoethanol, 5× RT buffer, and 25 mM dNTPs were all from the Invitrogen red module for cDNA synthesis. The primer was a 36-mer oligonucleotide (5′-CCAGCAGATGATGAACACACCGAGAACAATG-GCAAT-3′) complementary to a region of the D2 receptor cDNA extending from the 5′ end of the sequence encoding membrane-spanning domain VI to nucleotide 1074 of $D2_{415}$ (18). The cDNA synthesis was carried out for 2 hr at 42°C, then inactivated at 65–70°C for 10 min. The cDNA was stored at −20°C until use. The PCR was carried out in a volume of 100 μl including 2 μl of the cDNA reaction mixture, 69 μl of H_2O, 1 μl of forward primer and of reverse primer (≈1 μg each), 10 μl of 10× PCR buffer, 16 μl of 1.25 mM dNTPs, and 0.5 μl of *Taq* polymerase. PCR buffer, dNTPs, and *Taq* polymerase were from the Perkin–Elmer GeneAmp kit. The forward primer was the oligonucleotide used in the RT reaction. The reverse primer was a 39-mer oligonucleotide (5′-ACTCTGCTGGTCTATATCAAAATCTA-CATCGTCCTCCGG-3′) corresponding to a segment extending from base 612 in membrane-spanning domain V to base 650 in the third cytoplasmic loop. The two primers define a fragment of 462 bases in the sequence published by Bunzow *et al.* (18). This fragment was amplified by 40 cycles of PCR under the following conditions: 94°C for 1 min to denature DNA, 60°C for 2 min to anneal primers, and 72°C for 3 min for *Taq* polymerase extension. The products of the PCR amplification were visualized by electrophoresis of 40 μl of

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Abbreviation: RT, reverse transcription.

the reaction mixture in a 1% agarose gel in Tris/acetate buffer and staining with ethidium bromide.

RNA Blot Hybridization. A 36-base oligonucleotide (D2i, 5'-CGGTGCAGAGTTTCATGTCCTCAGGGTGGGTA-CAGT-3', corresponding to bases 728–763; ref. 16) was used to hybridize to the insert present in the long form of D2 receptor mRNA ($D2_{444}$). A junctional oligonucleotide (D2j, 5'-CTCGGCGGGCAGCATCCTTGAGTGGTGTCT-TCAGGT-3', corresponding to bases 704–739; ref. 18) was used for detection of the shorter form of D2 receptor mRNA ($D2_{415}$). Methods of RNA isolation, electrophoresis in formaldehyde/agarose gels, and transfer to Biotrans membrane were as described (23). Oligonucleotides were ^{32}P-labeled by T4 polynucleotide kinase (BRL). The specific activity of both radiolabeled oligonucleotides was consistently 4×10^8 cpm/μg. For slot blot hybridization, RNA samples (6 μg) were vacuum-dried, dissolved in 50 μl of 6.1 M formaldehyde in 10× standard saline citrate (SSC) at 65°C for 15 min, and brought to a volume of 200 μl with 15× SSC. Biotrans membrane was prewetted with 10× SSC and placed on a slot minifold apparatus (Schleicher & Schuell). Samples were loaded and vacuum-applied. Filters were baked under vacuum at 80°C for 1 hr, and the RNA was crosslinked to the membrane by exposure to UV light for 2 min. Hybridizations were carried out in 5× SSC/50% formamide at 42°C, followed by four 15-min washes at 55°C. Radioactive signals from blots were estimated with the LKB UltroScan XL soft-laser scanning densitometer. Areas under optical density peaks over a path encompassing the length of the entire slot were measured. Several exposures of each blot were made; those producing signals that fell in the range over which signal intensity was linearly related to the amount of RNA loaded were selected for densitometric analysis. This linear range was previously determined with a standard curve constructed by blotting defined dilutions of rRNA and hybridizing the blot with a 28S rRNA-encoding DNA probe.

***In Situ* Hybridization Histochemistry.** Oligonucleotide probes were tailed with [α-[^{35}S]thio]dCTP to a specific activity of 5×10^8 cpm/μg by terminal deoxynucleotidyltransferase. Tissue preparation and hybridization conditions were as described (24, 25). Paraformaldehyde-fixed tissue sections (20 μm thick) were collected on slides, and the slides were rinsed twice in phosphate-buffered saline after each pretreatment step prior to dehydration. The pretreatment steps consisted of (*i*) fixation in buffered 4% formaldehyde for 5 min at room temperature, (*ii*) digestion in proteinase K (50 μg/ml in 50 mM Tris/5 mM EDTA, pH 8) for 7.5 min at 37°C, (*iii*) postfixation in buffered 4% paraformaldehyde for 1 min at room temperature, (*iv*) acetylation (0.1 M triethanolamine/0.9% NaCl/0.25% acetic anhydride) for 10 min at room temperature, (*v*) dehydration in graded ethanol solutions followed by chloroform (10 min at room temperature) and rehydration to 95% ethanol, and (*vi*) air-drying. Prehybridization buffer consisted of 50% formamide, 0.75 M NaCl, 10 mM Pipes (pH 6.8), 10 mM EDTA, 250 mM dithiothreitol, 5× Denhardt's solution, 0.2% SDS, 10% dextran sulfate, and denatured tRNA at 500 μg/ml. Tissue sections on slides were covered with prehybridization buffer (750 μl) within a humidified Plexiglas box at 37°C. For hybridization, the prehybridization buffer was replaced by prehybridization solution to which labeled oligonucleotide had been added and thoroughly mixed. Slides were coverslipped, sealed with contact cement, and placed into a humidified box at 37°C. Posthybridization rinsing was taken to 0.2× SSC at 42°C, with 5 mM dithiothreitol included in all rinses. Slides were air-dried and processed for emulsion autoradiography using Kodak NTB-2 emulsion. Manual grain counting was used to compare relative differences in the abundance of $D2_{444}$ and $D2_{415}$ mRNA on the lesioned side of the brain vs. the non-lesioned side. "Grain clusters" were taken to represent isotopic emission from single cells (24, 25).

Intracerebral 6-Hydroxydopamine Injections. Lesions of ascending dopaminergic tracts were introduced as described (8, 9), by injection of 6-hydroxydopamine (8 μg of free base in 4 μl of 0.9% NaCl/0.1% ascorbic acid vehicle) into the left ventral tegmental area (2.6 mm anterior to the interaural plane, 1.0 mm lateral to the sagittal suture, and 7.8 mm ventral to the dura) 30 min after pretreatment with desmethylimipramine. Two weeks after surgery, the extent of dopamine depletion in the striatum was assessed by testing rats for their ability to orient to somatosensory stimuli. As described elsewhere (8, 9, 26), rats were touched on 11 specific areas on each body surface with a 4-g von Frey hair. Scores for orientation to each body surface were summed, and the ratio of right/left orientation was determined. Rats with a right/left ratio less than 0.25 have neostriatal dopamine depletions averaging greater than 96–98% (8, 26). Nine rats were determined to have extensive neostriatal dopamine depletions and were randomly assigned to one or two groups for *in situ* hybridization ($n = 4$) or *in vitro* determination of the density of D2 receptors ($n = 5$). Orientation toward stimuli presented to the right body surface was negligible for all rats, with a mean right/left ratio of 0.05 in rats used for determination of receptor density and 0.02 in rats used for *in situ* hybridization.

Binding of [^{3}H]Spiperone and Data Analysis. Binding of [^{3}H]spiperone was carried out as described (18) except that 40 nM ketanserin was added to all assays to prevent binding to 5-HT_2 serotonin receptors. The concentration of [^{3}H]spiperone ranged from 15 to 450 pM. K_D and B_{max} values were determined by nonlinear regression analysis using the program GRAPHPAD. Statistical comparison of values in the right and left hemispheres was carried out using a *t* test for paired means.

RESULTS

PCR Analysis of D2 Receptor mRNA. RNA was isolated from rat neostriatum, pituitary, cerebral cortex, cerebellum,

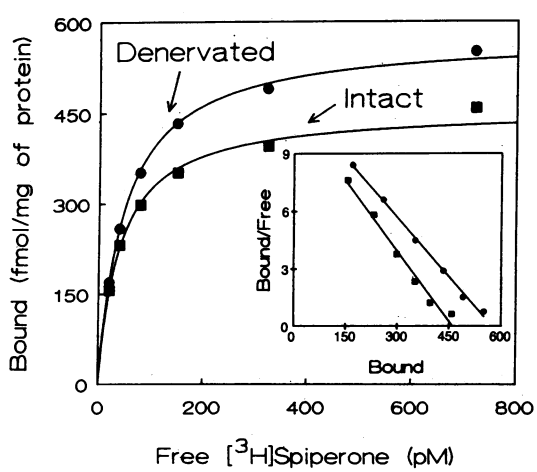

FIG. 1. Binding of [^{3}H]spiperone to rat neostriatal membranes. Results are shown from one of five experiments in which saturation analysis of the binding of [^{3}H]spiperone to denervated and intact striata was carried out. Data are plotted as amount of radioligand bound per mg of protein vs. the corrected free concentration (total added minus amount bound) of radioligand. B_{max} values in the experiment shown were 649 and 516 fmol per mg of protein in the denervated and intact neostriatum, respectively. K_D values were 48 and 40 pM, respectively. (*Inset*) Data have been transformed and plotted as radioligand bound (fmol/mg of protein)/free radioligand (nM) vs. radioligand bound (fmol/mg of protein). ●, Denervated neostriatum; ■, innervated neostriatum.

Table 1. Quantification of relative levels of $D2_{415}$ and $D2_{444}$ mRNAs by slot blot analysis

Tissue	$D2_{415}$	$D2_{444}$	$D2_{444}/D2_{415}$
Striatum	3.05	5.92	1.9
Pituitary	0.66	4.11	6.2
Midbrain	0.72	1.02	1.4
Anterior cortex	0.33	0.35	1.1
Posterior cortex	0.27	0.14	0.5
Hippocampus	0.48	0.45	1.1
Brainstem	0.53	0.42	1.3
Cerebellum	0.56	0.36	0.6
Kidney	0.30	0.36	1.2

Optical density measurements were determined from an 11-day exposure of identical slot blots probed with D2 receptor subtype-specific oligonucleotides radiolabeled to the same specific activity. At this exposure, the signals for hybridization to $D2_{444}$ mRNA in pituitary and striatum were beyond the linear range of the densitometer. Nevertheless, results are displayed here for the purpose of comparison to other tissues with lower expression. Accurate ratios for the two forms in pituitary and striatum, given in the text, were determined from a 16-hr exposure of the same blots.

and kidney. RT–PCR was performed on 2 μg of each RNA preparation and the products of the reaction were electrophoresed in an agarose gel (data not shown). A 460-base-pair (bp) band was seen in all tissues examined, but an additional, and usually more prominent, band of ≈550 bp was observed in all tissues except GH_4ZR_7 cells, which were transfected with a $D2_{415}$ cDNA (27). The 550-bp band was cloned into phage M13 and sequenced, and it is identical to $D2_{444}$ cDNA (15).

Table 2. Quantification of relative levels of $D2_{415}$ and $D2_{444}$ mRNAs by *in situ* hybridization

Tissue	$D2_{415}$	$D2_{444}$	$D2_{444}/D2_{415}$
Striatum	2.84 ± 0.29	5.51 ± 0.44	2.0 ± 0.1
Motor cortex	0.40 ± 0.08	0.18 ± 0.02	0.5 ± 0.1
Substantia nigra	2.57 ± 0.28	2.43 ± 0.45	1.0 ± 0.2
Hippocampus	0.57 ± 0.10	0.38 ± 0.08	0.7 ± 0.1
Cerebellum	0.99 ± 0.21	2.60 ± 0.78	2.6 ± 0.6

Optical density values (mean ± SEM) are shown for hybridization of D2j ($D2_{415}$) and D2i ($D2_{444}$) to rat brain. Whole-field densitometric measurements (n = 4) were made from the following regions: dorsal medial striatum, motor cortex, substantia nigra, hippocampal formation, and cerebellar cortex.

Regional Distribution of Alternatively Spliced mRNA for D2 Receptors. We designed oligonucleotide probes that would specifically hybridize with each of the two D2 receptor transcripts. Under the stringent hybridization conditions

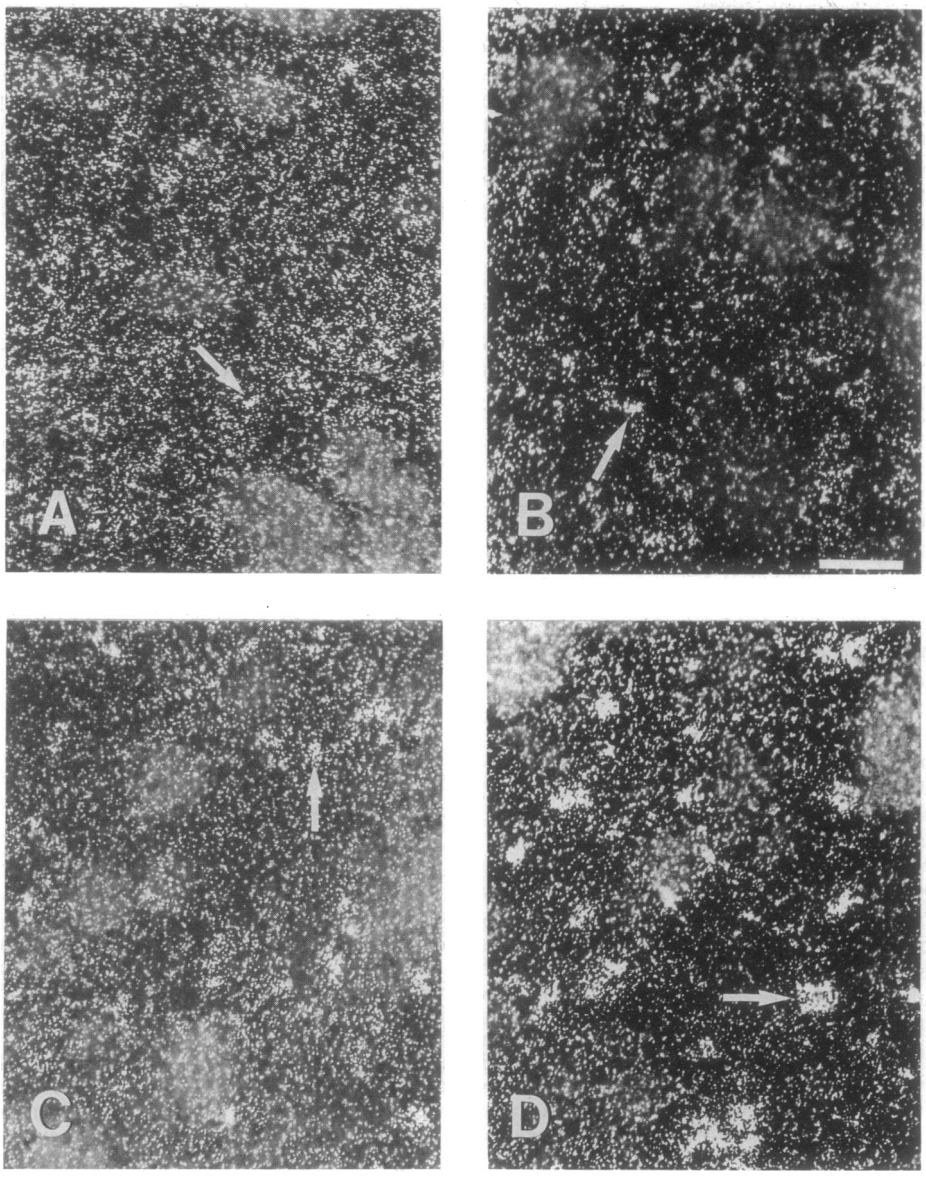

FIG. 2. Dark-field photomicrographs showing *in situ* hybridization of $D2_{415}$ and $D2_{444}$ mRNAs within striatal neurons. Bright clusters of grains, which were not observed after hybridization with sense probes, represent hybridization to cell bodies. One such cluster is indicated by the arrow in each panel. In *A* and *C*, adjacent tissue sections from the intact hemisphere of rats with unilateral destruction of the ascending dopaminergic system were hybridized with ^{35}S-labeled oligonucleotide probes specific for $D2_{415}$ (*A*) or $D2_{444}$ (*C*) receptor mRNA. In the denervated hemisphere, mRNA levels for both $D2_{415}$ (*B*) and $D2_{444}$ (D) were increased within neostriatal neurons. (Bar = 50 μm.)

used, the D2j probe hybridizes only to the shorter transcript, designated $D2_{415}$, whereas D2i hybridizes only to the longer transcript, designated $D2_{444}$. To demonstrate the specificity of D2i and D2j, gel-resolved fragments resulting from PCR amplification were transferred to a nitrocellulose membrane. D2i hybridized only to the upper band ($D2_{444}$), whereas D2j hybridized only to $D2_{415}$ (data not shown).

Slot blot analysis was carried out to quantify the relative levels of the alternatively spliced forms of D2 receptor mRNA in rat tissues and brain regions. Highest levels of D2 receptor mRNA were found in striatum, with high expression also observed in pituitary (Table 1). Moderate levels of D2 receptor message were found in the midbrain, and low levels in a number of other tissues, including cerebellum and hippocampus. There was no detectable hybridization of either probe to rRNA (data not shown). Distribution of the alternatively spliced variants was heterogeneous, in that D2 receptor mRNA in pituitary consisted almost exclusively of $D2_{444}$ ($D2_{444}/D2_{415}$ ratio = 6.0), whereas the level of $D2_{444}$ was approximately twice the level of $D2_{415}$ in rat striatum ($D2_{444}/D2_{415} = 2.4$). In several other areas the two transcripts were expressed in roughly equal proportions. Results for relative levels of $D2_{415}$ and $D2_{444}$ mRNAs obtained by *in situ* hybridization histochemistry were similar to those determined by slot blot analysis (Table 2). The greatest discrepancy between the two techniques was observed for values in the cerebellum, which could be due to the regional heterogeneity of D2 receptor distribution within the cerebellum (28).

Denervation-Induced Proliferation of D2 Receptors. The effect of denervation on the density of D2 receptors and levels of D2 receptor mRNA was determined 3 weeks after unilateral injection of 6-hydroxydopamine into the ventral tegmental area. The density of D2 receptors, determined by saturation analysis of the binding of [^{3}H]spiperone, was 400 ± 26 fmol/mg of protein in the intact neostriatum. In the denervated neostriatum, the density of D2 receptors was 509 ± 23 fmol/mg of protein, representing a mean increase of 29% over the number of receptors in the intact neostriatum ($P < 0.05$; Fig. 1). The K_D value for the binding of [^{3}H]spiperone in the intact neostriatum was 40 pM (95% confidence limit of the mean = 29–55 pM). This did not differ significantly from the K_D value determined in the denervated neostriatum, 57 pM (42–79 pM).

Increased Abundance of $D2_{415}$ and $D2_{444}$ mRNA After Denervation. As shown in Figs. 2 and 3, denervation increased the expression of mRNA for both $D2_{444}$ and $D2_{415}$ in neostriatum. Adjacent sections hybridized with either D2i or D2j showed qualitatively similar increases in the amount of hybridization in the denervated hemisphere. To quantify changes in D2 receptor gene expression, silver grains were counted on adjacent tissue sections from three animals. mRNA for both $D2_{415}$ and $D2_{444}$ was increased up to 2.4-fold following denervation (Fig. 3). Both variants were increased by similar amounts within an animal. Denervation induced a similar increase in all neurons within the analyzed region, as indicated by the similar standard error of the mean grains per neuron before and after denervation. In other brain regions, the abundance of D2 receptor mRNA was not altered by denervation (data not shown).

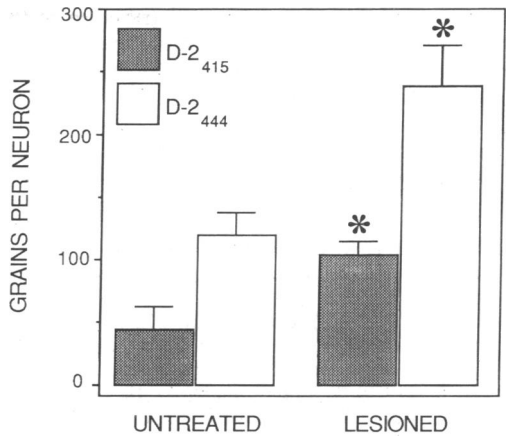

FIG. 3. Quantification of increased levels of D2 receptor mRNA levels following dopaminergic denervation. Manual grain counting from four different animals indicates that $D2_{444}$ receptor mRNA is normally twice as abundant as $D2_{415}$ message in the intact neostriatum. Both species of mRNA are increased ≈2-fold following 6-hydroxydopamine lesions of the ipsilateral ventral tegmental area (*, $P < 0.05$). Mean ± SEM of grains per neuron for the four groups are as follows: $D2_{415}$ receptor mRNA, untreated, 44 ± 19; $D2_{444}$, untreated, 119 ± 18; $D2_{415}$, lesioned, 104 ± 10; $D2_{444}$, lesioned, 238 ± 33.

DISCUSSION

Levels of expression of D2 receptor mRNA closely match the distribution of D2 receptors. Thus, the density of D2 receptors is high in the neostriatum and other nuclei of the basal forebrain, and low in regions such as cerebral cortex, hippocampus, and cerebellum (28–30). Interestingly, regulation of the processing of the primary transcript to produce the alternatively spliced forms of mRNA apparently varies between tissues, as reflected in the varying ratio of the mature transcripts. Thus, $D2_{444}$ RNA was expressed at 6-fold higher levels than $D2_{415}$ RNA in pituitary. $D2_{444}$ RNA was also expressed more abundantly in striatum. A number of other tissues, including hippocampus, kidney, and anterior cortex, expressed both transcripts at similar levels.

The demonstration by both slot blot and *in situ* hybridization that both $D2_{415}$ and $D2_{444}$ mRNAs were present in all brain regions in which D2 receptor mRNA was detected contrasts with some previous reports. Thus, in two reports (17, 20), $D2_{415}$ mRNA was not detected. Two other reports identified $D2_{415}$ mRNA in brain by RT–PCR but were unable to detect the mRNA in rat brain by *in situ* or Northern blot analysis (15, 16). Chio *et al.* (19) used subtype-selective junctional and insert probes, as did Dal Toso *et al.* (15), and were able by Northern blot analysis to detect $D2_{415}$ mRNA in rat neostriatum but not in other brain regions. On the other hand, our results are similar to results from recent studies in which mRNA distribution was determined by using the PCR (21, 22). Our ability to detect both forms of mRNA in many brain regions by hybridization may be due to the combination of our use of selective, relatively long oligonucleotide probes and quantitative slot blot hybridization.

Chronic administration of the D2 receptor antagonist haloperidol to rats increases the abundance of D2 receptor mRNA in the intermediate lobe of the pituitary, but not in the anterior lobe (31). Treatment with haloperidol increases the density of D2 receptors in rat neostriatum (32, 33) but may (34) or may not (35) increase neostriatal total D2 receptor mRNA levels. As described here, destruction of the ascending dopaminergic system resulted in an increased density of neostriatal D2 receptors, although the affinity of the receptors for radioligand was not significantly changed. The lesion-induced proliferation of receptors was associated with an increased abundance of mRNA for both $D2_{415}$ and $D2_{444}$. These findings confirm and extend previous work (36) indicating that denervation-induced proliferation of D2 receptors is due to an increased rate of receptor synthesis. The increased rate of synthesis apparently results from increased levels of D2 receptor mRNA. Furthermore, the $D2_{444}/D2_{415}$ ratio was not altered by denervation. Thus, the alternative splicing of the D2 receptor gene transcript to produce mRNA for $D2_{415}$ and $D2_{444}$ is regulated by tissue-specific factors, as

demonstrated by the heterogeneous distribution of the two variants, but the alternative splicing within the striatum is not regulated by the extent of innervation of the receptors by dopamine-containing terminals.

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