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The Effects of Haloperidol on Dopamine Receptor Gene Expression

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Haloperidol is a widely prescribed antipsychotic that acts as a dopamine D2 receptor antagonist. Chronic administration of haloperidol leads to an increase in striatal D2 receptor binding; however, studies examining striatal D2 receptor mRNA after haloperidol treatment report inconsistent results. This study examines the effects of haloperidol on dopaminoceptive striatal neurons, as well as dopamine D2 containing striatal inputs. Rats were injected subcutaneously with 2 mg/kg haloperidol twice daily for 7 days. A significant (36%) increase in D2 mRNA was observed in the anterior cingulate cortex. However, no changes were observed in the amounts of D1, D2, D3 mRNA, or D2 heteronuclear RNA (hnRNA) in the striatum or in the levels of D2 mRNA and hnRNA in the substantia nigra and ventral tegmental area. Thus, increased striatal D2 binding after haloperidol treatment may not be the result of altered D2 gene activity in the striatum or midbrain, but could result from an increase in D2 mRNA in cingulate corticostriatal neurons and/or a longer half-life for the D2 receptor protein in striatal neurons. Striatal proenkephalin mRNA increased significantly in the caudate-putamen (45%), nucleus accumbens (36%), and the olfactory tubercle (27%) while prodynorphin mRNA remained unaltered after haloperidol treatment. Since D2 receptor mRNA is generally colocalized with proenkephalin mRNA in striatal neurons, these results demonstrate what is likely a selective cellular increase in proenkephalin mRNA without a parallel increase in D2 mRNA.

INTRODUCTION

Haloperidol is a widely prescribed antipsychotic that acts as a dopamine D2 receptor antagonist in the central nervous system (60). Interestingly, the clinical effects of haloperidol do not occur rapidly, rather a period of 1–3 weeks of treatment is necessary, even though the pharmacological antagonism of the receptor is immediate. The therapeutic effects have been linked to an increase in dopamine receptor number which occurs with chronic exposure to the drug. Studies in rodent animal models indicate that there is approximately a 20% increase in striatal D2 receptor binding that begins during the first week of treatment and is maintained for months following withdrawal from a chronic treatment paradigm (10, 25, 33, 50, 52, 55, 56, 59, 71, 79, 80).

Several mechanisms can be hypothesized to explain the haloperidol-induced increase in striatal dopamine receptors. One possibility is that haloperidol may increase D2 receptor gene expression within striatal neurons. This increase in gene expression would then lead to increases in D2 receptor mRNA and protein. Several studies have described increases in D2 receptor mRNA following haloperidol treatment (3, 8, 13, 34, 54). However, many of these studies examine D2 mRNA levels after long term haloperidol treatment (14 days–6 months), time points that are later than the initial increase in D2 receptor protein expression. Shorter treatment paradigms have revealed that there are no changes in D2 mRNA levels within the time course of the initial increase in D2 receptor protein (25, 80).

A possible explanation for a lack of coordinated D2 binding and mRNA induction in the striatum relates to the issue of methodological sensitivity and/or mRNA pool turnover. Striatal neurons have a fairly large D2 mRNA pool. Therefore, it is possible that only a small change in the mRNA pool may be needed to induce the modest increase that has been reported in D2 binding. On the other hand, changes in the dynamics of the D2 mRNA pool may be difficult or impossible to detect using probes directed at the mRNA if the steady state mRNA level remains unchanged.

Another potential explanation for uncoordinated striatal D2 receptor binding and mRNA expression induction is provided by the receptor mRNA and binding site anatomy in the striatum. It is well known that structures outside of the striatum contribute D2 receptor binding sites to the striatum. Midbrain dopaminergic neurons, as well as many corticostriatal projections have D2 receptors on their axon terminals (17, 18, 19, 21, 23, 27, 44, 48, 58, 62, 65, 66, 72, 73, 75). Thus, increases in D2 gene expression in striatal afferent structures could result in changes in D2 receptor binding in the striatum.

Receptor multiplicity within the dopamine family of receptors leads to a final potential mechanism to explain paradoxical D2 receptor changes. Molecular cloning

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TABLE 1

Summary of Probes, Brain Regions, and Animals Utilized in This Study

Probe	Size (bases)	Specificity[a]	Reference (clone)	Regions[b]	Number of sections analyzed[c]	Levels[d]
D1 mRNA	530	Sequence encoding TM III–IV	82	CPu, Acb, Tu	4–6 per rat; 6 HAL rats and 6 VEH rats	B2.20–B0.70
D2 mRNA	495	Sequence encoding TM VI–VII	9	CPu, Acb, Tu	4–5 per rat; 5 HAL rats and 5 VEH rats	B2.20–B0.70
				Cingulate cortex	4–8 per rat; 5 HAL rats and 5 VEH rats	B3.70–B1.70
				Insular cortex	3–6 per rat; 5 HAL rats and 5 VEH rats	B2.70–B1.70
				Neocortex	4–5 per rat; 5 HAL rats and 5 VEH rats	B2.70–B-3.80
				SNc, VTA	4–5 per rat; 5 HAL rats and 5 VEH rats	B-4.80–B-6.04
D3 mRNA	325	Sequence encoding 3rd cyto loop	63	Acb, Tu	4–6 per rat; 6 HAL rats and 6 VEH rats	B2.20–B0.70
D2 hnRNA	425	Intron 7	9	CPu, Acb, Tu	4–6 per rat; 6 HAL rats and 6 VEH rats	B2.20–B0.70
				SNc, VTA	4–5 per rat; 5 HAL rats and 5 VEH rats	B-4.80–B-6.04
Prodyn mRNA	733	Exon 4	12	CPu, Acb, Tu	4–6 per rat; 6 HAL rats and 6 VEH rats	B2.20–B0.70
Proenk mRNA	693	Exon 3	81	CPu, Acb, Tu	4–6 per rat; 6 HAL rats and 6 VEH rats	B2.20–B0.70

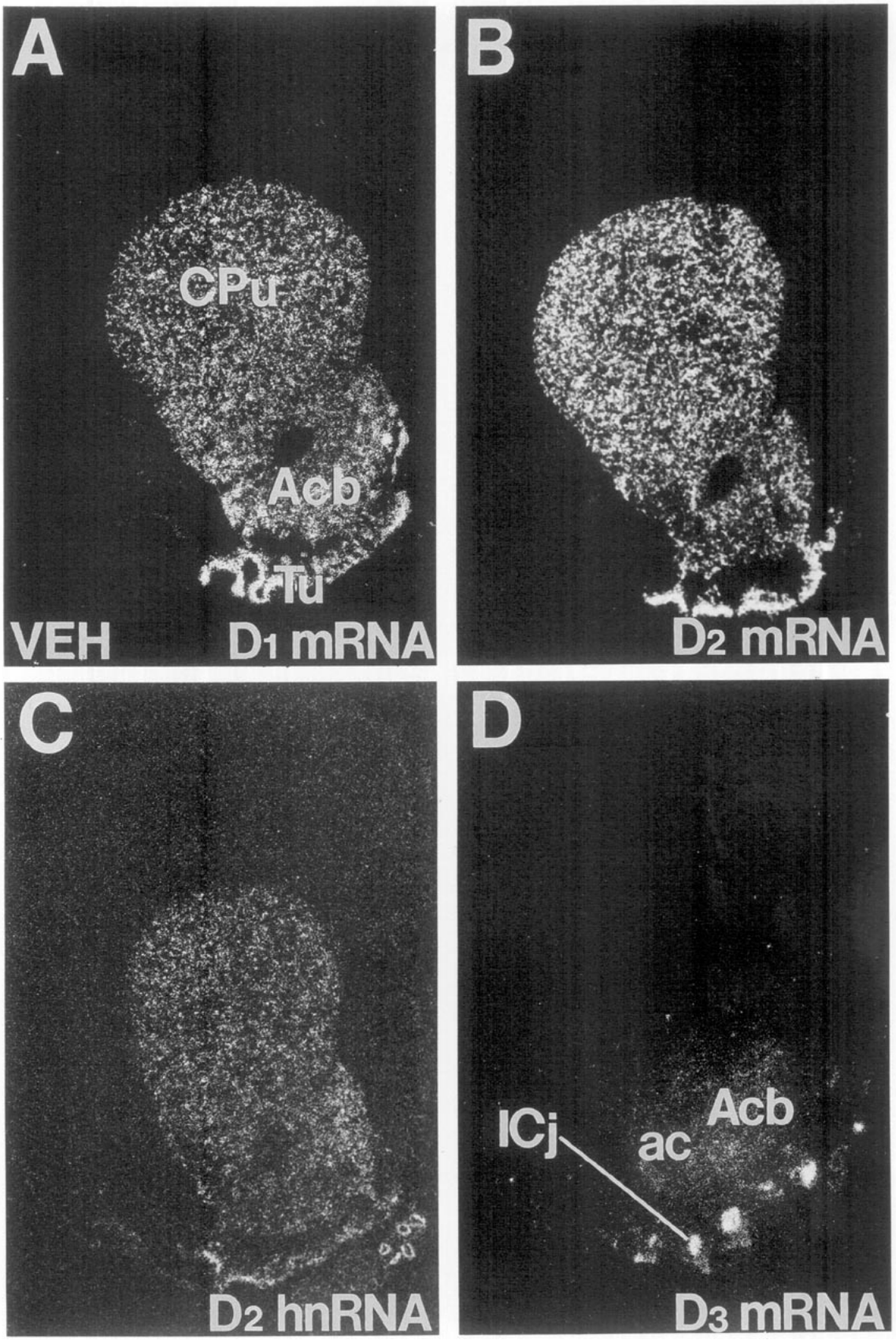
[a] TM, transmembrane domain.
[b] CPu, caudate-putamen; Acb, nucleus accumbens; Tu, olfactory tubercle; SNc, substantia nigra, pars compacta; VTA, ventral tegmental area.
[c] HAL, 7-day haloperidol-treated rat (see Materials and Methods); VEH, 7-day vehicle-treated rat (see Materials and Methods).
[d] All levels are noted in millimeters relative to bregma (B).

techniques have identified five dopamine receptors that can be differentiated based on their nucleotide and amino acid sequences. Pharmacologically, they can be segregated into two families. The D1 family contains the D1 and D5 receptors which have a similar D1-like pharmacology and are positively linked to G-proteins (15, 47, 67, 68, 82). The D2 family consists of the D2, D3, and D4 receptor subtypes. These receptors have a similar D2-like pharmacology and are negatively coupled to G-proteins (9, 26, 63, 65, 76). Given that the tritiated ligands used to measure increases in D2 binding also bind D3 and D4 dopamine receptors, the reported changes in D2 binding could reflect changes in D3 and/or D4 receptors.

Biochemical changes in the striatum in response to haloperidol are not limited to dopamine receptor changes. Previous studies indicate that the endogenous opioid peptide methionine–enkephalin is also increased in the striatum after chronic haloperidol treatment (1, 5, 11, 30, 31, 74). The mRNA that encodes the proenkephalin peptide also appears to increase in response to haloperidol treatment (1, 2, 46, 49, 70). This change in enkephalin may, in fact, occur in striatal cells that express the D2 receptor. Recent studies using anatomical techniques indicate that enkephalin peptides are frequently found within striatal neurons that express dopamine D2 receptors (24, 34). Dynorphin, another abundantly expressed opiate peptide, does not appear to be regulated by haloperidol and tends to colocalize with D1 receptors in striatal neurons (24). Although electrophysiological and other functional data do not indicate such a precise segregation of dopamine receptors (for review see 69), examining enkephalin and prodynorphin mRNAs provides a functional view of dopaminoceptive striatal efferent circuitry.

The goal of this set of studies is to broadly examine changes that occur in the striatum after 7 days of haloperidol treatment: (1) to clarify the mechanism for the haloperidol-induced increase in D2 receptor binding and (2) to follow neuropeptide mRNA changes in striatal

FIG. 1. Darkfield autoradiograms from *in situ* hybridizations of dopamine D1 receptor mRNA (A and E), D2 receptor mRNA (B and F), D2 receptor hnRNA (C and G), and D3 receptor mRNA (D and H) in coronal sections through the midstriatum. A–D are images made from vehicle-treated rats. E–H are images made from haloperidol-treated rats. All animals were given twice daily injections of 1% lactic acid (vehicle group) or haloperidol (2 mg/kg) for 7 days. ac, anterior commissure; CPu, caudate-putamen; Acb, nucleus accumbens; Tu, olfactory tubercle; ICj, islands of Calleja.


A
CPu
Acb
Tu
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D1 mRNA
B
D2 mRNA
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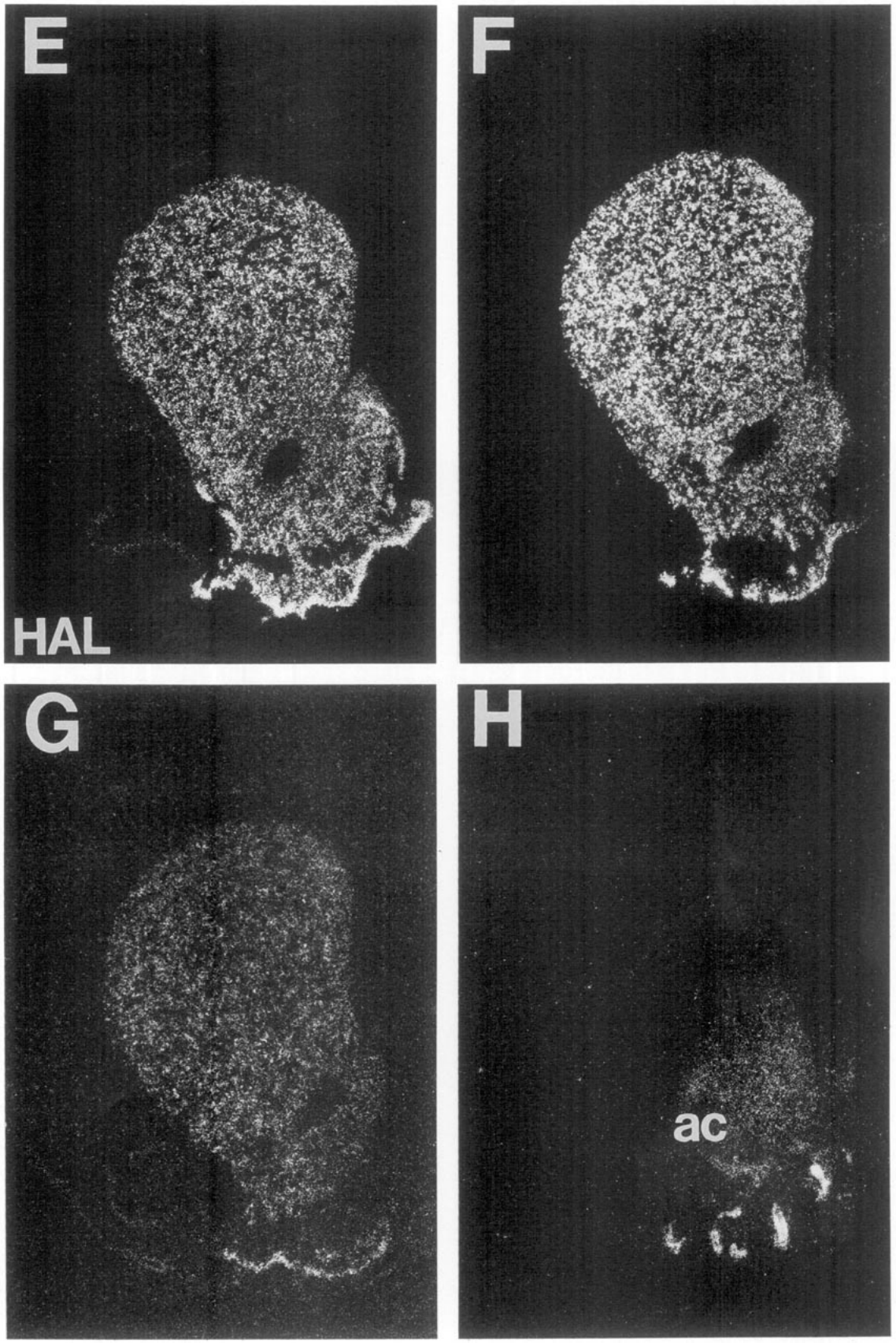
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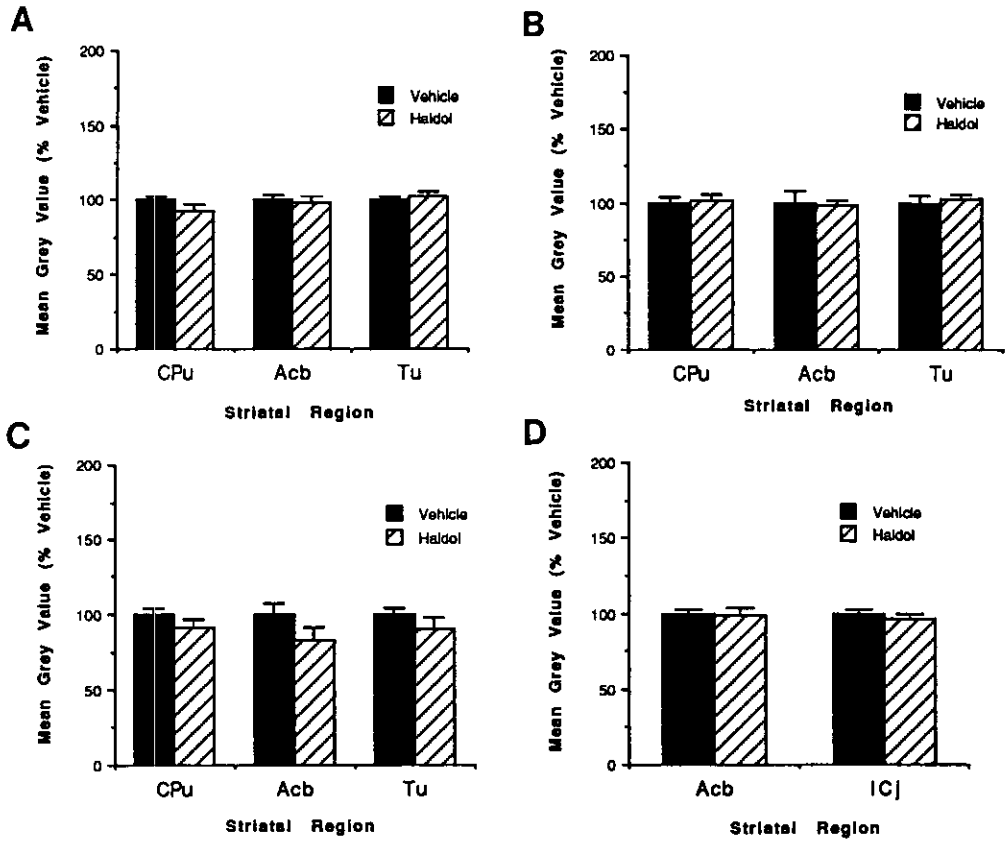


FIG. 2. A series of bar graphs showing relative amounts of D1 mRNA (A), D2 mRNA (B), D2 hnRNA (C), and D3 mRNA (D) in striatal subregions of vehicle-treated (black bars) and haloperidol-treated rats (hatched bars). Note all values are expressed as a percentage of vehicle-treated animals with all of the vehicle group values set at 100. CPu, caudate-putamen; Acb, nucleus accumbens; Tu, olfactory tubercle; ICj, islands of Calleja.

efferent circuitry. To accomplish this goal we have measured the level of D2 mRNA as well as its heteronuclear RNA (hnRNA) pool, *in situ*, in an attempt to address the issue of molecular sensitivity in an anatomical context. Since hnRNA is the short-lived precursor to mRNA, its pool size is quite small, thus hnRNA *in situ* hybridization provides the sensitivity to detect small changes in gene expression that may have been overlooked in studies that have examined D2 mRNA pools. Second, we have examined important D2 receptive striatal afferent regions for changes in D2 gene expression. To address a third hypothesis we have measured the levels of other known dopamine receptor mRNAs *in situ* to investigate the possibility of ligand specificity problems. Fourth and finally, to develop a picture of the responsiveness of the output side of the striatum, we measured prodynorphin mRNA and proenkephalin mRNA, two dopamine receptor-related molecules in the striatum.

MATERIALS AND METHODS

Drug Treatment

Adult male Sprague–Dawley (Charles River) rats were housed five or six per cage in a University laboratory animal medicine (ULAM) facility at the University of Michigan. The animals were weighed and given subcutaneous injections of either haloperidol (2 mg/kg) or 1% lactic acid (vehicle). Rats were injected twice daily at 9:00 and 17:00 h for 7 days.

Tissue Preparation

On the eighth day following the 9:00 injection, the rats were decapitated and the brains were rapidly removed, frozen in liquid isopentane at −30°C for 30 s, and then stored at −80°C until sectioning. At the time of sectioning the frozen brains were warmed to −20°C and mounted to a chuck with M-1 (Lipshaw) mounting medium. Fifteen-micrometer-thick coronal sections were cut using a Slee cryostat and the sections were thaw mounted onto polylysine-subbed slides and stored at −80°C.

In Situ Hybridization

Adjacent sections were processed for either D1, D2, D3, proenkephalin, or prodynorphin mRNA *in situ* hybridization or D2 intronic *in situ* hybridization. Briefly, the sections were removed from storage at −80°C and

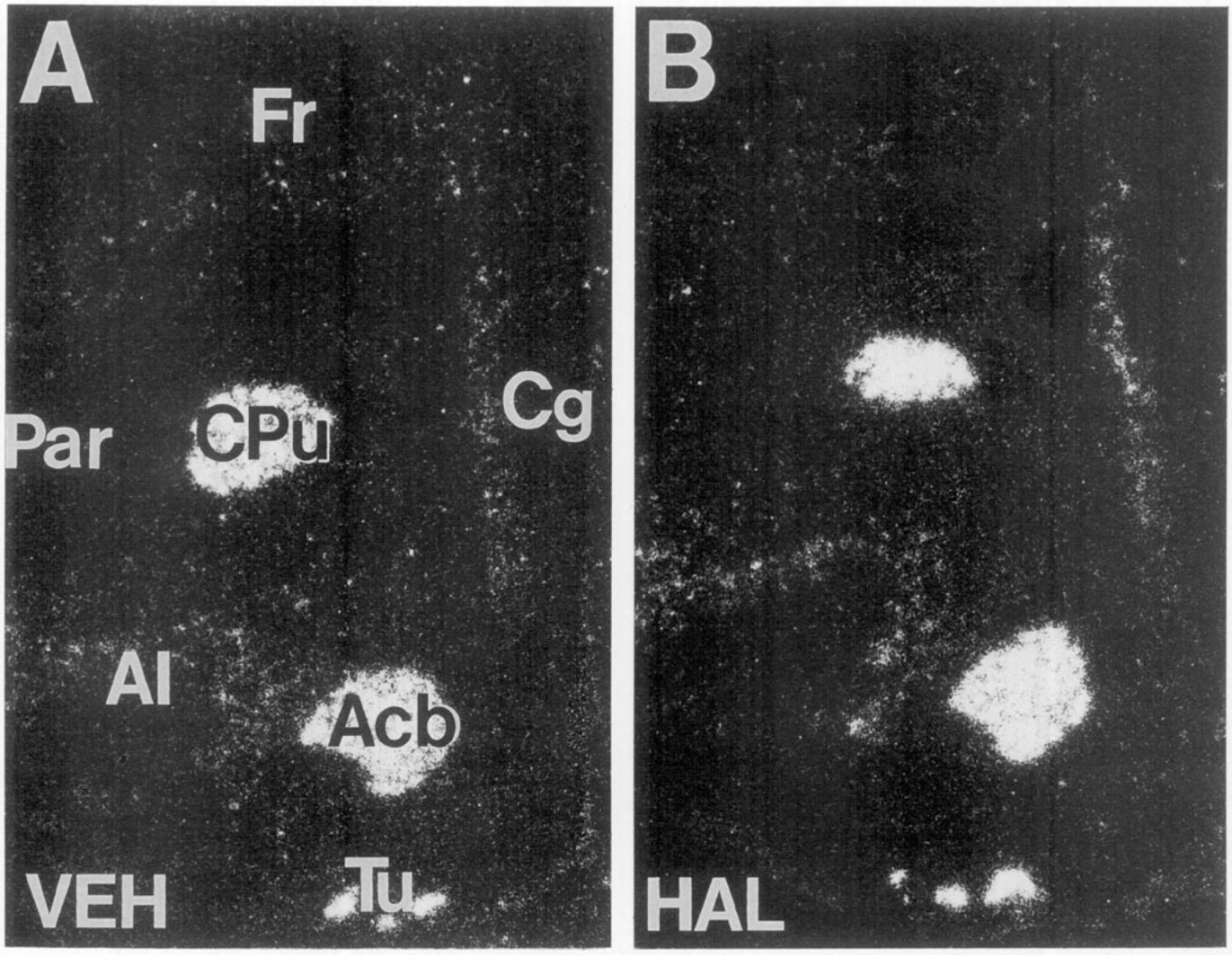


FIG. 3. Darkfield autoradiograms from *in situ* hybridizations of dopamine D2 receptor mRNA in the cerebral cortex of vehicle-treated (A) and haloperidol-treated (B) rats. All animals were given twice daily injections of 1% lactic acid (vehicle group) or haloperidol (2 mg/kg) for 7 days. Although no changes were observed in neocortical or insular cortical regions, a higher level of D2 mRNA expression was observed in the cingulate cortex of haloperidol-treated rats. Acb, nucleus accumbens; AI, anterior insular cortex; Cg, cingulate cortex; CPu, caudate-putamen; Fr, frontal cortex; Par, parietal cortex; Tu, olfactory tubercle.

fixed for 60 min in 4% formaldehyde at 22°C. The sections were then rinsed three times in 2× SSC (300 m*M* NaCl, 30 m*M* Na citrate, pH 7.2) and incubated in proteinase K (PK; 1 μg/ml in 100 m*M* Tris, pH 8.0, 50 m*M* EDTA) for 10 min at 37°C. Following PK treatment the sections were washed in water and then placed in 0.1 *M* triethanolamine (pH 8.0) to which acetic anhydride was added to a concentration of 1:400 and the sections were incubated with stirring at room temperature for 10 min. The sections were then rinsed in 2× SSC for 5 min, dehydrated in graded alcohols, and air dried.

^{35}S-Labeled cRNA probes were transcribed from cloned cDNA fragments shown in Table 1. It is important to note that the probes directed at D2 mRNA and D3 mRNA are complementary to regions of the mRNA that are not alternatively spliced; thus, total D2 RNA and D3 RNA pools were measured with these probes. Sections were hybridized overnight to the cRNA probes diluted in hybridization buffer (75% formamide; 10% dextran sulfate; 3× SSC, 50 m*M* Na_2PO_4, pH 7.4; 1× Denhardt's; 0.1 mg/ml yeast tRNA; 10 m*M* dithiothrietol). The probes were diluted in the hybridization buffer to a final concentration of 2×10^6 dpm/30 μl. Fifty microliters of the resulting hybridization solution was placed on two coronal sections, a coverslip was placed on the slide, and they were incubated overnight in a humid chamber at 55°C.

Following hybridization the slides were rinsed three times in 2× SSC and then treated with RNase A (200 μg/ml in 100 m*M* Tris, pH 8.0 and 0.5 *M* NaCl) for 60

min at 37°C. The sections were then rinsed in 2× SSC followed by room temperature washes in 1× SSC for 5 min, 0.5× SSC for 5 min, and 0.1× SSC for 5 min. Finally, the sections were washed in 0.1× SSC at 65°C for 60 min, rinsed with room temperature water, dehydrated in graded alcohols, air dried, and exposed to Kodak XAR-5 X-ray film. The *in situ* hybridizations were exposed for 1–7 days depending on the abundance of the target RNA.

Controls for riboprobe *in situ* hybridization have been described previously for the dopamine receptor mRNAs (38–40, 42), D2 receptor hnRNA (20), and prodynorphin and proenkephalin mRNAs (14, 57).

Image Analysis/RNA Quantitation

Computerized image analysis was used to quantify the amount of specific signal in tissue sections. X-ray exposures of *in situ* hybridizations were placed on a light table (Northern Lights) and images were captured using a Pulnix Model TM-745 video camera and a Macintosh IIci microcomputer equipped with an image capture card. A background field (a blank region of the X-ray film) was subtracted from the image to correct for possible inconsistencies in the illumination or camera field, and the corrected image was analyzed. Regions of interest were outlined with a cursor and the average gray value of pixels in this area of interest was calculated by the computer and saved. Table 1 lists the brain regions analyzed with each probe.

Statistics

Data are based on mean gray value measurements and are reported as a percentage of control. Error bars represent the standard error of the mean. One-way analysis of variance was used to determine if there were differences in mean gray values for regions of interest between treatment and control groups for each of the markers evaluated in this study. A P value less than 0.05 was considered significant.

RESULTS

D2 Gene Expression

D2 mRNA is most prominent in the corpus striatum. However, high levels of expression are observed in some afferent regions to the striatum such as the dopaminergic neurons of the ventral midbrain. Lower levels of D2 mRNA are found in other nondopaminergic afferents of the striatum, most prominently, the cerebral cortex.

After 7 days of haloperidol treatment no difference was observed in the level of striatal D2 mRNA between vehicle- and haloperidol-treated rats (Figs. 1B and 1F). The level of mRNA in the caudate-putamen (CPu), nucleus accumbens (Acb), and the olfactory tubercle (Tu) was measured indirectly using image analysis of X-ray autoradiograms. Image analysis confirmed no differences in D2 mRNA levels over any of the regions examined (Fig. 2B). In addition to the D2 mRNA pool, intronic *in situ* hybridization provided a measure of the D2 hnRNA pool in striatal neurons. Like the D2 mRNA pool, no changes were observed in striatal D2 hnRNA after haloperidol treatment (Figs. 1C and 1G). Semiquantitative image analysis confirmed that there were no differences in the amount of D2 hnRNA in the CPu, Acb, or Tu between vehicle- and haloperidol-treated rats (Fig. 2C).

Dopamine D2 receptor gene regulation was examined in afferents to the striatum that contain D2 receptor mRNA. It is likely that the cells in these regions, namely, the midbrain dopaminergic neurons and the cerebral cortex, send axons to the striatum that contain D2 receptors on their presynaptic terminals. Haloperidol-treated rats had 36% more D2 mRNA than vehicle-treated rats in the anterior cingulate cortex (Cg; Figs. 3 and 4). This difference did prove to be statistically significant by analysis of variance ($P = 0.0079$; $F = 12.35$). No differences were observed in the level of D2 mRNA between vehicle- and haloperidol-treated rats in the neocortex (Fr, Par) or insular cortex (AI; Figs. 3 and 4). In the cerebral cortex, D2 mRNA is much lower than the striatum or midbrain; however, D2 mRNA is found in layers II–III and V–VI in the neocortex and in the Cg and AI. Only D2 mRNA was measured in the cortex because cortical D2 hnRNA was below the level of detectability. D2 mRNA containing neurons in the midbrain are found in the substantia nigra, pars compacta (SNc), and the ventral tegmental area (VTA). Both D2 mRNA and hnRNA were measured in the SNc and VTA. No changes were observed in dopamine D2 recep-

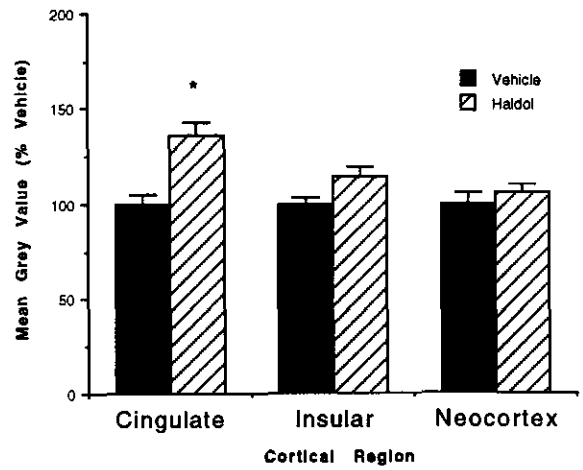


FIG. 4. A bar graph showing the relative amounts of D2 mRNA in cortical subregions of vehicle-treated (black bars) and haloperidol-treated rats (hatched bars). Note all values are expressed as a percentage of vehicle-treated animals with all of the vehicle group values set at 100. The asterisk indicates a statistically significant difference ($P < 0.05$).

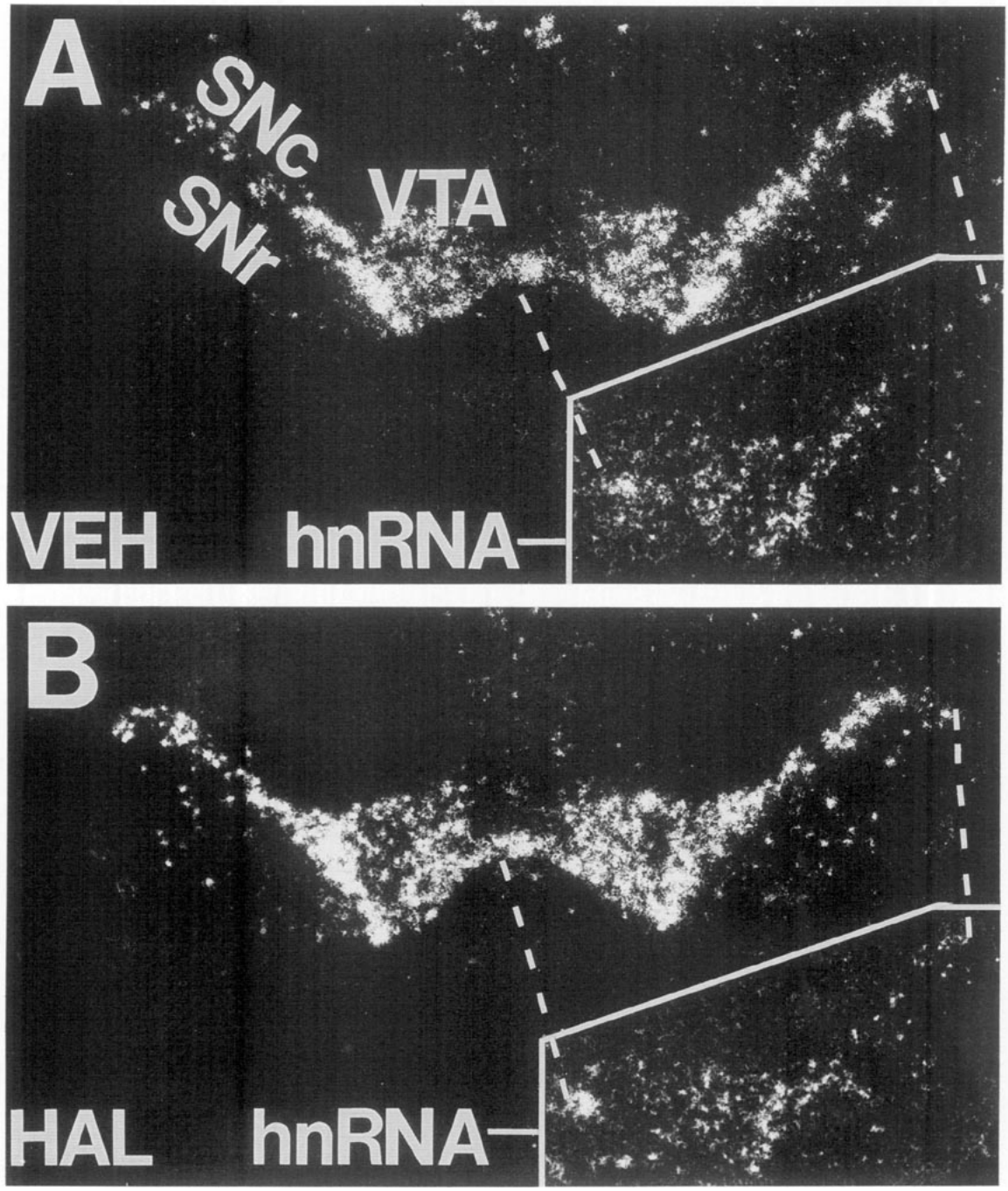


FIG. 5. Darkfield autoradiograms from *in situ* hybridizations of dopamine D2 mRNA (A and B) and hnRNA (A inset and B inset) in the substantia nigra and ventral tegmental area. (A) Images from the ventral midbrain of vehicle-treated rats and (B) haloperidol-treated rats. All animals were given twice daily injections of 1% lactic acid (vehicle group) or haloperidol (2 mg/kg) for 7 days. SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; VTA, ventral tegmental area.

tor mRNA or hnRNA levels in the SNc or VTA (Figs. 5 and 6).

Other Dopamine Receptor mRNAs

In the rat striatum, the prominent known dopamine receptors are D1, D2, and D3. D5 receptor mRNA is not expressed in the striatum, but is found in other rat brain regions (43). D4 receptor mRNA is found in the rat brain at extremely low levels and, thus, has not been anatomically characterized (A. Mansour, unpublished observations). Therefore, to examine the question of dopamine receptor subtype changes, in addition to D2 receptor mRNA and hnRNA, striatal dopamine D1 and D3

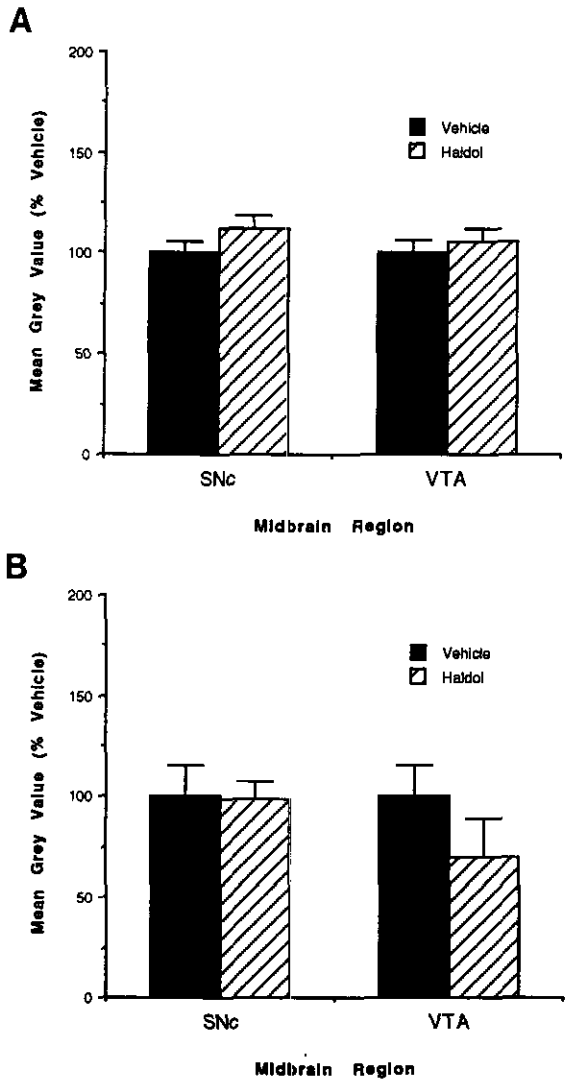


FIG. 6. Two bar graphs showing the relative amounts of D2 mRNA (A) and D2 hnRNA (B) in the substantia nigra, pars compacta, and ventral tegmental area of vehicle-treated (black bars) and haloperidol-treated rats (hatched bars). Note all values are expressed as a percentage of vehicle-treated rats with all of the vehicle group values set at 100. SNc, substantia nigra, pars compacta; VTA, ventral tegmental area.

receptor mRNA pools were measured using *in situ* hybridization. D1 receptor mRNA remained unchanged between vehicle and haloperidol rats (Figs. 1A, 1E, and 2A). Cells containing D3 mRNA were found mainly in the islands of Calleja (ICj) and the Acb. Haloperidol treatment did not induce a change in the level D3 receptor mRNA in the ICj or Acb (Figs. 1D, 1H, and 2D).

Opiate Peptide mRNAs

Since striatal D2 mRNA generally colocalizes with proenkephalin mRNA, and D1 mRNA with dynorphin mRNA, we examined the levels of these neuropeptide mRNAs after haloperidol treatment. Haloperidol-treated rats had higher levels of proenkephalin mRNA than vehicle-treated rats over all three areas of the striatum that were analyzed (Figs. 7B and 7D). A 45% difference was observed between vehicle- and haloperidol-treated rats CPu ($P = 0.0001$; $F = 45.83$; Fig. 8B). Haloperidol-treated rats had 36% more proenkephalin mRNA in the Acb than vehicle rats ($P = 0.0003$; $F = 28.12$; Fig. 8B). The Tu contained 27% more proenkephalin mRNA in haloperidol-treated rats than in vehicle rats ($P = 0.0003$; $F = 29.24$; Fig. 8B). No differences in striatal prodynorphin mRNA were observed between vehicle- and haloperidol-treated rats (Figs. 7A, 7C, and 8A). These data suggest that the haloperidol treatment paradigm used in these studies was effective in selectively potentiating proenkephalin mRNA without altering D2 mRNA or hnRNA, presumably in the same striatal cells.

DISCUSSION

D2 Receptor Expression in the Striatum

Binding experiments in the rat indicate that after 7 days of haloperidol treatment the number of D2 receptors in the striatum is significantly increased (10, 52, 71, 80). Several groups have examined D2 mRNA expression after haloperidol treatment. The results of these studies are, at first glance, contradictory. Some investigators report no change in D2 receptor mRNA following haloperidol treatment (25, 64, 77, 82), while others report increases in D2 mRNA (3, 8, 13, 34, 54). In general, longer treatment schedules resulted in increases in D2 mRNA, while shorter time courses (less than 14 days) did not alter D2 mRNA levels.

The experiments in this study extend short term data by examining D2 hnRNA levels in addition to D2 mRNA levels in a 7-day haloperidol treatment paradigm. The finding that D2 hnRNA, like the mRNA pool, did not change after 7 days of haloperidol treatment indicates that the large D2 mRNA pool did not mask a small change in transcription or an alteration of mRNA turnover. There are several reports of hnRNA regulation demonstrated with intronic *in situ* hybridization. An intron A specific proopiomelanocortin (POMC) probe was utilized to show a rapid increase and then decrease in anterior pituitary cell POMC hnRNA in response to adrenalectomy followed by dexamethasone treatment (22). *In vivo* hnRNA analysis in the brain has revealed rapid increases in corticotropin-releasing hormone in the medial parvicellular paraventricular hypothalamic nucleus in response to acute blockade of glucocorticoid synthesis (28) and an increase in the amount of arginine vasopressin hnRNA in the supraoptic nucleus after salt loading (29). All of these reports indicate that regulatory changes in the hnRNAs occurred more rapidly than changes in the corresponding mRNA pools. Thus, it appears from the D2 hnRNA and mRNA data, taken together with the existing literature, that increased D2

gene activity is not a likely explanation for the increase in striatal D2 receptors following 7 days of haloperidol treatment.

D2 Receptor Expression in Striatal Afferent Structures

The D2 receptor binding sites in the striatum are not all postsynaptically localized on striatal neurons. Several other brain areas send axons to the striatum that contain D2 receptors on their presynaptic terminals (17–19, 21, 23, 27, 44, 48, 58, 62, 65, 66, 72, 73, 75). The dopaminergic neurons of the midbrain substantia nigra and ventral tegmental area contain D2 receptor mRNA, and lesion studies suggest that these cells have D2 receptors on their axon terminals in the striatum. Although these cells could be a potential source of D2 receptor changes in the striatum, no differences were observed between haloperidol and control rats in the substantia nigra or ventral tegmental area for D2 mRNA or hnRNA.

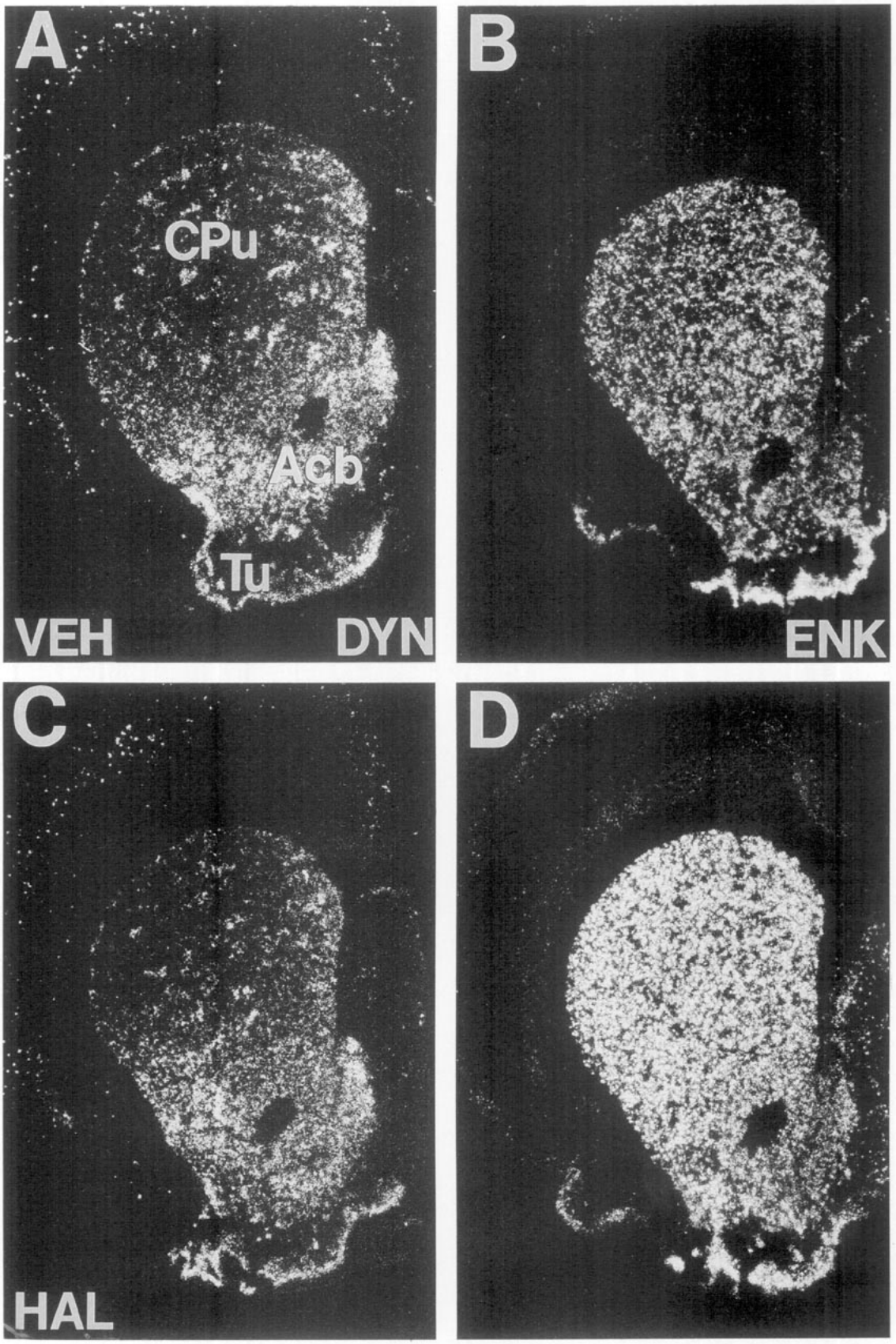
Cerebral cortical neurons also contain D2 mRNA. Although this D2 mRNA encodes receptors that remain in the cortex, it is likely that many of these D2 receptors are found in the corticostriatal axon terminals. D2 mRNA levels were measured in three broad areas of cortex that project to the striatum, the cingulate, insular, and neocortex. Haloperidol-treated rats were found to have significantly higher levels of D2 mRNA in their anterior cingulate cortex than the control rats. Unfortunately, D2 hnRNA levels could not be measured by *in situ* hybridization in any region of cerebral cortex because the level of D2 expression in the cortex is quite low leaving the level of D2 hnRNA below the limits of detection. No significant changes were observed in the insular or neocortex.

Although the anterior cingulate cortex contained more D2 mRNA in haloperidol-treated rats than in controls, it would not be reasonable to conclude that this increase is entirely responsible for the reported increase in D2 receptors in the striatum. First, the levels of D2 mRNA are very low in all regions of the cortex. This increase is a significant relative rise; however, the absolute change in mRNA is small and covers only a small region of cortex. In addition, the anterior cingulate cortex projects mainly to the nucleus accumbens and medial caudate putamen (61). Therefore, it would be difficult to envision anterior cingulate cortex projections being responsible for the broader changes in striatal D2 binding, but they could result in more compartmentalized changes that may occur in the nucleus accumbens or subregions of the caudate putamen.

It is interesting that haloperidol treatment resulted in changes in D2 mRNA levels in the anterior cingulate cortex, a subregion of the prefrontal cortex, given the recent attention that the human prefrontal cortex has received as a potentially important neural substrate in schizophrenia (4, 16, 78). The regions of cingulate cortex in which we observed an increase in D2 mRNA are part of an important corticoaccumbal projection (61). It is thought that a deficit in dopaminergic innervation of this pathway in humans results in enhanced striatal dopaminergic neurotransmission and schizophrenic behavior. Antipsychotics are known to induce the immediate early gene product, Fos protein, in this region of cingulate cortex, possibly in corticoaccumbal projection neurons (16, 53). Taken together these data indicate that this corticoaccumbens pathway may play an important role in the mediation of schizophrenic behavior by antipsychotics. Thus, understanding the biochemical responses of this pathway to these drugs may be important to understanding schizophrenia. Our data indicate that D2 mRNA levels are increased in the anterior cingulate cortex after 7 days of haloperidol treatment. These results support data indicating that chronic haloperidol treatment results in increased D2 receptor binding in the medial prefrontal cortex (37). On the other hand, another report has failed to detect a change in D2 binding after haloperidol. Liskowsky and Potter (36) examined D2 receptor binding in dissected frontal cortex after a 21-day haloperidol treatment paradigm. The failure of this study to detect an increase in binding may be due to the dissection method used to isolate cortical tissue. Given the D2 receptor mRNA increases only in a small region of frontal cortex, it is possible that any increases in D2 receptor binding in the cortex itself would be diluted by analyzing all of frontal cortex. Furthermore, if the D2 mRNA in the cortex encodes presynaptic receptors, one would expect a cortical contribution to striatal D2 receptor binding, a region where the authors did observe a significant increase in D2 binding. It is clear that careful subcompartmental analysis of the binding changes in the striatum is necessary to pursue the hypothesis of D2 receptor changes in specific corticostriatal pathways.

Other Dopamine Receptors

In addition to D2 receptor mRNA, D1 and D3 receptor mRNA levels were measured by *in situ* hybridization, to address the possibility that a dopamine receptor other than D2 might be responsible for the binding increases reported in the striatum. Since haloperidol does not bind to D1 receptors with high affinity, we did not expect changes in the D1 receptor mRNA pool. In fact, no changes in D1 mRNA levels were observed. However, the D3 receptor is a member of the D2 family of receptors and potentially could interact with haloperidol, especially at the doses used in most chronic haloperidol studies. Since classical D2 ligands like spiperone and raclopride do not differentiate between D2 and D3 receptor binding (35, 63), increases in D3 receptors could be misinterpreted as a D2 increase using these ligands in binding studies. Unfortunately, to date, no haloperidol studies have been performed using ligands


A
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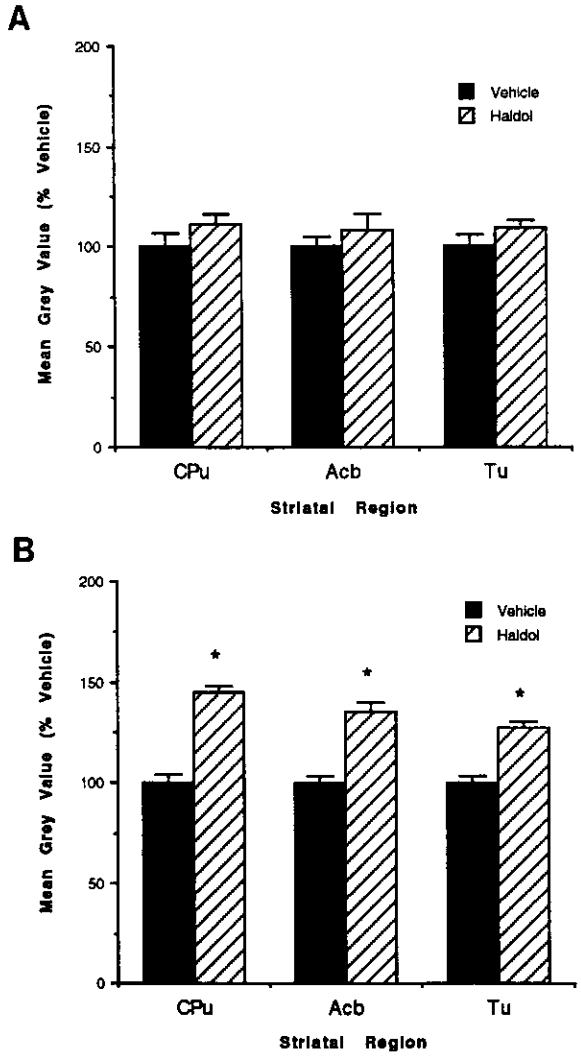

FIG. 8. Two bar graphs showing relative amounts of prodynorphin mRNA (A) and proenkephalin mRNA (B) in striatal subregions of vehicle-treated (black bars) and haloperidol-treated rats (hatched bars). Note all values are expressed as a percentage of vehicle-treated rats with all of the vehicle group values set at 100. Asterisks indicate statistically significant differences ($P < 0.05$).

like 7-hydroxy-DPAT that have higher specificity for the D3 receptor. In this study, we examined the D3 mRNA pool in the striatum by *in situ* hybridization. Since *in situ* hybridization unambiguously differentiates D2 from D3 receptor mRNAs, it allows evaluation of the nature of the dopamine receptor changes after haloperidol treatment. D3 mRNA levels were measured in the nucleus accumbens and islands of Calleja. No changes were observed after 7 days of haloperidol treatment in either region. Therefore, it seems unlikely that changes observed in D2 binding in the striatum can be explained by an increase in D3 receptors. Previous data would also predict this result, given the differing anatomical distributions of the D2 and D3 or D4 receptors in the striatum. D3 and D4 receptors are not typically found in striatal regions that show increases in dopamine receptor binding following haloperidol treatment (7, 41, 51, 63). However, haloperidol, especially at higher doses, may have actions at the D3 and D4 receptors, as well as the D2 receptor.

D2 Receptor Binding Changes with no mRNA Response

In order to explain an increase in D2 receptor B_{max} in the absence of an increase in mRNA, hnRNA, or changes in striatal afferent or related receptor mRNAs, one must envision either an increase in receptor protein translation or a decrease in receptor degradation. Experiments evaluating the effect of haloperidol on D2 receptor synthesis and stability have been performed using the irreversible antagonist *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroqinoline (EEDQ). The results of this study indicate that haloperidol decreases both the production and the degradation of dopamine D2 receptors. However, the rate of degradation was reduced more than the rate of synthesis, resulting in a higher steady state level of receptors (45). One would have to conclude based on the data from these studies, taken together with the EEDQ data, that D2 receptor stability must play an important role in the increase in striatal dopamine D2 receptor binding after short-term haloperidol treatment.

It is possible that D2 receptor stability is modulated through phosphorylation of specific amino acids in the receptor sequence. The beta adrenergic receptor which has served as a model for seven transmembrane receptor regulation, is phosphorylated by a specific receptor kinase referred to as beta adrenergic receptor kinase (BARK). The preferred substrate for this kinase is the agonist occupied receptor. When the receptor is agonist bound it becomes phosphorylated by BARK as well as protein kinase A and can then be down regulated by the cell (for review see 32). Since the agonist bound receptor is the preferred kinase substrate, antagonists protect the receptor from phosphorylation. Although these mechanisms are unknown for the D2 receptor, if the

FIG. 7. Darkfield autoradiograms from *in situ* hybridizations of prodynorphin mRNA (A and C) and proenkephalin mRNA (B and D) in coronal sections through the midstriatum. A and B are images made from vehicle-treated rats and C and D are images made from haloperidol-treated rats. All animals were given twice daily injections of 1% lactic acid (vehicle group) or haloperidol (2 mg/kg) for 7 days. No difference was observed in prodynorphin mRNA levels between vehicle- and haloperidol-treated rats; however, a higher level of proenkephalin mRNA was observed in the haloperidol group than in vehicle-treated rats. CPu, caudate-putamen; Acb, nucleus accumbens; Tu, olfactory tubercle.

beta adrenergic model applies to D2, haloperidol's antagonism of the receptor may lead to an increase in its stability by protecting it from receptor specific phosphorylation. Further studies into the mechanism of dopamine receptor regulation may shed light on haloperidol's paradoxical effect on the receptor and mRNA pool.

Opioid Peptide mRNA Expression

Finally, we have examined the effect of haloperidol treatment on mRNAs that encode two opiate peptides, dynorphin and enkephalin. Anatomical experiments in the striatum have shown that in general, D1 receptors are found on dynorphinergic neurons and D2 receptors are found on enkephalinergic neurons (24, 34). Although, recent functional studies indicate that dopamine receptors may colocalize in striatal neurons more often that previously thought (69), it is not surprising that enkephalin peptides have been shown to increase with haloperidol treatment, since both anatomical and functional data agree D2 receptors reside on enkephalinergic striatal cells. In this 7-day haloperidol paradigm, prodynorphin mRNA did not differ between haloperidol and control rats; however, enkephalin increased significantly. These data would indicate that antagonism of the D2 receptor changes the enkephalinergic tone in the striatum. Many of these enkephalinergic circuits are local; however, tracing studies indicate that the globus pallidus is a major target of enkephalinergic efferents of the striatum and dynorphinergic striatal outputs tend to target the substantia nigra (24). Thus, by altering the enkephalinergic tone in this system, haloperidol may change the flow of information through the striatopallidal pathway.

Summary

These studies indicate that 7-day haloperidol treatment, a paradigm that is reported to increase D2 receptor binding in the striatum, did not change the level of striatal D2 gene activity as measured by D2 hnRNA and mRNA levels in the striatum or in the midbrain dopaminergic neurons. Animals treated with haloperidol did have increased D2 mRNA in the anterior cingulate cortex, indicating that cells in this region respond more readily than striatal neurons. Cells in the cerebral cortex have small D2 mRNA pools and may be more likely to respond with transcriptional changes; however, the cingulate cortex was the only cortical region to respond to haloperidol treatment in this study. What makes its responsiveness unique from other cortical regions is not understood. In reference to corticostriatal circuitry, it seems unlikely that this small region of the prefrontal cortex could be responsible for the increases in D2 receptor protein in the striatum. Experiments in other laboratories indicate that haloperidol can increase the half-life of D2 receptors in striatal cells (45), which may be responsible for the observed increases in binding, at least in the initial phases of haloperidol treatment. It is clear from time course studies that there are no changes in D2 hnRNA or mRNA preceding the 7-day time point (20); however, data from other groups indicate that longer exposure to haloperidol results in an increase in D2 receptor mRNA. Perhaps with long-term haloperidol treatment (21 days or more), the changes observed in D2 receptor binding reflect an increase in D2 gene expression, indicating more complicated regulation of D2 than was first imagined.

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