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Differential expression of autoreceptors in the ascending dopamine systems of the human brain

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ABSTRACT **The tone and regulation of the brain dopaminergic projections are, in part, determined by the presence or absence of dopamine (DA) autoreceptors: rate of DA synthesis and turnover, as well as both pattern and rate of neuronal firing, are modulated by the expression and activity of these autoreceptors. The expression of dopaminergic receptors in the midbrain DA cell groups, presumably reflecting DA autoreceptors, was determined in the brains of the rat, Old World monkey, and human. In the rat, both the substantia nigra (A9) and the ventral tegmental area (A10) appear to express DA autoreceptors. In the monkey and human, however, only the projections arising from the substantia nigra express these receptors; the limbic projections originating in the ventral tegmental area lack this substrate for DA autoregulation. These results indicate that in the human, the nigrostriatal and mesocorticolimbic dopamine systems may be differentially autoregulated.**

The regulation of neurotransmitter synthesis and release is of central importance to the understanding of brain function. Dysregulation of these processes is likely involved in a number of neuropsychiatric conditions, and many psychotropic drugs exert their effects at this level of cellular physiology. One aspect of neurotransmitter regulation that has been appreciated is that a given transmitter can potentially regulate itself. This autoregulation is generally believed to be mediated via receptors for a given transmitter that are located on the cell synthesizing and releasing that transmitter. These so-called autoreceptors are presynaptically located and provide a "short-loop" level of regulatory feedback for a cell (1–7). This feedback tends to be inhibitory; thus the autoreceptor functions to maintain a set level of neurotransmitter synthesis, cell firing, or release (8–12).

Some dopamine (DA)-synthesizing cells synthesize autoreceptors (13, 14). The presence and functioning of DA autoreceptors are central to our understanding of certain aspects of the regulation of the brain DA systems. In those cells with functioning DA autoreceptors, DA appears to have a lower rate of synthesis and turnover, and these neurons manifest a lower rate and intensity of cellular firing, compared with cells without autoreceptors. Autoreceptor-expressing cells appear to have higher levels of responsiveness to various dopaminergic compounds. Interestingly, cells that lack DA autoreceptors appear to be affected by chronic DA antagonists (i.e., antipsychotic medications) differently than autoreceptor-expressing cells: those cells that lack autoreceptors do not develop either tolerance or depolarization inactivation after chronic antagonist treatment (15–20). DA autoreceptors can then be considered as one cellular mechanism for the modulation of the tone of a given system. Although extensively studied in lower animals, most work has concentrated on the rat. It has been assumed, however, that the human also expresses DA autoreceptors in brain (21, 22).

The two major ascending central nervous system DA projections originate in the midbrain (23). The nigrostriatal system originates from the substantia nigra (SN, cell group A9) and projects primarily to the basal ganglia, generally subserving motor functions. The mesocorticolimbic system arises from the DA-synthesizing cells in the ventral tegmental area (VTA, cell group A10) and sends axons to a number of rostral cortical and limbic sites. The mesocorticolimbic DA system is involved in the modulation of emotional states and other limbic functions. DA autoreceptors have been demonstrated and most thoroughly characterized in the rat nigrostriatal system, although they also exist in the rat mesocorticolimbic circuit (9, 10, 24–27). The autoreceptor appears to be exclusively a D_2-like DA receptor (28).

Most studies on the existence and nature of autoreceptors have used pharmacological and electrophysiological strategies, which have been possible to perform only in animal experiments. With the recent cloning of various DA system markers, however, a more direct anatomical approach is possible. *In situ* hybridization allows the determination of the distribution and concentration of specific mRNA molecules in an anatomical context. Although the peptide or protein that is the final product of translation may be located in many different regions of a given cell, the mRNA encoding it is restricted to the region of the cell body. In the case of receptor studies, this phenomenon is usefully exploited to determine their cells of origin. In the specific case of the DA systems, a given cell that normally synthesizes DA will require the expression of the mRNA and the final protein for tyrosine hydroxylase (TH), the key regulatory enzyme in the synthesis of DA. Thus, in the midbrain, the presence of TH mRNA serves as a marker for a DA-synthesizing cell. If a DA-synthesizing cell also expresses autoreceptors, these, too, are encoded by mRNA in the same cell because these autoreceptors are presynaptic. The development of these tools allows the question of DA autoreceptors to be directly addressed in the human brain by using postmortem tissue samples. We thus undertook this study to examine the nature of dopaminergic autoreceptors in the projections originating in the human midbrain.

METHODS

Tissue Preparation. Brain tissue was obtained from rats, monkeys, and humans for this study. Male Sprague–Dawley rats were sacrificed by decapitation. The brains were removed and frozen in isopentane (−30°C) for 30 sec. The brains of five Old World monkeys (both *Macaca mulatta* and *Macaca nemestrina*) were obtained from the Regional Primate Research Center at the University of Washington. These animals had been sacrificed as part of protocols

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Abbreviations: DA, dopamine; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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unrelated to this investigation that did not require study of the brain. Human brain samples from 23 individuals were obtained at autopsy, and a block of tissue containing the midbrain was excised and rapidly frozen on dry ice. Postmortem intervals of these samples ranged from 5 to 20 hr.

All tissue samples from all three species were maintained at −80°C until the time of processing. Tissue blocks were warmed to −20°C and cryostat sectioned (20 μm). Sections were thaw-mounted onto subbed microscope slides and stored at −80°C until processed for *in situ* hybridization.

***In Situ* Hybridization.** *In situ* hybridization was done in rat, monkey, and human brain with uridine 5′-[α-[^{35}S]thio]triphosphate-labeled RNA probes as we have described (29–32). The rat D_2 RNA probe was a 495-base probe, corresponding to the third intracytosolic loop and transmembrane domains VI and VII of the rat D_2 receptor (29, 30). This probe equally recognizes both the short and long isoforms of D_2 receptor mRNA. Rat D_3 and D_4 mRNAs were visualized by using 326- and 447-base RNA probes, respectively. Rat TH mRNA was visualized with an RNA probe generated from a 300-nt cDNA for TH cloned by PCR from a rat adrenal library.

For both monkey and human hybridizations, probes complemetary to human sequences were used. D_2 receptor mRNA was visualized with an RNA probe of 446 nt. This probe also equally labels both the short and long isoforms of D_2 receptor mRNA. D_3 and D_4 probes were 536 and 420 nt long, and TH mRNA was labeled with a 547-base probe.

To insure specificity of labeling, adjacent tissue sections were labeled with "sense"-strand controls, and other sections were pretreated with RNase A for all eight of the probes used (29–32). Specific hybridization was seen only in the "antisense"-labeled condition.

RESULTS AND DISCUSSION

We first examined the midbrain DA nuclei in the rat (Fig. 1). Using serial cryostat sections of brain at the level of the midbrain, alternate sections were subjected to *in situ* hybridization for either TH mRNA or D_2 receptor mRNA. TH mRNA was seen throughout the extent of the DA nuclei and was expressed at similar levels in both the SN and VTA. When adjacent sections were examined for the presence of D_2 receptor mRNA, a similar pattern was observed. Very high levels of D_2 receptor mRNA were seen in both the SN and VTA, as has been demonstrated in a number of reports (29, 30, 33–35). The ratio of the expression of TH mRNA to D_2 receptor mRNA in these two structures was also identical.

The VTA in the human brain is morphologically different from the corresponding structure in the rodent. In the human, DA-synthesizing cells are clustered more medially than seen in the rat but also extend more dorsally. A similar study was done in human brain tissue. Serial sections of midbrain tissue were prepared at the level of the red nucleus. *In situ* hybridization for TH mRNA revealed an extensive pattern of DA-synthesizing cells throughout the midbrain, consistent with previous reports (36) of the distribution of TH immunostaining in these areas. The SN was densely labeled for TH mRNA, although discrete cell clusters were clearly visible in the VTA, as well as in the retrorubral fields (A8). The nucleus

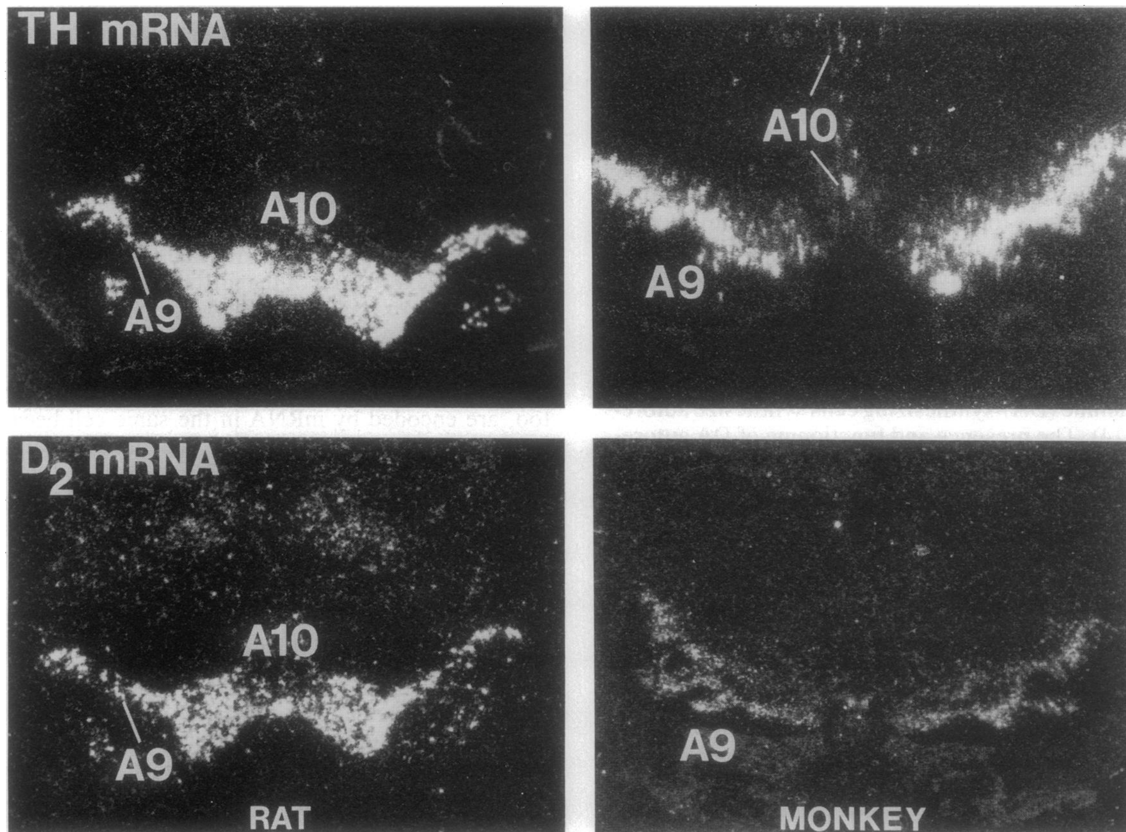

FIG. 1. *In situ* hybridization for TH and DA D_2 receptor mRNAs in the rat (*Left*) and Old World monkey (*Right*) midbrain. Serial, 20-μm sections of fresh frozen brain tissue were prepared for *in situ* hybridization and were hybridized with ^{35}S-labeled RNA probes. Labeled tissue sections were exposed to photographic film for 1–2 weeks. In the rat, abundant expression of TH mRNA is seen in both the SN (A9) and the VTA (A10). The distribution of D_2 receptor mRNA is identical to that of TH mRNA, with abundant expression in both A9 and A10. In the monkey, extensive labeling of A9 is seen for TH mRNA, and a lower density of labeling is seen in A10. D_2 receptor mRNA, however, is only seen in A9. In the rat, both A9 and A10 encode D_2 receptors serving as autoreceptors; in the monkey, however, autoreceptors are only encoded in A9 and are not encoded in A10.

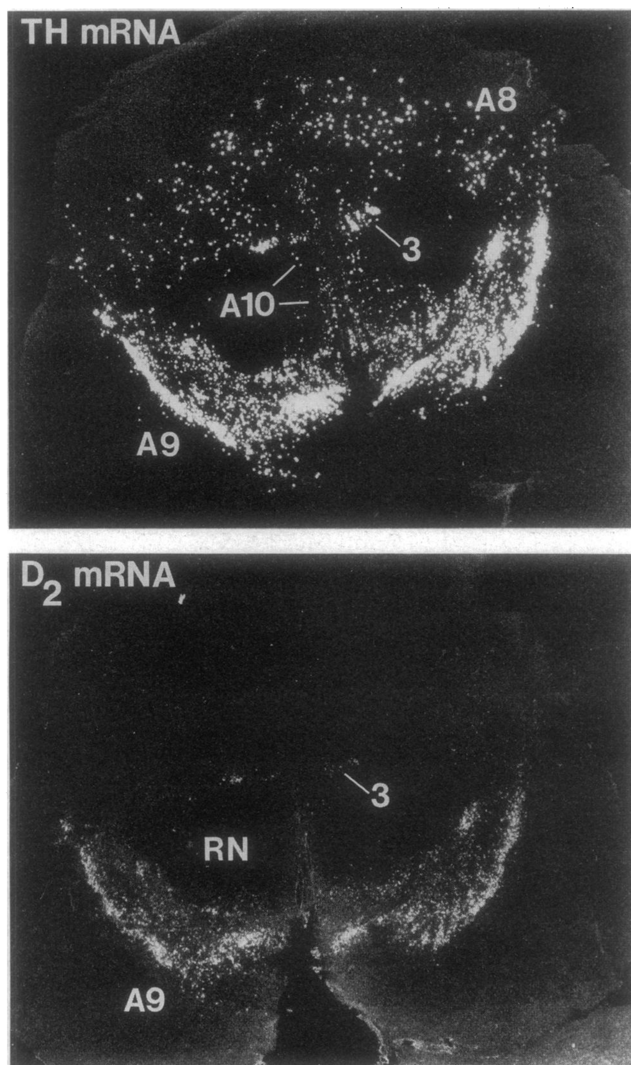

FIG. 2. *In situ* hybridization for TH and D_2 receptor mRNA in the human midbrain. (*Upper*) Twenty-micron sections were prepared from frozen, unfixed human brain tissue at the level of the red nucleus (RN), which can be seen as an unlabeled circular area. TH mRNA-positive cell clusters are apparent in the retrorubral fields (A8), as well as in A9 and A10. In addition, the oculomotor nucleus ("3") also expresses TH mRNA. (*Lower*) D_2 receptor mRNA can only be seen in A9 and in the oculomotor nucleus but cannot be seen in A8 and in A10. As in the case of the Old World monkey, it appears that in the human brain, DA autoreceptors are encoded by the SN but not by DA-synthesizing cells in the VTA or in the retrorubral area.

of the oculomotor nerve was also labeled; this nucleus has been previously shown to contain TH (36). In sharp contrast to observations in the rat, D_2 receptor mRNA was restricted in the human midbrain to those TH-positive cells in the SN as well as the oculomotor nucleus[†]. No significant expression of D_2 receptor mRNA was detected in either the VTA or the retrorubral fields (Figs. 2 and 3).

The most likely interpretation of the discrepancy of the distributions between TH and D_2 receptor mRNAs is that the SN in the human encodes DA receptors that subserve an autoreceptor function, whereas the VTA and retrorubral fields do not. Although the differences in neurochemical anatomy of these markers is striking, subsequent work will need to demonstrate on a cell-by-cell basis that, in fact, TH and D_2 receptor mRNAs are colocalized on a cellular level in addition to our demonstration of regional codistribution.

We investigated several other possibilities that might explain this discrepancy in the distributions of TH and D_2 receptor mRNAs. The images shown in the figures in this report were exposed to emulsion for 1–2 weeks; it is possible that the VTA and retrorubral fields express D_2 receptors but express them at lower levels than seen in the SN. These tissue sections were re-exposed to film for up to 3 mo. Despite this lengthy exposure time (sufficient to cause emulsion saturation of both the TH and D_2 receptor mRNA signals that had been seen with earlier exposure times), no D_2 receptor mRNA was detectable in the VTA or in the retrorubral fields. Based on these exposure times and the half-life of the radionucleotide used in the study, if D_2 receptor mRNA is expressed in either A8 or A10 in the human, it is present at levels at least two orders of magnitude lower than that seen in the SN.[‡]

Another possibility for the discrepancy seen in the distributions of TH and D_2 receptor mRNA could be that D_2 receptor mRNA is much more stable in the SN than in A8 or A10. A potential explanation for such a phenomenon might be some type of differential degradation of one or the other mRNA in one of these nuclei but not all of them. This possibility is unlikely, but given fairly lengthy postmortem intervals involved in the collection of human brain samples, it is a concern that should be addressed. To examine this possibility further, the same study was performed in the Old World monkey (Fig. 1). Serial sections of monkey brain tissue were prepared as the human samples had been handled, but postmortem intervals were considerably shorter and on the order of those for the rat. The anatomy of the DA cell groups in the Old World monkey midbrain is similar to that of the human. The same pattern of TH and D_2 receptor mRNA observed in the human was seen in monkey brain; although the SN appears to encode substantial levels of D_2 receptors, the VTA does not encode these receptors.

A final possibility considered was that the VTA and retrorubral fields were, in fact, expressing receptors capable of serving as autoreceptors, but they were not specifically D_2 DA receptors. Although the DA autoreceptor has been determined to be D_2-like, the recent cloning of at least five distinct DA receptors has added considerable complexity to the understanding of DA anatomy, pharmacology, and physiology (38–50). The DA receptors are now appreciated to cluster into two families of receptors, D_1-like and D_2-like. The D_1 and D_5 receptors compose the D_1-like receptors. The D_2-like receptors consist of D_2, D_3, and D_4. The D_3 and D_4 receptors are in many ways pharmacologically similar to the D_2 receptor, although there are some differences; the most notable may be that the D_4 receptor has a particularly high affinity for the atypical neuroleptic clozapine (39). These three receptors also differ considerably in their anatomical distributions: while the D_2 receptor is expressed in most regions of the brain associated with DA function, the D_3 and D_4 receptors are distributed in a more restricted fashion, with expression primarily in the limbic system (38, 47, 51). Given the D_2-like pharmacology and the primarily limbic distribution of the D_3 and D_4 receptors, a distinct possibility is that

[†]The oculomotor nucleus has not been associated with dopaminergic neurotransmission, although TH has been previously demonstrated in this structure (36), and both D_2 receptor mRNA (31) and binding sites (37) have been identified in this nucleus. Given that DA itself has not been found in this structure, the TH is likely related to adrenergic synthesis, and the D_2 receptors are probably postsynaptic.

[‡]This estimate assumes that a positive signal for D_2 receptor mRNA in the VTA could be detected if it were present at 10% of the corresponding level in the SN, which is conservative. An exposure time 13 times longer (i.e., 1 week vs. 3 mo) results in exposure to film of ≈10 times as much radiation (due to the half-life of ^{35}S of 87 days). Accordingly, the difference between the amount of D_2 receptor mRNA actually seen in the SN and what might be present in the VTA is at least 100-fold.

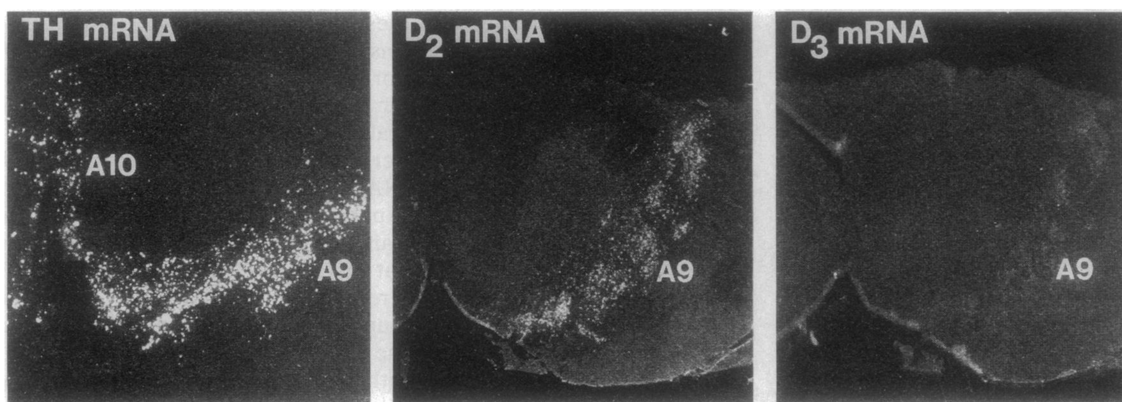

FIG. 3. *In situ* hybridization for TH, D_2, and D_3 receptor mRNA in the human midbrain. At this level of the midbrain, TH mRNA can be seen in both A9 and A10. In adjacent sections, D_2 receptor mRNA can be seen restricted to A9. D_3 receptor mRNA is also present in A9 but not in A10; however, the levels of expression are considerably lower than seen for D_2 receptor mRNA. No D_4 receptor mRNA was seen in these regions in the human brain.

the VTA is encoding DA autoreceptors, but they may be D_3 or D_4, and not D_2.

To examine this possibility, serial sections of rat, monkey, and human midbrain were examined for TH, D_2, D_3, and D_4 receptor mRNAs by *in situ* hybridization. D_3 receptor mRNA was seen in the SN of the human but appeared to be primarily restricted to the middle and lateral aspects of this nucleus (Fig. 3). The level of D_3 receptor mRNA was considerably lower than that of D_2; at most, the expression of D_3 receptor mRNA was 20% of the level of D_2 receptor mRNA. No D_3 receptor mRNA was seen in the SN of the monkey or rat or in the VTA in any of the three species studied. D_4 receptor mRNA could not be convincingly demonstrated in the midbrain of any of these species. Thus, it appears that the SN of the human expresses DA receptors that likely encode autoreceptors, which are primarily D_2, although a minor subset are D_3. The VTA and retrorubral area, however, although synthesizing DA, do not express D_2 (or D_3 or D_4) receptors serving an autoreceptor function.

Classically, the SN was viewed as giving rise to the nigrostriatal system, subserving motor functions, and the VTA projecting primarily to limbic structures. In general, this functional segregation continues to be the view of how these systems are organized, but recent work has revealed overlap in these projections (52–56). Most VTA fibers probably terminate in limbic regions in the monkey and human, but some likely have motor targets. Similarly, some of the nigral projections probably have limbic targets. The current results should not be construed to indicate that all limbic regions in the human and monkey brain lack DA autoreceptors; rather, those projections arising from A8 and A10 lack autoreceptors.

The expression of DA autoreceptors in the systems originating in the SN but not the VTA has significant impact on our understanding of brain DA function and dysregulation. Specifically, the presence of limbic autoreceptors encoded in the VTA has been invoked in several theories of DA dysregulation in schizophrenia. Further, several treatment strategies for schizophrenia have evolved, with specific therapeutic targeting of the presynaptic autoreceptor.

Schizophrenia has been viewed as a disorder of excess DA, especially in the limbic DA circuits (57–59). Although the mainstay of pharmacological treatment of schizophrenia and other psychotic states has been the blockade of postsynaptic D_2-like receptors, more recent attempts have used low doses of agonists at the (presumed) autoreceptor (60). The rationale is to stimulate the autoreceptor and thereby decrease the presynaptic synthesis and/or release of DA in these patients, thus effectively lowering dopaminergic tone. This strategy has been based on the belief that the mesocorticolimbic DA system expresses DA autoreceptors in the human, by analogy to the rat. It appears that this strategy may have been in error, given that the human appears not to express these autoreceptors in limbic structures. This finding helps to explain the actual observation of the lack of efficacy of low-dose DA agonist treatment of psychosis (61).

The relative lack of DA autoreceptors in the limbic, but not in the motor, systems of the human brain will necessitate a reconsideration of how some psychopathological states occur and how select psychotropic medications affect brain DA systems. For example, given that schizophrenia typically does not involve severe motor manifestations in the face of psychosis, it appears that any excess DA tone in the brain is likely restricted to the limbic system and is not a general, brain-wide effect. How a limbic-specific alteration in DA neurotransmission arises is not understood, but the lack of autoreceptors in the VTA, but not in the SN, suggests that this may be one mechanism for the differential regulation of DA systems in these functional circuits. At the simplest level, the limbic DA projections appear to lack this fundamental ability to self-regulate that the motor system possesses. This inability to effect short-loop feedback may well be a substrate for dopaminergic dysregulation seen in various neuropsychiatric conditions in which DA has been implicated.

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