

Dopaminergic Abnormalities in Select Thalamic Nuclei in Schizophrenia: Involvement of the Intracellular Signal Integrating Proteins Calcyon and Spinophilin

Sarah M. Clinton, Ph.D.

Hisham M. Ibrahim, M.D.

Kirk A. Frey, M.D., Ph.D.

Kenneth L. Davis, M.D.

Vahram Haroutunian, Ph.D.

James H. Meador-Woodruff, M.D.

Objective: While both thalamic abnormalities and dopaminergic dysregulation have been separately implicated in the pathophysiology of schizophrenia, little is known about the possible dysfunction of molecules associated with dopaminergic neurotransmission in the thalamus in this illness. In this study, the authors studied this question by measuring in postmortem brain the expression of molecules associated with dopaminergic neurotransmission.

Method: Using in situ hybridization and receptor autoradiography, the authors determined in schizophrenia and comparison subjects 1) thalamic expression of the transcripts encoding the five dopamine receptors; 2) binding to the dopamine D₁, D₂, and D₃ receptors; 3) monoaminergic innervation as assessed by binding to the vesicular monoamine transporter; and 4) tran-

scripts encoding three dopamine receptor-associated intracellular proteins (calcyon, spinophilin, and DARPP-32) that mediate integration of dopaminergic signaling with other neurotransmitter systems.

Results: Both calcyon and spinophilin transcripts were significantly elevated in schizophrenia subjects. Monoaminergic innervation, as well as dopamine receptor transcripts and binding sites, were unaffected in this illness.

Conclusions: These data indicate that there are dopaminergic abnormalities in the thalamus in schizophrenia but that they are at the level of intracellular integration of dopamine signaling with other neurotransmitter systems, likely including glutamate, in thalamic neurons.

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The dopamine hypothesis of schizophrenia is based largely on pharmacological evidence, including the observations that dopaminergic agonists can induce psychosis in healthy subjects or exacerbate psychotic symptoms in schizophrenia, and that D₂-like receptor antagonists effectively reduce psychotic symptoms. Subsequently, numerous investigators have examined myriad aspects of the dopamine system in schizophrenia (1–3). Dopamine is packaged into secretory vesicles by the vesicular monoamine transporter (VMAT) and released into the synaptic cleft, where it can activate one of five receptor subtypes (D₁–D₅). Dopamine receptors are classified as either “D₁-like” (D₁ and D₅) or “D₂-like” (D₂, D₃, and D₄) on the basis of their molecular structure and pharmacology; they couple to specific G proteins to regulate second messenger molecules (4).

D₁-like receptors primarily couple to G_s proteins to stimulate cAMP formation (5), but this also leads to increased levels of inositol phosphates (6) and intracellular calcium (7). One of the D₁ receptor-linked cAMP signaling pathways involves cAMP-dependent protein kinase, which phosphorylates DARPP-32 (dopamine- and cAMP-regulated phosphoprotein), a potent inhibitor of protein phosphatase-1 (8). The DARPP-32/protein phosphatase-1 cascade controls the phosphorylation states of myriad

downstream effectors and therefore exerts a powerful effect on neuronal function (8). Another recently identified transmembrane protein, calcyon, interacts with the D₁ receptor and mediates intracellular calcium mobilization, permitting “crosstalk” between expressed D₁ receptors and G_{q/11}-protein-coupled receptors such as metabotropic glutamate receptors and certain muscarinic cholinergic receptors (9).

D₂-like receptors couple to either G_i or G_o proteins to inhibit adenylyl cyclase and decrease cAMP production (4), modulate intracellular calcium levels and arachidonic acid release, and increase outward potassium currents (10). Yeast two-hybrid techniques have shown that the D₂ receptor binds to the protein spinophilin, which is enriched in dendritic spines and interacts with actin and protein phosphatase-1 (11). This D₂/spinophilin interaction may be important for building an intracellular signaling complex that modulates interactions between D₂ and neighboring receptors, especially the AMPA subtype of glutamate receptor, as well as their downstream effectors (11, 12). While most studies of dopaminergic abnormalities in schizophrenia have focused on the dopamine receptors, there has been recent interest in potential abnormalities of some of the intracellular proteins associated with the transduction of dopamine-mediated signaling (13, 14).

TABLE 1. Postmortem Characteristics of Schizophrenia and Comparison Subjects

Group	Gender	Age (years)	Postmortem Interval (minutes)	pH	Laterality	Cause of Death
Comparison subjects ^a						
1	F	86	280	6.5	Left	Unknown
2	M	70	482	6.0	Left	Lower gastrointestinal bleed
3	M	55	600	5.7	Left	Lymphoma
4	F	96	195	6.7	Left	Cardiopulmonary failure
5	F	90	250	6.0	Left	Cardiopulmonary failure
6	F	74	180	6.0	Right	Cardiopulmonary failure
7	F	82	230	5.8	Left	Cardiopulmonary failure
8	F	64	1,145	6.1	Left	Cardiopulmonary failure
Schizophrenia subjects ^b						
1	F	86	416	6.2	Right	Cardiac failure, pneumonia
2	M	61	212	6.5	Left	Cardiac failure
3	M	69	270	6.4	Right	Myocardial infarction
4	M	72	1,235	6.6	Left	Cardiopulmonary failure
5	M	63	372	5.9	Left	Cardiopulmonary failure
6	F	69	820	6.2	Left	Cardiopulmonary failure
7	M	68	335	6.8	Left	Cardiopulmonary failure
8	F	64	392	6.6	Left	Cardiopulmonary failure
9	M	61	169	6.4	Left	Cardiac failure
10	F	73	625	6.3	Right	Cardiopulmonary failure
11	F	79	1,225	7.1	Left	Cardiac failure
12	M	66	725	6.5	Right	Cardiac failure
13	F	76	1,270	6.1	Left	Cardiogenic shock

^a Mean age was 77.1 years (range=55–96); mean postmortem interval was 420 minutes (range=180–1145); mean pH was 6.1 (range=5.9–7.1).

^b Mean age was 69.8 years (range=61–86); mean postmortem interval was 620.5 minutes (range=169–1220); mean pH was 6.4 (range=5.7–6.7).

Superimposed on dopaminergic dysregulation in schizophrenia is the neuroanatomical circuitry that underlies this illness. While earlier hypotheses viewed schizophrenia as a disorder involving discrete brain regions, recent models often conceptualize schizophrenia as a disorder of the functional integrity of distributed circuitry (15, 16). The predominant neuroanatomical structures hypothesized to be abnormal in schizophrenia include the prefrontal cortex and other cortical and subcortical limbic regions. A critical neuroanatomical structure that links all these areas is the thalamus. The thalamus consists of discrete, topographically organized nuclei with reciprocal projections to and from limbic, sensory, and motor regions of the cortex. Further, much information reaching the cortex from subcortical areas converges on the thalamus before being distributed to cortical regions. While earlier views tended to relegate thalamic function to a simple relay station, thalamic nuclei play a pivotal role in gating and processing information sent to the cerebral cortex. Fibers from brain regions implicated in schizophrenia, including the prefrontal and cingulate cortices, hippocampus, and amygdala, terminate in several nuclei of the dorsal thalamus (e.g., dorsomedial, anterior, lateral dorsal, and central medial nuclei [17]). The fundamental plan of thalamic circuitry is straightforward: afferent and efferent neurotransmission of all dorsal thalamic nuclei is mediated by glutamate and, in turn, regulated by inhibitory GABAergic effects exerted by neurons of the reticular nucleus and by intrinsic interneurons of the thalamus. Monoaminergic and cholinergic afferents modulate neurotransmission in both the dorsal thalamus and reticular nucleus (17).

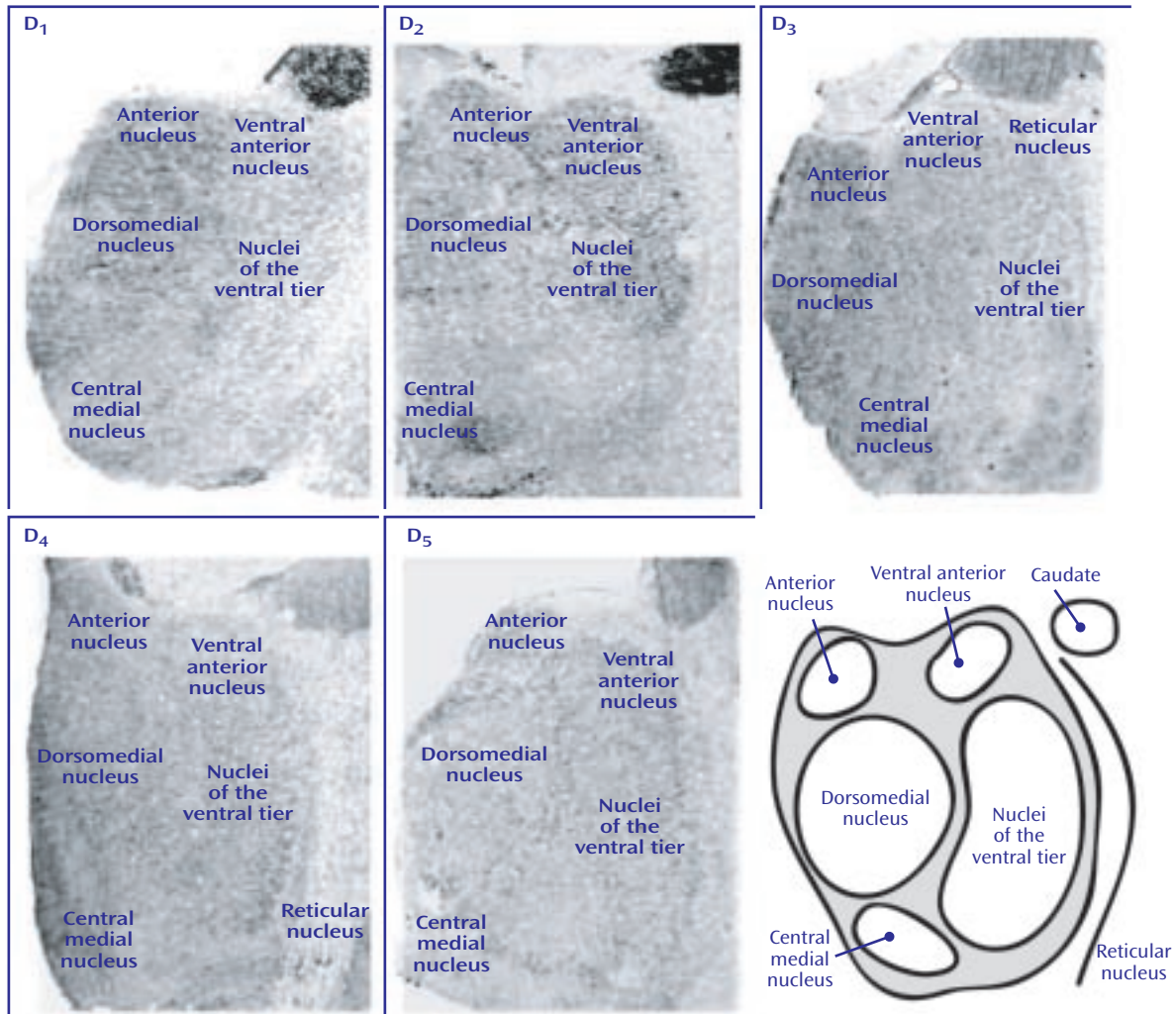
There is growing appreciation of thalamic abnormalities in schizophrenia. Multiple studies have detected structural and functional thalamic pathology in this illness. Postmortem and structural imaging studies have reported decreases in thalamic cell number and volume, while functional in vivo images studies have reported decreased thalamic metabolism as well as alterations in corticothalamic connectivity (18–26) (see reference 27 for review).

Both thalamic and dopaminergic dysfunction have been separately implicated in the pathophysiology of schizophrenia, but possible abnormalities of the dopamine system in the thalamus in this illness have been largely unexplored. While one study in the 1980s reported increased concentrations of dopamine in the thalamus of patients with schizophrenia (28), no studies to date have examined the expression of dopamine receptors or associated intracellular proteins in the thalamus in schizophrenia. Therefore, in this project we examined a number of molecules associated with dopaminergic neurotransmission in several thalamic nuclei in schizophrenia. We have determined the expression of dopamine receptor transcripts and receptor binding, binding to the vesicular monoamine transporter as a marker of afferent innervation, and transcripts encoding three intracellular proteins with a role in the integration of dopaminergic signaling at the intraneuronal level.

Method

Subjects

Thirteen subjects with schizophrenia and eight nonpsychiatrically ill individuals from the Mount Sinai Medical Center Brain

FIGURE 1. Dopamine Receptor mRNA Expression in Human Thalamus^a

^a All five transcripts are detected, but at low levels of expression. Note the much higher labeling for D₁ and D₂ mRNA in the tail of the caudate nucleus.

Bank were studied (Table 1). The cases in the present study are the same as those utilized in our previous reports on glutamate receptor and transporter expression in the thalamus in schizophrenia (29–33). Cases were classified as having schizophrenia if the presence of schizophrenic symptoms could be documented before age 40; the medical records contained evidence of psychotic symptoms and ≥ 10 years of psychiatric hospitalization with a diagnosis of schizophrenia; the DSM-III-R diagnosis of schizophrenia was agreed upon by two experienced clinicians; and neuropathological examination did not reveal Alzheimer's disease or other degenerative disorders. Subjects with a history of alcoholism or substance abuse were excluded. Normal comparison subjects had no history of any psychiatric or neurologic disorders and no discernible neuropathologic lesions. Next of kin consent was obtained for each subject. There were no significant between-group differences in age ($t=1.6$, $df=19$, $p=0.12$), postmortem interval ($t=1.2$, $df=19$, $p=0.25$), tissue pH ($t=2.3$, $df=19$, $p=0.15$), sex distribution ($\chi^2=1.7$, $df=1$, $p=0.19$), side of brain studied ($\chi^2=0.91$, $df=1$, $p=0.34$), or cigarette smoking history ($\chi^2=1.1$, $df=1$, $p>0.20$). At the time of death, six persons with schizophrenia were receiving typical antipsychotics, five had a mean drug-free period of 5.8 weeks ($SD=3.4$), and one had been drug free for many years.

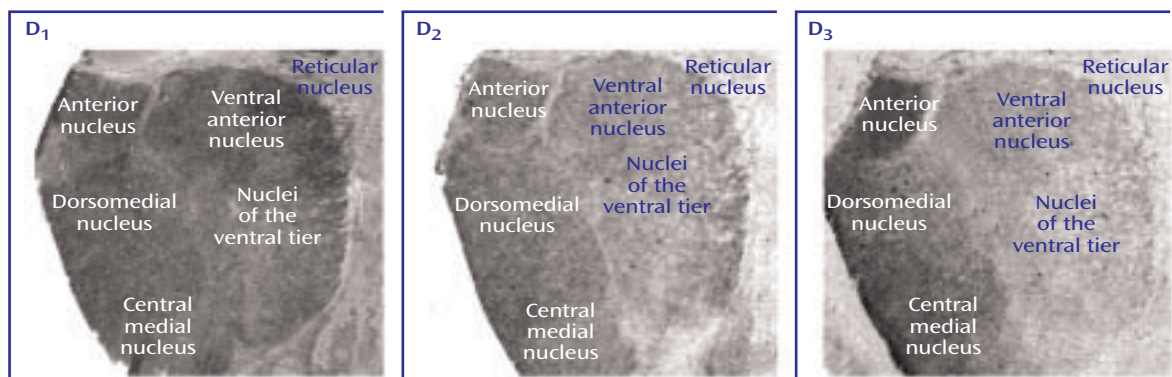
Tissue Preparation

Brains were prepared by slicing one hemisphere into 1-cm coronal slabs, which were snap frozen. Blocks containing thalamus were cryostat-sectioned (15 μm), and thaw-mounted onto poly-L-lysine-subbed microscope slides, dried, and stored at $-80^\circ C$. Two slides per subject were prepared for in situ hybridization for each probe. For receptor autoradiography, three slides per subject were used for each radioligand, two slides for total binding and the third to define nonspecific labeling.

In Situ Hybridization

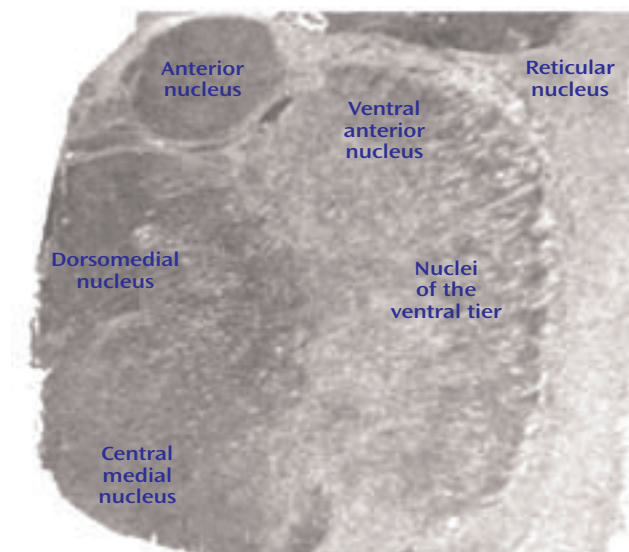
We performed in situ hybridization with riboprobes to determine the expression of transcripts encoding the five dopamine receptors and calcyon, spinophilin, and DARPP-32. For D₁–D₅ receptors, we used probes that we have previously described and validated (34–38). For the three intracellular proteins, we prepared new subclones. We amplified unique regions of calcyon (NCBI Genebank accession number AF225903, nucleotide coding region 120–509), DARPP-32 (AF233349, 12–274), and spinophilin (AF016252, 2423–2905) from a human brain cDNA library (EdgeBiosystems, Gaithersburg, Md.). Amplified cDNA segments were extracted (QIAquick Gel Extraction Kit, Qiagen, Valencia,

FIGURE 2. Dopamine Receptor Binding in Human Thalamus^a



^a Note the relatively homogeneous distribution of D₁ receptor binding across thalamic nuclei and the relative enrichment of both D₂ and D₃ binding in the more medial nuclei (anterior, dorsomedial, central medial). D₁ binding determined with [³H]SCH23390, D₂ with [³H]raclopride in the presence of 7-OH-DPAT, and D₃ with [³H]PD128907.

FIGURE 3. VMAT2 Binding in Human Thalamus^a



^a Note the strong enrichment in the more medial nuclei (anterior, dorsomedial, central medial), similar to the pattern seen for D₂ and D₃ receptor binding.

Calif.), subcloned (Zero Blunt TOPO PCR cloning kit, Invitrogen, Carlsbad, Calif.), and confirmed by nucleotide sequencing (Thermo Sequenase Radiolabeled Termination Cycle Sequencing Kit, USB, Cleveland). Linearized subclones were used to synthesize [³⁵S]UTP-labeled riboprobes for in situ hybridization, as we have described extensively in the past (34–38). To ensure the specificity of in situ hybridization signals, adjacent sections were incubated with sense strand probes for each of the dopamine receptors or associated intracellular protein and processed in parallel with antisense-labeled slides as described previously (34–38). One set of these slides was exposed to Kodak film for 7–60 days, and a second set was dipped in Kodak NTB-2 emulsion and stored at 4°C for 21–90 days before developing (39).

Receptor Autoradiography

Three slides (two for total and one for nonspecific binding) were used for each subject for each radioligand. Slides were removed from –80°C storage and warmed at room temperature for 15 minutes. For the dopamine receptors, slides underwent a binding pre-

wash at room temperature for 30 minutes. Slides were rinsed in deionized water and dried under a stream of cool air. Slides were then incubated for 2 hours in buffer containing radioligands at concentrations three times K_d, as determined for each ligand in this lab. Nonspecific binding was determined by adding an unlabeled compound to the ligand buffer mixture. After incubation, excess buffer was tapped off onto a paper towel, and slides were rinsed in fresh buffer twice at 4°C for 2 minutes. Slides were then briefly rinsed in deionized water at 4°C and dried under a stream of cool air. Slides were apposed to Amersham [³H]Hyperfilm.

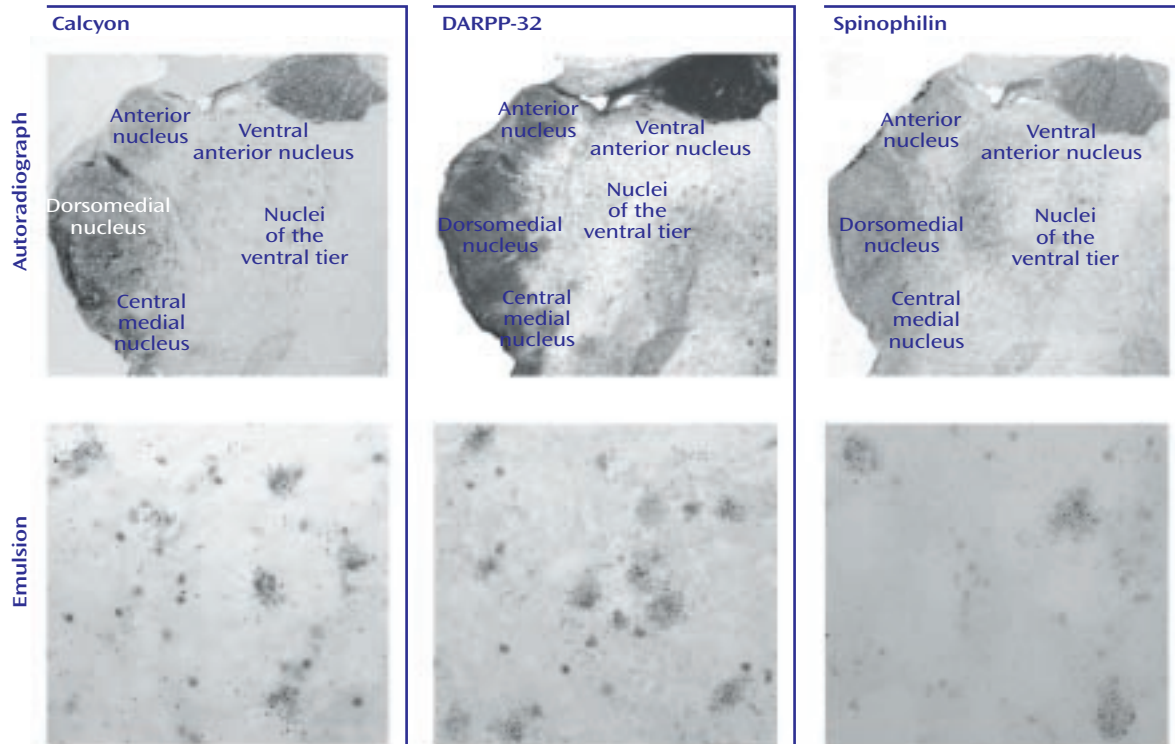
For D₁ receptor autoradiography, both pre- and postbinding washes were in 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, and 1 mM MgCl₂ buffer. The radioligand was 5 mM [³H]SCH23390 in the pre/postincubation buffer to which 5 μM ketanserin and 0.1% ascorbic acid were added. Nonspecific binding was determined using the same solution, to which 1 μM SCH23390 was added. For D₂ receptors, pre- and postbinding washes were in 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, and 100 μM pNHGG. Binding to the D₂ receptor was performed with 3 nM [³H] raclopride in the same buffer, with the addition of 50 μM 7-OH-DPAT. Nonspecific binding was determined in the presence of 1 μM spiperone. D₃ receptor binding was performed in 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. For visualization of D₃ receptors, 1 nM [³H] PD128907 was used, with nonspecific binding determined in the presence of 1 μM 7-OH-DPAT.

VMAT Autoradiography

Binding to VMAT2 was assayed by quantitative autoradiography of [³H]methoxytetraabenazine (MTBZ) binding as described previously (40). In brief, sections were prewashed for 5 min at 25°C in potassium phosphate buffer (137 mM KCl, 3 mM NaCl, 8 mM K₂HPO₄, 1.5 mM NaH₂PO₄, 1 mM EDTA; pH 8.0) followed by incubation in the same buffer containing 20 nM [³H]MTBZ for 30 minutes at 25°C. Slides were subsequently rinsed twice for 2 minutes each in buffer at 4°C, followed by a brief dip in distilled water at 4°C to remove excess buffer salts. Nonsaturable binding was assessed in adjacent tissue sections incubated in [³H]MTBZ together with 10 μM unlabeled tetraabenazine. Under these conditions, saturable MTBZ binding to VMAT2 displays a K_d of 3.9 nM in rat brain sections (40). Sections were air-dried and apposed to tritium-sensitive X-ray film for 2 weeks.

Data Analysis

The boundaries of thalamic nuclei were determined in each section based upon cellular and white matter patterns as defined by cresyl violet and gold chloride staining of sections from each

FIGURE 4. Intracellular Protein Transcript Expression in Human Thalamus^a

^a Top row shows film autoradiographs of sections treated with antisense strand riboprobes for each transcript. As in the case of other molecules associated with dopaminergic signaling in the thalamus, note the relative enrichment of transcripts encoding these intracellular proteins in more medial nuclei associated with limbic function (anterior, dorsomedial, central medial). Bottom row shows bright-field micrographs of emulsion-dipped slides labeled for each transcript, demonstrating cellular localization within the dorsomedial nucleus for each probe. Calcyon and DARPP-32 transcripts were present in both large glutamatergic relay neurons and small GABAergic interneurons. Spinophilin transcripts, on the other hand, were only expressed in the large relay cells.

subject, as we have previously described (29, 33). Thalamic nuclei were identified on the basis of descriptions of thalamic architecture (17). The following nuclei were identified for each subject: anterior, dorsomedial, ventral anterior, central medial, ventral, and reticular. In this study, the ventral nucleus corresponds to a grouping of several nuclei of the ventral tier. Images were digitized from films and analyzed by quantitative densitometry. For in situ hybridization images, gray scale values were obtained from each of the six thalamic nuclei, corrected for tissue background, and converted to optical density. These values are linear with concentration over the gray scale values found in this study. For receptor binding images, gray scale values for each nucleus were corrected for nonspecific binding and converted to optical density. Values from two sections for each subject were averaged and used for statistical analysis. Statistical analysis was performed for each probe by analysis of variance, with diagnosis and nuclei as the independent variables. The Kolmogorov-Smirnov test was used to ensure normality of all data, and Pearson product-moment correlations were used to determine relationships between continuously distributed variables. For all tests, $\alpha=0.05$.

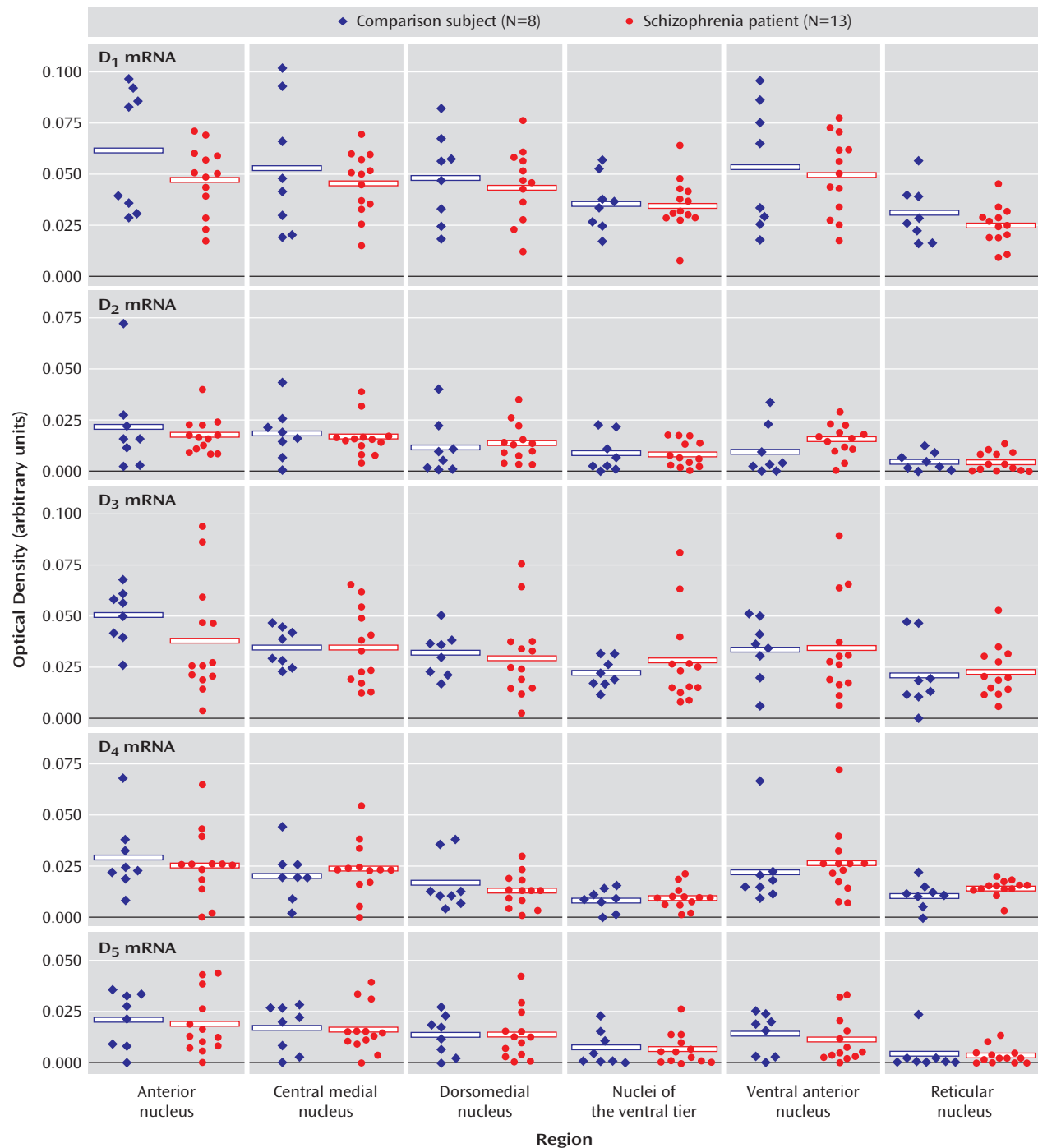
Results

Normal Distribution of Dopamine Receptor Transcripts in Human Thalamus

All five dopamine receptor transcripts were identified in thalamic nuclei, although at low levels of expression (Fig-

ure 1). Both D₁ and D₂ receptor mRNAs were present, but at considerably lower levels than in striatal structures. D₃, D₄, and D₅ receptor mRNAs were also present at low levels of expression, comparable to the very low levels of these three transcripts in the caudate and putamen.

There was significant heterogeneity of expression for each transcript across different thalamic nuclei. D₁ receptor mRNA differed across nuclei ($F=14.3$, $df=5$, 95 , $p<0.000001$); post hoc analyses indicated that this effect was due to higher levels of D₁ mRNA expression in the dorsomedial, anterior, central medial, and ventral anterior nuclei than in the reticular and ventral nuclei. D₂ receptor mRNA ($F=15.9$, $df=5$, 95 , $p<0.000001$) and D₅ receptor mRNA ($F=18.8$, $df=5$, 95 , $p<0.000001$) also differed across nuclei, with relatively higher levels in the anterior and central medial nuclei compared with the ventral tier and reticular nuclei. There was also heterogeneity of D₃ receptor mRNA expression ($F=10.1$, $df=5$, 95 , $p<0.000001$), which was primarily associated with high levels of D₃ transcripts in the anterior nucleus and very low levels in the reticular nucleus. Finally, D₄ receptor mRNA expression varied across nuclei ($F=9.31$, $df=5$, 95 , $p<0.000001$), with very low levels of expression in the ventral tier nuclei compared with other nuclei studied.

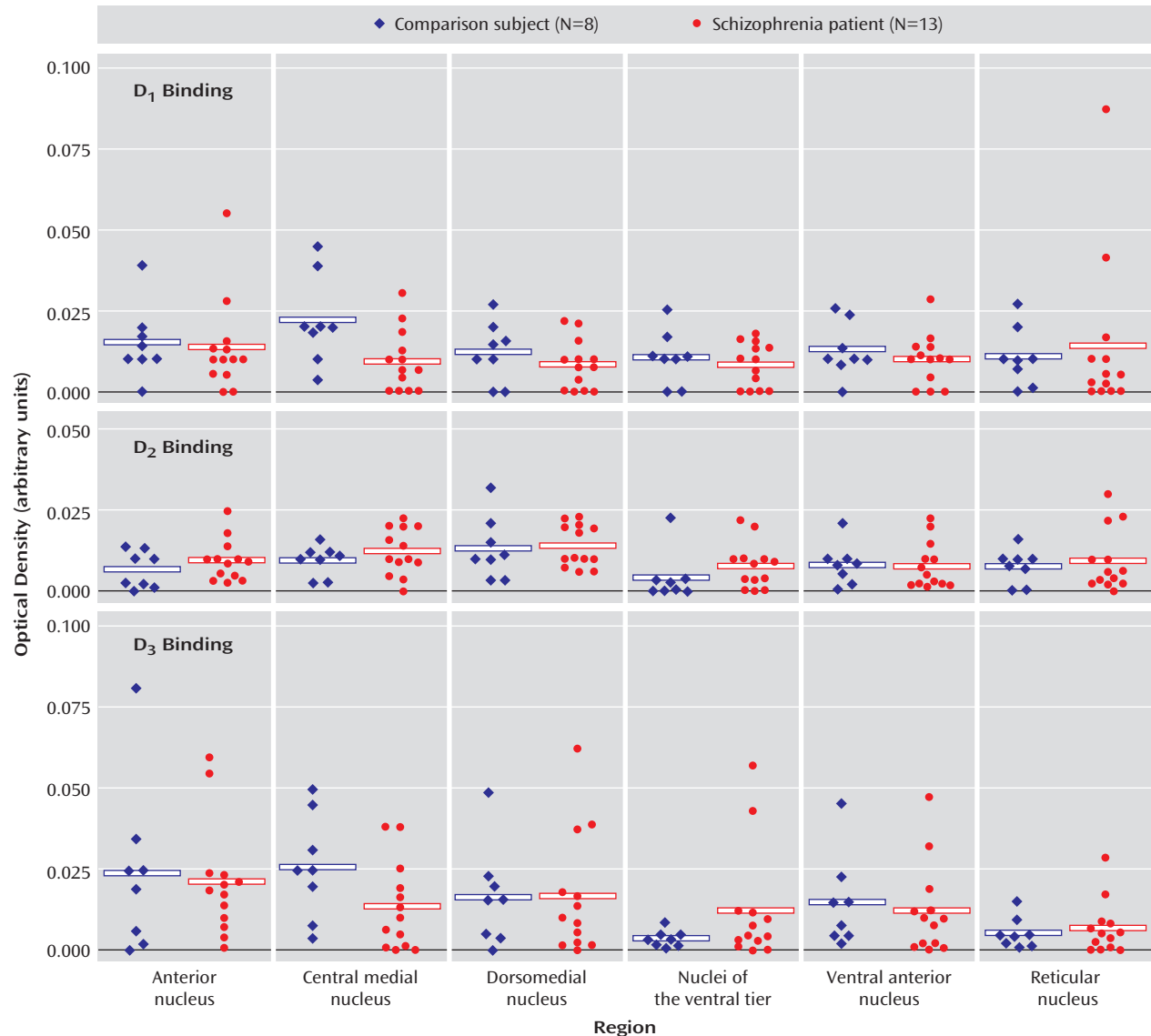
FIGURE 5. Dopamine Receptor Transcript Expression in Six Thalamic Nuclei in Schizophrenia and Comparison Subjects^a

^a There were no differences in the expression of these five transcripts between the persons with schizophrenia and comparison subjects.

Dopamine Receptor Binding in Human Thalamus

Binding to the D₁, D₂, and D₃ receptors was determined by receptor autoradiography (Figure 2). Similar to the dopamine receptor transcripts, significant main effects were identified for both D₂ and D₃ binding sites across thalamic nuclei. There was a main effect of nucleus for D₂ receptor binding ($F=4.1$, $df=5$, 95 , $p<0.005$) that was due to higher D₂ receptor binding in the dorsomedial nucleus

than in the other five nuclei. There was also a main effect of nucleus for D₃ receptor binding ($F=7.1$, $df=5$, 95 , $p<0.00005$). This variability of D₃ receptor binding was associated with decreased binding in the reticular nucleus compared with other nuclei as well as the ventral nuclei having lower D₃ receptor binding than the anterior and central medial nuclei. D₁ binding did not significantly vary across these nuclei.

FIGURE 6. Dopamine Receptor Binding in Thalamus in Schizophrenia and Comparison Subjects^a

^a There were no differences in levels of these three binding sites between the persons with schizophrenia and the comparison group.

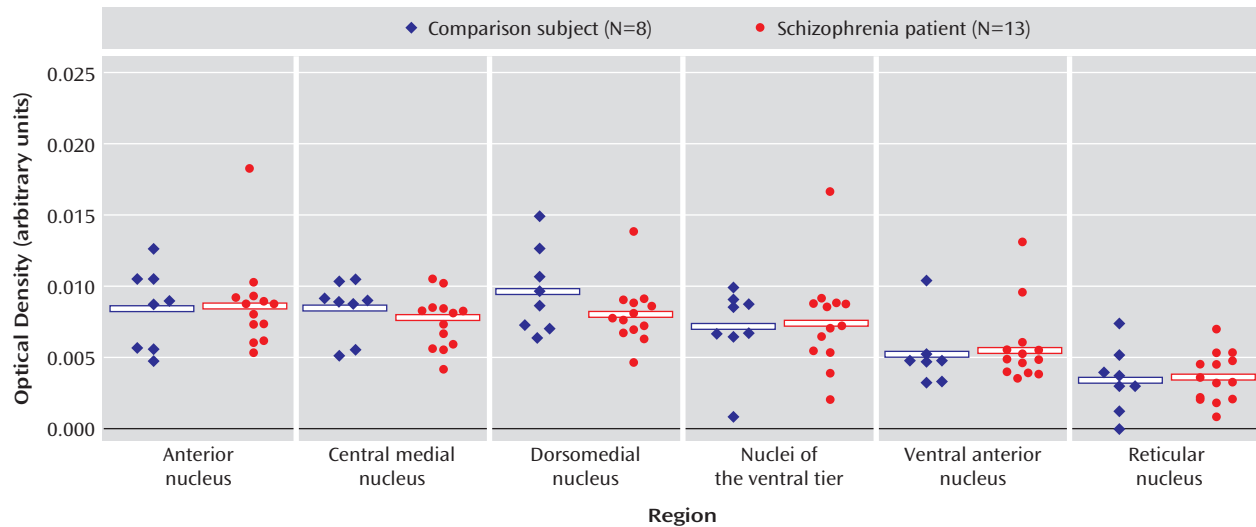
VMAT2 Binding in Human Thalamus

As a marker of presynaptic dopaminergic innervation, we investigated binding to the vesicular monoamine transporter, VMAT2 (Figure 3). Modest levels of VMAT2 binding were seen in all thalamic nuclei, with particular enrichment in the dorsomedial, central medial, and anterior nuclei. There was significant regional heterogeneity ($F=14.8$, $df=5$, 95 , $p<0.000001$), with enrichment in the more medial nuclei.

Normal Distribution of Transcripts Encoding Calcyon, DARPP-32, and Spinophilin in the Human Thalamus

Finally, we examined the expression of the transcripts encoding three intracellular molecules associated with

dopamine receptor-mediated neurotransmission: calcyon, spinophilin, and DARPP-32 (Figure 4). As in the case of other dopaminergic molecules, there was significant regional heterogeneity of the expression of these three transcripts across thalamic nuclei. In the case of calcyon, there was a main effect of nucleus ($F=22.2$, $df=5$, 95 , $p<0.000001$) that was associated with higher levels of calcyon mRNA being expressed in the anterior, dorsomedial, central medial, and ventral anterior nuclei compared with the ventral tier and reticular nuclei. Spinophilin ($F=12.5$, $df=5$, 95 , $p<0.000001$) and DARPP-32 ($F=44.5$, $df=5$, 95 , $p<0.000001$) transcripts were also heterogeneously expressed across the thalamus, with relatively lower levels expressed in the ventral tier and reticular nuclei compared with the more medial thalamic nuclei studied.

FIGURE 7. VMAT2 Binding in Thalamus in Schizophrenia and Comparison Subjects^a

^a There were no differences in levels of vesicular monoamine transporter in schizophrenia versus control subjects.

Dopaminergic Abnormalities in the Thalamus in Schizophrenia

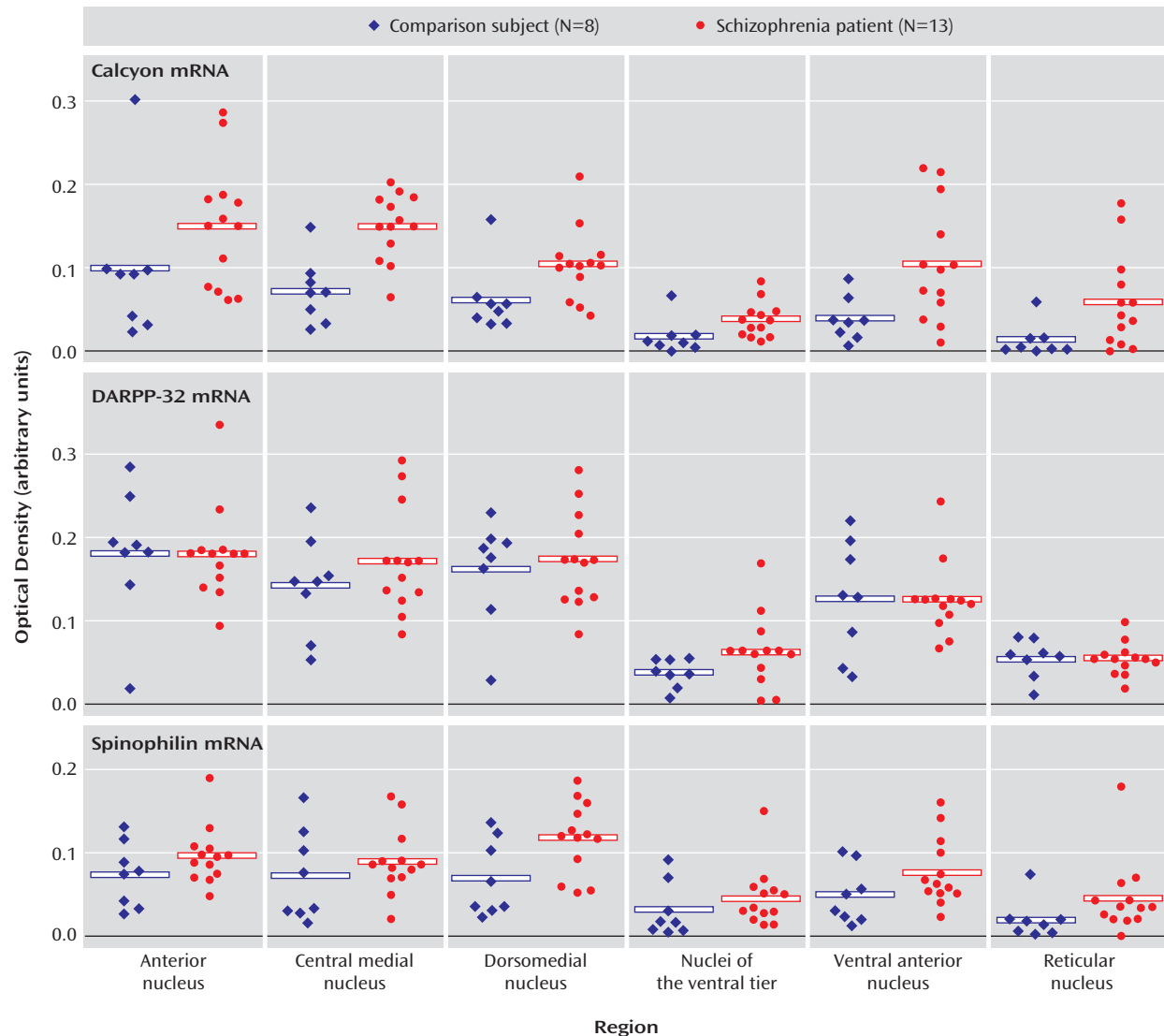
There were no significant main effects of diagnosis nor any significant diagnosis-by-nucleus interactions for any of the dopamine receptor transcripts (D_1 – D_5) (Figure 5). Receptor autoradiography studies showed that there were no significant main effects of diagnosis or significant diagnosis-by-region interactions for the D_1 – D_3 binding sites (Figure 6). Similarly, there was no difference in expression of VMAT2 binding between persons with schizophrenia and the comparison group (Figure 7). We did find a main effect of diagnosis for transcripts encoding two of the three dopamine receptor-interacting proteins (Figure 8). There was a main effect of diagnosis for calcyon expression ($F=8.65$, $df=1, 19$, $p<0.01$), which was associated with a nearly two-fold increase of calcyon transcript expression in the thalamus in schizophrenia relative to comparison subjects (Figure 8). We calculated total antipsychotic exposure for the 6 months preceding death, expressed as chlorpromazine equivalents for the subjects with schizophrenia, and found that calcyon mRNA levels in the central medial and dorsomedial nuclei negatively correlated with antipsychotic exposure (central medial nucleus: $r=-0.89$, $df=11$, $p=0.007$; dorsomedial nucleus: $r=-0.79$, $df=11$, $p=0.03$), but no other correlations were noted. Calcyon mRNA expression was not significantly correlated with tissue pH. We also found a main effect of diagnosis for spinophilin ($F=4.6$, $df=1, 19$, $p<0.05$) that was due to an elevation of spinophilin mRNA in all thalamic nuclei in schizophrenia relative to comparison subjects (Figure 8). This is similar to the pattern seen for calcyon, although the magnitude of the differences between comparison and schizophrenia subjects was not as great. There were no correlations between expression of this transcript and antipsychotic exposure or tissue pH. Finally, DARPP-32 was

not significantly different in schizophrenia (Figure 8). There were no significant diagnosis-by-region interactions for calcyon, spinophilin, or DARPP-32.

Discussion

These data indicate that dopaminergic neurotransmission in the thalamus is potentially impaired in schizophrenia. It is interesting that the expression of dopamine receptors is spared in the thalamus in schizophrenia, as is presynaptic innervation as determined by binding to the vesicular monoamine transporter. On the other hand, expression of both calcyon and spinophilin, intracellular molecules associated with the integration of dopaminergic neurotransmission with signaling from other neurotransmitter systems in the dendritic spine, are grossly abnormal. These data suggest that dopamine signaling is altered in the thalamus in schizophrenia, but at the level of intracellular signal integration.

Relatively little is known about the normal neurochemical anatomy of the dopamine system in the thalamus. Studies on rodents have revealed only sparse dopaminergic innervation to a few thalamic nuclei, including the dorsomedial nucleus (41–44). This is typically associated with low levels of dopamine receptors (45, 46) with several exceptions: increased densities of D_2 receptors have been noted in the anterior and lateral dorsal nuclei, and high densities of D_4 receptors were reported in the reticular nucleus (47–49). Studies on rodents and primates have also typically revealed low levels of transcripts encoding dopamine receptor subtypes in thalamus, although these earlier studies tend to have focused on other structures and only note thalamic expression in passing (39, 50–53). One study focused on human brain D_2 and D_3 dopamine receptor expression noted specific nuclear distributions in the thalamus (54). Our dopamine receptor data are consistent with

FIGURE 8. Intracellular Protein Transcript Expression in Thalamus in Schizophrenia and Comparison Subjects^a

^a Both calcitonin and spinophilin transcripts were significantly increased in schizophrenia.

most of these earlier reports: transcripts for all five of the dopamine receptors were detected in human thalamus but at very low levels of expression. Similarly, D₁, D₂, and D₃ receptor binding were all detected in the thalamus. It is interesting that both D₂ and D₃ binding were enriched in more medial, limbic-associated nuclei, which is consistent with earlier work showing relatively increased expression of D₂ and D₃ transcripts and binding in the anterior and dorsomedial nuclei of the human thalamus (54). Furthermore, our studies of VMAT2 binding and the three intracellular protein transcripts revealed a similar pattern of expression, with enriched levels expressed in medial and anterior nuclei. Taken together, these data support a role for dopaminergic modulation of human thalamic function that is more extensive in limbic-related nuclei.

While a large body of work has examined dopaminergic abnormalities in the striatum and prefrontal cortex in

schizophrenia, relatively little attention has been focused on alterations of molecules associated with this neurotransmitter in the thalamus. A few studies have suggested that dopamine levels are elevated in the thalamus in schizophrenia. Using high-performance liquid chromatography, one group found a substantial elevation of dopamine but not norepinephrine levels in the thalamus in schizophrenia (28, 55). These authors presented detailed maps for the thalami from selected comparison and schizophrenia patients, highlighting “dopamine hot spots” where dopamine/norepinephrine ratios were especially high (28). The thalami of comparison subjects in this work contained very little endogenous dopamine, but there was significant enhancement of dopamine/norepinephrine ratios in many regions of the thalami in schizophrenia subjects. The pattern of “dopamine hot spots” widely varied between the thalami from each of the pa-

tients, however, making these data difficult to interpret. Using a similar assay, this group found significant differences in thalamic dopamine levels within a group of rats that all were handled, housed, etc. under identical conditions, suggesting that technical matters may have confounded this experiment (56).

This group speculated that the observed increase in thalamic dopamine content might be due to increased dopaminergic innervation of the thalamus in schizophrenia (28). We have examined afferent monoaminergic innervation to the thalamus by measuring binding to VMAT2 as measured by MTBZ. VMAT2 is the transporter responsible for neuronal translocation of neurotransmitter monoamines from cytoplasm to synaptic vesicles and is expressed in neurons employing the neurotransmitter dopamine, norepinephrine, serotonin, or histamine (57–59). Unlike alternative markers of synaptic terminals, the expressed concentration of VMAT2 appears to reflect the density and integrity of presynaptic innervation and is relatively unaffected by counterregulatory and compensatory changes accompanying drug treatment. The vast majority of data supporting this interpretation are derived from study of the nigrostriatal dopaminergic projection. Experimental animals treated with a range of drugs affecting dopaminergic neurotransmission (including D_2 dopamine receptor agonists, D_2 antagonists, dopamine reuptake inhibitors, and dopamine metabolism inhibitors) do not significantly alter the expressed levels of VMAT2 in the striatum (59, 60) or in the cerebral cortex (61). Conversely, interventions associated with the irreversible lesions of nigrostriatal neurons or terminals (40, 62) result in reduced striatal VMAT2 binding levels, reflecting lesion severity (40). We interpret our present MTBZ data as a reflection of the distribution and density of presynaptic monoaminergic terminals in the thalamus. Given the striking parallels among VMAT2, D_2 , and D_3 binding sites and calcyon and DARPP-32 transcripts, we suspect that VMAT2 is to a large extent reflecting dopaminergic innervation in the thalamus. Our data suggest that there is no change in monoaminergic innervation to the thalamus in schizophrenia.

Similarly, there were no changes found for either dopamine receptor transcripts or binding sites in schizophrenia subjects. On the other hand, there were significant changes in the expression of two intracellular proteins that are enriched in the dendritic spine and link dopamine receptors to other effector pathways. The most dramatic change was noted for the transcript that encodes calcyon. Calcyon is a recently identified protein found to interact with the D_1 receptor, which may facilitate D_1 receptor-mediated increases in intracellular calcium. Yeast two-hybrid and coimmunoprecipitation studies have shown that calcyon binds intracellular C-terminal residues of the D_1 receptor, and immunocytochemical studies showed that this protein is widely expressed throughout the brain (9). By binding a C-terminal region of the D_1 receptor, calcyon appears to mediate calcium mobilization by permitting

“crosstalk” between D_1 -coupled G_s proteins and $G_{q/11}$ protein-coupled receptors, including metabotropic glutamate and muscarinic cholinergic receptors (9). D_1 receptor stimulation alone does not affect calcium levels, but application of a D_1 agonist after stimulating P2Y purinergic or M_1 muscarinic receptors triggered a large increase of intracellular calcium (9). Calcyon has also been shown to regulate the affinity state of the D_1 receptor (63). Therefore, calcyon may serve several functions in the neuron, including regulating D_1 receptor affinity for agonists as well as the integration of D_1 -mediated signaling with $G_{q/11}$ protein-coupled receptors. A recent study found that levels of expression of calcyon protein (expressed both per total protein and per neuronal DNA) were significantly increased in the dorsolateral prefrontal cortex of patients with schizophrenia (13). These results in the prefrontal cortex are consistent with our present data of a significant elevation of calcyon mRNA in the thalamus in schizophrenia.

Spinophilin was also significantly increased in the thalamus in schizophrenia. Yeast two-hybrid has been used to demonstrate an interaction between the third intracytoplasmic loop of the D_2 receptor and spinophilin, a protein enriched in dendritic spines and known to bind PP-1 and F-actin (64, 65). Spinophilin is also widely expressed in the brain but is particularly enriched in the hippocampus, striatum, and thalamus (64). Spinophilin may function, in part, as a protein phosphatase-1-targeting protein to concentrate protein phosphatase-1 in dendritic spines, where it would be in close proximity to postsynaptic targets such as *N*-methyl-D-aspartic acid (NMDA) and AMPA glutamate receptors and calcium/calmodulin-dependent kinase II (CamKII) (12, 64). Spinophilin may also impact synaptic transmission by regulating dendritic spine number and density (64, 66) by targeting protein phosphatase-1 to the spine microfilament network, which is dynamically modified by protein phosphatase-1 activity (67). Data from a spinophilin knockout mouse support both of these possibilities, as these animals show both changes in glutamatergic transmission and spine density (66). Although spinophilin has been found to interact with the D_2 receptor *in vitro*, this interaction has not yet been established *in vivo* (11). Since spinophilin has several binding sites, including a site to bind F-actin, protein phosphatase-1, and D_2 , as well as a PDZ domain, it may serve as a scaffold-adaptor protein for G-protein coupled receptors, which in turn could link receptors to the cytoskeleton and coordinate their associated signaling pathways. A recent study that examined levels of expression of spinophilin in the dorsolateral prefrontal cortex in schizophrenia did not reveal any significant differences (13). Our present data indicating increased spinophilin mRNA in the thalamus in schizophrenia suggest that brain region-specific alterations in levels of expression of spinophilin may occur in schizophrenia.

DARPP-32 levels were unchanged in schizophrenia. D_1 -type dopamine receptors primarily couple to G_s -G pro-

teins to stimulate cAMP formation, which activates cAMP-dependent protein kinase A. Protein kinase can phosphorylate numerous intracellular proteins, including the phosphoprotein DARPP-32, which plays a central role in neuronal function, particularly in brain regions that receive rich dopaminergic input (8). Many neurotransmitters, including dopamine, modulate the phosphorylation and/or dephosphorylation state of DARPP-32 (68). The phosphorylated form of DARPP-32 is a potent inhibitor of protein phosphatase-1, the predominant phosphatase used in neurons, which is particularly enriched in the postsynaptic density (69). The DARPP-32/protein phosphatase-1 cascade controls the phosphorylation states and activity of myriad downstream effectors and therefore exerts a powerful effect on neuronal function (8).

A previous study revealed that DARPP-32 expression was significantly reduced in the dorsolateral prefrontal cortex in schizophrenia (14). The same study also revealed that DARPP-32 was not reduced in patients with Alzheimer's disease who had been treated with haloperidol, suggesting that the decrease in DARPP-32 observed in schizophrenia is unlikely to be the result of antipsychotic treatment alone. Our finding of no change of DARPP-32 mRNA in schizophrenia is perhaps not unexpected. DARPP-32 is likely not transcriptually regulated in schizophrenia. Instead, changes in this molecule are likely translational or particularly at the level of phosphorylation state.

Our study has several limitations. These data are from elderly patients with schizophrenia, so generalization of our findings to younger patients should be made with caution. Another potential confounding variable that must be considered in this study, as in any study of postmortem brain in schizophrenia, is the possibility that any findings are due to past antipsychotic exposure. It is unlikely that our significant results are due to drug effects. Several studies in the macaque have examined the effects of haloperidol on expression of both calcyon and spinophilin, although only in the cortex. Chronic antipsychotic treatment did not alter expression of calcyon in the prefrontal cortex (13). On the other hand, spinophilin levels are decreased in the cortex following haloperidol treatment (13, 70), opposite to our findings in the thalamus in schizophrenia. In addition, we did not detect any positive correlations between expression of these transcripts and antipsychotic exposure. While we cannot completely rule out the possibility that our findings are associated with antipsychotic treatment, these data strongly suggest otherwise.

Many studies of neurotransmitter-mediated abnormalities in schizophrenia have focused on cell membrane expressed molecules, particularly neurotransmitter receptors. Our results suggest that there are dopaminergic abnormalities in the thalamus in schizophrenia but that they occur at the level of intracellular proteins that modulate dopamine receptor-related intracellular signaling events. These proteins, calcyon and spinophilin, link dopamine receptor-signaling pathways to other neu-

rotransmitter receptors (11, 12, 71, 72), so our findings suggest that in schizophrenia, the intracellular integration of dopamine signaling with other neurotransmitter systems in the dendritic spine is abnormal in the thalamus. Specifically, while calcyon and spinophilin appear to mediate intracellular interactions between dopamine and other neurotransmitters, both have been shown to promote dopamine-glutamate synergies (12, 71, 72). Our current data, along with our previously published observations of altered expression of NMDA receptors (29) and NMDA receptor-interacting intracellular molecules like PSD95 (33) in these same subjects, suggest that the neurochemical disturbances in thalamic circuits in schizophrenia may involve abnormalities of the molecules that integrate glutamatergic and dopaminergic signaling pathways in thalamic neurons.

Received May 27, 2003; revisions received April 22 and July 28, 2004; accepted Sept. 9, 2004. From the University of Michigan Mental Health Research Institute and the Departments of Psychiatry, Radiology, and Neurology; and the Department of Psychiatry, Mount Sinai School of Medicine, New York. Address correspondence and reprint requests to Dr. Clinton, Mental Health Research Institute, University of Michigan, 205 Zina Pitcher Place, Ann Arbor, MI 48109-0720; clinton@umich.edu (e-mail).

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