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CORTICAL LOCALIZATION OF DOPAMINE D4 RECEPTORS
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Using polyclonal antibody against dopamine D4 receptor we investigated cortical distribution of D4 receptors, with the special emphasis on regions of the prefrontal cortex. Prefrontal cortex is regarded as a target for neuroleptic drugs, and engaged in the regulation of the psychotic effects of various substances used in the experimental modeling of schizophrenia. Western blot analysis performed on samples from the rat cingulate, parietal, piriform cortices and also striatum revealed that antibody recognized one main band of approximately 40 kD, which corresponds to the predicted molecular weight of D4 receptor protein. In immunocytochemical studies we found D4 receptor-positive neurons in all regions of prefrontal cortex (cingulate, agranular/insular and orbital cortices) and all cortical regions adjacent to prefrontal cortex, such as frontal, parietal and piriform cortex. Substantial number of D4 receptor-positive neurons has also been observed within the striatum and nucleus accumbens. In general, a clear stratification of the D4 receptor-positive neurons was observed in the cortex with the highest density seen in layers II/III and V/VI. D4 immunopositive material was also found in the dendritic processes, particularly clearly visible in the layer II/III. At the cellular level D4 receptor immunoreactivity was seen predominantly on the periphery of the cell body, but a certain population of neurons with clear cytoplasmic localization was also identified. In addition to cortical distribution of D4 receptor-positive neurons we tried also to define types of neurons expressing D4 receptor protein. In double-labeling experiments, D4 receptor protein was found in nonphosphorylated neurofilament H-positive, calbindin-D28k-positive, as well as parvalbumin-positive cells. Since, used proteins are markers of certain populations of pyramidal neurons and GABA-ergic interneurons, respectively, our data indicate that D4 receptors are located on cortical pyramidal output neurons and their dendritic processes as well as on interneurons. Above localization indicates that D4 receptors are not only directly influencing excitability of cortical inter- and output neurons but also might be engaged in dendritic spatial and temporal integration, required for the generation of axonal messages. Additionally, our data show that D4 receptors are widely distributed throughout the cortex of rat brain, and that their cortical localization exceeds the localization of dopaminergic terminals.

Key words: *dopamine D4 receptors, prefrontal cortex, immunocytochemistry, neurofilament H, calbindin-D28k, parvalbumin.*

INTRODUCTION

In recent years there has been a growing interest in the physiology and pharmacology of dopamine D4 receptors (1—4). There have been several attempts to link schizophrenia, attention deficit disorder, or addiction with disturbances of the informational flow via dopamine D4 receptors, due to alternations in receptor density, mRNA levels, or appearance of particular D4 receptor isoforms (4—8). Dopamine D4 receptors, which were cloned by Van Tol (9) belong to the G-protein coupled receptor family of dopamine D2 receptors. Like all members of the D2 receptor family, D4 receptors inhibit cyclic AMP formation and are supposed to be localized both pre- and postsynaptically, moreover they are believed to mediate the effects of neuroleptic drugs (2, 3, 8, 10).

The interest in pharmacology of dopamine D4 receptors is mainly driven by observations that clozapine, an atypical and highly effective neuroleptic drug devoid of extrapyramidal side effects, has approximately 10 times higher affinity to D4 receptors than to any other dopaminergic receptors (11). What seems to be of particular importance is that the therapeutic doses of clozapine correlate well with its dissociation constant measured at the D4 receptor (11). These data support the role of dopamine D4 receptors as a potential target for antipsychotic drugs, although clinical data directly associating schizophrenia with dysfunction of dopamine D4 receptors are at least controversial (6, 8, 12). The putative therapeutic potential of D4 receptor antagonists is not limited to treatment of schizophrenia since there are also indications that dopamine D4 receptor antagonists might be effective in treatment of drug addiction. For example D4 receptor antagonists inhibit amphetamine-induced sensitization (13), which is supposed to maintain the forces of addiction as well as to be the critical element of psychostimulant induced psychoses (14). It is also apparent that the anti-addictive effects of D4 antagonists might somehow be associated with the involvement of D4 receptors in novelty seeking behavior, and possibly attention deficit disorders (7, 15).

Although above arguments indicate importance of dopamine D4 receptors, evaluation of their function and brain distribution is difficult due to lack of clear behavioral effects even after specific agonists and antagonists as well as lack of selective radiolabeled ligands and low abundance of their mRNA. Since the direct binding studies as well as *in situ* hybridization of D4 receptor mRNA are impossible currently, the only way to visualize D4 receptor and study its distribution is comparison of immunohistochemical studies using various antibodies specific to different epitopes of D4 receptor protein. It is known that antibodies labeling the various epitopes of neurotransmitter receptor protein, although specific in the Western blots and immunoprecipitation studies, dependently on the functional state of receptor and consequently epitope

structure, visualize cellular distribution of receptor more or less fragmentarily (16). Availability of the antibody labeling amino acid sequence of second extracellular loop of the dopamine D4 receptors, not used as yet, by other authors, inclined us to perform complementary study aimed at visualization of dopamine D4 receptors in rat prefrontal cortex (10, 17—20). However the main goal of the present study was not only the distribution of D4 receptor in the cortex, but the identification of types of neurons expressing D4 receptor protein. For this reason we used double-labeling immunohistochemical technique for colocalization of D4 receptors protein with parvalbumin, calbindin-D28k and nonphosphorylated neurofilament H, which are markers of GABA-ergic interneurons and pyramidal output neurons (21, 22).

We selected prefrontal cortex (cingulate, agranular/insular and orbital cortices) for the present study, because i) prefrontal cortex receives massive dopaminergic innervation (23—25), ii) atypical neuroleptics have high affinity to dopamine D4 receptors (11), and both atypical neuroleptics, as well as highly selective antagonists of D4 receptors induced anatomically specific activation of cFos proteins in the prefrontal cortex (26—28), and iii) changes in the neurotransmission of the prefrontal cortex are used in the experimental modeling of certain aspect of schizophrenia (29—30). Thus the precise localization and identification of dopamine D4 receptor-positive neurons in the above regions may be important for understanding their physiological role, as well as the possible mode of action of novel drugs operating via D4 receptors.

Although focus of this study was on the prefrontal cortex, we also visualized D4 receptor-positive neurons in adjacent cortical regions as well as the striatum.

MATERIALS AND METHODS

Animals

All experiments were carried out in Wistar male rats weighing 200—250 g. Rats were housed in groups (6 animals per cage) in an artificial light/dark cycle (12/12 hours, light on at 07:00), with free access to standard laboratory chow (LSM, Bacutil) and tap water.

Perfusion and sectioning

Rats were anaesthetized with sodium pentobarbital (100 mg/kg) and were transcardially perfused with ice cold saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Following a 2-hour postfixation period (4% paraformaldehyde in 0.1M PBS, as a fixative), 50 μ m thick sections were cut at the level of the frontal cortex, using a vibratome (Technical Products International); collected sections were selected for staining using the stereotaxic atlas of Swanson (31).

Dopamine D4 receptor immunohistochemistry

The brain sections were washed three times with 0.01 M PBS and incubated for 60 min in blocking buffer (PBS supplemented with 1% bovine serum albumin, and 2% normal goat serum, Vector Lab). Afterwards, the sections were incubated with primary site-specific anti-dopamine D4 receptor peptide (residues 176–185) polyclonal antibody (Calbiochem, catalogue number 324405). The antibody was diluted to the final concentration of 1:3000 in blocking solution (as above). Incubation was carried out for 48 hours at 4°C, with the continuous agitation. After several washes in 0.01 M PBS, the sections were further incubated for 60 min at room temperature with the secondary biotinylated, anti-rabbit antibody (Vector Lab), washed, and visualized using the Vectastain ABC Elite kit (Vector Lab). The reaction was visualized using (a) diaminobenzidine-glucose oxidase method (32), resulting in a brown reaction product or (b) tetramethylbenzidine-tungstate method (33, 34), resulting in the blue staining. The distribution of immunostaining was the same, regardless of chromogen used.

Double-labeling

For the double-staining experiments, after extensive washing, selected sections immunoreacted for D4 receptors were subjected to the subsequent staining as described above, briefly, they were further incubated with either parvalbumin (1:10 000 dilution; Sigma), calbindin-D28k (1:10 000 dilution; Sigma) or nonphosphorylated neurofilament H protein, clone SMI-32 (1:5 000 dilution; Sternberger Monoclonals Inc.) mouse monoclonal antibodies for 48 h at 4°C, reacted with biotinylated anti-mouse antiserum (1h) and reacted with a chromogen of different color than one used in the first immunostaining. Control experiments with antibody omission were also performed. When one primary antibody was omitted, only single staining was detected, when both were omitted, no staining was seen. The D4 receptor antiserum (Calbiochem), used in the present study was raised in rabbit, which was immunized with a synthetic peptide corresponding to a defined sequence within the human D4 receptor (ac-176-185) covalently attached to carrier protein. It has been characterized by the producer using ELISA, immunocytochemistry and western blot techniques. According to Calbiochem the antibody recognizes D4 receptor from human and rat and has no cross reactivity with any other dopaminergic receptors including D1, D2, D3, D5. The specificity and use of parvalbumin, calbindin-D28k and SMI-32 antisera have already been described (17, 21, 22, 35).

For data presentation and mapping of immunopositive material, digital images were captured using Spot II digital camera (Diagnostic Instruments) attached to an Optiphot II Nikon microscope. Photomontages of consecutive sections were composed using Photo Shop program (Adobe). Images were printed on a high quality photoprinter.

Western blots

For Western blot analysis, rat were sacrificed by decapitation and samples of the cingulate, parietal, piriform cortex, as well as striatum and nucleus accumbens were punched out from 1 mm thick coronal sections and homogenized in 2% SDS. Protein levels were determined using a BCA Protein Assay Kit (Sigma). Then samples were adjusted to contain final concentration of 50 mM Tris, pH 6.8, 2% SDS, 8% glycerol, 2% 2-mercaptoethanol with bromophenol blue as a marker and boiled for 5 min. Aliquots containing 40 µg of proteins per lane were separated on a 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad Mini-Protean II). The proteins were transferred to nitrocellulose, and incubated with the same anti-D4 receptor antibody as for the immunohistochemistry, diluted 1:2500. Blots were visualized by chemiluminescence (BM Chemiluminescence Western Blotting Kit, Boehringer Mannheim cat.no. 1520 709), and Kodak X-omat films.

The molecular weight of protein bands was estimated using Sigma Gel program and its algorithms evaluating the protein weight by comparing the migration of standards with the migration of proteins under investigation.

RESULTS

Immunoblot analysis of cortical D4 receptor protein

A site specific antibody directed against the extracellular segment (residues-176-185) of the D4 dopamine receptor recognized a single band on Western blots of rat cingulate, parietal and piriform cortex protein, as well as, in lysates from nucleus accumbens and striatum (*Fig. 1*). The band corresponded to approximately 40 kD, which is the predicted molecular weight of the rat dopamine D4 receptor protein. In control experiments no band was found in homogenate from rat liver and in BSA solution.

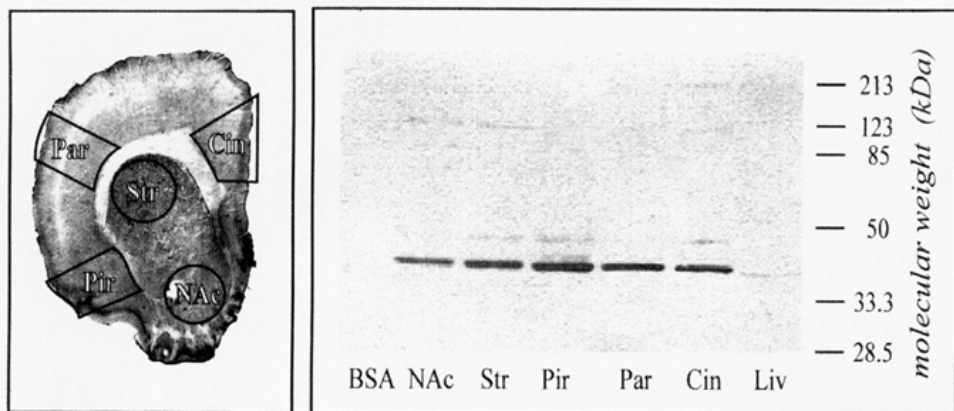


Fig. 1. Left panel — example of coronal brain section stained for the presence of dopamine D4 receptors with marked regions punched out for the Western blot analysis; Right panel — typical Western blot analysis of D4 receptor protein from the cingulate (Cin), parietal (Par) and piriform cortices (Pir), nucleus accumbens (NAc) and striatum (Str). Brain samples were separated together with the sample of bovine serum albumin (BSA) and rat liver (Liv) homogenates. Bars on the right lane correspond to molecular weight standards.

Cortical distribution of dopamine D4 receptor protein

D4 receptor immunoreactivity was observed in all regions of the frontal cortex including the prefrontal cortex (cingulate, agranular/insular and orbital cortices) and all adjacent regions such as the parietal and piriform cortices (*Figs 1* left and *2A, B, C*). A particularly great number of D4-positive cell bodies were noticed in the cingulate and piriform cortex (*Figs 1* left and *2A, C*).

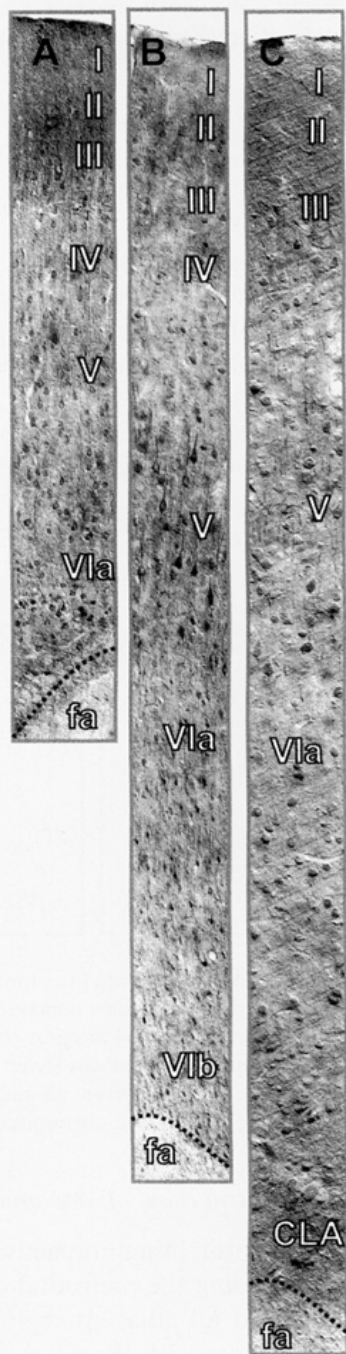


Fig. 2. Cortical distribution of D4-positive neurons in rat (**A**) cingulate cortex, (**B**) parietal cortex and (**C**) piriform cortex. Tangential orientation: corpus callosum is at the bottom, while surface of the brain or the medial line (in the case of cingulate cortex) is at the top. Numbers and abbreviations defined the specific cortical layer according to Swanson (31). Abbreviations: fa- corpus callosum, anterior forceps; CLA-claustrum.

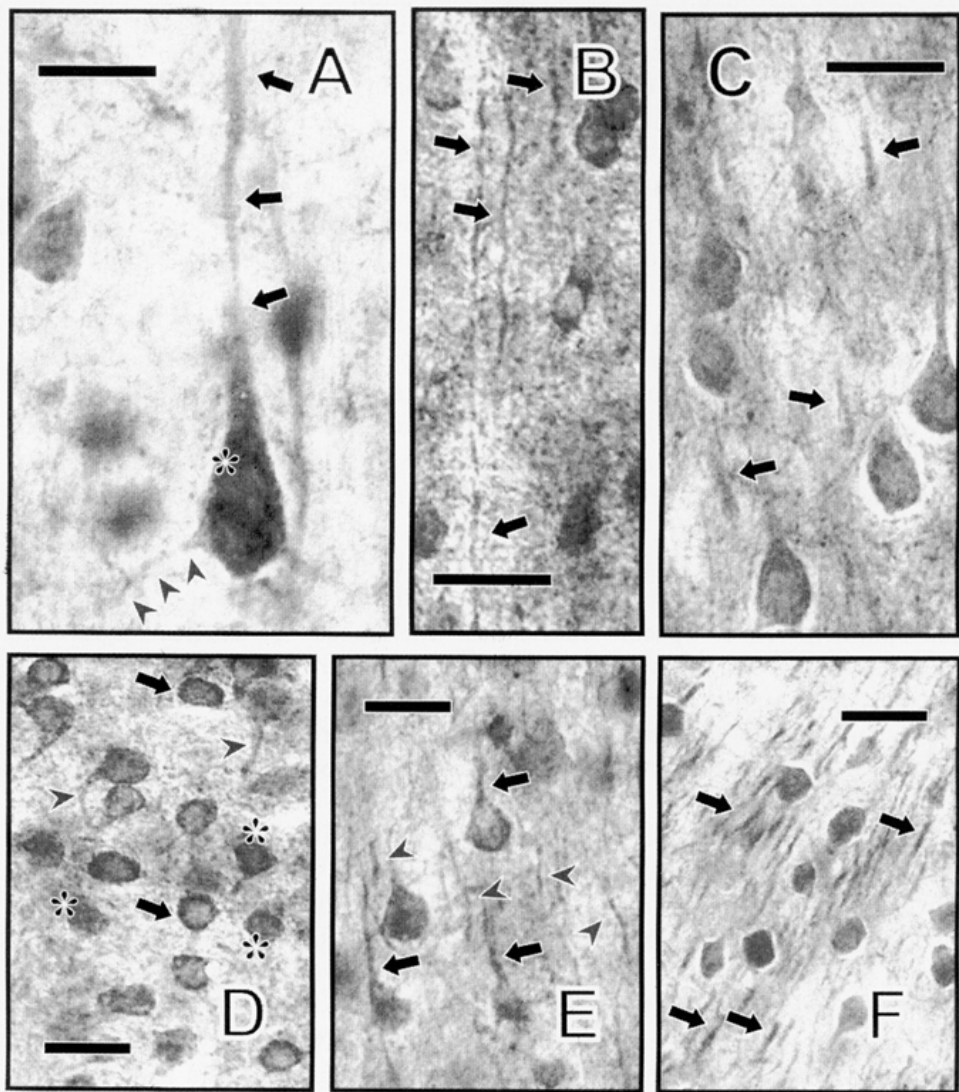


Fig. 3. Examples of D4-positive neurons in various regions of the rat cortex. (A) — neuron from the parietal cortex (layer V) with distinct morphology of pyramidal cells. Arrowheads show faint staining of basilar dendrites. Arrows show dendritic processes with the presence of D4 receptor immunoreactivity. (B) — D4-positive dendritic processes seen through layers V-II of the cingulate cortex. Indicated by arrows — examples of dendritic processes seen in layer IV. (C) — typical D4 receptor-positive neurons in layers II/III of the parietal cortex; arrows show short segments of dendritic processes. (D) — D4 receptor-positive neurons in deep layer (VI) of the cingulate cortex. Examples of neurons with relatively homogenous distribution of the immunopositive material in the cell body cytoplasm (asterisks), and neurons showing condensation of D4 receptor protein at the cell body periphery — arrows. Arrowheads indicate faint staining of dendrites. Note relatively high number of immunopositive cells in comparison with E. (E) — D4 receptor-positive neurons in deep layer VI of the parietal cortex. Emanating from cell bodies short segments of D4 receptor-positive dendrites (arrows), and only short segments of D4 receptor-positive dendrites (arrowheads). (F) — D4 receptor-positive neurons and short segments of dendrites (arrows) in layer II/III of piriform cortex. In A-F scale bar 3 μ m.

Stratification of D4 receptor-positive neurons was not clear in cingulate cortex but was prominent in parietal cortex (*Fig. 2A-C*). Overall we found the cellular localization of D4 receptors confined to the periphery of the cytoplasm and to the short initial fragment of apical dendrites protruding from the cell body (*Fig. 3A-F*). However, exceptions were seen in all regions of cortex examined, where individual neurons were usually found with relatively uniform distribution of immunoreactivity in the cytoplasm (*Fig. 3A-F*). In addition to D4 receptor-positive cell bodies, we also observed several short segments of apical dendrites immunopositive for the presence of D4 receptors in the parietal and piriform cortex (*Fig. 3C, E, F*). However, the most prominent dendritic processes containing D4 immunoreactivity were found in the cingulate cortex (*Fig. 3B*). Judging by the shape, size and cortical localization, D4 receptor immunopositive neurons can be defined mainly as cortical pyramidal neurons (*Fig. 3A*), which occasionally show some faint staining of basilar dendrites (*Fig. 3A*), and also interneurons (*Fig. 3B-F*).

Our suggestion was supported by double-labeling experiments where we found that D4 receptors are colocalized with nonphosphorylated neurofilament H, a specific marker of the subset of cortical pyramidal neurons (21) what proves that D4 receptors are present in pyramidal neurons, and on the dendritic processes of the pyramidal neurons (*Fig. 4*, upper panel). In double-labeled sections, the intracellular segmentation (in apical dendrites) of the D4 receptor immunoreactivity could be seen very clearly.

Double-labeling with antibodies against calcium binding proteins parvalbumin and calbindin-D28k, specific markers of the subpopulations of cortical interneurons (22) mainly GABA-ergic, confirmed that D4 receptors are also localized on GABA-ergic interneurons (*Fig. 4*, lower panels). Specifically, all parvalbumin-positive neurons were also positive for the presence of D4 receptor protein (*Fig. 4*, lower right panel D), also most of calbindin-D28k-positive neurons possessed D4 receptor protein, although occasionally single calbindin-D28k-positive neurons devoid of D4 receptors could also be found (*Fig. 4*, lower left panel D).

Considerably high amount of D4 receptor protein was also found in the dorsolateral part of striatum (even higher than in the parietal cortex) and nucleus accumbens. The clear staining of the periphery and cytoplasm of neurons was observed (*Fig. 5*), what corresponds well with the intensity of bands seen in Western blot analysis (*Fig. 1* left and right).

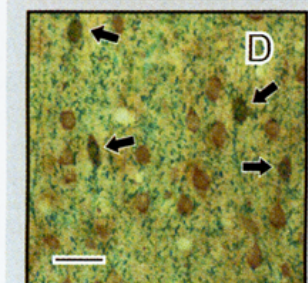
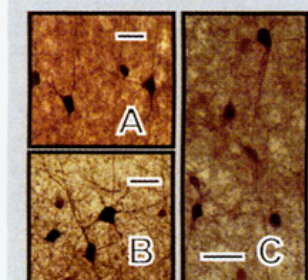
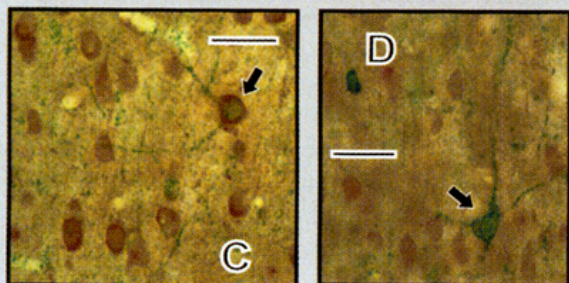
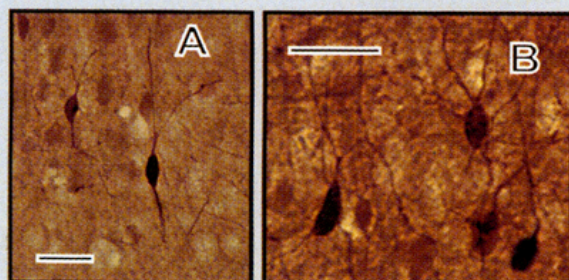
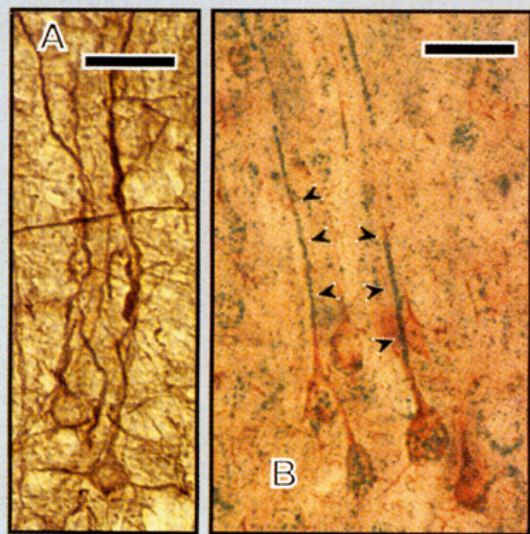


Fig. 4. Colocalization of D4 receptors with nonphosphorylated neurofilament H protein (upper panel), calbindin-D28k protein (lower, left panel), and parvalbumin protein (lower, right panel). **Upper panel:** (A) — examples of nonphosphorylated neurofilament H (SMI 32)-positive neurons in rat cingulate cortex; (B) — double-labeling procedure showing (arrowheads) colocalization of D4 receptors (blue) with nonphosphorylated neurofilament H protein (brown). Scale bar 3 μ m. **Lower, left panel:** (A,B) — examples of calbindin-D28k positive neurons in the rat cerebral cortex; (C, D) — double-labeling procedure showing: (C) — example (arrow) of localization of D4 receptors (brown) in calbindin-D28k-positive neurons (blue), (D) — example (arrow) of calbindin-D28k-positive, D4 receptor-negative neuron. Scale bar 3 μ m. **Lower, right panel:** (A, B, C) — examples of parvalbumin-positive neurons in the rat cerebral cortex; (D) — double-labeling procedure showing examples (arrows) of colocalization of D4 receptors (brown) with parvalbumin protein (blue). Scale bar 3 μ m.

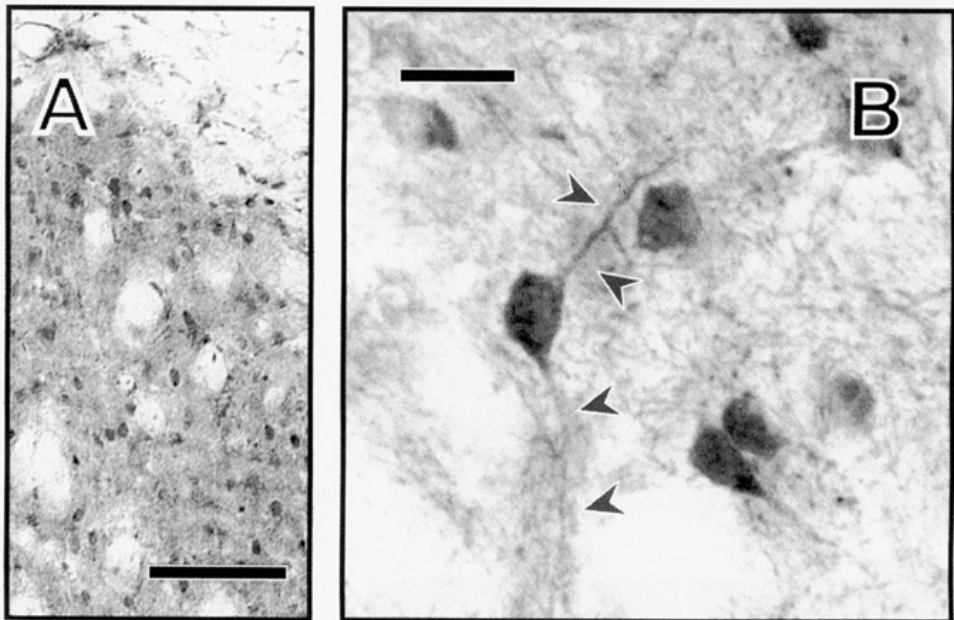


Fig. 5. Distribution of D4-positive neurons in (A) the upper part of the frontal striatum, close to the corpus callosum (scale bar 10 μ m). (B) high-power magnification of striatal D4-positive neurons. Note the presence of D4-positive material in dendritic processes of striatal neurons (arrowheads) and in the surrounding neuropil. Scale bar 3 μ m.

DISCUSSION

Our present study shows that dopamine D4 receptor immunoreactivity can be found in all regions of the frontal cortex and is distributed in both the cell bodies and in fine dendritic processes. Dopamine D4 receptor staining was present in both dopamine innervated areas of the cortex, as well as in regions which are presumably devoid of dopaminergic terminals (23, 24, 36). We observed a relatively great number of D4 receptor positive neurons in the striatum and nucleus accumbens; thus the apparent localization of D4 receptors does not corroborate the initial suggestion of their preferential localization in the prefrontal cortex — specifically the cingulate — where the majority of dopaminergic terminals of the mesocortical dopaminergic system are observed (17, 18, 25).

The D4 receptor-positive neurons seen in the present study, may be defined by shape and their layer-specific localization as pyramidal output neurons and interneurons, presumably GABA-ergic ones, what has been confirmed in colocalization experiments which showed that D4 receptors are colocalized

with specific marker of pyramidal neurons (neurofilament H) as well as with markers of GABA-ergic interneurons (parvalbumin and calbindin-D28k) (21, 22). Our data are in line with the results of the previous studies carried out on rat and primate brains using different antibodies (10, 17—19). More specifically, there are data indicating that in monkey brain dopamine D4 receptor protein is colocalized with parvalbumin and GABA, used as markers of cortical interneurons (10, 17). Moreover, some observations point out that the dopamine D4 receptor in rat cortex is also present in cortical output neurons — pyramidal ones which innervate rat substantia nigra (18). Described localization indicates that activation of D4 receptors may have both direct and indirect impact on the excitability of the cortical neurons which give the rise to the cortico-cortical or cortico-subcortical neuronal pathways (37). Possible direct effects, by the analogy to the primates brains, may engage dopaminergic terminals which make synaptic contacts with the dendritic spines of pyramidal neurons (38), where released dopamine *via* stimulation of D4 receptors (in addition to activation of D1 and D5 receptors (39) will lead, as it is speculated, to the subsequent activation of output neurons (37). On the other hand localization of D4 receptors on the cortical GABA-ergic interneurons, favors the second type of interaction between dopaminergic terminals and pyramidal neurons — indirect one. This model involves release of GABA after activation of D4 receptors on GABA-ergic interneurons and subsequent inhibition of the pyramidal neurons (37). The last hypothesis is supported by the observations indicating that dopaminergic terminals make synaptic contacts with parvalbumin-positive neurons (40, 41). Summing up, above localization of dopaminergic D4 receptors may suggest that the pharmacological effects of D4 receptor agonists and antagonists will be a result of both inhibitory and stimulatory influences. Above framework of anatomical colocalization of D4 receptors and receptors belonging to D1 dopamine family recently has been used to explain the mechanisms of working memory and its deficits, which as it is suggested may be a key phenomenon associated with the attention deficit disorders as well as cognitive dysfunction in schizophrenia (42).

At a cellular level, the receptor protein was mainly observed in the pericaryon periphery, however, there was also population of neurons which showed a relatively uniform distribution of the immunopositive material. The observed peripheral distribution of receptor protein is in line with an earlier suggestion that functional receptors are predominantly localized in the cell body periphery (43). On the other hand, it is tempting to speculate that the antibody used in the present study is able to visualize not only active, membrane receptors (seen on the cell surface), but also receptors which are for example internalized, so they are not in a functional state. It might be interesting to verify this speculation, in the future, since the appearance of

a great number of inactive receptor protein may account for the discrepancies between protein localization and distribution of receptor binding sites.

A widespread distribution of dopamine D4 receptors in several regions of rat frontal cortex, observed in our present study, is not surprising, since a similar distribution was already found in other species, including non-human primates (4, 10, 17–20), and in humans, using an *in situ* hybridization for mapping mRNA of D4 receptors (44). In monkey brain D4 receptors are mainly localized in layer V (45), while in human brain — depending on the cortex region — in layers III and V; nonetheless, regions without clear stratification but with apparent presence of D4 receptor mRNA were also found (10, 44, 45). On the other hand there is only certain overlap between protein distribution seen in immunocytochemical studies including the present one (10, 18, 19) and autoradiographic localization of D4 receptor binding site based on binding of [³H] NGD 94—1, a novel and highly specific antagonist of D4 receptors (46). Specifically, no binding has been found in the parietal cortex and striatum and only a moderate amount of binding was found in the cingulate and piriform cortex, where a particularly prominent labeling of D4 receptor protein was observed in immunohistochemical studies. So far the reason for the observed discrepancy is obscure. An explanation of the above inconsistency can be only speculative, one of the suggestions may assume that not all of recognized by antibody D4 receptor protein forms a functional receptor which is capable to bind specific antagonist.

Despite certain similarities, interspecies differences e.g. between monkey and rat should also be emphasized. For example in monkey brain only a very small amount of D4 receptor immunoreactivity in the striatum can be observed (17), while in our experiments quantity of D4 receptors was considerably larger, taking into account both the number of D4 receptor-positive neurons and the intensity of labeling in comparison with the background. The present results are deliberately compared with those obtained with monkeys and humans, since the distribution of D4 receptors or — more specifically — their binding sites, in the rat brain is still controversial (for comparison see: 46–48). Very low abundance of the D4 receptor mRNA in the rat brain does not permit its visualization at a cellular level; and that would be a procedure of choice which would verify the results of immunocytochemistry. Due to the lack of selective ligands, except for [³H]NGD 94—1, the distribution of D4 receptors measured by autoradiography gave, so far, inconsistent results, not only in the striatum and nucleus accumbens but also regarding their presence in various regions of the cerebral cortex (46–48).

We found that dopamine D4 receptor immunoreactivity is also present in subregions or specific layers of the cortex, which are devoid of dopaminergic terminals of the mesocortical dopaminergic system (24, 25, 36). This finding may indicate that in those regions of the rat cortex dopamine D4 receptors are

not functional because of the lack of dopaminergic synapses, however the volumetric transport of the neurotransmitter, i.e. dopamine may be taken into consideration (49, 50). On the other hand, in cell lines stably expressing D4.2R, D4.4R and D4.7R isoforms of D4 receptors, it has been found, that these receptors may bind (at a nM range) not only dopamine but also noradrenaline (51). These findings have been further confirmed by two functional tests using [³⁵S]GTP γ binding assay and the forskolin-induced accumulation of cAMP. These results indicate that noradrenaline, similarly like dopamine may bind as an agonist at dopamine D4 receptor (51). An interpolation of the above finding to *in vivo* physiology may suggest that in certain subregions of the cortex devoid of dopaminergic terminals, D4 receptors might be under noradrenergic control. Although functional consequences of this intriguing finding are not known, it has been speculated that such multisensitivity of dopamine D4 receptors to various catecholaminergic neurotransmitters may provide a novel mechanism of integration of catecholaminergic signaling (51).

We also found dopamine D4 receptor protein in dendritic processes, furthermore in double-labeling experiments we showed that those processes were emanating from pyramidal neurons. Additionally we observed that D4 receptor immunoreactivity was not uniformly distributed along the apical dendritic processes, but showed clear segmentation. In the identified by calcium binding proteins interneurons D4 receptors seem to be localized mainly in cell bodies. Localization of D4 receptors on fine dendritic processes gives first anatomical evidence for possible involvement of dopamine D4 receptors in the process of spatial and temporal integration of dendritic currents (37, 52). It is speculated that upon their activation, clusters of the receptors on fine dendritic processes, may influence the function of receptor-gated ion channels or alter intrinsic conductance via activation of various second and third messengers and in consequence regulate the excitability of neurons (37, 52). It has been suggested that such clusters of cortical dendritic receptors which regulate spatial and temporal dendritic currents may operate as a gating mechanism responsible for the efficacy of working memory and selective attention (35, 37). Although in the future it may be important to confirm the role of D4 receptors in the above phenomenon in experimental animals, the available clinical data — although still controversial — indicate that the pathology of attention disorders may be connected with the appearance of certain splice variants of dopamine D4 receptors (7).

An intense interest in the physiology of dopamine D4 receptors is fostered not only by heuristic aspects of the physiology of dopaminergic systems, but also by pharmacological attempts to find selective ligands of dopamine D4 receptors and to understand the mechanism of action of clozapine and other atypical neuroleptics which are supposed to exert their antipsychotic activity via, among the others, blockade of dopamine D4 receptors (8, 9, 29). One of the

interesting features of clozapine and other atypical neuroleptic is their anatomical selectivity for the prefrontal cortex (more specifically the cingulate cortex), as already has been indicated by the appearance of cFos proteins (26, 27). Initially, it has been suggested that the presence of these proteins in the cingulate cortex may be linked to the presence of D4 receptors there (26, 27). Our present study as well as some earlier studies with different antibodies against D4 receptor protein indicate that there is only slight overlap between cFos after clozapine and the distribution of D4 receptor protein, which may suggest that the presence of cFos proteins observed after atypical neuroleptics is not exclusively mediated by the blockade of D4 receptors, and that possibly other receptors are responsible for the specific selectivity of atypical neuroleptics for the prefrontal cortex (10, 19, 27). It seems of interest to find out in the future whether cFos after selective antagonists of D4 receptors is colocalized with D4 receptor protein.

Conclusions: Our present study shows a relatively high amount of dopamine D4 receptor-positive neurons in investigated regions of rat cerebral cortex, which are both dopamine innervated and devoid of dopaminergic terminals. D4 receptors are localized on cell bodies of interneurons and pyramidal neurons and on their dendritic processes, as well. The described distribution seems to be in line with the observed localization of mRNA encoding D4 receptors in human and monkey brains, with distinct neuronal stratification being found in various region of the cortex.

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