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Quantitative Analysis of the Dopamine D4 Receptor in the Mouse Brain

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The D4 receptor (D4R), a member of the dopamine D2-like receptor family, has been implicated in the pathophysiology of several diseases and has been the target of various investigations regarding its distribution and quantification. The brain distribution of the D4R has been well described in various species, but the quantification is still an issue of controversy, because no specific ligand is commercially available. To circumvent this difficulty we have performed a biochemical and autoradiographical study in brain samples obtained from mice lacking D4Rs and their wild-type siblings; comparison of their binding parameters allows a more accurate quantification of the members of the D2-like receptor family (D2, D3, and D4 receptors). We found that the distribution of D2-like receptors in mouse brain is similar to that of rat brain, i.e., caudate putamen, nucleus accumbens, olfactory tubercle, and hippocampus. The contribution of the D4R to the overall population of D2-like receptors is 17% in nucleus accumbens, 21% in caudate putamen and olfactory tubercle, and 40% in hippocampus. Based on our study we conclude that nemonapride probably binds to nondopaminergic sites that if not properly blocked may lead to overestimations of D4R levels. We observed that the experimental condition that better estimates the density of D4 receptors is the displacement of D2 and D3 [^{3}H]nemonapride binding sites with cold raclopride. J. Neurosci. Res. 59:202–208, 2000. © 2000 Wiley-Liss, Inc.

Key words: D4 knockout mice; benzamides binding; D2-type receptors

The neurotransmitter dopamine and its receptors have been the major target of investigations aimed at understanding the pathophysiology of schizophrenia, Parkinson's disease, Huntington's chorea, tardive dyskinesia, and affective disorders such as mania, among others (Baldessarini and Tarazi, 1996). Five dopamine receptor subtypes have been identified, which were divided into two subfamilies: the D1-like (D1 and D5) and the D2-like (D2, D3, and D4) receptor subtypes, based on their molecular and neuropharmacological similarities. Among the latter group, the D4R differs from the D2 and D3 receptors by showing high affinity for the atypical antipsychotic clozapine (Van Tol et al., 1991) and a distinct mRNA distribution localized mainly to limbic and cortical structures rather than to the nigrostriatal pathway (Mansour et al., 1991; O'Malley et al., 1992). These observations led to the hypothesis that the D4R might contribute to the pathophysiology of psychoses and therefore be a potential target for selective antipsychotic drugs (Seeman, 1995; Kahn and Davis, 1995). More recently the D4R has also been implicated in attention deficit hyperactivity disorder, mood disorder, and Parkinson's disease (Tarazi and Baldessarini, 1999).

Localization of the D4R protein has been achieved to date employing basically two different methodologies, tritiated ligand binding and immunocytochemistry. Most of the D4R ligand binding studies were performed employing the indirect method described by Seeman et al. (1993a) that involves the subtraction of binding sites defined with the selective D2/D3 antagonist [^{3}H]raclopride from total D2-like binding sites determined with the antagonist [^{3}H]nemonapride. This method was used both in biochemical binding assays (Seeman et al., 1993a,b; Sumiyoshi et al., 1995) and in quantitative autoradiography (Murray et al., 1995; Lahti et al., 1995; Defagot and Antonelli, 1997). An alternative binding method incorporates cold raclopride in the [^{3}H]nemonapride binding reaction at a concentration that blocks only D2 and D3 receptors (Tarazi et al., 1997). Combined data collected in these autoradiographic studies coincide in suggesting that the D4R is localized in hippocampus, frontal and entorhinal cortex and nucleus accumbens in both rat and human brains. Some discrepancies arise regarding the density of D4Rs in the basal ganglia. A novel custom-

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synthesized specific D4R ligand [^{3}H]NGD 94-1 was employed in two recent studies and showed that D4Rs are localized mainly in entorhinal cortex, hippocampus, and hypothalamus in both rat and human brains (Primus et al., 1997; Lahti et al., 1998). Other methods such as [^{3}H]clozapine binding failed to detect D4Rs (Flamez et al., 1994). In parallel, immunohistochemical studies showed that the D4Rs are present mainly in cerebral cortex, hippocampus, and thalamus in the monkey brain (Mrzljak et al., 1996) and in cerebral cortex, hippocampus, thalamus, and caudate putamen in the rat brain (Ariano et al., 1997; Defagot et al., 1997).

In spite of all the work done over the past several years, the quantification of the D4R remains controversial. The density of D4Rs was originally reported to be approximately 10% of the total population of D2-like receptors in human striatum (Seeman et al., 1993a). However, later reports that employed the subtraction method in human and rat brains showed a wide variety of D4R levels (Reynolds and Mason, 1995; Murray et al., 1995; Sumiyoshi et al., 1995; Lahti et al., 1995, 1996; Defagot and Antonelli, 1997), with values as high as 62% in human ventral caudate nucleus (Murray et al., 1995). The subtraction method would be reliable only if the tritiated ligands were specific for the dopaminergic receptors. However, it has been reported that [^{3}H]nemonapride binds to other nondopamine receptors, such as sigma and serotoninergic receptors (Tang et al., 1997; Helmeste et al., 1996, 1997). Therefore, the calculated abundance of D4Rs may be overestimated owing to nemonapride binding to non-D2-like receptors that raclopride is unable to displace.

Knockout mice lacking the expression of a particular receptor provide a useful tool to study its distribution and density. Rubinstein et al. (1997) produced mice lacking D4Rs by means of homologous recombination in embryonic stem cells. The mutation produced a truncated 131-amino-acid polypeptide, of which the first 91 residues correspond to the D4R N-terminus. The mutant mice displayed locomotor supersensitivity to ethanol, cocaine, and methamphetamine, and it was suggested that the D4R modulates motor behaviors as well as the activity of nigrostriatal dopamine neurons. To establish more precisely the experimental conditions to measure D4R binding sites using commercially available ligands, we compared biochemical and autoradiographic binding protocols in brain samples obtained from both mice lacking D4Rs and their wild-type siblings.

MATERIALS AND METHODS

Materials

[^{3}H]nemonapride and [^{3}H]raclopride were purchased from New England Nuclear Co. (Boston, MA). LKB Ultrofilm was obtained from LKB Instruments (Gaithersburg, MD) and S(−)-raclopride from Research Biochemicals International (RBI; Natik, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Mice lacking the D4R were originally generated in Portland, Oregon. Subsequently, breeding pairs were sent to Buenos Aires, Argentina, where a separate breeding colony was established. All mice tested were 8–12 week old F2s (129sv/Ola × C57B1/&J) that were intercrossed for more than 10 generations. Animals were housed in the same sex groups of five or six with free access to food and water and were used while weighing 25–30 g. The vivarium was maintained at 20–22°C on a 12 hr light/dark cycle (lights on at 0700).

Preparation of Synaptosomes

Synaptosomes were prepared as described by Alvarez Maubecin et al. (1995). D4R+/+ and D4R−/− mice were killed by decapitation and the brains were rapidly removed. Caudate putamen, hippocampus, and frontal cortex were dissected out on ice and homogenized in buffer containing 10 mM HEPES (pH 7.4), 0.32 M sucrose, and 1 mM $MgCl_2$ (1 g tissue/10 ml buffer) using a Teflon-glass homogenizer. The homogenate was centrifuged at 1,000*g* for 10 min. The supernatant was then centrifuged at 12,000*g* for 20 min, and the pellet (crude synaptosomal fraction) was resuspended in the same buffer solution and further purified in a Percoll gradient. Three discontinuous Percoll gradients were prepared in 9 ml rimmed polycarbonate tubes consisting of 2.3 ml Percoll (10%, 15%, 23%; vol/vol, in buffer containing 0.32 M sucrose, 1 mM $MgCl_2$ adjusted to pH 7.4 and centrifuged at 32,500*g* for 14 min at 4°C. The bands between 10% and 15% Percoll layers (pure synaptosomal fraction) were removed, slowly diluted (1:4) in binding buffer, and centrifuged at 28,000*g* for 15 min. The pellet was resuspended in the binding buffer (see below) to a final protein concentration of 5 mg/ml. Protein content was determined by the method of Lowry et al. (1951).

Ligand Binding

The saturation binding curves were carried out in concentrations of [^{3}H]nemonapride ranging from 0.05 nM to 1 nM and [^{3}H]raclopride ranging from 0.2 nM to 8 nM. Ligand binding assays were performed by incubating the synaptosomal preparation for 60 min at 22°C in a final volume of 500 μl binding buffer (50 mM Tris-HCl, pH 7.8, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1 mM EDTA). The reaction was stopped by adding 2 ml cold binding buffer and by rapid filtration through Whatman GF/B filters. The filters were washed twice with 2 ml of the same buffer and assayed for radioactivity in a liquid scintillation counter (45–50% efficiency). Specific binding was calculated by subtracting the binding observed in the presence of 50 μM sulpiride (nonspecific binding: 30–35%).

Autoradiographic Binding Assays

D4R+/+ and D4R−/− mice were anesthetized and perfused with ice-cold heparinized (1 U/ml) saline via cannula in the left cardiac ventricle. The brain was removed from the cranium and further chilled by immersion in 4°C saline for 3–5 min. The whole brain was frozen by immersion for 15–20 sec in freon (−40°C) and stored at −85°C. Sagittal and coronal cryosections (12 μm) were thawed mounted onto gelatin-coated slides and dried on a warm plate for 30 sec. Two sections were mounted per slide. The slides were stored dessicated at −85°C

for up to 1 month without significant changes in radioligand binding.

Slide-mounted mouse brain sections were preincubated for 20 min at room temperature in 25 mM Tris-HCl buffer, 100 mM NaCl, and 1 mM $MgCl_2$, pH 7.5. Sections were then incubated at room temperature for 60 min in fresh buffer with 1 nM [^{3}H]nemonapride (D2, D3, D4 radioligand) with or without 1 μM of S(−)-raclopride, to occupy D2/D3 sites selectively. Following incubation the sections were rinsed in the medium without ligand at 4°C for 10 min and dipped three times in cold distilled water. The sections were dried using a stream of air at room temperature and then placed on a warm plate (60°C) for 30 sec. Slides with tissue sections and radioactivity standards (14 sections of metacrylate plastic impregnated with tritium: 0.02–7.39 mCi/g; American Radiolabeled Chemical, St. Louis, MO) were apposed to LKB Ultrofilm at 4°C for 28 days for [^{3}H]nemonapride. Films were processed in Kodak Dektol developer for 4 min. Specific binding was calculated by subtracting the binding observed in the presence of 80 μM sulpiride (nonspecific binding 30–35%).

Analysis of Binding Assays

Data from binding were obtained by Scatchard analysis with a nonlinear least-squares curve-fitting program to obtain equilibrium dissociation constants (Kd) and maximal density of receptors (Bmax). A one-binding site model was the best fit for the data from both ligands. A microcomputer image analysis system (MCID; Imaging Research, St. Catherine's, Ontario, Canada) was used for quantitative analysis of film autoradiograms (Baskin et al., 1989; Baskin and Stahl, 1993). Typically, results were obtained from three or four mice. In an individual experiment, a minimum of two slides, each containing two brain sections, was incubated for a given experimental condition. For each tissue section, three to five optical density readings were made in each region of interest. A mean optical density value was obtained for each relevant anatomical area of a tissue section. The tritiated standards used were calibrated in terms of tissue-equivalent radioactivity with 12 μm liver slices labelled with [^{3}H]formaldehyde as described by Baskin et al. (1989). Combined results were initially calculated in fmol [^{3}H]nemonapride bound per square millimeter since the concentration of protein for each brain area scanned is unknown (Antonelli et al., 1989; Stahl and Baskin, 1990). Statistical analysis of the results shown in Figures 2 and 3 were processed by Student's *t* test, taking $P < 0.05$ as significant.

RESULTS

Biochemical Ligand Binding

Saturation binding experiments employing the biochemical filtration method were performed in synaptosomes obtained from a homogenate pool of caudate putamen, hippocampus, and frontal cortex of D4R+/+ and D4R−/− mice. By employing a concentration range of 0.05 nM to 1 nM for [^{3}H]nemonapride and 0.2 nM to 8 nM for [^{3}H]raclopride, the equilibrium parameters for both ligands were calculated in mice brain synaptosomes (Fig. 1). The Kd values for [^{3}H]nemonapride and [^{3}H]raclopride were 0.25 ± 0.01 nM and 2.3 ± 0.02 nM, respectively, in D4R+/+ mouse brains. In D4R−/− mouse brains the Kd values were 0.22 ± 0.01 nM and 2.0 ± 0.02 nM, respectively. In brain tissue taken from D4R+/+ mice the specific binding for [^{3}H]nemonapride and [^{3}H]raclopride was saturable, with mean Bmax values of 144.4 ± 3.2 and 115 ± 2.2 fmol/mg protein, respectively. Similarly Bmax values of 115.3 ± 2.2 fmol/mg protein for [^{3}H]nemonapride and 108 ± 1.8 fmol/mg protein for [^{3}H]raclopride were calculated from D4R−/− mouse brains. The difference between the Bmax values of both radioligands is assumed to represent D4-like binding sites in D4R+/+ mice (29.4 ± 1.0 fmol/mg protein). The Bmax difference in synaptosomes from D4R−/− mouse brains diminished 75% but still showed a remnant of 7.3 ± 0.4 fmol/mg protein. The Kd

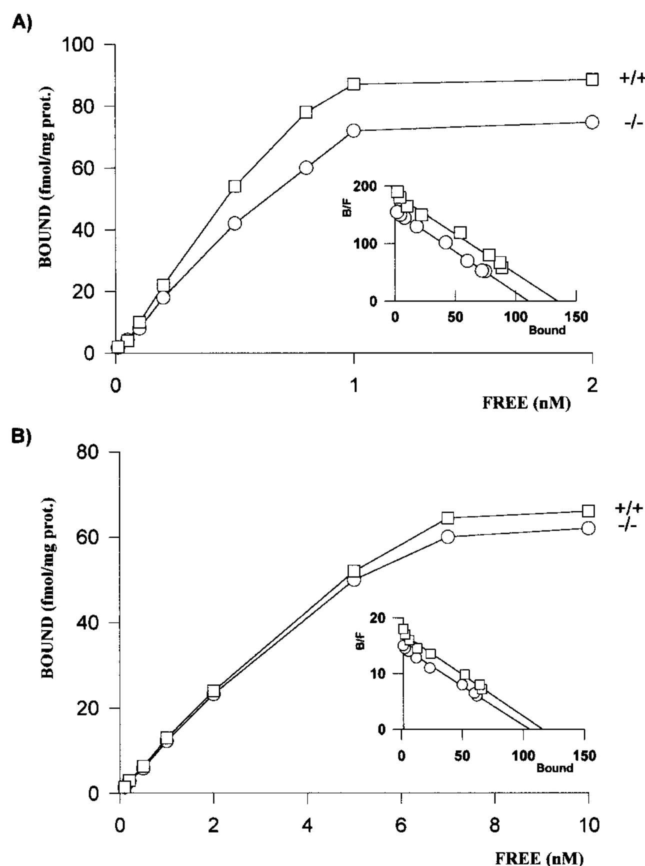

Fig. 1. Saturation binding curves and Scatchard analysis of specific binding of [^{3}H]nemonapride (**A**) and [^{3}H]raclopride (**B**) in D4R+/+ and D4R−/− mice. The data are from a representative experiment. Synaptosomes were prepared from a homogenate pool obtained from caudate putamen, hippocampus, and frontal cortex of D4R+/+ and D4R−/− mice. The binding assays were performed as described in Materials and Methods under standard conditions using 0.05 nM to 1 nM of [^{3}H]nemonapride and 0.2 nM to 8 nM of [^{3}H]raclopride. The ordinate indicates the amount bound expressed in fmol/mg prot. ± SD, and the abscissa indicates the concentrations of ligands. **Insets:** Scatchard plot of the data. B, bound; F, free.

TABLE I. Bmax and Kd for [^{3}H]Nemonapride and [^{3}H]Raclopride Specific Binding in D4R+/+ and D4R−/− Synaptosomes*

	Nemonapride (N)	Raclopride (R)	N − R
D4R+/+			
Kd (nM)	0.25 ± 0.01	2.3 ± 0.02	
Bmax (fmol/mg protein)	144.4 ± 3.2	115 ± 2.2	29.4 ± 1.0
D4R−/−			
Kd (nM)	0.22 ± 0.01	2.0 ± 0.02	
Bmax (fmol/mg protein)	115.3 ± 2.2	108 ± 1.8	7.3 ± 0.4

*Bmax and Kd values for [^{3}H]nemonapride and [^{3}H]raclopride were taken from Figure 1. The values of 29.4 ± 1.0 fmol/mg prot for D4R+/+ and 7.3 ± 0.4 fmol/mg prot for D4R−/− mice reflects the concentration of the putative D4 receptor determined by subtracting the corresponding Bmax of [^{3}H]raclopride from that of [^{3}H]nemonapride.

and Bmax values for [^{3}H]nemonapride and [^{3}H]raclopride specific binding are summarized in Table I.

Autoradiographic Binding Assays: Distribution of D2-Like Binding Sites in D4R+/+ and D4R−/− Mouse Brains

We have also studied the distribution of D2-like binding sites in D4R+/+ and D4R−/− mice using quantitative autoradiography at a saturating concentration of [^{3}H]nemonapride (1 nM). We found that [^{3}H]nemonapride binding sites in D4R+/+ mice were mainly detected in caudate putamen, nucleus accumbens, olfactory tubercle, and hippocampus showing a stronger signal in brain sections taken from wild-type mice in comparison to the signal detected in sections of D4 knockout mice. Figure 2 shows the binding levels expressed in fmol/mm^2 in the different brain areas of D4R+/+ and D4R−/− mice. A 21%, 17%, 21%, and 40% decrease was found in caudate putamen, nucleus accumbens, olfactory tubercle, and hippocampus, respectively, in D4R−/− mouse brain sections, although caudate putamen was the only area that reached statistical significance. These percentages can be also interpreted as the contribution of D4Rs to the total D2-like receptors present in the areas studied in mouse brain sections. The absolute values for D4R+/+ and D4R−/− were 0.61± 0.11 (12) and 0.48 ± 0.015 (10) for caudate putamen, 0.48 ± 0.03 (5) and 0.40 ± 0.09 (5) for nucleus accumbens, 0.39 ± 0.12 (6) and 0.31 ± 0.08 (6) for olfactory tubercle, and 0.05 ± 0.03 (9) and 0.03 ± 0.015 (5) for hippocampus, respectively.

[^{3}H]nemonapride binding was assessed in D4R−/− brain sections in the presence of 1 μM unlabelled raclopride (Fig. 3). We observed that [^{3}H]nemonapride binding was only partially inhibited by raclopride in all brain areas analyzed, including caudate putamen, nucleus accumbens, and olfactory tubercle (Fig. 3). The absolute values for D4−/− and D4−/− with raclopride were and 0.48 ± 0.015 (10) and 0.15 ± 0.02 (10) for caudate putamen, 0.4 ± 0.09 (5) and 0.15 ± 0.03 (4) for nucleus accumbens, and 0.31 ± 0.08 (6) and 0.18 ± 0.05 (3) for olfactory tubercle. The raclopride concentration employed is known to displace [^{3}H]nemonapride from D2, D3, and probably D4 receptors binding sites (Reynolds and Mason, 1994; Reynolds, 1996). Because our brain samples do not express D4 receptor, the remaining labelled sites (shaded bars in Fig. 3) are presumably nondopaminergic receptors.

Figure 4 shows the distribution of [^{3}H]nemonapride binding in sagittal brain sections taken from D4R+/+ (Fig. 4A) and D4R−/− mice (Fig. 4B). When raclopride 1 μM was present during the incubation reaction in brain sections from D4R−/− mice, a residual signal was still present in all brain areas analyzed (Fig. 4C).

DISCUSSION

In the present studies, we have employed brains taken from dopamine D4R−/− mice and from their D4R+/+ littermates to assess more clearly the overall contribution of this receptor subtype within the D2-like receptor family (D2R, D3R, and D4R). The radioreceptor binding assay method employed to calculate the D4-like binding sites that involves subtraction of the number of sites defined with [^{3}H]raclopride (D2/D3 receptor antagonist) from the total number of sites defined with [^{3}H]nemonapride (a D2/D3/D4 antagonist) was pioneered by Seeman et al. (1993a,b) and was later used by various groups both in biochemical binding techniques and in quantitative autoradiography (Murray et al., 1995;

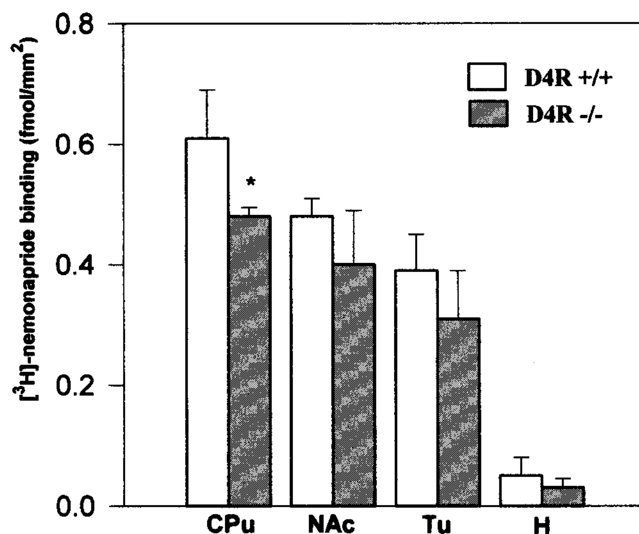

Fig. 2. Distribution of binding sites with affinity for [^{3}H]nemonapride D4R+/+ and D4R−/− brain by quantitative autoradiography. Mice brain sections were incubated with 1 nM of [^{3}H]nemonapride. Specific binding was analyzed on autoradiograms as described in Materials and Methods. Values are reported as mean ± SD obtained from four to nine optical density readings made in each region of interest. A mean optical density value was obtained for each relevant anatomical area of a tissue section. *$P < 0.05$, significantly different from D4R+/+ mice value (Student's *t* test). CPu, caudate putamen; NAc, nucleus accumbens; Tu, olfactory tubercle; H, hippocampus.

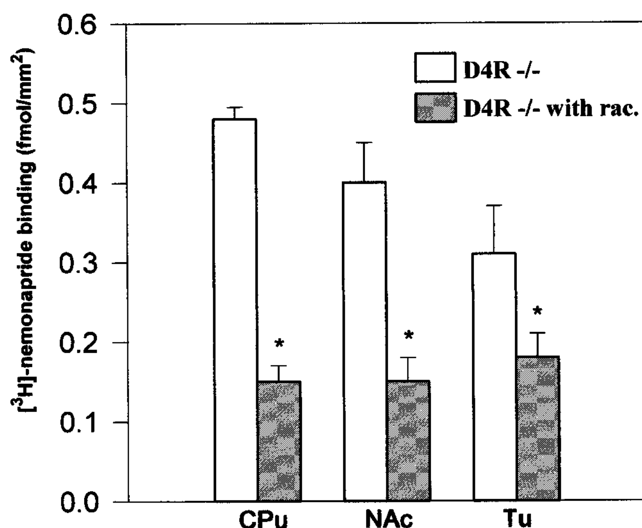

Fig. 3. [^{3}H]nemonapride binding in D4R−/− brain sections in presence of 1 μM of unlabeled raclopride. Mice brain sections obtained from D4R−/− mice were incubated with 1 nM of [^{3}H]nemonapride in the presence or absence of 1 μM of unlabeled raclopride. The specific binding was analyzed on autoradiograms as described in Materials and Methods. Values are reported as mean ± SD obtained from four to nine optical density reading made in each region of interest. *$P < 0.05$ significantly different from knockout mice value (Student's *t* test). CPu, caudate putamen; NAc, nucleus accumbens; Tu, olfactory tubercle.

Lahti et al., 1995; Defagot and Antonelli, 1997; Tarazi et al., 1997). Employing this methodology, we have determined the Kd and Bmax values for nemonapride and raclopride in synaptosomes from a pooled homogenate of caudate putamen, frontal cortex, and hippocampus obtained from D4R+/+ and D4R−/− mouse brains. An alternative approach to quantify D4 receptors is to employ [^{3}H]nemonapride in the presence of 300 nM or 1 μM of cold raclopride to block D2 and D3 receptors (Reynolds and Mason, 1994; Tarazi et al., 1997). We also used this approach to determine the distribution and the relative contribution of the D4 receptor in different brain areas through quantitative autoradiography.

The Kds obtained in D4R−/− mouse brain synaptosomes with both radioligands were similar to those obtained for D4R+/+ mice, indicating that the affinity of the radioligands for the D2-like dopaminergic receptors was not altered for the absence of one of the members of the family. Comparisons to Kd values reported in biochemical binding assays in rat caudate putamen show that our values are an order of magnitude higher for nemonapride binding and two times higher for raclopride binding (Kohler at al., 1985; Terai et al., 1989; Farooqui et al., 1991; Vile et al., 1995). The difference might be mainly related to the species difference and to the fact that we are employing a pool homogenate of frontal cortex, caudate putamen, and hippocampus.

As expected, the Bmax obtained with [^{3}H]nemonapride in brain tissue from D4R−/− mice was lower than that obtained in normal mice. Because raclopride has a very low affinity for D4Rs (Seeman et al., 1993a), the Bmax values obtained with this ligand were similar in both cases. The subtraction of the Bmax obtained with [^{3}H]raclopride from [^{3}H]nemonapride in D4R+/+ mice might represent the D4-like component, which is 20% of the total D2 type. The value obtained after subtracting the Bmax in D4R−/− mouse brain synaptosomes is 75% lower than that obtained in normal mice. We belive that the remaining 25% represents nondopaminergic sites that exhibit high affinity for nemonapride. It has been argued that [^{3}H]nemonapride is not a specific ligand for labelling dopamine receptors because it binds to sigma (Helmeste et al., 1996; Tang et al., 1997) and 5-HT1A serotoninergic receptors (Helmeste et al., 1997). In addition, the binding buffer composition seems to be an important factor. High Na^+ levels and Tris-HCl buffers enhance nemonapride binding to D2 receptors, whereas K^+ phosphate buffers favor sigma receptor binding (Ujike et al., 1996). In our hands, and in spite of using high Na^+ levels and a Tris-HCl buffer, we still find nondopaminergic binding components. Some authors routinely include the sigma receptor antagonist DTG 0.5 μM and the serotoninergic receptor blocker pindolol 0.1 μM (Lahti et al., 1995, 1996; Tarazi et al., 1997), although it seems that these antagonists do not completely block the nondopaminergic sites labelled by [^{3}H]nemonapride. In fact, Tarazi et al. (1997) report that the specific D4 antagonist L-745,870 displaces 74–83% of raclopride-insensitive binding of [^{3}H]nemonapride; the remaining 17–26% probably reflects the nondopaminergic sites. In our hands, these sites were inhibited by 16% with 0.5 μM DTG and 8% with 50 nM ketanserine, a serotoninergic antagonist (data not

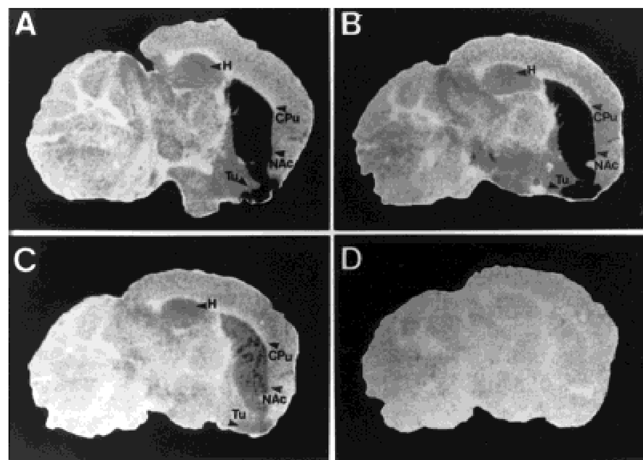

Fig. 4. Autoradiographic images of [^{3}H]nemonapride in mouse brain. Slide-mounted sagittal sections obtained from D4R+/+ (**A**) and D4R−/− (**B**) mice were incubated with 1 nM [^{3}H]nemonapride. **C:** D4−/− mouse brain sections incubated with 1 μM unlabeled raclopride. **D:** Nonspecific binding in the presence of 80 μM sulpiride. H, hippocampus; CPu, Caudate putamen; NAc, nucleus accumbens core; Tu, olfactory tubercle.

shown). A more thorough study is needed to define the identity of these nondopaminergic nemonapride binding sites in order to define the necessary blockers to include during the incubation.

To asses the D4R-like binding distribution in different brain areas, quantitative autoradiography studies were undertaken. [^{3}H]nemonapride binding was clearly detected in caudate putamen, nucleus accumbens, olfactory tubercle, and hippocampus, in the same rank order as that observed in the rat brain (Defagot and Antonelli, 1997). From sections obtained from D4R−/− mouse brains, we observed a 21% binding in the caudate putamen, 17% in the nucleus accumbens, 21% in the olfactory tubercle, and 40% in the hippocampus. We added unlabeled raclopride, at a concentration that inhibits D2, D3, and D4 receptors, when incubating with [^{3}H]nemonapride on D4R−/− mouse brain sections to observe a complete blockade of the binding. However, 25%, 31%, and 46% binding was still present in caudate putamen, nucleus accumbens, and olfactory tubercle, respectively, probably representing the nondopaminergic sites discussed above. This binding was not further blocked by L-745,870 a specific blocker of D4 receptor (data not shown), because there are no D4 receptors in the D4R−/− mouse brains.

Because no selective radioligand is commercially available for determining direct D4R concentration in brain, the issue of the contribution of the D4R subtype to the overall D2-like receptors remains controversial. Several authors have reported D4R concentrations, but there are still some discrepancies that seems to be mainly related to the methodology employed. Seeman et al. (1993a) and Sumiyoshi et al. (1995), employing a biochemical binding method and subtracting the two radioligand Bmax values, reported a 11% to 16% of D4Rs present in human striatum. Reynolds and Mason (1995) employing [^{125}I]epidepride found 11%. When the same subtraction method was employed with an autoradiographic approach, Lahti et al. (1996) reported 23%, 65%, 91%, 79%, 77%, and 88% of D4Rs in caudate putamen, substantia nigra, insula, hippocampus, and entorhinal and temporal cortices, respectively. At saturating concentrations of the ligands, the percentages reported were 46–62% in nucleus accumbens and caudate putamen (Murray et al., 1995), employing a autoradiographic approach. It should be pointed out that the density of D4Rs calculated with the subtraction method shows higher values than when the density is estimated with [^{3}H]nemonapride in the presence of cold raclopride. An estimation of D4R values calculated from the data reported by Tarazi et al. (1998) employing the first method shows values of 40%, 57.5%, and 57%, whereas, when the second method was employed, the values were 13%, 13%, and 16% in the same areas: medial caudate putamen, lateral caudate putamen, and nucleus accumbens, respectively. The difference between [^{3}H]nemonapride levels in D4R+/+ and D4R−/− mice seems to be a more accurate approach in determining the percentage of D4Rs; similar nondopaminergic receptor sites are presumably present in both brain types and disregarded after the subtraction. In our hands, and at saturating nemonapride concentrations, this difference is 17% in nucleus accumbens, 21% in caudate putamen and olfactory tubercle, and 40% in hippocampus, when assayed by quantitative autoradiography. From this point of view, quantification of the D4R-like population employing [^{3}H]nemonapride in the presence of cold raclopride is in better agreement with our data and seems to be a more accurate method than the two radioligand subtraction method.

In summary, comparing the results obtained in this study regarding the overall contributions of D4Rs to the D2R-like population, it can be suggested that in the absence of a commercially available specific D4R ligand the biochemical binding method that employs [^{3}H]nemonapride in the presence of cold raclopride is currently the most accurate method for quantifying D4Rs. Based on this consideration and subtracting the probable contribution of nondopaminergic sites, we conclude that in most of the prominent mouse forebrain structures the D4R contribution to D2R-like total population is between 17% and 40%.

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