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[^{3}H]Pramipexole: a selective radioligand for the high affinity dopamine D_2 receptor in bovine striatal membranes

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The characterization of [^{3}H]pramipexole binding to bovine striatal membranes is reported in full experimental detail. According to kinetic experiments, saturation and competition studies a single binding site can be selectively labeled which turned out to be the high affinity D_2 receptor. Addition of GPP(NH)P resulted in almost complete loss of specific binding. The bovine D_2 subtype shows high sequence identity with the human D_2 receptor indicating that the heterologous competition assays are of interest for the evaluation of neurotropic drug candidates. Using the representative D_2 agonists (+)-7-OH-DPAT, (−)-3-PPP and (*S*)-7-dipropylaminotetrahydroindolizine the same rank order of affinities was determined as described for rat striata labeled with [^{3}H]pramipexole, however, the Ki values turned out to be significantly higher. Furthermore, the system facilitates structure activity relationship studied on D_2 affinity modulating peptides. Using L-prolyl-L-leucyl-glycinamide as an example a significant increase of specific radioligand binding could be measured.

1. Introduction

Structure activity relationship (SAR) studies for the development of novel atypical neuroleptics [1] or antiparkinsonian drugs [2] require practical and efficient dopamine receptor assays [3]. For the investigation of competitively acting dopamine agonists at the D_2 receptor subtype [4] as well as for affinity modulating peptides [5–7], an *in vitro* system selectively labeling the D_2 subtype in the high affinity state is of special interest. Since it is known that recombinant G protein-coupled receptors vary in receptor/G protein stoichiometry [8] and lack intramembrane interactions which influence the ratio between high and low affinity state (e.g. with neurotensin receptors) [9], we were looking for a natural receptor system including a tissue and a radioligand of high selectivity and strength. Due to the high receptor density of the D_2 subtype, compared to D_3 and D_4 receptors [3], striatal membranes are known as a valuable source for D_2 binding experiments. Most of the previously described ligand binding studies at the high affinity D_2 receptor employed [^{3}H]NPA as a radioligand [10] which is difficult to handle due to a high non-specific membrane binding, or [^{3}H]7-OH-DPAT which shows remarkable affinity to sigma receptors as well [11]. Using [^{3}H]pramipexole labeled rat striatal membranes we have recently described SAR studies on D_2 autoreceptor agonists [4, 12–15]. Pramipexole binding and activation of recombinant D_2, D_3 and D_4 receptors was also examined [16]. In this paper, we report the characterization of [^{3}H]pramipexole binding to bovine striatal membranes, and the application as a specific tool for ligand displacement studies and for the investigation of affinity modulating peptides at the dopamine D_2 receptor.

2. Investigations and results

2.1. D_2 Receptor sequence alignment

Sequence alignment studies comparing the human D_2 receptor with the respective bovine and rat sequences were performed. All the used homology matrices (greer.homo, identity.homo, mutation.homo, physprop.homo, pmutation.homo and swiss.homo) yielded the same identity scores for the system human/rat (95.9% entire structures, 93.3% third loops) and human/bovine (96.6% entire structures, 96.9% third loops).

2.2. Kinetic experiments with [^{3}H]pramipexole

In order to investigate the binding properties of the system and to optimize the incubation time for equilibrium experiments kinetic studies were performed (Fig. 1). Studying the association rate of [^{3}H]pramipexole to bovine striatal membranes revealed that [^{3}H]pramipexole becomes bound to the membranes reaching an equilibrium within 30 min. Association data could be fitted using a mono-exponential model yielding a k_{+1} of $6.7 \times 10^7\ min^{-1}\ M^{-1}$ ($t_{1/2} = 2.6$ min). Dissociation rate was examined by adding 1 μM (+)-butaclamol to the assay medium. The dissociation occurs with a $t_{1/2}$ of 5.8 min ($k_{-1} = 0.12\ min^{-1}$). The K_D value for [^{3}H]pramipexole binding, calculated from the ratio of k_{-1} to k_{+1} is 1.8 nM, similar to values determined by saturation and competition experiments.

2.3. Saturation studies of [^{3}H]pramipexole binding

The binding of [^{3}H]pramipexole to bovine striatal membranes was examined using 8 concentrations of [^{3}H]pramipexole (0.1 nM–17 nM). Specific binding was obtained by calculating the difference between total binding and binding in the presence of (+)-butaclamol. The thus obtained concentration binding curve reveals saturable [^{3}H]pramipexole binding (Fig. 2). Nonlinear least-

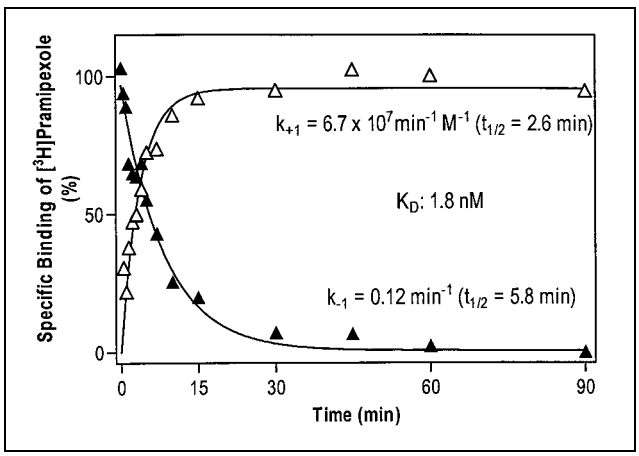

Fig. 1: Time course of association and dissociation of [^{3}H]pramipexole (0.5 nM). (Shown are means, n = 6)

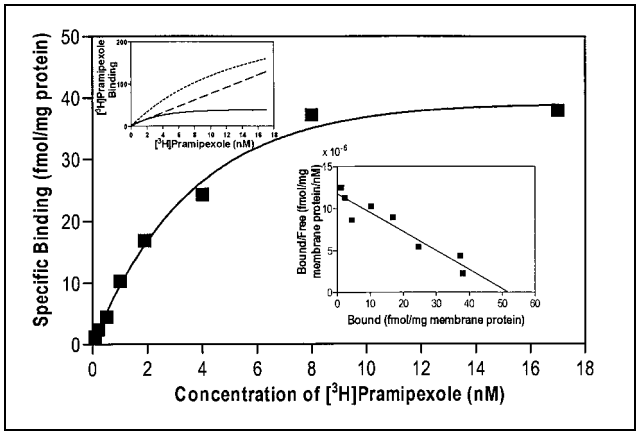

Fig. 2: Specific [^{3}H]pramipexole binding to bovine striatal membranes determined in a saturation experiment (····· total binding, – – – nonspecific binding, ——— specific binding) and the resulting Scatchard plot

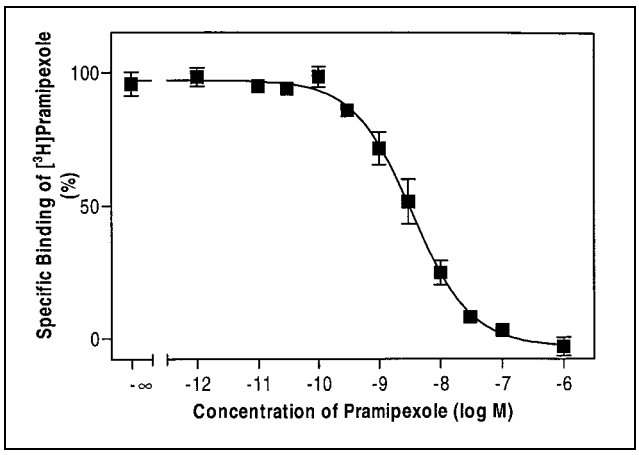

Fig. 3: Competition experiment with [^{3}H]pramipexole (0.5 nM) and unlabeled pramipexole yielding a K_D of 2.3 nM and a B_{max} of 69 fmol/ml membrane protein, $n_H = 0.95$. (Shown are means ± SEM, n = 4)

square fitting of the saturation isotherm yielded a K_D of 2.5 nM. The Scatchard plot of the data also indicated a single binding site and a B_{max} value of 51.9 fmol/mg membrane protein.

2.4. Competition experiments with [^{3}H]pramipexole

Binding of [^{3}H]pramipexole was also characterized by a competition experiment with unlabeled pramipexole. The radioligand was used in a concentration of 0.5 nM. Nonspecific binding was determined in the presence of (+)-butaclamol and amounted to 20% of the total. Labeling of a single binding site was indicated by the Hill coefficient ($n_H = 0.95$), which was not significantly different from unity (partial F-test, $p < 0.05$) (Fig. 3). The IC_{50} was determined with nonlinear regression analysis. Subsequent transformation according to Cheng-Prusoff [17] gave a K_D of 2.3 ± 0.29 nM ($n = 13$). In comparison, a K_D of 3.9 ± 0.2 nM is reported for cloned human D_2 receptors, heterologously expressed in HEK 293 cells [16]. According to DeBlasi [18] a B_{max} value of 69 ± 7.2 fmol/mg membrane protein ($n = 13$) was determined. Applying the described methodology for heterologous competition experiments allowed the determination of the affinity of D_2 receptor agonists. Using (+)-7-OH-DPAT, (−)-3-PPP and (*S*)-7-dipropylaminotetrahydroindolizine as representative examples Ki values of 3.5 nM, 52 nM and 150 nM, respectively, were obtained (Table 1). Interestingly, in all cases lower affinities were observed than for [^{3}H]pramipexole displacement employing rat striatal membranes (0.3 nM, 11.6 nM and 25 nM) [4, 14, 12].

Table 1: Affinity of D_2-ligands to D_2-receptors labeled with [^{3}H]pramipexole

	$D_{2\,bovine}$		$D_{2\,rat}$
(+)-7-OH-DPAT	3.5 ± 0.51	(n = 3)	0.3
(−)-3-PPP	52 ± 2.6	(n = 3)	11.6
(*S*)-7-Dipropylamino-tetrahydroindolizine	150 ± 35	(n = 3)	25

Ki values (nM) of competition experiments with [^{3}H]pramipexole

2.5. D_2/D_1 selectivity of [^{3}H]pramipexole binding

The affinity of pramipexole towards bovine D_1 receptors was investigated by a competition experiment using the selective D_1 antagonist [^{3}H]SCH23390 (0.3 nM) as a radioligand. Employing ligand concentrations up to 10^{-4} M significant binding of pramipexole could not be observed.

2.6. D_2/D_3 selectivity of [^{3}H]pramipexole binding

Pramipexole is known as an agonist with high affinity for dopamine D_2 as well as D_3 receptors [16]. To ensure that [^{3}H]pramipexole labels only D_2 receptors in striatal membranes, 100 μM GPP(NH)P, a non-hydrolyzable analogue of GTP, was added to the assay mixture. Specific binding of [^{3}H]pramipexole was reduced to less than 10%. This is consistent with the properties of agonist binding to dopamine D_2 receptors whereas only weak G protein-coupling is known for the D_3 receptor. Furthermore D_2 and D_3 receptors differ in thermal sensitivity [19]. According to the literature the D_3 receptor becomes denaturated by about 75% upon exposure to 53 °C for 2 min, while only 10% of the D_2 sites become denaturated under the same conditions. In our experiments, heating up the homogenate to 53 °C for 2 min had no effect on the binding of [^{3}H]pramipexole.

2.7. High affinity/low affinity selectivity of pramipexole binding

The ability of pramipexole to differentiate between high affinity and low affinity binding sites was investigated by observing the competition with the D_2 antagonist [^{3}H]spiperone. Agonist competition curves were best fitted to two populations of binding sites with two Ki values (12.4 nM and 4700 nM), one for the high and one for the low affinity state of the receptor and a distribution of 30% for the high affinity and 70% for the low affinity state complex. In comparison, an analogous competition experiment employing heterologously expressed rat D_2 receptors gave a Ki_{high} of 7.1 nM and Ki_{low} of 2710 nM as well as a 1 : 1 distribution of the two affinity states [16]. Addition

Table 2: Selectivity of pramipexole for high- and low-affinity binding states

	Ki_{high} (nM)	Ki_{low} (nM)
Pramipexole	12.4 ± 2.7 (n = 6) (30%)	4700 ± 1400 (n = 6) (70%)
Pramipexole $+10^{-4}$ M GPP(NH)P		4800 (n = 2)

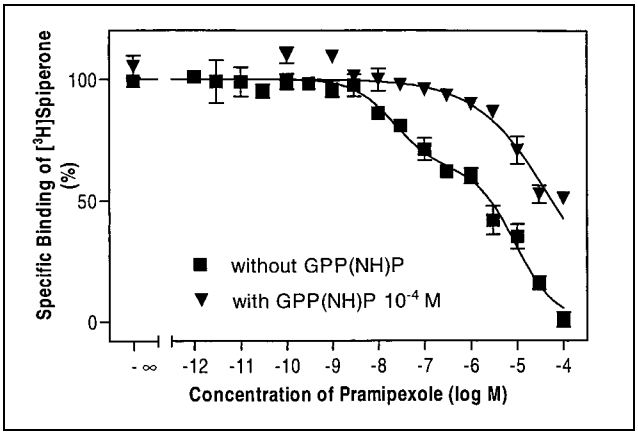

Fig. 4: Inhibition of [^{3}H]spiperone binding (0.5 nM) by pramipexole. (Shown are means ± SEM, n = 6)

of 100 μM GPP(NH)P resulted in a monophasic curve shifted to the right and a single Ki of 4800 nM (Table 2, Fig. 4).

[^{3}H]Spiperone binding at D_2 receptors was characterized by competition experiments with spiperone yielding a K_D of 1.7 ± 0.23 nM and a B_{max} value of 890 ± 103 fmol/mg membrane protein.

2.8. *Modulation of [^{3}H]pramipexole binding by PLG*

PLG (Pro-Leu-Gly-NH_2) has been shown to increase agonist binding to dopamine receptors by enhancing affinity and the number of high affinity binding sites [20]. Modulation of [^{3}H]pramipexole binding by PLG was investigated. Depending on the concentration of PLG (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) an increase of specific [^{3}H]pramipexole binding between 6.5% and 11.7% was observed. 10^{-7} M proved to be the PLG concentration exhibiting the maximal effect. The observed enhancement of [^{3}H]pramipexole binding in the presence of PLG was evaluated for significance using the one sample t-test for the concentrations 10^{-10} to 10^{-7} M. The enhancement proved to be significant ($p < 0.05$). Fig. 5 shows the extent of the increase of specific binding of [^{3}H]pramipexole.

3. Discussion

Bovine brain tissue was chosen for the binding experiments at the high affinity dopamine D_2 receptor because sequence alignment studies showed a 96% identity when compared to the human D_2 receptor. Especially, high identity could be observed for the third cytoplasmatic loop (96.9%) which is pivotal for G protein -coupling and, thus, for the high affinity ternary complex. In comparison, alignment of the systems human and rat gave a lower identity score (93.3%). Furthermore, bovine brains are cheap and easily available in sufficient amounts from slaughterhouses. For the receptor binding studies we dissected the striatal area which is known for a high D_2 receptor density compared to a weak expression of the D_3 subtype [3]. Since the antiparkinsonian drug pramipexole [21] is known for its high selectivity versus D_1, low affinity D_2, 5HT, sigma receptors [16, 22], which are also localized in striata, it should make possible specific and selective labeling of the high affinity D_2 receptor. In our experiments, selectivity of pramipexole high affinity agonist binding over low affinity binding was demonstrated by a competition experiment using [^{3}H]spiperone as a radioligand. Under these conditions, two populations of binding sites in a 30 : 70 ratio were identified. The observed Ki values differed by a factor of 380 (Ki_{high} = 12.4 nM, Ki_{low} = 4700 nM). This gives evidence for a utilization of [^{3}H]pramipexole as a radioligand selectively labeling the high affinity ternary complex. Furthermore, the high D_2/D_1 selectivity of pramipexole was demonstrated by a displacement experiment using the D_1 antagonist [^{3}H]SCH23390 when a specific pramipexole binding could not be observed in ligand concentrations up to 10^{-4} M. In practice, [^{3}H]pramipexole binding was characterized by kinetic studies yielding k_{+1} and k_{-1} values of 6.7×10^7 min^{-1} M^{-1} and 0.12 min^{-1}, respectively. In addition, saturation experiments and homologous competition studies were performed resulting in a K_D of 2.3 nM, and a B_{max} of 560 fmol/ml homogenate and a Hill coefficient of 0.95 indicating high affinity labeling of a single binding site. Agonist binding to D_2 receptors is characterized by binding to a G protein-coupled high affinity state. In presence of the nonhydrolyzable GTP analog GPP(NH)P the high affinity state is destabilized resulting in a strong reduction of specific binding [23]. In contrast, the D_3 receptor is characterized by a weak coupling to G proteins resulting in a insensitivity to addition of GPP(NH)P. Addition of GPP(NH)P to our membrane suspension reduced specific binding to less than 10%. This shows that [^{3}H]pramipexole labeled in fact the D_2 but not the D_3 subtype. This is confirmed by a comparison of K_D values (K_D = 2.3 nM for bovine striatal membranes, K_D = 3.9 nM for heterologously expressed human D_2, K_D = 0.5 for heterologously expressed human D_3). Competition experiments using the representative D_2 agonists (+)-7-OH-DPAT, (−)-3-PPP and (*S*)-7-dipropylaminotetrahydroindolizine showed the same rank order of affinities as described for rat striata labeled with [^{3}H]pramipexole, however, the Ki values turned out to be significantly higher. Using L-prolyl-L-leucyl-glycinamide as an example [5] a significant increase of specific radioligand binding could be measured. Thus, labeling of bovine striatal membranes with [^{3}H]pramipexole facilitates a practical and efficient evaluation of D_2 affinity modulating peptides.

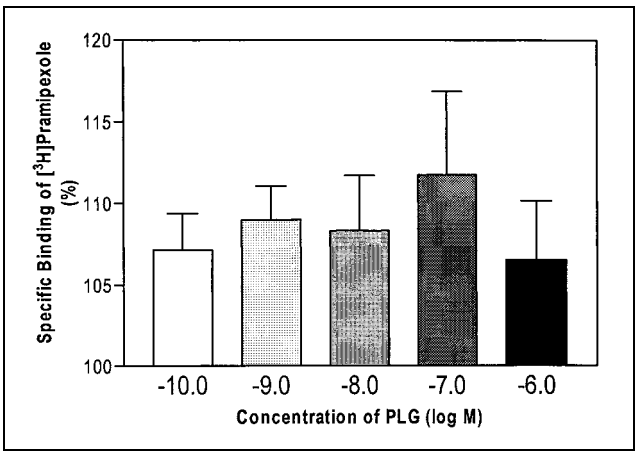

Fig. 5: Increase of specific binding of [^{3}H]pramipexole (0.5 nM) in the presence of increasing concentrations of PLG. (Shown are means ± SEM, n = 5)

4. Experimental

4.1. *Sequence alignment*

Dopamine D_2 receptor sequences (human/bovine/rat) including the classification of functional domains were taken from the GPCR Database at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany [24] (Accession numbers: rat P13953; human P14416; bovine P20288). Alignment and comparison of the entire protein structures and the third

cytoplasmic loops were done with the Needleman-Wunsch algorithm [25] using different homology matrices (default parametrization) as implemented in the SYBYL 6.4 package from Tripos after converting the appropriate loop sequences to PIR-format [26].

4.2. Membrane preparation

Fresh bovine brains were obtained from the local slaughterhouse, the striata were dissected out and stored at −80 °C. All steps were carried out at 4 °C unless otherwise specified. The striata were minced with a scalpel, washed twice with sucrose (2 vol 0.32 M) and then homogenized in a Waring Blendor homogenizer (20 vol sucrose). The resulting suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 1,000 g for 15 min. The supernatant was then centrifuged at 100,000 g for 1 h. The resulting pellet was resuspended with a Potter-Elvehjem homogenizer in Tris-HCl buffer (8 vol of 50 mM) containing 1 mM EDTA (pH 7.4, Tris-EDTA buffer) and centrifuged at 60,000 g for 20 min. The pellet was washed twice with Tris-EDTA buffer, homogenized and centrifuged as described above. Finally the pellet was suspended in Tris-EDTA buffer to give a protein concentration of 7–9 mg/ml, and homogenized with a Potter-Elvehjem homogenizer. The final suspension was stored at −80 °C in small aliquots.
On the day of use, the membranes were thawed, diluted with Tris-EDTA buffer containing $MgCl_2$ (5 mM), Dithiothreitol (0.1 mM), Bacitracin (100 μg/ml) and soybean trypsin inhibitor (5 μg/ml) as an incubation buffer, and used for binding assays.
Protein content was determined according to the method described by Lowry et al. [27] using bovine serum albumin as a standard.

4.3. [^{3}H]Pramipexole binding experiments

Assays were carried out by addition of membrane suspension (∼300 μg protein) to glass tubes containing incubation buffer, the competing ligand and water respectively and [^{3}H]pramipexole (0.5 nM final concentration). For competition experiments a range of concentrations of competitor from 10^{-12} M to 10^{-6} M was incubated with the radiolabeled ligand and membranes in incubation buffer. Non-specific binding was determined in the presence of (+)-butaclamol (1 μM). When the selectivity towards D_3 receptors was determined GPP(NH)P (100 μM) was added. Alternatively, the mixture was heated to 53 °C for 2 min. Samples were incubated for 2 h at 23 °C. Incubation was terminated by rapid filtration through Whatman GF/B filters (using a Brandel Harvester). The filters were rinsed with ice-cold Tris-EDTA buffer (3 × 5 ml), the radioactivity trapped on the filters was determined by placing the filters into a scintillation cocktail (ReadyProtein, Beckman) and counting them in a Beckman scintillation counter (LS 6500).
For saturation experiments aliquots of homogenate (250–300 μg) diluted in incubation buffer were incubated in the presence of 8 concentrations of [^{3}H]pramipexole (0.1 nM to 17 nM). Non-specific binding was determined in the presence of (+)-butaclamol (1 μM). After 2 h at 23 °C incubation was terminated by rapid filtration through Whatman GF/B filters. The filters were rinsed with Tris-EDTA buffer (3 × 5 ml) and counted as described above.
For association experiments [^{3}H]pramipexole (final concentration 0.5 nM) was added to the membrane suspension diluted in incubation buffer and stirred at 23 °C. Aliquots were taken over a period of 2 h and filtered, rinsed and determined as described above.
For dissociation experiments [^{3}H]pramipexole (final concentration 0.5 nM) was incubated with membrane suspension in incubation buffer and stirred for 30 min at 23 °C. Dissociation was started by adding (+)-butaclamol (final concentration 1 μM) to the incubation mixture. Aliquots were taken over a period of 2 h, filtered, rinsed and determined as described above.
The effect of PLG on radioligand binding was followed by including PLG at various concentrations in the assay mixture ([^{3}H]pramipexole 0.5 nM, incubation buffer, membrane suspension ∼300 μg protein). Non-specific binding was determined for each concentration of PLG in the presence of 1 μM (+)-butaclamol. Incubation and filtration were carried out as described for competition experiments.

4.4. [^{3}H]Spiperone binding experiments

[^{3}H]Spiperone binding assays were carried out in analogy to the assays with [^{3}H]pramipexole, using [^{3}H]spiperone in a concentration of 0.5 nM, 130–150 μg protein and 50 nM ketanserin for masking 5HT receptors. Non-specific binding was determined in the presence of (+)-butaclamol (1 μM).

4.5. [^{3}H]SCH23390 binding experiments

[^{3}H]SCH23390 binding assays were carried out in analogy to the assays with [^{3}H]pramipexole, using [^{3}H]SCH23390 in a concentration of 0.3 nM and 130–150 μg protein. The incubation buffer contained Tris (50 mM), NaCl (120 mM), KCl (5 mM). $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM). pH was adjusted to 7.4. Non-specific binding was determined in the presence of (+)-butaclamol (1 μM).

4.6. Data analysis

Data were analyzed using nonlinear regression analysis yielding the half maximum concentration (IC_{50}) and the slope factor n_H (Hill coefficient) for competition experiments. Kinetic experiments yielded the half-life time of association and dissociation $t_{1/2}$ and the rate constants k_{+1} and k_{-1} respectively. For the calculations "PRISM" was used (GraphPad Software San Diego, CA, USA). IC_{50} values were transformed to Ki values according to the equation of Cheng and Prusoff [17]. If not stated otherwise the data are expressed as means ± SEM.

4.7. Chemicals

[^{3}H]Pramipexole (67–76 Ci/mmol) was purchased from Amersham Buchler. [^{3}H]Spiperone (18.5 Ci/mmol) and [^{3}H]SCH23390 (81.4 Ci/mmol) were purchased from NEN Dupont. Pramipexole ((*S*)-2-amino-4,5,6,7-tetrahydro-6-propylaminobenzothiazole dihydrochloride) was a gift from Boehringer Ingelheim, Germany. (−)-3-PPP (*S*(−)-3-(3-Hydroxyphenyl)-*N*-propylpiperidine), 7-OH-DPAT (*R*(+)-2-Dipropylamino-7-hydroxy-1,2,3,4-tetrahydronaphthalene), SCH23390 (*R*(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine), ketanserin (3-[2-[4-(Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1*H*, 3*H*)-quinazolinedione) and (+)-butaclamol were purchased from RBI delivered by Biotrend Cologne, Germany. Bacitracin, soybean trypsin inhibitor, GPP(NH)P, spiperone (8-[4-(4-Fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one) and bovine serum albumin were purchased from Sigma. All other chemicals were of analytical grade and were obtained from commercial sources.

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