Neuroleptic Activity

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Contents

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General Considerations

Neuroleptics have been defined as therapeutics effective against schizophrenia. One has to bear in mind that the effect of certain drugs has not been predicted by pharmacological tests but has been found in clinical trials by serendipity. The clinical discoveries were followed by pharmacological studies in many laboratories (Courvoisier 1956).

Various studies have demonstrated the blockade of postsynaptic catecholamine receptors, especially D_2-receptors, to be the main mode of action of most neuroleptics. Several in vitro methods measure the receptor blockade by neuroleptics.

Pharmacological models in the development of antipsychotic drugs were reviewed by Costall et al. (1991).

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In Vitro Methods

D_1 Receptor Assay: [^{3}H]-SCH 23390 Binding to Rat Striatal Homogenates

Purpose and Rationale

Dopamine receptors are the primary targets in the development of drugs for the treatment of schizophrenia, Parkinson's disease, and Huntington's chorea (Seeman and Van Tol 1994).

Reviews on dopamine receptors and their subtypes were given by Baldessarini and Tarazi (1996; Missale et al. 1998) and by the NC-IUPHAR subcommittee on dopamine receptors (Schwartz et al. 1998).

Multiple dopamine receptors are known. Two groups are most studied, designated as D_1 and D_2. In the group of D_1-like dopamine receptors, the subtypes D_{1A} and D_5/D_{1B} have been described. To D_2-like dopamine receptors belong the D_{2S}, the D_{2L}, the D_3, and the D_4 receptor (Sokoloff et al. 1990; Civelli et al. 1991; Grandy et al. 1991; Van Tol et al. 1991; Lévesque et al. 1992; Baldessarini et al. 1993; Ginrich and Caron 1993; Todd and O'Malley 1993; Waddington and Deveney 1996).

D_1 receptors are positively linked to adenylate cyclase, and the D_2 receptor has been shown to be negatively linked to adenylate cyclase. For typical neuroleptic agents, like butyrophenones, a good correlation was found between D_2 receptor binding and clinically effective doses. Atypical neuroleptics, like clozapine, were found to be potent inhibitors of D_1 and D_4 receptor binding, renewing interest in these receptor types. The compound SCH 23390 was found to be selective for the D_1 receptor.

Procedure

Reagents

[N-Methyl-^{3}H] Sch 23390 (Amersham Lab., specific activity 67–73 Ci/mmol). For IC_{50} determinations, ^{3}HSch 23390 is made up to a concentration of 10 nM and 50 μl is added to each tube. This yields a final concentration of 0.5 nM in the assay.

d-Butaclamol (Ayerst Laboratories). A 1 mM stock solution is made and diluted 1:20.

20 μl are added to three tubes for the determination of nonspecific binding.

For the test compounds α, 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assays ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

Male Wistar rats are decapitated, brains rapidly removed, striata dissected, and weighed. The striata are homogenized in 100 volumes of 0.05 M Tris buffer, pH 7.7, using a Tekmar

homogenizer. The homogenate is centrifuged at 40,000 g for 20 min, and the final pellet is resuspended in the original volume of 0.05 Tris buffer, pH7.7, containing physiological ions (NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM, and $CaCl_2$ 2 mM).

Assay

50 µl	0.5 M Tris buffer, pH7.7, containing physiological ions
380 µl	H_2O
20 µl	Vehicle or butaclamol or appropriate concentration of test compound
50 µl	^{3}H-SCH 23390
500 µl	Tissue suspension

The tubes are incubated at 37 °C for 30 min. The assay is stopped by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. The filter strips are then washed three times with ice-cold 0.05 M Tris buffer, pH7.7, and counted in 10 ml Liquiscint scintillation cocktail.

Evaluation

Specific binding is defined as the difference between total binding and binding in the presence of 1 µM butaclamol. IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the average of duplicate determinations.

Modifications of the Method

Wamsley et al. (1992) recommended the radioactive form of a dopamine antagonist, [^{3}H] SCH39166, as ligand for obtaining selective labeling of D_1 receptors.

Sugamori et al. (1998) characterized the compound NNC 01–0012 as a selective and potent D_{1C} receptor antagonist.

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D_2 Receptor Assay: [3H]-Spiroperidol Binding

Purpose and Rationale

The neuroleptic compound haloperidol has been used as binding ligand to study the activity of other neuroleptics. The use of haloperidol has been superseded by spiroperidol. Dopamine receptor binding assays employing dopaminergic antagonists in mammalian striatal tissue, a dopamine-enriched area of the brain, have been shown to be predictive of in vivo dopamine receptor antagonism and antipsychotic activity. Significant correlations exist between neuroleptic binding affinities and their molar potencies in antagonism of apomorphine- or amphetamine-induced stereotypy, apomorphine-induced emesis in dogs, and antipsychotic activity in man. Spiroperidol is considered to be an antagonist specific for D_2 receptors.

Procedure

Tissue Preparation

Male Wistar rats are decapitated, their corpora striata removed, weighed, and homogenized in 50 volumes of ice-cold 0.05 M Tris buffer, pH7.7. The homogenate is centrifuged at 40,000 g for 15 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 40,000 g. The final pellet is then resuspended in Tris buffer containing physiological salts (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) resulting in a concentration of 10 mg/ml.

Assay

The membrane preparations are incubated with 3H-spiroperidol (0.25 nM) and various concentrations of test drug at 37 °C for 20 min. in a K/Na phosphate buffer (50 mM, pH7.2), followed by

cooling in an ice bath for 45 min. To determine nonspecific binding, samples containing 10 mM (+)-butaclamol are incubated under identical conditions without the test compound.

Bound ligand is separated by rapid filtration through Whatman GF/B glass fiber filters. The filters are washed three times with ice-cold buffer, dried, and shaken thoroughly with 3.5 ml scintillation fluid. Radioactivity is determined in a liquid scintillation counter. Specific binding is defined as the difference between total binding and the binding in the presence of 2.0 mM (+)-butaclamol.

Evaluation
The following parameters are determined:

- Total binding of ^{3}H-spiroperidol
- Nonspecific binding: binding of samples containing 2 mM butaclamol
- Specific binding: total binding nonspecific binding
- Percent inhibition: 100-specific binding as percentage of the control value

IC_{50} values are determined using at least 3–4 different concentrations of the test compound in triplicate. Results are presented as mean $\pm$ standard deviation.

Dissociation constants (K_d) are determined, using ^{3}H-spiroperidol concentrations ranging between 0.1 and 1.0 nM. Ki values (inhibitory constants) are calculated using the following equation:

$$K_i = \frac{IC_{50}}{1 + c/K_d}$$

c = ^{3}H-spiroperidol concentrations used to determine IC_{50}.

Standard values: K_i of haloperidol = 6.0 $\pm$ 1.2 nM.

Modifications of the Method
Two isoforms of the D_2 receptor were found by alternative splicing: the long (D_{2L}) and the short (D_{2S}) isoform (Dal Toso et al. 1989; Giros et al. 1989; Monsma et al. 1989; Itokawa et al. 1996).

Niznik et al. (1985) recommended [^{3}H]-YM-09151–2, a benzamide neuroleptic, as selective ligand for dopamine D2 receptors.

Hall et al. (1985) used [^{3}H]-eticlopride, a substituted benzamide, selective for dopamine D_2 receptors, for in vitro binding studies.

Radioactive ligands for the D_2 and the D_3 receptor were described by Seeman and Schaus (1991), Chumpradit et al. (1994), Booze and Wallace (1995), Gackenheimer et al. (1995), Seeman and van Tol (1995), and Van Vliet et al. (1996).

Vessotskie et al. (1997) characterized binding of [^{125}I]S(−)5-OH-PIPAT to dopamine D_2-like receptors.

Neve et al. (1992) used a special apparatus, the "cytosensor microphysiometer," which measures the rate of proton excretion from cultured cells (McConnell et al. 1991, 1992; Owicki and Parce 1992). In C_6 glioma cells and L fibroblasts expressing recombinant dopamine D_2 receptors, the dopamine D_2 receptor agonist, quinpirole, accelerated the rate of acidification of the medium dose-dependent up to 100 nM quinpirole. The response was inhibited by the D_2 antagonist spiperone. The D_2 receptor-stimulated acidification was due to transport of protons by a Na^+/H^+ antiporter which was verified by the inhibition with amiloride or methylisobutyl amiloride.

The isolated rabbit ear artery was recommended as a useful model to characterize dopamine D_2 agonists and antagonists (Hieble et al. 1985).

Human Recombinant Dopamine D_{2A} and D_{2B} Receptors
Hayes et al. (1992) described functionally distinct human recombinant subtypes of the dopamine D_2 receptor, D_{2A} and D_{2B}.

D_{2A} Receptor Binding
In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D_{2A} receptor is measured.

Twenty mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 2.0 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 mM haloperidol. Membranes

are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

D$_{2B}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D$_{2B}$ receptor is measured.

Fifteen mg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 37 °C. Nonspecific binding is estimated in the presence of 10 mM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

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Dopamine D$_2$ Receptor Autoradiography (^{3}H-Spiperone Binding)

Purpose and Rationale

Autoradiography of ^{3}H-spiperone binding sites using selective labeling conditions permits the visualization of the anatomical locations of D$_2$-dopamine receptors (Palacios et al. 1981). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Altar et al. 1984; 1985). Using autoradiographic techniques, it has been demonstrated that striatal D2 receptors are present on intrinsic neurons (Trugman et al. 1986; Joyce and Marshall 1987) and that the distribution of D$_2$ receptors within the striatum is not homogeneous (Joyce et al. 1985). Anatomically discrete interactions of drugs with D$_2$ receptors can be examined in vitro with inhibition experiments and ex vivo following acute or chronic drug treatment of the whole animal.

Since ^{3}H-spiperone labels serotonin-2 (5-HT$_2$) sites in many brain regions, a masking concentration of a 5-HT$_2$ receptor blocker, e.g., ketanserin, is included to selectively define binding to D$_2$ receptors. This is necessary if the test compound

inhibits 5-HT$_2$ binding or if the brain region of interest has a low D$_2$ receptor density.

The assay is used to determine potential anti-psychotic activity of compounds via direct interaction with the D$_2$ dopamine recognition site in discrete regions of the rat brain.

Procedure

Reagents
1a. 0.5 M Tris + 1.54 M NaCl, pH7.4.
1b. 0.05 M Tris + 0.154 M NaCl, pH7.4.
2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham (TRK.818).

For IC_{50} determinations, ^{3}H-spiperone is prepared at a concentration of 8 nM, and 0.55 ml is added to each slide mailer (yields a final concentration of 0.4 nM in the 11.0 ml assay volume).

For saturation experiments, ^{3}H-spiperone is prepared at a concentration of 20 nM. The final concentrations should range from 0.2 to 1.0 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Sulpiride is obtained from sigma. A stock solution of 5×10^{-4} M is made by dissolving the sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 10 μM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).
4. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin in 0.5 ml 1 N acetic acid and bringing the final volume to 10 ml with distilled water. The tartrate salt is water-soluble. This is further diluted to 5×10^{-6} M (50 μl q.s. to 10 ml). 0.22 ml is added to all mailers.
5. Test compounds (for IC_{50} determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentrations in the assay range from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation
Rat brain sections are collected from plates 9 (rostral nucleus accumbens) through plate 17 (caudal striatum) of *The Rat Brain Atlas in Stereotaxic Coordinates* by Paxinos and Watson.

1. For in vitro inhibition experiments, 3–5 sets of 10 slides are collected with 3–4 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 3–4 sections per slide.
3. For ex vivo inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay
1. Preparation of slide mailers (11.0 ml volume/ slide mailer).

Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover two sections. A proportional adjustment of the volumes to be pipetted is made.
(a) In vitro inhibition experiments

Separate mailers are prepared for total binding, nonspecific binding, and 7–8 concentrations of test compound. Ketanserin is included in all mailers to mask binding of [^{3}H]-spiperone to 5-HT$_2$ sites so that inhibition of binding is D$_2$-selective.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 0.4 nM final concentration
3.96 ml	Distilled water
0.22 ml	Ketanserin, 5×10^{-6} M, final concentration 100 nM or vehicle
0.22 ml	Test compound, final concentration 10^{-8} to 10^{-5} M or sulpiride 5×10^{-4}, final conc. 10 μM or vehicle

(b) Ex vivo inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including ketanserin to mask 5-HT_2 receptor binding.

(c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Ketanserin is not included in the mailers, in saturation experiments, since specific binding is defined as sulpiride-displaceable.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[3H]-spiperone, final concentration 0.2–1.0 nM
4.18 ml	Distilled water
0.22 ml	5×10^{-4} M sulpiride, final concentration 10 µM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH7.4 for 5 min, and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, rinsed in buffer 1b for 2×5 min, and dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter, and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually over night). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrofilm for 14–17 days.

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Binding to the D₃ Receptor

Purpose and Rationale

Sokoloff et al. (1990) reported molecular cloning and characterization of a dopamine receptor (D_3) as a potential target for neuroleptics. The D_3 receptor is localized in limbic areas of the brain which are associated with cognitive, emotional, and endocrine functions. Together with the D_{2S}, the D_{2L}, and the D_4 receptor, the D_3 receptor belongs to the group of D_2-like dopamine

receptors (Ginrich and Caron 1993). 7-[^{3}H] hydroxy-*N*,*N*-di-n-propyi-2-aminotetralin (Lévesque et al. 1992), R(+)-7-OH-DPAT (Baldessarini et al. 1993), and [^{125}I]trans-7-OHPIPAT-A (Kung et al. 1993) have been recommended as ligands for receptor binding studies.

Chio et al. (1993) compared the heterologously expressed D$_3$ dopamine receptors with D$_2$ receptors in Chinese hamster ovary cells.

Damsma et al. (1993) described R-(+)-7-OH-DPAT (R-(+)-7-hydroxy-2-(*N*,*N*-di-n-propylamino)tetralin) as a putative dopamine D$_3$ receptor ligand.

Functional correlates of dopamine D$_3$ receptor activation in the rat in vivo and their modulation by the selective agonist, (+)-S 14297, have been described by Millan et al. (1995).

Isoforms of the D$_3$ receptor have been described (Pagliusi et al. 1993).

Akunne et al. (1995) described binding of the selective dopamine D$_3$ receptor agonist ligand [^{3}H]PD 128907 = 4aR,10bR-(+)-trans-3,4,4a,10b-tetrahydro-4-n-propyl-2H,5H-[1] benzopyrano[4,3-b]1,4-oxazin-9-ol.

Procedure

Human dopamine D$_3$ receptor is expressed in Chinese hamster ovary cells. Cells are grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum. Cells are harvested by trypsin treatment (0.25 %) for 4–5 min and centrifugation at 2000 *g* for 5 min. They are homogenized with a Polytron in 10 mM Tris–HCl (pH7.5) containing 1 mM EDTA and are centrifuged at 35,000 *g* for 15 min. The pellet is then resuspended by sonication in a buffer containing 50 mM NaHepes, 1 mM EDTA, 50 µM 8-hydroxyquinoline, 0.005 % ascorbic acid, and 0.1 % bovine serum albumin (pH7.5) (incubation buffer). Membrane suspensions (15–25 µg protein) are added to polypropylene test tubes containing [^{3}H]7-OH-DPAT (7-[^{3}H] hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin) for the D$_3$ receptor assay. Competing drugs are dissolved in incubation buffer, the final volume being 1 ml. Tubes are incubated in triplicate for 1 h at room temperature. The incubations are stopped by rapid filtration under reduced pressure through Whatman GF/C glass filters coated with 0.1 % bovine serum albumin, followed by three rinses with 3–4 ml ice-cold buffer. Nonspecific binding is measured in the presence of 1 µM dopamine.

Evaluation

Saturation curves are analyzed by computer nonlinear regression using a one-site cooperative model to obtain equilibrium dissociation constants (K_D) and maximal density of receptors (B_{max}). Inhibition constants (K_i) are estimated according to the equation

$$K_i = IC_{50}/1 + L/K_D$$

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Binding to D_4 Receptors

Purpose and Rationale

Van Tol et al. (1991) reported cloning of the gene of a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. Together with the D_{2S}, the D_{2L}, and the D_3 receptor, the D_4 receptor belongs to the group of D_2-like dopamine receptors (Ginrich and Caron 1993). Recognition and characterization of this dopamine binding site may be useful in the design of new types of antipsychotic drugs.

Dopamine D_4 receptors have been localized in GABAergic neurons of the primate brain (Mrzljak et al. 1996).

Procedure

A plasmid construct of a 3.9-kb gene-cDNA hybrid subcloned into the expression vector pCD-PS is introduced into COS-7 cells by calcium phosphate-mediated transfection. Cells are cultivated and homogenized (Teflon pestle) in 50 mM Tris–HCl (pH7.4 at 4 °C) buffer containing 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM KCl, and 120 mM NaCl. Homogenates are centrifuged for 15 min at 39,000 g, and the resulting pellets resuspended in buffer at a concentration of 150–250 μg/ml. For saturation experiments, 0.25 ml of tissue homogenate are incubated in duplicate with increasing concentrations of [^{3}H]-spiperone (70.3 Cl mmol^{-1}; 10–3000 pM final concentration) for 120 min at 22 °C in a total volume of 1 ml. For competition binding experiments, assays are initiated by the addition of 0.25 ml membrane and incubated in duplicate with various concentrations of competing ligands (10^{-14}–10^{-3} M) and [^{3}H]spiperone (150–300 μM) either in the absence or the presence of 200 μM Gpp(NH)p for 120 min at 22 °C. Assays are terminated by rapid filtration through a Titertek cell harvester and filters then monitored for tritium. For all experiments, specific binding is defined as that inhibited by 10 μM (−)sulpiride.

Evaluation

Both saturation and competition binding data are analyzed by the nonlinear least-square curve-fitting program ligand run on a suitable PC.

Modifications of the Method

Human Recombinant Dopamine D$_{4,2}$, D$_{4,4}$, D$_{4,7}$, and D$_5$ Receptors

Van Tol et al. (1992) described multiple dopamine D$_4$ receptor variants in the human population.

Sunahara et al. (1991) reported the cloning of the gene for a human D$_5$ receptor.

Human Recombinant Dopamine D$_{4,2}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D$_{4,2}$ receptor is measured.

Fifteen μg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 μM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine D$_{4,4}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D$_{4,4}$ receptor is measured.

Twenty-five μg of membrane are incubated with [^{3}H]-spiperone at a concentration of 1.0 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 μM haloperidol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine D$_{4,7}$ Receptor Binding

In a radioligand binding assay, the binding of [^{3}H]-spiperone to membranes prepared from COS cells transiently expressing a recombinant human dopamine D$_{4,7}$ receptor is measured.

Fifteen μg of membrane is incubated with [^{3}H]-spiperone at a concentration of 0.7 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 μM haloperidol. Membranes

are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]-spiperone bound.

Human Recombinant Dopamine D$_5$ Receptor

In a radioligand binding assay, the binding of [^{3}H] SCH 23390 to membranes prepared from COS cells expressing a recombinant human dopamine D$_5$ receptor is measured.

First, 40 μg of membrane is incubated with [^{3}H]SCH 23390 at a concentration of 2 nM for 2 h at 25 °C. Nonspecific binding is estimated in the presence of 10 μM *cis*-flupentixol. Membranes are filtered and washed three times with binding buffer, and filters are counted to determine [^{3}H]SCH 23390 bound.

Several selective dopamine D$_4$ antagonists were described: Hidaka et al. (1996), Merchant et al. (1996), Rowley et al. (1996), and Birstow et al. (1997).

Some radioligands were proposed as being selective for dopamine D$_4$ receptors: [^{3}H]clozapine (Ricci et al. 1997a, b), [^{3}H]NGD 94–1 (Thurkauf 1997; Primus et al. 1997), and RBI-257 (Kula et al. 1997).

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Determination of Dopamine Autoreceptor Activity

Purpose and Rationale

The method describes the procedure to determine if a compound possesses autoreceptor blocking activity without the interference from postsynaptic effects. Striatal DOPA (3,4-dihydroxyphenylalanine), DOPAC (3,4-dihydroxyphenylacetic acid), and DA (dopamine) are quantitated following in vivo treatment with drug, apomorphine, gamma butyrolactone, and NSD-1015. Antipsychotic compounds that block striatal dopaminergic presynaptic autoreceptors are believed to possess a greater liability for producing EPS.

Procedure

Reagents

1. 0.1 M HCl
2. 1 N NaOH
3. 0.1 M perchloric acid (PCA) containing 4.3 mM EDTA
4. 2 mM solutions of DOPAC, DA, and DOPA in 0.1 M HCl, with 0.5 ml aliquots stored at −60 °C until use
5. Preparation of 2° standard mixture

   10 µM solution of DOPAC, DA, and DOPA diluted from reagent 4 with 0.1 M PCA/EDTA

   The 2° standard solution is used for the preparation of standard curves.

6. Mobile phase/MeOH-buffer (4: 96, v/v) buffer: 0.012 mM sodium acetate, 0.036 M citric acid, and 152 μM sodium octane sulfonate (mobile phase); methanol/buffer (80 ml + 1920 ml) filtered through a 0.2 pm nylon 66 filter

7. Preparation of dosing solutions

   (a) Apomorphine (2 mg/kg) is prepared in saline containing 1 % Tween 80 + 0.1 % ascorbic acid to prevent oxidation.

   (b) GBL (750 mg/kg) is prepared as a solution in saline containing 1 % Tween 80.

   (c) NSD-1015 (100 mg/kg) is prepared as a solution in saline containing 1 % Tween 80.

HPLC-Instrumentation

Consists of the following:

- Pump, model SP8810 (Spectra Physics)
- Injector, WISP 710B (Waters Associates)
- Detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA)
- Integrator, D-2000 (Hitachi), used as a backup for the data collection/integrator, CS 9000 (IBM) system
- Analytical column: C18-ODS Hypersil, 3 pm, 100×4.6 mm (Shandon)

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation at the predetermined time. The brain is rapidly removed; the striatum is dissected on ice and frozen on dry ice. The tissue is analyzed by HPLC the same day.

Tissue is homogenized in 500 μl 0.1 M PCA/EDTA. The homogenate is centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatant is transferred to 0.2 pm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

Five μl of the striatum homogenate is injected into the HPLC column.

HPLC flow rate is 1.5 ml/min; run time is 20 min. Helium flow is constant in mobile phase.

For protein analysis, 1.0 ml 1 N NaOH is added to the tissue pellet. The next day, the protein analysis is performed as described by Bradford (1976) using the BioRad Assay Kit.

Evaluation

Peak area is used for quantitation. The mg of protein and pmoles of DOPAC, DA, and DOPA are calculated from linear regression analyses using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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Dopamine-Sensitive Adenylate Cyclase in Rat Striatum

Purpose and Rationale

Agonist stimulation of dopamine D_1 receptors leads to increased cAMP formation mediated by a guanine nucleotide-binding regulatory protein. This effect is blocked by selective antagonists like SCH 23390.

Agonist stimulation of the dopamine D_2 receptor leads to a decreased cAMP formation mediated by a guanine nucleotide-binding protein. Apomorphine is a potent agonist with full intrinsic activity at D_2 receptors. Phenothiazines block both D_1 and D_2 receptors, whereas butyrophenones and related drugs are very potent antagonists at D_2 receptors.

Studies on cAMP formation may be useful for differentiation of antipsychotic drugs.

Procedure

Tissue Preparation

Male Wistar rats are sacrificed by decapitation, the brains removed, and the striata dissected out, and weighed. Striatal tissue from two rats is homogenized in 25 volumes of ice-cold 0.08 M Tris-maleate buffer, pH7.4, containing 2 mM EGTA. Protein content of an aliquot is determined. A 50 µl aliquot is used in the cyclase enzyme assay.

Enzyme Assay

The following volumes are placed in conical centrifuge tubes kept in an ice-water bath:

200 µl	Incubation buffer (equal amounts of 0.8 mM Tris-maleate, pH 7.4; 60 mM $MgSO_4$; 100 mM theophylline and 4 mM EGTA)
50 µl	1 mM dopamine HCl or water
25 µl	Test drug or water
125 µl	Distilled water
50 µl	Tissue homogenate

After incubation for 20 min at 0 °C, the enzyme reaction is started by addition of 50 µl of 15 mM ATP solution. The tube rack is placed in a shaking water bath preset at 30 °C for 2.5 min. The reaction is terminated by placing the tube rack in a boiling water bath for 4 min. Then, the tubes are centrifuged at 1000 g for 10 min.

A 25 µl aliquot of the supernatant in each tube is removed and the cAMP determined using a commercial RIA kit (Amersham).

Evaluation

Results are expressed as pmoles cAMP/mg protein of dopamine-stimulated versus nondopamine-stimulated level. Percentage inhibition of this dopamine-stimulated level by test drugs is calculated and IC_{50} values determined by log-probit analysis.

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α_1-Adrenergic Receptor Binding in Brain

Purpose and Rationale

The use of neuroleptic and antidepressant drugs is sometimes limited by their side effects, such as orthostatic hypotension and sedation. These side effects are attributed to blockade of central and peripheral adrenergic α-receptors. For neuroleptics the ratio between their dopamine antagonistic and their receptor antagonistic potencies should be taken into account rather than their absolute α-blocking effect. WB-4101 is a specific and potent antagonist of the α_1-adrenoreceptor, characterized in vitro in rat brain, heart, vascular smooth muscle, and gastrointestinal smooth muscle.

The in vitro [³H]-WB 4101 receptor binding assay quantitates the α-adrenergic blocking properties of psychoactive agents and is used to assess a compound's potential to cause orthostatic hypotension and sedation as well as primary blood pressure lowering effects through α_1-receptor blockade.

Procedure

Reagents

[Phenoxy-3-³H(N)]-WB 4101 = (2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane, New England Nuclear (specific activity 20–35 Ci/mmol).

For IC_{50} determinations, [³H]-WB 4101 is made up to a concentration of 2 nM in Tris buffer and 500 µl is added to each tube (yields a final concentration of 0.5 nM in the 2 ml assay).

L-norepinephrine bitartrate (Sigma Chemical Company). A 800 µM solution is prepared in Tris buffer and 250 µl is added to each of three tubes to determine nonspecific binding. This yields a final concentration of 100 µM in the 2 ml assay.

Test compounds: A 80 µM stock solution is made up in a suitable solvent and serially diluted with Tris buffer, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Usually, seven concentrations are studied for each assay.

Tissue Preparation

Male Wistar rats (100–150 g) are sacrificed by decapitation. The whole brain minus cerebellum is homogenized in 75 volumes of ice-cold 0.05 M Tris buffer, pH7.7. The homogenate is centrifuged at 40,000 g at 4 °C for 15 min. The supernatant is discarded and the pellet is rehomogenized in fresh Tris buffer and recentrifuged at 40,000 g at 4 °C for 15 min. The final pellet is resuspended in the original volume of ice-cold 0.05 M Tris buffer. The final tissue concentration in the assay is 10 mg/ml. Specific binding is approximately 80 % of total bound ligand.

Assay

1200 µl	Tissue suspension
500 µl	3H-WB 4101
250 µl	Vehicle (for total binding) or
800 µM	L-norepinephrine bitartrate (for nonspecific binding) or appropriate drug concentration

Sample tubes are kept in ice for additions, then vortexed, and incubated for 15 min at 25 °C. The binding is terminated by rapid vacuum filtration through Whatman GF/B filters, followed by three 5 ml washes with ice-cold 0.05 M Tris buffer. The filters are counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation

Specific WB 4101 binding is defined as the difference between the total binding and that bound in the presence of 100 µM norepinephrine. IC_{50} calculations are performed using computer-derived log-probit analysis.

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[^{3}H]Spiroperidol Binding to 5-HT$_2$ Receptors in Rat Cerebral Cortex

Purpose and Rationale

The purpose of this assay is to determine the anti-serotonin activity of neuroleptics, antidepressants, and antihypertensive compounds, by measuring the displacement of [^{3}H]spiroperidol from serotonergic antagonist binding sites in cerebral cortical membranes. The regulation of 5-HT2 receptor density by chronic antidepressant treatment is discussed in a separate protocol (see chapter "▶ Antidepressant Activity").

The receptor binding of serotonergic sites in the CNS has been investigated using [^{3}H]serotonin (5-HT) (Bennett and Snyder 1976), [^{3}H]LSD (Peroutka and Snyder 1979), and [^{3}H] spiroperidol (Peroutka and Snyder 1979; List and Seeman 1981; Leysen et al. 1978) as the radioligand. Receptor sites have been defined kinetically and classified as 5-HT$_1$ sites (labeled by [^{3}H]5-HT and displaced by agonists) and 5-HT$_2$ sites (labeled by [^{3}H]-spiroperidol and displaced by antagonists). [^{3}H]LSD labels both 5-HT1 and 5-HT2 binding sites (Peroutka and Snyder 1979). Of the brain regions tested, the frontal cerebral cortex contained the greatest density of 5-HT2 binding sites. Lesioning studies indicate that 5-HT$_2$ binding sites are postsynaptic and not linked to adenyiate cyciase (Peroutka et al. 1979).

The inhibition of 5-HT$_2$ binding correlates with the inhibition of quipazine-induced head twitch, which may reflect decreased behavioral excitation. The physiological and pharmacological role of these receptors is not clear. Although numerous neuroleptics and antidepressants of varying chemical structures are potent inhibitors of 5-HT$_2$ binding, there is no clear-cut relationship to the efficacy of these drugs. Methysergide and cyproheptadine are both potent inhibitors of 5-HT$_2$ binding without having neuroleptic or antidepressant effects. However, potent interaction with 5-HT$_2$ receptors may indicate a reduced potential for catalepsy, since methysergide blocks catalepsy induced by haloperidol (Rastogi et al. 1981). The interaction of serotonergic neurons with cholinergic neurons in the striatum (Samanin et al. 1978) may also be decreased by potent 5-HT2 antagonists. In addition, the ratio of activity at D2 and 5-HT2 receptors may be useful in the screening of atypical antipsychotic agents (Meltzer et al. 1989). Furthermore, it has been shown that ketanserin, a selective 5-HT$_2$ antagonist, is an effective hypotensive agent which blocks peripheral vascular 5-HT receptors.

5-HT$_2$ receptors have been subdivided into 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. The new 5-HT receptor classification has been

published by the VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin) (Hoyer et al. 1994). Further comments were given by Humphrey et al. (1993), Martin and Humphrey (1994), Saxena (1994), and Tricklebank (1996).

Several compounds with HT_{2A} antagonistic activity are described, such as trazodone (Clements-Jewery et al. 1980; Hingtgen et al. 1984; Stryjer et al. 2003), MDL 100,907 (Kehne et al. 1996; Moser et al. 1996), and sarpogrelate (Hayashi et al. 2003).

McCullough et al. (2006) described the $5\text{-}HT_{2B}$ antagonist and $5\text{-}HT_4$ agonist activities of tegaserod in the anesthetized rat.

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.7
   (a) 57.2 g Tris–HCl.
       16.2 g Tris base
       q.s. to 1 l (0.5 M Tris buffer, pH7.7)
   (b) Make a 1:10 dilution in distilled H_2O (0.05 M Tris buffer, pH7.7).
2. Tris buffer containing physiological ions
   (a) Stock buffer

NaCl	7.014 g
KCl	0.372 g
CaCl$_2$	0.222 g
MgCl$_2$	0.204 g

   q.s. to 100 ml in 0.5 M Tris buffer.
   (b) Dilute 1:10 in distilled H_2O.
   (c) This yields 0.05 M Tris–HCl, pH7.7; containing NaCl (120 mM), KCl (5 mM), CaCl$_2$ (2 mM), and MgCl$_2$ (1 mM).
3. [Benzene-^{3}H] spiroperidol (20–35 Ci/mmol) is obtained from New England Nuclear. For IC_{50} determinations, ^{3}H-spiroperidol is made up to a concentration of 30 nM in 0.01 N HCl and 50 μl added to each tube (yields a final concentration of 1.5 nM in the 1 ml assay).
4. Methysergide maleate is obtained from Sandoz. Methysergide maleate stock solution is made up to 0.25 mM for determination of nonspecific binding. The final concentration in

the assay is 5 μM, when 20 μl of the stock solution is added to the reaction tube.
5. Test compounds. For most assays, α 1 mM stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay, and higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Male Wistar rats are decapitated, and the cerebral cortical tissue is dissected, weighed, and homogenized in 50 volumes of 0.05 M Tris buffer, pH 7.7 (buffer 1b) with the Brinkman Polytron and then centrifuged at 40,000 g for 15 min. The supernatant is discarded and the pellet resuspended and recentrifuged as described above. This pellet is resuspended in 50 volumes of buffer 2b and stored in an ice bath. The final tissue concentration is 10 mg/ml. Specific binding is 7 % of the total added ligand and 50 % of total bound ligand.

Assay

50 μl	0.5 M Tris-physiological salts (buffer 2a)
380 μl	H_2O
20 μl	Vehicle (for total binding) or 0.25 mM methysergide (for nonspecific binding) or appropriate drug concentration
50 μl	[^{3}H] spiroperidol
500 μl	Tissue suspension

The samples are incubated for 10 min at 37 °C and then immediately filtered under reduced pressure using Whatman GF/B filters. The filters are washed with three 5 ml volumes of ice-cold 0.05 M Tris buffer, pH 7.7 mM methysergide.

Evaluation

IC_{50} calculations are performed using log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

Modification of the Method

The receptor binding properties of the 5-HT$_2$ antagonist ritanserin were reported by Leysen et al. (1985).

Preclinical characterization of a putative antipsychotic as a potent 5-HT$_{2A}$ antagonist was reported by Kehne et al. (1996).

Using [^{125}I]LSD and [^{3}H]5-HT binding assays, Siegel et al. (1996) characterized a structural class of 5-HT$_2$ receptor ligands.

[^{3}H]Ketanserin has been described as a selective ^{3}H-ligand for 5-HT$_2$ receptor binding sites (Leysen et al. 1981).

[^{3}H]RP 62203, a potent and selective 5-HT$_2$ antagonist, was recommended for in vivo labeling of 5-HT$_2$ receptors (Fajolles et al. 1992).

Other selective 5-HT$_2$ receptor radioligands were recommended:

[^{125}I]-EIL (radioiodinated D-(+)-N1-ethyl-2-iodolysergic acid diethylamide) (Lever et al. 1991); [^{3}H]MDL100,907 (Lopez-Gimenez et al. 1998).

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Serotonin 5-HT$_2$ Receptor Autoradiography (^{3}H-Spiperone Binding)

Purpose and Rationale

Autoradiography of ^{3}H-spiperone binding sites with selective labeling conditions permits the visualization of the anatomical locations of 5-HT$_2$ receptors (Palacios et al. 1981; Pazos et al. 1985; Altar et al. 1985). Quantitative measurements of the binding to receptors can be obtained with computer-assisted video analysis of the autoradiograms with a greater anatomical resolution and sensitivity than in membrane homogenates (Pazos et al. 1985; Altar et al. 1984). Using autoradiographic techniques, it has been demonstrated that there is a heterogeneous distribution of 5-HT$_2$ receptors, with much higher levels in telencephalic areas such as the neocortex and the claustrum than in meso- or metencephalic areas. Within the cortex, 5-HT$_2$ receptors are abundant in layers IV and V (Pazos et al. 1985). The high concentration of 5-HT$_2$ receptors in the frontoparietal motor area and the claustrum which connects to the motor cortex and other motor areas suggests a physiological role for

5-HT$_2$ receptors in some motor syndromes (Cadet et al. 1987; Costall et al. 1975; Kostowski et al. 1972). The high affinity of the atypical antipsychotic clozapine for 5-HT$_2$ receptors (Fink et al. 1984; Altar et al. 1986) and the downregulation of 5-HT$_2$ receptors following chronic administration of clozapine (Reynolds et al. 1983; Lee and Tang 1984; Wilmot and Szczepanik 1989) suggest that 5-HT$_2$ receptor interaction may be a significant factor in the lack of extrapyramidal side effects and tardive dyskinesias with its clinical use.

Since ^{3}H-spiperone labels a_1-noradrenergic sites in the cerebral cortex, a masking concentration of the a_1-blocker prazosin is included to selectively define binding to 5-HT$_2$ receptors (Morgan et al. 1984). This is necessary if the test compound also inhibits $a1$receptors which may be present in the brain region of interest.

The assay is used to determine the direct interaction of potential antipsychotic compounds with the serotonin-5-HT$_2$ recognition site in discrete regions of the rat brain either in vitro or after ex vivo treatment of the whole animal.

Procedure

Reagents

1a. 0.5 M Tris + 1.54 M NaCl, pH7.4.
1b. 0.05 M Tris + 0.154 M NaCl, pH7.4.
2. ^{3}H-spiperone (specific activity 70–90 Ci/mmol) is obtained from Amersham.
   – For IC_{50} determinations, ^{3}H-spiperone is made up to a concentration of 20 nM, and 0.55 ml is added to each slide mailer (yields a final concentration of 1.0 nM in the 11.0 ml assay volume).
   – For saturation experiments, ^{3}H-spiperone is made up to a concentration of 20 nM. The final concentration should range from 0.5 to 2.5 nM. Typically, six concentrations are used by adding 0.55 ml or less to each mailer (for smaller volumes, add water to bring total addition of 0.55 ml).
3. Methysergide is used to determine nonspecific binding in brain sections of the frontal cortex. Methysergide maleate is obtained from Sandoz. A stock solution of 2.5×10^{-4} M is

made by dissolving in distilled water. A volume of 0.22 ml of the stock solution is added to the nonspecific binding slide mailers (final concentration 5 μM). All other mailers receive 0.22 ml of vehicle (1 ml of 0.01 N acetic acid in a final volume of 10 ml with distilled water).

4. Ketanserin is used to determine nonspecific binding in those slide mailers containing sections with the nucleus accumbens and striatum. Ketanserin (free base or tartrate salt) is obtained from Janssen. A stock solution of 10^{-3} M is made by dissolving the ketanserin (free base) in 0.05 N acetic acid or the tartrate salt in distilled water. This is further diluted to 5×10^{-6} M (50 μM q.s 10 ml with distilled water). A volume of 0.22 ml is added to the slide mailers to give a final concentration of 100 nM.

5. Prazosin is used to mask α_1-receptors in cortical brain section.

   Prazosin HCl is obtained from Pfizer. A stock solution of 10^{-4} M is made by dissolving prazosin in 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. This is further diluted to 5×10^{-6} M (100 μM q.s 10 ml). A volume of 0.22 ml is added to those slide mailers to be used for cortical brain sections to give a final concentration of 100 nM.

6. Sulpiride is used to mask D_2 receptor binding in brain sections from the nucleus accumbens and striatum.

   Sulpiride is obtained from sigma. A stock solution of 10^{-4} M is made by dissolving sulpiride in 1.0 ml of 0.01 N acetic acid and bringing the final volume to 10 ml with distilled water. A volume of 0.22 ml is added to the appropriate slide mailers to give a final concentration of 10 μM.

7. Test compounds (for in vitro IC_{50} determinations). For most assays, a 5×10^{-3} M stock solution is made up in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Seven concentrations are used for each assay. Higher or lower concentrations may be used depending on the potency of the drug.

Tissue Preparation

Frontal cortical brain sections are collected from plates 5 through 8, and nucleus accumbens/striatal sections are collected from plates 9 (rostral n. accumbens) through plate 17 (caudal striatum) of "The Rat Brain Atlas in Stereotaxic Coordinates" by Paxinos and Watson.

1. For in vitro inhibition experiments, 3–5 sets of 10 slides are collected with 4–5 sections per slide.
2. For saturation experiments, 3–5 sets of 12 slides are collected with 4–5 six sections per slide.
3. For ex vivo inhibition experiments, a set of 8 slides is used, 4 for total binding and 4 for nonspecific binding.
4. For experiments in which the tissue sections will be swabbed and counted with scintillation fluid, two sections per slide are collected.

Assay

1. Preparation of slide mailers (11.0 ml volume/slide mailer).

   Note: If slides with sections are to be wiped for scintillation counting, a final volume of 6.5 ml is sufficient to cover two sections. A proportional adjustment of the volumes to be pipetted is made.

   (a) In vitro inhibition experiments

      Separate mailers are prepared for total binding, nonspecific binding, and 7–8 concentrations of test compound.

      1. For frontal cortical brain sections, prazosin is included in all mailers to mask the binding of [^{3}H]-spiperone to α_1-receptors, and nonspecific binding is defined with 5 μM methysergide.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 1.0 nM final concentration
3.96 ml	Distilled water
0.22 ml	Prazosin 5×10^{-6} M, final concentration 100 nM or vehicle
0.22 ml	Test compound, final concentration 10^{-8}–10^{-5} M or methysergide 2.5×10^{-4} M, final concentration 5 μM or vehicle

2. For brain sections with the nucleus accumbens and striatum in which there is negligible binding of [^{3}H]-spiperone to α_1-receptors, prazosin is not included. Since levels of 5-HT$_2$ receptors in these brain areas are low, 10 μM sulpiride is included in all mailers to mask the binding of [^{3}H]-spiperone to D$_2$ receptors.

Ketanserin, final concentration of 100 nM, is used to determine nonspecific binding since methysergide has a weak affinity for D$_2$ receptors (IC_{50} approximately 1–5 μM).

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, 1.0 nM final concentration
3.96 ml	Distilled water
0.22 ml	Sulpiride 5×10^{-4} M, final concentration 10 μM or vehicle
0.22 ml	Test compound, final concentration 10^{-8} to 10^{-5} M or ketanserin 5×10^{-5} M, final concentration 100 nM or vehicle

(b) Ex vivo inhibition experiments

Separate mailers are prepared for total and nonspecific binding, as described above, including sulpiride to mask D$_2$ receptor binding with brain sections through the nucleus accumbens and striatum and prazosin to mask α_1-receptors in cortical brain sections.

(c) Saturation experiments

Separate mailers are prepared for total and nonspecific binding at each radioligand concentration. Prazosin is not included in the mailers in saturation experiments, since specific binding is defined by methysergide which has negligible affinity for α_1-receptors.

5.50 ml	Buffer 1b
0.55 ml	Buffer 1a
0.55 ml	[^{3}H]-spiperone, final concentrations 0.5–2.5 nM
4.18 ml	Distilled water
0.22 ml	2.5×10^{-4} M methysergide, final concentration 5 μM or vehicle

2. Slides are air-dried for 10–15 min at room temperature, preincubated in 0.05 M Tris + 0.154 M NaCl, pH7.4 for 5 min, and further incubated for 60 min with [^{3}H]-spiperone. Slides are then rinsed with ice-cold solutions as follows: dipped in buffer 1b, 2×5 min rinsed in buffer 1b, dipped in distilled water.

Slides used for wipes: both sections are wiped with one Whatman GF/B filter, and radioactivity is counted after addition of 10 ml of scintillation fluid. Slides used for autoradiography: slides are dried under a stream of air at room temperature and are stored in a desiccator under vacuum at room temperature (usually overnight). Slides are then mounted onto boards, along with ^{3}H-standards (Amersham RPA 506).

In the dark room under safelight illumination (Kodak GBX-2 filter), slides are exposed to Amersham Hyperfilm or LKB Ultrafilm for 14–17 days.

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Binding to the Sigma Receptor

Purpose and Rationale

Sigma receptors, as a class of binding sites in the brain, were originally described as a subtype of the opiate receptors. Efforts to develop less addicting opiate analgesics led to the study of several benzomorphan derivatives which produce analgesia without causing the classical morphine-induced euphoria. Unfortunately, these compounds, like *N*-allylnormetazocine (SKF 10,047), produced a variety of psychotic symptoms. This psychotomimetic effect is thought to be mediated by sigma receptors. This binding site is sensitive to many neuroleptics, most notably the typical antipsychotic haloperidol, leading to the hypothesis that drug interactions with the sigma site may be a new approach for the discovery of novel antipsychotics which are not dopamine receptor antagonists. D$_2$ receptor antagonism is thought to be linked with the occurrence of extrapyramidal symptoms in the form of hyperkinesia and Parkinson symptoms or tardive dyskinesia, limiting the therapeutic use of traditional antipsychotic medication. It is hoped that ligands to the sigma receptor do not produce these adverse reactions. The sigma site is believed to be distinct from the binding site for the psychotomimetic drug phencyclidine.

Procedure

Reagents

(+)-SKF 10,047 is prepared as a stock solution of 5×10^{-3} M with distilled water. 130 µl added to the 6.5 ml assay yields a final concentration of 10^{-4} M.

³H-(+)-SKF 10,047 (specific activity 40 Ci/mmol) is obtained from New England Nuclear. A 200 nM stock solution is made up with distilled water for IC_{50} determinations. 325 µl added to each tube yields a final concentration of 10 nM in the 6.5 ml assay.

Test Compounds

A 5 mM stock solution is prepared in a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M.

Tissue Preparation

The assay utilizes slide-mounted cross sections of brain tissue from male Hartley guinea pigs. Whole brain sections of 10 pm thickness are obtained from the hippocampus, thaw-mounted onto gel-chrome alum subbed slides, freeze-dried, and stored at −70 °C until use. On the day of the assay,

the sections are thawed briefly at room temperature until the slides are dry and then used in the assay at a final volume of 6.5 ml.

Assay

Incubation solutions are prepared in plastic slide mailer containers as follows:

3.250 ml	0.05 M Tris buffer, pH 7.7
2.470 ml	Distilled water
0.325 ml	0.5 M Tris buffer, pH 7.7
0.130 ml	(+)-SKF 10,047 or vehicle
0.325 ml	[^{3}H](+)-SKF 10,047

Dried slides with tissue sections are added to the slide mailers and incubated at room temperature for 90 min. Non-bound radioligand is removed by rinsing the slides sequentially in two 5-min rinses in ice-cold 0.05 M Tris buffer and a dip in ice-cold distilled water. The sections are either swabbed with Whatman GF/B filters for scintillation counting of tissue-bound radioligand or exposed to tritium-sensitive film for autoradiography of the binding sites.

Evaluation

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M (+)SKF 10,047 and is typically 60–70 % of total binding. IC_{50} values for the competing drug are calculated by log-probit analysis of the data.

Modifications of the Method

[^{3}H]-(+)-pentazocine has been recommended as a highly potent and selective radioligand for μ receptors (de Costa et al. 1989; DeHaven-Hudkins et al. 1992).

Classification of sigma binding sites into α_1 and α_2 receptors has been proposed (Walker et al. 1990; Quirion et al. 1992; Abou-Gharbia et al. 1993).

Hashimoto and London (1993) characterized [^{3}H]ifenprodil binding to σ_2 receptors in rat brain.

Ganapathy et al. (1999) provided evidence for the expression of the type 1 sigma receptor in the Jurkat human T lymphocyte cell line.

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Simultaneous Determination of Norepinephrine, Dopamine, DOPAC, HVA, HIAA, and 5-HT from Rat Brain Areas

Purpose and Rationale

To measure the effects of potential antipsychotic drugs on catecholamines and indols, a quantitative method for the determination of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindolacetic acid (5HIAA), and 5-hydroxytryptamine (5-HT) from rat brain regions is used. These catecholamines and indols are measured in rat brain prefrontal cortex, nucleus accumbens, and striatum.

Procedure

Reagents

1. 0.1 M HCl.
2. 1 N NaOH.
3. 2 mM solutions of DOPAC, DA, and DOPA in 0.1 M HCl;
   0.5 ml aliquots are stored at −60 °C until use.
4. Preparation of 2° standard mixture
   – 10 µM solution of NE, DOPAC, DA, HVA, 5HIAA, and 5-HT (diluted from reagent 3) in mobile phase (reagent 5).

– The 2° standard solution is used for the preparation of standard curves.

5. Mobile phase/MeOH: buffer (7.5:92.5, v/v).

– Buffer: 0.07 M sodium acetate, 0.04 M citric acid, 130 μM EDTA, and 230 μM sodium octane sulfonate

– Mobile phase: methanol/buffer (150 ml + 1850 ml) is filtered through a 0.2 pm nylon 66 filter.

HMLC-Instrumentation

- Pump, model SP8810 (Spectra Physics).
- Injector, WISP 710B (Waters Associates).
- Detector, 5100A electrochemical with a 5011 analytical cell and 5020 guard cell (ESA).
- Integrator, D-2000 (Hitachi), used as a backup for the data collection/integrator, CS 9000 (IBM) system.
- Analytical column: C18-ODS Hypersil, 3 pm, 100 × 4.6 mm (Shandon).

Animal Treatment

Six rats per group (150–250 g) are dosed with 4–5 different concentrations of the putative antipsychotic drug; usual concentrations range from 0.03 to 30 mg/kg. At a predetermined time, usually 60 min, the rats are sacrificed.

Tissue Preparation

Following treatment with test drug, rats are sacrificed by decapitation. The brain is rapidly removed and placed on ice. The striatum, nucleus accumbens, and prefrontal cortex are dissected and placed in 1.5 ml microcentrifuge tubes. The tubes are capped and immediately placed in dry ice. The frozen brain sections are stored at −60 °C until HPLC analysis.

Tissue is homogenized in mobile phase (striatum, in 600 μl, nucleus accumbens and prefrontal cortex, in 300 μl). The homogenates are centrifuged for 6 min using a microcentrifuge (model 5413, Eppendorf). The supernatants are transferred to 0.2 pm microfilterfuge™ tubes and centrifuged for 6–8 min as before. The filtrate is transferred to WISP vials. Standards are included every 12–15 samples.

The following volumes are injected to the HPLC column:

- Striatum, 5 μl; nucleus accumbens, 20 μl; prefrontal cortex, 50 μl.
- HPLC flow rate is 1.0 mi/min; run time is 25 min.

  Helium flow is constant in mobile phase.

For protein analysis, 1 N NaOH is added to the tissue pellets as follows:

- Striatum: 1.0 ml
- Nucleus accumbens and prefrontal cortex: 0.5 ml

The next day, the protein analysis is run in duplicate with 5 μl of striatum and 20 μl of nucleus accumbens and prefrontal cortex as described by Bradford (1976) using the BioRad Assay Kit.

Evaluation

Peak area is used for quantitation. The mg of protein and pmoles of NE, DOPAC, DA, HVA, 5HIAA, and 5-HT are calculated from linear regression analysis using the corresponding standard curve. Final data are reported as pmoles/mg protein.

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Measurement of Neurotransmitters by Intracranial Microdialysis

Purpose and Rationale

Methods to measure neurotransmitters and their metabolites in specific areas of the brain by microdialysis were introduced by Ungerstedt and his group (Ungerstedt et al. 1982; Zetterström et al. 1982, 1983; Zetterström and Ungerstedt 1983; Ungerstedt 1984; Stähle et al. 1991; Lindefors et al. 1989; Amberg and Lindefors (1989) and by Imperato and di Chiara 1984, 1985). In brain dialysis, a fine capillary fiber is implanted in a selected brain area. Low molecular weight compounds diffuse down their concentration gradients from the brain extracellular fluid into a physiological salt solution that flows through the capillary fiber at a constant rate. The fluid is collected and analyzed.

Procedure

Several designs of dialysis probes have been used (Santiago and Westerink 1990; Kendrick 1991):

1. *Horizontal Probe*

    A straight tube (Vita Fiber, 3 × 50 Amicon) with an outer diameter of 0.34 mm and a molecular weight cutoff of 50,000 is used. The outer surface of the tube is porous and can easily be sealed by epoxy which is applied by passing the tube through a droplet of epoxy and then through a narrow hole corresponding to the outer diameter of the tube. The wall of the tube is sealed in this way except for the area where the dialysis is intended to take place. The length of this region can be varied from 2 to 8 mm depending upon which structure of the brain will be perfused. During the coating and all other handling of the tube, it is supported by a thin tungsten or steel wire inserted into its lumen. One end of the tube is glued into a steel cannula (6 mm long, outer diameter 0.64 mm).

    Male Sprague Dawley rats weighing 250–300 g are anesthetized with halothane and held in a stereotactic instrument. The animals are maintained under halothane anesthesia during the entire experiment.

    Holes are drilled bilaterally (5.7 mm below and 1.5 mm in front of bregma) in the temporal bones after the temporal muscles have been retracted from the bones and folded away.

    During the implantation, the cannula is held by the micromanipulator of the stereotactic instrument, and the dialysis tube is passed horizontally through the brain through the holes drilled on both sides of the skull. A polyethylene tubing carrying the perfusion fluid is connected to the steel cannula. The perfusate is collected at the other end.

2. *Loop Probe*

    The probe is made of a flexible cellulosic tubing (Dow 50, outer diameter 0.25 mm).

Both ends of the tube are inserted into 0.64 mm diameter steel tubes, one of which is bent in an angle. A very thin microsuture (0.1 mm in diameter) is inserted into the tube and positioned half between the steel tubes. Before implantation, the tube is moistened and bent in such a way that the two steel tubes are held closely together in the micromanipulator of the stereotactic instrument. A tungsten wire is inserted into the straight steel tube and passed down the lumen of the dialysis tube in order to stretch it and make it rigid enough to be implanted into the brain. The tube is implanted vertically, and the steel cannulae are attached to the skull by dental cement. The tungsten wire is removed before starting the experiment. The cellulosic tube is flexible enough to withstand the bending at the lower end. The microsuture keeps the bend open.

Loop-shaped or U-shaped microdialysis probes have been used by several authors, e. g., Ichikawa and Meltzer (1990), Jordan et al. (1994), Westerink and Tuinte (1985), and Auerbach et al. (1994).

3. *Vertical Probe*

The probe is sealed at one end by epoxy. The other end is glued into a 0.64-mm-diameter steel tube. A thin inner cannula made of a steel tube or a glass capillary carries the fluid to the bottom of the dialysis tube where it leaves the inner capillary and flows upwards and leaves the probe by a lateral tube. This vertical probe can also be coated with epoxy. It is especially suited for reaching ventral parts of the brain and performing dialysis in small nuclei of the brain.

A similar device has been described for continuous plasma sampling in freely moving rats by Chen and Steger (1993).

Most of the commercially available microdialysis probes are based on this principle.

4. *Commercially Available Microdialysis Probes*

The microdialysis probes CMA/10, manufactured by Carnegie Medicine, Stockholm, Sweden, consist of a tubular membrane (polycarbonate; length: 3 mm; outside diameter: 0.50 mm; and inside diameter: 0.44 mm)

glued to a cannula (outside diameter, 0.60 mm) and sealed with a glue at the tip (Stähle et al. 1991). The perfusion medium is carried to the dialyzing part of the probe by a thin cannula inside the probe. The medium leaves the inner cannula through two holes, flows back between the membrane and the inner cannula, and is collected at the outlet of the probe. The perfusion medium is delivered by means of a high precision microsyringe pump.

This probe was used by several authors, e. g., Wood et al. (1988), Benveniste et al. (1989), Rollema et al. (1989), Scheller and Kolb (1991), Wang et al. (1993), Kreiss and Lucki (1995), and Fink-Jensen et al. (1996).

CMA/11 probes were used by Boschi et al. (1995), Romero et al. (1996), and Gobert et al. (1997).

Dialysis fibers with a semipermeable membrane AN 69-HF, Hospal-Dasco, Bologna, Italy, were used by de Boer et al. (1994), Rayevsky et al. (1995), Arborelius et al. (1996), Gainetdinov et al. (1996), and Tanda et al. (1996).

Evaluation

Samples of the dialyzate are collected for different time intervals and analyzed for neurotransmitters. For the **evaluation of neuroleptics**, most authors measured dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC), and homovanillinic acid (HVA) by HPLC using appropriate detectors. See and Lynch (1996) analyzed dialysis samples for glutamate and GABA concentrations.

For the **evaluation of antidepressants**, the concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxy indole acetic acid (5-HIAA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), or noradrenaline (NA) were measured in the effluent by HPLC. Wood et al. (1988) and Egan et al. (1996) used 3-methoxytyramine accumulation as an index of dopamine release.

Critical Assessment of the Method

The results obtained from brain dialysis depend on at least three variables: type of probe, post-implantation interval, and whether

anesthetized or freely moving animals are used (Di Chiara 1990).

Several authors analyzed the diffusion processes underlying the microdialysis technique and described the limitations of the experiments (Jacobson et al. 1985; Amberg and Lindefors 1989; Benveniste et al. 1989; Scheller and Kolb 1991; Le Quellec et al. 1995).

As a matter of fact, brain microdialysis has been used for the evaluation of many drugs in various indications, such as:

- For neuroleptics by Ichikawa and Meltzer (1990), Meil and See (1994), Hernandez and Hoebei (1994), See et al. (1995), Schmidt and Fadayel (1995), Semba et al. (1995), Rayevsky et al. (1995), Fink-Jensen et al. (1996), See and Lynch (1996), Gainetdinov et al. (1996), Egan et al. (1996), and Klitenick et al. (1996)
- For **antidepressants** by de Boer et al. (1994), Jordan et al. (1994), Arborelius et al. (1996), Ascher et al. (1995), Auerbach et al. (1994), de Boer (1995, 1996), Casanovas and Artigas (1996), Gobert et al. (1997), Ichikawa and Meltzer (1995), Kreiss and Lucki (1995), Petty et al. (1996), Potter (1996), Romero et al. (1996), Sharp et al. (1996), and Tanda et al. (1996a, b)
- For studies in **Parkinson** models by Rollema et al. (1989) and Parsons et al. (1991)

Modifications of the Method

Ferraro et al. (1990) continuously monitored ethanol levels in the brain by microdialysis.

Hernandez and Hoebei (1994) performed simultaneous cortical, accumbens, and striatal microdialysis in freely moving rats.

Hegarty and Vogel (1995) assayed dopamine, DOPAC, and HVA in the brain of rats after acute and chronic diazepam treatment and immobilization stress.

Casanovas and Artigas (1996) implanted microdialysis probes simultaneously in six different brain areas of rats (frontal cortex, dorsal striatum, ventral hippocampus, dorsal hippocampus, dorsal raphe nucleus, median raphe nucleus).

Beneviste et al. (1984) determined extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis.

Boschi et al. (1995) showed that microdialysis of small brain areas in mice is feasible using the smallest commercially available probes.

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Use of Push–Pull Cannulae to Determine the Release of Endogenous Neurotransmitters

Purpose and Rationale

Originally reported by Gaddum (1961), the push–pull cannula has become recognized and utilized as a powerful tool in conjunction with sufficiently sensitive assays to measure low levels of neuroregulator release in distinct brain areas in vivo (Philippu 1984).

This method has been used for various purposes, e.g.:

- To perfuse the ventricles of the brain with drugs or to determine the release of labeled or endogenous compounds in the CSF (Bhattacharya and Feldberg 1958; Korf et al. 1976)
- To perfuse distinct brain areas with drugs and to study their effects on functions of the central nervous system (Myers et al. 1976; Bhargava et al. 1978; Ruwe and Myers 1978)
- To inject labeled monoamines or amino acids and to investigate the resting or induced release of radioactive compounds and their metabolites (Sulser et al. 1969; Strada and Sulser 1971; Kondo and Iwatsubo 1978)

- To perfuse distinct brain areas with labeled transmitter precursors and to determine the patterns of release of the newly synthesized transmitters (Philippu et al. 1974; Chéramy et al. 1977; Nieoullon et al. 1977; Gauchy et al. 1980)
- To perfuse distinct brain areas of anesthetized and conscious animals and to determine the release of endogenous neurotransmitters in the perfusate (Dluzen and Ramirez 1991)

Procedure

The superfusion of the hypothalamus of the conscious, freely moving rabbit has been described by Philippu et al. (1981) and Philippu (1984). Rabbits of both sexes are anesthetized with 40 mg/kg sodium pentobarbital i.p. Guide cannulae are mounted on a metal plate which is fixed on the skull with screws and dental cement. Some days after the operation, the guide cannulae are replaced with push–pull cannulae which are 4 mm longer than the guide cannulae, thus reaching the areas which are intended for superfusion. The push–pull cannulae are connected by tubing to two peristaltic pumps: one to push and another one to pull the fluid. The second pump is essential, because the superfusate is not directly collected from the side branch of the push–pull cannula but from tubing which is connected to the side branch. The superfusate is automatically collected every 10 s in fraction collectors.

Evaluation

The concentrations of neurotransmitters, e.g., epinephrine, norepinephrine, or dopamine, are determined with appropriate analytical methods (Wolfensberger 1984) before and after stimulation.

Modifications of the Method

Experiments in cats were described by Dietl et al. (1981) and in rats by Tuomisto et al. (1983).

The cortical cup technique for collection of neurotransmitters has been described by Moroni and Pepeu (1984).

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Fos Protein Expression in Brain

Purpose and Rationale

The proto-oncogene c-fos encodes a 55,000 mol wt, 380 amino acid phosphoprotein (FOS), which after translation in the cytoplasm reenters the nucleus and binds to DNA (Morgan and Curran 1989). C-fos induction can occur as a consequence of synaptic activation. An increase in fos immunoreactivity is associated with an increased metabolic demand on a neuron, i.e., a marker for neurons that is metabolically activated. Intermediate early genes such as c-fos have been tentatively classified or linked to third messengers, whose function is to produce a long-term effect on the recipient neuron.

Acute administration of antipsychotics induces c-fos expression in several areas of the rat forebrain as was shown with immunocytochemical methods (Dragunow et al. 1990; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994). Fos protein is believed to act as an initiator of long-term cellular changes (neural plasticity) in response to a variety of extracellular stimuli, including drugs (Graybiel et al. 1990; Rogue and Vicendon 1992). Typical (e.g., haloperidol) and atypical (e.g., clozapine) neuroleptic drugs have different antipsychotic effects and side effects. A differential FOS-protein induction in rat forebrain regions after haloperidol and clozapine treatment was found (Deutch et al. 1992; Fibiger 1994; Fink-Jensen and Kristensen 1994; Merchant et al. 1994; Sebens et al. 1995). The induction pattern of Fos-like immunoreactivity in the forebrain could serve as predictor of atypical antipsychotic drug activity (Robertson et al. 1994).

Procedure

Groups of 4–6 male Wistar rats weighing 350–450 g are injected subcutaneously with saline

(control) or with various doses of the standard drugs or compounds with putative antipsychotic activity. After 2 h, the animals are deeply anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital and perfused with 200 ml saline followed by 200 ml of 4 % paraformaldehyde in phosphate buffer solution (PBS). Each brain is removed immediately after perfusion and placed in fresh fixative for at least 12 h.

After the post-fixative period, 30-pm sections are cut from each brain using a vibratome. Several antisera to detect Fos can be used, such as a sheep polyclonal antibody directed against residues 2–16 of the N-terminal region of the Fos molecule or a polyclonal antiserum raised in rabbits against Fos peptide (4–17 amino acids of human Fos).

Sections are washed three times with 0.02 mM PBS and then incubated in PBS containing 0.3 % hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections are then washed three times in PBS and incubated in PBS containing 0.3 % Triton X-100, 0.02 % azide, and Fos primary antisera (diluted 1:200) for 48 h. The sections are then washed three times with PBS and incubated with a biotinylated rabbit antisheep secondary antibody (diluted 1:200) for 1 h. The sections are washed three times with PBS and incubated for 1 h with PBS containing 0.3 % Triton X-100 and 0.5 % avidin-biotinylated horseradish peroxidase complex. After three washes in PBS, the sections are rinsed in 0.1 M acetate buffer, pH6.0. Fos immunoreactivity is revealed by placing the sections in a solution containing 0.05 % 3,3'-diaminobenzidine, 0.2 % ammonium nickel sulfate, and 0.01 % H_2O_2. The reaction is terminated with a washing in acetate buffer. The sections are mounted on chrome-alum-coated slides, dehydrated, and prepared for microscopic observation.

Drug-induced changes in Fos-like immunoreactivity are quantified by counting the number of immunoreactive nuclei in the medial prefrontal cortex, nucleus accumbens, medial and dorsolateral striatum, and the lateral septal nucleus. The number of Fos-positive nuclei is counted with a 550×550 pm grid placed over each of these regions with a $100 \times$ magnification.

Typical and atypical antipsychotics can be classified on the basis of difference between Fos-like immunoreactivity in the nucleus accumbens and lateral striatum. For this purpose, the data are corrected for the effects which are produced by the injection procedure itself. The injection-corrected value for the dorsolateral striatum is subtracted from the corresponding accumbal value for each drug dose.

This manipulation yields a value termed the atypical index, i.e., number of Fos-positive neurons in the nucleus accumbens minus the number in the lateral striatum = atypical index. A negative index indicates the probability of side effects, like extrapyramidal syndrome, exerted by the typical neuroleptics, a positive value to be devoid of it.

Evaluation

A one-way analysis of variance is performed on the cell count data for each dose and the corresponding vehicle control. If the analysis of variance is significant, multiple comparisons are performed by using the Newman–Keuls test.

Modifications of the Method

Graybiel et al. (1990) reported a drug-specific activation of c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum by amphetamine and cocaine.

Deutch et al. (1991) found that stress selectively increases Fos protein in dopamine neurons innervating the prefrontal cortex.

Gogusev et al. (1993) described modulation of c-fos and other proto-oncogene expression by phorbol diester in a human histiocytosis DEL cell.

Deutch et al. (1995) studied the induction of Fos protein in the thalamic paraventricular nucleus as locus of antipsychotic drug action.

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Neurotensin

General Considerations on Neurotensin and Neurotensin Receptors

Neurotensin is a 13-amino acid peptide originally isolated from calf hypothalamus (Carraway and Leeman 1973). It is secreted by peripheral and neuronal tissues and produces numerous

pharmacological effects in animals, suggesting **analgesic** (Coguerel et al. 1988; Clineschmidt and McGuffin 1977; Smith et al. 1997), **wound healing** (Brun et al. 2005), **cardiovascular** (Carraway and Leeman 1973; Schaeffer et al. 1998; Seagard et al. 2000), **endocrine** (Rostene and Alexander 1997), **hypothermic** (Bissette et al. 1976; Benmoussa et al. 1996; Tyler-McMahon et al. 2000), and **antipsychotic** (Nemeroff 1986; Sarhan et al. 1997; Feifel et al. 1999; Kinkead et al. 1999; Cusack et al. 2000) actions. Neurotensin is even considered to be an endogenous neuroleptic (Ervin and Nemeroff 1988; Gully et al. 1995). Radke et al. (1998) studied synthesis and efflux of neurotensin in different brain areas after acute and chronic administration of typical and atypical antipsychotic drugs.

Neurotensin affects **gastrointestinal functions**, such as stimulating the growth of various gastrointestinal tissues (Feurle et al. 1987), modulating pre- and postprandial intestinal motility (Pellissier et al. 1996), inhibiting gastric acid secretion (Zhang et al. 1989a), stimulating responses in rat stomach strips (Quirion et al. 1980), inducing contractile responses in intestinal smooth muscle (Unno et al. 1999), and maintaining gastric mucosal blood flow during cold water restraint (Zhang et al. 1989b; Xing et al. 1998).

Neurotensin acts as a **growth factor** on a variety of normal and cancer cells (Wang et al. 2000).

Like other neuropeptides, neurotensin is synthesized as part of a larger precursor which also contains neuromedin N, a six amino acid neurotensin-like peptide belonging to the gastrin-releasing peptide/bombesin family (see J.3.1.8).

Several peptidic and non-peptidic neurotensin agonists and antagonists have been synthesized and analyzed in pharmacological tests as potential drugs mainly in psychopharmacology (Gully et al. 1995, 1996, 1997; Azzi et al. 1996; Castagliuolo et al. 1996; Chapman and See 1996; Mule et al. 1996; Hong et al. 1997; Johnson et al. 1997; Sarhan et al. 1997; Betancur et al. 1998; Gudasheva et al. 1998; Schaeffer et al 1998; Kitabgi 2002). Furthermore, inhibitors

of neurotensin-degrading enzymes were described (Bourdel et al. 1996). Binder et al. (2001) reviewed neurotensin and dopamine interactions.

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Neurotensin Receptor Binding

Purpose and Rationale

Neurotensin interacts with two cloned receptors that were originally differentiated on the basis of their affinity to the antihistaminic drug levocabastine (Schotte et al. 1986). The high sensitive, levocabastine-insensitive rat neurotensin receptor (NTR1) was cloned first (Tanaka et al. 1990) and shown to mediate a number of peripheral and central neurotensin responses, including the neuroleptic-like effects of the peptide (Labbé-Jullié et al. 1994). The human NTR1 has been cloned from the colonic adenocarcinoma cell line HT29 (Vita et al. 1993) and shown to consist of a 416 amino acid protein that shares 84 % homology with rat NTR1. A second human NTR1 receptor differing only in one amino acid has been cloned from substantia nigra by Watson et al. (1993).

The lower-affinity, levocabastine-sensitive neurotensin receptor (NTR2) was cloned by Chalon et al. (1996) and Mazella et al. (1996) and characterized by Yamada et al. (1998). Studies by Dubuc et al. (1999) indicate that NTR2 mediates neurotensin-induced analgesia.

A third neurotensin receptor (NTR3) was cloned from a human brain cDNA library (Mazella et al. 1998; Vincent et al. 1999; Mazella 2001; Mazella and Vincent 2006). It is identical with sortilin, a receptor-like protein, cloned from human brain (Petersen et al. 1997, 1999). The NT3/gp95/sortilin protein is a transmembrane

neuropeptide receptor which does not belong to the superfamily of G-protein-coupled receptors.

Gully et al. (1997) described a binding assay for the neurotensin1 receptor.

Procedure

Cell Culture
CHO cells transfected with cDNA of the human neurotensin receptor cloned from HT 29 cells (h-NTR1-CHO cells) are cultured at 37 °C in modified Eagle's medium without nucleosides, containing 10 % fetal calf serum, 4 mM glutamine, and 300 μg /ml geneticin (G418), in a humidified incubator under 5 % CO_2 in O_2. The colonic adenocarcinoma HT 29 cell line (American Type Culture Collection, Rockville, MD) is cultured under similar conditions in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10 % fetal calf serum, 4 mM glutamine, 200 IU/ml penicillin, and 200 mg/ml streptomycin. One week after seeding, confluent monolayer cultures are washed three times with 3 ml PBS and harvested by enzymatic dissociation with trypsin. After dilution with PBS, cells are resuspended in the same culture medium at a density of 5×10^4 cells/ml and are plated into 35-mm diameter, fibronectin-coated Petri culture dishes.

Membrane Homogenate Preparation and Binding Assay
Whole brains of male Sprague Dawley rats albino guinea pigs or cell pellets are homogenized in 10 volumes of ice-cold 50 mM Tris–HCl buffer (pH7.4) for 30 s, using a polytron homogenizer (setting 5). After 20 min. centrifugation at 30,000 g, the pellet is washed; centrifuged again under the same conditions; resuspended in a storage buffer containing 50 mM Tris–HCl (pH7.4), 1 mM EDTA, 0.1 % BSA, 40 mg/l bacitracin, 1 mM 1,10-orthophenanthroline, and 5 mM dithiothreitol; and stored as aliquots in liquid nitrogen until used.

Aliquots of membranes (10, 50, 300, and 500 μg of protein for h-NTR1-CHO cells, HT 29 cells, rat brain, and guinea pig brain, respectively) are incubated for 20 min at 20 °C in the incubation buffer (0.5 ml final volume) containing appropriate concentrations of [^{125}I-Tyr^3] neurotensin (25–100 pM) and unlabeled drugs. After incubation, the assay medium is diluted with 4 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) supplemented with 0.1 % BSA and 1 mM EDTA, and the mixture is rapidly filtered under reduced vacuum through Whatman GF/B glass fiber filters that have been pretreated with 0.1 % polyethyleneimine. The filters are washed under the same conditions three times and radioactivity is measured. Nonspecific binding is determined in the presence of 1 μM unlabeled neurotensin. All experiments are performed in triplicate, and data are expressed as the mean ± SEM of at least three separate determinations.

Evaluation
The IC_{50} is the value of ligand that inhibits 50 % of the specific binding and is determined using an iterative nonlinear regression program (Munson and Rodbard 1980).

Modifications of the Method
Cusack et al. (1995) studied species selectivity of neurotensin analogs at the rat and two human NTR1 receptors.

Lugrin et al. (1991) produced a series of pseudopeptide analogs of neurotensin by systematically replacing peptide bonds in neurotensin with CH_2NH bonds. The compounds were screened in vitro for agonist or antagonist activity and for metabolic stability.

Le et al. (1997) cloned the human neurotensin receptor gene and determined the structure.

Labbé-Jullié et al. (1998) attempted to identify residues in the rat NTR1 that are involved in binding of a nonpeptide neurotensin antagonist.

Souazé et al. (1997) and Najimi et al. (1998) studied the effects of a neurotensin agonist and showed in human colonic adenocarcinoma HT 29 cells after short incubation an increase, after prolonged exposure a decrease of mRNA levels, and in the human neuroblastoma cell line CHP 212 a high-affinity neurotensin receptor gene activation.

Ovigne et al. (1998) described a monoclonal antibody specific for the human NTR1.

Nouel et al. (1999) found that both NT2 and NT3 neurotensin receptor subtypes were expressed by cortical glial cells in culture.

Cusack et al. (2000) developed a neurotensin analog, NT34, which can distinguish between rat and human neurotensin receptors and exhibits more than a 100-fold difference in binding affinities.

Neuromedin N, a peptide belonging to the gastrin-releasing peptide/bombesin family (see chapter "▶ Pharmacological Effects on Gastric Function"), shows a high affinity to brain neurotensin receptors and is rapidly inactivated by brain synaptic peptidases (Checler et al. 1990).

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Genetically Altered Monoamine Transporters

Monoamine transporters, such as the dopamine transporter, 5-hydroxytryptamine transporter, and noradrenaline transporter, in the plasma membrane provide effective control over the intensity of monoamine-mediated signaling by recapturing neurotransmitters released by presynaptic neurons (Gainetdinov et al. 2002). These transporters act also as molecular gateways for neurotoxins (Uhl and Kiayama 1993; Miller et al. 1999; Vincent et al. 1999).

Takahashi et al. (1997) found that heterozygote animals of VMAT2 knockout mice display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity.

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Dopamine Transporter Knockout Mice

Many drugs exert their psychotropic action via dopamine transporters (Amara and Kuhar 1993; Giros and Caron 1993).

Dopamine transporter knockout mice, which are generated by disruption of the gene encoding the dopamine transporter by homologous recombination (Giros et al. 1996; Sora et al. 1998), have a distinct biochemical and behavioral phenotype. At the neurochemical level, the homoeostasis of dopamine-containing neurons is altered markedly, including disrupted clearance of dopamine, an elevated extracellular concentration of dopamine, and dramatically decreased intraneuronal storage of dopamine (Jones et al. 1998; Gainetdinov et al. 1998; Benoit-Marand et al. 2000).

In response to the elevated dopamine-mediated tone, both presynaptic and postsynaptic dopamine receptors are downregulated (Giros et al. 1996), but although autoreceptor functions are lost (Jones et al. 1999), some postsynaptic responses appear to be enhanced (Gainetdinov et al. 1999a; Fauchey et al. 2000).

Dopamine transporter knockout mice are hyperactive (Gainetdinov et al. 1999b; Spielewoy et al. 2000) and have a much reduced body size (Bossé et al. 1997). These animals have cognitive deficits (Gainetdinov et al. 1999a, b), disrupted sensorimotor gating (Ralph et al. 2001), and sleep dysregulation (Wisor et al. 2001). Dopamine transporter knockout mice appear to provide a model of some aspects of manic behavior (Ralph-Williams et al. 2003).

Abnormalities in skeletal structure (Bliziotes et al. 2000) and altered regulation of gastrointestinal tract motility (Walker et al. 2000) are also observed.

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Serotonin Transporter Knockout Mice

The serotonin transporter has a key role in regulating the intensity of 5-HT-mediated transmission and is the primary target for several antidepressants and psychostimulants (Amara and Kuhar 1993; Bengel et al. 1996).

Disruption of 5-HT uptake in serotonin transporter knockout mice increases the extracellular concentration of 5-HT sixfold and reduces intracellular concentration by 60 % – 80 % (Fabre et al. 2000).

Holmes et al. (2003) found that mice lacking the serotonin transporter exhibit 5-HT_{1A} receptor-mediated abnormalities in tests for anxiety-like behavior.

Lira et al. (2003) reported altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin-transporter-deficient mice.

Marked desensitization of both presynaptic and postsynaptic 5-HT_{1A} receptors is observed in electrophysiological studies (Gobbi et al. 2001).

There is a significant decrease in 5-HT_{1A} receptor binding sites, mRNA, and protein in some, but not all, 5-HT-containing brain areas. Altered hypothermic and neuroendocrine responses to 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) are also reported (Li et al. 1999).

Thermal hyperalgesia in mice after chronic constrictive sciatic nerve injury was absent in serotonin transporter-deficient mice (Vogel et al. 2003).

Decreases in 5-HT_{1A} and 5-HT_{1B} receptor coupling are observed, accompanied by disruption of the neurochemical responses to the 5-HT1A receptor agonist ipsapirone and the $5\text{-HT}_{1A}/5\text{-HT}_{1D}$ receptor agonist GR127935 (Fabre et al. 2000).

The hyperlocomotor effect of MDMA, but not that of high doses of d-amphetamine, is disrupted in serotonin receptor knockout mice (Bengel et al. 1996).

In double knockout mice that lack the dopamine transporter and have no or one copy of the gene that encodes the serotonin transporter, no place preference for cocaine was observed (Sora et al. 2001).

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Noradrenaline Transporter Knockout Mice

The noradrenaline transporter has a role similar to that of the dopamine transporter and the serotonin transporter with respect to noradrenaline-mediated transmission (Blakely et al. 1994).

Noradrenaline-transporter knockout mice have been generated using homologous recombination (Xu et al. 2000).

Wang et al. (1999) reviewed genetic approaches to studying norepinephrine function using knockout of the mouse norepinephrine transporter gene.

The prolonged synaptic lifetime of noradrenaline in noradrenaline transporter knockout mice results in elevation of the extracellular concentration of noradrenaline and depletion of the intraneuronal stores. In addition, in noradrenaline transporter knockout mice, the α_1-adrenoceptor decreased in the hippocampus (Xu et al. 2000), although α_{2A}-adrenoceptor density did not change in the spinal cord (Bohn et al. 2000).

Noradrenaline-transporter knockout mice have a lower body weight and reduced locomotor responses to novelty. In the tail-suspension test used for screening antidepressant drugs, noradrenaline transporter knockout mice behaved like antidepressant-treated, wild-type animals, and no additional effects of the antidepressants desipramine, paroxetine, and bupropion were observed in mutant mice in this test (Xu et al. 2000).

In the tail-flick assay, morphine induced greater analgesia in noradrenaline transporter knockout mice compared with wild-type mice (Bohn et al. 2000).

In synaptosomes from the frontal cortex of noradrenaline transporter knockout mice, cocaine and nisoxetine had no inhibitory effect on the uptake of dopamine, whereas in the nucleus accumbens, the effectiveness of cocaine was somewhat reduced. Uptake of dopamine in brain

regions that have low levels of dopamine transporter may depend primarily on the noradrenaline transporter (Morón et al. 2002).

Locomotor responses to cocaine and amphetamine are elevated in noradrenaline knockout mice, and chronic administration of cocaine did not induce further sensitization. The enhanced responses to psychostimulants in noradrenaline transporter knockout mice correlate with the suppression of presynaptic dopamine function and supersensitivity to postsynaptic D2 and D3 receptors (Xu et al. 2000).

Haller et al. (2002) studied behavioral responses to social stress in noradrenaline transporter knockout mice.

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In Vivo Tests

Golden Hamster Test

Purpose and Rationale

"Innate behavior" of many species including man has been described by Lorenz (1943, 1966). The "golden hamster test" (Ther et al. 1959) uses the innate behavior of this species (*Mesocricetus auratus*) for differentiation between neuroleptic and sedative – hypnotic activity. The aggressive behavior of male golden hamsters is suppressed by neuroleptics in doses which do not impair motor function.

Procedure

Ten to 20 male golden hamsters with an average weight of 60 g are crowded together in Makrolon[R] cages for at least 2 weeks. During this time, the animals develop a characteristic fighting behavior. For the test, single animals are placed into glass jars of 2 l. In this situation, the hamsters assume a squatting and resting position during the day. If the animals are touched with a stick or a forceps, they wake up from their daytime sleep and arouse immediately from the resting position. If one tries to hold the hamster with a blunted forceps, a characteristic behavior is elicited: The hamster throws himself onto his back, tries to bite and to push the forceps away with his legs, and utters angry shrieks. Touching the animals is repeated up to six times followed by punching with the forceps. Only animals responding to the stimulus with all three defense reactions (turning, vocalizing, and biting) are included into the test.

The test compounds are applied either subcutaneously, intraperitoneally, or orally. Six animals are used for each dose.

Evaluation

The stimuli are applied every 20 min for 3 h. The number of stimuli until response is recorded. Furthermore, the suppression of the defense reactions (turning, biting, and vocalizing) is evaluated. An animal is regarded to be completely "tamed" if all defense reactions are suppressed even after

punching with the forceps at least once during the test period.

After each stimulation, the "tamed" animal is placed on an inclined board with 20° inclination. Normal hamsters and hamsters tamed by neuroleptics are able to support themselves or to climb on the board. Impaired motor function causes sliding down. This experiment is repeated three times after each testing of the defense reactions. An animal's coordination is considered to be disturbed if it falls three times during two tests of the experiments.

For each dose, the number of tamed hamsters and the number of animals with impaired motor function are recorded. Using different doses, ED_{50} values can be calculated for the taming effect and for impairment of motor function.

The ED_{50} values of taming were 1.5 mg/kg for chlorpromazine s.c. and 0.2 mg/kg for reserpine s.c. Much higher doses (ten times of chlorpromazine and five times of reserpine) did not elicit motor disturbances. On the contrary, while ED_{50} values of 10 mg/kg phenobarbital s.c. and 180 mg/kg meprobamate p.o. for the taming effect were found, these doses already caused severe motor disturbances. The taming dose of diazepam was 10 mg/kg p.o. which already showed some muscle-relaxing activity. The term "neuroleptic width" indicates the ratio between the ED_{50} for taming and the ED_{50} for motor disturbances. Only for neuroleptic drugs are ratios found between 1:5 and 1:30.

Critical Assessment of the Method

The method has the advantage that neuroleptics can easily be differentiated from sedative and hypnotic drugs. Anxiolytics with pronounced muscle-relaxing activity also show no significant differences between taming and impaired motor function. Moreover, the method has the advantage that no training of the animals and no expensive apparatus are needed.

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Influence on Behavior of the Cotton Rat

Purpose and Rationale

The "cotton-rat test" is another attempt to use the innate behavior as described for several animal species by Lorenz (1943, 1966) for the differentiation of psychotropic drugs (Vogel and Ther 1960). The cotton rat (*Sigmodon hispidus*) is a very shy animal which conceals himself at any time. This innate flight reflex is suppressed by centrally active drugs. Simultaneous evaluation of motor function allows the differentiation between neuroleptic and sedative drugs.

Procedure

Cotton rats are bred in cages equipped with a clay cylinder of 20 cm length and 10 cm diameter. This cylinder is used by the animals for hiding, sleeping, and breeding. Moreover, the animals which bite easily can be transported from one cage to another just by closing the cylinder on both ends. For the test, young animals with a body weight of 40 g are used. Young animals are as shy as the old ones but less vicious. Nevertheless, leather gloves have to be used for handling of cotton rats. Normal cages ($25 \times 30 \times 20$ cm) with a wire lid are used. A tunnel of sheet metal (half of a cylinder) 20 cm long and 7 cm high is placed into the cage. The cotton rats hide immediately in this tunnel. If the tunnel is lifted and placed on another site of the cage, the cotton rats immediately hide again.

Three rats are placed in one cage and tested for their behavior. Selective shaving of the fur enables the observer to recognize each animal. If the rats behave as described, they are then treated with the test compound subcutaneously or orally. At least six animals divided in two cages are used for each dose of test compound or standard. Fifteen min after application of the drug, the test period of three h is started. The tunnel is lifted and placed to another site. If the animals do not show the immediate flight reflex, an airstream of short duration is blown through the wire lid. If the animal still does not respond with the flight reflex, it is considered to be positively influenced. Afterwards, the animal is placed on an inclined board with 35° of inclination and tested for disturbance of motor coordination. A normal animal is able to climb upwards. If coordination is disturbed, the rat slides down.

Evaluation

The test procedure is repeated every 15 min over a period of 3 h. The animals which show at least one suppression of the flight reflex during the test period are counted as well as those who slide down on the inclined board. Using different doses, ED_{50} values are calculated for both parameters. The ratio between these two ED_{50} values is regarded as "neuroleptic width" which is 1:20 for chlorpromazine and 1:30 for reserpine, whereas ratios of 1:2 for phenobarbital and 1:1.5 for meprobamate indicate the absence of neuroleptic activity.

Critical Assessment of the Method

The method allows the differentiation of drugs with neuroleptic activity against other centrally active drugs. No training of the animals and no expensive equipment are necessary.

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Artificial Hibernation in Rats

Purpose and Rationale

Giaja (1938, 1940, 1953, 1954) studied the effects of reduced oxygen tension and cold environment on rats. The animals were placed in hermetically closed glass vessels which were submerged in ice water. Due to the respiratory activity, the oxygen tension diminishes and the carbon dioxide content increases. Under the influence of cooling and of hypoxic hypercapnia, the rectal temperature falls to 15 °C, and the animal is completely anesthetized and immobilized. The rat can survive in this poikilothermic state for more than 20 h. Complete recovery occurs after warming up. This kind of artificial hibernation was augmented by chlorpromazine (Courvoisier et al. 1953; Giaja and Markovic-Giaja 1954). Vogel (1959) and Ther et al. (1959, 1963) used these observations for evaluation of neuroleptics and opioid analgesics.

Procedure

Male Wistar rats weighing 100–150 g are deprived of food with free access to tap water overnight. The test compounds are injected subcutaneously 15 min prior to the start of the experiment. First, the rats are placed in ice-cold water to which surfactant is added in order to remove the air from the fur for 2 min. Then, the animals are placed into hermetically closed glass vessels of 750 ml volume which are placed into a refrigerator at 2 °C temperature. During the following hour, the vessels are opened every 10 min for exactly 10 s, allowing some exchange of air and reducing the carbon dioxide accumulation. At each time, animals are removed from the glass vessel and observed for signs of artificial hibernation which are not shown by control animals under these conditions. Treated animals, lying on the side, are placed on the back and further examined. An animal is considered positive, when it remains on the back, even if the extremities are stretched out. In this state, cardiac and respiration frequency are reduced, and the rectal temperature has fallen to 12–15 °C. The rigor of the musculature allows only slow movements of the extremities. The animals recover completely within a few

hours if they are brought to their home cages at room temperature. Artificial hibernation is induced dose-dependent by neuroleptics of the phenothiazine type and by some opioid analgesics like meperidine and methadone. In contrast, morphine shows only slight activity.

Evaluation

Various doses are applied to groups of ten animals. Percentage of positive animals is calculated for each group, and ED_{50} values with confidence limits are estimated according to Litchfield and Wilcoxon.

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Catalepsy in Rodents

Purpose and Rationale

Catalepsy in rats is defined as a failure to correct an externally imposed, unusual posture over a prolonged period of time. Neuroleptics which have an inhibitory action on the nigrostriatal dopamine system induce catalepsy (Costall and Naylor 1974; Chermat and Simon 1975; Sandberg et al. 1986), while neuroleptics with little or no nigrostriatal blockade produce relatively little or no cataleptic behavior (Honma and Fukushima 1976). Furthermore, cataleptic symptoms in rodents have been compared to the Parkinson-like extrapyramidal side effects seen clinically with administration of antipsychotic drugs (Duvoisin 1976).

Procedure

Groups of six male Sprague Dawley or Wistar rats with a body weight between 120 and 250 g are used. They are dosed intraperitoneally with the test drug or the standard. Then, they are placed individually into translucent plastic boxes with a wooden dowel mounted horizontally 10 cm from the floor and 4 cm from one end of the box. The floor of the box is covered with approximately 2 cm of bedding material. White noise is presented during the test. The animals are allowed to adapt to the box for 2 min. Then, each animal is grasped gently around the shoulders and under the forepaws and placed carefully on the dowel. The amount of time spent with at least one forepaw on the bar is determined. When the animal removes its paws, the time is recorded, and the rat is repositioned on the bar. Three trials are conducted for each animal at 30, 60, 120, and 360 min.

Evaluation

An animal is considered to be cataleptic if it remains on the bar for 60 s. Percentage of cataleptic animals is calculated. For dose–response curves, the test is repeated with various doses and more animals. ED_{50} values can be calculated. A dose of 1 mg/kg i.p. of haloperidol was found to be effective.

Critical Assessment of the Method

The phenomenon of catalepsy can be used for measuring the efficacy and the potential side effects of neuroleptics.

Modifications of the Method

Catalepsy induced by neuroleptic drugs can also be measured by the **PAW test**, which measures increase in forelimb and hindlimb retraction time in rats (Ellenbroek et al. 1987, 2001; Ellenbroek and Cools 1988, 2000; Prinssen et al. 1994, 1995).

The test is performed 30 min after intraperitoneal injection of test drug. Male Wistar rats weighing 220–300 g are placed on a Perspex platform (30 × 30 cm with a height of 20 cm) containing two holes for the forelimbs (40 mm) and two for the hindlimbs (50 mm), and a slit for the tail. The distance between the right and left forelimb holes is 15 mm, and the distance between forelimb and hindlimb holes is 55 mm. The rat is held behind the forelimbs, and the hindlimbs are gently placed in the holes. The forelimb retraction time and the hindlimb retraction time are defined as the time the animal needs to withdraw one forelimb and one hindlimb, respectively. The average forelimb retraction time and hindlimb retraction time (the mean of three measurements) is calculated for each rat.

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Pole Climb Avoidance in Rats

Purpose and Rationale
The pole-climb avoidance paradigm is an avoidance escape procedure used to separate neuroleptics from sedatives and anxiolytics. Whereas sedative compounds suppress both avoidance and escape responding at approximately the same doses, neuroleptic drugs reduce avoidance responding at lower doses than those affecting escape responding (Cook and Catania 1964).

Procedure
Male rats of the Long-Evans strain with a starting body weight of 250 g are used. The training and testing of the rats is conducted in a $25 \times 25 \times 40$ cm chamber that is enclosed in a dimly lit, sound-attenuating box. Scrambled shock is delivered to the grid floor of the chamber. A 2.8-kHz speaker and a 28-V light are situated on top of the chamber. A smooth stainless-steel pole, 2.5 cm in diameter, is suspended by a counterbalance weight through a hole in the upper center of the chamber. A microswitch is activated when the pole is pulled down 3 mm by a weight greater than 200 g. A response is recorded when a rat jumps on the pole and activates the microswitch. The rat cannot hold the pole down while standing on the grid floor because of the counterbalance tension and cannot remain on the pole any length of time because of its smooth surface. The activation of the light and the speaker together is used as the conditioning stimulus. The conditioning stimulus is presented alone for 4 s and then is coincident with the unconditioned stimulus, a scrambled shock delivered to the grid floor, for 26 s. The shock current is maintained at 1.5 mA. A pole climb response during the conditioned stimulus period terminates the conditioned stimulus and the subsequent conditioned and unconditioned stimuli. This is considered an avoidance response. A response during the time when both the conditioned and unconditioned stimuli are present terminates both stimuli and is considered an escape response. Test sessions consist of 25 trials or 60 min, whichever comes first. There is a minimum intertrial interval of 90 s. Any time remaining in the 30 s allotted to make the pole climb is added to the 90 s intertrial interval. Responses during this time have no scheduled consequences; however, rats having greater than ten intertrial interval responses should not be used in the experiment. Before testing experimental compounds, rats are required to make at least 80 % avoidance responses without any escape failures.

Evaluation
Data are expressed in terms of the number of avoidance and escape failures relative to the respective vehicle control data. ED_{50} values can be calculated using different doses.

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Footshock-Induced Aggression

Purpose and Rationale

The test as described by Tedeschi et al. (1959) using mice which fight after footshock-induced stimulation is useful to detect neuroleptics but also shows positive effects with anxiolytics and other centrally effective drugs. The method has been used by several authors to test drugs with neuroleptic activity. The test is described in chapter "Tests for Anxiolytic Activity".

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Brain Self-Stimulation

Purpose and Rationale

In several species, electrical stimulation of selected brain loci produces effects which are positively reinforcing and pleasurable (Olds and Milner 1954; Olds 1961, 1972). Most of the data available have been obtained from experiments using rats with electrodes chronically implanted in the median forebrain bundle at the level of hypothalamus. Minute electrical pulses sustain a variety of operant behaviors such as lever pressing. Neuroleptics have been shown to be potent blockers of self-stimulation (Broekkamp and Van Rossum 1975; Koob et al. 1978; Gallistel and Freyd 1987). Conversely, compounds that facilitate catecholaminergic transmission such as d-amphetamine and methylphenidate will increase responding for such stimulation.

Procedure

Male Wistar rats (350–400 g) are anesthetized with 50 mg/kg pentobarbital i.p. and their heads placed on a level plane in a Kopf stereotactic instrument. A midline incision is made in the scalp and the skin held out of the way by muscle retractors. A small hole is drilled in the scull with a dental burr at the point indicated by the stereotactic instrument for the structure it is desired to stimulate. Using bregma as a reference point, the electrode (Plastic Products MS303/1) is aimed at the medium forebrain bundle according to the atlas of Paxinos and Watson (1986), using the coordinates of AP $= -0.8$ mm, Lat $= +2.8$ mm, and DV $= -7.2$ mm below dura. The assembly is then permanently affixed to the scull using stainless-steel screws and bone cement.

After a minimum of 10 days for recovery, the animals are trained to bar press for electrical stimulation on a continuous reinforcement schedule in a standard operant box outfitted with a single lever. The reward stimulus is a train of biphasic square-wave pulses generated by a Haer stimulator (Pulsar 4i). The parameters are set at a pulse duration of 0.5 ms with 2.5 ms between each pulse pair. The train of pulses may vary between 16 and 30/s, and the intensity of the pulses that are delivered range from 0.1 to 0.5 mA using the lowest setting that will sustain maximal responding. After consistent baseline responding is obtained for five consecutive 30-min session, the animals are ready for testing with standard agents. Compounds are administered 60 min. prior to testing. All data are collected on both cumulative recorders and counters.

Evaluation

The number of drug responses is compared to the number of responses made during each animal's 30-min control session on the preceding day, which is considered to be equal to 100 %. Testing various doses, ED_{50} values with 95 % confidence limits can be calculated.

Critical Assessment of the Method

Since there is sufficient evidence that self-stimulation behavior is maintained by catecholamines, the method gives indirectly insight into the catecholaminergic facilitating or blocking properties of a compound. Active neuroleptic drugs inhibit the self-stimulation behavior in very small doses. The relative potency observed in this test of clinically efficacious drugs parallels their potency in the treatment of schizophrenia.

Modifications of the Method

Reinforcing brain stimulation by electrodes placed in the medial forebrain bundle of rats is decreased after lesion of the internal capsule in the region of the diencephalic–telencephalic border. This decrement in rewarding processing can be reversed by antidepressant drugs (Cornfeldt et al. 1982).

Depoortere et al. (1996) used electrical self-stimulation of the ventral tegmental area to study the behavioral effects of a putative dopamine D_3 agonist in the rat.

Anderson et al. (1995) examined the interaction of aversive and rewarding stimuli in self-stimulating rats in terms of duration and direction. The rats were implanted with two moveable electrodes, one in a region supporting self-stimulation (the ventral tegmental area) and another in a region supporting escape (the nucleus reticularis gigantocellularis).

Kokkinidis et al. (1986) used amphetamine withdrawal for a behavioral evaluation. Mice implanted with stimulating electrodes in the lateral hypothalamus demonstrated stable and reliable rates of self-stimulation responding. After exposure to a chronic schedule of amphetamine treatment, response rates were severely depressed.

Post-amphetamine depression of self-stimulation from the substantia nigra can be reversed by cyclic antidepressants (Kokkinidis et al. 1980).

Moreau et al. (1992) reported that antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation in rats.

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Prepulse Inhibition of Startle Response

Purpose and Rationale

Prepulse inhibition is a model of sensorimotor gating which can be assessed in both animals and humans using the startle reflex response. When a fixed startle-eliciting stimulus (i.e., the pulse) is preceded by 30–500 ms by a weak, non-startle-eliciting stimulus (i.e., the prepulse), the magnitude of the startle response is significantly reduced to the pulse alone. Schizophrenic patients have decreased prepulse inhibition relative to normal control subjects, and this is thought to reflect an impairment in their ability to filter irrelevant sensory stimuli (Braff and Geyer 1990; Geyer 1998). Similar reductions in prepulse inhibition are produced in rats by administration of psychotomimetic drugs such as the dopamine agonists amphetamine and apomorphine or the noncompetitive NMDA antagonists phencyclidine and dizocilpine (MK801) (Mansbach and Geyer 1989; Swerdlow et al. 1998; Geyer et al. 2001; Rowley et al. 2001; Weiss and Feldon 2001; Pouzet et al. 2002). Most antipsychotics tested are able to antagonize prepulse inhibition disruption produced by dopamine antagonists, whereas prepulse inhibition disruption by NMDA antagonists may be selectively sensitive to antipsychotics with atypical features (Bakshi and Geyer 1995; Bubenikova et al. 2005; Fox et al. 2005). Haloperidol failed to block the effects

of phencyclidine and dizocilpine prepulse inhibition of startle (Keith et al. 1991).

Feifel et al. (Feifel and Reza 1999; Feifel et al. 1999a, b) tested the effects of a neurotensin agonist on prepulse inhibition of startle in rats.

Procedure

Male Sprague Dawley rats were treated with various doses of test compound or saline s.c. Immediately afterwards, rats receive a second s.c. injection consisting of 2 mg/kg d-amphetamine, or 0.5 mg/kg apomorphine, or 0.1 mg/kg dizocilpine or saline. Then, 10 min later, animals were placed in special startle chambers (SR-LAB, San Diego Instruments, San Diego, Calif., USA). Startle chambers consist of a Plexiglas cylinder 8.2 cm in diameter, resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure housed in a sound-attenuated room exposed to 70-dB background noise. After a 5-min acclimation period, acoustic stimuli were presented via a speaker mounted 24 cm above the animal. Acoustic stimuli consisted of a 120-dB pulse by itself (pulse alone) or a 120-dB pulse preceded by 100 ms by prepulses 3, 5, and 10 dB above background noise. There was an average of 15 s between stimuli. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced the motion within the cylinder. Startle amplitude was defined as the degree of motion detected by this accelerometer. Each rat was tested on four separate occasions separated by 7 non-test days. On each test day, the dose of test compound was kept constant, but the specific psychotomimetic agent was alternated across test days in a counterbalanced fashion.

Evaluation

Prepulse inhibition was calculated as the percentage of the pulse-alone startle amplitude using the following formula: [1 (startle amplitude after prepulse–pulse pair/startle amplitude after pulse only)] $\times$ 100. Analysis of data was then carried out using a three-factor repeated-measures analysis of variance (ANOVA). Significant factor results from the ANOVA were followed up with separate one-way ANOVAs for each psychotomimetic agent and then, when indicated, with individual group mean comparisons using post hoc t-tests for multiple comparisons using the Bonferroni method.

Modifications of the Method

Sipes and Geyer (1995) studied the disruption of prepulse inhibition of the startle response in the rat by DOI [(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride], which is mediated by 5-HT_{2A} receptors. The authors suggested that studies of the serotonergic substrates of prepulse inhibition may provide a model of the possible serotonergic role in the sensorimotor gating abnormalities in patients with schizophrenia and with obsessive-compulsive disorder.

Ellenbroeck et al. (1998) described the effects of an early stressful life event on sensorimotor gating in adult rats.

Andersen and Pouzet (2001) compared the effects of acute versus chronic treatment with typical or atypical antipsychotics on d-amphetamine-induced sensorimotor gating deficits in rats.

Heidbreder et al. (2000) used the prepulse inhibition of acoustic startle for behavioral, neurochemical, and endocrinological characterization of the early social isolation syndrome.

Krebs-Thomson et al. (2001) reported that postweanling handling attenuates isolation-rearing disruption of prepulse inhibition in rats.

Weiss et al. (2001) studied the dissociation between the effects of preweaning and/or postweaning social isolation on prepulse inhibition and latent inhibition in adult Sprague Dawley rats.

Dirks et al. (2003) reported reversal of startle gating deficits in transgenic **mice** overexpressing corticotropin-releasing factor by antipsychotic drugs.

Andreasen et al. (2006) studied the effect of nicotinic agents on prepulse inhibition (PPI) in mice using a startle response/PPI system from TSE Systems, Bad Homburg, Germany.

Lind et al. (2004) described prepulse inhibition of the acoustic startle reflex in pigs and its disruption by d-amphetamine.

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N40 Sensory Gating

Purpose and Rationale

The N40 auditory-evoked potential has been used to develop an animal model for the study of sensory gating mechanisms (Boutros et al. 1997a; Boutros and Kwan 1998). The method has been applied to evaluation of psychotropic compounds (Adler et al. 1986; Boutros et al. 1994, 1997b). Bickford-Wimer et al. (1990) localized one possible source of the N40 waveform to the CA3 region of the hippocampus.

Fox et al. (2005) used the N40 sensory gating model in mice for evaluation of a potential antipsychotic drug.

Procedure

Male DBA/2 mice were stereotaxically implanted with tripolar stainless-steel wire head stages for EEG recordings in the CA3 region of the hippocampus. The mice were first anesthetized with a solution of 2.8 % ketamine, 0.28 % xylazine, and 0.05 % acepromazine. Three access holes for the electrodes were made at AP −1.8 mm from the bregma, and in a plane perpendicular to the suture, ML 0.6 (cortical electrode), 1.6 (reference electrode), and 2.6 mm electrode directed at the hippocampus). The depth of the hippocampal electrode tip was DV 1.65–1.70 mm below the surface of the cortex. The depths of the cortical and reference electrodes were DV 0.5 mm from the surface of the skull, resulting in contact, but not penetration, of the cortical tissue. The tripolar electrode was lowered into position with a stereotaxic electrode holder and affixed using cyanoacrylic gel and dental acrylic and two anchor screws. Mice were allowed to recover for 3 days before commencement of the experiments. Awake mice were recorded in acoustically isolated chambers. Flexible tethers and electrical swivels were used to convey EEG signals to differential AC EEG amplifiers and allowed the mice free movement within the chambers. The EEG was amplified 1000 × with a 50- to 60-Hz notch filter engaged, and high- and low-pass filters were set at 1 and 100 Hz, respectively. Hippocampal auditory-evoked potentials were generated by presentation of 60 sets of 3 kHz-paired tone bursts from a speaker within the recording chamber at a distance of 15–20 cm to the mouse. The first tone of the pair is referred to as the conditioning stimulus, and the second is referred to as the test stimulus. The duration of both the condition and test stimuli was 5 ms, with 0.5 s between the stimuli and 20 s between pairs. Data acquisition software recorded EEG signals 100 ms before and for 899 ms after the initial conditioning stimulus. The software averaged the 60-paired responses into one composite-evoked response. Various

doses of test drug were administered i.p. 20–30 min before mice were placed into the recording chambers and initiation of auditory-evoked potential recording. Recording of paired auditory potentials continued for two 20-min sessions, each comprised of 60 paired stimuli. Each mouse was administered every treatment dose and a control vehicle treatment in a balanced order on separate days with at least 48 h between treatments. This within-subject design allowed each mouse to serve as its own control. The hippocampal response to auditory stimuli was identified as the highest positive peak deflection in the ongoing EEG at a latency of 10–20 ms after the stimulus (P20), followed by the lowest negative peak deflection in the ongoing EEG at 20–45 ms after the stimulus (N40). The difference in amplitude between P20 and N40 was defined as the N40 amplitude in microvolts.

Evaluation

N40 amplitudes were determined for both the averaged conditioning and test-evoked potentials, and a ratio was derived between the two responses by dividing the test amplitude by the conditioning amplitude (T/C ratio).

Modifications of the Method

Flack et al. (1996) studied sensory gating in a computer model of the CA3 neural network of the hippocampus.

Stevens et al. (1998) investigated changes in auditory information processing after kainic acid lesions in adult rats used as a model of schizophrenia.

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Latent Inhibition

Purpose and Rationale

Latent inhibition has been recommended as an animal model of schizophrenia (Feldon and Weiner 1992; Swerdlow et al. 1996; Vaitl and

Lipp 1997; Moser et al. 2000; Bender et al. 2001). Latent inhibition refers to the retarded acquisition of a conditioned response that occurs if the subject being tested is first preexposed to the to-be-conditioned stimulus without the paired unconditioned stimulus. Because the "irrelevance" of the to-be-conditioned stimulus is established during non-contingent preexposure, the slowed acquisition of the conditioned stimulus-unconditioned stimulus association is thought to reflect the process of overcoming this learned irrelevance. Latent inhibition has been reported to be diminished in acutely hospitalized schizophrenia patients. Several authors used the latent inhibition model in rats to test psychotropic compounds (Solomon et al. 1981; Feldon and Weiner 1991; Moran and Moser 1992; De la Casa et al. 1993; Lacroix et al. 2000; Alves et al. 2002). Trimble et al. (2002) tested the effects of selective D1 antagonists on latent inhibition in the rat.

Procedure

Animals
Male Sprague Dawley rats weighing 300–400 g were housed two to a cage under a 12-h reversed cycle lighting with food and water ad libitum. All experimental manipulations were carried out in the dark phase of the dark/light cycle.

Apparatus
Modified metal Skinner boxes (24.5 × 24.5 × 21 cm measured from a raised grid floor) were located in darkened, sound-insulated, ventilated outer boxes. A removable water bottle was located on one side of each Skinner box through a hole of 1.0 cm diameter, positioned 2 cm above the grid floor. When water was not required, the water bottle was removed. Licks at the spout of each water bottle were recorded using a lickometer (model 453, Campden Instruments, London, UK). The preexposed stimulus was a flashing light (10 s duration with three light flashes per second) situated in the middle of the roof of each Skinner box. The grid floor consisted of steel bars (0.5 cm in diameter) spaced 1 cm apart. Shock generators with scramblers were calibrated to produce 0.5-mA shocks via the grid floor.

Procedure
Rats were randomly assigned to experimental groups and were allocated to a particular Skinner box. They had experience of only that box for the duration of the experiment. After adaptation to the housing conditions for 1 week, rats were placed immediately on a 23-h water deprivation schedule that continued until the end of the experiments. Food remained freely available.

Baseline Days (Days 15–19)
After 7 days on the water deprivation period, 5 days of pretraining commenced. Each rat was placed in a Skinner box for 15 min. The water bottle was present and each rat could drink freely. After the baseline session was over, each rat was returned to its home cage and allowed access to water for 45 min.

Preexposure (Day 20)
With the water bottle removed, each rat was placed in a Skinner box. Rats received ten stimulus (flashing house-light) presentations of 10 s duration (three light flashes per second) with a fixed stimulus interval of 50 s. Afterwards the rats were returned to their home cages and allowed access to water for 1 h.

Conditioning (Day 21)
With the water bottle removed, each rat was placed in a Skinner box. Then, 5 min later, each rat received the first of two light footshock pairings. House-light parameters were identical to those of the preexposure period. The house-light was immediately followed by the footshock (0.5 mA, 1 s). The second light-shock pairing was given 5 min later. After the conditioning period had terminated, animals were returned to their home cages and allowed access to water for 1 h.

Re-baseline Day (Day 22)
With the water bottle present, each rat was placed in a Skinner box and allowed to drink as in the baseline sessions.

Test Day (Day 23)
With the water bottle present, each rat was placed in a Skinner box and allowed to drink. When each rat

completed 75 licks, the flashing house-light was presented and continued until 5 min had elapsed from stimulus onset. Time bins of 30-s duration commenced from the time of stimulus presentation, and the number of licks made by each rat within every time bin was recorded. This measure allowed the pattern of drinking over the course of stimulus presentation to be shown. The amount of suppression of licking for each rat was assessed using a suppression ratio calculated from the time (in seconds) to complete licks 51–75 (pre-stimulus) divided by the time (in seconds) to complete licks 51–100 (pre-stimulus + stimulus on). A suppression ratio of 0.01 indicates total suppression of licking (no latent inhibition), while a ratio of 0.5 indicates no change in licking rate from the pre-stimulus period to the stimulus-on period (latent inhibition).

DRUG Treatment
Test drugs or vehicle was administered by subcutaneous injection in various doses 30 min prior to preexposure and conditioning.

Evaluation
Times to complete licks and the suppression ratios were analyzed independently using a 2×6 ANOVA with main factors of preexposure and drugs.

Modifications of the Method
Lehmann et al. (1998) studied the long-term effects of repeated maternal separation on three different latent inhibition paradigms.

Pouzet et al. (2004) reported that latent inhibition is spared by NMDA-induced ventral hippocampal lesions, but is attenuated following local activation of the ventral hippocampus by intracerebral NMDA infusion.

Bethus et al. (2005) examined the effects of prenatal stress and gender in latent inhibition.

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Tests Based on the Mechanism of Action

Amphetamine Group Toxicity

Purpose and Rationale

It is well known that aggregation of mice in small cages greatly enhances the toxicity of amphetamine. The death rate can be reduced by pretreatment with neuroleptics. This phenomenon is generally accepted as an indicator of neuroleptic activity. The increased toxicity results from increased behavioral activation due to aggregation inducing an increase of circulating catecholamines. The mechanism can be understood by the fact that amphetamine is an indirectly acting sympathomimetic amine that exerts its effects primarily by releasing norepinephrine from storage sites in the sympathetic nerves. After administration of high doses of amphetamine, mice exhibit an elevated motor activity which is highly increased by aggregation. This increased behavioral activation is followed by death within 24 h in 80–100 % of control animals. Neuroleptics reduce this death rate. In contrast, non-neuroleptic sympatholytics and psychosedative agents like the barbiturates do not produce a dose-related protection. Moreover, anxiolytic agents like benzodiazepines are also found to be ineffective in the prevention of amphetamine group toxicity.

Procedure

Ten male mice of the NMRI-strain are used for each group. They are dosed with the test compound or the standard either orally or intraperitoneally and all placed in glass jars of 18 cm diameter. Untreated animals serve as controls. The test has to be performed at room temperature of 24 °C. Thirty min after i.p. or 1 h after oral administration, the mice receive 20 mg/kg d-amphetamine subcutaneously. The mortality is assessed 1, 4, and 24 h after dosing.

Evaluation

The mortality of amphetamine-only treated animals is at least 80 %. If less than 80 % die due to low ambient temperature, the test has to be repeated. The estimation of ED_{50} values for protection and their confidence limits are calculated by probit analysis of the data using the number of dosed versus the number of surviving animals. Doses of 10 mg/kg chlorpromazine p.o. and 1 mg/kg haloperidol have been found to be effective.

Critical Assessment of the Method

The amphetamine group toxicity test has been used by many investigators and has been found to be a reliable method for detecting neuroleptic activity.

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Inhibition of Amphetamine Stereotypy in Rats

Purpose and Rationale

Amphetamine is an indirect acting sympathomimetic agent which releases catecholamines from

its neuronal storage pools. In rats the drug induces a characteristic stereotypic behavior. This behavior can be prevented by neuroleptic agents.

Procedure

Groups of six Wistar rats with a body weight between 120 and 200 g are used. They are injected simultaneously with d-amphetamine (10 mg/kg s. c.) and the test compound intraperitoneally and then placed individually in stainless-steel cages (40 × 20 × 18 cm). The control groups receive d-amphetamine and vehicle. Stereotypic behavior is characterized by continuous sniffing, licking or chewing and compulsive gnawing. The animals are observed 60 min after drug administration. An animal is considered to be protected, if the stereotypic behavior is reduced or abolished.

Evaluation

The percent effectiveness of a drug is determined by the number of animals protected in each group. A dose–response is obtained by using ten animals per group at various doses. ED_{50} values can be calculated. The standard neuroleptic drugs have the following ED_{50} values: chlorpromazine 1.75 mg/kg i.p. and haloperidol 0.2 mg/kg i.p.

Critical Assessment of the Method

Inhibition of amphetamine-induced stereotypies in rats can be regarded as a simple method to detect neuroleptic activity. However, this may reflect the effects in the corpus striatum which are thought to be responsible for the Parkinsonism-like side effects of neuroleptics.

Modifications of the Method

Ljungberg and Ungerstedt (1985) described a rapid and simple behavioral screening method for simultaneous assessment of limbic and striatal blocking effects of neuroleptic drugs. A low dose of 2 mg/kg d-amphetamine i.p. induces both increased locomotion, thought to reflect an increased dopamine transmission in the nucleus accumbens, and weak stereotypies, thought to reflect an increased dopamine transmission in the neostriatum. The behavior is measured in a combined open-field apparatus with holes on the bottom to measure nose-pocking and registration of time spent in the corners. Neuroleptics with less propensity to induce unwanted extrapyramidal side effects can be differentiated from classical drugs with more extrapyramidal adverse reactions.

Segal and Kuczenski (1997) described an escalating dose "binge" model of amphetamine psychosis. Rats were exposed to escalating doses of amphetamine (1.0–8.0 mg/kg) before multiple daily injections of relatively high doses of the drug (8 mg/kg every 2 h × 4 injections).

Atkins et al. (2001) described stereotypic behaviors in mice selectively bred for high and low methamphetamine-induced stereotypic chewing.

Machiyama (1992) recommended chronic methylamphetamine intoxication in **Japanese monkeys** (*Macaca fuscata*) as a model of schizophrenia in animals.

Ellenbroek (1991) described the ethological analysis of **Java monkeys** (*Macaca fascicularis*) in a social setting as an animal model for schizophrenia.

Sams-Dodd and Newman (1997) described the effects of the administration regime on the psychotomimetic properties of d-amphetamine in the Squirrel monkey (*Saimiri sciureus*).

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Inhibition of Apomorphine Climbing in Mice

Purpose and Rationale

Administration of apomorphine to mice results in a peculiar climbing behavior characterized initially by rearing and then full-climbing activity, predominantly mediated by the mesolimbic dopamine system (Costall et al. 1978). The ability of a drug to antagonize apomorphine-induced climbing behavior in the mouse has been correlated with neuroleptic potential (Protais et al. 1976; Costall et al. 1978).

Procedure

Groups of ten male mice (20–22 g) are treated i.p. or orally with the test substance or the vehicle and placed individually in wire-mesh stick cages. Thirty min afterwards, they are injected s.c. with 3 mg/kg apomorphine. Ten, 20, and 30 min after apomorphine administration, they are observed for climbing behavior and scored as follows:

0 = four paws on the floor
1 = four feet holding the vertical bars
2 = four feet holding the bars

Evaluation

The average values of the drug-treated animals are compared with those of the controls, and the decrease is expressed as percent. The ED_{50}-values and confidence limits are calculated by probit analysis. Three dose levels are used for each compound and the standard with a minimum of ten animals per dose level.

Critical Assessment of the Test

Similar to the enhancement of compulsive gnawing of mice after apomorphine by antidepressant drugs, the suppression of climbing behavior of mice after apomorphine can be used for testing neuroleptic drugs. The test has been modified by various authors.

In contrast to other strains of mice, apomorphine climbing is not induced in DBA2 mice unless subchronic manipulations of brain dopamine transmission are performed (Duterte-Boucher and Costentin 1989).

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Inhibition of Apomorphine Stereotypy in Rats

Purpose and Rationale

Apomorphine induces a stereotyped behavior in rats, characterized by licking, sniffing, and gnawing in a repetitive, compulsive manner, which is an indication of striatal dopaminergic stimulation (Anden et al. 1967; Ernst 1967; Costall and Naylor 1973). Compounds which prevent apomorphine-induced stereotypy antagonize dopamine receptors in the nigrostriatal system (Ljungberg and Ungerstedt 1978; Tarsy and Baldessarini 1974). Furthermore, antagonism of this behavior is predictive of propensity for the development of extrapyramidal side effects and tardive dyskinesias (Klawans and Rubovits 1972; Tarsy and Baldessarini 1974; Christensen et al. 1976; Clow et al. 1980).

Procedure

For screening, groups of six male Wistar rats with a body weight between 120 and 200 g are used. The test drug or the standard is administered i.p. 60 min. prior to apomorphine dosage. Apomorphine HCl is injected s.c. at a dose of 1.5 mg/kg. The animals are placed in individual plastic cages. A 10 s observation period is used to measure the presence of stereotypic activity such as sniffing, licking, and chewing 10 min after apomorphine administration. An animal is considered protected if this behavior is reduced or abolished.

Evaluation

The percent effectiveness of a drug is determined by the number of animals protected in each group. With a group size of ten animals, dose–response curves are obtained and ED_{50} values calculated. ED_{50} values were found to be 0.2 mg/kg s.c. for haloperidol and 5.0 mg/kg for chlorpromazine, whereas clozapine was ineffective even at high doses.

Modifications of the Methods

Puech et al. (1978) studied the effects of several neuroleptic drugs on hyperactivity induced by a low dose of apomorphine in mice.

Apomorphine induces stereotypic behavior in a variety of species including pigeons. The symptoms in pigeons are manifested as pecking against the wall of the cage or on the floor. Akbas et al. (1984) described a method registering the pecking after apomorphine by a microphone, amplification through a pulse preamplifier, and registration with a polygraph. The effect of apomorphine was dose-dependent decreased by yohimbine and neuroleptics.

Stereotyped behavior in guinea pigs induced by apomorphine or amphetamine consisting in continuous gnawing and sniffing of the cage floor was described by Klawans and Rubovits (1972) and used as an experimental model of tardive dyskinesia.

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Yawning/Penile Erection Syndrome in Rats

Purpose and Rationale

Yawning is a phylogenetically old, stereotyped event that occurs alone or associated with stretching and/or penile erection in humans and in animals from reptiles to birds and mammals under different conditions (Argiolas and Melis 1998). The yawning–penile erection syndrome can be induced in rats by apomorphine and other dopamine autoreceptor stimulants (Ståhle and Ungerstedt 1983; Gower et al. 1984) and can be antagonized by haloperidol and other dopamine antagonists. Antagonism against this syndrome

can be regarded as indication of antipsychotic activity (Furukawa 1996).

Besides the **dopaminergic** system in this behavior (Mogilnicka and Klimek 1977; Baraldi et al. 1979; Benassi-Benelli et al. 1979; Nickolson and Berendsen 1980; Gower et al. 1984, 1986; Dourish et al. 1985; Doherty and Wisler 1994; Kurashima et al. 1995; Bristow et al. 1996; Fujikawa et al. 1996a; Asencio et al. 1999) also the **serotonergic** (Baraldi et al. 1977; Berendsen and Broekkamp 1987; Berendsen et al. 1990; Protais et al. 1995; Millan et al. 1997), the **cholinergic** (Yamada and Furukawa 1980; Fujikawa et al. 1996b), the **GABAergic** (Zarrindast et al. 1995), the **NO system** (Melis et al. 1995, 1996, 1997a, b), and **steroid** as well as **peptide hormones** (Bertolini and Baraldi 1975; Bertolini et al. 1978; Holmgren et al. 1980; Berendsen and Nickolson 1981; Berendsen and Gower 1986; Gully et al. 1995) are involved (Argiolas and Melis 1998).

Procedure

Naive male Wistar rats, weighing 220–280 g, are housed under controlled 12 h light–dark cycle with free access to standard food pellets and tap water. Rats are pretreated with subcutaneous injection of the antagonist 30 min prior to injections of the agonist, such as apomorphine (0.02 to 0.25 mg/kg s.c.) or physostigmine (0.02 to 0.3 mg/kg s.c. or i.p.). After administration of the agonist, rats are placed in individual transparent Perspex cages. A mirror is placed behind the row of observation cages to facilitate observation of the animals for penile erections and yawns. Yawning is a fixed innate motor pattern characterized by a slow, wide opening of the mouth. A penile erection is considered to occur when the following behaviors are present: repeated pelvic thrusts immediately followed by an upright position and an emerging, engorged penis which the rats proceed to lick while eating the ejaculate. The number of penile erections and yawns is counted for 30 min following the last injection.

Evaluation

The results are expressed as the mean number of yawns and of penile erections per group $\pm$ SEM. The statistical significance is determined by comparing the results of each group with the results of the relevant control group using a nonparametric rank sum test.

Critical Assessment of the Method

Ferrari et al. (1993) published some evidence that yawning and penile erection in rats underlie different neurochemical mechanisms. Nevertheless, the procedure can be regarded as a useful behavioral tool to study putative antipsychotic activity of new compounds.

Modifications of the Method

Two sublines of Sprague Dawley rats were bred for high- and low-yawning frequency in males (Eguibar and Moyaho 1997).

Apomorphine produced more yawning in Sprague Dawley rats than in F344 rats (Tang and Himes 1995).

Sato-Suzuki et al. (1998) evoked yawning by electrical or chemical stimulation in the paraventricular nucleus of anesthetized rats.

The yawning–penile erection syndrome in rats can be elicited by injections of 50 ng NMDA or AMPA (Melis et al. 1994, 1997b) into the paraventricular nucleus of the hypothalamus or intracerebroventricular injection of 50 ng oxytocin (Melis et al. 1997a) or ACTH (Genedani et al. 1994; Poggioli et al. 1998) or α-MSH (Vergoni et al. 1998).

Champion et al. (1997) and Bivalacqua et al. (1998) studied the effect of intracavernosal injections of adrenomedullin and other peptide hormones on penile erections in **cats**.

Dopaminergic influences on male sexual behavior of **rhesus monkeys** were studied by Pomerantz (1990, 1992).

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Inhibition of Mouse Jumping

Purpose and Rationale

Lal et al. (1975) described a jumping response in mice after administration of L-dopa in amphetamine pretreated animals where the number of jumps can be objectively counted. The mouse jumping is due to dopaminergic overstimulation similar to that seen in rats when stereotypy is induced by higher doses of amphetamine. The phenomenon can be blocked by neuroleptics.

Procedure

Male CD-1 mice weighing 22–25 g are injected with 4 mg/kg d-amphetamine sulfate, followed 15 min later by an i.p. injection of 400 mg/kg L-dopa. The mice spontaneously begin to jump at a high rate. A median of 175 jumps can be observed in these mice during 60 min. Since mice do not show any jumping after saline administration, the responses after drug administration are specific and can be measured automatically through a pressure-sensitive switch closure or properly positioned photoelectric beam disruptions. Test compounds are administered 60 min prior to L-dopa injection.

Evaluation

Jumps of mice treated with test drugs or standard are counted and expressed as percentage of jumps in amphetamine-/L-dopa-treated animals. Using various doses, ED_{50} values with 95 % confidence limits are calculated.

Critical Assessment of the Method

The method has been found to be sensitive and rather specific for neuroleptic drugs.

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Antagonism Against MK-801-Induced Behavior

Purpose and Rationale

Dizocilpine (MK-801), a noncompetitive NMDA antagonist, induces a characteristic behavior in rat and mice, which is regarded as a model of psychosis (Andiné et al. 1999). In mice MK-801 induces a characteristic stereotypy marked by locomotion and falling behavior through both dopamine-dependent and dopamine-independent mechanisms (Carlson and Carlson 1989; Verma and Kulkarni 1992). Antipsychotic agents dose-dependently antagonize this MK-801-induced behavior.

Procedure

Male CD-1 mice (20–30 g) are individually placed in activity boxes lined with wire-mesh flooring and allowed to acclimate for 60 min. The animals are then dosed with compounds 30 min prior to subcutaneous administration of MK-801 at 0.2 mg/kg. The mice are observed for locomotion and the presence of falling behavior 15 min following MK-801 administration.

Evaluation

ED_{50} values and 95 % confidence limits are calculated by the Litchfield and Wilcoxon method.

Modifications of the Method

Deutsch and Hitri (1993), Rosse et al. (1995), Deutsch et al. (2002, 2003), and Mastropaolo et al. (2004) described methods to measure the MK-801-induced explosive behavior in mice, called "popping."

Farber et al. (1996) showed that neuroleptic drugs can prevent neuronal vacuolization and necrosis induced by MK-801 (Fix et al. 1993).

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Phencyclidine Model of Psychosis

Purpose and Rationale

Phencyclidine (PCP)-induced symptoms in rats are considered as a model of psychosis (Ogawa et al. 1994; Halberstadt 1995; Steinpreis 1996; Abi-Saab et al. 1998; Sams-Dodd 1998a; Jentsch and Roth 1999; Phillips et al. 2000; Farber 2003; Morris et al. 2005).

PCP-induced symptoms can be antagonized by neuroleptic drugs (Witkin et al. 1997; Sams-Dodd 1998b; Javitt et al. 2004).

Cartmell et al. (1999) found that metabotropic glutamate receptor agonists selectively attenuate phencyclidine versus d-amphetamine motor behaviors in rats.

Procedure

Behavior of male Sprague Dawley rats weighing 250–300 g was monitored while in transparent, plastic shoebox cages of the dimensions $45 \times 25 \times 20$ cm, with 1 cm depth of wood clips as bedding, and a metal grill on the top of the cage. Motor monitors consisted of a rectangular rack of 12 photobeams arranged in an 8×4 formation. Shoe boxes were placed inside these racks, enabling the activity of the rat to be monitored in a home-cage environment. The lower rack was positioned at a height of 5 cm, which allowed the detection of PCP-induced head movements in addition to movements of the body of the rat. Rearing activity was detected by a second rack placed 10 cm above the first. Rats were placed in the cage for an acclimation period of 30 min, and then removed, administered the test compounds s.c. or sterile water, and then returned to the same cages. After 30 min, the rats were given an s.c. injection of PCP or amphetamine

or sterile water and once again returned to the cages. Motor activity was monitored for the following 60 min resulting in the measurement of three different parameters: ambulations (pattern of beam breaks indicating that the animal had relocated its entire body), fine movements (nonambulatory beam breaks), and time at rest. An indication of rearing activity was detected in the upper rack of photobeams.

Evaluation

Data were analyzed by a one-way ANOVA, and then post hoc comparisons for each dose group versus control or PCP alone or PCP and test compound were made using Newman–Keuls multiple comparison test.

Modifications of the Method

Furuya et al. (1998) investigated the effects of a strychnine-insensitive glycine site antagonist on the hyperactivity and the disruption of prepulse inhibition induced by phencyclidine (PCP) in rats.

Redmond et al. (1999) tested the effects of acute and chronic antidepressant administration on PCP-induced locomotor hyperactivity.

Boulay et al. (2004) tested a putative atypical antipsychotic for improvement of social interaction deficits induced by PCP in rats.

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Inhibition of Apomorphine-Induced Emesis in the Dog

Purpose and Rationale

The blockade of centrally acting dopaminergic mechanisms is considered to play a major role in suppression of psychotic reactions in schizophrenia. Apomorphine, regarded as a direct dopaminergic agonist, produces a pronounced emetic effect in dogs, and the blockade of apomorphine emesis is used as an indication of dopaminergic blockade. However, although both antiemetic activity and antipsychotic activity are thought to be due to dopaminergic blockade, the sites of action are in different brain areas, and there is a lack of complete correlation of these activities.

Procedure

Adult beagle dogs of either sex are used in treatment groups of three to nine dogs/dose. The dogs are given the test compounds in a gelatin capsule; they are then dosed with 0.15 mg/kg apomorphine s.c. at various intervals after administration of the test compound. The dogs are first observed for overt behavioral effects, e.g., pupillary response to light, changes in salivation, sedation, tremors, etc.; then, after the administration of apomorphine, the dogs are observed for stereotypic sniffing, gnawing, and the emetic response. Emesis is defined as retching movements followed by an opening of the mouth and either attempted or successful ejection of stomach content.

Evaluation

If the experimental compound is antiemetic in the primary screen, the dose is progressively lowered to obtain a minimal effective dose or an ED_{50} value. The ED_{50} values for haloperidol and chlorpromazine were found to be 0.06 mg/kg p.o. and 2.0 mg/kg p.o., respectively. Clozapine was not effective at doses between 2 and 10 mg/kg. p.o.

Critical Assessment of the Method

The method has been extensively used by several laboratories. However, since nonclassical neuroleptics like clozapine did not show pronounced activity, the test has been abandoned. Moreover, tests in higher animals like dogs are limited due to regional regulations.

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Purposeless Chewing in Rats

Purpose and Rationale

Purposeless chewing can be induced in rats by directly acting cholinergic drugs or cholinesterase inhibitors (Rupniak et al. 1983), which can be blocked by antimuscarinic agents. The chewing behavior has been proposed to be mediated through central M_2 receptors rather than via

central M_1 sites (Stewart et al. 1989). Chewing can also be induced by chronic administration of neuroleptics in rats (Clow et al. 1979; Iversen et al. 1980). Purposeless chewing is mediated by dopaminergic and nicotinic mechanisms.

Procedure

Male albino rats are housed 10 per cage at room temperature and kept on a 12 h light–dark cycle. For the experiments, rats are placed individually in a large glass cylinder (height 30 cm, diameter 20 cm) at $21 \pm 1°C$ and allowed to habituate for 15 min before injection of drugs. The antagonists, e.g., sulpiride or mecamylamine as standards, are given at different doses 30 min before treatment either with 0.01 mg/kg nicotine or 1 mg/kg pilocarpine i.p. Number of chewings are counted by direct observation immediately after drug administration. The results are presented as number of chews in a 30-min period.

Evaluation

Analysis of variance (ANOVA), followed by Newman–Keuls tests, are used to evaluate the significance of the results obtained. $P < 0.05$ is considered as significant.

Modifications of the Method

Tremulous jaw movements induced by tacrine (Cousins et al. 1999) can be antagonized by antipsychotic drugs (Betz et al. 2005; Ishiwarii et al. 2005).

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Models of Tardive Dyskinesia

Purpose and Rationale

Tardive dyskinesia is a severe side effect of traditional neuroleptics affecting a considerable number of patients probably based on a genetic disposition, being characterized by involuntary movements of the oral region. Various authors used rats as animal model for tardive dyskinesia,

either after treatment with reserpine (Waddington 1990; Neisewander et al. 1994; Bergamo et al. 1997; Queiroz and Frussa-Filho 1999; Andreassen and Jorgensen 2000; Casey 2000; Van Kampen and Stoessl 2000; Calvente et al. 2002; Abílio et al. 2003; Peixoto et al. 2003) or haloperidol (Takeuchi et al. 1998; Harvey and Nel 2003; Naidu et al. 2003; Burger et al. 2005). Several authors compared the effects of different neuroleptics (See and Ellison 1990; Tamminga et al. 1994) or studied potential antagonistic effects (Takeuchi et al. 1998; Queiroz and Frussa-Filho 1999; Abílio et al. 2003; Naidu et al. 2003; Peixoto et al. 2003).

Burger et al. (2005) found that ebselen attenuates haloperidol-induced orofacial dyskinesia and oxidative stress in rat brain.

Procedure

Male Wistar rats weighing 270–320 g were injected s.c. once a week with 12 mg/kg haloperidol decanoate for 4 weeks. Another group was pretreated with 30 mg/kg ebselen and received in addition to haloperidol every other day an i.p. injection of 30 mg/kg ebselen.

The animals were observed for the quantification of orofacial dyskinesia just before haloperidol administration and on the 7th, 14th, 21st, and 28th day after the first administration of haloperidol.

Rats were placed individually in cages (20 × 20 × 19 cm) containing mirrors under the floor and behind the back wall of the cage to allow behavioral quantification when the animal was faced away from the observer. To quantify the occurrence of oral dyskinesia, the incidence of tongue protrusions, vacuous chewing movements frequency, and the duration of facial twitching were recorded for 15 min. Observers were blind to drug treatment.

Evaluation

Data were analyzed by a three-way ANOVA, followed, when appropriate, by univariate analysis and Duncan's post hoc test.

Modifications of the Method

Several authors used monkeys (*Cebus apella* or *Macaca speciosa*) to evaluate the effect of neuroleptics to induce tardive dyskinesia-like symptoms (Gunne and Barany 1979; Domino 1985; Werge et al. 2003).

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Single-Unit Recording of A9 and A10 Midbrain Dopaminergic Neurons

Purpose and Rationale

Interactions with central nervous system dopamine pathways are crucial for the expression of antipsychotic effects seen with clinically effective neuroleptics. These interactions also have a role in the expression of several of the neurological side effects seen with these agents. Extracellular single-unit recording techniques of rat A9 (substantia nigra) and A10 (ventral tegmental area) dopamine neurons show that after acute treatment with neuroleptics, the number of spontaneously firing cells is increased in both areas. After repeated treatment (21 days), a decrease was found with all neuroleptics in the A10 neurons, whereas in the A9 cell, only compounds with clinically evident extrapyramidal side effects induced a decrease. Clozapine which is believed not to produce extrapyramidal side effects resulted in the depolarization inactivation of A10 neurons but not A9 cells. The method provides a prediction of a compound's antipsychotic potential as well as potential neurological side effects (Chiodo and Bunney 1983).

Procedure

Male Wistar rats weighing 280–360 g are anesthetized with chloral hydrate intraperitoneally. The animal is mounted in a stereotaxic apparatus (Kopf, model 900). The cranium is exposed, cleaned of connective tissue, and dried. The skull overlying both the substantia nigra (A9: anterior (A) 3000–3400 μm, lateral (L) 1800–2400 μm from lambda) and the ventral tegmental area (A10: A 3000–3400 μm, L 400–1000 μm from lambda) (Paxinos and Watson 1986) is removed. Using the dura as point of reference, a micropipette driven by a hydraulic microdrive is lowered through the opening of the skull at vertical 6000–8500 μm. Spontaneously firing dopamine neurons within both the substantia nigra and the ventral tegmental area are counted by lowering the electrode into twelve separate tracks (each track separated from the other by 200 μm) in each region. The sequence of these tracks is kept constant, forming a block of

tissue which can be reproducibly located from animal to animal.

Extracellular neuronal signals are sampled using a single barrel micropipette approximately one μm at its tip and filled with 2 M NaCl saturated with 1 % pontamine sky blue dye (in vitro impedance between 5 and 10 MΩ). Electrical potentials are passed through a high-impedance preamplifier, and the signal is sent to a window discriminator which converts potentials above background noise levels to discrete pulses of fixed amplitude and duration. Only cell whose electrophysiological characteristics match those previously established for midbrain dopamine neurons are counted. In an anesthetized rat, a neuron is considered to be dopaminergic if it displays a triphasic positive–negative–positive spike profile of 0.4 to 1.5 mV amplitude and 2.5 ms duration, firing in an irregular pattern of 3 to 9 Hz with occasional bursts characterized by progressively decreasing spike amplitude and increasing spike duration.

At the end of each experiment, the location of the last recorded track tip is marked by passing 25 microampere cathodal current through the recording micropipette barrel for 15 min in order to deposit a spot of dye. The rat is sacrificed, and the brain is then removed, dissected, and frozen on a bed of dry ice. Frozen serial sections (20 μm in width) are cut, mounted and stained with cresyl violet, and examined using a light microscope.

Animals pretreated with vehicle prior to neuronal sampling serve as controls. For animals that are used in an acute single-unit dopamine neuron sampling assay, test compounds are administered intraperitoneally 1 h prior to the beginning of dopamine neuron sampling. For animals used in a chronic single-unit dopamine sampling assay, the compounds are administered once a day for 21 days, and dopamine neuron sampling is started 2 h after the last dose on the 21st day.

Evaluation

Drug treatment groups are compared to vehicle groups with a one-way ANOVA with a post hoc Newman–Keuls analysis for significance.

Modifications of the Method

Nyback et al. (1975) tested the influence of tricyclic antidepressants on the spontaneous activity of norepinephrine-containing cells of the locus coeruleus in anesthetized rats.

Scuvée-Moreau and Dreese (1979) studied the effect of various antidepressant drugs on the firing rate of locus coeruleus and dorsal raphe neurons of the anesthetized rat with extracellular microelectrodes.

Using the method of single-unit recording of spontaneous firing of locus coeruleus neurons in rats, Cedarbaum and Aghajanian (1977) studied the inhibition by microiontophoretic application of catecholaminergic agonists.

Marwaha and Aghajanian (1982) examined in single-unit studies the actions of adrenoceptor antagonists at *alpha-1* adrenoceptors of the dorsal raphe nucleus and the dorsal lateral geniculate nucleus and *alpha-2* adrenoceptors of the nucleus locus coeruleus.

Mooney et al. (1990) studied the organization and actions of the noradrenergic input to the superior colliculus of the hamster using microiontophoretic techniques together with extracellular single-unit recording.

Bernardini et al. (1991) studied in vitro with brain slices of mice the amphetamine-induced and spontaneous release of dopamine from A9 and A10 cell dendrites.

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In Vivo Voltammetry

Purpose and Rationale

Various groups (Lane et al. 1979, 1987, 1988; Blaha and Lane 1983, 1984, 1987; Crespi et al. 1984; Marsden et al. 1984; Maidment and Marsden 1987a, b; Armstrong-James and Millar 1979, 1984; Kawagoe et al. 1993) described in vivo voltammetry as an electrochemical technique that uses carbon fiber microelectrodes stereotactically implanted in brain areas to monitor monoamine metabolism and release. De Simoni et al. (1990) reported on a miniaturized optoelectronic system for telemetry of in vivo voltammetric signals in freely moving animals.

Procedure

Carbon fiber working electrodes are made from pyrolytic carbon fibers supported in a pulled glass capillary (Armstrong-James and Millar 1979; Sharp et al. 1984) and electrically pretreated for simultaneous recording of ascorbic acid DOPAC and 5-HIAA (Crespi et al. 1984).

Male Sprague Dawley rats weighing 270–340 g are anesthetized with a 2–3 % halothane O_2/NO_2 mixture (1:1) and held in a stereotactic frame. Reference and auxiliary electrodes

are positioned on the surface of the dura through 1 mm holes drilled in the cranium and held in place with dental cement. Holes, approx. 2 mm in diameter, are drilled in the cranium above the left or right nucleus accumbens and contralateral anterior striatum, and the underlying dura is broken with a hypodermic needle. A working electrode is lowered in one of the above regions and cemented in place. A second electrode is then implanted in the remaining structure. The coordinates, measured from the bregma, are as follows: nucleus accumbens–rostrocaudal +3.4 mm, mediolateral ± 1.4 mm, dorsoventral −7 mm, striatum–rostrocaudal +2.8 mm, mediolateral ± 2.6 mm, and dorsoventral −5.5 mm.

Drugs are injected subcutaneously. Voltammograms are recorded using a Princeton Applied Research 174A polarographic analyzer alternatively from each region every 5 min and after a 1 h stabilization period.

Evaluation

Voltammetric data are expressed as percentage changes from preinjection control values using the mean of the last six peak heights before administration of drug as the 100 % value. However, statistical analysis of the data is carried out on the absolute peak heights using a paired Student's *t*-test to compare six preinjection control peak heights with those after administration of drug at selected time points.

Modifications of the Method

Swiergiel et al. (1997) constructed voltammetric probes from stainless steel and fused silica tubing sheathing carbon fibers and compared them with commercially available glass-sealed IVEV-5 electrodes. This type of electrodes can be easily manufactured and does not require any special equipment.

Parada et al. (1994, 1995) described a triple-channel swivel suitable for intracranial fluid delivery and microdialysis experiments which can be equipped with three electrical channels for in vivo voltammetry and measurement of intracranial temperature with a thermocouple.

Frazer and Daws (1998) used electrodes coated with a perfluorinated ion exchange resin (Nafion) to assess serotonin transporter function in vivo by **chronoamperometry** whereby voltage is applied to the electrode in a pulsed manner and the current obtained measured as a function of time.

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Genetic Models of Psychosis

The Heterozygous Reeler Mouse

Purpose and Rationale

Reelin is an extracellular matrix protein secreted by GABAergic interneurons that, acting through pyramidal neuron integrin receptors, provides a signal for dendritic spine plasticity. The gene responsible for a mouse mutant strain is called reeler (D'Arcangelo and Curran 1998; Lombroso and Goldowitz 1998; Fatemi 2001; Pappas et al. 2003). Heterozygous reeler mice that exhibit a 50 % downregulation of reelin expression replicate the dendritic spine and GABAergig defects described in human schizophrenia (Larson et al. 2003). The heterozygote reeler mouse was recommended as a model for the development of a new generation of antipsychotics (Tueting et al. 1999; Rowley et al. 2001; Costa et al. 2002). This view has been challenged by Podhorna and Didriksen (2004).

Tomasiewicz et al. (1993) and Wood et al. (1998) proposed **NCAM-180 knockout mice** with a deletion of the neural cell adhesion molecule variant (NCAM-180) displaying increased lateral ventricle size and a reduced prepulse inhibition of startle response as model for schizophrenia.

Dirks et al. (2003) reported reversal of startle gating deficits in **transgenic mice overexpressing corticotropin-releasing factor** by antipsychotic drugs.

Van den Buuse (2003) showed deficient prepulse inhibition of acoustic startle in **Hooded-Wistar** rats compared with Sprague Dawley rats, suggesting that the Hooded-Wister line could be a useful genetic animal model to study the interaction of glutaminergic and dopaminergic mechanisms in anxiety and schizophrenia.

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The Hooded-Wistar Rat

Purpose and Rationale

Van den Buuse (2003), Lodge et al. (2003), and Martin et al. (2004) suggested that the Hooded-Wistar line (fawn-hooded rats) could be a useful genetic animal model to study the interaction of glutamatergic and dopaminergic mechanisms in anxiety and schizophrenia.

Broderick (2002) compared hippocampal serotonin and norepinephrine release during open-field behavior in Sprague Dawley animals with the Fawn-Hooded animals model of depression.

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General Considerations on Neurotensin and Neurotensin Receptors

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