

STEREOSPECIFIC BINDING OF ^3H -PHENCYCLIDINE IN BRAIN MEMBRANES

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Summary

Phencyclidine (PCP) displaceable binding of ^3H -PCP to glass-fiber filters was eliminated and total binding markedly reduced by initial treatment of the discs with 0.05% polyethyleneimine. Assessed with treated filters, unlabeled PCP displaced ^3H -PCP in both rat and pigeon brain membranes with an EC50 of 1 μM . Of similar high inhibitory potency were dextrorphan, levorphanol, SKF 10047 and ketamine, while morphine, naloxone and etorphine had EC50 values higher than 1 mM. Using the dissociative anesthetic dexoxadrol and its inactive isomer levoxadrol as displacing agents, stereospecific binding of ^3H -PCP was obtained in rat and pigeon brain membranes. The markedly higher potency of dexoxadrol, relative to levoxadrol, in displacing bound ^3H -PCP is compatible with behavioral data for these enantiomers. However, they were equipotent in displacing ^3H -PCP bound to glass-fiber filters in the absence of tissue. Heat denaturation, but not freezing, abolished stereospecific binding of ^3H -PCP, which was also absent in rat liver membranes. The stereospecific binding component in brain displayed biphasic saturability at 60-70 nM and 300-400 nM, respectively.

Within the last 3 years evidence has been presented for the existence of specific binding sites for PCP in brain membranes (1-3). However, some of the initially presented findings (1,2) were questioned in light of possible artifacts introduced by the employed methodology. It was shown that ^3H -PCP substantially binds to glass-fiber filters used in the rapid filtration assay (4). Furthermore, unlabeled PCP and other compounds displaced ^3H -PCP bound to filters or brain membranes with a similar rank order of potency. Subsequently it was argued (5) that the extensive filter binding notwithstanding, the originally presented data (2) did reveal statistically significant interaction of ^3H -PCP with brain membranes. Displaceable binding of ^3H -PCP was also described in homogenates of peripheral organs, but the binding affinities, contrary to that assessed in brain membranes, did not correlate with the pharmacological activity of PCP measured in the rotarod test (3).

The biological significance of binding sites for pharmacological agents demonstrated in vitro is often debated. In particular, there is a lingering controversy regarding possible methodological artifacts in characterizing a specific PCP receptor. Both of these issues depend on the criteria for assessing the specificity of ligand binding. The latter aspect was a major aim in a recent study on opiate receptor binding (6). After alleviating the interaction of ^3H -PCP with glass-fiber filters (7), we have now examined the properties of ^3H -PCP binding in brain membranes, focusing on the specificity of the process.

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Materials and Methods

Materials. Polyethyleneimine (PEI) was obtained as a 50% aqueous solution from Sigma Chemical Co. A 0.5% stock solution was prepared, and its pH adjusted to 7.4 with HCl. As needed for filter treatment, this solution was diluted to 0.05%. All PEI solutions were stored at room temperature. Dexoxadrol and levoxadrol were kindly provided by Dr. J. Collins, The Upjohn Company. d-Morphine was obtained as a gift from Dr. K. Rice, N.I.H. Radiolabeled PCP (piperidyl-3,4-³H) with a specific activity of 43 Ci/mole was obtained from New England Nuclear. The solution of free base in ethanol was evaporated under nitrogen at room temperature, and the residue was taken up in water, adjusted to pH 5 with HCl. The specific activity of the solution was adjusted with unlabeled PCP to 17 Ci/mole. This solution was stored at 4°. In initial experiments, ³H-PCP with a specific radioactivity of 17 Ci/mole, obtained from the National Institute on Drug Abuse, was used.

Preparation of tissue. Male Sprague-Dawley rats (200 g) or White Carneaux pigeons (500 g) were decapitated, the brains quickly excised and transferred to 2-4°. The brain was briefly washed in 50 mM Tris. HCl, pH 7.4, blotted with filter paper, and the cerebrum dissected and weighed. The tissue was disintegrated with 100 parts of the cold buffer for 1 min at power output 6.5 of a Brinkman Polytron Homogenizer, Model PT-35. The homogenate was centrifuged at 20,000 x g for 15 min in the cold. The supernatant was decanted and the pellet suspended with the original volume of fresh buffer using a Dounce all-glass homogenizer. Aliquots of this suspension were frozen at -70°. On the day of an experiment a sufficient number of aliquots were quickly thawed, briefly dispersed in a Dounce homogenizer, and kept in ice. Membranes from rat liver were prepared as described above for brain.

Treatment of filters. Prior to their use, Whatman GF/C glass-fiber filters were washed in a beaker, by swirling and decanting, with 3 x 250 ml of deionized water to remove adhering fine particulate matter (8). Subsequently, the filters were swirled with 2 x 100 ml of a 0.05% aqueous solution of PEI, pH 7.4, and soaked in another 100 ml of the same solution for approximately 5 min. The filters were then aligned on a stainless steel mesh to await filtration. All the steps of the filter treatment were carried out at room temperature.

Binding assay. In designing this assay various methodological aspects of known importance for opiate receptor binding were considered (6,8). The assay of ³H-PCP binding was carried out in 8 ml polyethylene tubes, and the assay mixture consisted of 400 µl of Tris. HCl buffer, pH 7.4, or membrane suspension (0.6 mg protein/ml); 50 µl H₂O or unlabeled drug solution; and 50 µl of ³H-PCP (17 Ci/mole). Appropriate dilutions of unlabeled drugs and of ³H-PCP were made from stock solutions stored at -20° and 4°, respectively. To reach equilibrium in binding, the tubes were incubated for 2 hours at 0° (ice-water), then placed on ice and filtered as follows. A previously treated filter was briefly immersed into 0.05% PEI and then dried on the filtration apparatus for approximately 50 sec. Using a Cornwall syringe, 4 ml of ice-cold buffer were rapidly added into a tube, and its content immediately filtered. After repeating this procedure once, the filter was directly washed with another 4 ml aliquot of the ice-cold buffer. The entire filtration of a sample required approximately 8 sec. The filters were placed in counting vials and covered with 1 ml absolute ethanol. Subsequently, 10 ml of a dioxane-xylene-naphthalene scintillation mixture were added (8), and the tubes subjected to liquid scintillation counting. The counting efficiency was 45%, as determined with ³H₂O as internal standard. Protein was determined according to Lowry *et al* (9).

Results

Glass-fiber filters not subjected to the initial PEI treatment displayed substantial binding of ^3H -PCP that was displaceable by unlabeled PCP (Fig. 1, left graph). With treated filters, the "specific", i.e., PCP-displaceable binding was virtually eliminated, and total binding markedly reduced. In addition, the filter treatment decreased the variability of the data.

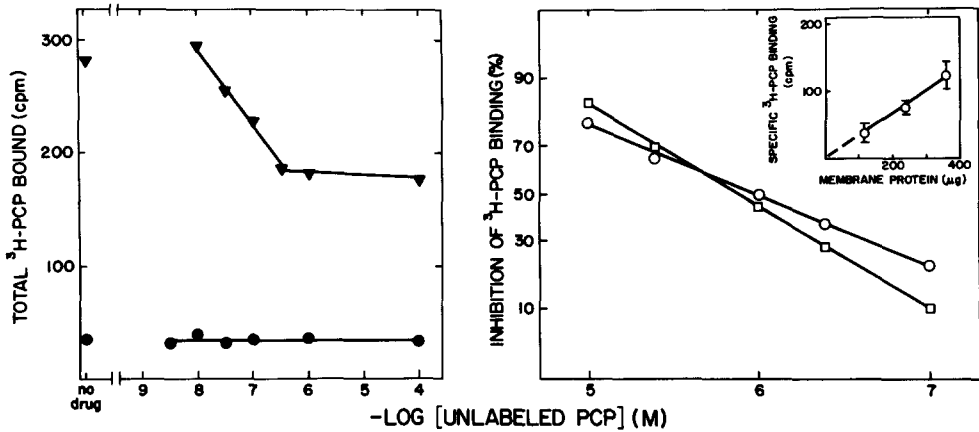


FIG. 1

Displacement of ^3H -PCP bound to glass-fiber filters and to brain membranes. The assay medium containing 5 nM ^3H -PCP and various concentrations of its unlabeled form, but in the absence of tissue, was incubated as described under Materials and Methods. The assay mixture was then filtered through Whatman GF/C filters, previously washed in deionized water with (●) and without (▲) additional soaking in 0.05% PEI (left graph). In other experiments, rat (○) or pigeon (◻) brain membranes were incubated as described with 5 nM ^3H -PCP and various concentrations of unlabeled PCP. The assay mixture was filtered through GF/C filters initially treated with PEI. The binding of 5 nM ^3H -PCP in the presence of various concentrations of unlabeled PCP was related to the maximal displaceable binding, obtained with 100 μM PCP. The results are presented as log-probit plots of the binding data (right graph). The inset depicts the tissue linearity of specific ^3H -PCP binding in rat brain membranes. Presented are mean values and \pm S.E.M. of triplicate observations.

The displaceable ^3H -PCP binding in rat and pigeon brain membranes displayed tissue linearity and had similar pharmacological specificity (Fig. 1 and Table I). Etorphine, morphine, naloxone as well as hexamethonium had little effect on specific ^3H -PCP binding. Atropine was just somewhat more potent, while dextrorphan, levorphanol, d- and l- SKF 10047 and ketamine had EC_{50} 's in part approximating those of unlabeled PCP in displacing ^3H -PCP (Table I). In studies on opiate receptor binding, etorphine, naloxone, SKF 10047, levorphanol and morphine, but not dextrorphan, were of high potency in displacing ^3H -etorphine in rat brain membranes (10, 11).

TABLE I

Potency of various compounds in displacing bound ^3H -PCP
in rat brain membranes

Drug	Inhibition of specific ^3H -PCP binding EC ₅₀ (μM)
Phencyclidine	1.0
Phencyclidine	1.3 (pigeon brain)
Dexoxadrol	0.5
Levoxadrol	39.2
Dextrorphan	5.6
Levorphanol	6.7
(+) SKF 10047	10.2
(-) SKF 10047	27.0
Ketamine	36.3
Atropine	863.0
d-Morphine	>1000
l-Morphine	>1000
Etorphine	>1000
Naloxone	>1000
Hexamethonium	>1000

Aliquots of the membrane preparation were incubated under conditions described in Materials and Methods with 5 nM ^3H -PCP and 5 different concentrations of each listed compound. The concentrations yielding 50% inhibition of specific ^3H -PCP binding were obtained from log-probit plots of the data as described in the legend to Fig. 1.

A differential effect on ^3H -PCP binding in brain membranes from both rat and pigeon was displayed by the enantiomers of α -dioxadrol (Fig. 2). The d-isomer, dexoxadrol, was a significantly more potent inhibitor than its levorotatory counterpart, levoxadrol (Fig. 3, left and middle graphs). This stereospecific effect on ^3H -PCP binding was not observed in rat liver membranes, and was also absent in the binding of ^3H -PCP to untreated glass-fiber filters. Exposure of the membrane preparations from rat and pigeon brain to 90° for 5 min. abolished the differential effect of the enantiomers (Fig. 3, right graph).

The stereospecific binding component of ^3H -PCP in brain membranes was saturable (Fig. 4). Specific binding in these experiments was defined as the difference between ^3H -PCP bound in the presence of 10 μM levoxadrol and dexoxadrol, respectively. While specific ^3H -PCP binding became saturable at 70-80 nM, at higher concentrations an additional binding isotherm became apparent (Fig. 4, inset) The two approximate concentrations of PCP at which half-maximal saturation occurred were 30 nM and 80 nM.

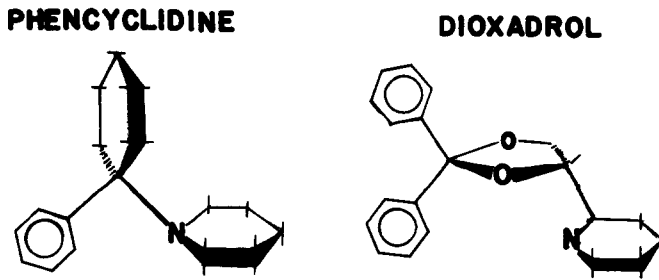


FIG. 2

Structural features of phencyclidine and dioxadrol

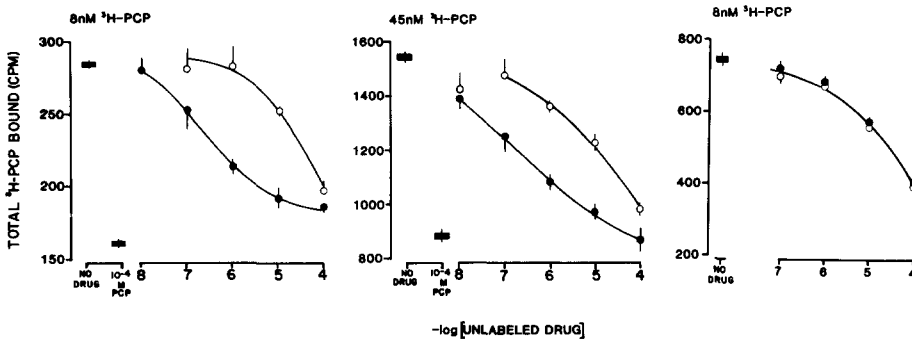


FIG. 3

Stereospecificity in the displacement of bound ^3H -PCP in rat brain membranes. Aliquots of rat brain membranes were incubated with 8 nM (left graph) and 45 nM (middle graph) ^3H -PCP and different concentrations of levoxadrol (○) or dexoxadrol (●) as described under Materials and Methods. Membrane binding of ^3H -PCP at both concentrations was also determined in the absence and presence of 100 μM unlabeled PCP. The difference in bound ^3H -PCP obtained under the latter two conditions was considered as maximal displaceable or "specific" binding. In separate experiments (right graph), the suspension of rat brain membranes was initially heated to 90° for 5 min, cooled to room temperature and dispersed in a Dounce homogenizer. The assay conditions for ^3H -PCP binding were as in the experiments depicted in the left graph. In all experiments shown in this figure PEI treated GF/C filters were used. Statistical aspects were as described in the legend to Fig. 1.

Discussion

The method of rapid filtration offers convenience and good precision in quantitating radiolabeled drug binding to particulate fractions of tissue. The procedure has been widely used to study ligand-receptor interactions, including the opiate (12, 13) and the adrenergic (14) receptors. Binding of the radiolabeled drugs to the employed filter material has been recognized as a disturbing phenomenon, underlined by the stereospecific interaction of opiates with glass-fiber filters (15). Specific treatments of the filters have been described to decrease opiate binding (8), and various methodological approaches were evaluated in assessing the specificity of ligand binding to opiate receptor (6). With this background we were interested in the controversy regarding ^3H -PCP binding, as determined by the method of rapid filtration.

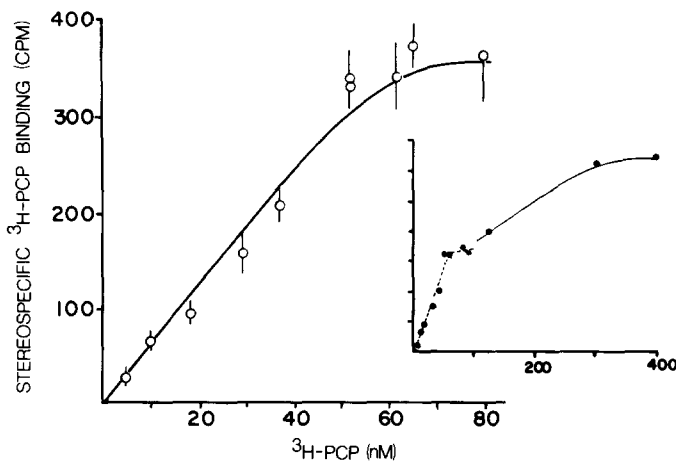


FIG. 4

Saturability of stereospecific ^3H -PCP binding in brain membranes. Aliquots of rat brain membrane suspension were incubated as described under Materials and Methods with different concentrations of ^3H -PCP up to 400 nM (inset). Each concentration of the radiolabeled ligand was incubated in the presence of 10 μM levoxadrol and dexodadrol, respectively. The difference in bound ^3H -PCP obtained under the latter two conditions was defined as stereospecific binding, and is plotted on the ordinate. Again, PEI treated filters were used, and the statistical aspects were as described in the legend to Fig. 1.

Our findings confirmed the considerable extent of binding of ^3H -PCP to untreated glass-fiber filters. However, when assayed with filters treated with polyethyleneimine to reduce binding of ^3H -PCP by more than 90%, a specific, i.e., PCP-displaceable, binding component of ^3H -PCP was determined in brain membranes. While specific binding of ^3H -PCP to glass-fiber filters was eliminated, its binding to brain membranes was not affected by the filter treatment. At any

given concentration of ^3H -PCP its specific binding was reduced to zero when extrapolated to the condition of no tissue, i.e., 0 mg protein. The pharmacological specificity of the ^3H -PCP binding component was similar in both rat and pigeon brain membranes, and resembled that reported previously (1,2,16). A direct comparison of the respective numerical values for the EC_{50} 's is difficult in view of the different experimental conditions applied, e.g., the concentration of ^3H -PCP. Potent opiate agonists such as morphine and etorphine failed to displace bound ^3H -PCP. On the other hand, dextrorphan, but not d-morphine, both inactive as opiates, markedly inhibited ^3H -PCP binding to brain membranes. At first glance, the potency of levorphanol seemed contradictory to the findings obtained with the other opiates and dextrorphan. However, in the presence of a potent narcotic antagonist such as naltrexone, levorphanol displays PCP-like discriminative and cataleptic properties in pigeons (J. GRIPPO, R.E. SOLOMON, S. HERLING and J.H. WOODS, manuscript in preparation). Thus, by blocking the narcotic effects of levorphanol, its functional similarity to PCP, and to dextrorphan, in vivo is revealed. Such a relationship has indeed been observed in course of the interaction of these compounds at ^3H -PCP binding sites in brain membranes (Table I). It is of interest to note that among the enantiomers tested the dextrorotatory isomers were of higher potency. This difference was also observed with d- and l-morphine, although both compounds displayed only marginal potency.

Of particular interest was the differential effect of the enantiomers of dioxadrol in displacing membrane bound ^3H -PCP. Dexoxadrol was 80-fold more potent than its l-isomer, levoxadrol, in displacing the radiolabeled ligand in both rat and pigeon brain membranes. This property of the enantiomers is in agreement with behavioral evidence for the differing pharmacological properties of these compounds observed in the pigeon (17) and monkey (18, 19). In these studies dexoxadrol, in contrast to levoxadrol, displayed phencyclidine-like behavioral properties. The differential effects of dexoxadrol and levoxadrol add a novel approach in assessing specificity of ^3H -PCP binding. It resembles the principle introduced for characterizing the interaction of ligands with the opiate receptor, i.e., the use of enantiomeric displacing drugs to define stereospecific binding (20). The biological relevance of the stereospecific binding component was underlined by its heat sensitivity, presence in the brains of two species, and its absence in liver membranes. It was also absent in untreated glass-fiber filters, i.e., the enantiomers of α -dioxadrol were of equal potency in displacing ^3H -PCP bound to that material. The notion that the stereospecific binding component of ^3H -PCP represents receptor-related phenomenon is further supported by the heat sensitivity of saturable binding observed at several ligand concentrations. However, in view of the presence of an additional binding component of lower affinity, further kinetic resolution of ligand association and dissociation will be necessary to arrive at meaningful values for the binding parameters. In particular, the concentration of displacing drugs relative to that of the radiolabeled ligand has to be optimized for the subsequent Scatchard analysis of the binding data (6). The concentration of PCP for half-maximal saturation of binding (30 nM) was lower than the corresponding values of 80 nM (1), 250 nM (2) and 46 nM (16) reported previously for the nonspecific binding of ^3H -PCP. In this respect it is of interest to note that half-maximal saturation of the low affinity binding component in our study occurred at approximately 80 nM (Fig. 4).

The treatment of the glass-fiber filters by polyethyleneimine served as an important tool in this study. By markedly reducing total, and virtually eliminating specific binding of ^3H -PCP to filters, it alleviated interference of that interaction in assessing binding of the ligand to brain membranes. Furthermore, by reducing nonspecific binding and data variability, the use of treated filters contributed to the resolution of multiple ^3H -PCP binding.

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