Dopamine D2^{High} Receptors Stimulated by Phencyclidines, Lysergic Acid Diethylamide, Salvinorin A, and Modafinil

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KEY WORDS glutamate agonist; dopamine agonist; phencyclidine; ketamine; MK801; D2^{High} receptor; schizophrenia

ABSTRACT Although it is commonly stated that phencyclidine is an antagonist at ionotropic glutamate receptors, there has been little measure of its potency on other receptors in brain tissue. Although we previously reported that phencyclidine stimulated cloned-dopamine D2Long and D2Short receptors, others reported that phencyclidine did not stimulate D2 receptors in homogenates of rat brain striatum. This study, therefore, examined whether phencyclidine and other hallucinogens and psychostimulants could stimulate the incorporation of [^{35}S]GTP-γ-S into D2 receptors in homogenates of rat brain striatum, using the same conditions as previously used to study the cloned D2 receptors. Using 10 μM dopamine to define 100% stimulation, phencyclidine elicited a maximum incorporation of 46% in rat striata, with a half-maximum concentration of 70 nM for phencyclidine, when compared with 80 nM for dopamine, 89 nM for salvinorin A (48 nM for D2Long), 105 nM for lysergic acid diethylamide (LSD), 120 nM for R-modafinil, 710 nM for dizocilpine, 1030 nM for ketamine, and >10,000 nM for S-modafinil. These compounds also inhibited the binding of the D2-selective ligand [^{3}H]domperidone. The incorporation was inhibited by the presence of 200 μM guanylylimidodiphosphate and also by D2 blockade, using 10 μM S-sulpiride, but not by D1 blockade with 10 μM SCH23390. Hypertonic buffer containing 150 mM NaCl inhibited the stimulation by phencyclidine, which may explain negative results by others. It is concluded that phencyclidine and other psychostimulants and hallucinogens can stimulate dopamine D2 receptors at concentrations related to their behavioral actions. **Synapse 63:698–704, 2009.** © 2009 Wiley-Liss, Inc.

INTRODUCTION

Because phencyclidine elicits a psychosis-like syndrome (Goff and Coyle, 2001; Javitt and Zukin, 1991; Jentsch and Roth, 1999), it is important to determine its receptor basis of action. Although it is often stated that phencyclidine is an NMDA (N-methyl-D-aspartic acid) receptor antagonist (Koek et al., 1989; Wong et al., 1986), there is little information on the receptor selectivity of phencyclidine.

Phencyclidine stimulates cloned dopamine D2 receptors in vitro (Seeman and Guan, 2008; Seeman et al., 2005) with similar or higher affinities compared with the NMDA receptor (Kapur and Seeman, 2002; Seeman and Lasaga, 2005; Seeman et al., 2005). Although one publication reported that phencyclidine had no affinity for cloned D2Long receptors (Jordan et al., 2006), it was later shown that the hypertonic buffer (with 150 mM NaCl) used in that study completely inhibited the stimulation by phencyclidine (Seeman and Guan, 2008).

Another report, moreover, found that phencyclidine did not stimulate the incorporation of [^{35}S]GTP-γ-S into rat striatal homogenate (Odagaki and Toyoshima, 2006). These latter findings were important to reexamine, because of the reported different findings of phencyclidine action on the cloned D2Long receptors. The present report indicates that phencyclidine consistently and potently stimulates the incorporation of

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Received 4 November 2008; Accepted 24 November 2008
DOI 10.1002/syn.20647
Published online in Wiley InterScience (www.interscience.wiley.com).

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[^{35}S]GTP-γ-S into rat striatal homogenate, an effect that is blocked by the D2-selective antagonist, S-sulpiride.

In addition to examining the role of D2 receptors in the action of phencyclidine, the present project examined whether other psychostimulants, including ketamine, LSD, and the potent hallucinogen salvinorin A, also stimulated D2 receptors in brain striata. Using cloned-dopamine D2 receptors, previous reports showed that LSD acted at the high-affinity state of the D2 receptor (Seeman, 2004), whereas salvinorin A was a potent kappa opioid agonist (Chavkin et al., 2004; Roth et al., 2002). The present data show that these compounds also stimulate dopamine D2 receptors in brain homogenized striata.

The direct actions of R- and S-modafinil on dopamine D2 receptors were also examined in this study, because earlier reports indicated that D2 receptors presumably had a significant role in the in vivo actions of modafinil (Korotkova et al., 2007; Qu et al., 2008; Wu et al., 2008).

MATERIALS AND METHODS

Incorporation of [^{35}S]GTP-γ-S into homogenates of rat striata

The striata were removed from Sprague-Dawley rat brains (that had been stored at −70°). The striata were homogenized in buffer (4 mg frozen tissue per ml buffer), using a teflon-glass homogenizer (with the piston rotating at 500 rpm) and 10 up and down strokes of the glass container. The buffer contained 50 mM Tris-HCl (pH 7.4 at 20°C), 1 mM EDTA, 5 mM KCl, 1.5 mM $CaC1_2$, 4 mM $MgC1_2$, and 120 mM NaCl. The homogenate was not washed, centrifuged, or preincubated because previous work found that 30–50% of the D2 receptors were lost by these procedures (Seeman et al., 1984).

The effect of dopamine, phencyclidine (Sigma-Aldrich Co., St. Louis, MO), phencyclidine congeners (Chaudieu et al., 1989), ketamine, MK801, LSD (Sandoz Pharmaceuticals, Basel, Switzerland), salvinorin A (Sigma-Aldrich Co.), and R- and S-modafinil (Toronto Research Chemical Co., Ontario, Canada) on the incorporation of [^{35}S]GTP-γ-S was measured as previously described (Kapur and Seeman, 2002; Seeman et al., 2005; Seeman and Guan, 2008). Unless otherwise specified, the homogenized tissue was suspended in assay buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 120 mM NaCl, and 10 μM GDP). The final incubation glass test tube (12 × 75 mm) received 0.25 ml of drug (dopamine, a phencyclidine, ketamine, MK801, or salvinorin A), 0.25 ml of [^{35}S]GTP-γ-S (1250 Ci/mmol, final concentration of 0.2–0.3 nM; PerkinElmer Life Sciences, Boston, MA), and 0.5 ml of tissue suspension. Unless otherwise indicated, the reaction mixture was incubated for 30 min in a 30°C water bath. The reaction was terminated by rapid filtration, using a 12-well cell harvester (Titertek, Skatron, Lier, Norway) and buffer-presoaked glass fiber filter mats (Whatman GF/C). After filtering the incubate, the filter mat was rinsed with buffer for 15 s (7.5 ml buffer). The filters were pushed out and placed in scintillation minivials (7 ml, 16 × 54 mm; Valley Container, Bridgeport, CT). The minivials received 4-ml each of scintillant (Research Products International Corp., Mount Prospect, IL) and were monitored for radioactivity 6 h later in a Beckman LS5000TA scintillation spectrometer. The specific binding of [^{35}S]GTP-γ-S was defined as total binding minus that in the presence of 10 μM S-sulpiride. The definition of 100% incorporation of [^{35}S]GTP-γ-S was that caused by the presence of 10 μM dopamine.

Postmortem human striata

The phencyclidine-stimulated incorporation of [^{35}S]GTP-γ-S was also examined in postmortem control human striata (caudate nucleus and/or putamen) obtained from the National Neurological Research Specimen Bank (Los Angeles, CA). The tissues were shipped frozen to Toronto and kept at −70°C until used. The postmortem human striata were from individuals who had died of non-neurological diseases at ages 63 (case LA2371 male, caudate nucleus), 65 (case LA2607 male, putamen), and 80 (case LA2519 male, putamen).

Inhibition of [^{3}H]domperidone binding to dopamine D2 receptors

Drug potencies to inhibit the binding of [^{3}H]domperidone to the high- and low-affinity states of the dopamine D2 receptor were obtained on homogenates of rat striata and on human cloned D2Long receptors (in CHO cells; Liu et al., 2000).

The dopamine D2 receptors in the striata were measured with [^{3}H]domperidone (2 nM final concentration; custom synthesized as [phenyl-^{3}H(N)]domperidone; 41.4 Ci/mmol; Moravek Radiochemicals, Brea, CA; Seeman et al., 2003). Each incubation tube (12 × 75 mm, glass) received, in the following order, 0.5 ml buffer, containing a range of drug concentrations, with or without a final concentration of 10 μM S-sulpiride (to define nonspecific binding to the dopamine D2 receptors), 0.25 ml [^{3}H]domperidone (generally 1.8 nM as the final concentration in the incubation tube), and 0.25 ml of tissue homogenate. Each concentration of drug was tested in duplicate. The tubes, containing a total volume of 1 ml, were incubated for 2 h at room temperature (20°), after which the incubates were filtered, using a 12-well cell harvester (Titertek, Skatron, Lier, Norway) and buffer-presoaked glass fiber filter mats (Whatman GF/C). After filtering the

incubate, the filter mat was rinsed with buffer for 15 s (7.5 ml buffer), and the filters were processed as detailed above. The specific binding of [^{3}H]domperidone was defined as total binding minus that in the presence of 10 μM S-sulpiride. The drug dissociation constants (Ki) at the high- and low-affinity states of the D2 receptor were calculated using the Cheng-Prusoff Equation (1973).

Independent saturation of dopamine D2 receptors, using a range of [^{3}H]domperidone concentrations, revealed a [^{3}H]domperidone dissociation constant (Kd) of 0.48 ± 0.08 nM (n = 6) for the rat homogenized striata and 0.35 ± 0.018 nM (n = 14) for the D2Long receptors in CHO cells.

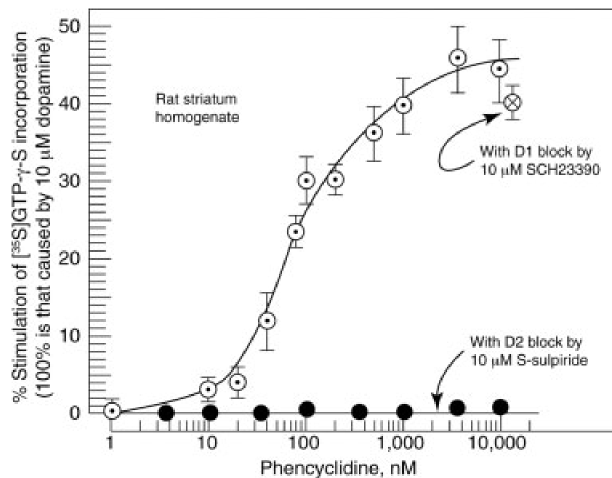

Fig. 1. Phencyclidine stimulated the incorporation of [^{35}S]GTP-γ-S into homogenates of rat striata (n = 4). This effect was blocked by the presence of 10 μM S-sulpiride but not by SCH23390.

RESULTS

Phencyclidine consistently stimulated the incorporation of [^{35}S]GTP-γ-S into the homogenates of rat brain striata. The maximum amount of incorporation elicited by phencyclidine was 45.5 ± 4% (mean ± s.e.; Fig. 1), using the value of 100% for the effect obtained with 10 μM dopamine. The phencyclidine concentration for half-maximum incorporation was 70 ± 30 nM (mean ± s.e.; n = 4), compared with 80 ± 41 nM (mean ± s.e.; n = 7) for dopamine (Table I).

Using postmortem human striata, the maximum incorporation caused by phencyclidine was 49 ± 4.7% (mean ± s.e.; n = 3), with 144 ± 71 nM being the phencyclidine concentration for half-maximum incorporation, compared with 78 ± 52 nM for dopamine (Table I). However, because the postmortem human striata had been stored at −70°C for many years (average of 11 years), the absolute amount of dopamine-induced stimulation of [^{35}S]GTP-γ-S incorporation was only an increase of 2000 DPM/filter, compared with 4000 DPM/filter for rat striata (baseline binding was generally 12,000–25,000 DPM/filter for both tissues).

The phencyclidine concentration of 70 ± 30 nM for half-maximum stimulation of [^{35}S]GTP-γ-S incorporation in rat striata is of the same order of magnitude as that of 20 ± 10 nM found for the human cloned D2Long receptor in the absence of NaCl (Seeman and Guan, 2008). These D2-stimulating concentrations of phencyclidine also match the phencyclidine concentrations of 10 to 100 nM that occupy the high-affinity state of the D2 receptor (i.e., the D2^{High} state) (Seeman, 2004; Seeman and Guan, 2008).

As shown in Figure 1, the presence of 10 μM S-sulpiride, a D2-selective blocking compound, completely

TABLE I. Hallucinogen stimulation of [^{35}S]GTP-gamma-S incorporation

Drug	Striatum or clone	Concentration for 50% stimulation nM n = 5–9	Maximum level of stimulation %[a] n = 5–9	Ki at D2^{High} using [^{3}H]domperidone nM n = 4–5
Dopamine	Rat	80 ± 40	100%	1.75[d]
Dopamine	Human	78 ± 52	100%	~2[d]
Phencyclidine	Rat	70 ± 30[b]	46 ± 4%	4[c]
p-OH-PCP*	Rat	84 ± 8	41 ± 4%	147 ± 10
m-OH-PCP	Rat	103 ± 9	36 ± 5%	91 ± 20
o-OH-PCP	Rat	362 ± 189	40 ± 6%	103 ± 28
Phencyclidine	Human	144 ± 71	49 ± 4.7%	Not done
Salvinorin A	Rat	89 ± 11	44 ± 2%	10
Salvinorin A	D2Long	48 ± 4	62 ± 4%	4.6
R-Modafinil	Rat	120 ± 20	48 ± 2%	16
S-Modafinil	Rat	No effect	No effect	>10,000
LSD	Rat	105 ± 36	36 ± 3%	0.8[e]
Dizocilpine	Rat	710 ± 190	29 ± 6%	24[c]
Ketamine	Rat	1033 ± 329	27 ± 3%	8[c]

*PCP = Phencyclidine.
[a]Blocked in the presence of 10 μM S-sulpiride.
[b]Compares with 20 nM phencyclidine for PCP on D2Long clone (Ref. c).
[c]From Seeman and Guan, 2008.
[d]From Seeman et al., 2003.
[e]From Seeman, 2004.

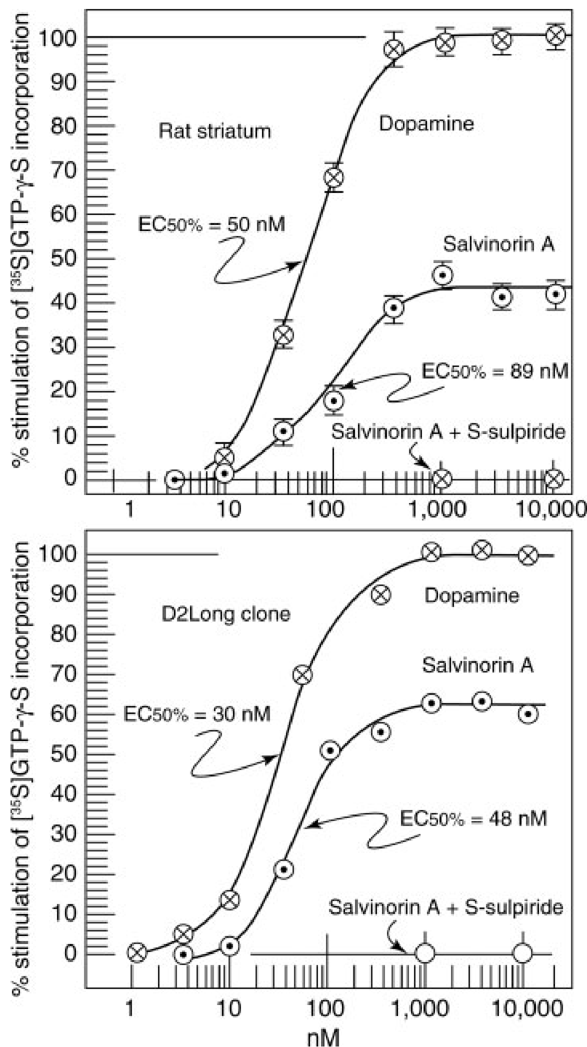

Fig. 2. The potent hallucinogen salvinorin A stimulated the incorporation of [^{35}S]GTP-γ-S into homogenates of rat striata and into the D2Long clone.

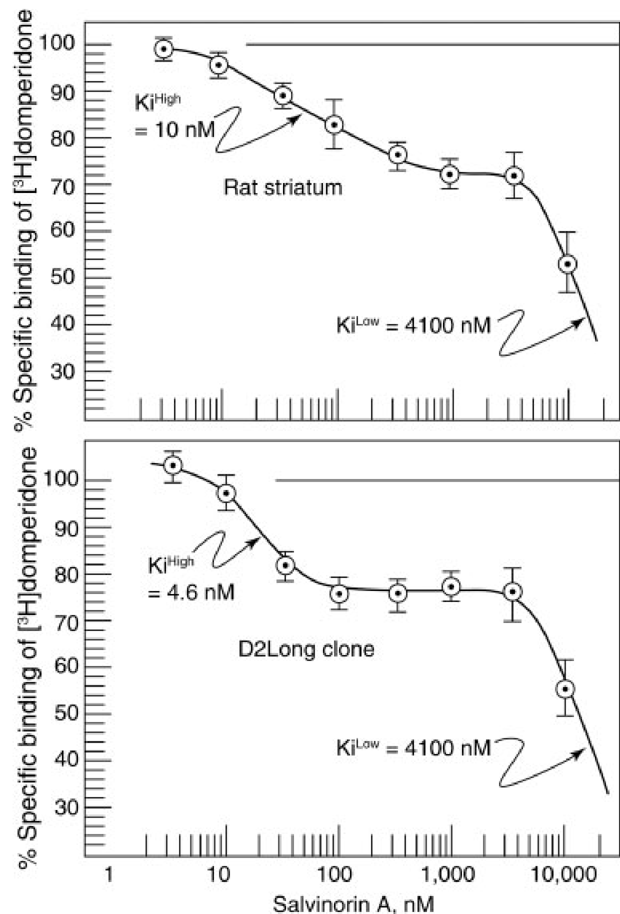

Fig. 3. Salvinorin A inhibited the binding of the D2-selective ligand [^{3}H]domperidone to the high- and low-affinity states of D2 receptors in rat brain homogenized striata (top) and in human cloned D2Long receptors (bottom).

inhibited the phencyclidine-induced incorporation of [^{35}S]GTP-γ-S. However, the presence of 10 μM SCH23390, a D1-selective blocking compound, had no effect on the phencyclidine-stimulated incorporation (Fig. 1).

To determine the possible mode of phencyclidine attachment to the D2 receptor, three phencyclidine-related compounds were studied (Chaudieu et al., 1989). The substitution of a hydroxyl group in the *para*-, *meta*-, or *ortho*- positions on the phenyl ring of phencyclidine showed that the *para*-substituted compound was the most potent at D2^{High} in this series (Table I).

For further comparison with phencyclidine, other hallucinogens and psychostimulants were tested for their ability to stimulate the incorporation of [^{35}S]GTP-γ-S into the rat striatal tissue. The maximum incorporation of [^{35}S]GTP-γ-S caused by ketamine and by dizocilpine (MK 801) was 27 and 29%, respectively, using the value of 100% for 10 μM dopamine. The ketamine and dizocilpine concentrations for half-maximum stimulation of incorporation of [^{35}S]GTP-γ-S were 1033 ± 329 nM and 710 ± 190 nM, respectively (Table I).

The maximum incorporation of [^{35}S]GTP-γ-S caused by lysergic acid diethylamide (LSD) was 36%, compared with 100% for 10 μM dopamine. The LSD concentration for half-maximum stimulation of incorporation of [^{35}S]GTP-γ-S was 105 ± 36 nM.

Salvinorin A is a potent hallucinogen and considered to target the kappa opioid receptor (Chavkin et al., 2004; Roth et al., 2002). Salvinorin A had EC50% values of 48 nM to 89 nM for its actions at the D2 receptor (Fig. 2 and Table I). The salvinorin A effects were blocked by 10 μM S-sulpiride.

Because salvinorin A readily stimulated the incorporation of [^{35}S]GTP-γ-S in striatum and in cloned D2Long receptors and was blocked by the D2-selective S-sulpiride, it was necessary to examine whether salvinorin A could also inhibit the binding of the D2-selective ligand, [^{3}H]domperidone, to rat striata and

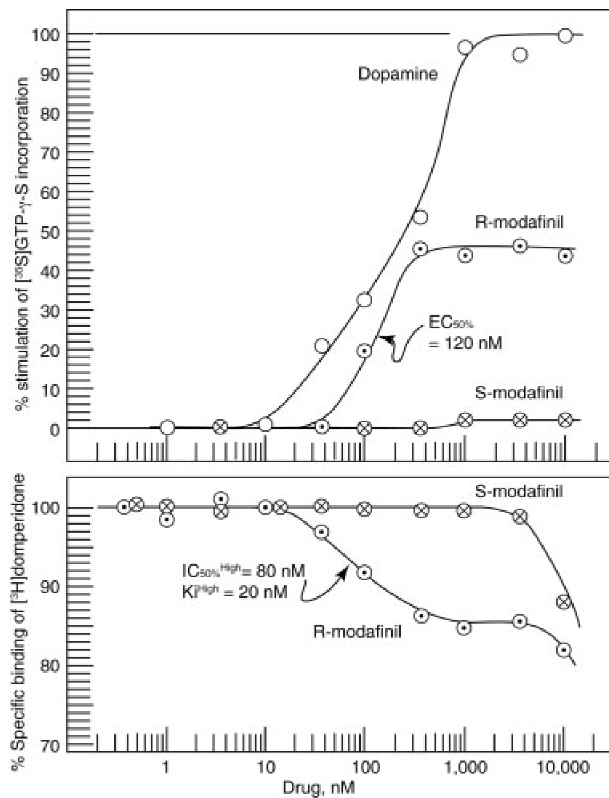

Fig. 4. Representative experiment showing that R-modafinil targeted dopamine D2 receptors. Top: R-modafinil, but not S-modafinil, stimulated the incorporation of [^{35}S]GTP-γ-S (0.3 nM) into homogenates of rat striata, an effect inhibited by 10 μM S-sulpiride. Baseline level of [^{35}S]GTP-γ-S binding was 12,000 DPM/filter. Bottom: R-modafinil, but not S-modafinil inhibited the specific binding of [^{3}H]domperidone to D2^{High} receptors in homogenates of rat striata with a Ki^{High} of 20 nM. Nonspecific binding defined in presence of 10 μM S-sulpiride.

to human cloned D2 receptors. The results show that salvinorin A had dissociation constants of 10 nM at D2^{High} and 4100 nM at D2$_{Low}$ in rat striata (Fig. 3). Similar values were found using the D2Long clone (Fig. 3, bottom).

To examine the possibility that salvinorin A may have been stimulating kappa receptors in the D2-expressing CHO cells, the effect of morphine sulfate on [^{35}S]GTP-γ-S stimulation was tested on the cloned D2Long receptors. However, using concentrations up to 10,000 nM, morphine sulfate did not stimulate the incorporation of [^{35}S]GTP-γ-S.

The suggestion by Korotkova et al. (2007) and by Qu et al. (2008) that the psychostimulant modafinil may act via dopamine D2 receptors was tested. R-Modafinil stimulated the incorporation of [^{35}S]GTP-γ-S with an EC50% of 120 nM, while targeting the D2^{High} receptor, using [^{3}H]domperidone, with an IC50%^{High} of 80 nM and an average Ki^{High} value of 16 nM (Fig. 4). S-Modafinil was not active in these experiments, in keeping with the reported lack of in vivo activity.

For all the hallucinogens tested, the presence of 200 μM GN (guanylylimido-diphosphate) prevented the incorporation of [^{35}S]GTP-γ-S, suggesting that they were acting as agonists at the high-affinity state of D2.

DISCUSSION

In summary, therefore, this study showed that phencyclidine, ketamine, MK801, lysergic acid diethylamide, and salvinorin A stimulated dopamine D2 receptors in the rat striatal tissue, based on the fact that S-sulpiride, but not SCH23390, selectively inhibited the drug-induced incorporation of [^{35}S]GTP-γ-S, and that these compounds have significant affinity for the D2^{High} receptor (Figs. 2 and 3; Seeman and Guan, 2008).

It was a surprise to find that the hallucinogen salvinorin A stimulated the incorporation of [^{35}S]GTP-γ-S by 44% (Table I), an effect that was completely blocked in the presence of the D2-selective S-sulpiride (10 μM). Considering that salvinorin A has been highlighted to be selective for kappa opioid receptors (Chavkin et al., 2004; Roth et al., 2002), the present data were surprising. In fact, however, the EC50% of salvinorin A to stimulate [^{35}S]GTP-γ-S incorporation in rat brain striata (blocked by S-sulpiride) was 89 nM (Table I), somewhat more potent than its EC50% of 235 nM to stimulate [^{35}S]GTP-γ-S incorporation in guinea pig brain caudate nucleus membranes, using 10 μM of the kappa opioid U69,593 to define 100% (Roth et al., 2002).

Previous reports had ruled out any dopamine receptor action of salvinorin A, based on its apparent inability to inhibit the binding of 0.2 nM [^{3}H]methylspiperone to cloned D2 receptors (Chavkin et al., 2004; NIMH-PDSP; Roth et al., 2002).

Recent work, however, found that [^{3}H]spiperone-type ligands, as well as the [^{3}H]raclopride ligand, in contrast to [^{3}H]domperidone, are not sensitive to the low-concentration range of dopamine, namely 1 to 100 nM, corresponding to the D2^{High} state (Seeman et al., 2003). In fact, although the data are not shown here, we found that phencyclidine, salvinorin A, and R-modafinil, only inhibited the binding of [^{3}H]raclopride at concentrations higher than 8000–10,000 nM.

Considering that the D2^{High} receptor appears to be the functional state of the D2 receptor (George et al., 1985; McDonald et al., 1984), the potent values of 4.6–10 nM for the salvinorin A dissociation constant at D2^{High} suggests that the D2^{High} state of the receptor may well be central to the action of salvinorin A in vivo. In fact, the dissociation constants and EC50% values of 4.6–10 nM and 89 nM, respectively, for the salvinorin A actions at the D2 receptor are well within the expected concentration range in the plasma after an oral dose of 0.5–1 mg (Chavkin et al., 2004; Roth et al., 2002). Because there are no nitro-

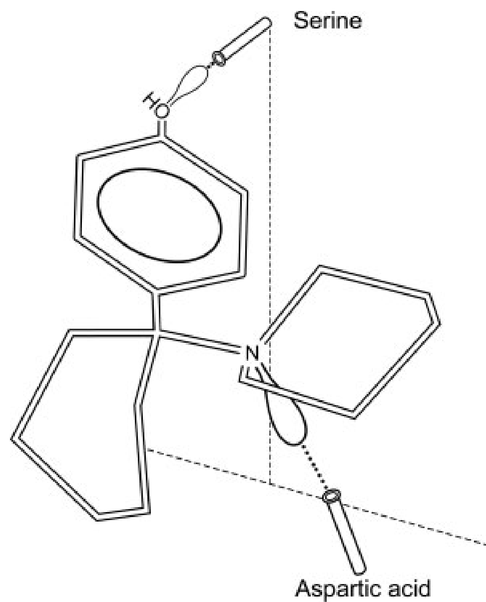

Fig. 5. The amino acid residues of aspartic acid and serine in the D2 tetrahedral model (Seeman et al., 1985) would serve to hydrogen-bond with the nitrogen atom and the *para*-hydroxy substituent, respectively, of *para*-hydroxy-PCP.

gen atoms on salvinorin A that could attach to the D2 receptor, it is likely that the attachment groups are the methoxy group (at position 18; Kane et al., 2008) and the ketone oxygen (position 17; Kane et al., 2008) of salvinorin A that can hydrogen bond to the D2 receptor, as demonstrated in the tetrahedral model of the D2 receptor (Seeman et al., 1985). That is, the methoxy group would hydrogen bond with the serine residue of the D2 receptor, whereas the ketone oxygen would hydrogen bond with the aspartate residue of the D2 receptor (Seeman et al., 1985). In fact, by its action on the D2^{High} state of the D2 receptor, salvinorin A may potentiate the D2-sensitizing action of quinpirole (Beerepoot et al., 2008).

The same amino acid residues of aspartate and serine in the D2 tetrahedral model (Seeman et al., 1985) would serve to hydrogen-bond with the nitrogen atom and the *para*-hydroxy substituent, respectively, of *para*-hydroxy-PCP (Fig. 5).

Although there is no precise correlation between the EC50% values of the four phencyclidines with their Ki values at D2^{High}, phencyclidine itself is the most potent of the four compounds and had the lowest EC50% and Ki^{High} values (Table I). Although the *p*-hydroxy-phencyclidine was marginally more potent than *m*-hydroxy-phencyclidine in stimulating [^{35}S]GTP-γ-S incorporation (Table I), *m*-PCP was more potent than *p*-PCP on the rotarod test (Chaudieu et al., 1989). However, no simple relation is expected between behavioral effects and molecular potencies, because these compounds act on both the glutamate and dopamine neural pathways.

Because the report of Odagaki and Toyoshima (2006) indicated that phencyclidine did not stimulate the incorporation of [^{35}S]GTP-γ-S in homogenates of rat striata, their experimental conditions were repeated, but the tissue was not washed because of the known loss of D2 receptors (Seeman et al., 1984). However, using their final incubation conditions, phencyclidine still stimulated the incorporation of [^{35}S]GTP-γ-S, albeit much reduced when their condition of 150 mM NaCl was in the buffer (data not shown). The inhibitory action of 150 mM NaCl on the phencyclidine-induced incorporation was previously described for the cloned D2Long and D2Short receptors (Seeman and Guan, 2008). It is also possible that the washing and enrichment of striatal membranes done by Odagaki and Toyoshima (2006) may have discarded D2 receptors that may be more sensitive to the phencyclidine-induced incorporation of [^{35}S]GTP-γ-S. In addition, the rat striatal tissue used by Odagaki and Toyoshima (2006) had been homogenized and washed in buffer containing 10% sucrose, which is known to inhibit certain properties of dopamine D2 receptors such as receptor internalization (Ko et al., 2002).

The results further show that the glutamate ionotropic antagonists phencyclidine, ketamine, and dizocilpine not only stimulated the incorporation of [^{35}S]GTP-γ-S but also bound to the agonist high-affinity state of D2 receptors, this latter state removed by the presence of guanylylimidodiphosphate.

Although a physiological level of 120 mM NaCl permitted the phencyclidine-induced incorporation of [^{35}S]-GTP-γ-S (Fig. 1), the fact that 150 mM NaCl markedly inhibited phencyclidine-induced stimulation may explain other findings (Jordan et al., 2006; Odagaki and Toyoshima, 2006) that phencyclidine had no affinity for D2 receptors.

The half-maximum potency of 70 nM for phencyclidine in stimulating the [^{35}S]GTP-γ-S incorporation is somewhat lower than the dissociation constants of 97–313 nM for phencyclidine at the ionotropic receptor labeled by [^{3}H]MK801 (summarized by Seeman and Guan, 2008). This observation suggests that the in vivo action of phencyclidine involves a dopamine agonist action at D2 receptors, and may underlie the psychotic component of phencyclidine action, as previously analyzed (Seeman and Guan, 2008).

Because phencyclidine has a D2 agonist action, and because haloperidol is relatively selective in blocking dopamine D2 receptors, it is important to note that haloperidol blocks the clinical psychotic actions of phencyclidine in nonschizophrenia individuals who had recently ingested phencyclidine (Castellani et al., 1982a,b; Giannini et al., 1984, 1984–85, 2000). In animals as well, haloperidol and remoxipride (which is highly D2-selective) blocked the motor stimulation elicited by phencyclidine or by dizocilpine without eliciting catalepsy (Ögren and Goldstein, 1994).

Although the present data suggest that there is a dopamine-agonist component in the action of phencyclidine, it is important to note that the behavioral effects of phencyclidine may result from a combined and synergistic action of glutamate antagonism and dopamine agonism (Seeman and Guan, 2008). As observed by Jentsch and Roth (1999), "it is important to point out that phencyclidine cannot be argued to support a single "transmitter hypothesis" of schizophrenia."

ACKNOWLEDGMENTS

The authors thank Sandoz Pharmaceuticals Inc. (now Novartis) for donating lysergic acid diethylamide and Neuréva (France) for providing the phencyclidine congeners. They also thank Drs. J.M. Kamenka, R. Chicheportiche, and A. Privat for synthesizing and providing the phencyclidine congeners. This work was supported by the Ontario Mental Health Foundation, the Dr. Karolina Jus estate, the Medland family, the O'Rorke family, the Rockert family, the Essel Foundation, Constance E. Lieber and Stephen Lieber. Postmortem brain tissue was donated by the National Neurological Research Specimen Bank, VA West Los Angeles Healthcare Center, Los Angeles, CA 90073 (sponsored by the NINDS/NIMH, the National Multiple Sclerosis Society, the Hereditary Disease Foundation, the Comprehensive Epilepsy Program, the Tourette Syndrome Association, the Dystonia Medical Research Foundation, and the Veterans Health Services and Research Administration, Department of Veterans Affairs), courtesy of Dr. Wallace W. Tourtellotte.

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