Haloperidol regulates the binding of guanine nucleotides to synaptic membranes through the NMDA receptor

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The NMDA receptor is believed to be important in a wide range of nervous system functions including neuronal migration, synapse formation, learning and memory. In addition, it is involved in excitotoxic neuronal cell death that occurs in a variety of acute and chronic neurological disorders. Besides agonist/coagonist sites, other modulator sites, including the butyrophenone site, may regulate the NMDA receptor. It has been shown that many neuronal modulator mechanisms may be co-coordinated by a group of binding proteins that both clusters NMDA receptors and links them to signalling pathways within the cell. We have found that 5'-guanylylimidodiphosphate (Gpp(NH)p) inhibited the binding of [^{3}H]haloperidol to both the cortical and hippocampal synaptic membranes with high affinity and, reciprocally, haloperidol reduced the binding of [^{3}H]Gpp(NH)p to the membranes. Both effects are abolished by addition of anti-p21^{Ras}. Affinity-purified preparations of the NMDA receptor, which were immunoprecipitated by anti-p21^{Ras} contained only the ε2 (NR2A/NR2B) subunits of NMDA receptors and nNOS. These data suggest that the possible proteins participating in the formation of the macromolecular signalling complexes in postsynaptic density may be nNOS and p21^{Ras}.

Keywords: haloperidol, NMDA receptor, nNOS, p21^{Ras}

1. INTRODUCTION

The NMDA receptor is believed to be important in a wide range of nervous system functions including neuronal migration, synapse formation and learning and memory. In addition, it is involved in the excitotoxic neuronal cell death that occurs in a variety of acute and chronic neurological disorders (see [21] for a review).

It has been shown that many neuronal modulator mechanisms may be co-coordinated by a group of binding proteins that both clusters NMDA receptors and links them to signalling pathways within the cell. The NR2A and NR2B subunits of the receptor bind to the SH2 domains of phospholipase C in a tyrosine phosphorylation-dependent manner [11]. Members of the PSD-95/SAP90 family will also cluster NMDA receptors when co-expressed in transfected cells and are associated with the NMDA receptor in neuronal membranes [14,18]. PSD-95 also binds to non-inducible nitric oxide synthase (nNOS) by interaction of its PDZ domain with a PDZ domain in nNOS [2] thus suggesting that PDZ domains may participate in the assembly of macromolecular signalling complexes involving NMDA receptors and one or more signalling molecules at the postsynaptic density.

One of the possible signalling proteins participating in the formation of the macromolecular signalling complexes in postsynaptic density may be p21^{Ras} [20]**.** It has been found that a p21^{Ras}-activating protein, SynGAP (GTPase activating protein), is exclusively localized at synapses and associated with the PSD95 and NMDA receptors [7, 13]. It has also been shown that NMDA receptor stimulation leads to activation of p21^{Ras} through generation of nitric oxide (NO) in a cGMP-dependent pathway via nNOS [24]. These data suggest that p21^{Ras} may also participate in the formation of supramolecular complexes with NMDA receptors.

Besides its agonist/coagonist sites, the NMDA receptor is regulated by other modulator action, including voltage-dependent blocking of the channel by Mg^{2+}, voltage-independent actions of Zn^{2+}, the redox state of the receptor, and sites for arachidonic acid, ethanol, neurosteroids, pH and polyamines [23]. It has been found that haloperidol, a therapeutically useful antipsychotic drug, inhibits neuronal NMDA responses and has neuroprotective effects against NMDA-induced neurotoxicity [12,19]. Results from Whittemore et al. [22] suggest that a noncompetitive allosteric modulator site expressed by isoforms of the receptor containing the NR1/NR2B subunit mediates haloperidol's action on the NMDA receptor. The ligand binding experiments of [8], as well as point mutation studies [3] showed that haloperidol interacts with polyamine sensitive sites of the NR2B subunits.

Haloperidol induces the expression of immediate early genes [15] and genes associated with synaptic plasticity [9]. NMDA receptor antagonists diminished the c-fos mRNA increase produced by haloperidol, indicating that the NMDA receptor is involved in this process [17, 25]. However, the signalling pathway whereby haloperidol induces the expression of genes through the NMDA receptor is unknown. In this study we have tried

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to demonstrate that the action of haloperidol may be mediated by $p21^{Ras}$, which forms a macromolecular complex with the NMDA receptor.

2. MATERIALS AND METHODS

Membrane preparations from rat cortex or hippocampus were obtained after tissue homogenization in 20 volumes of ice-cold 0.32 M sucrose, containing 0.1 M phenylmethylsulphonyl fluoride (PMSF), 3 mM EDTA, 5 units/ml aprotinin and 5 µg/ml pepstatin A. The homogenate was centrifuged at 1000 *g* for 10 min, the pellet was removed, and the supernatant was centrifuged at 20000 *g* for 20 min. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4 buffer, containing 0.1 mM PMSF, 2 mM EDTA, 5 µg/ml pepstatin and 5 units/ml aprotinin (buffer A) to yield a suspension of 5 mg protein/ml and frozen at −40 °C until use.

Solubilization was carried out using 1% sodium deoxycholate at a detergent/protein ratio = 4/1 (mg/mg) in buffer A during 1 hour at 4 °C followed by centrifugation at 100000 g for 1 hour. The final supernatant was dialyzed against 20 mM Tris-HCl (pH 7.4) containing 2 mM EDTA and 0.1 mM PMSF (buffer B) and was applied on either dextrorphan-Sepharose or trihexyphenidyl (THP)-Sepharose columns (1 x 10 cm) pre-equilibrated in buffer A. The columns were washed with 40 ml buffer A and matrix-binding proteins were eluted by 100 mM dextrorphan or by 100 mM phencyclidine in buffer A. The eluates were dialyzed against buffer B and their binding activity was determined.

[^{3}H]haloperidol binding to synaptic membranes, and the solubilized or affinity-purified preparations, were determined in buffer A, containing 200–300 µg/ml protein and 5 nM [^{3}H]haloperidol. Nonspecific binding was calculated after addition 0.5 mM of haloperidol to the medium. The incubation was carried out during 1 hour at 4 °C and the mixture was filtered through Whatman GF/B filters pretreated with 0.05 polyethylenimine. [^{3}H]Gpp(NH)p binding assays were carried out in the presence of 5 nM [^{3}H]Gpp(NH)p as described for [^{3}H]haloperidol binding. 1 mM Gpp(NH)p was used to define the nonspecific binding. Radioactivity retained on the filter was determined by liquid scintillation spectrophotometry.

The binding of THP and dextrorphan to Sepharose 4B was carried out as Egly et al. [10] suggested for triflufenazine immobilization [6]. Protein concentration was determined using a dye-binding method (Bio-Rad).

3.RESULTS

3.1 Binding experiments

The guanine nucleotide analogue, 5'-guanylylimidodiphosphate (Gpp(NH)p), was examined for its effect on ^{3}H-haloperidol binding to rat cortical and hippocampal synaptic membranes. It was found that Gpp(NH)p inhibits the binding of ^{3}H-haloperidol to the both cortical (IC_{50}= 1012.8 nM, data not shown) and hippocampal (IC_{50}= 900.6 nM) synaptic membranes with high affinity (fig.1). On the contrary haloperidol reduces the binding

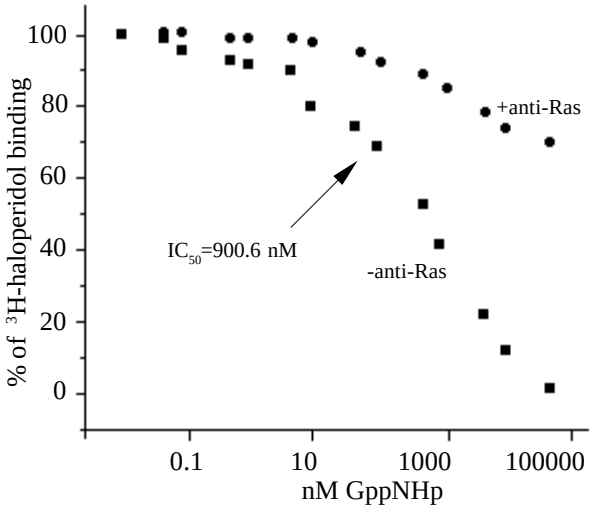

Figure 1. Inhibition of 5 nM [^{3}H]haloperidol binding by GppNHp in rat hippocampal synaptic membranes in the presence (•) and absence (▪) of anti-Ras. 100 % binding corresponds to the specific binding of the control in the absence of GppNHp. Binding was carried out as described in the experimental procedures. The curve is representative of three independent experiments. Specific binding in the absence of GppNHp (dpm ± S.E.M.) from [^{3}H]haloperidol was 8097 ± 421.

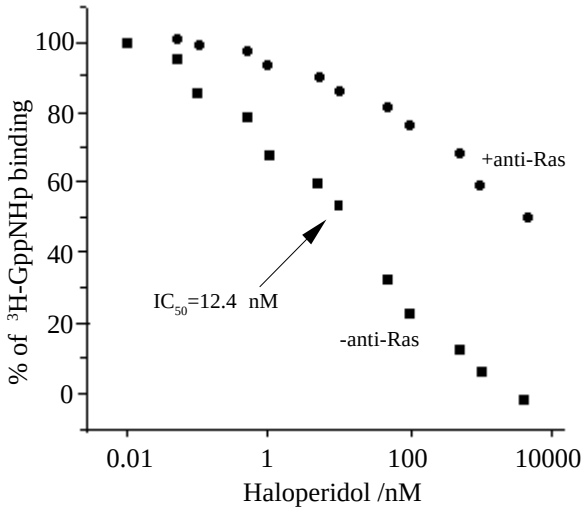

Figure 2. Inhibition of 5 nM [^{3}H]GppNHp binding by haloperidol in rat hippocampal synaptic membranes in the presence (•) and absence (▪) of anti-Ras. 100 % binding corresponds to the specific binding of the control in the absence of haloperidol. Binding was carried out as described in the experimental procedures. The curve is representative of three independent experiments. Specific binding in the absence of haloperidol (dpm ± S.E.M.) from [^{3}H]GppNHp was 2773 ± 248.

of [^{3}H] Gpp(NH)p to the membranes (fig. 2). Both effects are abolished by addition of anti-p21^{Ras}.

In the next series of experiments the effects of other NMDA receptor and sigma antagonists on the binding of [^{3}H] Gpp(NH)p to the hippocampal synaptic membranes were examined. We found that out of the series of NMDA antagonists tested, haloperidol is significantly more potent at decreasing [^{3}H]Gpp(NH)p binding. Phencyclidine (PCP),MK-801((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) and dextrorphan were less potent than haloperidol (Table 1). These data demonstrate that the action of haloperidol on the binding of guanine nucleotide with synaptic membranes is specific and can be directed at a system of p21^{Ras}.

Table 1. The inhibition of [^{3}H]GppNHp binding[a] to synaptic membranes, and solubilized and affinity-purified preparations.

Agent	IC_{50} [b] (nM)		
	Membranes	Solubilized preparation	Affinity-purified preparation 1
Haloperidol	12.4 ± 3.65	35.8 ± 5.28	245.8 ± 37.4
Phencyclidine	432.5 ± 62.5	679.9 ± 47.3	1789.9 ± 176.3
SKF 10 047	568.2 ± 67.4	981.5 ± 82.5	5877.7 ± 679.1
Dextrorphan	1578.6 ± 142.8	3756.4 ± 427.8	9241.5 ± 834.5
Pentazocine	2898.7 ± 284.3	4227.1 ± 492.3	8714.8 ± 878.4
MK-801	3487.9 ± 427.1	7890.5 ± 770.1	7721.9 ± 793.2
Glutamate	> 10 000	> 10 000	> 10 000

[a]100 % binding corresponds to the specific binding of 5nM [^{3}H]GppNHp in the absence of drugs. Binding was measured as described in the experimental procedures. Specific binding in the absence of drugs (dpm ± S.E.M.) from [^{3}H]GppNHp was 2773 ± 248 for membranes, 946±85 for the solubilized preparation and 234± 37 for the affinity-purified preparation.
[b] Mean ± SEM calculated from 3 independent experiments..

3.2 Purification and characterization of haloperidol binding proteins

It has been shown previously that many neuronal modulator mechanisms may be co-coordinated by a group of binding proteins that both clusters NMDA receptors and links them to signalling pathways within the cell [21]. The possible signalling proteins participating in the formation of the macromolecular signalling complexes in postsynaptic density may be nNOS and p21^{Ras} [20]. For identification of the target protein for haloperidol in the supramolecular complex of NMDA receptors the solubilization and affinity chromatography was performed. For this purpose, the final supernatant followed solubilization by 1% sodium deoxycholate (see Materials and Methods) and subsequent centrifugation was applied either on THP-Sepharose or dextrorphan-sepharose columns. Proteins binding THP-Sepharose or dextrorphan-Sepharose from columns were eluted by 10 μM phencyclidine or by 10 μM dextrorphan respectively. SDS-PAG electrophoresis has shown that eluates from THP-sepharose (preparation 1) contain 5 major protein fractions with molecular weights of 23000, 48000, 56000, 11500 and 125000, and eluates from dextrorphan-sepharose (preparation 2) contain 6 protein fractions with molecular weights of 23000, 32000, 48000, 56000, 11500, 125000 (data not shown). As the dextrorphan has higher affinity to a sigma receptor, is possible that the additional protein in the preparation 2 is one of the sigma binding proteins.

Pharmacological specificities of solubilized and both affinity-purified preparations were studied by competition binding experiments. MK-801 was found to have the highest affinity for the proteins of solubilized preparations, while pentazocine, a sigma opiate agonist, the lowest (data not shown). This shows that sodium deoxycholate solubilizes NMDA-glutamate receptors highly effectively, but sigma-binding proteins relatively ineffectively. The pharmacological profile of the affinity-purified preparations does not significantly differ by specificity from the solubilized preparations, indicating that the pharmacological specificity of NMDA receptors does not change during affinity chromatography.

It was also revealed that both preparation 1 and preparation 2 bound [^{3}H] GppNp and did not lose sensitivity to haloperidol. The data for haloperidol are presented in Table 1; similar results are obtained for the other drugs. In spite of the fact that the IC_{50} is in this case lower against the membranous preparation, the specificity of interaction with the drugs is the same (data not shown). Thus it is possible to conclude that the supramolecular complex of NMDA- receptor after affinity chromatography contains proteins binding in the same way as haloperidol and GppNHp.

3.3 Immunoprecipitation of the NMDA receptor

For identification of proteins in the supramolecular complex of NMDA receptor both affinity-purified preparations were immunoprecipitated by anti-Ras and analyzed by Western-blot. It was found that only the ε2 (NR2A/NR2B) subunits of the NMDA receptors were present in both immunoprecipitated preparations. In addition it was revealed that both preparations contained nNOS and did not contain the ε1 subunit of the NMDA receptor and SAP (fig. 3).

Thus, it is possible to conclude that the supramolecular complex obtained after dextrorphan-Sepharose and THP-Sepharose chromatography contains the NR2A/NR2B subunits of the NMDA receptor, p21Ras and nNOS. Furthermore this macromolecular complex is sensitive to haloperidol and binds guanine nucleotides.

Figure 3. Co-immunoprecipitation of NR2A/NR2B subunits of NMDA-receptor and nNOS by anti-Ras. Preparations 1 and 2 were precipitated using the Ras antibody, separated on SDS-PAGE, and analyzed using immunoblots probed with nNOS, NR2A/NR2B subunits and SAP antibodies as desribed in "Materials and Methods". Lanes 1 and 2 – nNOS in preparations 1 and 2 respectively; lanes 3 and 4 – NR2A/NR2B in preparations 1 and 2 respectively; lane 5 – SAP(PSD-95). The blot is representative of three independent experiments.

4. DISCUSSION

Several PDZ-containing proteins have been shown to interact closely with ion-channel linked receptors (see for review [5]). PSD-95 and PSD-93 were reported to interact directly with the C-terminal domains of two different subunits (NR2A and NR2B) of the NMDA receptor [1,14]. The multivalent PDZ-containing protein CIPP (channel interacting PDZ-domain protein) was shown to bind to all four NR2 subunits [16]. On the other hand nNOS and synaptic Ras-GTPase activating protein (synGap),which have been reported [7,13] to interact with PDZ-containing proteins, could further contribute to a complex protein scaffold. Our data has shown that the affinity-purified NMDA receptor preparation was immunoprecipitated by anti-Ras and contained nNOS. The protein complex does not contain SAP, however, and this fact indicates that the interaction of Ras and nNOS with the NR2B subunit is possibly carried out by other proteins, such as PSD-93 [1], CIPP [16] or SAP102 [13].

Ras signalling at the postsynaptic membrane may be involved in the modulation of excitatory synaptic transmission [20]. On the basis of our results it is possible to conclude that the macromolecular complex containing NR2B and Ras may be directly regulated by haloperidol. As shown in fig. 1, haloperidol decreases the binding of ^{3}H-GppNp with synaptic membranes in a dose-dependent manner. Neither PCP, MK-801, dextrorphan nor pentazocine change the binding of ^{3}H-GppNp with synaptic membranes, which suggests that haloperidol binds elsewhere than at the PCP-site of the NMDA receptor. Ilyin et al. [12] indicated that haloperidol selectively inhibits NMDA receptors composed of NR1 and NR2B subunits by acting at a noncompetitive allosteric site distinct from the glutamate-, glycine- and PCP-binding sites, but related to the ifenprodil binding sites. Brimecombe et al. [3] found an NR2B point mutation affecting the haloperidol and CP101 (ifenprodil analogue) sensitivity of a single recombinant NMDA receptor. Together, these observations suggest that the unique effect of haloperidol on ^{3}H-GppHp binding (and possibly on the activity of $p21^{Ras}$) may be mediated by the haloperidol/ifenprodil binding sites of the NMDA receptor. Accordingly, it is possible to conclude that regulation of the Ras-signalling pathway via the NMDA receptor may be carried out by polyamine (ifenprodil) sites.

REFERENCES

1. Brenman, J.E., Chao, D.S., Gee, S.H., et al. Interaction of nitric oxid synthase with the post synaptic density protein PSD-95 and alpa-1-syntrophin mediated by PDZ domains. *Cell* **84** (1996) 757–767.
2. Brenman, J.E., Christopherson, K.S., Graven, S., et al. Cloning and characterization of post synaptic density 93, a nitric oxide synthase interacting protein. *J.Neurosci.* **16** (1996) 7407–7415.
3. Brimecombe, J.C., Gallagher, M.J., Lynch, D.R., Aizenman, E. An NR2B point mutation affecting haloperidol and CP101, 606 sensitivity of single recombinant N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Therapeutics* **286** (1998) 627–34.
4. Boegman, R.J., Vincent, S.R. Involvement of adenosine and glutamate receptors in the induction of c-fos in the striatum by haloperidol. *Synapse* **22** (1996) 70–77.
5. Carner, C.C., Nash, J., Huganir, R.L. PDZ domains in synapse assembly and signaling. *Trends Cell. Biol.* **10** (2000) 274–280.
6. Charbonneau, H., Porath, J. Purification of plant calmodulin by flufenazin-sepharose affinity chromatography. *Biochem. Biophys. Res. Comm.* **90** (1979) 1039–1047.
7. Chen, H.J., Rojas-Soto, M., Oquni, A., Kennedy, M.B. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron* **20** (1998) 895–904.
8. Coughenour, L.L., Cordon, J.J. Characterization of haloperidol and trifluperidol as subtype-selective N-methyl-D-aspartate receptor antagonists using [^{3}H]TCP and [^{3}H]Ifenprodil binding in rat brain membranes. *J. Pharmacol. Exp. Therapeutics* **280** (1997) 584–592.
9. Eastwood, S.I., Heffernan, J., Harrison, P.J. Chronic haloperidol treatment affects the expression of synaptic and neuronal plasticity-associated genes. *Mol. Psychiatry* **2** (1997) 322–329.
10. Egly, J.-M., Porath, J. Change transfer and water mediated chromatography. II. Adsorption of nucleotides and related compounds in acriflavin-Sephadex. *J. Chromatogr.* **168** (1979) 35–47.
11. Gurd, J.W., Bisson, N. The N-methyl-d-aspartate receptor subunits NR2A and NR2B bind to the SH2-domains of phospholipase C. *J. Neurochem.* **69** (1997) 623–30.
12. Ilyin, V.I., Whittemore, E.R., Guastella, J., et al. Subtype-specific inhibition of NMDA receptors by haloperidol. *Mol. Pharmacol*, **50** (1996) 1541–1550.
13. Kim, J.H., Liao, D., Lau, L.F., Huganir, R.L. SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron* **20** (1998) 683–691.

14. Kornau, H.-C., Schenker, L.T., Kennedy, M.B., Seeburg, P.H. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269** (1995) 1737–1740.
15. Kouradi, C., Heckers, S. Haloperidol-induced Fos expression in striatum is dependent upon trancription factor cyclic AMP response element binding protein. *Neuroscience* **65** (1995) 1051–1061.
16. Kurschner, C., Mermelstein, P.G., Holden, W.T., Surmeier, D.J. CIPP, a novel multivalent PDZ domain protein, selectively interacts with kir4.0 family members, NMDA receptor subunits, neurexins, and neurogulins. *Mol. Cell Neurosci*. **11** (1998) 161–172.
17. Morinobu, S., Strausbaugh, H., Terwilliger, R., Duman, R.S. Regulation of c-fos and NGF-A by antidepressant treatment. *Synapse* **25** (1997) 313–320.
18. Neithammer, M., Kim, E., Sheng, M. Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J. Neurosci.* **16** (1996) 2157–2163.
19. Nishikawa, H., Hashino, A., Kume, R., et al. Involvement of direct inhibition of NMDA receptors in the effects of -receptor ligands on glutamate neurotoxicity in vitro. *Eur. J. Pharmacol.* **404** (2000) 41–48.
20. Orban, P.C., Chapman, P.F., Brambilla, R. Is the Ras-MAPkinase signalling pathway necessary for long-term memory formation? *Trends Neurosci.* **22 (**1999) 38–44.
21. Ozawa, S., Kamiya, H., Tsuzuki, K. Glutamate receptors in the mammalian central nervous system. *Prog. Neurobiol.* **54** (1998) 581–618.
22. Whittemore, E.R., Ilyin, V.I., Woodward, R.M. Antagonism of N-methyl-D-aspartate receptors by σ-site ligands: potency, subtype selectivity and mechanisms of inhibition. *J. Pharmacol. Exp. Therapeutics* **282** (1997) 326–338.
23. Yamakura, T., Shimoji, K. Subunit- and site-specific pharmacology of the NMDA receptor channel. *Prog. Neurobiology* **59** (1999) 279–298.
24. Yun, H-Y., Gonzalez-Zulueta, M., Dawson, V.L., Dawson, T.M. Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of $p21^{ras}$. *Proc. Natl Acad. Sci*. **95** (1998) 5773–5778.
25. Ziolkowska, B., Hollt, V. The NMDA receptor antagonist MK-801 markedly reduces the induction of c-fos gene by haloperidol in the mouse striatum. *Neurosci. Lett.* **156** (1993) 39–42.

APPENDIX

Alphabetical list of abbreviations:

Gpp(NH)p: 5'guanylylimidodiphosphate;
EDTA: ethylenediaminetetraacetic acid;
MK-80: (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cycloheptene-5,10-imine;
NMDA: N-methyl-D-aspartate;
nNOS: neural nitric oxide synthase;
PMSF: phenylmethylsulphonylfluoride;
PSD-95: postsynaptic density protein;
SAP-90: synapse associated protein;
THP: trihexyphenidylhydrochloride.