European College of Neuropsychopharmacology
ecnp
21st ECNP Congress, 30 August - 3 September 2008, Barcelona, Spain

Some molecular aspects of haloperidol's neurotoxicity

E. Zhuravliova, T. Barbakadze, N. Natsvlishvili, D.G. Mikeladze

Department of Biochemistry, I.Beritashvili Institute of Physiology, Tbilisi, Georgia

ABSTRACT

One of the most important problems of modern pharmacology and particularly neuropharmacology, is design of drugs without any side effects. From this regard the study of molecular mechanisms, underlining these undesirable effects of neuroleptic therapeutics, gets a great importance. One of the target molecules of above-mentioned drugs in central nervous system is N-metyl-D-aspartate (NMDA) glutamate receptor. 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 of agonist/coagonist sites, other modulator sites, including butyrophenone site may regulate the N-methyl-D-aspartate receptor. It has been shown that haloperidol, an antipsychotic neuroleptic drug, interacts with the NR2B subunit of NMDA receptor and inhibits NMDA response in neuronal cells.

We found that NMDA receptor was co-immunoprecipitated by anti-Ras antibody and this complex, beside NR2 subunit of NMDA receptor contained haloperidol-binding proteins, nNOS and Ras-GRF. Furthermore, we have shown that haloperidol induces neurotoxicity of neuronal cells via NMDA receptor complex, accompanied by dissociation of Ras-GRF from membranes and activation of c-Jun-kinase. Inclusion of insulin prevented relocalization of Ras-GRF and subsequent neuronal death. Haloperidol-induced dissociation of Ras-GRF leads to inhibition of membrane-bound form of Ras protein and changes downstream regulators activity that results in the initiation of the apoptotic processes via the mitochondrial way. Our results suggest that haloperidol induces neuronal cell death by the interaction with NMDA receptor, but through the alternative from glutamate excitotoxicity signaling pathway.

INTRODUCTION

- The NMDA receptor (NMDAR) is believed to be important in a wide range of nervous system functions including neuronal migration, synapse formation, learning and memory. In addition, NMDAR is involved in excitotoxic neuronal cell death that occurs in a variety of acute and chronic neurological disorders [*Waxman and Lynch, 2005*]. Among the many regulatory proteins activated by calcium entering cells through NMDARs are Ras-GTPases (H-, N- and K-Ras) [*Sheng and Kim, 2002*], which can be activated by a wide array of upstream signals, including Ras-GRF proteins.
- It has been found that haloperidol, a therapeutically useful antipsychotic drug, inhibits neuronal NMDA responses and has neuroprotective effects against NMDA-induced neurotoxicity [*Ilyin et al., 1996; Nishikawa et al., 2000*]. However, there are some evidence that haloperidol induces oxidative toxicity of neuronal cells by activation of NF-kB [*Post et al., 1998*] and apoptosis through p38 mitogen-activated protein kinase/c-Jun-NH2-terminal protein kinase pathway [*Noh et al., 2000*]. Oxidative toxicity of haloperidol has emerged as pathogenic events of extrapyramidal side effects including tardive diskinesia, which imposes the major limitation on the use of this class of drugs [*Marsden and Jenner, 1980; Diederich and Goetz, 1998*]. It is clear that both neuroprotective and neurotoxic effects of haloperidol depend on cellular localization and the abundance of haloperidol sensitive NR2B subunits that transmit the extracellular signals through various downstream effectors. In this regard, most important targets of haloperidol may be proteins that directly interact with NR2B subunits [*Krapivinski et al., 2003*] and transmit extracellular signals by allosterically regulated protein–protein interactions.
- The purpose of present study is the clarification the role of protein-protein interactions with NMDA receptor and Ras protooncoprotein during haloperidol-induced neurotoxicity and identification of possible down-stream regulators.

METHODS

- **Cerebrocortical Cultures.** Primary cortical cultures of mixed neurons and glia were derived from newborn Wistar rats. Briefly, after dissociation in 0.027% trypsin, cerebral cortical cells were plated in either 96-well plates or 35-mm dishes that had been coated previously with 15 g/ml poly-L-lysine. Cells were seeded (10^6/ml) in serum-free Dulbecco's modified Eagle medium (DMEM) containing 40 g/ml gentamycin and 60 g/ml penicillin. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 6–7 days.
- **MTT reduction cell viability assay.** The effects of 1 mM glutamate, 10 mM MK-801, 10 mM haloperidol, 10 mM DTG and 100 ng/ml insulin on cell viability was assessed by a colorimetric assay based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue-colored formazan product by mitochondrial succinate dehydrogenase [*Abe and Matsuki, 2000*]. Additions were made directly to the glia/neuron culture medium for 24 h.
- **Cytotoxicity Detection Assay: Lactate Dehydrogenase Test.** Cell death was estimated by the measurement of lactate dehydrogenase (LDH) [*Miyamoto et al., 1989*] released into the medium by dead or damaged cells after 24-hr treatment with different agents.
- **Solubilization and purification of NMDAR complexes.** Membrane preparations from rat cortex or hippocampus was obtained after tissue homogenization in 20 vol ice-cold 0.32 M sucrose and then two-step centrifugation at 1000 g and 20000 g. Solubilization was performed by 1% sodium deoxycholate at detergent/protein ratio = 4/1 (mg/mg) during 1 h at 4°C and then centrifugation at 100,000 g for 1 h was followed. Final supernatant was dialyzed and then applied on dextrorphan-Sepharose column (1 cm x 10 cm) and matrix-binding proteins were eluted by 100 mM dextrorphan. The eluates were dialyzed B and their binding activity was determined. Binding of dextrorphan to Sepharose 4B was carried out by *Egly and Porath* [1979].
- **Binding experiments.** [3H]Gpp(NH)p binding to solubilized or affinity-purified preparations were determined in buffer A, containing 200–300 mg/ml protein and 5 nM [3H]Gpp(NH)p. Non-specific binding was calculated after addition 0.5 mM of Gpp(NH)p into the medium. The incubation was carried out during 1 h at 4°C and the mixture was filtered through Whatman GF/B filters pretreated with 0.05 polyethylenimine. Radioactivity retained on the filter was determined by liquid scintillation spectrophotometer.
- **Immunoprecipitation and Immunoblotting.** ε2 and ε1 subunits, PSD-95, NOS1 and Ras-GRF were immunoprecipitated and assessed by Western blotting. Secondary immunolabeled bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and analyzed by densitometric scanning. The intensities of the bands were within the linear range of the amount of protein loaded. Equal protein loading was confirmed by using b-actin as an internal standard.
- **Measurements of Farnesylated Ras.** The amount of farnesylated Ras was determined according to the method of *Goalstone et al.* [1998] with slight modification.
- **Assay for NO synthesis.** Synthesis of NO was determined by assay of culture supernatants for nitrite [*Kolker et al., 2001*]. Briefly, 300 ml of culture supernatant was allowed to react with 100 ml of 10% Griess reagent and 2.6 ml deionized water, and incubated at room temperature for 30 min. The optical density of the assays samples was measured spectrophotometrically at 548 nm. Fresh culture medium served as the blank in all experiments.
- **Data Analysis.** The numerical data from each experiment were analyzed separately and treated by one-way analysis of variance (ANOVA). When a significant effect was observed by the ANOVA, Student's *t*-test was also used to compare the samples.

RESULTS

- Addition of 10 mM haloperidol to the cells reduces cell survival and preincubation with 1 mM glutamate increases the toxic effects.
- The action of haloperidol was not eliminated neither by MK-801, nor sigma2 antagonist DTG, suggesting that pro-apoptogenic effect of haloperidol does not mediated by sigma-sites, or by channel proteins of NMDAR.
- Insulin in doze-dependent manner reduces the haloperidol-induced apoptosis and increases cell surviving.

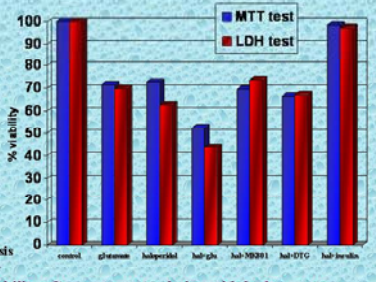


Fig. 1 Cell viability after exposure to haloperidol, glutamate, sigma ligands or insulin for 24 h as assessed by the MTT and LDH tests

- Haloperidol is significantly more potent at decreasing [3H]Gpp(NH)p binding (IC50 = 12.5 nM).
- Phencyclidine (PCP), MK-801 and dextrorphan were less potent than haloperidol at decreasing [3H]Gpp(NH)p binding.

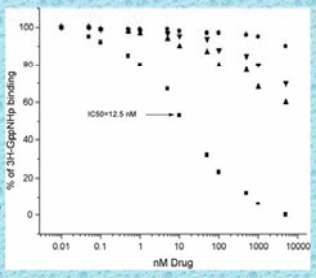


Fig.5 Inhibition of 5 nM [3H]GppNHp binding by MK-801 (●), pentazocine (▲), SKF-10 047 (▼) and haloperidol (■) in affinity-purified preparation of NMDA receptor.

- Both glutamate and haloperidol induce translocation of cytochrome c from mitochondria into cytosol, implying that pore-forming voltage-dependent anion channel (VDAC) open in both cases.
- Haloperidol decreases the content of membrane-bound Ras-GRF, whereas the levels of farnesylated Ras did not change.

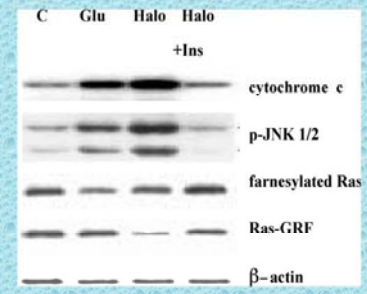


Fig. 2 Effect of glutamate, haloperidol and insulin on the levels of cytoplasmic cytochrome c, farnesylated Ras, membrane-bound Ras-GRF and phospho-JNK.

- It was found that only the ε2 (NR2A/NR2B) subunits of the NMDA receptors were present in immunoprecipitated preparations.
- In addition, it was revealed that this preparation contained Ras-GRF and nNOS and did not contain the ε1 subunit of the NMDA receptor and PSD-95.

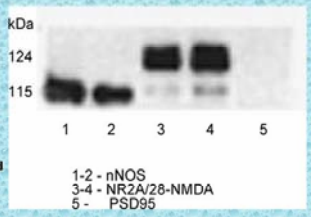


Fig. 6 Co-immunoprecipitation of NR2A/NR2B subunits of NMDA receptor and nNOS by anti-Ras.

- Treatment of cell by glutamate increases the production of NO.
- Addition of haloperidol to the cells reduces the glutamate-induced production of nitric oxide.
- Moreover, in the presence of haloperidol endogenous production of NO was reduced also.
- Thus, the cytotoxic effect of haloperidol is not mediated by activation of NO-synthase and correspondingly by increase of [Ca2+]i, suggesting that this process differs from glutamate-induced cell death.

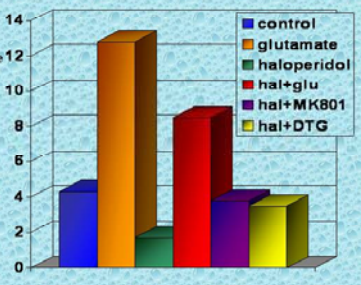


Fig. 3 NO production after treatment of cells by glutamate, haloperidol, MK-801 and DTG

- Guanine nucleotide analogue, 5'-guanylylimidodiphospate (Gpp(NH)p) inhibits the binding of 3h-haloperidol to the both cortical (IC50= 1012, 8 nm) and hippocampal (IC50= 900,6 nm) synaptic membranes with high affinity (fig. 4).
- On the contrary haloperidol reduces the binding of 3H Gpp(NH)p to the membranes (fig. 4). Both effects were abolished by addition of anti- p21Ras.

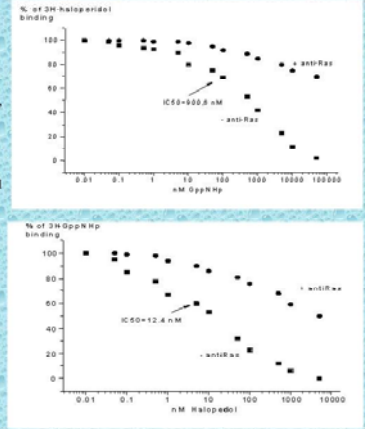
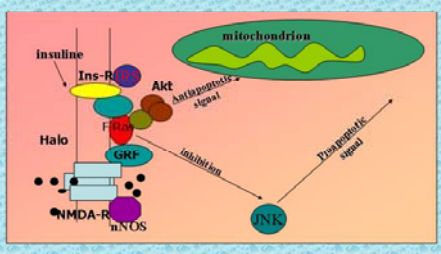


Fig. 4 Haloperidol and Gpp(NH)p binding to synaptic membrane

CONCLUSION:

- We found that in our experimental conditions with immature primary neuronal/glial cells haloperidol as well as glutamate induces apoptosis.
- The cytotoxic effect of haloperidol is not mediated by activation of NO-synthase and correspondingly by increase of [Ca^{2+}], suggesting that this process differs from glutamate-induced cell death.
- Ras-GRF1 specifically binds only the NR2B subunit of NMDAR and occupation of modulatory sites of NMDAR by haloperidol reduces Ras guanine nucleotides exchange activity. Such negative cooperation may be result of dissociation of Ras-GRF from supramolecular complexes of NMDAR.
- Ras-GRF is recruited to the membranes in response to cellular activation by insulin receptors.
- In contrast to glutamate haloperidol activates phosphorylation of JNK and induce JNK-dependent translocation of cytochrome c from mitochondria.
- Our results suggest, that haloperidol-induced dissociation of Ras-GRF leads to inhibition of membrane-bound form of Ras and decreases downstream regulators activities that results in the initiation of the apoptotic processes via JNK activation and release of apoptogenic factors from mitochondria.



REFERENCES

1. Abe, K., Matsuki, A., 2000. **Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and LDH release using MTT.** *J. Neurosci. Res. 38, 325–329.*
2. Diederich, N., Goetz, C., 1998. **Drug-induced movement disorders.** *Neurol. Clin. 16, 125–139.*
3. Goalstone M.J., Leitner W., Wall E., Dolgonos L., Rother K.I, Aceili D., Draznin B. **Effect of insulin on farnesyltransferase.** *JBC 1998; 37; 22892-22896.*
4. Ilyin, V.I., Whittemore, E.R., Guastella, J., Weber, E., Woodward, R.M., 1996. **Subtype-selective inhibition of N-methyl-D-aspartate receptors by haloperidol.** *Mol. Pharmacol. 50, 1541–1550.*
5. Kolker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G., 2001. **Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons.** *Pediatr. Res. 50,76–82.*
6. Krapivinski, G., Krapivinski, I., Mansuian, Y. et al. 2003. **The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1.** *Neuron 40(4), 775–784.*
7. Marsden, C., Jenner, P., 1980. **The pathophysiology of extrapiramidal side effects of neuroleptic drugs.** *Psychol. Med. 15, 679–692.*
8. Nishikawa, H., Hashino, A., Kume, T., Katsuki, H., Kaneko, S., Akaike, A., 2000. **Involvement of direct inhibition of NMDA receptors in the effects of s-receptor ligands on glutamate neurotoxicity in vitro.** *Eur. J. Pharmacol. 404, 41–48.*
9. Noh, J., Kang, H.J., Kim, E.Y., Sohn, S., Chung, Y.K., Kim, S.U., Owan, B.J., 2000. **Haloperidol-induced neuronal apoptosis: role of p38 and c-Jun-NH2- terminal protein kinase.** *J. Neurochem. 75, 2327–2334.*
10. Post, A., Holsboer, F., Behl, C., 1998. **Induction of NF-kB activity during haloperidol-induced oxidative toxicity in clonal hippocampal cells: suppression of NF-kB and neuroprotection by antioxidants.** *J. Neurochem. 18, 8236–8246.*
11. Sheng, M., Kim, M.J., 2002. **Postsynaptic signaling and plasticity mechanisms.** *Science 298, 776–780.*
12. Waxman, E.A., Lynch, D.R., 2005. **N Methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease.** *Neuroscientist 11, 37–49.*